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TNFR-Associated Factor 2 Deficiency in B Lymphocytes Predisposes to Chronic Lymphocytic Leukemia/Small Lymphocytic Lymphoma in Mice

Gema Pérez-Chacón,* David Llobet,†,1 Constanza Pardo,*-2 José Pindado,†,3 Yongwon Choi,‡ John C. Reed,† and Juan M. Zapata*‡

We have previously shown that transgenic (tg) mice expressing in B lymphocytes both BCL-2 and a TNFR-associated factor 2 (TRAF2) mutant lacking the really interesting new gene and zinc finger domains (TRAF2DN) develop small lymphocytic lymphoma and chronic lymphocytic leukemia with high incidence (Zapata et al. 2004. Proc. Nat. Acad. Sci. USA 101: 16600–16605). Further analysis of the expression of TRAF2 and TRAF2DN in purified B cells demonstrated that expression of both endogenous TRAF2 and tg TRAF2DN was negligible in Traf2DN-tg B cells compared with wild-type mice. This was the result of proteasome-dependent degradation, and rendered TRAF2DN B cells as bona fide TRAF2-deficient B cells. Similar to B cells with targeted Traf2 deletion, Traf2DN-tg mice show expanded marginal zone B cell population and have constitutive p100 NF-κB2 processing. Also, TRAF3, X-linked inhibitor of apoptosis, and Bcl-xL expression levels were increased, whereas cellular inhibitors of apoptosis 1 and 2 levels were drastically reduced compared with those found in wild-type B cells. Moreover, consistent with previous results, we also show that TRAF2 was required for efficient JNK and ERK activation in response to CD40 engagement. However, TRAF2 was deleterious for BCR-mediated activation of these kinases. In contrast, TRAF2 deficiency had no effect on CD40-mediated p38 MAPK activation but significantly reduced BCR-mediated p38 activation. Finally, we further confirm that TRAF2 was required for CD40-mediated proliferation, but its absence relieved B cells of the need for B cell activating factor for survival. Altogether, our results suggest that TRAF2 deficiency cooperates with BCL-2 in promoting chronic lymphocytic leukemia/small lymphocytic lymphoma in mice, possibly by specifically enforcing marginal zone B cell accumulation, increasing X-linked inhibitor of apoptosis expression, and rendering B cells independent of B cell activating factor for survival. The Journal of Immunology, 2012, 189: 1053–1061.

Tumor necrosis factor receptor-associated factors (TRAFs) constitute a family of trimeric adapter proteins that interact with the cytosolic regions of various members of the TNF-family receptors and with components of TLRs complexes. TRAFs function as docking molecules for kinases and other proteins involved in TNFR and TLR signaling. Furthermore, different members of the TRAF family also catalyze ubiquitination of various target proteins via their intrinsic E3 ubiquitin ligase activity. Thus, TRAFs can control the extent of the response by catalyzing the conjugation of a substrate with either lysine 48- or lysine 63-linked polyubiquitin chains, with differing consequences in terms of proteasome-dependent protein degradation and protein activation, respectively (1–3). Gene ablation studies in mice have demonstrated a critical role for different TRAF family members in regulating signaling by many TNFRs, and deregulation of these pathways has been shown to cause several autoimmune and inflammatory diseases, as well as cancer (reviewed in Ref. 4). Chronic lymphocytic leukemia (CLL) is the most common leukemia in the Western world, and it is characterized by the gradual accumulation of quiescent, apoptosis-resistant B cells (5). Although CLL is seemingly a very uniform disease, new molecular data have highlighted unexpected heterogeneity among patients. This is also reflected in the variability of the clinical progression of this leukemia, with patients suffering an indolent disease that does not require immediate treatment, patients with aggressive disease, and patients who develop resistance to current therapy. Predisposing genetic factors have been described, and they include trisomy 12, del(13q14), del(17p13), del(11q23), and chromosomal translocations. In this report, we describe that TRAF2 deficiency cooperates with BCL-2 in promoting chronic lymphocytic leukemia/small lymphocytic lymphoma in mice, possibly by specifically enforcing marginal zone B cell accumulation, increasing X-linked inhibitor of apoptosis expression, and rendering B cells independent of B cell activating factor for survival.

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G.P.-C. performed research and analyzed data. D.L., C.P., and J.P. performed research. Y.C. contributed reagents. J.C.R. contributed reagents, analyzed data, and helped in the writing of the paper. J.M.Z. designed the experiments, performed research, analyzed data, and wrote the paper.

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Abbreviations used in this article: BAFF, B cell activating factor; cIAP, cellular inhibitor of apoptosis; CLL, chronic lymphocytic leukemia; FO, follicular; IAP, inhibitor of apoptosis; MZ, marginal zone; NIK, NF-κB-inducing kinase; RING, really interesting new gene; SLL, small lymphocytic lymphoma; TRAF, TNFR-associated factor; TRAF2DN, TRAF2 mutant lacking the RING and zinc finger domains; tg, transgenic; WT, wild-type; XIAP, X-linked IAP.

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treatments. A need, therefore, exists to identify the critical modulators of CLL B cell growth and survival toward the goal of identifying new targets for therapeutic invention.

Previous results from our group demonstrated that double-transgenic (tg) mice expressing in B cells both a mutant Traf2 lacking the N-terminal region of the protein (Traf2DN) encompassing the really interesting new gene (RING) and zinc finger domains and also the antiapoptotic protein BCL-2 develop CLL/small lymphocytic lymphoma (SLL) with high incidence (>85%) in adulthood (9–16 mo) (6). The cooperation between Traf2DN and BCL-2 in promoting CLL/SLL was supported by the lack of significant incidence (<5%) of leukemia/lymphoma in single-tg mice carrying only the Traf2DN or the BCL-2 transgenes. B cells from these mice demonstrated a reduced rate of spontaneous apoptosis and resistance to apoptosis induced by chemotherapeutic drugs, but no increased proliferation, thus implying that resistance to apoptosis rather than deregulation of proliferation is responsible for the B cell accumulation in these mice.

Given the structural similarities between mutant Traf2DN and Traf1, both having highly conserved TRAF domains but lacking a RING domain, we proposed that Traf2DN might mimic Traf1 function and that the Traf2DN-tg mice might recapitulate the increased Traf1 expression observed in CLL cells from patients (7). In this report, we have further studied the role of Traf2DN in B cell transformation. We show that Traf2DN-tg B cells have very reduced expression of both Traf2 and Traf2DN compared with wild-type (WT) B cells, as a result of constitutive proteasome-dependent degradation, thus rendering B cell-specific Traf2DN-tg mice effectively equivalent to B cell-specific Traf2-deficient mice. We show that Traf2 deficiency in B cells enforces marginal zone (MZ) B cell accumulation and overcomes the dependency of B cells on B cell activating factor (BAFF) for survival, confirming previous results (8, 9). Traf2 deficiency also alters the expression levels of different proteins, including X-linked inhibitor of apoptosis (XIAP). This results suggest that unbridled BAFF signaling, XIAP upregulation, and BCL-2 provide nonredundant and complementary protection against apoptosis to MZ B cells and, when working in tandem, predispose human and mouse B cells to CLL and SLL.

Materials and Methods

tg mice

Lymphocyte-specific tg mice expressing a 1D4-epitope–tagged Traf2 deletion mutant lacking the N-terminal 240 aa encompassing the RING and zinc finger domains (Traf2DN) (10), B cell-specific BCL-2-tg mice mimicking the (14:18)(q32;21) translocation involving BCL-2 and IgH found in human follicular (FO) lymphomas (11), and Traf2DN/BCL-2 double-tg mice (6) have been described. The animal protocols were approved by the Institutional Animal Care and Use Committees from the hosting institutions. All tg mice in the study were genotypically heterozygous.

Genotyping

DNA was isolated from tg mouse tails using the Maxwell 16 system and the Maxwell 16 system DNA purification kit (Promega Ibérica, Madrid, Spain). A total of 300 ng DNA was used for amplification. Transgenes were amplified using GoTaq polymerase (Promega) and the following primers for Traf2 (forward, 5′-GACCGAGACAGATTGGACCC-3′; reverse, 5′-GCA CATAGGAAGATTGGCC-3′) and BCL-2 (forward, 5′-CTAGAGGATTGC TTTACGGGCCCTC-3′; reverse, 5′-ACCTGAGGAGACGGTACC-3′).

Reagents and Abs

Abs used were against Traf2 (C-20 and N-19), Traf3 (C-20), Traf1 (N-19), IgG1 (C-21), RELA (C-20), Santa Cruz Biotechnology, Santa Cruz, CA); BCL-XL, XIAP (BD Transduction Laboratories, Franklin, NJ); MCL-1 (Rockland, Gilbertsville, PA); JNK, phospho-JNK, ERK, phospho-ERK, p38, phospho-p38, AKT, phospho-AKT, p100/p52 NFκB2 (Cell Signaling Technologies, Danvers, MA); cellular inhibitors of apoptosis 1 and 2 (sIAP1/2), c-REL (R&D Systems, Abingdon, U.K.); and β-ACTIN (Sigma-Aldrich, St Louis, MO). Abs against human and mouse BCL-2 have been described previously (12). Rabbit polyclonal Abs against the cytosolic region of CD40 were prepared in the laboratory. Proteasome inhibitor MG-132 was from Calbiochem (La Jolla, CA). Bortezomib was kindly provided by Millenium Pharmaceuticals (Cambridge, MA).

Isolation and activation of B cells

Spleens from tg mice and WT littermates were mechanically processed, and mononuclear cells were isolated by Ficoll density centrifugation (Lymphocyte-M; Cedarlane Laboratories, Burlington, NC). B cells were isolated by negative magnetic selection using the StemSep mouse B cells enrichment kit (StemCells Technologies, Vancouver, CA), following the manufacturer’s specifications. B cells were resuspended in RPMI 1640 medium supplemented with 10% FCS (Hyclone, Logan, UT), 50 μM 2-ME, 100 μU/ml penicillin, 100 μg/ml streptomycin, 2 mm l-glutamine, and oxaloacetate, pyruvate, and insulin (OPT) media supplement (Sigma-Aldrich). Purified B cells (3–6×10^6) from age-matched sex-matched WT, and Traf2DN-tg littermates were left untreated or treated with the indicated concentrations of CD40L, BAFF, IL-4 (R&D Systems), and antimouse IgM (μ-chain specific; F(ab’)_2 fragment; Jackson Laboratories, West Grove, PA) for the indicated times.

Flow cytometry

Lymphocytes isolated as described earlier were incubated with 50 μg/ml hemocyanin-γ-globulin to block FcRs. Then 10^5 to 5×10^6 cells were incubated with a combination ofallocytoxycyanin-, FITC-, or PE-conjugated Abs recognizing various surface markers. After 1-h incubation at 4°C, cells were washed in high-glucose DMEM (without phenol red; Irvine Scientific, Santa Ana, CA) containing 3% FCS. Flow cytometry analysis was accomplished using a FACScalibur equipped with detectors for four colors (BD Biosciences, San Jose, CA).

Cell lysates and protein quantification

Cells were lysed in complete Laemmli buffer (0.125 M Tris pH 6.8; 4% SDS and 20% glycerol) supplemented with a mixture of protease inhibitors (Complete; Roche Diagnostics, Mannheim, Germany) and phosphatase inhibitors (PhosSTOP; Roche Diagnostics). Lysates were sonicated and protein concentration was determined by the bicinchoninic acid method (Pierce, Rockford, IL.).

SDS-PAGE and immunoblotting

Protein samples (10–20 μg/concentration) were prepared as described previously were supplemented with 10% 2-ME and 0.004% bromophenol blue, and SDS-PAGE analysis was performed as described previously (13). Abs were detected by ECL (Pierce) and either exposed on film or captured using the Gel Logic 1500 Image System and the Molecular Imaging Soft 4× Kodak software (Kodak Molecular Imaging Systems, New Haven, CT).

RNA isolation and RT-PCR

Purified B cells (5×10^6) were lysed in TRIzol (Invitrogen Life Technologies, Grand Island, NY) and RNA was purified using the RNAeasy kit (Qiagen Ibérica, Madrid, Spain), following the manufacturer’s instructions. Reverse transcription and PCR was carried out using the Superscript One-Step RT-PCR with platinum Taq kit from Invitrogen and primers for mouse Traf1 (forward, 5′-CTCAACAAAGGAGTGGAGGC-3′; R 5′-CGTGTGTTCTTGCTCTGCGTACC-3′), Traf2 (forward, 5′-GTTCTCTGCGAATTCCACACC-3′; reverse, 5′-CCAAATCTGCTCTGCTGTAAGACG-3′), Traf3 (forward, 5′-CTCGGCTACACATCATTAFTACCC-3′; reverse, 5′-CGAGTGCCAAGATCTTTCCACACC-3′), and Gapdh (forward, 5′-TGTACCAAAATGCGAGTTC-3′; reverse, 5′-CATTGAGGATGGAAGATGGG-3′).

B cell proliferation and survival

Purified B cells (5×10^5 per condition) were incubated in 96-well plates with the indicated stimuli for 72 h at 37°C in an atmosphere of 95% air and 5% CO2. Cell survival and proliferation were determined using either the CellTiter-Glo Luminescent Cell Viability Assay from Promega or by incubating the cells in each well with 0.5 μCi [3H]thymidine for 12 h before harvesting the cells.

Results

Traf2DN-tg B cells lack endogenous Traf2 expression

The Traf2 deletion mutant encoded by the Traf2DN transgene lacks the RING and zinc fingers domains required for the E3
ubiquitin ligase activity of TRAF2. As such, this truncated TRAF2 protein was conceived as a dominant inhibitor of endogenous, full-length TRAF2 (10). TRAF2DN expression was detectable by immunoblotting in spleen extracts from mice harboring the Traf2DN transgene (Traf2DN-tg and Traf2DN/BCL-2 double-tg mice), although it could not be detected in extracts from spleens isolated from WT and BCL-2-tg mice (Fig. 1A, top). However, as previously shown (6), endogenous TRAF2 expression was significantly reduced in splenocytes from Traf2DN-tg and Traf2DN/BCL-2 double-tg mice compared with splenocytes from mice that did not contain the Traf2DN transgene (WT and BCL-2; Fig. 1A, bottom). To further investigate these differences in TRAF2 expression, we purified B cells from Traf2DN-tg splenocytes and assessed the levels of expression of endogenous TRAF2 and mutant TRAF2DN. As shown in Fig. 1B, Traf2DN-tg B cells did not express significant levels of endogenous TRAF2 compared with WT B cells. Surprisingly, TRAF2DN expression was negligible in Traf2DN-tg B cells, thus indicating that Traf2DN-tg B cells had very reduced levels of both endogenous TRAF2 and mutant TRAF2DN expression (Fig. 1B). In contrast, TRAF3 expression was higher in Traf2DN-tg B cells compared with WT B cells (see later), whereas no differences in IxB expression were observed (Fig. 1B). Consistent with this result, communciprecipitation experiments showed that activated CD40 failed to recruit TRAF2 in B cells isolated from Traf2DN-tg mice, whereas TRAF2 was readily recruited to CD40 in B cells from WT mice (Fig. 1C). In contrast, TRAF3 was recruited to the activated CD40 in B cells from both Traf2DN-tg mice and WT littermates (Fig. 1C).

To rule out that failure to detect TRAF2 was not caused by posttranslational modifications of this protein resulting in epitope masking, we used several anti-TRAF2 Abs against different regions of the molecule, but all failed to detect TRAF2 in B cells extracts from the Traf2DN-tg mice. Representative results obtained using two anti-TRAF2 polyclonal Abs against the C or the N terminus of the molecule are shown in Fig. 2A.

We next assessed the levels of Traf2 mRNA in B cells from Traf2DN-tg and WT mice. As shown in Fig. 2B, Traf2 mRNA levels were similar in both Traf2DN-tg and WT B cells, thus indicating that failure to express TRAF2 by Traf2DN-tg B cells was not the result of nonproductive Traf2/Traf2DN recombination and/or disruption of endogenous Traf2 gene transcription. These results prompted us to evaluate whether the expression of other members of the TRAF family was also altered in the Traf2DN-tg B cells. In this regard, we observed weak but consistent Traf1 mRNA expression in WT B cells. However, Traf1 mRNA was absent in Traf2DN-tg B cells (Fig. 2A). This was in sharp contrast with the levels of Traf1 mRNA, which were significantly higher in Traf2DN-tg B cells compared with those in WT B cells (Fig. 2B). This result suggests that Traf1 mRNA transcription is upregulated in Traf2DN-tg B cells, although it does not result in Traf1 accumulation.

We have previously described that TRAF1 expression is upregulated in lymphocytes on activation (7). Therefore, to elucidate whether TRAF1 could accumulate in activated Traf2DN-tg B cells, we incubated B cells from these mice and from WT littermates with anti-μ Ab to engage the BCR. Indeed, as shown

![FIGURE 1.](http://www.jimmunol.org/) Endogenous TRAF2 is not expressed in B cells from Traf2DN-tg mice. (A) Splenocytes isolated from age-matched WT, Traf2DN-tg (T2DN), BCL-2-tg, and Traf2DN/BCL-2 double-tg (+/+) mice were lysed in Laemmli buffer, sonicated, and analyzed by SDS-PAGE and immunoblotting. Protein samples were normalized for protein content (25 μg) and blotted with anti-TRAF2 Abs. Cell lysate from the B cell line Ramos was used as control. Two different exposures of the same immunoblot are shown. (B) Purified B cells from spleens of Traf2DN-tg mice and WT littermates were analyzed as in (A), performing immunoblotting with Abs recognizing TRAF2, TRAF3, and IxB. (C) Splenocytes (10^7) isolated from Traf2DN-tg mice and WT littermates were preincubated on ice for 10 min with 6 μg/ml of an agonist rat anti-mouse CD40 mAb (Caltag, Burlingame, CA), followed by incubation at 37°C for the times indicated. Then, CD40+ cells were isolated using anti-rat Ab crosslinked to magnetic beads (Invitrogen, Carlsbad, CA). The recovered CD40+ B cells were lysed in isotonic buffer containing 1% Triton X-100. CD40-immunocomplexes were purified using a magnet and boiled for 3 min in Laemmli buffer. Immunocomplexes were analyzed by SDS-PAGE and immunoblotting using anti-TRAF3 and anti-TRAF2 Abs (Santa Cruz). L, Total splenocytes lysate (14 μg).

![FIGURE 2.](http://www.jimmunol.org/) Patterns of expression of TRAF family members in B cells from WT and Traf2DN-tg mice. (A) Splenocytes and purified B cells from spleens from Traf2DN-tg mice and WT littermates were lysed in Laemmli buffer. A total of 14 μg protein from each sample was analyzed by SDS-PAGE and immunoblotting with Abs indicated. (B) Total RNA was extracted from purified B cells from WT and Traf2DN-tg mice, and 1 μg RNA from each sample was used for retrotranscription followed by specific PCR amplification of the indicated cDNAs. (C) Purified B cells from Traf2DN-tg and WT splenocytes were left untreated or activated with 100 μg/ml anti-μ Ab for the indicated times. At the end of the incubation period, cells were collected and lysed in Laemmli buffer. Lysates from 10^6 B cells from each condition were analyzed by SDS-PAGE and immunoblotting.
in Fig. 2C, TRAF1 upregulation was evident after 60 min of activation in both WT and Traf2DN-tg B cells, indicating no impediment of TRAF1 expression in activated TRAF2-deficient B cells. In contrast, expression of endogenous TRAF2 was not restored in BCR-activated Traf2DN-tg B cells, which would be consistent with our previous results (7) indicating that TRAF2 expression is not upregulated in activated B cells (Fig. 2C). In contrast, TRAF3 protein levels were higher in Traf2DN-tg B cells compared with WT B cells (Fig. 2A), whereas no differences in the amounts of Traf3 mRNA were observed in B cells from both sources (Fig. 2B). TRAF3 accumulation in TRAF2-deficient B cells would be consistent with previous results from Hostager and coworkers (14) showing that TRAF3 is ubiquitinated by TRAF2 and subsequently degraded. Finally, IκBα (Fig. 2A), Gapdh mRNA (Fig. 2B), and β-ACTIN (Fig. 2C) were used as RNA and protein loading controls, showing comparable levels of expression in Traf2DN-tg and WT B cells.

**TRAF2 depletion in Traf2DN-tg B cells is caused by proteasome-dependent degradation**

Because TRAF2 is a substrate for K48-ubiquitination (15), we next assessed whether lack of TRAF2 expression in Traf2DN-tg B cells could be the result of proteasome-dependent degradation. Indeed, incubation of Traf2DN-tg B cells with the proteasome inhibitors bortezomib and MG-132 resulted in the accumulation of endogenous TRAF2 (Fig. 3). Furthermore, the expression of Traf2DN transgene was also increased in the presence of both proteasome inhibitors (Fig. 3). This result suggests that expression of mutant TRAF2DN (which lacks the RING finger domain required for E3 ubiquitin ligase activity, but which retains the TRAF domain and coiled-coil segments that mediate trimerization of TRAF2 molecules) alters endogenous TRAF2 protein homeostasis by promoting proteasome-dependent degradation. As a control for the inhibitory effect of bortezomib and MG-132 on proteasome activity, we measured levels of IκBα, a known proteasome substrate, finding that this protein accumulated in B cells in the presence of both proteasome inhibitors (Fig. 3).

**B cell differentiation and B cell responses to CD40L and BAFF are altered in Traf2DN-tg mice**

Previously, it was reported that genetically engineered mice containing homozygous targeted Traf2 genes restricted to B cells show expanded MZ B cell populations and exhibit impaired responses to CD40 and BAFF-R engagement (8). Consistent with the idea that the Traf2DN-tg B cells are indeed TRAF2-deficient B cells, spleens from Traf2DN-tg mice also have an expanded B cell population characterized by the expression of IgM<sup>MZ</sup> CD21<sup>hi</sup>CD23<sup>lo</sup>, which is consistent with a splenic MZ phenotype (Fig. 4A) (6). In this regard, we have previously described (7) that TRAF2 expression is absent in MZ lymphocytes, whereas it is expressed in germinal center lymphocytes, thus suggesting that TRAF2 activity might favor FO B cell differentiation, survival, or proliferation, to the detriment of the MZ B lineage.

Interestingly, survival of resting Traf2DN-tg B cells in culture was remarkably increased compared with that of WT B cells (p = 0.001; Fig. 4B). In contrast, incubation of WT B cells with BAFF (TNFSF13B) promoted their survival in culture, whereas BAFF only slightly increased Traf2DN-tg B cell survival (p = 0.12). Notably, survival of WT B cells in the presence of BAFF was comparable with that of BAFF-treated Traf2DN-tg B cells (p = 0.67). Furthermore, BAFF failed to provide additional survival advantages to Traf2DN-tg B cells activated with anti-μ, in contrast with WT B cells (Fig. 4C). Altogether, these results suggest that Traf2DN-tg B cells are not dependent on BAFF for survival, most likely because Traf2DN-tg B cells have constitutively activated survival pathways that circumvent the requirement for BAFF, confirming previous results using B cell-specific Traf2<sup>−/−</sup> mice (8, 9). Furthermore, we observed that induction of B cell proliferation by CD40 engagement was significantly reduced in the Traf2DN-tg B cells compared with WT B cells (Fig. 4D), thus confirming that Traf2 is required for efficient CD40-mediated B cell proliferation (8, 14).

**Constitutive p100 NFκB2 processing in Traf2DN-tg B cells**

It was previously described that targeted deletion of Traf2 caused constitutive NF-κB2 activation in B cells (8, 9) and in other cell types (9, 16, 17). Consistent with prior results, we found that Traf2DN-tg B cells showed increased p100 NF-κB2 processing to active p52 (Fig. 5A, 6A). Interestingly, constitutive activation of NF-κB2 has been shown to promote MZ B cell differentiation (18), and BAFF-mediated MZ B cell expansion is reportedly dependent on NF-κB2 activity (19). Consequently, the accumulation of MZ B cells in the Traf2DN-tg mice might be explained by this constitutive NF-κB2 activity. In contrast, IκBα degradation in response to CD40 activation was similar in B cells from the Traf2DN-tg mice and WT littermates (Fig. 5A), and no differences in IκBα expression were noticed regardless the presence or absence of TRAF2 (Fig. 2A), which is consistent with what has been described in B cells with targeted Traf2 deletion (14, 20). The levels of the NF-κB subunits c-REL and RELA were also similar in WT and Traf2DN-tg B cells (Fig. 6A).

**TRAF2 is differentially required for MAPK activation in response to CD40 and BCR stimulation**

To examine signaling alterations in B cells lacking TRAF2, we analyzed the activation of certain MAPKs in splenic B cells from Traf2DN-tg mice and WT littermates in response to CD40 and BCR engagement. As shown in Fig. 5A, CD40-mediated activation of JNK was significantly reduced in Traf2DN-tg B cells compared with WT B cells, confirming previous results (10). Moreover, activation or ERK1/2 was also significantly reduced in B cells lacking TRAF2. In contrast, CD40-mediated p38 MAPK activation was similar irrespective of the presence or absence of TRAF2 (Fig. 5A). These differences in MAPK activation were not the result of differential expression of JNK, ERK, and p38, which was similar in Traf2DN-tg and WT B cells (Fig. 5). Altogether, these results are consistent with those found in Traf2<sup>−/−</sup> B cells (20).

![FIGURE 3](http://www.jimmunol.org/Downloaded from http://www.jimmunol.org/). The expression of endogenous TRAF2 and mutant TRAF2DN is recovered by proteasome inhibition. B cells from Traf2DN-tg mice (2.5 × 10<sup>6</sup> cells/condition) were left untreated or incubated either with bortezomib (2.5 or 10 ng/ml) for 18 h or with MG-132 (100 or 300 nM) for 6 h. Then B cells were lysed in Laemmli buffer and sonicated. A total of 10 μg protein from each sample was analyzed by SDS-PAGE and immunoblotting using Abs against TRAF2, IκBα, and β-ACTIN.
BCR-mediated MAPK activation was also affected in B cells by TRAF2. Specifically, when anti-μ mAb was used to trigger BCR activation of B cells isolated from the Traf2DN-tg mice, we observed increased BCR-mediated ERK activation and significantly reduced p38 MAPK activation in these cells compared with WT B cells (Fig. 5B). In contrast, the level of JNK phosphorylation triggered by BCR engagement was similar regardless of the presence or absence of TRAF2 (Fig. 5B). However, although JNK phosphorylation returned to basal levels 60 min after BCR engagement in WT B cells, it remained heavily phosphorylated in Traf2DN-tg B cells (data not shown). In contrast, BCR-mediated AKT activation was also independent of TRAF2 (Fig. 5B).

**TRAF2 deficiency in B cells is associated with decline of cIAP1/cIAP2 expression and upregulation of XIAP**

We next assessed the effect of TRAF2 deficiency on the expression of various proteins relevant to CLL that are implicated in the control of apoptosis, including members of the BCL-2 and inhibitor of apoptosis (IAP) families. First, the analysis of the expression levels of BCL-2 and MCL-1 proteins in B cells isolated from the spleens of Traf2DN-tg mice and WT littermates by immunoblotting failed to show any significant difference (Fig. 6A). However, BCL-XL protein expression was upregulated in B cells from Traf2DN-tg mice (Fig. 6A), consistent with prior descriptions of Traf2−/− B cells (9). Furthermore, analysis of expression of IAP family members cIAP1, cIAP2, and XIAP by immunoblotting showed differential results depending on the presence or absence of TRAF2. Thus, cIAP1 and cIAP2 were absent in B cells from the Traf2DN-tg mice, although they were detected in WT B cells (Fig. 6A). This result is in agreement with Csomos and coworkers (21, 22), who have reported that cIAP1 and cIAP2 undergo autoubiquitination and subsequent proteasome degradation in the absence of TRAF2. In contrast, expression of caspase inhibitor XIAP was significantly upregulated in B cells lacking TRAF2 (Fig. 6A). Interestingly, cIAP1 has been shown to bind XIAP and to induce its proteasomal degradation by ubiquitin-dependent and -independent pathways (23, 24). Therefore, depletion of cIAP1 might underlie XIAP upregulation in the Traf2DN-tg B cells.

Next, we asked whether these differences in protein expression observed in Traf2DN-tg B cells were also found in Traf2DN/BCL-2 double-tg mice that had developed CLL/SLL. For that purpose, heterozygous Traf2DN-tg and BCL-2−/− mice were crossed to produce litters representing the four possible genotypes. Once Traf2DN/BCL-2 double-tg mice (+/+ ) had developed overt CLL/SLL, characterized by high B cells counts in blood (>107 B cells/ml) and severe splenomegaly and lymphadenopathy, as de-
pressed the human BCL-2 transgene (Fig. 6B). WT and BCL-2 expression, whereas Traf2 (+/-) and XIAP, and had comparable levels of p100 NF-kB cells expressed similar amounts of TRAF2, TRAF1, cIAP1/2, and ubiquitination in response to receptor activation (28). Although signaling through TNFR2 induces cIAP1-dependent degradation as a result of TRAF2-mediated ubiquitination of TRAF2 (27), activation of CD30 triggers cIAP1 degradation as a result of TRAF2-mediated ubiquitination of cIAP1 (21). In contrast, other reports have described the ability of TRAF2 to activate cIAP1 and cIAP2 by catalyzing their K63-ubiquitination in response to receptor activation (28). Although seemingly contradictory, these results illustrate the complexity of TRAF biology, where variations in mechanisms can reflect pancreatic B cells but variable expression of BCL-XL, ranging from the levels found in WT B cells to those found in Traf2/DN-tg and BCL-2-tg B cells (Fig. 6B). In contrast, Traf2/DN/tg double-tg lymphoma cells showed a pattern of protein expression similar to Traf2/DN-tg B cells, including constitutive p100 NF-kB processing, absence of detectable TRAF2, TRAF1, and cIAP1/2, and increased XIAP expression. Altogether, these results suggest that inhibition of cIAP1/2 expression, p100 NF-kB2 activation, and XIAP upregulation may contribute to the increased resistance to apoptosis observed in TRAF2-deficient cells.

Discussion

The Traf2/DN-tg mouse model was engineered to overexpress a TRAF2 deletion mutant lacking the RING and zinc fingers domains (Traf2/DN) (10). This mutant was conceived as a dominant inhibitor of TRAF2 because it lacks the RING domain required for E3 ubiquitin ligase activity, but still contains the TRAF domain, thus retaining the ability to interact with TNFR family members and to trimerize with endogenous TRAF2 (26). However, our results indicate that Traf2/DN-tg B cells not only fail to accumulate mutant Traf2/DN protein, but also lack expression of endogenous TRAF2 protein, effectively turning Traf2/DN-tg B cells into bona fide Traf2-deficient B cells.

These results strongly suggest that expression of the E3 defective Traf2/DN mutant in B cells enforces continuous basal ubiquitination and subsequent proteasome-mediated degradation of both endogenous TRAF2 and mutant Traf2/DN in B cells, as indicated by the restoration of TRAF2 and Traf2/DN expression in Traf2/DN-tg B cells upon incubation with the proteasome inhibitors bortezomib and MG-132. Although it remains unclear what protein is responsible for TRAF2 ubiquitination, a likely candidate is TRAF2 itself. Indeed, it has been described that on receptor activation, TRAF2 can catalyze its own K48-ubiquitination, thus targeting TRAF2 for proteasome-dependent degradation (15). TRAF2 ubiquitination could also be catalyzed by cIAP1 and cIAP2, which interact with TRAF2 and are recruited to various members of the TNFR family on activation. However, although signaling through TNFR2 induces cIAP1-dependent Traf2/DN degradation (27), activation of CD30 triggers cIAP1 degradation as a result of TRAF2-mediated ubiquitination of cIAP1 (21). In contrast, other reports have described the ability of TRAF2 to activate cIAP1 and cIAP2 by catalyzing their K63-ubiquitination in response to receptor activation (28). Although seemingly contradictory, these results illustrate the complexity of TRAF biology, where variations in mechanisms can reflect...
The role of TRAF2 in control of B cell homeostasis is well established. The original description of the \textit{Traf2}\textsuperscript{TNF}\textsuperscript{-tg} mice that we used demonstrated that disrupting TRAF2 function causes splenomegaly and lymphadenopathy as a result of polyclonal expansion of B cells (10). We confirmed those observations and further demonstrated a role for TRAF2 as a B cell tumor suppressor, showing that disruption of TRAF2 function cooperates with BCL-2 to develop CLL/SLL (6). Additional evidence of the role of TRAF2 in B cell homeostasis was obtained by Brink and coworkers (22) describing that, in the absence of TRAF2, cIAP1 and cIAP2 undergo auto-ubiquitination and subsequent degradation. In further support of this hypothesis, we have confirmed that proteasome inhibition causes cIAP1/2 protein accumulation in \textit{Traf2}\textsuperscript{TNF}\textsuperscript{-tg} B cells (data not shown). Furthermore, cIAP1 depletion might underlie XIAP upregulation in \textit{Traf2}\textsuperscript{TNF}\textsuperscript{-tg} B cells, because it has been shown that cIAP1 targets XIAP for proteasomal degradation by ubiquitin-dependent and -independent pathways (23, 24).

In support of a role for BAFF in CLL/SLL causative factors is the fact that BAFF cooperates with various oncogenes to promote CLL/SLL in mice. Indeed, either mice with B cells having constitutively activated BAFF-mediated pathways (as is the case of \textit{Traf2}\textsuperscript{TNF/BCL-2} double-tg mice) or mice with B cells continuously exposed to high levels of circulating BAFF (\textit{Baff/Tcl-1} and \textit{Baffc-Myc} double-tg mice) (32, 33) develop CLL/SLL.

It is interesting to mention that, similar to \textit{Baff}\textsuperscript{-tg} mice (32–34), deregulation of NF-\kappaB2 activation predisposes to either autoimmunity or CLL/SLL. In this regard, Zhang and coworkers (35) developed \textit{tg} mice expressing in lymphocytes p80HT, a lymphoma-associated NF-\kappaB2 mutant (36). These mice displayed a marked expansion of peripheral B cell populations and developed SLL. B cells from these mice were also resistant to apoptosis induced by cytokine deprivation and mitogenic stimulation. However, the same group also developed \textit{tg} mice overexpressing in B cells p52, the proteolytic product of p100 NF-\kappaB2 normally produced on activation (37). These mice did not develop SLL, but they were predisposed to inflammatory autoimmune disease. These results place NF-\kappaB2 at the crossroads of autoimmunity and CLL/SLL in B cells, both of which are known to be promoted by defects in apoptosis. However, our results with the \textit{Traf2}\textsuperscript{TNF}\textsuperscript{-tg} mice showed that constitutive NF-\kappaB2 activation may underlie MZ B cell expansion and might contribute to BAFF-independent B cell survival, but it is not sufficient to promote either B cell transformation or overt B cell-dependent autoimmunity in this model.

Notably, TRAF2 deficiency also has an impact in the signaling cascades activated by the BCR and by various members of the TNFR-family. Thus, we have observed significant differences in early MAPK activation by the BCR, with higher ERK and reduced p38 MAPK activation in the absence of TRAF2. It remains unclear how TRAF2 would control early BCR-mediated signaling, but altered BCR signaling in the TRAF2-deficient B cells might underlie the absence of autoimmune symptoms in these mice. However, these differences in BCR signaling might be the result of MZ B cell expansion, because it is well established that FO B cells and MZ B cells have different requirements for BCR signaling, but altered BCR signaling in the TRAF2-deficient B cells might underlie the absence of autoimmune symptoms in these mice. However, these differences in BCR signaling might be the result of MZ B cell expansion, because it is well established that FO B cells and MZ B cells have different requirements for BCR activation (38).

Our data also indicate that, in the absence of TRAF2, CD40 engagement failed to efficiently activate JNK and ERK, although p38 MAPK activation and I\kappaB\alpha degradation remained intact, confirming previous reports (14, 20). We also showed that CD40L-mediated B cell proliferation was significantly reduced in the \textit{Traf2}\textsuperscript{TNF}\textsuperscript{-tg} B cells compared with that of WT B cells. Interestingly, recent evidence (39) has shown that CLL patients can be segregated in two distinct functional subsets according to their dependency on CD40L for leukemic expansion, with CD40L-independent CLL patients having a shorter time to progression. Thus, the CLL/SLL developed by the \textit{Traf2}\textsuperscript{TNF/BCL-2} double-tg mice might be the mouse counterpart of this CD40L-independent human CLL subset.

In this regard, it is interesting to compare the \textit{Traf2}\textsuperscript{TNF/BCL-2} mouse model with the mechanisms believed to underlie the pathogenesis of human CLL. Most cases of CLL have aberrantly high levels of BCL-2 expression because of loss of gene encoding
microRNAs (miRs) that suppress expression of this antiapoptotic gene. Loss of the BCL-2-targeting miRs (miR15a and 16-1) as a consequence of 13q14 deletions is the most common genetic lesion thus far identified in CLL, occurring in >50% of cases. Our mouse model clearly recapitulates this circumstance. B cells from our Traf2DN/Bcl-2 double-tg mice also contain increased levels of XIAP. High levels of XIAP expression have been found in malignant B cells from CLL patients (40), and targeting XIAP expression has been shown to induce apoptosis of CLL cells in culture (41–43). We also observed a decline in cIAP1 and cIAP2 protein levels in murine Traf2DN/Bcl-2 double-tg B cells. In this regard, inactivating mutations in the human BIRC3 gene encoding cIAP2 have been found in CLL (44) and in other B lymphoid malignancies (45–47), and BIRC3/MALT1 translocations are causative of mucosal-associated lymphoid tissue lymphoma (30). Thus, this mouse model also recapitulates additional features of human CLL.

Currently, there is no evidence of mutations of the Traf2 gene in CLL patients (45, 48, 49). However, there might be alternative mechanisms to inhibit Traf2 function leading to B cell transformation. In this regard, Thomas and coworkers (50) have determined that transformation of B lymphocytes constitutively activates cell survival pathways and inactivates Bcl-2 (51). Finally, it is noteworthy that Traf1 is overexpressed in CLL cells, with higher Traf1 levels correlating with the development of refractory disease (7). Traf1 is the only member of the Traf family that lacks the RING domain; therefore, it lacks the ability to function as an E3 ubiquitin ligase. Traf1 can form hetero- 

References


