The tumor suppressor gene CYLD was identified in patients with familial cylindromatosis, which is an autosomal dominant predisposition to multiple neoplasms of the skin appendages. The tumors are believed to arise from the eccrine or apocrine cells of the skin (1). Approximately 70% of the cylindromas exhibit loss of heterozygosity on chromosome 16q containing the CYLD gene (2–6).

Sequence analysis of the CYLD gene predicts a protein with several functional domains, including three CAP-Gly domains and a deubiquitination domain at the C terminus (7–9). In vitro studies showed that CYLD contains binding sites for TRAF2 and NEMO (7), and that the deubiquitinating activity of CYLD is directed to lysine 63 (K63)–linked ubiquitin (Ub) (7). Linkage of Ub through K63 assembles a new molecular platform, allowing the recruitment of proteins involved in signal transduction (10–12), whereas Ub chains linked through K48 destined for proteasomal degradation (13, 14). Suppression of CYLD expression by RNA interference causes an increase in the activity of the transcription factor NF-κB, which is activated by TNFα signaling, thus inhibiting apoptosis (7, 8, 15). The increase of NF-κB activation in CYLD knockdown cells was attributed to defective removal of K63 Ub chains.

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from TRAF2 and NF-κB essential modulator (NEMO) by CYLD (7–9).

In a mechanistically analogous manner to its effect on TRAF2, CYLD was found to inhibit signaling from Toll-like receptor 2 (TLR2) by removing K63-linked Ub from TRAF6 and TRAF7 (16). Interestingly, it was shown that after TLR2 activation, CYLD inhibits the phosphorylation of TRAF6 and TRAF7, which, in turn, suppresses MKK3 and MKK6, leading to reduced MAP kinase p38 phosphorylation (17). In addition to p38 signaling, CYLD negatively regulates the stress-activating MAP kinase family, the JNK kinases. CYLD inactivation resulted in hyperactivation of JNK upon TNFα, IL-1β, LPS, and anti-CD40 treatment (17). This hyperactivation is believed to be a result of negatively regulating the activation of MKK7, which is the kinase responsible for JNK activation (17).

To investigate the physiological role of CYLD in vivo, we generated mouse strains with targeted modifications in the CYLD gene. In addition to a complete knockout of CYLD, exon 7 of the CYLD gene was flanked by loxP sites in a second mouse strain to result in conditional, excessive, and solitary expression of a shorter splice variant of the CYLD protein, hereafter termed sCYLD. The sCYLD protein is devoid of both TRAF2 and NEMO binding sites. In this article, we present data describing the profound alterations in the immune system of these mice (CYLD ex7/8), which is characterized by lymphomegaly and splenomegaly and a striking increase in B cell numbers. Overexpression of sCYLD further bestows B lymphocytes with increased IgG1 production and enhances survival capabilities of the B cell.

RESULTS

Generation of CYLDex7/8 mutant mice

CYLD mutant mice were generated applying standard gene-targeting techniques in mouse embryonic stem cells (Fig. S1, available at http://www.jem.org/cgi/content/full/jem.20070318/DC1). Using Cre/loxP technology, three different mouse strains were generated. The first strain, CYLDneo, contains the neo resistance gene upstream of exon 7, decreasing transcription of CYLD (Fig. S1). The second strain, CYLD FL, harbors two loxP sites in a second mouse strain to result in conditional, excessive, and solitary expression of a shorter splice variant of the CYLD protein, hereafter termed sCYLD. The sCYLD protein is devoid of both TRAF2 and NEMO binding sites. In this article, we present data describing the profound alterations in the immune system of these mice (CYLDex7/8), which is characterized by lymphomegaly and splenomegaly and a striking increase in B cell numbers. Overexpression of sCYLD further bestows B lymphocytes with increased IgG1 production and enhances survival capabilities of the B cell.
recombination through transient transfection of the targeted embryonic stem cells with a Cre-expressing plasmid (Fig. S1). CYLD<sup>ex7/8</sup> mice were born at the expected Mendelian frequencies and survived normally when housed under pathogen-free conditions.

Germline deletion of exon 7 should lead to splicing from exon 6 to 8 resulting in an out-of-frame translation of CYLD (Fig. 1 A). RT-PCR was applied to cDNA from mouse embryonic fibroblasts (MEFs) of the indicated genotypes using primers located in exon 6 and 9 of the CYLD transcript to verify the absence of the WT allele in CYLD<sup>ex7/8</sup> MEFs. This analysis revealed an unexpected shorter amplified product besides the expected band from the full-length transcript (FL-CYLD) in WT MEFs. This shorter product represents an alternative splice variant of CYLD lacking exons 7 and 8, termed sCYLD. CYLD<sup>ex7/8</sup> MEFs are devoid of the full-length transcript, but express the sCYLD splice variant excessively (Fig. 1 B). Using CYLD-specific antibodies, sCYLD protein could be detected in different tissues and cells of WT animals (Fig. 1 C). To investigate whether deletion of WT CYLD (FL-CYLD) in CYLD<sup>ex7/8</sup> mice results in higher expression levels of sCYLD, protein extracts were prepared from purified B cells of WT and CYLD<sup>ex7/8</sup> mice, revealing high expression of sCYLD in CYLD<sup>ex7/8</sup> cells compared with WT cells (Fig. 1 D).

Previously, CYLD was identified as a deubiquitinating enzyme, removing K63-conjugated Ub molecules from TRAF2, TRAF6, and NEMO (7–9). However, the putative TRAF2 and NEMO binding sites are absent in the sCYLD protein (Fig. 1 E). To verify whether sCYLD is, indeed, unable to bind TRAF2, HeLa cells were transfected with expression plasmids encoding either FL-CYLD or sCYLD together with TRAF2-encoding or control plasmids. As shown in Fig. 1 F, FL-CYLD protein, but not sCYLD, coimmunoprecipitated with TRAF2, indicating that in contrast to FL-CYLD, sCYLD does not bind TRAF2. However, in an 8% SDS-PAGE, the difference in size between FL-CYLD and sCYLD could not be unambiguously demonstrated (Fig. 1 F). In agreement, immunoprecipitated TRAF2 from extracts of CYLD<sup>ex7/8</sup> MEFs was highly ubiquitinated compared with TRAF2 immunoprecipitated from WT cells (Fig. 1 G).

To investigate whether sCYLD is still able to function as a deubiquitinating enzyme, similar transfection experiments were applied to determine whether both CYLD proteins are capable of removing K63-conjugated Ub from Bcl-3, which is a novel substrate for CYLD (18). The Bcl-3 binding site in CYLD is predicted to reside outside the area absent in sCYLD (Fig. 1 E). We cotransfected HeLa cells with expression vectors encoding His-tagged Ub and Flag-tagged Bcl-3, together with plasmids coding either for FL-CYLD, or for a catalytically inactive form of CYLD (C/S-CYLD) or for sCYLD. Fig. 1 H shows that both sCYLD and FL-CYLD are able to remove Ub from Bcl-3. Collectively, our results demonstrate that sCYLD is not able to deubiquitinate TRAF2, but still removes K63-linked Ub from Bcl-3.

Lymphoid system defects in CYLD<sup>ex7/8</sup> mice

CYLD<sup>ex7/8</sup> mice exhibited considerably larger spleens, LNs, and Peyer’s patches (Fig. 2 A and not depicted). Careful microscopic examination revealed a disorganized structure of the spleen, where the distinct B cell and T cell zones of the follicle were not uniform as in WT spleens (Fig. 2 B). Importantly, such abnormalities of the secondary immune organs were not reported in mice with complete inactivation of the CYLD gene (18) (unpublished data).

The CYLD<sup>ex7/8</sup> mice displayed massively enlarged secondary lymphoid organs as early as 4 wk of age, whereas other tissues appeared normal (unpublished data). To investigate the mechanisms underlying these enlarged lymphoid organs, we examined lymphocyte populations of BM, spleen, LNs, and peritoneal cavity (PC). Analysis of B220<sup>+</sup> cells in the BM of CYLD<sup>ex7/8</sup> mutant mice shows a small decrease in the percentage of the immature B lymphocyte compartments (Fig. 3 A, top). To analyze early B cell development, B220<sup>+</sup>IgM<sup>−</sup> cells were analyzed for the expression of the pro-B cell marker, c-Kit. We observed an increase in the pro-B cell fraction, indicating that deletion of the FL-CYLD leads to a partial block in early B cell differentiation (Fig. 3 A, bottom).

Strikingly, CYLD<sup>ex7/8</sup> mice, but not heterozygous and control mice, demonstrate a dramatic accumulation of B cells in all secondary lymphoid organs tested (Fig. 3 B and Table I; not depicted for Peyer’s patches). The proportion and absolute number of immature B cells was reduced in CYLD<sup>ex7/8</sup> mice (Fig. 3 C and Table I), although they displayed significantly higher total B cell numbers (Table I).

As seen in Fig. 3 D and Table I, the proportion, as well as the absolute number, of marginal zone (MZ; CD21<sup>hi</sup>CD23<sup>lo</sup>) B cells were analyzed for the expression of the pro-B cell marker, c-Kit. We observed an increase in the pro-B cell fraction, indicating that deletion of the FL-CYLD leads to a partial block in early B cell differentiation (Fig. 3 A, bottom).

Figure 2. Enlarged spleen and Peyer’s patches (PP) in CYLD<sup>ex7/8</sup> mice. (A) Spleens and PP dissected from WT and CYLD<sup>ex7/8</sup> mice were compared. Ruler indicates the size of the organs (centimeters). (B) Cryostat sections from WT and CYLD<sup>ex7/8</sup> spleens were immunostained for B and T cell follicles with anti-B220 (blue) and anti-CD3e (brown). Bars, 500 μm.
and follicular (CD21+CD23−) B cells were increased by two- to threefold in CYLDex7/8 mice compared with WT mice. Interestingly, expression of CD21 and CD23 by B cells isolated from CYLDex7/8 mice was elevated compared with control B cells (Fig. 3 D). The latter was accompanied by an increase in cell size of the CYLD mutant B cells (unpublished data). To analyze the structure of MZ B cells, spleen sections from CYLDex7/8 and littermate control mice were stained with anti-MOMA1 antibody, which binds to metallophilic macrophages, marking the border of the MZ. As shown in Fig. 3 E, MOMA1+ cells can be detected in the spleens of CYLDex7/8; however, the MZ appeared to be disordered and discontinuous compared with WT spleens. Furthermore, the amount of MOMA1+ cells was increased without forming a closed ring around the follicle. Notably, the B cell region located outside of the MOMA1+ cells corresponding to the MZ B cells was larger in the mutant spleens compared with controls (Fig. 3 E). Interestingly, the expansion of MZ and follicular B cells was accompanied by a decrease in the proportion and absolute number of B-1a (CD19highCD5−CD23−) and B-1b (CD19highCD5−CD23−) B cells in the PC (Fig. 3 F and Table I). These results suggest that either overexpression of sCYLD or disruption of FL-CYLD is critical for regulating the maturation of BM B cells and the distribution of peripheral B cells.

To address whether these differences in the B cell compartment resulted from the overexpression of sCYLD, we analyzed another CYLD mutant strain, CYLDex8, in which the neomycin resistance gene was introduced into the first coding exon of the CYLD gene, thus preventing its expression (18).
Table I. Lymphocyte population sizes

<table>
<thead>
<tr>
<th>Spleen</th>
<th>B cells</th>
<th>Immature</th>
<th>MZ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genotype</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT (14)</td>
<td>46.7 (4.8)</td>
<td>7.7 (0.8)</td>
<td>2.4 (0.3)</td>
</tr>
<tr>
<td>CYLD&lt;sup&gt;ex7/8&lt;/sup&gt; (9)</td>
<td>49.3 (6.6)</td>
<td>6.2 (0.9)</td>
<td>2.9 (0.4)</td>
</tr>
<tr>
<td>CYLD&lt;sup&gt;ko&lt;/sup&gt; (14)</td>
<td>111.1 (17.3)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.8 (0.7)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>10.3 (1.6)&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>CYLD&lt;sup&gt;ko&lt;/sup&gt; (5)</td>
<td>11.1 (10)</td>
<td>10.1 (0.9)&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>CD19-Cre/CYLD&lt;sup&gt;FL/FL&lt;/sup&gt; (6)</td>
<td>4.2 (4.1)</td>
<td>4.8 (0.7)</td>
<td></td>
</tr>
<tr>
<td>Genotype</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT (4)</td>
<td>1.4 (0.3)</td>
<td>0.9 (0.2)</td>
<td></td>
</tr>
<tr>
<td>CYLD&lt;sup&gt;ex7/8&lt;/sup&gt; (4)</td>
<td>1.6 (0.1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CYLD&lt;sup&gt;ko&lt;/sup&gt; (4)</td>
<td>2.1 (0.2)&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
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<tr>
<td>CYLD&lt;sup&gt;ko&lt;/sup&gt; (5)</td>
<td>0.3 (0.1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD19-Cre/CYLD&lt;sup&gt;FL/FL&lt;/sup&gt; (5)</td>
<td>0.8 (0.2)</td>
<td></td>
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</tbody>
</table>

The mean size of lymphocyte populations was calculated based on their frequency as determined by flow cytometry (Fig. 2). Total numbers (× 10<sup>6</sup>) are indicated (the SEM is shown in parentheses). Data are the mean of the number of mice indicated in parentheses after the mice genotypes. Values are the mean ± the SEM. 

<sup>a</sup>P ≤ 0.001 versus control.

Fig. S2 A (available at http://www.jem.org/cgi/content/full/jem.20070318/DC1) shows that the B cell development in the BM was unchanged in CYLD<sup>ko</sup> mice. Furthermore, in contrast to CYLD<sup>ex7/8</sup> mice, CYLD<sup>ko</sup> mice showed neither differences in B/T cell ratio nor an elevation of MZ and follicular B cells in the spleen nor a decrease of B-1 B cells in the PC (Fig. S2, B–D). Moreover, B cell numbers in these mice were comparable to WT B cell numbers (Table I). Thus, we conclude that the absence of the FL-CYLD, in tandem with the overexpression of sCYLD, is responsible for the increased proportion of B cells in the lymphoid organs of CYLD<sup>ex7/8</sup> mice.

Skewed humoral immune response of CYLD<sup>ex7/8</sup> mice

To determine whether the expansion of B cells in CYLD<sup>ex7/8</sup> mice affects the immune response, we evaluated serum antibody levels in CYLD<sup>ex7/8</sup> and WT mice. The sera of CYLD<sup>ex7/8</sup> mice contained significantly lower IgM and IgG3 antibody titers compared with WT littersmates, whereas all other antibody classes, including IgG1, IgG2a, IgG2b, and IgA, were elevated in CYLD<sup>ex7/8</sup> mice (Fig. 4 A). The latter antibody isotypes are more frequently secreted by class-switched B cells after encountering T cell–dependent (TD) antigens. Therefore, we next investigated TD antigen responses of CYLD<sup>ex7/8</sup> B cells and control B cells. To this end, mice were challenged with nitrophenol-conjugated chicken γ-globulin (NP-CG) and immune responses were measured by ELISA. Upon in vivo challenge with NP-CG, CYLD<sup>ex7/8</sup> mice developed significantly lower antibody titers of both IgM and IgG3 isotypes, whereas secretion of NP-specific IgG1 and IgG2a antibodies was enhanced compared with WT mice (Fig. 4 B). In addition, CYLD<sup>ex7/8</sup> mice developed higher titers of NP-specific IgA antibodies, whereas the NP-specific IgE antibody levels were similar to the levels in WT mice (Fig. 4 B, top). Previously, it was shown that WT mice harbor a relatively high number of NP-specific precursor cells (∼1%) (19). To test whether these findings hold true for another TD antigen with lower precursor frequency, we immunized mice with KLH. CYLD<sup>ex7/8</sup> mice developed significantly higher levels of KLH-specific IgG1 antibodies after immunization compared with WT mice (Fig. S3 A, available at http://www.jem.org/cgi/content/full/jem.20070318/DC1). CYLD<sup>ex7/8</sup> mice and littermate controls were also immunized with the T cell–independent (TI) antigen NP-Ficoll, resulting in similar levels of NP-specific IgM and IgG3 antibodies in CYLD<sup>ex7/8</sup> and WT mice (Fig. 4 C).

To examine whether the high IgG1 antibody levels observed in nonimmunized mice, as well as in the immunized mutant mice, were a consequence of increased antibody production by plasma cells or whether CYLD<sup>ex7/8</sup> B cells switch more efficiently to other antibody classes, B cells from WT and CYLD<sup>ex7/8</sup> mice were isolated and, subsequently, incubated for 5 d ex vivo with optimal concentrations of LPS and IL-4. A higher proportion of CYLD mutant B cells switched to the IgG1<sup>+</sup> isotype compared with WT B cells (Fig. 4 D). Overall, these results were strengthened by an increased proportion of PNA<sup>+</sup>Fas<sup>+</sup> B cells in CYLD<sup>ex7/8</sup> Peyer’s patches, which is indicative of germinal center B cells (Fig. 4 E). The hyper TD antigen-specific response observed in CYLD<sup>ex7/8</sup> mice suggests that sCYLD is important for negatively regulating TD, but not TI, responses.

The B cell phenotype in CYLD<sup>ex7/8</sup> mice is B cell intrinsic

As all cells in CYLD<sup>ex7/8</sup> mice lacked exon 7, it was unclear whether the defect in B cells was intrinsic to B cells or secondary
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because of defects in other cell types. Therefore, we crossed CYLD<sup>FL/FL</sup> mice to CD19-Cre mice (20, 21), leading to Cre-mediated excision of exon 7 of CYLD solely in B cells. The resulting mice, CD19-Cre/ CYLD<sup>FL/FL</sup> exhibited enlarged secondary immune organs, including spleen, LNs, and Peyer’s patches similar to the CYLD<sup>ex7/8</sup> mice that harbor the mutation in the germline (unpublished data). To analyze B cell development in these mice, BM cells were stained for B220 and IgM. As seen in Fig. 5 A (top), BM cells of CD19-Cre/ CYLD<sup>FL/FL</sup> mice show a small decrease in the percentage of immature cells, similar to BM of CYLD<sup>ex7/8</sup> mice. B220<sup>-</sup>IgM<sup>-</sup> cells were gated and analyzed for the expression of the pro-B cell marker, c-Kit. As for the CYLD<sup>ex7/8</sup> mice, an accumulation of c-Kit<sup>+</sup>–positive cells in the BM of CD19-Cre/ CYLD<sup>FL/FL</sup> was detected (Fig. 5 A, bottom). Importantly, also in CD19-Cre/ CYLD<sup>FL/FL</sup> mice, the total number of B cells was increased by two- to threefold (Fig. 5 B and Table I) compared with WT mice. All other characteristics of the B lymphocytes detected in CYLD<sup>ex7/8</sup> mice were also manifested in CD19-Cre/ CYLD<sup>FL/FL</sup> mice, including a reduction in the percentage of transitional B cells, a reduction of immature B cells (Fig. 5 C), and an increase in the total number of MZ B cells (Fig. 5 D and Table I). Finally, in both CD19-Cre/ CYLD<sup>FL/FL</sup> and CYLD<sup>ex7/8</sup> mice, the ratio of B-1 to B-2 B cells in the PC was inverted (Fig. 5 E). Together, these data indicate that the B cell defects seen in the CYLD<sup>ex7/8</sup> mice are cell autonomous.

**Overexpression of sCYLD results in prolonged B cell survival**

Bcl-2 transgenic mice exhibit enhanced B cell survival in vitro; this is associated with an increased size of secondary immune organs and B cell compartments in vivo (22), which is similar to CYLD<sup>ex7/8</sup> mice. To examine the survival capacities, B cells from CYLD<sup>ex7/8</sup> and WT mice were cultured in enriched medium and monitored daily for survival by cell counting and FACS analysis. The CYLD mutant B cells exhibited prolonged survival compared with WT B cells (Fig. 6 A, top).
assessed by quantitative real-time PCR. CYLD ex7/8 B cells express fivefold more Bcl-2 mRNA compared with WT B cells (Fig. 6 D). These results were further verified at protein level by intracellular staining with Bcl-2–specific antibody (Fig. 6 D). In addition, we found that primary fibroblasts isolated from CYLD ex7/8 mutant mice are more resistant to TNFα/H9251–induced apoptosis in the absence of protein synthesis (Fig. 6 E). Therefore, it is tempting to speculate that the deletion of the FL-CYLD, concomitant with the overexpression of sCYLD, conveys increased resistance to apoptosis, leading to the enlarged B cell compartment in CYLD ex7/8 mice.

Next, we determined the responses of CYLD ex7/8 and WT B cells to activating stimuli by incubating CFSE-labeled B cell cultures with optimal concentrations of anti-IgM F(ab’)2 and anti-CD40 antibodies, or with anti-RP105, LPS, or BAFF. As shown in Fig. 6 F, B cells from CYLD ex7/8 mice responded similarly to the different stimuli compared with WT B cells. Although the addition of BAFF to culture medium resulted in a more activated status of the mutant B cells compared with the WT B cells (Fig. 6 D), supplementing the culture with BAFF together with LPS failed to increase proliferation (Fig. 6 F). Therefore, we conclude that sCYLD contributes to the survival capacity of B cells, but does not affect B cell proliferation.

Elevated expression of NF-κB and TRAF proteins in resting CYLD ex7/8 B cells

Previously, CYLD was shown to act on different proteins involved in the NF-κB (7–9) and mitogen-activated protein kinase (MAPK) signaling pathways (17, 24). To study whether the enlargement of the B cell compartment in CYLD ex7/8
its processed form, p52, were only slightly increased (Fig. 7 A). Furthermore, we could show that the RelB protein levels were also highly increased in the cytoplasm, as well as the nucleus, of CYLD ex7/8 B cells (Fig. 7 B). The elevated protein levels of TRAF2, TRAF3, p100, RelB, and IκB/κH9260 in CYLD ex7/8 B cells likely resulted from increased protein stability rather than from increased transcriptional activity, as quantitative real-time PCR revealed no significant differences in relative expression of these genes (Fig. S5, available at http://www.jem.org/cgi/content/full/jem.20070318/DC1).

To investigate whether the noncanonical NF-κB pathway is affected in CYLD ex7/8 B cells, purified B lymphocytes of control and CYLD ex7/8 mutant mice were cultivated with optimal concentrations of BAFF for the indicated time points (Fig. 7 C). Mice results from alterations in the expression levels of proteins involved in the NF-κB signaling pathway, whole-cell lysates of purified B cells from three CYLD ex7/8 mutant mice and three control mice were subjected to Western blot analysis. This analysis revealed increased protein levels of TRAF2 in the CYLD mutant B cells compared with WT B cells (Fig. 7 A). Additionally, the protein levels of TRAF3 and NEMO were determined, showing that TRAF3 levels were increased, whereas NEMO levels were unchanged (Fig. 7 A). The levels of 1κBα protein were markedly elevated in CYLD ex7/8 B cells, although its phosphorylation on conserved serine residues was unaltered. Most interestingly, NfkB2/p100 protein levels primarily involved in the noncanonical NF-κB pathway were dramatically increased, whereas the levels of
At all time points analyzed, we found increased p100 levels, but no significant changes in the levels of its processed form, p52, in CYLD<sup>Δ7/8</sup> B cells when compared with controls (Fig. 7 C).

To measure canonical NF-κB activity in CYLD<sup>Δ7/8</sup> B cells, splenic B cells were stimulated with anti-CD40, LPS, and anti-BCR (Fig. 7 D). We did not observe significant differences in IkBα degradation upon triggering of CYLD<sup>Δ7/8</sup> and WT B cells with the aforementioned stimuli (Fig. 7 D). Similarly, we did not detect changes in IkBα degradation and phosphorylation after anti-BCR treatment (Fig. 7 D). As a direct measure of NF-κB activity, we performed electrophoretic mobility shift assay (EMSA) using NF-κB consensus-binding oligonucleotides with nuclear extracts from resting B cells stimulated with optimal concentrations of anti-BCR (Fig. 7 E). Nonstimulated CYLD<sup>Δ7/8</sup> B cells exhibited detectable NF-κB activation, which was not observed in WT B cells (Fig. 7 E). However, the NF-κB–activating response to anti-IgM was unaltered at the different time points of stimulation in CYLD<sup>Δ7/8</sup> B cells compared with WT B cells. This result suggests that the basal activity of NF-κB may reflect an increased response to a yet unknown endogenous activator.

To estimate the effect of the sCYLD in B cells on MAPK signaling, we performed cytometric bead assay (CBA) to determine quantitatively phosphorylated MAPKs, which can be visualized using FACS analysis. In this assay, LPS stimulation resulted in increased phosphorylation of MAPK p38 in CYLD<sup>Δ7/8</sup> B cells compared with WT B cells (Fig. 7 F). Similar results were observed by Western blotting of anti-BCR–stimulated B cells (Fig. 7 G). In contrast, we observed...
reduced phosphorylation of ERK1 and ERK2 upon LPS stimulation (Fig. 7 F). This could be further confirmed with Western blot analysis (Fig. 7 G).

In keratinocytes isolated from CYLDko mice, Bcl-3 associates with the NF-κB proteins p52 and p50 and promotes increased transactivation of cyclin D1 compared with WT cells (18). Although we have shown that sCYLD is able to interact with Bcl-3 and to remove K63-conjugated Ub, we were interested to see whether Bcl-3 is present in the nuclei of B cells from CYLDex7/8 mice without activation. To this end, we prepared cytosins of CYLDex7/8, CYLDex7/8wt, CYLDko, and WT B cells. Fig. 8 (A and B) demonstrates that Bcl-3 was predominantly located in the nucleus, or in close proximity to the nuclei, of untreated CYLDex7/8 B cells, whereas in WT B cells, Bcl-3 was primarily found in the cytoplasm. Calculating the percentage of B cells of the various genotypes containing nuclear Bcl-3 revealed that 60% of the CYLDex7/8 B cells contain nuclear Bcl-3, whereas B cells from WT, CYLDex7/8wt, and CYLDko exhibit 8, 3, and 2.5% of Bcl-3 in the nucleus, respectively (Fig. 8 C). Furthermore, we could corroborate these results using Western blot analysis of extracts from CYLDex7/8 mice (Fig. 8 D). By quantitative real-time PCR, we could show that the increased protein levels of Bcl-3 in the B cells of the CYLDex7/8 mice are not a result of higher Bcl-3 gene expression (Fig. S5). Interestingly, mice that are deficient for CYLD do not show spontaneous translocation of Bcl-3 to the nucleus. These results are reminiscent of the previously published Bcl-3 transgenic mice (25), in which overexpression of Bcl-3 in B cells resulted in lymphadenopathy, splenomegaly, and altered immunoglobulin production, similar to what was observed in CYLDex7/8 mice.

DISCUSSION

In this article, we report the function of a splice variant of CYLD devoid of the TRAF2 and NEMO binding sites. Expression of this shorter form of CYLD (sCYLD) with a simultaneous deficiency of the full-length (FL) protein gave rise to a dramatic accumulation of mature B lymphocytes in all secondary lymphoid organs in vivo. This B cell accumulation is a consequence of increased survival, rather than increased proliferation, putatively resulting from aggravated Bcl-2 expression caused by various alterations in signal transduction pathways, such as NF-κB and MAPK signaling. In contrast, mice that are entirely devoid of CYLD (CYLDko mice) do not show any abnormalities in the B cell compartment. Therefore, we reason that sCYLD has other functions in B cell homeostasis than FL-CYLD. Under physiological conditions FL-CYLD expression might be diminished, whereas the expression of sCYLD is elevated. Indeed, we detected various expression levels of both CYLD forms in different tissues and cell types. Therefore, we suggest that also

Figure 8. Bcl-3 accumulation in CYLDex7/8 B cells. (A) Cytospins were prepared from B cells isolated from CYLDex7/8, CYLDex7/8wt, CYLDko, and WT mice and immunostained with anti–Bcl-3 and counterstained with Hoechst 33258. Shown are unstimulated B cells (top) and B cells treated with anti–BCR (bottom). Bar, 5 μm. (B) Same as in A, but showing more cells. Bar, 5 μm. (C) Quantification of nuclear Bcl-3 from unstimulated B cell cytosins from the indicated genotypes. For each individual genotype, 400 cells were counted and quantified for nuclear Bcl-3 localization. The frequency of nuclear Bcl-3 localization in CYLDex7/8 B cells was statistically significant (P < 0.001) compared with B cells isolated from WT, CYLDex7/8wt, and CYLDko mice. (D) Lysates of MACS-purified B cells from the indicated genotypes subjected to Western blot analysis using Bcl-3 antibody. Duplicates for each mouse strain are shown. Actin was used as a loading control.
under normal conditions, the unique function of sCYLD can be manifested.

The CYLD-deficient mice generated by Reiley et al. (26) demonstrate an elevated number of B lymphocyte, which is explained by a compensatory effect caused by the reduction in the T-lymphocyte number (26). In CYLD<sup>ex7/8</sup> mutant mice, the generalized accumulation of mature B cells seems to be B cell intrinsic because CD19-Cre/CYLD<sup>Ft/Lt</sup> mice, in which only the B cells express sCYLD and concomitantly lack the FL-CYLD protein, show a dramatically enlarged B cell compartment. We further observed that CYLD<sup>ex7/8</sup> mice have an increased number of Igκ-positive B cells compared with WT mice. This might be caused by the partial block in B cell development, which is demonstrated by an increase in the number of c-Kit–positive B cells in the BM. A block during B cell development may allow the developing B cells to edit their light chain locus, as seen in the process of B cell editing (27), which, indeed, results in higher numbers of λ-positive B cells.

We observed increased protein levels of several molecules involved in the NF-κB signaling pathway in CYLD<sup>ex7/8</sup> B cells, including IkBα. However, the degradation and phosphorylation of IkBα is as efficient in the CYLD<sup>ex7/8</sup> B cells as in the WT B cells upon stimulation, showing that the canonical NF-κB activation is unaltered. Consistently, the relative expression of IkBα, p100, and RelB, which are all under the regulation of the NF-κB pathway, was unaltered as determined by quantitative real-time PCR, thus pointing to increased protein stability resulting from differences in post-translational modifications. Nevertheless, EMSA revealed elevated binding of nuclear transcription factors to the NF-κB consensus probe in unstimulated B cells.

We could demonstrate elevated protein levels of TRAF2 and TRAF3 in CYLD<sup>ex7/8</sup> B cells, which is a consequence of impaired posttranslational modification because relative expression revealed by real-time PCR was unaltered. Consistently, TRAF2 was highly ubiquitinated in CYLD<sup>ex7/8</sup> MEFs, presumably by K63-linked Ub chains, as shown by Western blot analysis. Therefore, it is tempting to speculate that K63-ubiquitinated TRAF2 exhibit increased protein stability and possibly have impaired functionality. This is supported by the aggravated TRAF3 protein levels in CYLD<sup>ex7/8</sup> B cells because TRAF2 was shown to be a negative regulator of TRAF3 upon stimulation with CD40 (28). B lymphocytes deficient for TRAF2 demonstrate a phenotype similar to that of CYLD<sup>ex7/8</sup> B cells with increased TRAF3 protein levels, but also show constitutive processing of p100 to p52, which is contrary to CYLD<sup>ex7/8</sup> B cells (29). In contrast, however, TRAF3-deficient cells also display constitutive processing of p100 to p52, resulting in embryonic lethality. This phenotype can be rescued in vivo by simultaneous deficiency of p100 (30). Therefore, elevated TRAF3 levels should lead to increased p100 levels. Consistently, CYLD<sup>ex7/8</sup> B cells exhibit increased p100 protein levels, but not elevated processing to p52. In addition, elevated TRAF2 and TRAF3 levels might promote MAPK p38-mediated isotype switching to IgG1, as essential functions for these adaptor molecules in CD40-induced class switch recombination and Ig production were reported (31, 32). Indeed, CYLD<sup>ex7/8</sup> B cells switch more efficiently to IgG1, but whether this is a result of the p38 hyperactivation observed in CYLD<sup>ex7/8</sup> B cells or a result of impaired ERK activation still needs further investigation.

Furthermore, CYLD<sup>ex7/8</sup> B cells exhibit a significant accumulation of Bcl-3 in the nucleus that cannot be explained by an inability of sCYLD to associate with Bcl-3, as we could show that sCYLD binds and removes K63-linked ubiquitin chains from Bcl-3. Western blot analysis of B lymphocytes using Bcl-3 antibody revealed aggravated Bcl-3 protein levels, whereas on the RNA level, expression of Bcl-3 was unaltered in CYLD<sup>ex7/8</sup> B cells. Ultimately, CYLD<sup>ex7/8</sup> mice show a very similar phenotype to mice overexpressing Bcl-3 in B lymphocytes (25). Both transgenic mice show lymphadenopathy and splenomegaly and exhibit distorted follicles in the spleen, resulting from an enlarged B cell compartment. Further, both mouse strains showed an increase in IgG1 and decreased levels of IgM and IgG3 in the sera compared with WT mice. In addition, enhanced B cell survival, accumulation of MZ B cells, as well as increased B cell size, and the increased expression of CD21/CD23 observed in CYLD<sup>ex7/8</sup> mice are also found in Bcl-3 transgenic mice.

The elevated expression of Bcl-2 in CYLD<sup>ex7/8</sup> B cells may be a consequence of the dramatic overexpression of the NF-κB proteins p100 and RelB, which might activate transcription of Bcl-2 in a complex with Bcl-3 upon signaling through the noncanonical NF-κB pathway (33). Thus, nuclear Bcl-3 in combination with p100 can induce the expression of Bcl-2 (33) and might account for the elevated B cell survival observed in the CYLD<sup>ex7/8</sup> mice, as shown previously for Bcl-2 transgenic mice (22, 34). Concomitantly, CYLD<sup>ex7/8</sup> B cells are less dependent on BAFF signaling by a yet unsolved mechanism. This was demonstrated by ineffective BAFF blocking by TACI-Ig. Collectively, our data suggest that the newly identified splice variant sCYLD regulates B cell survival mediated through a variety of signaling disarrangements.

**MATERIALS AND METHODS**

**Generation of CYLD mutant mice.** The generation of CYLD mutant mice is described in the Supplemental materials and methods. All animal experiments were in accordance with the guidelines of the Central Animal Facility Institute of the University of Mainz.

**RNA analysis.** For RNA analysis, total RNA was isolated using Trizol reagent (Invitrogen) according to the manufacturer's instruction. The following primers were used for RT-PCR amplification of mouse CYLD: P1, 5′-CTTGGCCCGCTGAGTGA-3′; P2, 5′-TTGAAATGCGCT-3′. Quantitative real-time PCR of Bcl-2, p100, RelB, IkBα, TRAF2, TRAF3, and Bcl-3 was performed using primers from Qiagen, as described on their homepage (https://www1.qiagen.com/GeneGlobe/Default.aspx).

**Immunostaining of purified B cells.** Cytospins of purified B cells were fixed in acetone for 10 min at −20°C and stained for Bcl-3 according to standard methods, using polyclonal rabbit IgG Bcl-3 antibody. All incubation steps of the staining procedure were performed at room temperature.
The cytospins were counterstained with Hoechst 33258. Cytospins were analyzed by confocal microscopy.

**Histological analysis.** For histological staining of B and T cells and germinal centers, frozen 6-μm spleen sections were thawed, air dried, fixed in acetone, and stained for 1 h at room temperature in a humidified chamber with biotinylated rat anti-CD19 (BD Biosciences), rat anti-B220, anti-CD3e, and anti-MOMA-1, respectively (eBioscience), followed by horseradish peroxidase–conjugated secondary antibody and alkaline phosphatase–conjugated streptavidin.

**Flow cytometry.** Single-cell suspensions were prepared from the different organs. Red blood cells of LN and spleen were lysed in cell suspension with tris-ammonium chloride, pH 7.2. Cells were incubated with combinations of antibodies to cell surface determinants, conjugated to PE, FITC, Cy-Cyochrome, or biotin. Antibodies specific to the following surface markers were purchased from BD Biosciences: CD35, CD19, CD21 (766), CD23, HSA (CD24), CD25, IgD0,2, CD43, and IgD. Anti-CD45R (B220; clone RA3-6B2) and anti-IgM (R33-24-12) were prepared in our laboratory. Biotinylated cells were visualized with streptavidin conjugated to PE or Cy-Cyochrome (BD Biosciences). Bcl-2 was visualized using anti-Bcl-2 antibody (Santa Cruz Biotechnology) and secondary donkey anti-rabbit sera conjugated to FITC (Jackson ImmunoResearch Laboratories). All numbers were acquired on a FACS Calibur (BD Biosciences), and results were analyzed with CellQuest software (BD Biosciences). Absolute numbers of splenocyte subpopulations were calculated based on their percentage and the total number of splenocytes.

**Immunostaining and transfection.** The coding regions of murine Bcl-3, TRAF2, FL-CYLD, or CYLD were cloned after RT-PCR using specific primers from the total RNA of activated B cells. PCR products were cloned in pcDNA3.1 vector (Invitrogen). HeLa cells and MEFs were transfected with the different constructs or histidine-Ub (His-Ub), as described in Fig. 1, and expressed using LipofectAmine 2000 (Invitrogen). For anti-Bcl-3 or anti-TRAF2 immunoprecipitation, lysates were precleaned for 30 min at 4 ° C. The protein content was determined and compensated for equal content in all supernatants. For testing protein input, a small part of the resulting lysates was gel separated and immunoblotted (protein input), and the remaining part was used for immunoprecipitation. Immunoblots were developed with the ECL Plus reagent (GE Healthcare) according to the manufacturer’s guidelines.

**Immunization.** 8-wk-old WT and CYLD<sup>+/−</sup> mice were immunized intraperitoneally with 20 μg of NP28-CCG or 100 μg of KLH for TD response, or 20 μg of NP-Ficol for TI responses. On days 0, 7, 14, 21, and 28, serum was collected from peripheral blood. Circulating antibodies were measured by isotype- and antigen-specific ELISA. Captured antibodies were detected with enzyme-conjugated secondary antibodies.

**TACI-Ig treatment.** CYLD<sup>+/−</sup> and control mice were treated via i.p. injection with 20 μg of TACI-Ig (Alexis Biochemicals) or human IgG (Hu-Ig) twice a week for a total of 1 wk.

**B cell preparation and B cell proliferation assay.** Total mouse splenic B cells were purified through negative depletion with CD134 beads (Miltenyi Biotech). The purity (>95%) was determined by flow cytometry. For in vitro proliferation studies, B cells were labeled with CFSE, and 2 x 10<sup>5</sup> cells/well were incubated in RPMI medium plus 10% FCS or untreated or treated with 5 μg/ml anti-CD40, 5 μg/ml anti-IgM, 2 μg/ml LPS, and 5 ng/ml LPS plus IL-4 with and without 100 ng/ml BAF4 for 4 d.

**Measurement of TNFα-induced cell death.** MEFs (10<sup>4</sup> per well) were plated onto 6-well plates and cultured for 24 h in DME containing 10% FCS. Subsequently, the cells were stimulated with either 10 ng/ml TNFα with and without 300 ng/ml cycloheximide. Triplicate samples of TNFα-induced cell death were measured by FACS analysis using Topro3 and Annexin V.

**Western blot.** To prepare whole-cell lysates, B cells were lysed in 1% Triton X–100 with protease inhibitors. MACS-purified B cells were prepared. Protein lysates (30 μg) were separated by 10% SDS-PAGE and subsequently transferred to PVDF membranes. Protein blots were probed with antibodies to phospho-IkBα, IκBα, phospho-ERK, ERK, phospho-p38, p38, p100, and Bcl-2 (all from Cell Signaling Technology), Bcl-3, NEMO, TRAF2, TRAF3, and RelB (from Santa Cruz Biotechnology), and with antibodies to actin (Sigma-Aldrich), AKT, or H2B (Cell Signaling Technology) as internal loading controls. CYLD-specific antibodies were prepared by immunization of rabbits with recombinant CYLD fragments. Nuclear extracts were prepared using high-salt buffer, and proteins were subjected to Western blotting. CBA was performed according to the manufacturer’s guidelines.

**Statistical analysis.** Values are presented as the means ± the SEM or SD, with the number of independent experiments. Statistical differences were determined using the Student’s t test.

**Online supplemental material.** Fig. S1 shows the targeting strategy to generate the CYLD<sup>+/−</sup> mice. Fig. S2 describes the B cell population of CYLD<sup>−/−</sup> mice. Fig. S3 describes the KLH-specific immune responses of CYLD<sup>+/−</sup> mice and class switch recombination in culture. Fig. S4 shows the B cell populations in CYLD<sup>−/−</sup> mice treated with TACI-Ig and Fig. S5 summarizes the relative RNA expression of different molecules using real-time PCR. The online version of this article is available at http://www.jem.org/cgi/content/full/jem.20070318/DC1.

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**REFERENCES**


SUPPLEMENTAL MATERIALS AND METHODS

The generation of CYLD<sup>ex7/8</sup> mice. The CYLD<sup>ex7/8</sup> mouse strain was generated using standard gene targeting techniques (1). In brief, a Cyld-targeting vector flanking exon 7 by loxP sites was generated by inserting the short arm of homology, the exon 7, and the long arm of homology into the pRapidFlirt (unpublished data) containing the loxP sites, the FRT-flanked neomycin resistance gene, and the TK gene from herpes simplex as negative selection markers. The 2.5-kb short arm was amplified from C57BL/6 genomic DNA via PCR, digested with BglII, and subsequently cloned into the BamHI site of pRapidFlirt. The resulting plasmid was used for the insertion of the 600-bp loxP-flanked sequence into the SbfI site, consisting of a part of intron 7, the exon 7, and part of intron 8. This 600-bp fragment was PCR amplified from C57BL/6 genomic DNA, digested with SbfI, and cloned into the pRapidFlirt. The resulting plasmid was used for the finalization of the CYLD<sup>ex7/8</sup> targeting vector by inserting the long arm into the Xhol site. The long arm was amplified by PCR from C57BL/6 genomic DNA. All PCR fragments and plasmids were verified by sequencing. The targeting vector was then linearized and electroporated into V6.5 embryonic stem cells. Recombinant ES cells were identified by Southern blot analysis and were injected into CB20 blastocysts to generate the CYLD<sup>neo/+</sup> mice. In addition, this clone was used for in vitro deletion of the loxP-flanked exon 7 and the neomycin resistance gene by Cre-mediated recombination transfecting with a Cre-expressing plasmid. One of the Cre-deleted clones was injected into tetraploid blastocysts to generate germline CYLD<sup>ex7/8/wt</sup> mice. All mice were backcrossed to C57BL/6 mice 10 times and kept in a specific pathogen-free barrier.

Induced class switch recombination. Using MACS beads, B cells were isolated from wild-type, as well as CYLD<sup>ex7/8</sup> mice. The B cells were incubated for 5 d in the presence of 10 μg/ml LPS and 20 ng/ml IL-4. Thereafter, the B cells were analyzed for the expression of IgG1 by flow cytometry.

REFERENCE