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Elevated Choline Kinase α-Mediated Choline Metabolism Supports the Prolonged Survival of TRAF3-Deficient B Lymphocytes

Samantha Gokhale,*† Wenyun Lu,‡§‖ Sining Zhu,*† Yingying Liu,* Ronald P. Hart,*‖ Joshua D. Rabinowitz,*†§‖ and Ping Xie,*†§‖

Specific deletion of the tumor suppressor TRAF3 from B lymphocytes in mice leads to the prolonged survival of mature B cells and expanded B cell compartments in secondary lymphoid organs. In the current study, we investigated the metabolic basis of TRAF3-mediated regulation of B cell survival by employing metabolomic, lipidomic, and transcriptomic analyses. We compared the polar metabolites, lipids, and metabolic enzymes of resting splenic B cells purified from young adult B cell–specific Traf32/2 mice and littermate control mice. We found that multiple metabolites, lipids, and enzymes regulated by TRAF3 in B cells are clustered in the choline metabolic pathway. Using stable isotope labeling, we demonstrated that phosphocholine and phosphatidylcholine biosynthesis was markedly elevated in Traf32/2 mouse B cells and decreased in Traf3-reconstituted human multiple myeloma cells. Furthermore, pharmacological inhibition of choline kinase α, an enzyme that catalyzes phosphocholine synthesis and was strikingly increased in Traf32/2 B cells, substantially reversed the survival phenotype of Traf32/2 B cells both in vitro and in vivo. Taken together, our results indicate that enhanced phosphocholine and phosphatidylcholine synthesis supports the prolonged survival of Traf32/2 B lymphocytes. Our findings suggest that TRAF3-regulated choline metabolism has diagnostic and therapeutic value for B cell malignancies with TRAF3 deletions or relevant mutations. The Journal of Immunology, 2020, 204: 000–000.

TRAF3, a member of the TRAF family, regulates the signal transduction pathways of a diverse array of immune receptors, including the TNFR superfamily, TLRs, NOD-like receptors, RIG-I-like receptors, and cytokine receptors (10, 18, 19). Specifically in B lymphocytes, TRAF3 directly binds to two receptors pivotal for B cell physiology, the BAFFR and CD40, which are required for B cell survival and activation, respectively (20, 21). Specific deletion of the Traf3 gene in B lymphocytes results in severe peripheral B cell hyperplasia in mice due to the prolonged survival of mature B cells independent of the principle B cell survival factor BAFF (4, 5). This effect of TRAF3 deficiency in B cells eventually leads to spontaneous development of splenic MZL and B1 lymphomas at high incidence by 18 mo of age (8). These in vivo findings are consistent with the frequent deletions and

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Abbreviations used in this article: AEC, adenylate energy charge; B-Traf32/2, B cell–specific Traf32/2; Chk2, Chk2 kinase; Cho, choline; DAG, diacylglycerol; Dgkα, diacylglycerol kinase α; DLBCL, diffuse large B cell lymphoma; Eln, ethanolamine; Faah, fatty acid amide hydrolase; Gdpd3, glycerophosphodiester phosphodiesterase domain containing 3; G6P, glucose-6-phosphate; HL, Hodgkin lymphoma; LC, lipid chromatography; LC-MS, liquid chromatography–mass spectrometry; LMC, littermate control; Lpcat, lysophosphatidylcholine acyltransferase; MAG, monoacylglycerol; MM, multiple myeloma; MZL, marginal zone lymphoma; NF-κB, noncanonical NF-κB; NIK, NF-κB-inducing kinase; PC, phosphatidylcholine; P-Cho, phosphocholine; P-DMEM, phosphomethylmethanolamine; PFP, pemtose phosphate pathway; TRAF, TNFR-associated factor.

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inactivating mutations of the TRAF3 gene identified in human B cell neoplasms, demonstrating the tumor suppressive role of TRAF3 in mature B lymphocytes.

The signal transduction pathway underlying TRAF3-mediated regulation of B cell survival has been elucidated in previous studies. It was found that, in the absence of stimulation, TRAF3 constitutively binds to NF-κB–inducing kinase (NIK) (the upstream kinase of the noncanonical NF-κB [NF-κB2] pathway) and TRAF2, whereas TRAF2 also constitutively associates with cIAP1/2 (18, 22). In this complex, cIAP1/2 induces K48-linked polyubiquitination of NIK, thereby targeting NIK for proteasomal degradation and thus inhibiting NF-κB2 activation (23–26). Upon BAFF or CD154 stimulation, trimeredized BAFFR or CD40 recruits TRAF3, TRAF2, and cIAP1/2 to the plasma membrane, releasing NIK from the TRAF3–TRAF2–cIAP1/2 complex and allowing NIK to accumulate in the cytoplasm (26–28). Accumulated NIK protein subsequently induces the activation of IKKα and NF-κB2, and activated NF-κB2, in turn, promotes the expression of anti-apoptotic proteins of the Bcl-2 family (such as Bcl-2, Bcl-xL, and Mcl-1) to induce B cell survival (18, 22, 29). Specific depletion of TRAF3, TRAF2, or cIAP1/2 in B cells all results in similar phenotype in mice, with BAFF-independent constitutive NF-κB2 activation and prolonged survival of mature B lymphocytes (4, 5, 28). However, it remains unclear whether this pathway regulates cellular metabolism to control B cell survival.

In the current study, we aimed to investigate the metabolic basis of the tumor suppressor TRAF3-mediated regulation of B cell survival. To address this, we first employed unbiased metabolome and lipidome screening approaches to compare the metabolism of resting splenic B cells purified from young adult B cell–specific Traf3+/+ (B-TRAF3+) and littermate control (LMC) mice. To understand how TRAF3 regulates B cell metabolism, we performed transcriptome profiling to identify metabolic enzymes downstream of TRAF3 signaling. Our results revealed that multiple metabolites, lipids, and enzymes regulated by TRAF3 in B cells are clustered in the choline (Cho) metabolic pathway. Using stable isotope labeling, we demonstrated the increased biosynthesis of phosphocholine (P-Chol) and phosphatidylcholine (PC) in Traf3+/+ B cells. To assess the functional importance of elevated Cho metabolism, we examined the effects of two Cho metabolic inhibitors, Mito-Cho (an analog of Cho that prevents Cho biosynthesis of phosphocholine [P-Cho] and phosphatidylcholine [PC] in the choline metabolic pathway) and Daudi were purchased from American Type Culture Collection (Manassas, VA) (30). All mouse and human B lymphoma cell lines were cultured as previously described (30).}

**Reagents and Abs**

Tissue culture supplements, including stock solutions of sodium pyruvate, L-glutamine, nonessential amino acids, and HEPES (pH 7.55), were from Invitrogen (Carlsbad, CA). Trimethyl-D9-Cho ([D9]H3-Cho) was purchased from Cambridge Isotope Laboratories (Tewksbury, MA). MN58B and RSM932A were obtained from Abiobius (Gloucester, MA). Abs against Chk2 were from Abcam (Cambridge, MA). Polyclonal rabbit Abs to Traf3 (H122) were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-actin Ab was from Chemicon (Temecula, CA). HRP-labeled secondary Abs were from Jackson ImmunoResearch Laboratories (West Grove, PA).

**Splice Cell**

Mouse splenic B cells were purified using anti-mouse CD43–coated magnetic beads and a MACS Separator (Miltenyi Biotec), following the manufacturer’s protocols as previously described (4). The purity of the isolated B cell population was monitored by FACS analysis, and cell preparations of >98% purity were used for metabolomic, lipidomic, and transcriptomic analyses as well as protein preparation. An aliquot of purified splenic B cells were cultured ex vivo in mouse B cell medium (4) for 24 h before metabolomic and lipidomic analyses.

**Water-soluble polar metabolite and lipid profiling using liquid chromatography–mass spectrometry**

Traf3+/+ and LMC splenic B cells were quickly washed twice with PBS. Ten million Traf3+/+ and LMC splenic B cells were centrifuged at 12,000 rpm at 4°C for 5 min to obtain a pellet. Separate pellets were used to extract water-soluble polar metabolites and lipids, respectively. To extract water-soluble metabolites, 184 μl of methanol/H2O (80:20) + 0.5% formic acid at dry ice temperature was added to the pellet, vortexed for 5 s, and was allowed to sit on dry ice for 10 min. Then 16 μl of 15% NH4HCO3 was added to neutralize the solution. The final solution was kept at –20°C for 15 min, and the resulting mixture was transferred into an Eppendorf tube and centrifuged at 13,200 rpm at 4°C for 15 min. The supernatant was taken for liquid chromatography–mass spectrometry (LC-MS) analysis. Liquid chromatography (LC) separation was achieved using a Vanquish UHPLC System (Thermo Fisher Scientific, San Jose, CA) and an Xbridge BEH Amide Column (150 × 2 mm, 2.5-μm particle size; Waters, Milford, MA). Solvent A is water/acetonitrile (95:5) with 20 mM ammonium acetate and 20 mM ammonium hydroxide at a pH of 9.4, and solvent B is acetonitrile. The gradient is 0 min, 90% B; 2 min, 90% B; 3 min, 75% B; 7 min, 75% B; 8 min, 70% B; 9 min, 70% B; 10 min, 50% B; 12 min, 50% B; 13 min, 25% B; 14 min, 25% B; 16 min, 0% B; 21 min, 0% B; 21.5 min, 90% B; and 25 min, 90% B (31). Total running time is 25 min at a flow rate of 150 μl/min. For all experiments, 5 μl of extract was injected with column temperature at 25°C. The Q-Exactive Plus Mass Spectrometer was operated in both negative and positive mode scanning m/z 70–1000 with a resolution of 140,000 at m/z 200. The mass spectrometry parameters were as follows: sheath gas flow rate, 28 (arbitrary units); auxiliary gas flow rate, 10 (arbitrary units); sweep gas flow rate, 1 (arbitrary unit); spray voltage, 3.3 kV; capillary temperature, 320°C; S-lens radio frequency level, 65; automatic gain control target, 3E6; and maximum injection time, 500 ms.

To extract lipids, 1 ml of 0.1 M HCl in methanol/H2O (50:50) was added to the pellet and set in a −20°C freezer for 30 min. Afterwards, 0.5 ml of chloroform was added to the mixture and vortexed, then set on ice for 10 min. Samples were centrifuged at 13,200 rpm for 10 min, and the chloroform phase at the bottom was transferred to a glass vial as the first extract, using a Hamilton syringe. Another 0.5 ml of chloroform was added to the remaining material, and the extraction was repeated to get a second extract. The combined extract was dried under nitrogen flow and redissolved in 200 μl of methanol/chloroform/2-propanol (1:1:1). Lipids were analyzed on a Q-Exactive Plus Mass Spectrometer coupled to a Vanquish UHPLC System (Thermo Fisher Scientific). Each sample was analyzed twice using the same LC gradient but with a different ionization mode on the mass spectrometer to cover both positively and negatively charged species. The LC separation was achieved on an Agilent Poroshell 120 EC-C18 Column (150 × 2.1 mm, 2.7-μm particle size) at a flow rate of 150 μl/min. The gradient was 0 min, 25% B; 2 min, 25% B; 4 min, 65% B; 6 min, 100% B; 20 min, 100% B; 21 min, 25% B; and 27 min, 25% B (32). Solvent A is 1 mM ammonium acetate + 0.2% acetic acid in methanol/H2O (10:90). Solvent B is 1 mM ammonium acetate + 0.2% acetic acid in methanol/2-propanol (9:2). Other mass spectrometry parameters were the same as above.
Data analyses were performed using MAVEN Software, which allows for sample alignment, feature extraction, and peak picking (33). Extracted ion chromatograms for each metabolite were manually examined to obtain its signal, using a custom-made metabolite library.

Stable isotope labeling analysis using LC-MS

To analyze kinetic Cho flux into metabolic pathways, purified splenic B cells were cultured in RPMI medium containing 80 \( \mu \)g/ml of trimethyl-D<sub>3</sub>-Cho (\(^{1}H_{3}\)Cho). At the indicated time points, cells were centrifuged at 12,000 rpm at 4°C for 5 min to obtain a pellet. To extract water-soluble metabolites, 16 \( \mu \)l of methanol/H<sub>2</sub>O (80:20) + 0.5% formic acid at dry ice temperature was added to the pellet, vortexed for 5 s, and set on dry ice for 10 min. Then 16 \( \mu l \) of 15% NH<sub>4</sub>HCO<sub>3</sub> was added to neutralize the solution. The final solution was set at ~20°C for 15 min, and the resulting mixture was transferred into an Eppendorf tube and centrifuged at 13,200 rpm at 4°C for 15 min. The supernatant was taken for LC-MS analysis of water-soluble metabolites and their labeled forms, as described above. In addition, P-Cho (P0378; Sigma-Aldrich) standards in 80% methanol were used for polar metabolites and lipids (34).

ATP measurement using an ATP Determination Kit

To specifically measure ATP concentrations, fresh lysates of purified splenic B cells were prepared using ice-cold 1.5% TCA in Mammalian Cell PE LB Lysis Buffer (G-Biosciences, St. Louis, MO) and subsequently neutralized with 20 mM Tris-HCl, pH 7.5 (35). ATP concentrations in lysates were determined using a luciferase-luciferin–based bioluminescence assay with an ATP Determination Kit (Molecular Probes, Eugene, OR), following the manufacturer’s protocol. Briefly, 10 \( \mu l \) of lysates was added into 90 \( \mu l \) of luciferase reagent and measured on a luminometer (Glomax 20/20; Promega, Fitchburg, WI) (35). ATP values were normalized as nanomoles of ATP per 10<sup>6</sup> cells (35).

Transcriptomic microarray analysis

Total cellular RNA was extracted from splenic B cells purified from young adult (8–12 wk-old) LMC and B-Traf3<sup>+/−</sup> mice using TRIzol reagent (Invitrogen), according to the manufacturer’s protocol. RNA quality was assessed on an RNA Nano Chip using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA) (36). The mRNA was amplified with a TotalPrep RNA Amplification Kit with a T7-oligo(dT) primer, following the manufacturer’s protocol. Briefly, 10 \( \mu l \) of lysates was added into 90 \( \mu l \) of luciferase reagent and measured immediately on the dual luciferase reporter system (Glomax 20/20). Results were analyzed with Illumina GenomeStudio v2011.1, background corrected, and variance stabilized in R/Bioconductor (La Jolla, CA). Results were extracted with Illumina GenomeStudio (La Jolla, CA). Results were extracted with Illumina GenomeStudio (La Jolla, CA). Results were extracted with Illumina GenomeStudio (La Jolla, CA).

TranscriptMan assays of the transcript expression of identified enzyme-encoding genes

dNA was prepared from RNA using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA). Quantitative real-time PCR of specific genes was performed using corresponding TaqMan Gene Assay Kit (Applied Biosystems), as previously described (36, 40). Briefly, real-time PCR was performed using TaqMan primers and probes (FAM-labeled) specific for mouse Chka, lysophosphatidyl-dihylocine acyltransferase (Lpcat) 1, fatty acid amid hydrolase (Fah), glycrophosphodiester phosphodiesterase domain containing 3 (Gpdcd3), phosphatidylglycerol (PlgD). Each reaction also included the probe (VIC-labeled) and primers for mouse Actb, which served as an endogenous control. Reactions were performed on a 7500 Fast Real-Time PCR System (Applied Biosystems). Relative mRNA expression levels of each gene were analyzed using the Sequence Detection Software (Applied Biosystems) and the comparative cycle threshold method, as previously described (36, 40).

Protein extraction and immunoblot analysis

Total protein lysates were prepared as described (36). Proteins were separated by SDS-PAGE and immunoblotted with Abs to Chka, TRAF3, and actin. Immunoblot analysis was performed as previously described (8, 30). Images of immunoblots were acquired using a low-light imaging system (LAS-4000 mini; FUJIFILM Medical Systems USA, Stamford, CT) (8, 30).

MTT assay

Mouse B lymphoma cell lines (1 × 10<sup>6</sup> cells per well) and human MM or B lymphoma cell lines (3 × 10<sup>5</sup> cells per well) were plated in 96-well plates in the absence or presence of serial dilutions of Chk inhibitors. At the indicated time point after treatment, cell viability and proliferation were measured using the MTT assay, as described (41). Possible influences caused by direct MTT-inhibitor interactions were excluded by studies in a cell-free system.

Transduction of human MM cells with a lentiviral TRAF3 expression vector

The full-length coding cDNA sequence of human TRAF3 was cloned from the 293T cell line using reverse transcription PCR. Primers used for the cloning of human TRAF3 are hTRAF3-F (5’-CCT AAA ATG GAG TCG AGT AAA AAG-3’) and hTRAF3-R (5’-TTA TCA GGC ATG CCC A-3’). The TRAF3 cDNA was subcloned into the lentiviral expression vector pUB–eGFPP-Thyl.1 (42) (generously provided by Dr. Z. Chen, University of Miami, Miami, FL) by replacing the eGFP coding sequence with the TRAF3 coding sequence. The resultant pUB–TRAF3–Thyl.1 lentiviral vector was verified by DNA sequencing. Lentiviruses of pUB–TRAF3–Thyl.1 and an empty vector pUB-Thyl.1 were packaged, and lentiviral titers were determined as previously described (30, 43). Human MM 8226 cells were transduced with the packaged lentiviruses at a multiplicity of infection of 1:5 (cell/virus) in the presence of 8 µg/ml polybrene (30, 43). Transduction efficiency of cells was analyzed at day 3 posttransduction using Thy1.1 immunofluorescence staining followed by flow cytometry. Transduced cells were subsequently analyzed for cell cycle distribution, Chk expression, and Cho metabolism. For cell cycle analysis, transduced cells were fixed at day 4 or 7 posttransduction with ice-cold 70% ethanol. Cell cycle distribution was subsequently determined by propidium iodide staining followed by flow cytometry, as previously described (4, 44).

In vivo administration of MN58B and RMS932A

Gender-matched, young adult LMC and B-Traf3<sup>+/−</sup> mice were injected i.p. with a Chka inhibitor, MN58B or RMS932A, at 2 mg/kg per mouse or vehicle control three times a week for 4 wk. Mouse spleen size was subsequently determined, and splenic B cell populations were analyzed by flow cytometry.

Flow cytometry

Single-cell suspensions were made from mouse spleens. Immunofluorescence staining and FACS analyses were performed as previously described (4, 8). Erythrocytes from spleens were depleted with ACK lysis buffer. Cells (1 × 10<sup>6</sup>) were blocked with rat serum and Fc receptor–blocking Ab (2.4G2) and incubated with various Abs conjugated to FITC, PE, PerCP, or allophycocyanin for multiple color fluorescence surface staining. Analyses of cell surface markers included Abs to CD45R (B220), CD3, CD21, and CD23 (BioLegend, San Diego, CA). Listmode data were acquired on a FACScalibur (Becton Dickinson, Mountain View, CA). The results were analyzed using the FlowJo Software (Tree Star, San Carlos, CA).

Statistics

Statistical analyses were performed using Prism Software (GraphPad, La Jolla, CA). For direct comparison of the levels of polar metabolites, lipids, or transcripts between LMC and Traf3<sup>+/−</sup> B cells, statistical significance was determined with the unpaired t test for two-tailed data. The p values < 0.05 are considered significant, p values < 0.01 are considered very significant, and p values < 0.001 are considered highly significant.

Results

TRAF3-mediated regulation of polar metabolites

To explore the metabolic basis of the prolonged survival of Traf3<sup>+/−</sup> B cells, we performed a metabolomic screening by LC-MS. We
compared the steady-state levels of water-soluble polar metabolites in LMC and Traf3−/− splenic B cells purified from gender-matched young adult (8- to 12- wk-old) mice by magnetic sorting, either directly ex vivo or after culture for 1 d. Comparison of direct ex vivo B cells revealed the metabolic differences between the two genotypes in the presence of endogenous B cell survival factor BAFF, whereas comparison of postculture B cells allowed us to identify the metabolic changes of the two genotypes in response to in vitro culture in the absence of BAFF. Our results showed that 16 metabolites were significantly upregulated in Traf3−/− B cells (Supplemental Fig. 1A, 1B). We have analyzed splenic B cells derived from three female and three male mice of each genotype and did not detect significant gender difference in the effects of TRAF3 ablation on polar metabolites. Both female and male Traf3−/− B cells exhibited consistent elevation of these 16 metabolites.

Interestingly, we found that P-Chol and phosphodiethanolamine (P-DEtn), two precursors of phospholipids, were significantly increased in Traf3−/− B cells both directly ex vivo (day 0) and at day 1 after culture (Fig. 1A). Five glucose metabolite intermediates were also significantly upregulated in Traf3−/− B cells, including glucose-6-phosphate (G6P), dihydroxyacetone phosphate (DHAP), glyceraldehyde-3-phosphate (GAP3), ribose-5-phosphate (ribose-5-P), and sedoheptulose-7-phosphate (STP) (Supplemental Fig. 1C). Surprisingly, although G6P is the convergence point of the glycolytic and pentose phosphate pathways (PPP) (45), we only detected an increase in metabolites of the nonoxidative PPP. In contrast, metabolites of aerobic glycolysis (such as 3-phosphoglyceric acid [3PG], pyruvate [PYR], and lactate), the TCA cycle (such as citrate, α-ketoglutarate, and malate), and the oxidative PPP (such as 6-phosphogluconolactone [6PGL], 6-phosphogluconic acid [6PG], and NADPH) were not significantly different between Traf3−/− and LMC B cells (Supplemental Fig. 1C). It is possible that some changes in metabolites may have been missed in our metabolic study, especially given the evidence that some metabolites are unstable during the LC-MS run time (46, 47). However, it is notable that the primary role of the nonoxidative PPP is to generate ribose-5-P, the molecular backbone of ribonucleotide biosynthesis (45, 48–50). Consistent with this notion, we detected an increase of nine ribonucleotides in Traf3−/− B cells, including UMP, CMP, GMP, AMP, CDP, UDP, GDP, ADP, and inosine (Supplemental Fig. 1C). It is intriguing that no significant differences were observed in the concentrations of the ribonucleotide triphosphates (Supplemental Fig. 1C). Based on the adenylate energy charge (AEC) index (51), LMC B cells had an increased AEC at day 1 after culture because of drastically decreased AMP concentrations (Supplemental Fig. 1D). This increased AEC might predispose LMC B cells to apoptosis, as a similar increase of AEC was observed in cerebellar granule cells en route to apoptosis (52). We also directly measured ATP concentrations in splenic B cells using an ATP Determination Kit. Our results showed that ATP concentrations were slightly lower in LMC B cells than in Traf3−/− B cells at day 1 after culture, but this difference is not statistically significant (Supplemental Fig. 1D).

Taken together, our results of polar metabolites indicate that TRAF3 regulates the Cho metabolism, nonoxidative PPP, and ribonucleotide metabolic pathways in B cells.

**Regulation of lipid metabolism by TRAF3**

In light of the observed upregulation of two precursors of phospholipids and the known pivotal importance of phospholipids in maintaining cell survival (53, 54), we sought to identify lipids and phospholipids that are regulated by TRAF3 in B cells. We performed an LC-MS–based lipidomic screening to compare the steady-state levels of lipids between LMC and Traf3−/− splenic B cells purified from gender-matched young adult mice. We detected 169 lipid species, among which 11 lipids were significantly elevated and eight lipids were significantly decreased in Traf3−/− B cells as compared with LMC B cells (Supplemental Fig. 2A, 2B). Interestingly, two PC species (32:2 and 34:3) and five phosphatidylethanolamine species (32:1, 34:2, 36:3, 46:10, and 36:6) were markedly elevated in Traf3−/− B cells both directly ex vivo and at day 1 after culture (Fig. 1B, 1C). Three phosphatidylinositol species (36:4, 38:4, and 38:5) and one galactosylceramide (Gal-Cer 18:3) were increased in Traf3−/− B cells only at day 1 after culture but not directly ex vivo (Supplemental Fig. 2C). In contrast, four diacylglycerol (DAG) species (DAG_NH4 34:0, 36:0, 38:0, and 40:1) and two monounsaturated glycerol (MAG) species (MAG_Na 16:0 and 18:0) were significantly decreased in Traf3−/− B cells at day 0 but not at day 1 after culture, whereas two ceramides (d18:1/26:4 and d18:1/26:5) were moderately decreased in Traf3−/− B cells only at day 1 after culture (Supplemental Fig. 2C). Given the prolonged survival phenotype of Traf3−/− B cells both in vivo in mice and in vitro in culture (4, 5), the consistent elevation of the two PC species and five phosphatidylethanolamine species in Traf3−/− B cells both directly ex vivo and at day 1 after culture highlights the potential importance of these phospholipids in Traf3−/− B cell survival.

**Metabolic enzymes regulated by TRAF3**

To understand how TRAF3 regulates cellular metabolism in B cells, we set out to identify metabolic enzymes regulated by TRAF3. We performed a transcriptome profiling using LMC and Traf3−/− splenic B cells purified from gender-matched young adult mice. We identified 101 genes differentially expressed between LMC and Traf3−/− splenic B cells, including 65 upregulated genes and 36 downregulated genes in Traf3−/− B cells (the cutoff fold of changes are as follows: 2-fold up or down, p < 0.05 (National Center of Biotechnology Information Gene Expression Omnibus accession number: GSE113920) (https://www.ncbi.nlm.nih.gov/geo/). Functional clustering and signaling pathway analyses by Ingenuity (http://www.ingenuity.com) revealed that 17 metabolic enzymes were up- or downregulated at least 2-fold in Traf3−/− B cells (Supplemental Fig. 3A). These include eight enzymes involved in Cho and phospholipid metabolism, four enzymes in nucleotide and DNA metabolism, three enzymes in glycolysis and glyceraldehyde dihydroxyacetone, and two enzymes in amino acid metabolism (Supplemental Fig. 3B). Moreover, we detected an additional 29 metabolic enzymes that were up- or downregulated 1.5- to 2-fold in Traf3−/− B cells (p < 0.05; Supplemental Fig. 3C, 3D). By integrating information across our metabolomic, lipidomic, and transcriptomic datasets, we identified a major clustering of TRAF3-mediated regulation of B cell metabolism in the interconnected Cho and ethanolamine (EtN) metabolic pathways (Fig. 1E).

We prioritized the metabolic enzymes identified by the microarray analysis according to their potential importance in the Cho and EtN metabolic pathways. We selected six enzyme-encoding genes altered in Traf3−/− B cells for further verification by quantitative real-time PCR. Our results verified the transcript changes of the examined enzyme genes in Traf3−/− B cells (Fig. 1D). Most relevant to the upregulation of PC and phosphatidylethanolamine metabolism was increased Cicla and Lpcat1, as well as decreased Gdpl3. The decreased DAG and MAG may relate to lower Dyka and upregulated Faah (Fig. 1D, 1E). These results suggest that TRAF3 regulates the expression of metabolic enzymes in B cells to control PC and phosphatidylethanolamine metabolism.

**TRAF3-mediated regulation of the Kennedy pathway of the P-Chol–PC synthesis**

The two major pathways of the phospholipid PC synthesis are de novo biosynthesis (also termed the Kennedy pathway) and sequential
methylation of phosphatidylethanolamine (Fig. 1E) (54–56). Chkα, also called Etn kinase, is the first enzyme of the Kennedy pathway. It directly phosphorylates both Cho and Etn with higher activity on Cho (Fig. 1E) (54–56). In this study, we demonstrated that Chkα was remarkably enhanced at the protein level in Traf3−/− splenic B cells by Western blot analysis (Fig. 2A). Chkα overexpression is thought to be responsible for the elevated P-Cho and PC phenotype in most cases of human breast, lung, and prostate cancers (55, 57). We likewise detected significantly elevated levels of P-Cho and PC in premalignant Traf3−/− B cells (Fig. 1A, 1B). In this context, we next

![FIGURE 1. TRAF3 inhibited PC and phosphatidylethanolamine metabolic pathways in B cells. Splenic B cells were purified from gender-matched, young adult (8- to 12-wk-old) LMC or B-Traf3−/− mice. Water-soluble polar metabolites (A) or lipids (B and C) were extracted from cells directly ex vivo (day 0) or after culture for 1 d and then analyzed by LC-MS as described in Materials and Methods. (A) Increased levels of P-Cho and P-DMEtn in Traf3−/− B cells. (B and C) Elevated levels of PC and phosphatidylethanolamine species in Traf3−/− B cells. (D) Verification of transcript regulation of selected metabolic enzymes identified by the microarray analysis. Total cellular RNA was prepared from purified splenic B cells, and cDNA was synthesized by reverse transcription. Quantitative real-time PCR was performed using TaqMan assay kits specific for mouse Chka, Lpcat1, Faah, Gdpd3, Plcd3, or Dgka. Relative mRNA levels were analyzed using the ΔΔcycle threshold method and normalized using β-actin mRNA as an endogenous control. Results shown in (A)–(D) are mean ± SD (n = 6, including three female and three male samples for each genotype). (E) Pathway schematics showing TRAF3-mediated regulation of the interconnected PC and phosphatidylethanolamine metabolism in B cells. Enzymes are denoted in italic font in the schematics. Metabolites, lipids, and metabolic enzymes that were regulated by TRAF3 are indicated in red (for those upregulated in Traf3−/− B cells) or blue (for those downregulated in Traf3−/− B cells). *p < 0.05 (significantly different; t test), **p < 0.01 (very significantly different; t test), ***p < 0.001 (highly significantly different; t test) between LMC and Traf3−/− B cells. The Journal of Immunology 5
performed stable isotope labeling to determine if the increased P-Cho is generated from de novo synthesis or from catabolism of Cho-containing metabolites and if the Kennedy pathway contributes to the elevation of PC species. We cultured LMC and Traf3−/− splenic B cells purified from gender-matched young adult mice in mouse B cell medium containing the stable isotope tracer trimethyl-D9-Cho ([2H]9-Cho) (58). We subsequently analyzed the labeled and unlabeled polar metabolites of cultured cells at different time points using LC-MS. We found that Traf3−/− splenic B cells exhibited significantly increased incorporation of the stable isotope tracer D9-Cho into P-Cho (Fig. 2B). We also compared the labeled and unlabeled lipids of the cells at different time points using LC-MS (58). We detected a significantly higher levels of D9-Cho–labeled PC species (32:2 and 34:3) in Traf3−/− B cells than in LMC B cells at 24 h after culture (Fig. 2C). Collectively, these stable isotope labeling data indicate that the biosynthesis of P-Cho–PC is elevated in Traf3−/− B cells.

**Chka inhibitors suppressed the survival of TRAF3-deficient B cells in culture**

We next asked if Chka expression is consistently upregulated in TRAF3-deficient malignant B cells and if elevated Chka-mediated Cho metabolism is important for oncogenic B cell survival. To address this, we analyzed Chka expression levels in two Traf3−/− mouse B lymphoma cell lines, 27-9 and 105-8, that were previously derived from aging B-Traf3−/− mice (8, 30). Our results showed that upregulation of Chka expression was maintained in these two Traf3−/− mouse B lymphoma cell lines at a similarly high level as that detected in premalignant Traf3−/− splenic B cells (Fig. 3A). To investigate the functional importance of elevated Cho metabolism, we tested the effects of two pharmacological inhibitors of Chka, MN58B (IC50: 1.4 μM) and RSM932A (IC50: 1 μM)
(59–61), on the survival of these Traf3−/− mouse B lymphoma cells. Our results obtained by MTT assay demonstrated that both MN58B and RSM932A potently inhibited the survival and proliferation of Traf3-deficient mouse B lymphoma cell lines 27-9 and 105-8 cells in a dose-dependent manner (Fig. 3C). These results suggest that Traf3 inactivation–induced Chkα upregulation is important for the survival of cultured Traf3-deficient mouse B lymphoma cells.

To strengthen the clinical relevance of our findings obtained from Traf3-deficient mouse B cells, we next examined the expression of CHKA in three patient-derived MM cell lines that contain biallelic deletions or inactivating mutations of Traf3, including 8226, KMS11, and LP1 (6). Our results revealed that the expression of CHKA was upregulated in all three Traf3-deficient human MM cell lines to a varied extent (3- to 7-fold as compared with normal B cells) (Fig. 3B), suggesting that Traf3 inactivation also upregulates CHKA expression in human B cells. Of particular interest, the two Chkα inhibitors MN58B and RSM932A also suppressed the survival and proliferation of all these Traf3-deficient human MM cell lines in a dose-dependent manner (Fig. 3D). Thus, elevated Chkα-mediated Cho metabolism appears to play an indispensable and causal role in the survival phenotype of both mouse and human Traf3-deficient malignant B cells in culture.

To determine if elevation of Chkα-mediated Cho metabolism is ubiquitously critical for malignant B cell survival, we also examined several Traf3-sufficient B cell lines for comparison. We found that Chkα expression was ubiquitously upregulated (~2- to 9-fold increase as compared with normal B cells) in all the Traf3-sufficient B cell lines examined, including the mouse B lymphoma cell lines A20.2J and m12.4.1 as well as human Burkitt’s lymphoma cell lines Ramos and Daudi (Fig. 3A, 3B). Thus, Chkα expression may be upregulated in malignant B cells via either Traf3 inactivation–dependent or Traf3-independent pathways. Interestingly, however, inhibition of Chkα by MN58B was not effective, and RSM932A had minimal effects on suppressing the survival and proliferation of Traf3-sufficient mouse B lymphoma cell lines A20.2J and m12.4.1 cells (Fig. 3C). In contrast, both MN58B and RSM932A were effective in inhibiting the survival and proliferation of Traf3-sufficient human Burkitt’s lymphoma cell line Ramos cells. Furthermore, it is perplexing that MN58B failed to inhibit the survival and proliferation of another Burkitt’s lymphoma cell line, Daudi, although this cell line was susceptible to RSM932A treatment (Fig. 3D). Taken together, the responses of different Traf3-sufficient malignant B cell lines to the Chkα inhibitors varied considerably from cell line to cell line, which likely reflects the differences in their oncogenic mutation landscapes and the presence or absence of redundant Chkα-independent Cho metabolic pathways.

Reconstitution of Traf3 inhibits the Kennedy pathway of the P-Cho–PC biosynthesis in human MM cells

To further verify that Traf3 regulates Chkα-mediated Cho metabolism in human B cells, we selected a patient-derived MM cell line 8226 that contains biallelic deletions of the Traf3 gene to conduct Traf3 reconstitution experiments (6). We transduced 8226 cells using a lentiviral expression vector, pUB–Traf3–Thy1.1, to reconstitute the expression of Traf3 proteins. Cells transduced with an empty lentiviral vector, pUB-Thy1.1, were used as a negative control in these experiments. Transduction efficiency of the lentiviral vectors was >99% in human MM 8226 cells, as demonstrated by FACS analysis (Fig. 4A). Reconstitution of Traf3 expression induced cellular apoptosis in ~50% of the transduced 8226 cells at day 7 posttransduction, as revealed by cell cycle analysis (Fig. 4B). Interestingly, reconstitution of Traf3 expression markedly decreased the protein levels of Chkα in the transduced 8226 cells at day 3 posttransduction, as shown by Western blot analysis (Fig. 4C).

We next performed stable isotope labeling to assess if reconstitution of Traf3 expression inhibits the Kennedy pathway of P-Cho–PC biosynthesis. We cultured the transduced 8226 cells in human B cell medium containing the stable isotope tracer trimethyl-D3-Cho at day 3 posttransduction. We subsequently analyzed the labeled and unlabeled polar metabolites as well as phospholipids at different time points using LC-MS. Similar to that observed in mouse splenic B cells, reconstitution of Traf3 expression significantly decreased the pool size of P-Cho and two downstream phospholipid PC species (32:2 and 34:3). This was associated with slower production of labeled P-Cho and PC lipids (32:2 and 34:3) from the D3-labeled Cho tracer (Fig. 4D, 4E). We noticed that the labeling fractions of both P-Cho and the two PC species did not significantly change between Traf3-transduced and the control vector–transduced cells, suggesting that the same metabolic pathways are being used in both cases. Therefore, the differences that we detected in the pool size of P-Cho and the two PC species were due to different production rates of these molecules between Traf3-transduced and the control vector–transduced cells. Together, our results demonstrate that reconstitution of Traf3 inhibited Chkα expression and the Kennedy pathway of P-Cho–PC biosynthesis as well as survival in transduced human MM 8226 cells.

Pharmacological inhibitors of Chkα reversed the phenotype of the B cell compartment in B-Traf3−/− mice

Our in vitro data regarding the roles of Traf3 in Chkα-mediated Cho metabolism in human MM cells (Fig. 4) and the effects of Chkα inhibitors on the survival of Traf3-deficient mouse B lymphoma and human MM cells (Fig. 3) prompted us to further evaluate the in vivo effects of these inhibitors on the B cell hyperplasia phenotype in B-Traf3−/− mice. We administered MN58B or RSM932A to gender-matched, young adult LMC and B-Traf3−/− mice via i.p. injection at 2 mg/kg per mouse three times a week for 4 wk and then analyzed the spleen size and splenic B cell compartment of the treated mice. Our results demonstrated that in vivo administration of either MN58B or RSM932A substantially decreased the spleen size as measured by spleen weight in B-Traf3−/− mice but not in LMC mice (Fig. 5A). Flow cytometric analysis revealed that treatment with MN58B or RSM932A partially reduced the percentage and drastically decreased the numbers of splenic B cells in B-Traf3−/− mice but not in LMC mice (Fig. 5B, 5C). Furthermore, treatment with MN58B or RSM932A remarkably decreased the percentage and numbers of the splenic marginal zone B cell subset and also dramatically reduced the numbers of the follicular B cell subset in B-Traf3−/− mice, which was not observed in LMC mice (Fig. 5B, 5C). In contrast, MN58B or RSM932A did not significantly affect splenic T cell numbers in B-Traf3−/− or LMC mice (Fig. 5C). In summary, administration of pharmacological inhibitors of Chkα substantially reversed the B cell hyperplasia phenotype observed in B-Traf3−/− mice, suggesting that elevated Chkα-mediated P-Cho and PC biosynthesis contributes to the prolonged survival of Traf3−/− B cells in vivo.

Discussion

Targeted therapies are currently the focus of anticancer research and are the core concept of precision medicine. Interestingly, targeting cancer metabolism has recently emerged as a promising strategy for the development of selective and effective anticancer drugs (62). Defining metabolic pathways regulated by critical oncogenes and tumor suppressor genes is required for such therapeutic
FIGURE 3. Chkα inhibitors MN58B and RSM932A suppressed the survival of TRAF3-deficient mouse and human malignant B cells in culture. (A) Upregulation of Chkα expression in TRAF3-deficient mouse B lymphoma cell lines 27-9 and 105-8 as well as TRAF3-sufficient mouse B lymphoma cell lines A20.2J and m12.4.1. (B) Upregulation of Chkα expression in TRAF3-deficient human MM cell lines 8226, KMS11, and LP1 as well as TRAF3-sufficient human Burkitt’s lymphoma cell lines Ramos and Daudi. Chkα expression was determined by quantitative real-time PCR using TaqMan assays specific for mouse (A) and human (B) Chkα, respectively, and results shown are mean ± SD (n = 4). (C) Mouse B lymphoma cell lines 27-9, 105-8, A20.2J, and m12.4.1 were treated with various concentrations (1:2 serial dilutions) of MN58B or RSM932A for 24 h. (D) Human MM or Burkitt’s lymphoma cell lines 8226, KMS11, LP1, Ramos, and Daudi cells were treated with various concentrations (1:2 serial dilutions) of MN58B or RSM932A for 72 h. Total viable cell numbers were subsequently determined by MTT assay. Viable cell curves shown (C and D) are the results of at least three independent experiments with duplicate samples in each experiment (mean ± SEM).
exploitation. In the current study, we elucidated the metabolic pathways regulated by a new tumor suppressor, TRAF3, in B lymphocytes using metabolomic, lipidomic, and transcriptomic analyses. We found that multiple polar metabolites, lipids, and enzymes regulated by TRAF3 in B cells are clustered in the P-Cho, PC, and phosphatidylethanolamine biosynthesis pathways (Fig. 1E).

Aberrant Cho metabolism has been recognized as a cancer hallmark associated with oncogenesis, invasion, and metastasis as well as responsiveness to therapy in human cancers (55, 57). The metabolic pathways of PC and phosphatidylethanolamine are closely interconnected, and moderate perturbations of PC and phosphatidylethanolamine metabolism have profound effects on cell viability and apoptosis (63). Consistent with these previous findings, our results showed that premalignant Traf3−/− B cells contained markedly elevated levels of P-Cho, P-DMEtn, PC, and phosphatidylethanolamine as well as Chkα, the first enzyme of de

FIGURE 4. Reconstitution of TRAF3 expression inhibited the P-Cho–PC biosynthesis in human MM cells. The human MM cell line 8226 cells were transduced with a lentiviral expression vector of TRAF3 (pUB–TRAF3–Thy1.1) or an empty lentiviral expression vector (pUB-Thy1.1). (A) Transduction efficiency. Transduced 8226 cells were analyzed by Thy1.1 staining and flow cytometry at day 3 posttransduction. Dashed profiles show the untransduced cells (a negative control of the Thy1.1 staining), and gated populations (Thy1.1+) indicate cells that were successfully transduced with the lentiviral expression vector. (B) Representative FACS histograms of cell cycle analysis. Cell cycle distribution of transduced 8226 cells was analyzed by propidium iodide staining and flow cytometry at day 4 and 7 posttransduction. Gated populations indicate apoptotic cells (sub-G0: DNA content < 2n) and proliferating cells (S/G2/M phase: 2n < DNA content ≤ 4n). (C) Expression of Chkα and TRAF3 determined by Western blot analysis. Total cellular proteins were prepared at day 3 posttransduction and then immunoblotted for Chkα, TRAF3, and actin. (D and E) Analysis of Cho metabolism by stable isotope labeling. At day 3 posttransduction, transduced 8226 cells were cultured in human B cell medium containing 80 μg/ml of trimethyl-D9-Cho. D9-labeled and unlabeled P-Cho (D) and PC species (E) were analyzed at the indicated time points by LC-MS. (D) Decreased incorporation of D9-Cho into P-Cho in pUB–TRAF3–Thy1.1 (TRAF3)–transduced 8226 cells. (E) Reduced incorporation of D9-Cho into two PC species in TRAF3-transduced 8226 cells. The composition of the D9-labeled and unlabeled P-Cho or PC species of each sample is shown in the bar graphs (top panel), and the kinetic increase of the D9-labeled P-Cho or PC species of each transduce cell type is shown in the curves (bottom panel). Results shown are mean ± SEM (n = 3). *p < 0.05 (significantly different; t test), **p < 0.01 (very significantly different; t test) between TRAF3-transduced and the control vector pUB-transduced cells.
novo PC and phosphatidylethanolamine biosynthesis (the Kennedy pathway). Interestingly, Chka expression was ubiquitously up-regulated in a variety of TRAF3-deficient as well as TRAF3-sufficient malignant B cell lines examined in the current study, including mouse B lymphoma cell lines, human MM, and Burkitt’s lymphoma cell lines. We also surveyed the public gene expression databases of human cancers at Oncomine (http://www.oncomine.org) and learned that the expression of CHKA is significantly increased in human DLBCLs (64–66). Overexpression of Chka has been shown to cause an elevated P-Cho and PC phenotype and thus has been identified as a therapeutic target in breast, lung, and prostate cancers as well as T cell lymphoma and leukemia (55, 57, 67, 68). In this study, our stable isotope labeling experiments demonstrated increased biosynthesis of P-Cho and PC in Traf3−/− mouse B cells as well as decreased biosynthesis of P-Cho and PC in TRAF3-reconstituted human MM cells. Interestingly, pharmacological inhibition of Chka reversed the survival phenotype of TRAF3-deficient B cells both in vitro and in vivo. Taken together, our findings indicate that elevated Chka-mediated Cho metabolism supports the aberrantly prolonged survival of Traf3−/− B cells. Therefore, our study established, to our knowledge, a novel connection between the tumor

![Figure 5](http://www.jimmunol.org/)
suppressor TRAF3 and the known oncocgenic Chk2–P–Cho–PC metabolic pathway in premalignant and malignant B cells.

PC and phosphatidyethanolamine are the most abundant phospholipids in cell membranes and play a dual role as structural components and substrates for lipid second messengers, such as phosphatidic acid and DAG (69). We detected significantly increased levels of multiple species of PC and phosphatidyethanolamine but decreased levels of their catabolic products, such as DAG and MAG in Traf3−/−B cells. In addition to Chk2, we also discovered TRAF3-mediated regulation of several other enzymes that catalyze the anabolic or catabolic pathways of phospholipids in B cells, including Lpact1, Faaah, Gdpl3, Pldc3, Dgka, Lacc1, and Pip5k1b. Among these, upregulation of Lpca1t1 and Faaah, as well as downregulation of DGKA and PIP5K1B, has been documented in human cancers (70–75). In particular, the key enzyme Mthfd1 in B cells, including TRAF3-mediated regulation of several other enzymes as well as downregulation of Pip5k1b and Faah, has been recognized as an inhibitor of phospholipase A2 (PLA2) (76), the catabolic enzyme of the Lands Cycle that converts lysophosphatidylcholine (LPC) to PC, has been shown to play important roles in cancer pathogenesis and progression (70–72). Interestingly, Anxa4, a gene robustly upregulated in Traf3−/−B cells, has been recognized as an inhibitor of phospholipase A2 (PLA2) (76), the catabolic enzyme of the Lands Cycle that mediates the breakdown of PC and phosphatidylethanolamine (Fig. 1E). Thus, simultaneous upregulation of both Lpact1 and Anxa4 would likely promote the synthesis of PC and inhibit the breakdown of PC and phosphatidylethanolamine, which may also contribute to the increased PC and phosphatidylethanolamine levels detected in Traf3−/−B cells. Collectively, our results revealed the complex interactions between TRAF3 and phospholipid metabolic pathways associated with survival regulation in B cells.

We noted that another major effect of TRAF3 on B cell metabolism was clustered in the nonoxidative PPP and ribonucleotide metabolic pathways (Supplemental Fig. 4). In line with the increased levels of G6P, nonoxidative PPP metabolites, and ribonucleotides, we detected upregulation of the key enzyme of glycogen breakdown Pgy1 and two enzymes of ribonucleotide biosynthesis (Mthfd1 and Adssl1) as well as downregulation of two enzymes involved in ribonucleotide catabolism (Ubp1 and Pdeo2a) in Traf3−/−B cells. Relevant to our observation, Mambetsari et al. (77) reported that a glucose transporter (Glut1) and an enzyme hexokinase II (HKII) that converts glucose to G6P are upregulated in Traf3−/−B cells, although not detected in our microarray analysis. Therefore, both elevated glycogen breakdown and increased glucose uptake and conversion may lead to increased nonoxidative PPP metabolism in Traf3−/−B cells. Corroborating our finding, MTHFD1, an enzyme of one-carbon metabolism that is essential for de novo purine biosynthesis, is upregulated in a variety of human B cell malignancies, including DLBCL, follicular lymphoma, B cell acute lymphoblastic leukemia, B cell chronic lymphocytic leukemia, Burkitt’s lymphoma, HL, and MM (Oncomine) (65, 66, 78, 79). A common polymorphism of MTHFD1 R653Q (c.1958 G → A) in the synthetase domain impairs purine synthesis, and the AA genotype of MTHFD1 R653Q (c.1958 G → A) increases levels of multiple species of PC and phosphatidylethanolamine in B cells, including Lpact1, Faah, Gdpl3, Pldc3, Dgka, Lacc1, and Pip5k1b. Among these, upregulation of LPCAT1 and FAHH, as well as downregulation of DGKA and PIP5K1B, has been documented in human cancers (70–75). In particular, the key enzyme of the Lands Cycle that converts lysophosphatidylcholine (LPC) to PC, has been shown to play important roles in cancer pathogenesis and progression (70–72).

In summary, our study has contributed to a better understanding of the metabolic mechanisms underlying aberrant survival of B lymphocytes induced by inactivation of the tumor suppressor TRAF3. We demonstrated that elevated Cho metabolism via the Chk2-driven de novo Kennedy pathway plays an important causal role in the survival phenotype of Traf3−/−B cells. Our findings support that the Chk2–P–Cho–PC metabolic pathway has diagnostic and therapeutic value for B cell malignancies. For example, changes in P–Cho cellular levels can be noninvasively monitored in patients by in vivo imaging using Cho analogue tracers [19F]-Cho or [13C]-Cho in positron emission tomography/computed tomography (82). Our study supports the use of such a Cho metabolism–based approach for early detection of B cell malignancies and for the assessment of malignant B cell responses to therapy. Furthermore, inhibition of Cho metabolism by Chk2 inhibitors or other drugs has the potential to improve the efficacy of standard chemotherapy, radiation therapy, and immunotherapy in B cell malignancies, especially in patients with TRAF3 deletion or relevant mutations.

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Disclosures
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References


