

TRAF2 deficiency in B lymphocytes predisposes to chronic lymphocytic leukemia/small lymphocytic lymphoma in mice¹

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ABSTRACT

We have previously shown that transgenic mice expressing in B lymphocytes both Bcl-2 and a TRAF2 mutant lacking the RING and zinc finger domains (TRAF2DN) develop small lymphocytic lymphoma (SLL) and chronic lymphocytic leukemia (CLL) with high incidence. Further analysis of the expression of TRAF2 and TRAF2DN in purified B cells demonstrated that expression of both endogenous TRAF2 and transgenic TRAF2DN was negligible in TRAF2DN-tg B cells compared to wild-type mice. This was the result of proteasome-dependent degradation, and rendered TRAF2DN B cells as *bona fide* TRAF2 deficient B cells. In agreement to what has been described in B cells with targeted TRAF2 deletion, TRAF2DN-tg mice show expanded marginal zone B cell population and have constitutive p100 NF- κ B2 processing. Also, TRAF3, XIAP and Bcl-X_L expression levels were increased, while cIAP1/2 levels were drastically reduced compared to 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 BAFF for survival. Altogether, our results suggest that TRAF2 deficiency cooperates with Bcl-2 in promoting CLL/SLL in mice, possibly by specifically enforcing MZ B cell accumulation and rendering B cells independent of BAFF for survival.

INTRODUCTION

TNF-Receptor Associated Factors (TRAFs) constitute a family of trimeric adapter proteins that interact with the cytosolic regions of various members of the Tumor Necrosis Factor (TNF)-family receptors (TNFRs) and with components of Toll-Like Receptors (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 poly-ubiquitin 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 (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 do not require immediate treatment, patients with aggressive disease, and patients that develop resistance to current treatments. A need, therefore, exists to identify the critical modulators of CLL B cell growth and survival towards 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 RING and zinc finger domains and also the anti-apoptotic protein Bcl-2 develop CLL/small lymphocytic lymphoma (SLL) with high incidence (>85%) in adulthood (9-16 months) (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 to wild-type 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 MZ B cell accumulation and overcomes the dependency of B cells on BAFF for survival, confirming previous results (8, 9). These results suggest that unbridled BAFF signaling and Bcl-2 provide non-redundant 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

Transgenic mice. Lymphocyte-specific transgenic mice expressing a 1D4-epitope tagged TRAF2 deletion mutant lacking the N-terminal 240 amino acids 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 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 transgenic mice in the study were genotypically heterozygotes.

Genotyping. DNA was isolated from transgenic mouse tails using the Maxwell[®] 16 system and the Maxwell[®] 16 system DNA purification kit (Promega Biotech Iberica, Spain). A total of 300 ng of DNA was used for amplification. Transgenes were amplified using GoTaq polymerase (Promega) and the following primers for TRAF2 (F 5'-GACCAGGACAAGATTGAGGC-3' and R 5'-GCACATAGGAATTCTTGGCC-3') and Bcl-2 (F 5'-TTAGAGAGTTGCTTTACGTGGCCTC-3' and R 5'-ACCTGAGGAGACGGTGACC 3').

Reagents and antibodies. Antibodies used were against TRAF2 (C-20 and N-19), TRAF3 (C-20), TRAF1 (N-19), I κ B α (C-21), RelA (C-20) (Santa Cruz Biotech, Santa Cruz, CA), Bcl-x_L, 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 NF κ B2 (Cell Signaling Technologies, Danvers, MA), cIAP1/2, c-Rel (R&D Systems, Abingdon, UK) and β -actin (Sigma-Aldrich, St Louis, MO). Antibodies against human and mouse Bcl-2 have been described (12). Rabbit polyclonal antibodies 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 wild-type (WT) littermates were mechanically processed and mononuclear cells were isolated by Ficoll density centrifugation (Lympholyte[®]-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 μ M 2-mercaptoethanol, 10 U/ml of penicillin, 100 μ g/ml of streptomycin, 2 mM L-glutamine, and oxaloacetate, pyruvate and insulin (OPI) media supplement (Sigma-Aldrich). Purified B cells ($3-6 \times 10^6$) from age-matched, sex matched WT and TRAF2DN-tg littermates were left untreated or treated with the indicated concentrations of CD40L, B cell activating factor (BAFF), IL-4 (R&D systems) and anti-mouse IgM (μ -chain-specific; F(ab')₂ fragment) (Jackson Laboratories, West Grove, PA) for the indicated times.

Flow cytometry. Lymphocytes isolated as described above were incubated with 50 μ g/ml human γ -globulin to block Fc receptors. Then, 10^5 to 5×10^5 cells were incubated with a combination of allophycocyanin (APC)-, FITC- or PE-conjugated antibodies 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 4 colors (BD Biosciences, San Jose, CA).

Cell lysates and protein quantification: Cells were lysed in incomplete Laemmli buffer (0.125M Tris pH 6.8; 4% SDS and 20% glycerol) supplemented with a cocktail 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 (BCA) method (Pierce, Rockford, IL).

SDS-PAGE and immunoblotting. Protein samples (10-20 μ g/condition) prepared as described above were supplemented with 10% β -mercaptoethanol and 0.004% bromophenol blue and SDS-PAGE analysis was performed as described (13). Antigens were detected by enhanced chemiluminescence (Pierce) and either exposed on film or captured using the Gel Logic 1500 Image System and the Molecular Imaging Soft 4X 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 RNeasy kit (Qiagen Iberica, 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 (F 5'-CTCAACAAGGAAGTGGAGGC-3'; R 5'-CGGTTGTTCTGGTCAAGTAGC-3'), TRAF2 (F 5'-GTTCTCTGCAGATCCACACC-3'; R 5'-CCATATCTGCTTGTGTAGAAGGC-3'), TRAF3 (F 5'-CCTGGCACACATCTTATAGCC-3'; R 5'-CGAGTCCAAGATCCTTCACC-3') and GAPDH (F 5'-TGTCAGCAATGCATCCTGC-3'; R 5'-CATCGAAGGTGGAAGAGTGG-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 hours at 37 °C in an atmosphere of 95% air and 5% CO₂. Cell survival and proliferation was determined using either the CellTiter-Glo[®] Luminescent Cell Viability Assay from Promega or by incubating the cells in each well with 0.5 μCi of [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), while 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 to 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, B cells were purified from TRAF2DN-tg splenocytes and the levels of expression of endogenous TRAF2 and of TRAF2DN-tg were assessed. As shown in Fig. 1B, TRAF2DN-tg B cells did not express significant levels of endogenous TRAF2 compared to 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 to WT B cells (see below), while no differences in I κ B α expression was observed (Fig. 1B). Consistent with this result, coimmunoprecipitation experiments showed that activated CD40 failed to recruit TRAF2 in B cells isolated from TRAF2DN-tg mice, while 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, several anti-TRAF2 antibodies against different regions of the molecule were used, but all failed to detect TRAF2 in B cells extracts from the TRAF2DN-tg mice. Representative results obtained using two anti-TRAF2 polyclonal Ab 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 non-

productive *traf2/traf2DN* recombination and/or by 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 expression in WT B cells. However, TRAF1 was absent in TRAF2DN-tg B cells (Fig. 2A). This was in sharp contrast to the levels of TRAF1 mRNA, which were significantly higher in TRAF2DN-tg B cells compared to 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 upon 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 mAb to engage the BCR. Indeed, as shown 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 TRAF2 was not restored in BCR-activated TRAF2-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 to WT B cells (Fig. 2A), while 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, I κ B α (Fig. 2A), GAPDH mRNA (Fig. 2B) and β -actin (Fig. 2C) were used as protein and RNA 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

Since 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 the TRAF2DN mutant (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 I κ B α , 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 marginal zone (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^{high}CD21^{high}CD23^{low}, which is consistent with a splenic MZ phenotype (Fig. 4A and (6)). In this regard, we have previously described (7) that TRAF2 expression is absent in MZ lymphocytes, while it is expressed in germinal center lymphocytes, thus suggesting that TRAF2 activity might favor follicular 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 longer than WT B cells ($p = 0.001$) (Fig. 4B). In contrast, incubation of WT B cells with B cell activating factor (BAFF; TNFSF13B) promoted their survival in culture, while BAFF only slightly increased TRAF2DN-tg B cells survival ($p = 0.12$). Notably, survival of WT B cells in the presence of BAFF was comparable to 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 to 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*^{-/-} 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 to WT B cells (Fig. 4D), thus confirming that TRAF2 is required for efficient CD40-mediated B cell proliferation (8, 14).

Constitutive p100 NF- κ B2 processing in TRAF2DN-tg B cells

It was previously described that targeted deletion of *TRAF2* caused constitutive NF- κ B2 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- κ B2 processing to active p52 (Fig. 5A and 6A). Interestingly, constitutive activation of NF- κ B2 has been shown to promote MZ B cell differentiation (18) and BAFF-mediated MZ B cell expansion is reportedly dependent on NF- κ B2 activity (19). Consequently, the accumulation of MZ B cells in the TRAF2DN-tg mice might be explained by this constitutive NF- κ B2 activity. In contrast, I κ B α 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 I κ B α expression were noticed regardless the presence or absence of TRAF2 (Fig. 6A), consistently to what has been described in B cells with targeted TRAF2 deletion (14, 20). The levels of the NF- κ B subunits c-Rel and RelA were also similar in WT and TRAF2DN-tg B cells (Fig. 6A).

TRAF2 is differentially required for Mitogen Activated Protein Kinases (MAPKs) activation in response to CD40 and B Cell Receptor (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 to WT B cells, confirming previous results (10). Moreover, activation of 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 to those found in TRAF2^{-/-} B cells (20).

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 to WT B cells (Fig. 5B). In contrast, the level of JNK phosphorylation triggered by BCR engagement was similar regardless the presence or absence of

TRAF2 (Fig. 5B). However, while JNK phosphorylation returned to basal levels 60 min after BCR engagement in WT B cells, it remained heavily phosphorylated in TRAF2DN-tg B cells (not shown). In contrast, BCR-mediated AKT activation was also independent of TRAF2 (Fig. 5B).

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

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 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-X_L protein expression was upregulated in B cells from TRAF2DN-tg mice (Fig. 6A), consistent with prior descriptions of TRAF2-deficient 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, while 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).

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-tg 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 ($> 10^7$ B cells/ml) and severe splenomegaly and lymphadenopathy, as described (6, 23), B cells were purified from the spleen of these mice and also from age-matched WT (-/-), TRAF2DN-tg (+/-), and Bcl-2-tg (-/+) littermates. As expected, mice bearing the TRAF2DN transgene (+/- and +/+) lacked endogenous TRAF2 expression, while Bcl-2-tg mice (-/+ and +/+) strongly expressed the human Bcl-2 transgene (Fig. 6B). WT and Bcl-2-tg B cells expressed similar amounts of TRAF2, TRAF1, cIAP1/2 and XIAP, and had comparable levels of p100 NF- κ B2 processing. However, Bcl-2-tg B cells showed increased expression of Bcl-X_L and reduced levels of Mcl-1. TRAF2DN/Bcl-2-

double tg B cells had slightly reduced Mcl-1 levels compared to WT B cells but variable expression of Bcl-X_L, ranging from the levels found in WT B cells to those found in TRAF2DN-tg and Bcl-2-tg B cells (Fig. 6B). In contrast, TRAF2DN/Bcl-2-double tg lymphoma cells showed a pattern of protein expression similar to TRAF2DN-tg B cells, including constitutive p100 NF- κ B2 processing, absence of detectable TRAF2, TRAF1, and cIAP1/2, and increased XIAP expression. Altogether, these results suggest that p100 NF- κ B2 activation, upregulation of XIAP, and inhibition of cIAP1/2 expression may contribute to the increased resistance to apoptosis observed in TRAF2-deficient B cells.

DISCUSSION

The TRAF2DN-tg mouse model was engineered to overexpress a TRAF2 deletion mutant lacking the RING and zinc fingers domains (TRAF2DN) (10). This mutant was conceived as a dominant inhibitor of TRAF2 since 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 (24). However, our results indicate that TRAF2DN-tg B cells not only fail to accumulate mutant TRAF2DN protein, but also lack expression of endogenous TRAF2 protein, effectively turning TRAF2DN-tg B cells into *bona fide* TRAF2-deficient B cells.

These results strongly suggest that expression of the E3 defective TRAF2DN mutant in B cells enforces continuous basal ubiquitination and subsequent proteasome-mediated degradation of both endogenous TRAF2 and mutant TRAF2DN in B cells, as indicated by the restoration of TRAF2 and TRAF2DN expression in TRAF2DN-tg B cells upon incubation with the proteasome inhibitors bortezomib and MG-132. While it remains unclear what protein is responsible for TRAF2 ubiquitination, a likely candidate is TRAF2 itself. Indeed, it has been described that upon 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 2, which interact with TRAF2 and are recruited to various members of the tumor necrosis factor receptor (TNFR) family upon activation. However, while signaling through TNFR2 induces cIAP1-dependent TRAF2 degradation (25), activation of CD30 triggers cIAP1 degradation as a result of TRAF2-mediated ubiquitination of cIAP (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 (26). Although seemingly contradictory, these results illustrate the complexity of TRAF biology, where variations in mechanisms can reflect differences in cell types, specific ligand/receptor signaling systems, and the strength of the activation signal.

In any event, it remains an open question whether TRAF2 ubiquitination and degradation in TRAF2DN-tg B cells are dependent on receptor activation. In this regard, it should be expected that if TRAF2 degradation was stimulus-dependent, culturing TRAF2DN-tg B cells *in vitro* in the absence of any stimulus should restore TRAF2 accumulation, but we have failed to detect TRAF2 or TRAF2DN in cultures of non-activated TRAF2DN-tg B cells (data not shown). Interestingly, we also discovered an absence of cIAP1 and cIAP2 proteins in TRAF2DN-tg B cells. This result is

consistent with previous data from Csomos and coworkers (22) describing that in the absence of TRAF2, cIAP1 and cIAP2 undergo autoubiquitination and subsequent degradation. In further support of this hypothesis, we have confirmed that proteasome inhibition causes cIAP1/2 protein accumulation in TRAF2DN-tg B cells (data not shown).

The role of TRAF2 in control of B cell homeostasis is well established. The original description of the TRAF2DN-tg mice that we employed 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 (8, 9) using mice with *traf2*-deficient B cells. These B cell-specific *traf2*^{-/-} mice also developed splenomegaly and lymphadenopathy as a result of the expansion of mature B lymphocytes, in particular, cells with a MZ phenotype, as we have further confirmed in this report. Indeed, around 50% of splenic B cells from the TRAF2DN-tg mice have a MZ phenotype.

B cell expansion in both TRAF2DN-tg mice and B cell specific-*traf2*^{-/-} mice is the result of increased B cell survival rather than by deregulated proliferation. Our results concur with those of Gardam and coworkers (9) in that TRAF2-deficiency renders B cells independent of BAFF for their survival. Indeed, *in vitro* survival of non-stimulated TRAF2DN-tg B cells was similar to that of BAFF-treated WT B cells, and no significant increase in TRAF2DN B cells survival was achieved by culturing these cells in the presence of BAFF. Furthermore, we previously showed that TRAF2DN-tg B cells had reduced sensitivity to apoptosis induced by dexamethasone and fludarabine compared to WT B cells (6), further supporting that TRAF2 deficiency provides survival advantages to B cells.

We have observed constitutive processing of p100 NF- κ B2 into yield active p52 in TRAF2DN-tg B cells, confirming previous results from Grech and coworkers (8) in *traf2*^{-/-} B cells. Both MZ differentiation (18) and BAFF-independent increased survival of TRAF2-deficient B cells are likely consequence of this constitutive NF- κ B2 activation. In this regard, the molecular mechanism controlling NF- κ B2 activation in B cells has been previously elucidated, showing that, in resting B cells, p100 NF- κ B2 processing is prevented by maintaining NIK levels low. The mechanism underlying NIK downregulation involves its interaction with TRAF3, which acts as a bridge bringing NIK to the TRAF2/cIAP complex and thereby allowing cIAP1/2 to ubiquitinate

NIK, thus promoting its degradation by the proteasome. In contrast, activation of the BAFF-R with BAFF triggers p100 NF- κ B2 processing by recruiting TRAF3 to the cytosolic tail of the activated receptor and promoting TRAF2/cIAP-dependent TRAF3 degradation, thus preventing NIK degradation. Therefore, TRAF2, TRAF3 and cIAP1/2 work in tandem to repress NF- κ B2 activation in the absence of appropriate stimuli, thus tightly controlling B cell homeostasis and differentiation, as has been shown *in vivo* in a variety of genetically modified mouse models (8, 9, 20, 26-29).

In support of a role for BAFF in CLL/SLL etiology 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 it is the case of TRAF2DN/Bcl-2-double tg mice) or mice with B cells continuously exposed to high levels of circulating BAFF (BAFF/TCL-1 and BAFF/c-Myc-double tg mice (30, 31) develop CLL/SLL.

It is interesting to mention that Zhang and coworkers (32) developed transgenic mice expressing in lymphocytes p80HT, a lymphoma-associated NF- κ B2 mutant (33). 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, although constitutive NF- κ B2 activation in the TRAF2DN-tg mice may underlie MZ B cell expansion and might contribute to BAFF-independent B cell survival in the TRAF2DN-tg mice, constitutive NF- κ B2 activation is not sufficient to promote B cell transformation, as indicated by the lack of any significant incidence of B leukemia/lymphoma in the TRAF2DN-tg mice. Notably, TRAF2 deficiency also has an impact in the signaling cascades activated by various members of the TNFR-family and by the BCR. Thus, our data indicate that in the absence of TRAF2, CD40 engagement failed to efficiently activate JNK and ERK, although p38 MAPK activation and I κ B α degradation remained intact, confirming previous reports (14, 20). However, we also observed significant differences in early MAPK activation by the BCR. In this case, it is unclear whether TRAF2 is actually controlling early BCR-mediated signaling or these results reflect differences in BCR signaling by distinct B cell populations. In this regard, it is well established that follicular B cells (FO) and MZ B cells have different requirements for BCR activation (34).

It is interesting to compare the TRAF2DN/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 due to loss of gene encoding microRNAs (miRs) that suppress expression of this anti-apoptotic 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 elevated levels of XIAP. High levels of XIAP expression have been found in malignant B cells from CLL patients (35), and targeting XIAP expression has been shown to induce apoptosis of CLL cells in culture (36-38). We also observed a decline in cIAP1 and cIAP2 protein levels in murine TRAF2DN/Bcl-2 B cells. In this regard, inactivating mutations in the human *BIRC3* gene encoding cIAP2 have been found in CLL (39) and in other B lymphoid malignancies (40-42), and *BIRC3/MALT1* translocations are causative of mucosal associated lymphoid tissue lymphoma (28). Thus, the mouse model also recapitulates additional features of human CLL.

To present, there is no evidence of mutations of the *TRAF2* gene in CLL patients (40, 43, 44). However, there might be alternative mechanisms to inhibit TRAF2 function leading to B cell transformation. In this regard, Thomas and coworkers (45) have shown that phosphorylation of TRAF2 at Ser⁵⁵ (within the RING domain), which is constitutively found in Hodgkin's lymphoma samples, is deleterious for TRAF2 activity (46). 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 lacking the RING domain and therefore it lacks the ability to function as an E3 ubiquitin ligase. TRAF1 can form heterotrimers with TRAF2 (46) and there is evidence supporting a role for TRAF1 as both a positive and negative regulator of TRAF2 activities [Reviewed in (47)]. These results suggest a role for TRAF1 in the etiology of this disease, maybe as a downregulator of TRAF2 function. Further research on the role of TRAF1 in CLL/SLL is warranted.

In summary, our results indicate that TRAF2 deficiency cooperates with Bcl-2 in promoting CLL/SLL in mice, possibly by specifically enforcing MZ B cell accumulation and rendering B cells independent of BAFF for survival.

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FOOTNOTES

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Abbreviations used in this paper: BAFF, B cell activating factor; cIAP, cellular inhibitor of apoptosis; CLL, chronic lymphocytic leukemia; FO, follicular; MZ, marginal zone; NIK, NF- κ B inducing kinase; RING, really interesting new gene; SLL, small lymphocytic leukemia; TNFR, TNF receptor; TRAF, TNFR associated factor; TRAF2DN, TRAF2 lacking the N-terminal region (RING and zinc finger domains); Tg, transgenic; WT, wild-type; XIAP, X-linked inhibitor of apoptosis.

Disclosures: GP-C performed research and analyzed data. DL, CP and JP performed research. YC contributed reagents. JCR contributed reagents, analyzed data and helped in the writing of the paper. JMZ designed the experiments, performed research, analyzed data and wrote the paper. The authors declare no conflicting financial interest.

FIGURE LEGENDS

Figure 1. Endogenous TRAF2 is not expressed in B cells from TRAF2DN 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 μ g) and blotted with anti-TRAF2 antibodies. 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 antibodies recognizing TRAF2, TRAF3 and I κ B α . **C.** Splenocytes (10^7) isolated from TRAF2DN-tg mice and WT littermates were pre-incubated on ice for 10 min with 6 μ g/ml of an agonist rat anti-mouse CD40 mAb (Caltag, Burlingame, CA), followed by incubation at 37 °C for the times indicated. Then, CD40 positive cells were isolated using anti-rat Ab crosslinked to magnetic beads (Invitrogen, Carlsbad, CA). The recovered CD40 positive 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 μ g).

Figure 2. 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 μ g protein from each sample was analyzed by SDS-PAGE and immunoblotting with the antibodies indicated. **B.** Total RNA was extracted from purified B cells from WT and TRAF2DN-tg mice and 1 μ g RNA from each sample was used for retro-transcription 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 μ g/ml of anti- μ mAb 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.

Figure 3. The expression of endogenous TRAF2 and TRAF2DN-tg is recovered by proteasome inhibition. B cells from TRAF2DN mice (2.5×10^6 cells per 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 μ g of protein from each sample were analyzed by SDS-PAGE and immunoblotting using antibodies against TRAF2, I κ B α and β -actin.

Figure 4. Expansion of MZ B cells and reduced dependence on BAFF for B cell survival in the TRAF2DN-tg mice. **A.** Analysis of B lymphocyte populations in the spleens of TRAF2DN-tg mice and WT littermates. Three-color flow cytometry analysis was performed to determine the different splenic B lymphocyte populations. Gating was performed on the lymphocyte population for B220/CD3 staining and isotype control analyses or in the B220⁺ population in the case of CD23/CD21, IgM/IgD and IgM/CD21 analyses. Splenocytes were analyzed from a representative 6 months old TRAF2DN-tg mouse and a WT littermate. **B.** Analysis of BAFF dependence on survival of TRAF2DN-tg and WT B cells. Purified B cells (5×10^4) from spleens of WT and TRAF2DN-tg mice were left untreated or incubated with 100 ng/ml of recombinant mouse BAFF for 72 h. Then, cell viability was assessed. Figure shows mean \pm SEM ($n = 3$). Unpaired *t* Test (for comparison between BAFF-treated WT and TRAF2DN-B cells) and Wilconxon's test (for comparison between untreated and BAFF-treated TRAF2DN-tg B cells) were used for statistical analysis. **C.** Effect of BCR and BAFFR activation in proliferation of B cells from WT and TRAF2DN-tg mice. Purified B cells (5×10^4) from spleens of WT ($n = 7$) or TRAF2DN-tg mice ($n = 3$) were left untreated or activated with anti- μ mAb (10 μ g/ml, F(ab)₂ fragment) and co-stimulated with 100 ng/ml of purified mouse Flag-BAFF or left without any further stimulus for 68 h. Then ³H-Thymidine (0.5 μ Ci/well) was added to the culture and cells were harvested 10 h later. ³H-methyl-thymidine incorporation was measured and fold induction was determined by calculating the proliferation ratio between untreated and treated B cells (in duplicate) for each mouse. **D.** Effect of CD40L and IL-4 on proliferation of B cells from WT and TRAF2DN-tg mice. Purified B cells (5×10^4) from spleens of WT and TRAF2DN-tg mice were left untreated (control) or incubated with IL-4 alone (1 or 4 ng/ml), CD40L alone (1 μ g/ml) or a mixture of IL-4 (4 ng/ml) with either 100 ng/ml or 1 μ g/ml of CD40L for 72 h. Then, increase in cell number was

determined using the CellTiter-Glo[®] Luminescent Assay from Promega. Statistical analysis shows mean \pm SD (n = 3).

Figure 5. Analysis of CD40- and BCR-mediated signal transduction pathways in WT and TRAF2DN-tg B cells. Purified B cells (panel A, 4×10^6 ; panel B, 2×10^6) from WT and TRAF2DN-tg mice were left untreated (t = 0) or treated either with 1 μ g/ml of CD40L (A) or with 100 μ g/ml of anti- μ F(ab')₂ mAb (B) for the indicated times (t). Cell lysates were prepared in Laemmli buffer, sonicated and protein concentration was determined. A total of 10 μ g of protein from each sample was analyzed by SDS-PAGE and immunoblotting using antibodies against the indicated antigens.

Figure 6. Analysis of the effect of TRAF2 deficiency on the expression of NF- κ B subunits and anti-apoptotic proteins relevant to CLL. B cells were purified from (A) spleens of WT and TRAF2DN-tg mice or (B) WT (-/-), TRAF2DN-tg (+/-), Bcl-2-tg (-/+) or TRAF2DN/Bcl-2-double tg (+/+) mice (12-16 months old). TRAF2DN/Bcl-2-double tg mice in the figure have developed overt CLL/SLL. B cells (4×10^6 cell per sample) were lysed, sonicated and protein concentration was determined. A total of 12 μ g of protein (or 20 μ g for cIAP1/2 detection) of each sample were analyzed by SDS-PAGE/immunoblotting using the specific antibodies indicated in the figure. Each lane represents one individual mouse of each genotype.











