Deubiquitinating enzymes (DUB) form a family of cysteine proteases that digests ubiquitin chains and reverses the process of protein ubiquitination. Despite the identification of a large number of DUBs, their physiological functions remain poorly defined. Here we provide genetic evidence that CYLD, a recently identified DUB, plays a crucial role in regulating the peripheral development and activation of B cells. Disruption of the CYLD gene in mice results in B cell hyperplasia and lymphoid organ enlargement. The CYLD-deficient B cells display surface markers indicative of spontaneous activation and are hyperproliferative. The CYLD-deficient B cells display surface marker gene expression that is consistent with a more activated state. Consistently, in vitro work demonstrates that CYLD inhibits the activation of NF-κB and MAP kinases (MAPKs) by Toll-like receptors (TLRs) and tumor necrosis factor receptors (8–11). Ubiquitination of these signaling molecules appears to serve as a mechanism that activates their signal transduction functions (12). Consistently, in vitro work demonstrates that CYLD inhibits the activation of NF-κB and MAP kinases (MAPKs) by Toll-like receptors (TLRs) and tumor necrosis factor receptors (8–11, 13, 14). However, how CYLD regulates signal transduction under physiological conditions is still poorly understood. Recent studies using CYLD knock-out (CYLD−/−) mice suggest that the signaling function of CYLD is complex, which may involve distinct target proteins in different cell types and signaling pathways (5, 15). For example, the loss of CYLD in primary macrophages has no significant effect on the activation of

Ubiquitination is a posttranslational mechanism that regulates the degradation and biological function of diverse proteins (1, 2). Protein ubiquitination is catalyzed by well-defined enzymatic machinery, composed of an ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzymes (E2), and ubiquitin-protein ligases (E3). Recent studies on the E3 ubiquitin ligases demonstrate an important role for protein ubiquitination in the regulation of immune responses (3). In particular, ubiquitination is involved in the development, activation, and differentiation of lymphocytes. Defects in E3 ubiquitin ligases are associated with severe immunological disorders, such as the loss of immunological tolerance and development of autoimmunity (3). Emerging evidence suggests that protein ubiquitination is a tightly controlled and reversible process that is counter-regulated by deubiquitinating enzymes (DUBs), a family of cysteine proteases digesting ubiquitin chains (4). Like the E3s, the DUBs exist in large numbers, thus suggesting a high level of functional diversity and substrate specificity in their functions (4). However, despite the extensive studies on E3s, the physiological functions of DUBs are poorly defined. We have recently described the function of a DUB, CYLD, in regulating thymocyte development (5). CYLD positively regulates thymic TCR signaling and is required for the generation of CD4 and CD8 mature thymocytes (5). These findings provide the first example for how a DUB can function in the adaptive immune system. However, it is unclear whether CYLD also regulates other aspects of immune function, particularly the activation and homeostasis of lymphocytes.

CYLD was originally identified as a tumor suppressor mutated in familial cylindromatosis (6), an autosomal dominant predisposition to benign tumors of the skin appendages (7). More recent in vitro work suggests that CYLD functions as a DUB of tumor necrosis factor receptor-associated factors and the regulatory subunit of IkB kinase (IKK-γ) (8–11). Ubiquitination of these signaling molecules appears to serve as a mechanism that activates their signal transduction functions (12). Consistently, in vitro work demonstrates that CYLD inhibits the activation of NF-κB and MAP kinases (MAPKs) by Toll-like receptors (TLRs) and tumor necrosis factor receptors (8–11, 13, 14). However, how CYLD regulates signal transduction under physiological conditions is still poorly understood. Recent studies using CYLD knock-out (CYLD−/−) mice suggest that the signaling function of CYLD is complex, which may involve distinct target proteins in different cell types and signaling pathways (5, 15). For example, the loss of CYLD in primary macrophages has no significant effect on the activation of...
NF-κB induced by tumor necrosis factor-α and TLR ligands (5). On the other hand, CYLD modulates the signaling function of a protein tyrosine kinase, Lck, in thymocytes (5) and the nuclear translocation of an NF-κB coactivator protein, Bcl-3, in keratinocytes (15). Clearly, the precise signaling role of CYLD, especially that in the regulation of NF-κB, warrants further studies.

NF-κB represents a family of transcription factors that regulates diverse genes involved in the activation and survival of lymphocytes (16). In mammals, the NF-κB family includes RelA, RelB, c-Rel, NF-κB1 (or p50), and NF-κB2 (or p52), which form different homo- and heterodimers. The NF-κB members are normally sequestered in the cytoplasm as inactive complexes by physical interaction with specific inhibitors, including IκBα and related proteins (17). Activation of NF-κB involves phosphorylation-triggered degradation of IκBα and nuclear translocation of NF-κB complexes, particularly the p50/RelA and p50/c-Rel dimers. A multisubunit IKK complex responds to diverse cellular stimuli and mediates the phosphorylation of IκBα (18). In addition to this canonical pathway of NF-κB activation, a noncanonical pathway exists that mediates activation of two specific NF-κB members, RelB and NF-κB2 (17). Accumulating evidence suggests that the deregulated activation of NF-κB can cause severe immunological disorders, such as lymphoid malignancies and autoimmunity (19–21). As such, both the basal and the inducible activity of NF-κB are likely subject to negative mechanism of regulation, although the physiological negative regulators of NF-κB remain poorly defined.

In the present study, we show that CYLD plays a critical role in preventing uncontrolled NF-κB activation in B cells. Consistently, CYLD-deficient B cells are hyperproliferative when stimulated in vitro and display elevated levels of antigen responses in vivo. The CYLD−/− mice develop B cell hyperplasia and lymphoid organ abnormalities, which can be further exacerbated when these animals are challenged with antigens. We further show that CYLD also regulates peripheral B cell development since the loss of CYLD results in abnormal production of marginal zone B cells. These findings establish CYLD as a key regulator of B cell activation and development and reveal a physiological function of CYLD in NF-κB regulation.

MATERIALS AND METHODS

Mice—Clyd knock-out mice were generated as described (5). Cyld+ /− mice were intercrossed to generate Cyld− /− and Cyld+ /+ littermates. Genotyping was performed by PCR using tail DNA and the following primers: Cyld forward primer 1, 5’-CCA GGC ACT TTG AAT TGC TGT C-3’; Cyld reverse primer 1, 5’-CGT TCT TCC CAG TAG GGT GAA G-3’; Cyld reverse primer 2, 5’-GCA TGC TCC AGA CTG CTT GTG-3’. When the three primers were used together, the PCR yielded a 209-bp product for Cyld+ /+ mice, a 209- and a 255-bp product for Cyld− /− mice, and a 255-bp product for Cyld+ /+ mice. Unless specified, mice were housed in specific pathogen-free cages and monitored periodically for the lack of common pathogens. For studies that involved housing of mice under conventional conditions, age- and sex-matched CYLD− /− and wild-type mice were transferred from ventilated cages to conventional cages and housed for 6 weeks. Animal experiments were in accordance with protocols approved by the Pennsylvania State University College of Medicine Institutional Animal Care and Use Committee.

Antibodies and Reagents—The anti-CYLD antibody was generated by injecting rabbits with a glutathione S-transferase fusion protein containing an N-terminal region of human CYLD (amino acid 136-301). Phospho-IκBα (Ser-32) antibody was from Cell Signaling. Antibodies for actin (C-2), IκKB (H470), tubulin (Tu-02), p50 (C-19), c-Rel (sc-70), and RelB (C-19) were purchased from Santa Cruz Biotechnology, Inc. Fluorescence-labeled anti-mouse antibodies used in flow cytometry included activated protein C-anti-C19Rp (AA4.1), APC-anti-CD3 (145–2C11), PE.CY7-anti-CD19 (1D3), FITC-anti-CD21 (7G6), PE-anti-CD23 (B3B4), FITC-anti-CD80 (16-10A1), PE-anti-CD86 (GL1), FITC-anti-IgD (11–26c.2a), and PerCP-Cy5.5-anti-IgM (R6–60.2). Anti-C19Rp, anti-CD80, and anti-CD86 were purchased from eBioscience, and the rest of the conjugated antibodies were from BD Biosciences. Unconjugated anti-IgM and anti-CD40, used for B cell stimulation, were purchased from Jackson ImmunoResearch and BD Biosciences, respectively. Sheep red blood cells (SRBC) and human recombinant BAFF were purchased from Cocalico Biologicals, Inc. and BIOSOURCE, respectively. GST-IκKB was cloned by inserting a cDNA fragment encoding amino acids 166–197 of human IκKB into pGEX-4T vector (Amersham Biosciences). Recombinant protein was produced in Escherichia coli and purified using GST-Sepharose. Cycloheximide was obtained from Sigma, and all other antibodies and reagents have been described previously (5, 22, 23).

Flow Cytometry—Bone marrow cells were prepared as described previously (24). Spleen and mesenteric lymph node (MLN) cell suspensions were prepared by gentle homogenization using a tissue homogenizer. Mononuclear cells were isolated by centrifugation over lymphocyte separation medium (Cellogel). Peritoneal cells were isolated by flushing the peritoneal cavity using 10 ml of PBS. Flow cytometry was performed as described previously (5). The data shown in Fig. 3D were collected using FACS Calibur, and all the other data were generated using FACS Canto. For analyses of in vitro cultured B cells, the cells were incubated for 48 h in Iscove’s media either in the presence or in the absence of BAFF (100 ng/ml) and then subjected to flow cytometry.

Cell Proliferation Assays—B cells were purified from splenocytes using anti-B220-conjugated magnetic beads (Milteny Biotec) and were stimulated in 4 replicate wells of 96-well plates (1 × 106 cells/well) with anti-IgM (10 μg/ml), anti-CD40 (2 μg/ml), or LPS (3 μg/ml). After the indicated times of stimulation, the cells were labeled for 5 h with [3H]thymidine for proliferation assays based on thymidine incorporation.

For the carboxyl fluorescent succinimidyl ester (CFSE) cell proliferation assay, purified splenic B cells were washed once with PBS (pH 7.4, warmed to 37°C) and incubated with CFSE (1.25 μg/ml in PBS) for 10 min at 37°C. After two washes with Iscove’s medium, the cells were stimulated as described above followed by flow cytometry to measure the CFSE intensity.

Mouse Immunization, Immunohistochemistry, and Antibody Analyses—Mice were injected intraperitoneally with 0.2 ml of SRBC (1 × 109/ml in PBS) and sacrificed 6 days later. Spleens
Regulation of B Cell Activation by CYLD

were frozen in Tissue-Tec OCT compound (VWR International) using liquid nitrogen precooled 2-methilbutane. The frozen tissues were stored at −70 °C until processed to produce 6–8-µm cryostat sections. The sections were stained with rat anti-mouse B220 (eBioScience) followed by biotinylated anti-rat immunoglobulin (Vector Laboratories) or with biotin-conjugated hamster anti-mouse CD3 (eBioScience), biotin-conjugated peanut agglutinin (Vector Laboratories). The immunostaining were then detected with peroxidase-conjugated streptavidin using diaminobenzidine as chromagen (VECTORSTAIN Elite ABC kit, Vector Laboratories).

For analyses of antibody responses, mice were injected intraperitoneally with 0.2 ml of nitro-phenol-conjugated keyhole limpet hemocyanin (NP-KLH) or nitro-phenol-conjugated LPS (NP-LPS) (0.1 mg/ml in PBS). Sera were collected at the indicated times after immunization and subjected to ELISA to detect NP-specific antibodies using the SBA Clonotyping system, which amplifies the 32P-radiolabeled antigen using liquid nitrogen prechilled 2-methylbutane. The frozen tissues were stored at −70 °C until processed to produce 6–8-µm cryostat sections. The sections were stained with rat anti-mouse B220 (eBioScience) followed by biotinylated anti-rat immunoglobulin (Vector Laboratories) or with biotin-conjugated hamster anti-mouse CD3 (eBioScience), biotin-conjugated peanut agglutinin (Vector Laboratories). The immunostaining were then detected with peroxidase-conjugated streptavidin using diaminobenzidine as chromagen (VECTORSTAIN Elite ABC kit, Vector Laboratories).

IB and EMSA—Purified B cells were stimulated with anti-IgM (2.5 µg/ml) or LPS (2.5 µg/ml) for the indicated times.

TOTAL RNA was isolated from purified MLN B cells using the TRI reagent (Molecular Research Center, Inc.). Semiquantitative RT-PCR was performed using the following primers to amplify murine CD23, Iκbα and Gapdh, CD23 forward, 5′-GTG AGG ACT GTG TGA TGA TGC-3′; CD23 reverse, 5′-GAG GAG AAA TCC AGA AGA GTG-3′; Iκbα forward, 5′-CTG TTT GTG AAA CTG AAG AGC TG-3′; Iκbα reverse, 5′-CTT CAC AAA AGC AAC ATA GTG GC-3′; Gapdh forward, 5′-CTC ATG ACC ACA GTG CAT GCC ATC-3′; Gapdh reverse, 5′-CTG CTT CAC CTT CTT GAT GTC-3′.

RESULTS

CYLD−/− Mice Display Lymphoid Organ Abnormalities and B Cell Hyperplasia—To investigate the role of CYLD in regulating immune system function, we began by analyzing the peripheral lymphoid organs of the CYLD−/− mice and wild-type mice. As early as 8 weeks of age, the CYLD−/− mice displayed striking enlargement of the MLNs, and this abnormality became even more profound at older ages (Fig. 1A). On the other hand, the CYLD−/− and wild-type mice did not show obvious size differences in other lymph nodes or the Peyer’s patches, and only a small percentage of the mice had slightly enlarged spleens (data not shown). Thus, a prominent lymphoid abnormality of the CYLD−/− mice is the enlargement of MLNs.

To examine the effect of CYLD on lymphocyte homeostasis, we performed flow cytometry analyses to measure the frequency of B and T cells in MLNs. The CYLD−/− MLNs exhibited a profound increase in the percentage of B cells and a reduction in the percentage of T cells (Fig. 1B). The absolute number of MLN B cells was even more drastically increased in the CYLD−/− animals (Fig. 1C) due to the severe lymphadenopathy (Fig. 1A). We previously reported that the spleen of CYLD−/− mice contained more B cells and reduced numbers of T cells (5). Consistently, flow cytometry analyses of multiple animals revealed significantly higher frequency and numbers of B cells and reduced frequency and numbers of T cells in the spleens of CYLD−/− mice (Fig. 1D and data not shown).

In addition to the mainstream B cells (B2 cells), we also analyzed the frequency of B1 cells, which are predominantly located in the peritoneal cavity. The CYLD−/− mice only
showed a slight increase in this population of B cells in the peritoneal cavity (Fig. 1E) and no difference in spleen and MLNs (data not shown). Taken together, these results suggest that the loss of CYLD causes hyperplasia of mainstream B cells and abnormalities of peripheral lymphoid organs, especially MLNs.

**CYLD Plays a Minor Role in Regulating B Cell Development in the Bone Marrow**—Peripheral B cells are derived from immature B cells generated in the bone marrow. Because of the peripheral B cell hyperplasia in CYLD−/− mice, we examined whether the loss of CYLD resulted in elevated generation of immature B cells in the bone marrow. Flow cytometry analyses of bone marrow CD19+ cells (B cells) detected three major populations: the early stages of developing B cells (ProPre B cells, IgM−IgD−), the immature B cells (IgM+IgD−), and the recirculating mature B cells (IgM+IgD+). The CYLD−/− mice did not produce more immature B cells but rather had a moderate reduction in this population of B cells (Fig. 2, A and B). This result suggests that CYLD plays a minor and positive role in B cell development at the immature B stage. Thus, the peripheral B cell hyperplasia of CYLD−/− mice was not due to the overproduction of immature B cells within the bone marrow.

**CYLD Regulates Marginal Zone B Cell Development**—In the spleen, immature B cells go through transitional stages and eventually become follicular mature B cells or marginal zone B cells (28). To examine how the loss of CYLD affects peripheral B cell maturation, we analyzed the splenic B cell populations based on their defined surface markers (28). Young CYLD−/− mice (8 weeks) did not display profound alterations in B cell development.
maturation (Fig. 2, C and D), although they had a slight reduction in the CD21\textsuperscript{lo}CD23\textsuperscript{lo} T1 cells (Fig. 2D). Additionally, we detected a small increase in the CD21\textsuperscript{hi}CD23\textsuperscript{lo} marginal zone B cell population in these mutant animals (Fig. 2D). Interestingly, the increase in marginal zone B cells became much more prominent in older CYLD\textsuperscript{−/−} mice (14 weeks), as assessed based on the staining of both CD21/CD23 (Fig. 2E) and another marginal zone B cell marker, CD1d (Fig. 2F). These results suggest a role for CYLD in regulating the peripheral development of B cells to marginal zone population.

Spontaneous Activation of B Cells in CYLD\textsuperscript{−/−} Mice—We next examined whether the B cell hyperplasia in CYLD\textsuperscript{−/−} mice was associated with abnormal B cell activation. This possibility was first indicated in our analyses of B cell maturation markers.
Although the CYLD deficiency in young mice did not profoundly alter the frequency of transitional and mature B cell subpopulations in the spleen (Fig. 2D), the CYLD−/− splenic B cells displayed considerably higher intensity of CD23 and CD21 (Fig. 3A, Total, dotted lines). This abnormality occurred primarily in follicular B cells (Fig. 3A, FO) but not marginal zone B cells (Fig. 3A, MZ). Since follicular B cells contain both mature and transitional populations, we further identified the CD21/CD23 overexpressing cells based on the expression of AA4.1. Loss of CYLD caused CD21/CD23 up-regulation in both transitional (AA4.1 positive) and mature (AA4.1 negative) B cells (Fig. 3A, bottom panel, and data not shown). Further, this abnormality was also detected on B cells isolated from the MLNs (Fig. 3B). Parallel RT-PCR analyses showed that the CYLD−/− B cells expressed substantially higher levels of CD23 mRNA than the wild-type B cells, suggesting a role for CYLD in regulating CD23 gene expression (Fig. 3C).

Since CD21 and CD23 have been implicated in B cell activation and humoral immune responses (29–32), the findings described above, together with the B cell hyperplasia, suggest the possibility that the loss of CYLD may lead to abnormal B cell activation. To further confirm this possibility, we analyzed the expression of two other known B cell activation markers, CD80 and CD86, which function as costimulatory molecules modulating the activation of T and B cells (33, 34). As expected, wild-type B cells expressed low levels of CD80 and CD86 (Fig. 3D, solid lines). In contrast, the CYLD−/− B cells expressed markedly higher levels of CD80 and CD86 (Fig. 3D, dotted lines), thus further suggesting the activation phenotype of these mutant B cells. The spontaneous activation of CYLD−/− B cells was also indicated by their larger size, as demonstrated by the forward scatter analysis in flow cytometry (Fig. 3E). Taken together, these results suggest a key role for CYLD in maintaining the naive phenotype of B cells and provide an explanation for the B cell hyperplasia in CYLD−/− mice.

Hyperresponsiveness of CYLD−/− B Cells—As a more direct approach to determine the effect of CYLD deficiency on B cell activation, we analyzed the proliferative response of the CYLD−/− B cells to stimulation via different receptors. Thymidine incorporation assays revealed that the CYLD−/− splenic B cells had significantly higher proliferative ability than the wild-type B cells when stimulated with the BCR inducer anti-IgM (Fig. 4A). The CYLD−/− B cells were also hyperresponsive to LPS (Fig. 4A), a key costimulatory molecule that mediates B cell activation by helper T cells (36).

To further confirm the hyperproliferative phenotype of CYLD−/− B cells, we analyzed the division rate of CYLD−/− and control B cells using the CFSE labeling technique (Fig. 4B). Consistent with the thymidine incorporation results, the CYLD−/− B cells displayed significantly higher proliferation ability than the control B cells when stimulated with anti-IgM (Fig. 4B, middle panel, dotted line). After 48 h of anti-IgM stimulation, the majority of the CYLD−/− B cells had undergone five or more cell cycles, whereas most of the wild-type B cells had four or fewer divisions. The CYLD−/− B cells displayed even more drastic hyperproliferative ability when stimulated with LPS (Fig. 4B, bottom panel). Thus, the CYLD deficiency causes hyperresponses of B cells to BCR and TLR stimulations.

Antigen Exposure Causes Exacerbated Lymphoid Organ Abnormalities and Elevated B Cell Responses in CYLD−/− Mice—To determine the effect of CYLD deficiency on in vivo immune responses, we immunized the CYLD−/− mice with SRBC, which are frequently used as a model antigen for analyzing B cell expansion and germinal center formation. Interestingly, upon SRBC immunization, the CYLD−/− mice, but not wild-type mice, developed splenomegaly (Fig. 5A). This abnormality was associated with prominent enlargement of B cell follicles in the white pulp of the spleen (Fig. 5B, B220), although the size of T cell zones was comparable between the CYLD−/− and wild-type mice (Fig. 5B, CD3). The mutant spleen also contained larger and increased numbers of germinal centers, indicating heightened expansion of B cells (Fig. 5B, PNA). Since the CYLD−/− B cells were hyperresponsive to LPS in vitro (Fig. 4), we also immunized the mice with the T-independent antigen NP-LPS. As seen with SRBC, NP-LPS immunization caused prominent splenomegaly in CYLD−/− mice (Fig. 5A). We next examined whether the CYLD−/− mice developed splenomegaly when they were exposed to natural pathogens. Age- and sex-matched mutant and wild-type mice were transferred to conventional housing conditions, which allowed exposure of the animals to common pathogens within the food and air. Indeed,
Regulation of B Cell Activation by CYLD

after 6 weeks of conventional housing, over 50% of the CYLD−/− mice, but none of the wild-type mice, developed severe splenomegaly (Fig. 5A). The MLNs of these mutant animals were also further enlarged when compared with the mice housed under specific pathogen-free conditions (data not shown). These results suggest that immune responses cause exacerbated lymphoid abnormalities in CYLD−/− mice and further emphasize a role for CYLD in controlling B cell activation and homeostasis.

Deregulated B cell activation can cause abnormal immune responses through different mechanisms, such as activation of T cells and aberrant production of antibodies (37). Analysis of serum Ig level in unimmunized mice did not reveal significant differences between the wild-type and CYLD−/− mice (Fig. 5C). We next examined how the loss of CYLD affected the antigen-specific antibody responses by immunizing the mice with NP-conjugated T-independent (LPS) and T-dependent (KLH) antigens. Consistent with the hyperactivation of CYLD−/− B cells by LPS in vitro, the CYLD−/− mice mounted elevated levels of anti-NP-LPS IgM responses than the wild-type mice, although this T-independent antigen did not induce significant responses of other antibody isotypes (Fig. 5D). In response to NP-KLH, the CYLD−/− mice produced markedly more IgG1 and IgG2a than the wild-type mice (Fig. 5B). Since the production of IgG1 and IgG2a is critically dependent on CD80 and CD86 (38), this result is in agreement with the hyperexpression of CD80 and CD86 on CYLD−/− B cells. The basal immunoglobulin level of CYLD−/− mice was similar to that of the wild-type mice. Taken together with the data presented in Fig. 3, these findings suggest that CYLD plays a critical role in negative regulation of B cell responses in vivo.

Loss of CYLD Results in Constitutive Activation of NF-κB in B Cells—To understand the molecular mechanism by which CYLD negatively regulates B cell responses, we examined the effect of CYLD deficiency on BCR signaling using purified splenic and lymph node B cells. We detected a moderate enhancement of extracellular signal-regulated kinase (ERK) MAPK activation in CYLD−/− B cells (data not shown). More prominently, the activation of NF-κB induced by anti-IgM and LPS was significantly enhanced in CYLD−/− B cells (Fig. 6A, compare lanes 2 and 3 with lanes 5 and 6). Notably, the CYLD−/− B cells also exhibited a markedly higher level of basal NF-κB activity than the wild-type B cells (Fig. 6A, compare lanes 1 and 4). This finding suggested that the loss of CYLD might promote constitutive activation of NF-κB. To assure that the hyperbasal activation of NF-κB in CYLD−/− B cells was not due to the in vitro incubation, we prepared nuclear extracts from the CYLD−/− and wild-type B cells immediately after purification and repeated the EMSA. Consistent with prior reports (39), the wild-type B cells isolated from both mesenteric lymph nodes and spleen exhibited constitutive NF-κB activity (Fig. 6B, lanes 1 and 3). Importantly, the constitutive NF-κB activity was markedly elevated in the CYLD−/− B cells (lanes 2 and 4). Thus, in agreement with their hyperproliferative phenotype, the CYLD−/− B cells had aberrant activation of NF-κB.

To further examine the mechanism of NF-κB constitutive activation in CYLD−/− B cells, we performed antibody supershift assays to analyze the composition of the active NF-κB complexes. The C2 NF-κB complex appeared to contain mostly p50 since it was completely shifted by the anti-p50 antibody but did not appreciably react with the other anti-NF-κB antibodies (Fig. 6C). On the other hand, C1 partially reacted with all NF-κB members, including p50, RelA, c-Rel, as well as the noncanonical NF-κB members, p52 and RelB. These immune reactions were specific since neither C1 nor C2 reacted with a preimmune serum (Fig. 6C, lane 1).

IB assays were then carried out to examine whether the loss of CYLD affected the expression level of NF-κB members. The CYLD−/− and wild-type B cells expressed comparable amounts of canonical NF-κB members (p50, RelA, c-Rel) (Fig. 6D). In contrast, the noncanonical NF-κB members, p100 and RelB, were significantly induced in the absence of CYLD (Fig. 6D). On the other hand, the CYLD deficiency did not enhance the processing of p100 since the level of p52 was only slightly increased.
in CYLD−/− B cells (Fig. 6D). The up-regulation of p100 and RelB was consistent with the constitutive activation of NF-κB since the genes encoding these noncanonical NF-κB members are under the regulation of NF-κB (40, 41). Thus, the loss of CYLD causes posttranslational activation of canonical NF-κB, which in turn appears to mediate hyperexpression of noncanonical NF-κB members.

CYLD Deficiency Promotes Chronic Phosphorylation and Degradation of IκBα in B Cells—Since the hyperactivation of NF-κB under unstimulated conditions is a major feature of CYLD−/− B cells, we investigated the underlying mechanism by examining the fate of the NF-κB inhibitor IκBα. The steady level of IκBα was significantly lower in CYLD−/− cells when compared with wild-type cells (Fig. 7A). However, this result was not due to the reduction in IκBα gene expression since the level of IκBα mRNA was even higher in the mutant B cells (Fig. 7B). The existence of a higher amount of IκBα mRNA in CYLD−/− B cells was consistent with the hyperactivation of NF-κB as the expression of IκBα gene is under the control of NF-κB (42). These findings suggested that IκBα might be undergoing chronic degradation and resynthesis in CYLD−/− B cells. Indeed, inhibition of protein synthesis by cycloheximide led to rapid loss of IκBα in CYLD−/− B cells but only a slight reduction in the wild-type B cells (Fig. 7C, top panel). We noticed that the IκBα from CYLD−/− B cells migrated as doublet bands on SDS gels when the cell lysates were prepared in the presence of phosphatase inhibitors (Fig. 7C, top panel, lane 4). We thus examined whether the loss of CYLD resulted in constitutive phosphorylation of IκBα. A weak basal level of IκBα phosphorylation was detected in wild-type B cells (Fig. 7C, middle panel, lane 1). Interestingly, however, a markedly higher level of phospho-IκBα was detected in CYLD−/− B cells (Fig. 7C, middle panel, lane 4) despite the lower level of total IκBα protein in these mutant cells (Fig. 7C, top panel, lane 4). A selective IKK inhibitor, PS1145, blocked the chronic phosphorylation of IκBα and prevented the loss of IκBα in cycloheximide-treated CYLD−/− B cells (Fig. 7C, top and middle panels, lane 6), suggesting the requirement of IKKβ-mediated IκBα phosphorylation in triggering the chronic degradation of IκBα. Indeed, parallel in vitro kinase assays revealed hyperactivation of IKKβ in CYLD−/− B cells (Fig. 7D). Thus, the CYLD deficiency promotes constitutive activation IKKβ and degradation of IκBα.

Loss of CYLD Bypasses the Requirement of BAFF for CD23 Induction—BAFF receptor provides a major signal in B cells that targets the activation of noncanonical NF-κB (43, 44). Engagement of BAFF receptor by its ligand BAFF stimulates both B cell survival and up-regulation of CD21 and CD23 (45). BAFF-induced B cell survival is mediated through nuclear exclusion of protein kinase C-δ (46), although how BAFF induces CD21/CD23 expression is not completely understood. Since the CYLD−/− B cells displayed hyperepression of CD21 and CD23 (Fig. 3) and aberrant activation of the canonical and noncanonical NF-κBs (Fig. 6), we examined whether the loss of CYLD bypasses the requirement of BAFF for CD21/CD23 expression. We focused on CD23 since it was more drastically
with activated phenotypes. These symptoms are exacerbated when the mice are exposed to antigens, suggesting the involvement of abnormal immune reactions in triggering the lymphoid abnormalities. Indeed, when stimulated in vitro, the CYLD−/− B cells are hyperproliferative and exhibit aberrant signaling properties, particularly the activation of NF-κB. Since the CYLD−/− B cells exhibit spontaneous NF-κB activation and express surface activation markers, CYLD appears to be required for maintaining the naive phenotype of B cells.

CYLD is known to positively regulate thymic TCR signaling and thymocyte development (5). Our present work suggests that like E3 ubiquitin ligases, the DUB CYLD may possess different cellular targets and mediate multiple functions in the immune system. In thymocytes, a major target of CYLD is the Src kinase Lck. By physically interacting with Lck and inhibiting Lck ubiquitination, CYLD facilitates the recruitment of active Lck to its downstream target ZAP-70 and thereby promotes TCR-proximal signaling. Although B cells have an Lck homologue, Lyn, we have not been able to demonstrate the binding of CYLD to Lyn under endogenous conditions (data not shown). Further, CYLD is not required for B cell development or BCR signaling. It is more likely that CYLD targets a different molecule involved in NF-κB activation in B cells. Although the precise mechanism by which CYLD regulates NF-κB in B cells requires further investigations, we have obtained evidence that the major NF-κB inhibitor, IκBα, undergoes chronic phosphorylation and degradation in CYLD−/− B cells. Further, the IκBα degradation appears to be mediated through its phosphorylation by IKKβ since a selective IKK inhibitor is able to block the degradation of IκBα and since IKKβ is constitutively activated in CYLD−/− B cells. Prior in vitro studies suggest that CYLD inhibits the ubiquitination of the IKK regulatory subunit, IKKγ, and negatively regulates NF-κB activation by innate immune stimuli (8–10), although this function of CYLD has not been confirmed using primary innate immune cells (5). The results of the current study raise the intriguing question of whether IKKγ serves as a target of CYLD in primary B cells. Unfortunately, we were unable to detect endogenous CYLD-IKKγ physical interaction in primary B cells (data not shown), and others failed to detect this molecular interaction in cell lines (10). We also did not detect hyperubiquitination of IKKγ in CYLD−/− B cells (data not shown). Whether these results are due to technical challenge with endogenous proteins or CYLD functions through novel targets remain to be further studied. Nevertheless, our data suggest that chronic phosphorylation of IκBα by IKKβ is a mechanism that mediates the aberrant activation of NF-κB caused by CYLD deficiency. Given the critical role of NF-κB in regulating lymphocyte activation (16), the constitutive NF-κB activity likely contributes to the activated phenotype of CYLD−/− B cells.

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