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Elevated Choline Kinase \(\alpha\)-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 Traf3\(\text{−/−}\) 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 Traf3\(\text{−/−}\) mouse B cells and decreased in Traf3-reconstituted human multiple myeloma cells. Furthermore, pharmacological inhibition of choline kinase \(\alpha\), an enzyme that catalyzes phosphocholine synthesis and was strikingly increased in Traf3\(\text{−/−}\) B cells, substantially reversed the survival phenotype of Traf3\(\text{−/−}\) B cells both in vitro and in vivo. Taken together, our results indicate that enhanced phosphocholine and phosphatidylcholine synthesis supports the prolonged survival of Traf3\(\text{−/−}\) 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.

A berrant B cell survival is an important contributing factor to the pathogenesis of B cell malignancies, which comprise >50% of blood cancers and >80% of lymphomas (1–3). We and others have recently identified TNFR-associated factor (TRAF) 3, a cytoplasmic adaptor protein, as a critical regulator of cell survival and tumor suppressor in mature B lymphocytes (4–9). Deletions and inactivating mutations of the TRAF3 gene are some of the most frequently occurring genetic alterations in a variety of human B cell malignancies (9, 10), including multiple myeloma (MM, 17%) (6, 11), gastric marginal zone lymphoma (MZL, 21%), splenic MZL (10%) (12, 13), diffuse large B cell lymphoma (DLBCL, 14%) (14), B cell chronic lymphocytic leukemia (13%) (15), Hodgkin lymphoma (HL, 15%) (16), and Waldenström’s macroglobulinemia (5%) (17).

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...
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 noncanoncal 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-κB 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-κB, 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 deletion 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 transcriptional 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 phosphorylcholine (PC) in Traf3−/− B cells. To assess the functional importance of elevated Cho metabolism, we examined the effects of two Cho metabolites, phosphocholine (P-Cho) and phosphatidylcholine (PtdCho), on B cell survival.

**Materials and Methods**

**Mice and cell lines**

Traf3lox/loxCD19Cre (B-Traf3−/−) and Traf3lox/lox (LMC) mice were generated as previously described (4). All experimental mice for this study were produced by breeding of Traf3loxtfl mice with Traf3floxtflCD19cre mice. All mice were kept in specific pathogen-free conditions in the Animal Facility at Rutgers University and were used in accordance with NIH guidelines and under an animal protocol (Protocol No. 08-048) approved by the Animal Care and Use Committee of Rutgers University. Equal numbers of male and female mice were used for this study. Traf3−/− mouse B lymphoma cell line A20.2.1 was generously provided by Dr. G. Bishop (University of Iowa, Iowa City, IA) (30). Traf3−/− mouse B lymphoma cell line M12.4.1 as well as human Burkitt’s B lymphoma cell lines Ramos 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-D3-Cho (1H23-Cho) was purchased from Cambridge Isotopes Laboratories (Tewksbury, MA). MN58B and RSM932A were obtained from Aobious (Gloucester, MA). Abs against Chkα 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).

**Spleenic B cell purification, culture, and stimulation**

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 used to establish ex vivo 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 24 h, 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 C18 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. 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; 16 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 (2:98). 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 μg/ml of trimethyl-D_{3}-Cho ([1^{3}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, 1.5 ml of methanol/tri-HCl (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 μl of 15% NH_{4}HCO_{3} 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-Chol (Sigma-Aldrich) standards in 80% methanol at concentrations of 0.5, 1.5, and 5 μM were run separately to estimate the concentrations of P-Chol in biological samples.

To extract lipids, 1 ml of chloroform was added to the remaining pellet and vortexed, then set on ice for 10 min. Samples were centrifuged at 13,200 rpm for 10 min, and the chloroform phase was transferred to a glass vial. The concentrations of 0.5, 1.5, and 5 μM were run separately to estimate the concentrations of lipid species in biological samples.

**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 Lysis Buffer (B-G Biosciences, St. Louis, MO) and subsequently neutralized with 20 nM Tris-HCl, pH 7.5 (35). ATP concentrations in lysates were determined using a luciferase-luciferin–based bioluminescence assay with an ATP Determination Kit (Triton Benzoquinone dihydrogen, Karlsruhe, Germany), according to the manufacturer’s protocol. Briefly, 10 μl of lysates was added into 90 μl of luciferase reagent and measured immediately based on the maximal light intensity of the resulting bioluminescence in a luminometer (Glomax 2020, Promega, Fitchburg, WI) (35). A calibration curve with serial dilutions of ATP standards was prepared in each experiment. Data were normalized as nanomoles of ATP per 10{sup}6 cells (35).

**Transcriptoric microarray analysis**

Total cellular RNA was extracted from splenic B cells purified from young adult (8- to 12-wk-old) LMC and B-Traf3^{−/−} 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-oligoDT primer, following the manufacturer’s instructions (Ambion), and microarray analysis was carried out with the Illumina Sentrix Mouse Ref-8 24K Array at the Burnham Institute (La Jolla, CA). Results were extracted with Illumina GenomeStudio v2011.1, background corrected, and variance stabilized in R/Bioconductor using the lumi package (37, 38) and modeled in the limma package (39). The microarray analysis results have been deposited in the National Institutes of Health Gene Expression Omnibus database under the accession number GSE113920 (http://www.ncbi.nlm.nih.gov/geo/).

**TuqMan assays of the transcript expression of identified enzyme-encoding genes**

cDNA 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 TuqMan Gene Assay Kit (Applied Biosystems), as previously described (36, 40). Briefly, real-time PCR was performed using TuqMan primers and probes (FAM-labeled) specific for mouse Chka, lysophosphatidylcholine acyltransferase (Lpcat) 1, fatty acid amide hydrolase (Fah), glycerophosphodiester phosphodiesterase domain containing 3 (Gpd3), glyceroldehyde-3-phosphate dehydrogenase (Gad), and acetyl CoA synthetase (Acetyl) 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 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, Stanford, CT) (8, 30).

**MTT assay**

Mouse B lymphoma cell lines (1 × 10{sup}6 cells per well) and human MM or B lymphoma cell lines (3 × 10{sup}5 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 MTI 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 CCG CAG A-3′). The TRAF3 cDNA was subcloned into the lentiviral expression vector pUB–eGFP–Thy1.1 (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–Thy1.1 lentiviral vector was verified by DNA sequencing. Lentiviruses of pUB–TRAF3–Thy1.1 and an empty vector pUB-Thy1.1 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^{−/−} mice were injected i.p. with a Chka inhibitor, MN58B or RSM932A, 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^{6}) 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^{−/−} 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 Chk inhibitors was analyzed using the Sequence Detection Software (Applied Biosystems) and the comparative threshold method, as previously described (36, 40).
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 absence 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-Cho and phosphodimethyl ethanolamine (P-DMEth), 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 metabolic 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 metabolomic 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 O-36:1) 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 dicylglycerol (DAG) species (DAG_NH 34:0, 36:0, 38:0, and 40:1) and two monoyglycerol (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 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 glycosylation, and two enzymes in amino acid metabolism (Supplemental Fig. 3B). Moreover, we detected an additional 29 metabolic enzymes that were up- or downregulated at least 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 was increased Cnka and Lpcat1, as well as decreased Gpd3. 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-Cho–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](http://www.jimmunol.org/Downloadedfrom)
performed stable isotope labeling to determine if the increased P-Chol 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<sup>−/−</sup> splenic B cells purified from gender-matched, young adult LMC and B-Traf3<sup>−/−</sup> mice. Upregulation of Chkα protein in Traf3<sup>−/−</sup> B cells analyzed by Western blot analysis. Total cellular proteins were prepared from purified splenic B cells, separated by SDS-PAGE, and immunoblotted for Chkα, followed by TRAF3 and β-actin. Results shown are representative of three independent experiments. Analysis of Cho metabolism by stable isotope labeling. Purified splenic B cells were cultured in mouse B cell medium containing 80 μg/ml of trimethyl-D<sub>9</sub>-Cho ([2H<sub>9</sub>]-Cho) for the indicated time periods. The D<sub>9</sub>-labeled and unlabeled water-soluble metabolites (B) and lipids (C) of cultured splenic B cells were subsequently analyzed using LC-MS. (B) Increased incorporation of D<sub>9</sub>-Cho into P-Chol in Traf3<sup>−/−</sup> B cells. (C) Elevated incorporation of D<sub>9</sub>-Cho into PC species in Traf3<sup>−/−</sup> B cells. The composition of the D<sub>9</sub>-labeled and unlabeled P-Chol or PC species of each sample is shown in the bar graphs (left panel), and the kinetic increase of the D<sub>9</sub>-labeled P-Chol or PC species of each genotype of cells is shown in the curves (right panel). *p < 0.05 (significantly different; t test), **p < 0.01 (very significantly different; t test) between LMC and Traf3<sup>−/−</sup> B cells.

**FIGURE 2.** Elevated Chkα and Cho metabolism in Traf3<sup>−/−</sup> B cells. Splenic B cells were purified from gender-matched, young adult LMC and B-Traf3<sup>−/−</sup> mice. (A) Upregulation of Chkα protein in Traf3<sup>−/−</sup> B cells analyzed by Western blot analysis. Total cellular proteins were prepared from purified splenic B cells, separated by SDS-PAGE, and immunoblotted for Chkα, followed by TRAF3 and β-actin. Results shown are representative of three independent experiments. (B and C) Analysis of Cho metabolism by stable isotope labeling. Purified splenic B cells were cultured in mouse B cell medium containing 80 μg/ml of trimethyl-D<sub>9</sub>-Cho ([2H<sub>9</sub>]-Cho) for the indicated time periods. The D<sub>9</sub>-labeled and unlabeled water-soluble metabolites (B) and lipids (C) of cultured splenic B cells were subsequently analyzed using LC-MS. (B) Increased incorporation of D<sub>9</sub>-Cho into P-Chol in Traf3<sup>−/−</sup> B cells. (C) Elevated incorporation of D<sub>9</sub>-Cho into PC species in Traf3<sup>−/−</sup> B cells. The composition of the D<sub>9</sub>-labeled and unlabeled P-Chol or PC species of each sample is shown in the bar graphs (left panel), and the kinetic increase of the D<sub>9</sub>-labeled P-Chol or PC species of each genotype of cells is shown in the curves (right panel). *p < 0.05 (significantly different; t test), **p < 0.01 (very significantly different; t test) between LMC and Traf3<sup>−/−</sup> 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<sup>−/−</sup> mouse B lymphoma cell lines, 27-9 and 105-8, that were previously derived from aging B-Traf3<sup>−/−</sup> mice (8, 30). Our results showed that upregulation of Chka expression was maintained in these two Traf3<sup>−/−</sup> mouse B lymphoma cell lines at a similarly high level as that detected in premalignant Traf3<sup>−/−</sup> 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)
The efficiency of the lentiviral vectors was transduced with an empty lentiviral vector, pUB-Thy1.1, were 8226 cells using a lentiviral expression vector, pUB–TRAF3–

To conduct TRAF3 reconstitution experiments (6). We transduced the constitution of TRAF3 expression induced cellular apoptosis in 8226 cells, as demonstrated by FACS analysis (Fig. 4A). 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-Cholysis. We cultured the transduced 8226 cells in human B cell medium containing the stable isotope tracer trimethyl-D<sub>3</sub>-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-Chol and two downstream phospholipid PC species (32:2 and 34:3). This was associated with slower production of labeled P-Chol and PC lipids (32:2 and 34:3) from the D<sub>3</sub>-labeled Cho tracer (Fig. 4D, 4E). We noticed that the labeling fractions of both P-Chol 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-Chol 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 Chk<sub>a</sub> expression and the Kennedy pathway of P-Cholysis 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<sup>−/−</sup> 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<sup>−/−</sup> mice. We administered MN58B or RSM932A to gender-matched, young adult LMC and B-Traf3<sup>−/−</sup> 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<sup>−/−</sup> 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<sup>−/−</sup> 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<sup>−/−</sup> 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<sup>−/−</sup> or LMC mice (Fig. 5C).

In summary, administration of pharmacological inhibitors of Chkα substantially reversed the B cell hyperplasia phenotype observed in B-Traf3<sup>−/−</sup> mice, suggesting that elevated Chkα-mediated P-Cholysis and PC biosynthesis contributes to the prolonged survival of B-Traf3<sup>−/−</sup> 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. Chk inhibitors MN58B and RSM932A suppressed the survival of TRAF3-deficient mouse and human malignant B cells in culture. (A) Upregulation of Chka 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 Chka 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. Chka expression was determined by quantitative real-time PCR using TaqMan assays specific for mouse (A) and human (B) Chka, 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 cells 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 Traf3/2 B cells contained markedly elevated levels of P-Cho, P-DMEtn, PC, and phosphatidylethanolamine as well as Chk, 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 Chk and TRAF3 determined by Western blot analysis. Total cellular proteins were prepared at day 3 posttransduction and then immunoblotted for Chk, 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 Traf32/2 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 Chk-mediated Cho metabolism supports the aberrantly prolonged survival of Traf32/2 B cells. Therefore, our study established, to our knowledge, a novel connection between the tumor

FIGURE 5. Chka inhibitors MN58B and RSM932A substantially reversed the B cell hyperplasia phenotype in B-Traf32/2 mice. Gender-matched, young adult LMC and B-Traf32/2 mice were administered i.p. with a Chka inhibitor, either MN58B or RSM932A, at 2 mg/kg per mouse or vehicle control three times a week for 4 wk. Spleen size and B cell compartment were analyzed at 2 d after the last injection. (A) Chka inhibitors reduced the spleen weights of B-Traf32/2 mice. (B) Representative FACS profiles of mouse splenocytes. B cells and T cells were identified by B220 and CD3 staining, respectively. Splenic B cell subsets were further differentiated by the markers CD21 and CD23. Marginal zone (MZ) B cell subset was identified as B220+CD21+CD23low, and follicular (FO) B cell subset was identified as B220+CD21intCD23+. (C) The percentages and numbers of splenic B cells, B cell subsets, and T cells analyzed by FACS. Effects of MN58B or RSM932A treatment were compared with the vehicle control group for each genotype. Results shown are mean ± SD (n = 6, including three female and three male samples for each genotype). *p < 0.05 (significantly different; t test), **p < 0.01 (very significantly different; t test), ***p < 0.001 (highly significantly different; t test), ns, not significantly different (t test, p ≥ 0.05) between MN58B or RSM932A treated and the vehicle control group.
suppressor TRAF3 and the known oncogenic Chkx–P-Chol--PC metabolic pathway in premalignant and malignant B cells.

PC and phosphorylcholine 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 phosphorylcholine but decreased levels of their catabolic products, such as DAG and MAG in the Traf3−/− B cells. In addition to Chkx, we also discovered that TRAF3-mediated regulation of several other enzymes that catalyze the anabolic or catabolic pathways of phospholipids in B cells, including Lpcat1, Faah, Gpdp3, Pldc3, Dgka, Lacc1, and Pip5k1b. Among these, upregulation of Lpcat1 and Faah, as well as downregulation of Dgka and Pip5k1b, has been documented in human cancers (70–75). In particular, the key enzyme in 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 the 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 phosphorylcholine (Fig. 1E). Thus, simultaneous upregulation of both Lpcat1 and Anxa4 would likely promote the synthesis of PC and inhibit the breakdown of PC and phosphorylcholine, which may also contribute to the increased PC and phosphorylcholine levels detected in the Traf3−/− B cells. Collectively, our results reveal the complex interactions between TRAF3 and phospholipid metabolic pathways associated with survival regulation in B cells.

We noticed that another major effect of TRAF3 on the 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 Pygl and two enzymes of ribonucleotide biosynthesis (Mthfd1 and Adsl1) as well as downregulation of two enzymes involved in ribonucleotide catabolism (Upb1 and Pdeo2a) in the 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 the 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 the 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 G1958A is associated with a decreased risk of B cell acute lymphoblastic leukemia and non-HL (80, 81). It is thus likely that the increased nonoxidative PPP metabolism and ribonucleotide biosynthesis may also contribute to the prolonged survival of the Traf3−/− B cells.

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 Chkx-driven de novo Kennedy pathway plays an important causal role in the survival phenotype of the Traf3−/− B cells. Our findings support that the Chkx–P-Chol–PC metabolic pathway has diagnostic and therapeutic value for B cell malignancies. For example, changes in PC cellular levels can be noninvasively monitored in patients by in vivo imaging using Cho analogues tracers [19F]-Cho or [1C]-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 Chkx 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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