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Negative Regulation of CD40-Mediated B Cell Responses by E3 Ubiquitin Ligase Casitas-B-Lineage Lymphoma Protein-B

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It has been documented that CD40 is essential for B cell function. Casitas-B-lineage lymphoma protein-b (Cbl-b), an adapter protein and ubiquitin ligase, has been shown to regulate the activation of T and B cells through their Ag receptors. In this study, we report that CD40-induced B cell proliferation is significantly augmented in mice lacking Cbl-b. Furthermore, Cbl-b−/− mice display enhanced thymus-dependent Ab responses and germinal center formation, whereas introduction of CD40 deficiency abolishes these effects. Hyper thymus-dependent humoral response in Cbl-b mice is in part due to an intrinsic defect in B cells. Mechanistically, Cbl-b selectively down-modulates CD40-induced activation of NF-κB and JNK. Cbl-b associates with TNF receptor-associated factor 2 upon CD40 ligation, and inhibits the recruitment of TNF receptor-associated factor 2 to the CD40. Together, our data suggest that Cbl-b attenuates CD40-mediated NF-κB and JNK activation, thereby suppressing B cell responses. The Journal of Immunology, 2007, 179: 4473–4479.

CD40, a member of the TNF receptor superfamily, plays central roles in the homeostatic regulation of B cell functions (1). Activated CD4+ T cells express CD40L, and the interaction of CD40 on B cells with the CD40L on activated CD4+ T cells represents the key event in the initiation of B cell proliferation, differentiation, isotype switching, up-regulation of surface molecules contributing to Ag presentation, development of the germinal center (GC), and the humoral memory response to thymus-dependent (TD) Ags (1). CD40-CD40L interactions induce association of TNF receptor-associated factor (TRAF)-2, -3, and -6 with CD40, and activate IKKβ and then NF-κB (2, 3). CD40 ligation also activates the noncanonical NF-κB2 processing pathway, that is, IKKα leads to the phosphorylation and processing of p100 resulting in the formation of an active RelB/p52 heterodimer (4). The other knowing signaling events occurring upon CD40 ligation include activation of MAPKs such as ERK, JNK, and p38 MAPK, and PI3K (5, 6).

Casitas-B-lineage lymphoma protein-b (Cbl-b) belongs to the Cbl family, which consists of a protein tyrosine kinase (PTK)-binding (TKB) domain, linker, and RING finger domains, and extensive proline-rich regions in their COOH-terminal halves. It is now understood that Cbl-b functions as an E3 ubiquitin (Ub) ligase with a RING finger that recruits an Ub-conjugating enzyme (E2) and the TKB domain that recognizes target proteins for Ub conjugation (7, 8). The absence of Cbl-b in mice results in the hyper-activation of autoreactive T and B cells, leading to the development of spontaneous lupus-like disease (9), indicating a critical role of Cbl-b in the regulation of T and B cell activation and, hence, the maintenance of a balance between immunity and tolerance. B cells lacking Cbl-b show augmented proliferation in response to IgM stimulation (9), suggesting that Cbl-b regulates BCR-mediated signaling. In support of this notion, Cbl-b may negatively regulate BCR signaling in mature mouse B cells through the ubiquitination of Syk (10). However, it is largely unknown whether and how Cbl-b modulates CD40-mediated B cell function. In this study, we attempted to investigate the role of Cbl-b in CD40-mediated B cell function. Our data show that Cbl-b negatively regulates CD40-mediated B cell responses in vitro and in vivo. Cbl-b associates with TRAF-2 upon CD40 ligation, thus suppressing the recruitment of TRAF-2 to CD40, which leads to activation of NF-κB and JNK.

Materials and Methods

Mice

Wild-type (WT), CD40−/−, and Rag-1−/− BALB/c mice were purchased from The Jackson Laboratory. Cbl-b−/− mice were described previously (9), and have been backcrossed to a BALB/c background for 12 generations. Cbl-b−/− mice were crossed with CD40−/− mice and the offspring were intercrossed to generate Cbl-b−/−/CD40−/− mice. All mice were used for experiments at ages of 6 to 10 wk.

Reagents

Anti-mouse IgM F(ab′)2 were purchased from Zymed Laboratories. Abs against Cbl-b (sc-8006), p52 (sc-298), TRAF-2 (sc-876), TRAF-3 (sc-1828), and TRAF-6 (sc-7221) were purchased from Santa Cruz Biotechnology. Phospho-AbS were purchased from Cell Signaling Technology. A murine B cell isolation kit was obtained from Miltenyi Biotec. The fluorescence conjugated and nonfluorescence conjugated mAbs were purchased from BD Biosciences. MG-132 was obtained from Sigma-Aldrich. CD40L (CRC 803B) was purchased from Cell Sciences.

B cell isolation and activation

Splenic B cells from WT, CD40−/−, and Cbl-b−/− mice were isolated (purity >95% as determined by FACS analysis of B220 cell surface expression) using a B cell isolation kit using negative selection (Miltenyi Biotec). Contaminating T cells were <1% as determined by CD3 staining.
For in vitro activation, B cells (2 × 10^7/ml) were stimulated for various time periods indicated by CD40L or anti-CD40 mAbs. The cells were lysed in 1% Nonidet P-40 lysis buffer or radiimmunoprecipitation assay buffer (11, 12) where indicated.

**Assays of B cell proliferation and survival**

Splenetic B cells from WT and Cbl-b−/− mice were cultured for 56 h at 37°C in round-bottom 96-well plates with F(ab′)2 anti-IgM with or without anti-CD40 mAb as indicated. The cells were pulsed with 1µCi [3H]thymidine, and harvested 16 h later. The radioactivity was quantitated as previously described (11, 12). For viability assays, B cells were stimulated as above and cell death was measured through FACS analysis after 72 h of culture by double staining with propidium iodide (PI) and FITC-conjugated-annexin V. The percentage of cells in the double-negative quadrant was recorded as the viable fraction.

**Immunoprecipitation and Western blotting**

The conditions for immunoprecipitation and immunoblotting were described previously (11, 12).

**Detection of surface receptors and activation markers by flow cytometry**

Splenocyte suspensions were incubated with anti-CD32/CD16 (EBO, Calbiochem) in CFA (Sigma-Aldrich). For secondary responses, KLH-primed mice were challenged with 50 µg TNP-Ficoll (Biosearch Technologies). We bledd tails and prepared serum on days 7, 14, and 28 after primary immunization. Titers of KLH-specific IgG1, IgG2b, and IgM Abs, or TNP-specific IgG1 and IgM Abs were assessed using sandwich ELISA (Becton Dickinson Associates).

**Adoptive transfer**

For Rag-1−/− reconstitution experiments, CD4+ T cells were purified from spleens and lymph nodes via negative selection and sorted for CD45+CD42L−/− naive cells. Splenic B cells were isolated via negative selection and sorted for CD19+ cells. Five × 10^6 naive CD4+ T cells and 10^7 CD19+ cells from WT or Cbl-b−/− mice in different combinations were transferred i.v. into Rag-1−/− hosts, which were permitted to equilibrate 30 days after transfer to avoid effects of homeostatic proliferation (13). The mice were immunized with KLH as above and sacrificed at day 14.

**Immunohistochemistry**

Mice immunized with KLH in CFA were sacrificed on day 28. The spleens were collected and frozen, and 5-µm thick sections were cut. The frozen sections of spleens were immunostained with biotinstained-PNA followed by avidin-biotin complex-immunoalkaline phosphatase for GC and FITC-labeled anti-B220 followed by HRP-conjugated anti-FITC for B cells.

**NF-κB activity assay**

A TransAM ELISA was used to determine the nuclear activity of the p65 subunit (Active Motif). In brief, the nuclear extracts of WT and Cbl-b−/− B cells (2 × 10^6) treated with 2 µg/ml CD40L were prepared as previously described (14). Ten µg of nuclear protein diluted in lysis buffer was loaded in triplicate onto a 96-well plate coated with an oligonucleotide containing an NF-κB p65 consensus binding site (5′-GGGACTTTCC-3′). The wells were rinsed with washing buffer, and a NF-κB p65 Ab was added to each well at a dilution of 1/1000. After rinsing, a final incubation with HRP-conjugated secondary Ab resulted in a colorimetric reaction, which was then quantified by spectrophotometry at 450 nm with a reference wavelength of 655 nm.

**Statistical analysis**

Statistical analysis was done by two-tailed Student’s unpaired t test. Results are shown as the mean ± SD. A difference between experimental groups was considered significant when the p value was <0.05.

**Results**

Cbl-b−/− B cells display hyperproliferation and increased survival in response to CD40 stimulation

It has now been shown that Cbl-b−/− B cells are hyperproliferative in response to IgM stimulation (9), suggesting that Cbl-b may negatively regulate the threshold for B cell activation. To extend this finding, we measured proliferation of splenic B cells from naive WT and Cbl-b−/− BALB/c mice in response to IgM and CD40 stimulation. Cbl-b−/− B cells proliferated highly in response to stimulation of IgM and CD40 (Fig. 1A, upper panel), suggesting that Cbl-b regulates not only BCR- but also CD40-induced B cell activation. Consistent with this observation, CD40 ligation promoted cell cycle progression more in Cbl-b−/− B cells than WT B cells (Fig. 1A, lower panel). Note that the expression of BCR, CD40, CD69, B7-1, and I-Ek was comparable between WT and Cbl-b−/− B cells (Fig. 1B), suggesting that heightened B cell proliferation induced by BCR or CD40 does not result from enhanced expression of BCR or CD40 receptor, or an activated phenotype.

**FIGURE 1.** Cbl-b−/− B cells are hyperproliferative and display an increased rate of survival in response to CD40 ligation. A, Purified B cells from WT and Cbl-b−/− mice were stimulated with various concentrations of F(ab′)2 anti-IgM or anti-CD40, or constant F(ab′)2 anti-IgM with various concentrations of anti-CD40 at 37°C for 72 h, and B cell proliferation was determined by [3H]thymidine incorporation (upper panel). The cell cycle in CD40L-activated WT and Cbl-b−/− B cells was examined by PI staining and flow cytometry. Histograms represent the mean fluorescence intensity of PI incorporation. Numbers represent the percentage of cells in each phase (lower panel). B, WT and Cbl-b−/− B cells were stained with Abs against CD69, I-Ek, B7-1, and CD40, and analyzed by flow cytometry. C, WT and Cbl-b−/− B cells were cultured for 3 days with anti-CD40 (8 µg/ml), and the B cell survival was determined by Annexin-V and PI staining, and was defined as Annexin-V−PI− cells. D, WT and Cbl-b−/− B cells were stimulated with anti-CD40 for 0, 24, 48, and 72 h, and lysed. The cell lysates were blotted with anti-Bcl-xL and anti-actin, respectively. Data shown in this figure are pooled from two experiments.
It has been shown that CD40−/− and CD40L−/− mice showed defective T cell-dependent B cell activation, proliferation, and survival (1). To test the role of Cbl-b in CD40-mediated B cell survival, resting mature B cells were purified from spleen and cultured with or without anti-CD40 Ab for 3 days. The B cell survival was determined by Annexin-V and PI staining, and was defined as Annexin-V−/PI− cells. As shown in Fig. 1C, the absence of Cbl-b led to a significant enhancement of B cell survival induced by CD40 ligation. This result suggests that increased B cell survival may contribute to the aberrant CD40-mediated B cell proliferation in Cbl-b−/− mice. To better understand the capacity of CD40 ligation to elicit significant survival in Cbl-b−/− B cells, we compared the ability of anti-CD40 treatment to regulate the induction of Bcl-xL, a prosurvival protein which has been implicated in CD40-mediated B cell survival (15, 16) in WT and Cbl-b−/− B cells. Cbl-b deficiency greatly enhanced CD40-induced Bcl-xL expression in B cells (Fig. 1D).

**Cbl-b exerts its regulatory role in TD- and TI-humoral immune responses**

To investigate the role of Cbl-b in CD40-mediated B cell function in vivo, we measured TD humoral responses in WT, Cbl-b−/−, CD40−/−, and Cbl-b−/−CD40−/− mice. To this end, the mice were challenged with a TD Ag, KLH, and specific Ab responses, and GC formation were monitored. Consistent with previous reports (17, 18), CD40 function was dispensable for IgM production in response to KLH immunization but was required for Ig class switching for the production of IgG1 and IgG2b as CD40−/− mice produced very low levels of KLH-specific IgG1 and IgG2b Abs (Fig. 2A). In contrast, primary KLH immunization elicited a strong TD immune response resulting in increased production of anti-KLH IgG1 and IgG2b Abs in Cbl-b−/− mice. We note that IgG2a production was undetectable in all groups of mice immunized with KLH (data not shown). Overall secondary responses to KLH were...
Cbl-b DOWN-REGULATES CD40-INDUCED B CELL RESPONSES

Cbl-b selectively modulates CD40-mediated activation of NF-κB and JNK but not ERK, p38 MAPK, and Akt during B cell activation

CD40 ligation in B cells is known to activate multiple signaling pathways including NF-κB, MAPKs, and PI3K (5, 6). We then determined which signaling pathways are perturbed in the absence of Cbl-b. Loss of Cbl-b resulted in the hyperphosphorylation of CD40-induced IkBα and JNK, but phosphorylation of ERK, p38 MAPK, and Akt was comparable between WT and Cbl-b−/− B cells (Fig. 3A). To further confirm CD40-induced NF-κB activation is regulated by Cbl-b, the NF-κB p65 binding activity was assayed using an ELISA kit. NF-κB p65 binding activity was significantly enhanced in B cells lacking Cbl-b upon CD40 ligation (Fig. 3B). Because CD40 triggers both canonical and noncanonical NF-κB pathway (16), we then detected whether Cbl-b also affected noncanonical NF-κB pathway. Cbl-b−/− B cells showed equal p100 processing to p52 in response to CD40 ligation (Fig. 3C). These data implicate a negative regulatory role of Cbl-b in canonical but not noncanonical NF-κB activation pathway. Taken together, our data indicate that Cbl-b negatively regulates CD40-induced activation of NF-κB and JNK in B cells.

Cbl-b associates with TRAF-2 upon CD40 ligation and regulates the recruitment of TRAF-2 to CD40 in B cells

The recruitment of TRAFs to the CD40 cytoplasmic domain is essential for CD40-mediated activation of multiple signaling pathways including NF-κB and JNK/p38 MAPK, thus regulating B cell responses (19, 20). The enhanced recruitment of TRAF-2 to CD40 in TRAF−/− B cells upon CD40 ligation suggests that TRAF-3 may exert its suppressive effects on CD40 signaling by competing with TRAF-2 for binding to CD40 (20). To elucidate the molecular mechanism of Cbl-b's action, we investigated whether Cbl-b can associate with TRAF-2, -3, or -6 upon CD40 ligation. To this end, WT B cells were stimulated with CD40L for 5, 15, and 30 min at 37°C and lysed. The cell lysates were immunoprecipitated with anti-TRAF-2, anti-TRAF-3, and anti-TRAF-6, and blotted with anti-Cbl-b. Interestingly, we found that CD40 ligation induced association of Cbl-b with TRAF-2, which became evident at 15–30 min after stimulation. No association between Cbl-b and TRAF-3 or
TRAF-6 was observed in WT B cells before and after CD40 ligation (Fig. 4A). These data raised a possibility that Cbl-b may selectively regulate the recruitment of TRAF-2 to CD40. Indeed, the absence of Cbl-b indeed resulted in enhanced and sustained association of TRAF-2 with the CD40, whereas very little TRAF-3 was found to bind to CD40 in Cbl-b−/− B cells (Fig. 4B). We failed to detect TRAF-6 in CD40 immunoprecipitates (data not shown). Note that CD40 was found in both TRAF-2 and TRAF-3 immunoprecipitates upon CD40 ligation, suggesting that at least two distinct complexes, namely CD40-TRAF-2-Cbl-b and CD40-TRAF-3, are formed.

Cbl-b promotes CD40-induced TRAF-2 ubiquitination and degradation in primary B cells

Both TRAF-2 and Cbl-b are RING finger E3 Ub ligases, and TRAF-2 undergoes self-ubiquitination (21). We then hypothesized that Cbl-b may target TRAF-2 for ubiquitination and subsequent degradation. To visualize endogenous TRAF-2 ubiquitination, we treated WT and Cbl-b−/− B cells with MG-132, a proteasome inhibitor, to block proteasome-mediated degradation, allowing ubiquitinated proteins to accumulate in the cytoplasm. Using this approach, we clearly showed that CD40-induced TRAF-2 ubiquitination was defective in B cells lacking Cbl-b (Fig. 5A). We should note that TRAF-3 also underwent ubiquitination upon CD40 ligation (Fig. 5A). Consistent with these observations, TRAF-2 and TRAF-3, but not TRAF-6 underwent degradation upon CD40 ligation in a time-dependent manner in WT B cells, and this degradation was significantly inhibited in B cells lacking Cbl-b (Fig. 5B).

Discussion

In this study, we analyzed the role of Cbl-b in CD40-mediated B cell responses and signaling. Our results show that CD40-induced B cell proliferation in vitro and TD humoral responses are significantly increased in Cbl-b−/− mice (Fig. 1A and 2, A and B). HyperTD humoral response in Cbl-b−/− mice is in part due to an intrinsic defect in B cells (Fig. 2D). Introduction of CD40 mutation abrogates hyperTD humoral immune responses in Cbl-b−/− mice (Fig. 2, A and B), supporting the notion that Cbl-b functions as a major negative mediator in CD40 signaling pathway in B cells. Our data indicate that Cbl-b selectively affects CD40-mediated NF-κB and JNK activation possibly via inhibiting TRAF-2 degradation, thus regulating the recruitment of TRAF-2 to the CD40 (Figs. 4 and 5).

We show that Cbl-b−/− mice display heightened KLH-specific IgG production and GC formation in response to TD Ag (Fig. 2, A and B). These data suggest that Cbl-b is indeed involved in regulation of TD humoral responses. However, it was not defined whether the hyperTD Ab responses in Cbl-b−/− mice are B cell intrinsic, or are secondary to hyperactivity of T cells. The evidence that Cbl-b−/− B cells are hyperproliferative in response to CD40 ligation in vitro and that Cbl-b−/− mice display hyperhumoral responses induced by TI Ag challenge suggests an intrinsic defect in
Cbl-b−/− B cells. The direct evidence confirming the intrinsic B cell defect in Cbl-b−/− mice comes from the Rag-1−/− mice reconstitution experiments. Rag-1−/− mice reconstituted with WT T cells plus Cbl-b−/− B cells produced more IgG1 than those receiving WT B cells together with WT T cells. These data clearly indicate that there is a B cell intrinsic defect in Cbl-b−/− mice. However, the hyperTD Ab responses in Cbl-b−/− mice cannot be ascribed to B cells only because Rag-1−/− mice receiving Cbl-b−/− T cells and WT B cells produced the modest levels of IgG1.

This notion is further supported by the evidence that loss of Cbl-b in both T and B cells acts synergistically to increase IgG1 production. Therefore, the hyperTD Ab production in Cbl-b−/− mice results from intrinsic defects in both T and B cells which lead to enhanced interaction between CD40 in B cells and CD40L in activated T cells.

It was reported that loss of Cbl-b rescues impaired T cell proliferation and IL-2 production in CD28−/− mice (9, 22). We then tested whether this is the case with genetic inactivation of Cbl-b in CD40−/− mice. CD40 mutation completely abrogates heightened TD responses in Cbl-b−/− mice (Fig. 2, A and B), indicating that Cbl-b exerts its negative regulatory role in TD responses via a CD40-dependent manner, and Cbl-b is one of the important negative regulators in CD40-signaling pathway in B cells. Therefore, our data suggest that manipulating the levels of Cbl-b expression in B cells may have potential therapeutical use in Ab-mediated autoimmune disease such as myasthenia gravis. Indeed, blocking CD40-CD40L interaction completely suppresses the development of experimental autoimmune myasthenia gravis (23, 24).

The recruitment of TRAFs to the CD40 cytoplasmic domain is essential for CD40-mediated B cell responses. CD40 transduces signals via these adapter proteins to mediate the activation of multiple signaling pathways including NF-κB and JNK/p38 MAPK, which in turn phosphorylate and activate downstream transcription factors (19, 20). Although a TRAF-2-deficient B cell line has been shown to retain CD40-mediated activation of the canonical NF-κB pathway (25), TRAF-2 is essential for CD40-mediated activation of this pathway in primary B cells (26, 27). These studies indicate that the signaling milieu in B cell lines differs significantly from that of primary B cells. TRAF-3 has been shown to play a negative regulatory role in CD40 signaling. TRAF-3−/− B cells display enhanced CD40-mediated JNK activation. Furthermore, the recruitment of TRAF-2 to CD40 is increased in TRAF-3−/− B cells upon CD40 ligation, suggesting that TRAF-3 may exert its suppressive effects on CD40 signaling by competing with TRAF-2 for binding to CD40 (20). The evidence that Cbl-b associates with TRAF-2 but not with TRAF-3 and TRAF-6, suggests that Cbl-b may regulate the recruitment of TRAF-2 to CD40. Indeed, the absence of Cbl-b led to increased recruitment of TRAF2 but not TRAF-3 to the CD40 (Fig. 4B).

The association of Cbl-b with TRAF-2 suggests that Cbl-b may target TRAF-2 for ubiquitination. Indeed, treating primary B cells with MG-132 which allows ubiquitinated proteins to accumulate in the cytoplasm, we clearly show that CD40-induced TRAF2 ubiquitination is impaired in B cells lacking Cbl-b (Fig. 5A). Although the structural requirement for Cbl-b-TRAF-2 association has not been characterized, based upon the structural analysis of both Cbl-b and TRAF-2, we predict that Cbl-b may interact with TRAF-2 via its TKB domain or ubiquitin-associated domain with tyrosine residues or Ub chains of TRAF-2. Currently, the experiments designed to investigate how Cbl-b associates with TRAF-2 and regulates TRAF-2 degradation are underway.

We also showed that TRAF-3 degradation is impaired in Cbl-b−/− B cells upon CD40 ligation in a manner similar to TRAF-2. However, we could not detect the physical association of Cbl-b with TRAF-3 before and after CD40 ligation. It has been shown that CD40-induced TRAF-3 degradation is dependent upon TRAF-2 (26, 28). Given that Cbl-b only transiently associates with TRAF-2 and CD40 upon CD40 ligation, one might speculate that TRAF-2 that dissociates from CD40 and Cbl-b may then target TRAF-3 for ubiquitination. Our data also suggest that TRAF-2 may have higher affinity to CD40 than TRAF-3.

In conclusion, our data strongly indicate that B cells lacking Cbl-b display an intrinsic defect in CD40 signaling pathway. Cbl-b exerts its negative regulatory function on TD humoral responses via a CD40-dependent manner. Mechanistically, Cbl-b associates with TRAF-2, and regulates the recruitment of TRAF-2 to CD40 which leads to activation of NF-κB and JNK.

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Disclosures

The authors have no financial conflict of interest.

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