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A20 and CYLD Do Not Share Significant Overlapping Functions during B Cell Development and Activation

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The ubiquitin-editing enzyme A20 (TNFAIP3) and the deubiquitinase CYLD are central negative regulators of NF- κ B signaling. Both can act by removing nonproteolytic K63-linked polyubiquitin chains from an overlapping set of signaling molecules. In B cells, A20 deficiency results in hyperactivity, loss of immune homeostasis, inflammation, and autoimmunity. The reported consequences of CYLD deficiency are controversial, ranging from an absence of effects to dramatic B cell hyperplasia. These differences could be due to varying compensation for the loss of CYLD function by A20. Therefore, to explore potential overlapping physiological functions between A20 and CYLD, we generated and characterized A20/CYLD double-deficient B cells. Interestingly, the lack of both A20 and CYLD did not exacerbate the developmental defects and hyperresponsive activity of A20-deficient B cells. In addition, the extent of B cell activation after *in vitro* stimulation with anti-CD40, LPS, and CpG was comparable in B cells lacking A20/CYLD and A20 alone. However, in response to BCR cross-linking, we observed small but reproducible additive effects of the lack of A20 and CYLD. Taken together, our results demonstrate that A20 and CYLD do not share significant functions during B cell development and activation. *The Journal of Immunology*, 2012, 189: 4437–4443.

The NF- κ B pathway plays an important role in many physiological processes including innate and adaptive immunity, cell survival, and proliferation. NF- κ B-activating signals can be delivered from immune cell-surface receptors such as TNFR, BCR, CD40, and TLRs. Tight control of these signals is required to maintain immune cell homeostasis and prevent persistent activation of NF- κ B, which may lead to chronic inflammation, autoimmunity, and tumorigenesis.

The modification of key signaling molecules such as RIP1, TNFR-associated factor (TRAF) 6, or MALT1 with polyubiquitin chains has emerged as an essential regulatory mechanism of NF- κ B activation. Linkage with polyubiquitin chains via lysine 48 (K48) results in proteosomal degradation of the target protein. In contrast, nondegradative and regulatory functions are mediated through K63 and linear polyubiquitin chains that serve as scaffold molecules to recruit different kinase complexes. Ubiquitination is

reversible and counterregulated by deubiquitinating enzymes (summarized in Refs. 1, 2). Several deubiquitinases (DUBs) have been reported to negatively regulate NF- κ B. Among them are A20, CYLD, Cezanne, and USP21 (3–6).

A20, encoded by the TNF- α -inducible gene 3 (*Tnfaip3*), is a ubiquitin-modifying enzyme that negatively regulates K63-linked ubiquitination events and induces protein degradation via K48-linked polyubiquitin chains. Gene inactivation studies in mice established A20 as the central negative regulator of multiple NF- κ B-activating signaling pathways. Lack of signal containment in A20-deficient mice results in severe inflammation and lethality that is triggered by MyD88-dependent TLR signaling initiated by the commensal flora (7, 8). Cell type-specific deletion of A20 in immune cells and other tissues like intestinal epithelial cells and skin further confirmed its crucial role in the maintenance of tissue homeostasis and to prevent inflammatory diseases including autoimmunity (9–15). In B cells, loss of A20 causes hyperreactivity, general immune activation, and the production of autoantibodies (10, 11, 16).

In line with these studies, polymorphisms and mutations in the A20 gene locus are strongly associated with human autoimmune diseases (17–21). In contrast, CYLD mutations predispose to familial cylindromatosis, which is characterized by the development of benign tumors of skin appendages (22).

Using murine knockout (KO) models, the DUB CYLD was reported to be involved in a wide range of physiological processes including immune cell function, osteoclastogenesis, spermatogenesis, and tumorigenesis (23). The consensus mechanism of these functions is CYLD's specificity for removing K63-linked polyubiquitin chains from substrates, thereby controlling different pathways like NF- κ B, MAPK, and Wnt signaling (24–28). Most of the known molecular targets of CYLD's DUB activity are involved in NF- κ B signaling pathways.

Studies using a number of independently generated CYLD-deficient mice came to different conclusions regarding the cell type-specific roles of CYLD in the negative regulation of NF- κ B. In particular, the role of CYLD in B cell function is controversial.

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Abbreviations used in this article: DUB, deubiquitinase; GC, germinal center; HL, Hodgkin lymphoma; KO, knockout; mLN, mesenteric lymph node; MZP, marginal zone precursor; TRAF, TNFR-associated factor.

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Jin and colleagues (29) found massive hyperplasia and expansion of marginal zone B cells in CYLD-deficient mice and increased responses of CYLD-deficient B cells in response to activation. Similar effects were caused by the expression of a truncated CYLD lacking exons 7 and 8 (30). However, in another study, CYLD deficiency did not affect peripheral B cell numbers, but increased NF- κ B activation after stimulation (31). In accordance, B cells developed normally in CYLD-deficient mice employed in the current study (32). The lack of a B cell phenotype in our CYLD-deficient mouse model suggested the possibility of compensatory mechanisms by redundant proteins. It is, for example, conceivable that dysregulation of B cell homeostasis is caused by truncated CYLD rather than through the absence of full-length CYLD. Truncated forms of CYLD could exert dominant-negative functions by interfering with the action of redundant proteins.

As mentioned above, genetic studies in both human (17, 19, 21, 22) and mice (23, 33) have revealed different consequences of lack of A20 or CYLD function. Interestingly, however, >60% of EBV-negative classical Hodgkin lymphoma (HL) cases contain mono- or biallelic losses of A20 function, and 35% of classical HL cases display decreased CYLD copy numbers (34). Strikingly, the HL line KM-H2 has completely lost both A20 and CYLD expression due to biallelic mutations in both genes (34). In addition, it is remarkable that both A20 and CYLD share a set of signaling factors such as TRAF2, TRAF6, RIP1, and NF- κ B essential modulator as molecular targets (4, 5). Therefore, A20 represents a valid candidate protein that could compensate for the loss of CYLD function in B cells. To test this hypothesis, we generated A20/CYLD double-deficient B cells and studied the development of several B cell subsets in CD19Cre/A20^{F/F}CYLD^{-/-} mice, their response to B cell mitogens, and the impact of the combined loss of both DUBs on NF- κ B activation.

In our studies, we did not uncover general functions for CYLD in B cell differentiation and activation. More importantly, compound loss of A20 and CYLD did not exacerbate the effects of A20 deficiency, with the possible exception of B cell activation in response to BCR cross-linking *in vitro*. Therefore, the discrepancy of the various reported effects of CYLD deficiency is not functional compensation by A20, and this issue awaits further experimental clarification.

Materials and Methods

Mice

All mouse strains employed in this study are published and were originally generated using C57BL/6 embryonic stem cells or backcrossed to C57BL/6 at least six times (11, 32). Mice were housed in a specific pathogen-free environment in the animal facility of the Max Planck Institute of Biochemistry, Martinsried, Germany, and all animal procedures were approved by the Regierung of Oberbayern.

Flow cytometry

Single-cell suspensions were prepared and stained as published (35) with the following mAbs conjugated to FITC, PE, PerCP, allophycocyanin, or biotin: AA4.1 (AA4.1), B220 (RA3-6B2), CD1d (1B1), CD19 (eBio1D3), CD21 (2D6), CD23 (B3B4), CD25 (PC61.5), CD38 (90), CD5 (53-7.3), IgM (II/41), CD95 (15A7), CD86 (GL-1), CD80 (16-10A1), IL-6 (MP5-20F3) (all from eBioscience), and PNA (Vector Laboratories). Dead cells were excluded from analysis by 7-aminoactinomycin D or ethidium monoazide bromide staining. All samples were acquired on an FACSCalibur or FACSCanto II (BD Pharmingen), and results were analyzed with FlowJo software (Tree Star). For intracellular cytokine staining, cells were treated for 5 h at 37°C with 10 nM brefeldin A (Applichem), incubated with Fc-block (eBioscience), washed, and surface-stained prior to fixation with 2% paraformaldehyde and permeabilization with 0.5% saponin.

In vitro cultures

For *in vitro* culture, cells were purified by MACS depletion of CD43-expressing cells (>85–90% pure; Miltenyi Biotec). Final concentrations

of the stimuli for cellular activation were (unless otherwise indicated): 2.5 μ g/ml anti-CD40 (HM40-3; eBioscience), 10 μ g/ml anti-IgM (Jackson ImmunoResearch Laboratories), 0.1 μ M CpG (Alexis Biochemical), and 20 μ g/ml LPS (Sigma-Aldrich). ELISAs were conducted using Ab pairs to IL-6 (BD Biosciences) according to the manufacturer's instructions. To monitor cellular division, B cells were labeled in 1 ml 2.5 μ M CFSE (Molecular Probes) in PBS per 10⁷ cells at 37°C for 10 min.

EMSAs

Purified B cells were stimulated and lysed in whole-cell lysis buffer (20 mM HEPES [pH 7.9], 350 mM NaCl, 20% glycerol, 1 mM MgCl₂, 0.5 mM EDTA, 0.1 EGTA, 1% Nonidet P-40, 0.5 M NaF, 1 M DTT, 1 M β -glycerophosphate, 200 mM Na vanadate, and 25 \times Protease Inhibitor Mixture [Roche]) according to standard protocols. EMSAs were performed by using a [³²P]-dATP-labeled, dsNF- κ B oligonucleotide probe (5'-CAGGGCTGGG-GATCCCCATCTCCACAGG-3'). The samples were separated on native polyacrylamide gels prior to autoradiography. For EMSA supershift assays, whole-cell lysates were preincubated for 30 min on ice with anti-p50, anti-p65, or anti-c-Rel Abs (all from Santa Cruz Biotechnology).

Statistics

For comparisons of three and more groups, one-way ANOVA was used. The *p* values are presented in figure legends when a statistically significant difference was found.

Results

Loss of CYLD does not exacerbate the defects in A20-deficient B cell homeostasis

To study the consequences of the loss of both A20 and CYLD function in B cells, constitutive CYLD^{-/-} KO mice (32) were intercrossed with CD19Cre/A20^{F/F} mice (11), allowing B lineage-specific ablation of A20. In the resulting CD19Cre/A20^{F/F}CYLD^{-/-} mice, the CYLD deficiency was not restricted to the B lineage. However, in CYLD-deficient mice, B cell development was indistinguishable from wild-type mice, showing that complete CYLD deficiency does not affect the generation of B cells (Fig. 1A) (30). CYLD-deficient mice were also intercrossed with CD19cre mice to control for the heterozygous ablation of CD19 and the expression of the Cre recombinase.

We first assessed the impact of the combined deletion of A20 and CYLD on B cell development. To our surprise, loss of both DUBs did not exacerbate the defects in B cell subset differentiation caused by absence of A20 alone (11). The following developmental effects caused by absence of A20 in B cells were unchanged by additional absence of CYLD: 1) reduced proportions of mature recirculating B cells in the bone marrow (Fig. 1A); 2) increased proportions of transitional and reduced proportion of splenic mature B cells (Fig. 1B, *first panel*); 3) the expansion of CD23⁺ marginal zone precursor (MZP) B cells (Fig. 1B, *third panel*); and 4) reduced percentages of B1, in particular B1a cells, in the peritoneal cavity (Fig. 1C).

In contrast, we observed that constitutive absence of CYLD caused a slight reduction in splenocyte numbers, which was not affected by the additional absence of A20 in B cells (Supplemental Fig. 1). A20 deficiency in B cells induces the expansion of myeloid and T cells, resulting in splenomegaly and chronic inflammation (11). It seems possible that loss of CYLD function in myelomonocytic cells, which impairs their responses to inflammatory stimuli (36), counteracts the inflammation caused by A20-deficient B cells to some extent. This could explain the reduction of splenocyte numbers in CD19Cre/A20^{F/F}CYLD^{-/-} mice compared with CD19Cre/A20^{F/F} mice.

A20/CYLD deficiency does not enhance formation of spontaneous germinal centers compared with loss of A20 alone

In previous studies, we and others (10, 11, 16) reported that A20-deficient B cells are hyperresponsive to stimulation caused by

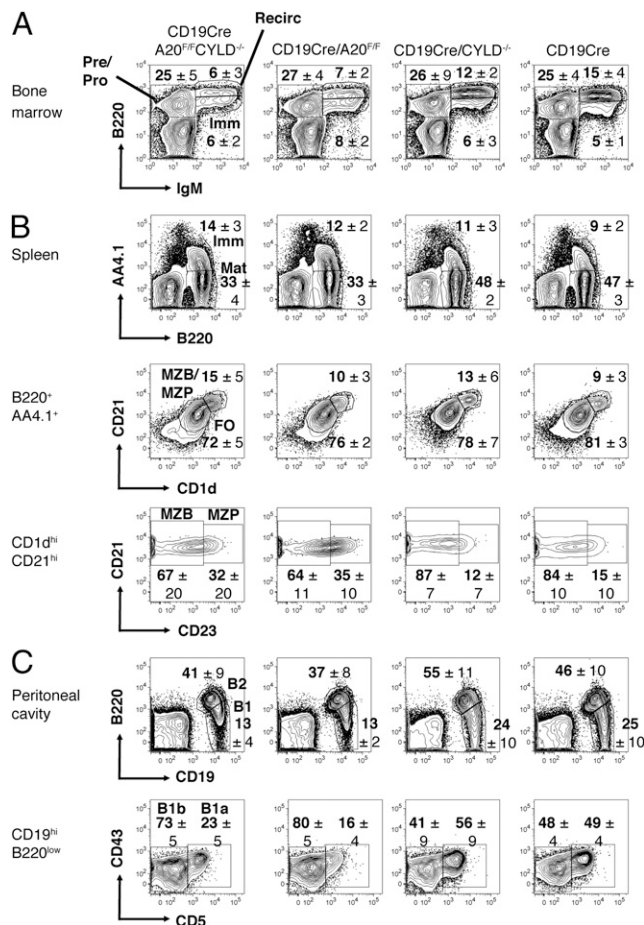


FIGURE 1. A20/CYLD-deficient B cells display similar developmental defects as A20-deficient B cells. **(A)** Proportions of pre/pro- ($B220^{+}IgM^{-}$), immature ($B220^{lo}IgM^{+}$), and mature/recirculating ($B220^{hi}IgM^{+}$) B cells of lymphocytes in the bone marrow. Numbers indicate mean and SD of five to six mice per genotype. **(B)** Proportions of transitional (Imm; $B220^{+}AA4.1^{+}$) and mature (Mat; $B220^{+}AA4.1^{-}$) B cells of total lymphocytes (*top panel*) and of follicular (FO; $CD1d^{hi}CD21^{int}$) and marginal zone (MZ)/MZP ($CD1d^{hi}CD21^{high}$) B cells of $B220^{+}$ B cells (*middle panel*) in the spleen. *Bottom panels*, Proportions of MZ ($CD1d^{hi}CD21^{hi}CD23^{lo}$) and MZP ($CD1d^{hi}CD21^{hi}CD23^{hi}$) B cells of $B220^{+}$ B cells. Numbers indicate mean and SD of nine mice per genotype. **(C)** Proportions of peritoneal B2 ($CD19^{+}B220^{+}$) and B1 ($CD19^{high}B220^{low}$) cells of total lymphocytes (*top panel*) and of B1a ($CD19^{high}B220^{low}CD5^{+}$) and B1b ($CD19^{high}B220^{low}CD5^{-}$) cells of total B1 cells (*bottom panel*). Numbers indicate mean and SD of nine mice per genotype.

enhanced NF- κ B signaling due to lack of negative regulation. Given that CYLD has been suggested to also negatively regulate NF- κ B in multiple cell types and was shown to restrict B cell activation (1), we asked whether combined loss of A20 and CYLD would cause additive effects during B cell activation.

To address this question *in vivo*, we studied spontaneous germinal center (GC) formation in the spleen and the GALT. Spontaneous GC formation was not enhanced in spleen and GALT of naive CYLD-deficient mice compared with control mice (Fig. 2). In contrast, naive $CD19Cre/A20^{F/F}CYLD^{-/-}$ and $CD19Cre/A20^{F/F}$ mice displayed the same slightly increased proportions of spontaneous splenic GC B cells compared with CYLD-deficient and control mice, although the differences did not reach statistical significance (Fig. 2). Similarly, the increased activation of A20-deficient B cells by bacterial Ags to form GCs in the mesenteric lymph nodes (mLN) and Peyer's patches was comparable in mice lacking both A20 and CYLD in B cells (Fig. 2).

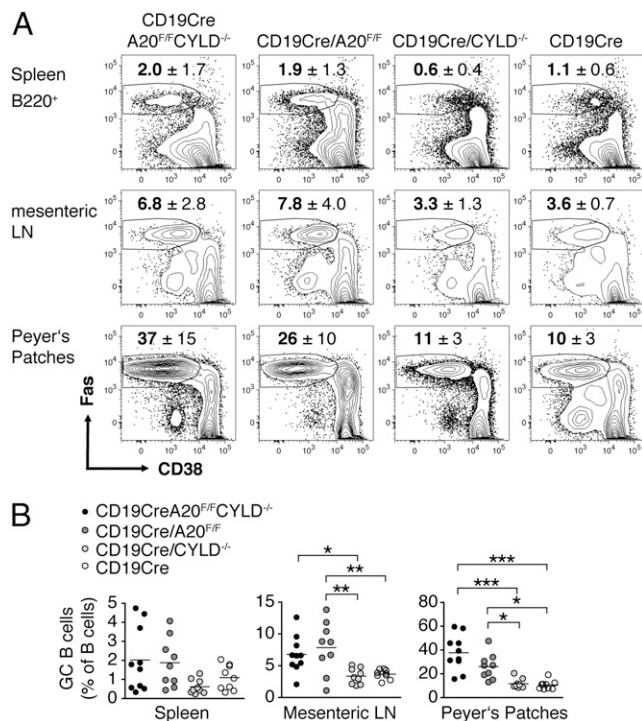


FIGURE 2. A20/CYLD deficiency in B cells enhances spontaneous GC formation to the same extent as A20 deficiency alone. **(A)** Proportions of GC B cells ($B220^{+}Fas^{hi}CD38^{lo}$) of total B cells in spleen (*top panel*), mLN (*middle panel*), and Peyer's patches (*bottom panel*); numbers indicate mean and SD of 9–10 mice per genotype. **(B)** Proportions of GC B cells in spleen, mLN, and Peyer's patches depicted as individual data points; bars indicate mean of 9–10 mice per group [same as in (A)]. * $p < 0.05$, ** $p < 0.001$, *** $p < 0.0001$, one-way ANOVA.

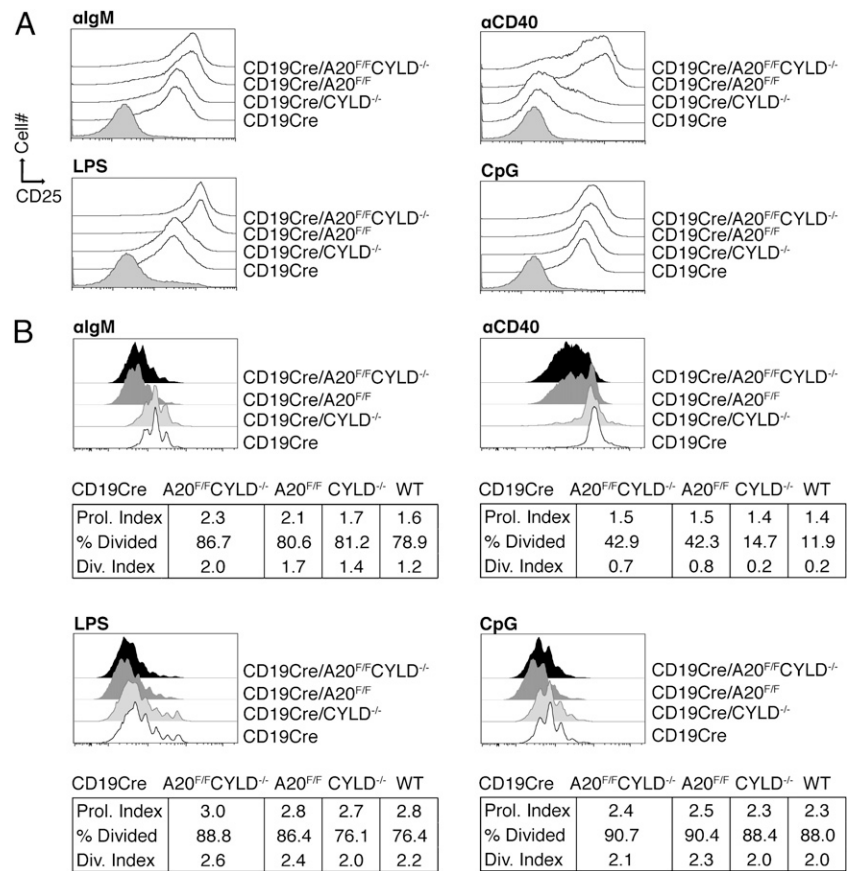
A20/CYLD deficiency does not enhance B cell activation and proliferation significantly more than absence of A20

We further compared B cell responses of A20/CYLD-deficient and A20-deficient B cells by measuring their activation, proliferation, and cytokine secretion in response to stimulation with B cell mitogens (anti-IgM, anti-CD40, LPS, and CpG) *in vitro*. Upon stimulation, B cells upregulate activation markers (CD25, CD80, CD86, MHC class II, and Fas) that were already slightly increased in resting A20/CYLD-deficient and A20-deficient B cells compared with CYLD-deficient and control B cells (Fig. 3A, Table I). These data are consistent with the finding that the spontaneous GC formation rate in $CD19Cre/A20^{F/F}CYLD^{-/-}$ and $CD19Cre/A20^{F/F}$ mice was comparable with each other but significantly higher than in $CD19Cre/CYLD^{-/-}$ and control mice. A20/CYLD-deficient B cells proliferated *in vitro* to a similar extent as A20-deficient B cells in response to CD40 and CpG, judged by the calculation of different parameters of cellular division using CFSE dilution assays. In contrast, BCR cross-linking and LPS stimulation slightly enhanced the proliferation of A20/CYLD-deficient B cells compared with A20-deficient B cells. These observations support our previous finding that CYLD does not majorly contribute to the negative regulation of B cell response in the presence or absence of A20.

A20/CYLD-deficient B cells produce more IL-6 in response to BCR cross-linking, but not in response to the engagement of CD40 and TLRs

We recently demonstrated that A20 negatively controls canonical NF- κ B activation in response to BCR cross-linking and CD40 and

FIGURE 3. A20/CYLD-deficient B cells display similar pattern of hyperresponsiveness as A20-deficient B cells. **(A)** Expression level of the B cell activation marker CD25 after overnight stimulation with anti-IgM, anti-CD40, LPS, or CpG compared with the resting condition of control B cells (gray-filled histogram). The histograms are representative of two to three independent experiments. **(B)** CFSE proliferation assay: histograms show CFSE intensities 3 d after stimulation. The tables under each histogram depict the proliferation index (Prol. Index, average number of divisions of the proliferating cells), the percentage of dividing cells (% Divided, the proportion of cells that initially started to divide), and the division index (Div. Index, average number of divisions of all cells); values were calculated with the FlowJo software (Tree Star). Values represent means of four independent experiments.



TLR stimulation. In addition, we showed that the expression of IL-6, which is a direct target of NF- κ B, correlates with the strength of NF- κ B signaling in B cells (11). Therefore, we evaluated the expression of IL-6 by ELISA (Fig. 4A) and intracellular FACS (Fig. 4B) of stimulated A20/CYLD-deficient and A20-deficient B cells compared with CYLD-deficient and control B cells. Both A20/CYLD and A20 deficiency alone led

to an equivalent increase in the production of IL-6 after stimulation with anti-CD40, LPS, or CpG in B cells (Fig. 4A). In contrast, BCR cross-linking caused elevated amounts of IL-6 (Fig. 4A) in A20/CYLD-deficient B cells compared with A20-deficient B cells. These results were in agreement with the observation that A20/CYLD deficiency enhanced the proliferation of B cells after BCR stimulation. Taking the median fluorescence

Table I. A20/CYLD-deficient and A20-deficient B cells express similar levels of activation markers

Stimuli	Genotype	CD25	CD80	CD86	MHC Class II	Fas
Resting	CD19Cre/A20 ^{F/F} CYLD ^{-/-}	25	36	14	1241	51
	CD19Cre/A20 ^{F/F}	25	38	17	965	57
	CD19Cre/CYLD ^{-/-}	18	27	11	547	36
	CD19Cre	18	28	9	573	33
Anti-IgM	CD19Cre/A20 ^{F/F} CYLD ^{-/-}	621	63	519	3051	111
	CD19Cre/A20 ^{F/F}	552	63	704	3051	127
	CD19Cre/CYLD ^{-/-}	340	45	737	2436	72
	CD19Cre	292	47	538	2890	63
Anti-CD40	CD19Cre/A20 ^{F/F} CYLD ^{-/-}	396	91	355	3220	396
	CD19Cre/A20 ^{F/F}	487	102	519	2813	403
	CD19Cre/CYLD ^{-/-}	39	42	130	1999	59
	CD19Cre	32	42	60	2227	45
LPS	CD19Cre/A20 ^{F/F} CYLD ^{-/-}	1104	165	777	2763	205
	CD19Cre/A20 ^{F/F}	1165	179	791	2525	217
	CD19Cre/CYLD ^{-/-}	319	56	704	2288	129
	CD19Cre	267	56	514	2738	95
CpG	CD19Cre/A20 ^{F/F} CYLD ^{-/-}	414	98	328	2571	106
	CD19Cre/A20 ^{F/F}	441	91	368	2414	103
	CD19Cre/CYLD ^{-/-}	346	57	382	1894	82
	CD19Cre	281	55	259	2267	68

Median fluorescence intensities of the B cell activation markers CD25, CD80, CD86, MHC class II, and Fas after overnight stimulation with anti-IgM, anti-CD40, LPS, or CpG compared with the resting condition of control B cells. Values represent the means of two to three independent experiments.

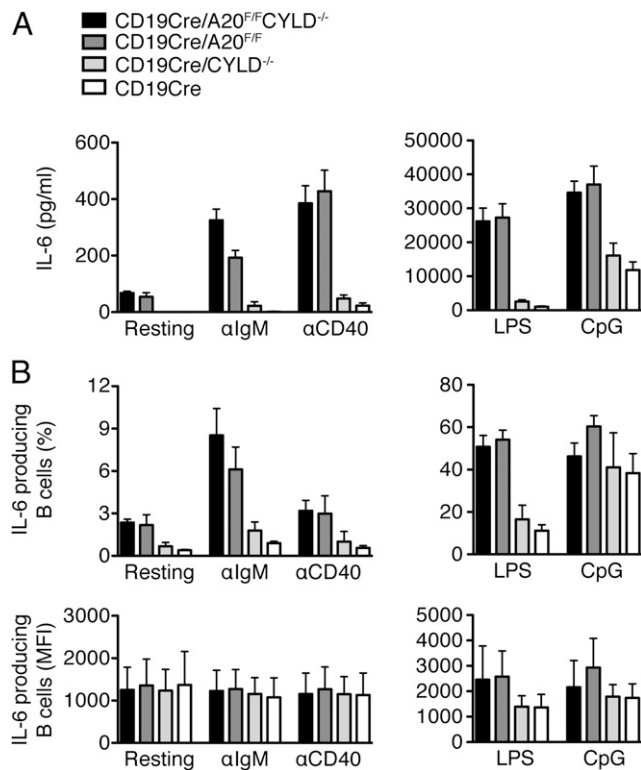


FIGURE 4. Proinflammatory IL-6 production is comparable between A20/CYLD-deficient and A20-deficient B cells. **(A)** Measurement of IL-6 production in overnight-stimulated B cells by ELISA. Bars represent means and SD of four independent experiments. **(B)** IL-6 production in overnight stimulated and brefeldin A-treated B cells assessed by intracellular FACS. *Top panel*, Percentage of IL-6-producing B cells. *Bottom panel*, Mean fluorescence intensity (MFI) of IL-6-producing B cells represents IL-6 amount per IL-6-producing cell. Bars depict means and SD of three independent experiments.

intensity as an estimate for the amount of IL-6 made per IL-6-producing cell suggests that B cells of all genotypes produce similar amounts of IL-6 in response to anti-IgM and anti-CD40 (Fig. 4B, *bottom panel*). Therefore, the differences between the genotypes most likely relate to the proportion of activated cells, rather than an increase in IL-6 secretion per cell. In contrast, stimulation with CpG and more so with LPS increased the IL-6 production per cell and the proportion of IL-6-producing cells (Fig. 4B) in both A20/CYLD-deficient and A20-deficient compared with CYLD-deficient and control B cells. However, the

differences were entirely due to the lack of A20, whereas the loss of CYLD had essentially no effect.

Enhanced proliferation and IL-6 production in A20/CYLD-deficient compared with A20-deficient B cells in response to BCR cross-linking correlates with enhanced NF- κ B activation

With two stimuli, we observed slightly enhanced in vitro responses in A20/CYLD-deficient compared with A20-deficient B cells: anti-IgM (proliferation and IL-6 production) and LPS (proliferation). To determine whether these enhanced responses correspond to increased NF- κ B activation, we performed EMSAs from B cells stimulated with anti-IgM (Fig. 5A) and LPS (Fig. 5B). NF- κ B DNA-binding complexes were verified by EMSA supershift assays using anti-p50, anti-p65, or anti-c-Rel Abs. The supershift assays reveal increased DNA-binding of mostly of p50/c-Rel and to a lesser extent of p50/p65 subunits following stimulation with anti-IgM or LPS (Supplemental Fig. 2).

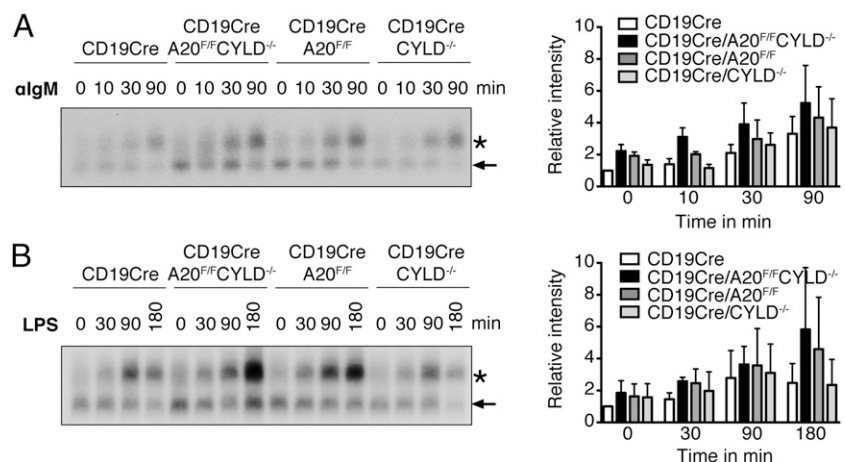
We observed enhanced BCR cross-linking-induced NF- κ B DNA binding in A20/CYLD-deficient and A20-deficient B cells at all investigated time points (10, 30, and 90 min) (Fig. 5A). In addition, A20/CYLD-deficient B cells displayed more robust NF- κ B activation compared with A20-deficient B cells, with the strongest difference noted 10 min following stimulation (Fig. 5A). These data demonstrate that anti-IgM-induced NF- κ B activity is slightly enhanced in A20/CYLD-deficient B cells compared with A20-deficient B cells. Possible explanations for this finding could be increased signal strength in individual B cells, the activation of a larger proportion of cells, or a combination of both.

In contrast, TLR4-mediated NF- κ B signaling (Fig. 5B) yielded no consistent difference between A20/CYLD-deficient and A20-deficient B cells. These findings are in line with the comparable LPS-induced IL-6 production in A20/CYLD and A20-deficient B cells.

Discussion

In this study, we addressed potential overlapping physiological functions of the ubiquitin-editing enzyme A20 and the DUB CYLD in B cells. By ablating A20 and CYLD in B cells, we demonstrate that loss of both proteins does not exacerbate the impaired B cell homeostasis and in vivo B cell hyperresponsiveness that we previously reported for mice lacking A20 specifically in B cells. Mice with A20/CYLD deficiency or A20 deficiency in B cells display equally severe B cell developmental defects. Similarly, A20/CYLD-deficient and A20-deficient B cells exhibit comparable degrees of in vitro responses to B cell mitogens and NF- κ B activation.

FIGURE 5. NF- κ B activation in A20/CYLD-deficient compared with A20-deficient, CYLD-deficient, and control B cells in response to BCR cross-linking and LPS treatment. EMSA of anti-IgM-induced **(A)** and LPS-induced **(B)** NF- κ B activation. The specific bands (asterisk) indicating NF- κ B p50/c-Rel and p50/p65 heterodimer DNA-binding (*left panels*) were quantified and normalized to controls at time point 0. The lower bands (arrow) represent p50 homodimers. Bars depict means and SD of the relative band intensities, derived from four (A) or three (B) independent experiments (*right panels*).



Thus, our work indicates that A20 and CYLD do not functionally overlap in any significant fashion during B cell development and activation. The possible exception might be B cell responses and NF- κ B activation after BCR cross-linking. However, we did not observe any significant consequences of the additive effects of A20 and CYLD deficiency on signaling downstream of the BCR in vivo. Because it is unclear to what extent BCR cross-linking accurately reflects recognition of Ag by the BCR in vivo, we did not pursue this observation in more detail.

Given that A20 and CYLD both contain DUB functions of K63-linked polyubiquitin chains and target similar molecular substrates including TRAF2, TRAF6, NF- κ B essential modulator, and RIP1, it is surprising that both proteins do not functionally overlap. Key differences that could account for this lack of functional overlap are their molecular mode of action and CYLD's specificity to hydrolyze K63-linked (37) and linear polyubiquitin chains (38). In contrast, A20 does not act as a processive DUB for K63-linked polyubiquitin chains but (39) effectively cleaves entire K63-linked polyubiquitin chains from substrates such as TRAF6, thereby demonstrating specificity for particular polyubiquitinated substrates (40). In addition, A20 can limit NF- κ B activation through noncatalytic mechanisms including lysosomal targeting of TRAF2 (41) and direct I κ B kinase inhibition (42). In vivo, A20 is involved both in the addition of K48-linked polyubiquitin chains to induce the proteasomal degradation of various target proteins (43, 44) and the removal of K63-linked ubiquitin chains to terminate signaling (8, 43, 45, 46).

Another difference between A20 and CYLD include their distinct temporal expression and regulation. A20 depends functionally on its inducible expression upon signal-induced NF- κ B activation (47), whereas CYLD is constitutively expressed. However, in response to mitogens and TNF- α , CYLD's DUB function is transiently inactivated by I κ B kinase-mediated phosphorylation (48). Thus, it has been proposed that A20 and CYLD may regulate NF- κ B activation at different phases (23). A20 function is crucial to terminate signal-induced NF- κ B activation (7, 49). In contrast, CYLD acts constitutively to prevent spontaneous NF- κ B activation (48).

In addition, different cell type-specific cofactor requirement and/or substrate-specific molecular mode of action could also explain the missing functional overlap between A20 and CYLD during the signal transduction pathways initiated upon B cell activation.

In contrast to another KO model (29) and the expression of a truncated CYLD protein (30), we did not observe major effects in our CYLD-deficient B cells with respect to cell numbers in mice, differentiation, and activation. Our findings are in agreement with the analysis of a third independently generated CYLD-KO mouse strain (31). It is also worth mentioning that our study is the only one that uses mice exclusively on the C57BL/6 genetic background. It cannot be excluded that some of the observed differences to the other studies are in part due to effects of the C57BL/6-129 mixed genetic background employed there (50).

In this study, we clearly demonstrate that the phenotypic differences between the mouse models are not due to a compensation for some of CYLD's functions by A20 in mice. Collectively, we conclude that A20 and CYLD do not significantly cooperate in the regulation of B cell development and activation.

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Disclosures

The authors have no financial conflicts of interest.

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