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Protein Kinase CK2 Regulates B-cell Development and Differentiation

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Abstract

Protein kinase CK2 (also known as Casein Kinase 2) is a serine/threonine kinase composed of two catalytic subunits (CK2α and/or CK2α') and two regulatory CK2β subunits. CK2 is overexpressed and overactive in B-cell acute lymphoblastic leukemia and diffuse large B-cell lymphomas, leading to inappropriate activation of the NF-κB, JAK/STAT and PI3K/AKT/mTOR signaling pathways and tumor growth. However, whether CK2 regulates normal B-cell development and differentiation is not known. We generated mice lacking CK2α specifically in B-cells (using CD19-driven Cre recombinase). These mice exhibited cell-intrinsic expansion of marginal zone B (MZB) cells at the expense of transitional B (TrB) cells, without changes in follicular B (FoB) cells. TrB cells required CK2α to maintain adequate B-cell receptor (BCR) signaling. In the absence of CK2α, reduced BCR signaling and elevated Notch2 signaling activation increased MZB cell differentiation. Our results identify a previously unrecognized function for CK2α in B-cell development and differentiation.

Introduction

B-cells are central to humoral immunity, producing pathogen-reactive antigen-specific antibodies (1, 2). Based on phenotype, developmental program and functional properties, B-cells are divided into B1 and B2 cell lineages. B1 cells derive from fetal progenitors, predominate in the pleural and peritoneal cavities, and contribute most serum immunoglobulin M (IgM), including during early phases of infection (3). B2 cells develop in the bone marrow from common lymphoid progenitor cells, then pass through pro-B, pre-B, and immature B-cell stages. Newly formed immature B-cells migrate from the bone marrow to the spleen as transitional B (TrB) cells, where they further mature into follicular B (FoB) or marginal zone B (MZB) cells (4). FoB cells circulate among lymphoid organs and give rise to germinal-center B-cells that undergo somatic hypermutation in T-cell-dependent responses (5). MZB cells localize in the splenic marginal zone, and provide a first-line-of-defense by rapidly producing antibodies in response to infection by blood-borne viruses and encapsulated bacteria (3, 6).

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TrB cells instructed to become FoB or MZB cells in the spleen depend on B-cell receptor (BCR) and Notch2 signaling (4, 7). BCR signaling strength drives B-cell subset fate determination (4). Weak BCR signaling seems to induce MZB cell development whereas relatively strong BCR signaling favors FoB cell development (4). Notch2 is another crucial B-cell fate determination factor, particularly for MZB cells. MZB cell instruction requires triggering of Notch2 on developing B-cells by the Notch2 ligand Delta-like 1 (Dll1) expressed by splenic venules in the red pulp and marginal zone (8–10). Aside from BCR and Notch2 signaling, B-cell maturation requires B-cell activating factor (BAFF) and NF-κB signaling (4).

Protein kinase CK2 is a highly conserved serine-threonine kinase present in cells as a tetramer consisting of two catalytic subunits (CK2α and/or CK2α') and two regulatory subunits (CK2β) (11, 12). The catalytic subunits CK2α and CK2α' (encoded by different genes *Csnk2a1* and *Csnk2a2*, respectively) are closely related and share similar enzymatic characteristics. However, functional distinctions between CK2α and CK2α' have been reported. First, global deletion of CK2α is embryonic lethal (13), while CK2α' knockout mice have viable offspring (14), suggesting that CK2α has the capacity to partially compensate for CK2α'. Stably transfected tumor cell lines with catalytically inactive forms of CK2α or CK2α' also provide evidence for functional differences between CK2α and CK2α'. Cell proliferation and viability were compromised by induced expression of catalytically inactive CK2α' (15). By comparison, induced expression of catalytically inactive CK2α in these cells was without effect on proliferation or viability, suggesting that CK2α' may have unique functions associated with the control of proliferation or viability. Therefore, CK2α and CK2α' share many similarities, but do have some functional differences. CK2 phosphorylates serine or threonine residues specified by acidic side chains in many proteins, including growth factor receptors, transcription factors, and cytoskeletal proteins (16, 17). CK2 has been primarily studied in cancers, with aberrant expression and high kinase activity in many cancers, promoting tumor survival and growth (18, 19). In tumor cells, CK2 sustains pro-survival and proliferative signaling cascades that depend on NF-κB, PI3K/AKT/mTOR and/or JAK/STAT signaling, which are also centrally important in lymphocyte biology (20). Our previous studies provided the first evidence that CK2 is critical for activating JAK/STAT signaling in tumor cells (21, 22). Growing evidence suggests CK2 involvement not only in cancer cells, but also in regulating both innate and adaptive immune cell functions (23). Ulges et al., and our group demonstrated that CK2 activity promotes $CD4^+$ Th17 and Th1 cell differentiation, and inhibits $Foxp3^+$ Treg-cell generation (24–27). CK2β is also involved in the suppressive function of $CD4^+$ $Foxp3^+$ Tregs against allergy-promoting Th2 cells (28). CK2 is critical for monocyte-derived dendritic cells to mature and produce cytokines necessary to polarize effector T-cells in response to allergic contact dermatitis-triggering allergens (29). Inhibiting CK2 disrupts myeloid cell differentiation in the tumor microenvironment, leading to substantial augmentation of antitumor activity by immune therapy (30). A recent study demonstrated the suppressive effects of CK2 on inflammatory myeloid cell responses, rendering host tissues more susceptible to bacterial expansion during infection (31). Whether CK2 has specific functions in B-cell development and differentiation has never been examined.

Here, we examined CK2α specifically in B-cells *in vivo*. We demonstrate that CK2 expression and kinase activity are induced and/or enhanced upon B-cell activation. To assess the B-cell specific CK2α function *in vivo*, we generated mice with B-cell specific conditional deletion of CK2α by crossing gene-floxed C*snk2a1*$^{fl/fl}$ mice with mice expressing Cre recombinase specifically in the B-cell lineage (CD19-Cre) (32). Deleting CK2α in B-cells led to MZB cell accumulation in the spleen accompanied by decreased TrB cells, without change in FoB cells. CK2α biases TrB cell development into MZB cells through regulating the magnitude of BCR and Notch2 signaling. Our data demonstrate that CK2α is an important regulator of B-cell development and differentiation, specifically, differentiation of TrB cells into MZB cells.

Materials and Methods

Mice.

B6.PEPBOY.CD19$^{Cre/+}$ mice (32) were obtained from Dr. F. Lund (University of Alabama at Birmingham, UAB). C*snk2a1*$^{fl/fl}$ mice were obtained from Dr. H. Rebholz (33). C*snk2a1*$^{fl/fl}$CD19$^{Cre/+}$ conditional knockout (CK2α-cKO) mice were created by crossing C*snk2a1*$^{fl/fl}$ mice to CD19$^{Cre/+}$ mice (32). C*snk2a1*$^{+/+}$ CD19$^{Cre/+}$ wild-type (WT) mice were used as controls. All animals including Rag1$^{-/-}$ mice and C57BL/6 mice were bred and maintained under specific pathogen-free conditions in the animal facility at UAB. Experiments were performed using male and female mice between 8- and 12-weeks of age. All experimental procedures involving animals were reviewed and approved by the UAB Institutional Animal Care and Use Committee.

Flow Cytometry.

Single-cell suspensions obtained from spleen, bone marrow (one femur and one tibia), and peritoneal cavity were counted using a hemocytometer and stained in FACS buffer (DPBS supplemented with 2% FCS) for 30 min on ice and incubated with fluorescence-labeled antibodies for 30 min at 4°C. The following antibodies were used in this study (all Biolegend except where noted otherwise): anti-CD19 BV650/APC/BV786 (clone 6D5); anti-B220 APC-Cy7/BV510 /BV605 (clone RA3-6B2); anti-CD23 PE/BV421 (clone B3B4); anti-CD21/35 PerCP-Cy5.5 (clone 7E9); anti-CD93 APC (clone AA4.1); anti-IgM PE-Cy7 (clone RMM-1); anti-IgD FITC/BV605 (clone 11-26c.2a); anti-CD24 Pacific Blue (clone M1/69); anti-CD43 APC (clone S11); anti-CD249 PE (clone BP-1, BD Bioscience); anti-CD38 APC-Cy7 (clone 90); anti-FAS Alexa 647 (clone Jo2); anti-CD138 PE/BV421 (clone 281-2); anti-CD45.1 Alexa Fluor 488/PerCP-Cy5.5 (clone A20); anti-CD45.2 Alexa 647/APC-Cy7 (clone 104); anti-CD11b FITC (clone M1/70); anti-CD5 PerCP-Cy5.5 (clone 53-7.3); anti-ADAM10-PE (clone 139712, R&D Systems); anti-CD79a (pY182) Alexa 647 (clone D1B9, Cell Signaling Technology); anti-Syk (pY352) Alexa 647 (clone 65E4, Cell Signaling Technology); anti-BTK (pY223) BV421(clone N35-86, BD Bioscience); anti-PLC-γ2 (pY759) Alexa 647(clone K86-689.37, BD Bioscience); and anti-ERK1/2 (pT202/pY204) Alexa 488 (clone 20A, BD Bioscience). For live- versus dead-cell discrimination, the Live/Dead Fixable Blue Stain Kit (L23105; ThermoFisher Scientific) or Live/Dead Fixable Aqua Stain Kit (L34957; ThermoFisher Scientific) was utilized. Stained cells were

acquired on an LSRII flow cytometer (BD Biosciences), and data were analyzed using FlowJo software (Tree Star, Inc).

For intracellular staining, spleen B-cells were purified by negative selection using CD43 Dynabeads (11422D; ThermoFisher Scientific), routinely to 90-95% purity, washed, and suspended in 2% FCS- RPMI. Cells were warmed at 37°C for 30 min. and incubated at 37°C with 10 μg/ml anti-IgM (Jackson ImmunoResearch) for the indicated time points. Subsequent surface and intracellular staining was performed using the BD Cytofix/ Cytoperm protocol.

Confocal Imaging.

Confocal imaging was performed on spleen sections. Spleens were harvested, embedded in Frozen Tissue Media and snap-frozen in dry ice and 100% ethanol bath followed by cryosectioning. Eight um thick spleen frozen sections were dried in air for 20 seconds before fixation in ice cold acetone for 15 min. and rehydration with 1% BSA for 10 min.. Tissues were blocked with 10% normal rat serum at room temperature for 30 min., stained with purified rat anti-mouse CD169 (MOMA-1, Bio-Rad) plus biotin-anti-B220 (RA3-6B2, BD Biosciences), and then with Alexa Fluor 488-conjugated goat polyclonal anti-rat IgG secondary antibody plus Alexa Fluor 555-streptavidin (Invitrogen). Images were taken using a Nikon A1 confocal microscope at the UAB High Resolution Imaging Facility.

In Vitro B-cell Stimulation.

Purified spleen B-cells were cultured in B-cell medium (RPMI 1640 with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 10 mM HEPES and 50 μM β-mercaptoethanol) followed by stimulation with 10 μg/ml LPS (Sigma-Aldrich), 100 ng/ml CD40L (R&D) plus 10 ng/ml IL-4 (Biolegend), or 10 μg/ml anti-IgM plus 10 ng/ml IL-4 for 1-3 days.

Immunoblotting.

B-cells were lysed in RIPA buffer, protein lysates were separated by electrophoresis, transferred to a nitrocellulose membrane, and blotted with anti-CK2α (abcam, Cambridge, MA), CK2β (abcam, Cambridge, MA), CK2α' (Santa Cruz Biotechnology) and β-Actin (Sigma-Aldrich) Abs, as previously described (24, 25).

CK2 Kinase Assay.

The CycLex CK2 Assay/Inhibitor Screening Kit (MBL International Corporation) was used to assess CK2 kinase activity. Cells were lysed, and both catalytic subunits (CK2α and CK2α') were immune-precipitated. Resulting lysates were assayed for CK2 kinase activity according to manufacturer's instructions.

TrB Cell Stimulation on OP9-Dll1 Cells.

OP9-Dll1 cells originally from Dr. Juan Carlos Zuniga-Pflucker (34) were obtained from Dr. Robert Welner (UAB). Four × 10^4 OP9-Dll1 cells were seeded in 24-well plates in 1-ml medium (α-MEM supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin) to reach 80% confluence. $CD93^+$ transitional B-cells were enriched from

splenocytes by first staining with CD93-APC, then using magnetic-bead enrichment with anti-APC beads and LS columns (both from Miltenyi) according to manufacturer's instructions. Enriched $CD93^+$ TrB cells (5×10^5), routinely 85% pure, were resuspended in B-cell media and added to the OP9-Dll1 cells. Non-adherent cells were harvested and analyzed for MZB cell phenotype by flow cytometry 3 days later. To inhibit Notch2 signaling, 25-μM DAPT (N-[N-(3,5-difluorophenacetyl)-l-alanyl]-S-phenylglycine t-butyl ester; Sigma) (35), which interferes with γ-secretase-mediated Notch protein cleavage, was added to the wells.

RNA Isolation, RNA Sequencing, and Quantitative RT-PCR.

RNA sequencing was performed as described (24). Briefly, MZB cells were sorted from WT and CK2α-cKO mice. Total RNA was extracted from FACS-sorted B cells using the miRNeasy Mini Kit (Qiagen, Venlo, Netherlands) according to manufacturer's protocols and submitted to GENEWIZ (South Plainfield, NJ) for RNA sequencing (RNA-seq) and bioinformatics analysis. RNA sequencing data was submitted to the Gene Expression Omnibus (GEO) Repository under accession number GSE165140 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE165140). Genes with an FDR less than 0.05 with a fold-change more than 1.5 or less than −1.5 were considered differentially expressed genes (DEG). Further pathway analysis was performed by Gene Set Enrichment Analysis (GSEA) available through the Broad Institute.

For quantitative RT-PCR (qRT-PCR) analysis, 1,000-2,000 ng RNA was reverse-transcribed into cDNA using M-MLV Reverse Transcriptase (Promega) as described (24). cDNA was subjected to qRT-PCR using TaqMan primers (Thermo Fisher Scientific). Relative gene expression was calculated according to the ΔΔ threshold-cycle (Ct) method.

Mixed Bone-Marrow Chimeric Mouse Generation.

Five-10×10^6 bone-marrow cells from CD45.1 WT or CK2α-cKO mice and CD45.2 C57BL/6 mice were mixed in a 1:1 ratio and i.v. injected into lethally irradiated CD45.2 $Rag1^{-/-}$ mice. Chimeric mice were euthanized 8 weeks later to analyze reconstitution of MZB, FoB, and TrB cells. Reconstitution was calculated as the ratio of CD45.1 WT or CK2α-cKO to CD45.2 WT cells.

Adoptive Transfer of TrB Cells.

TrB cells ($CD93^+$) were sorted from CD45.1 WT or CK2α-cKO mice. One $\times 10^6$ TrB cells were mixed with 10×10^6 CD45.2 C57BL/6 splenocytes and injected i.v. into CD45.2 $Rag1^{-/-}$ recipient mice. Recipient $Rag1^{-/-}$ mice were sacrificed 5 days post-transfer to evaluate splenic MZB cell differentiation.

Statistical Analysis.

Statistical differences for comparison between two groups were determined by Student's t-test using GraphPad Prism Software. Multiple comparisons were performed by one-way ANOVA. *p* values are indicated as: * $p<0.05$, ** $p<0.01$, *** $p<0.001$. All error bars represent mean ± SD.

Results

B-cell Activation Induces CK2 Expression and Kinase Activity.

To study how CK2 may regulate B-cell development and differentiation, we first examined CK2α, CK2β, and CK2α' expression patterns during B-cell activation. In wild-type naïve B-cells, upon stimulation *in vitro* with the T-cell independent stimulus LPS, and the T-cell dependent stimuli CD40L plus IL-4, or anti-IgM antibody plus IL-4, expression of CK2α, CK2β and CK2α' was strongly induced (Fig. 1A). Consistent with immunoblot results, intracellular staining revealed increased CK2α protein expression upon stimulation by LPS in a time-dependent manner (Fig. 1B). Increased protein expression also correlated with increased *Csnk2a1* (CK2α), *Csnk2b1* (CK2β) and *Csnk2a2* (CK2α') mRNA levels upon LPS stimulation compared with untreated B cells (Fig. 1C). Aside from subunit expression, a significant increase in overall CK2 kinase activity occurred upon LPS activation (Fig. 1D). These results demonstrate induction of CK2 protein expression and kinase activity upon B-cell activation, and suggest potential CK2 involvement in B-cell functions.

CK2α Deficiency Promotes MZB Cell Expansion.

Globally deleting CK2α is embryonically lethal (13). To determine CK2α involvement during B-cell development, we first crossed C*snk2a1*$^{fl/fl}$ mice with mice bearing Cre recombinase under control of the endogenous B-cell specific *Cd19* locus (32). The resulting C*snk2a1*$^{fl/fl}$CD19$^{Cre/+}$ (CK2α-cKO) mice were viable and underwent normal development with no signs of systemic or organ-specific inflammation, and had specific deletion of CK2α in B-cells. In the splenic B-cell compartment, deletion of CK2α was confirmed by qRT-PCR analysis (Fig. S1A) and a significant reduction in CK2 kinase activity was observed in B-cells from CK2α-cKO mice (Fig. S1B). Detailed characterization of the bone-marrow B-lineage cells demonstrated a decreased frequency of pre-B cells [Hardy Fraction (Fr) D] ($B220^+IgM^+CD43^-$) and an increased frequency of mature circulating B-cells (Fr F) ($B220^+IgM^+IgD^+CD43^-$) (Figs. S1C and S1D), and by absolute cell counts, we observed a reduced number of pre-B cells (Fr D) (Fig. S1E). Thus, CK2α deletion slightly impacts bone-marrow B-cell development. We next evaluated the impact of CK2α deletion on peripheral immune-cell development. CK2α-cKO mice exhibited an increase in spleen weight (Fig. 2A) and total splenocytes (Fig. 2B). CK2α-cKO mice had comparable frequencies of total $CD19^+B220^+$ B-cells (Fig. 2C) and $CD3^+$ T-cells (including $CD4^+$ and $CD8^+$ T-cells) compared with WT mice (Fig. S2A). Because of the increase in total splenocytes, the number of B-cells (Fig. 2C) and $CD4^+$ T-cells (Fig. S2B) was increased in CK2α-cKO mice. CK2α-cKO mice had a similar distribution of naïve ($CD44^-CD62L^+$) and effector T-cells ($CD44^+CD62L^-$), as detected by CD44 and CD62L expression (Fig. S2C).

Among B-cells, CK2α-cKO mice exhibited increased MZB cells and decreased TrB cells, with no effect on FoB cells (Figs. 2D–H). The localization of CK2α-cKO B-cells within the splenic microenvironment was examined. We stained spleen sections from WT and CK2α-cKO mice with B220 antibody to detect B-cells and CD169 antibody, which recognizes metallophilic macrophages located at the border between the follicle (Fo) and marginal zone (MZ) areas. In WT mice, the majority of B-cells were localized in the Fo area, whereas in CK2α-cKO mice, more B-cells were localized in the MZ area (Fig. 2I). Thus, B-cells

developing in CK2α-cKO mice show an abnormal distribution within the splenic microenvironment that is skewed toward an MZ localization. Moreover, we analyzed IgM and IgD expression on MZB cells and found that expression of IgM on MZB cells was comparable between WT and CK2α-cKO mice, while expression of IgD was significantly lower in CK2α-cKO MZB cells compared to WT MZB cells (Fig. 2J). Interestingly, upon LPS stimulation, we found MZB cells from CK2α-cKO mice that differentiated into CD138+ blasts were significantly reduced compared to MZB cells from WT mice (Figs. S3A–B), which suggests that CK2α promotes MZB cell differentiation into plasma cells. Characterization of B-cell subsets in the peritoneal cavity revealed no differences in peritoneal B1a ($CD19^+CD11b^+CD5^+$), B1b ($CD19^+CD11b^+CD5^-$), and B2 cells ($CD19^+CD11b^-CD5^-$) (Figs. 2K and 2L).

MZB Cell Increase in CK2α-cKO Mice is B-cell Intrinsic.

To determine whether increased MZB cells in CK2α-cKO mice was B-cell intrinsic or caused by changes in the stromal structures or other hematopoietic cells, we created mixed bone-marrow chimeras by lethally irradiating CD45.2 T-cell and B-cell deficient $Rag1^{-/-}$ recipient mice and reconstituting them with equal numbers of CD45.2 WT and CD45.1 WT or CK2α-cKO BM cells. Recipient mice were characterized 8 weeks post-transfer to investigate chimerism with B-cell development stages (Fig. 3A). Splenic $CD23^{hi}CD21/35^{lo}$ FoB cells were completely chimeric, and $CD21^{hi}CD23^{lo}$ MZB cells were generated mostly from CD45.1 CK2α-cKO hematopoietic cells (Figs. 3B and 3C). These findings demonstrate that enhanced MZB cell differentiation in CK2α-cKO mice is not due to micro-environmental changes, but rather is cell-intrinsic. We next evaluated whether TrB cells in the spleen might be affected. There was an underrepresentation of spleen TrB cells of the CD45.1 CK2α-cKO genotype (Fig. 3C). Collectively, increased MZB cells in CK2α-cKO mice is cell-intrinsic, and this enhancement starts from TrB cell stages, which may indicate that signals instructing TrB cells to become MZB cells are increased in the absence of CK2α.

CK2α Restricts TrB Cell Differentiation into MZB Cells.

To examine how CK2α deletion in B-cells leads to higher MZB cell numbers, we quantified cell proliferation and survival. To measure proliferation, WT and CK2α-cKO mice were injected with BrdU (a thymidine analogue incorporated into DNA during S phase) 22 h pre-sacrifice. We observed no difference in $BrdU^+$ FoB and MZB cells in CK2α-cKO mice compared with WT mice (Fig. 4A). Because there was no evidence of altered cycling under homeostatic conditions, we next analyzed cell survival. Splenic FoB and MZB cells exhibited comparable Caspase-3 staining, a measure of apoptosis (Fig. 4B). Together, these results show that CK2α deletion in B-cells promotes MZB cell accumulation without altering proliferation or cell survival.

To further address whether CK2α expression limits MZB cell differentiation, we performed adoptive-transfer experiments into $Rag1^{-/-}$ mice. We purified $CD45.1^+CD93^+$ TrB cells, and these were injected together with a ten-fold greater number of CD45.2 wild-type splenic cells, to avoid homeostatic proliferation that might bias toward MZB development (Figs. 4C and 4D). Five days post-transfer, we studied the fate of transferred CD45.1 spleen cells by

flow cytometry. WT and CK2α-cKO TrB cells gave rise to both FoB and MZB cells, whereas CK2α-cKO TrB cells gave rise to a higher frequency of MZB cells (Figs. 4C and 4D), suggesting that CK2α regulates TrB cells committed to become MZB cells. We also analyzed surface markers of TrB cells from WT and CK2α-cKO mice. We found that IgM expression was significantly lower in TrB cells from CK2α-cKO mice, while the expression levels of IgD, CD21 and CD23 are comparable between WT and in CK2α-cKO mice (Supplemental Fig. 4A). ADAM10 surface expression on TrB cells has been shown to mark commitment to the MZB cell fate (36). We analyzed ADAM10 expression in TrB cells. Interestingly, ADAM10 expression was lower in CK2α-cKO TrB cells (Supplemental Fig. 4B), which suggests that increased MZB cells in CK2α-cKO mice is independent of ADAM10 expression.

CK2α Regulates MZB Cell Differentiation Through BCR and Notch2 Signaling.

TrB cell development into FoB versus MZB cells depends, in part, on the strength of BCR signaling, and also requires Notch2 signaling (4). To address involvement of these signaling pathways relative to *in vivo* B-cell CK2 biology, we first examined whether BCR signaling is affected in CK2α-cKO TrB cells. When exposed to anti-IgM, CK2α-cKO TrB cells showed impaired BCR signaling as demonstrated by reduced levels of multiple phosphorylated intermediates, including CD79a, Syk, BTK, PLCγ 2, and ERK1/2 (Figs. 5A and 5B).

To address the mechanism of how CK2α regulates MZB differentiation, spleen MZB cells were isolated from WT and CK2α-cKO mice, and subjected to RNA sequencing. A total of 129 genes were significantly increased, and 67 genes were significantly decreased in CK2α-cKO MZB cells compared to WT MZB cells (Fig. 6A). GSEA of the data revealed that differentially expressed genes (DEG) were associated with the Notch-signaling pathway (Fig. 6B). CK2α-cKO MZB cells express higher levels of genes associated with Notch signaling pathways like *Heyl*, *Hes5* and *Dtx1* (Fig. 6C) (37). We also analyzed Notch2 target-gene expression levels in TrB cells from WT and CK2α-cKO mice by qRT-PCR, and found significantly higher expression levels of *Heyl*, *Hes5*, and *Dtx1* in TrB cells from CK2α-cKO mice compared to WT mice (Fig. 6D). To assess if Notch2-mediated differentiation of MZB cells from TrB cells was enhanced in CK2α-cKO mice, $CD93^+$ TrB cells from WT and CK2α-cKO mice were cultured on OP9 cells stably transfected with Dll1 (OP9-Dll1 cells), which stimulate Notch2 signaling through Delta-like-1 ligand expression. After 3 days of culture, a significant enhancement of $CD21/35^+IgM^+$ MZB cell differentiation was observed from TrB cells from CK2α-cKO mice compared to WT mice (32% vs 20%) (Fig. 6E). Accordingly, an inhibitor of Notch signaling (DAPT, 25 μM) mitigated *in vitro* MZB development (Fig. 6E). These results suggest that CK2α sustains BCR signaling, and negatively regulates Notch2 signaling, acting as a master regulator of MZB cell differentiation.

Discussion

Herein we identify a novel biological function of protein kinase CK2 in homeostasis of mature B-cell development. We found that CK2 expression and kinase activity were strongly

induced upon *in vitro* B-cell activation, and deletion of CK2α in B-cells led to aberrant enhanced accumulation of MZB cells. This accumulation of CK2α-deficient MZB cells was associated with reduced BCR signaling and elevated activation of Notch2 signaling in CK2α-deficient TrB cells. Our results identify the unrecognized involvement of CK2α in B-cell development and differentiation.

Previous studies demonstrated that CK2 is involved in malignant lymphocyte biology and acts as a pro-survival and signaling-boosting kinase, both in precursor and mature B-cell tumors (20, 38). CK2 is overexpressed, hyperactive, and essential for PI3K/PTEN/AKT signaling cascade activation in B-acute lymphoblastic leukemia (ALL) (39). Increased CK2 expression in B-ALL phosphorylates Ikaros and impairs Ikaros function (40). In mature B-lymphoid tumors including B-cell chronic lymphocytic leukemia (B-CLL), mantle-cell lymphoma (MCL) and diffuse large B-cell lymphoma (DLBCL), CK2 is overexpressed and essential for cell growth (19, 41). CK2 also positively regulates STAT3 and NF-κB-dependent signaling in MCL cells (42). We report, for the first time, that mouse CK2α is integral to normal B-cell development. Interestingly, deletion of CK2α in B-cells resulted in increased cellularity of the spleen. We found that $CD4^+$ T-cells, but not $CD8^+$ T-cells, were significantly increased in CK2α-cKO mice, while B-cells were dramatically increased in CK2α-cKO mice. Among B-cells, CK2α-cKO mice exhibited increased MZB cells and decreased TrB cells, with no effect on FoB cells. It has been reported that MZB cells are far superior to FoB cells in inducing $CD4^+$ T-cell expansion (43). Although it is not known whether naive MZB cells are able to efficiently activate $CD4^+$ T-cells, it has been shown that freshly isolated MZB cells from naive animals exhibit high levels of CD80 and CD86, indicative of previous antigenic experience (43). Therefore, more MZB cells could lead to $CD4^+$ T-cell expansion in CK2α-cKO mice.

B-cell specific deletion of CK2α increased MZB cell differentiation, but not that of FoB cells, which raises questions about how CK2α selectively affects one cell fate over another. MZB cell accumulation in the absence of CK2α may be caused by two possible mechanisms: enhanced lineage commitment from TrB cell to MZB cells and/or high survival of MZB cells. We did not find any evidence indicating reduced apoptosis or excessive proliferation of MZB cells in the absence of CK2α. When we performed adoptive-transfer experiments, we found that CK2α-cKO TrB cells gave rise to a higher frequency of MZB cells, suggesting that CK2α regulates TrB cells to become MZB cells. Also, in mixed bone-marrow chimeras, MZB cells were generated mostly from CK2α-cKO hematopoietic cells, while there was an underrepresentation of spleen TrB cells of the CK2α-cKO genotype. Therefore, increased MZB cells in CK2α-cKO mice start from TrB cell stages. TrB cells face a decision to mature into either FoB or MZB cells. It has been reported that BCR signaling causes immature type I TrB cells to become receptive to Notch ligands via Taok3-mediated surface expression of ADAM10 (36). BCR signaling strength has a critical role in determining TrB cell maturation into MZB or FoB cell compartments. Wen *et al.* demonstrated that the promotion of MZB cell development by exposure to a defined transgenic antigen, with FoB cell generation in its absence, indicates a BCR signal strength requirement that is higher for MZB cells than for FoB cell development (44). However, Pillai *et al.* proposed that relatively strong BCR signals favor the development of FoB cells, while weak BCR signals induce MZB cell development (4, 45, 46). Relatively strong BCR

signals render TrB cells in the follicle impervious to the presence of Dll1-mediated triggering of Notch2, whereas weak BCR signaling may enhance expression of one or more components of the Notch2-signaling pathway (4, 45, 46). Therefore, signals through BCR, Notch2, possibly BAFF, and signals involved in migration and retention of MZB cells may contribute cooperatively to the development of MZB cells. In this study, we found that deleting CK2α was associated with impaired BCR signaling in TrB cells, and decreased expression of IgM on CK2α-cKO TrB cells could be one of the reasons for impaired BCR signaling in TrB cells from CK2α-cKO mice. These data indicate CK2α is required for sustaining adequate BCR and BCR signaling to regulate B-cell differentiation. Others reported that CK2 inhibition with CX-4945, a specific CK2 inhibitor (47), impaired AKT phosphorylation and intracellular Ca^{2+} mobilization upon BCR engagement in DLBCL cells, and that CX-4945 acted synergistically with either SYK or BTK inhibitors to induce DLBCL cell death (48). However, the underlying molecular mechanisms by which CK2 directly or indirectly regulates BCR signaling requires further study.

Notch2 signaling is another important factor determining the differentiation fate of mature B-cells into FoB versus MZB cells (8, 10). Notch is a target of phosphorylation by CK2, and this phosphorylation negatively regulates Notch function (49, 50). Inhibiting CK2 by siRNA or by pharmacological inhibition restored Notch signaling in myeloid cells and substantially improved their differentiation (50). In our study, we demonstrate that in B-cells *in vivo*, CK2α negatively regulates the Notch2-signaling pathway, because in the absence of CK2α, Notch2 signaling was enhanced. This enhanced Notch2 signaling then seems to promote MZB cell differentiation, thereby linking CK2α to the signaling pathway critical for MZB cell differentiation.

In summary, our data identify a previously unrecognized function of protein kinase CK2α in B-cell development and differentiation, especially a critical function in MZB cell development. We demonstrate that CK2α is vital for sustaining adequate BCR signaling, and suppression of Notch2 signaling in B-cells, because in the absence of CK2α, weak BCR signaling and enhanced Notch2 signaling promotes MZB cell differentiation (Fig. 7). Thus, CK2α is an important regulator of MZB cell development and differentiation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Footnote:

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Key points:

1. CK2 expression and kinase activity are induced upon *in vitro* B-cell activation.
2. Deletion of CK2α in B-cells leads to aberrant enhanced accumulation of MZB cells.
3. CK2α sustains BCR signaling and suppresses Notch2 signaling in B-cells.

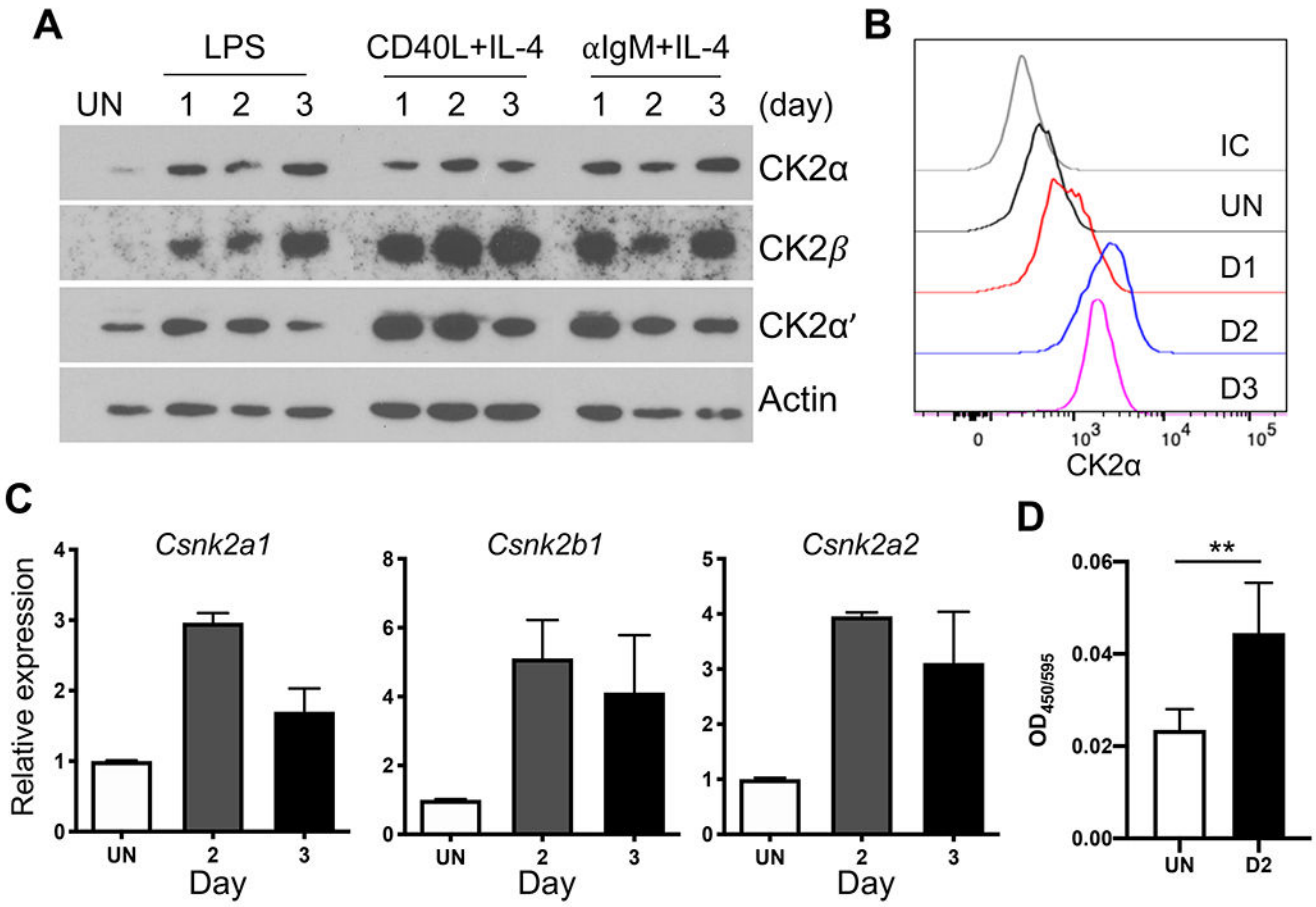

Figure 1. B-cell Activation Induces CK2 Expression and Kinase Activity.

(A) Immunoblotting analysis of CK2α, CK2β, and CK2α' in B-cells left untreated (UN) or activated with LPS (10 μg/ml), CD40L (100 ng/ml) plus IL-4 (10 ng/ml), or αIgM (10 μg/ml) plus IL-4 (10 ng/ml) for 1-3 days. Actin serves as a loading control. **(B)** Intracellular staining of CK2α in B-cells left untreated (UN) or activated for 1-3 days with LPS (10 μg/ml). IC, isotype control. **(C)** RNA was extracted from B-cells left untreated (UN) or activated for 2 or 3 days with LPS (10 μg/ml) and qRT-PCR performed using primers for *Csnk2a1*, *Csnk2b1* and *Csnk2a2*. **(D)** At day 2, untreated and LPS-activated B-cells were assayed for CK2 kinase activity. Values shown as the mean ± SD. ** $p < 0.01$, Student's t-test. n=3.

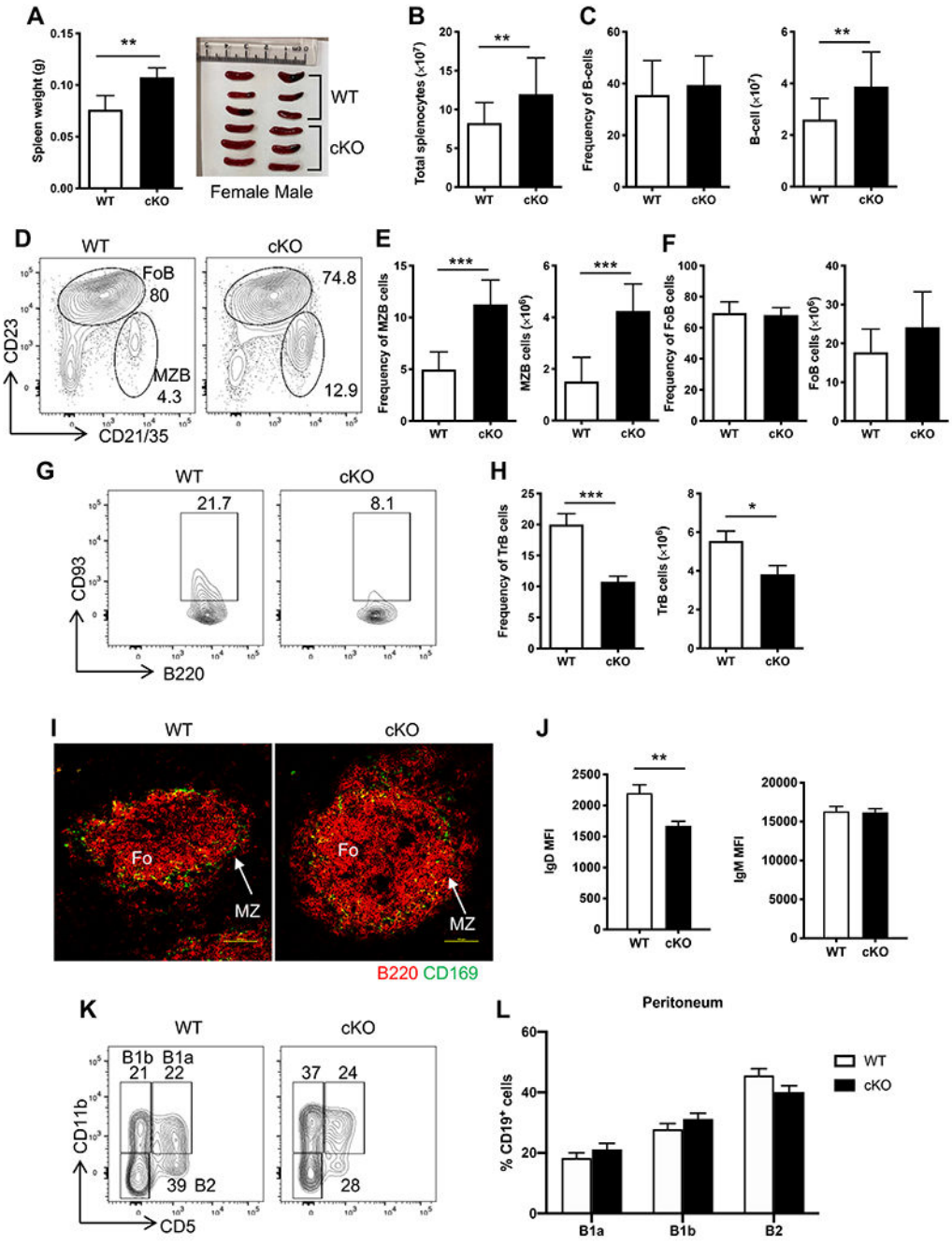

Figure 2. CK2α Deficiency Promotes Marginal-Zone B (MZB) Cell Increases.

(A) Spleen weights from WT and CK2α-cKO mice. **(B)** spleen cells from WT and CK2α-cKO mice were counted. **(C)** Percentage and number of $B220^{+}CD19^{+}$ B-cells in the spleens of WT and CK2α-cKO mice. **(D)** Representative flow-cytometry staining of spleens for $CD21/35^{hi}CD23^{lo}$ MZB cells and $CD21/35^{-}CD23^{hi}$ FoB cells. **(E, F)** Percentage and absolute number of MZB cells **(E)** and FoB cells **(F)** within splenic $B220^{+}CD19^{+}$ B-cells in WT and CK2α-cKO mice. **(G)** Representative flow-cytometry staining of spleens for CD93 expression on total B-cells. **(H)** Percentage and absolute number of $CD93^{+}$ TrB cells in the

spleens of WT and CK2α-cKO mice. **(I)** Spleen sections from WT and CK2α-cKO mice (8 weeks old) were analyzed for CD169 and B220 expression by immunofluorescent staining. Bar, 100 μm**. (J)** Quantification of IgM and IgD expression on MZB cells from WT and CK2α-cKO mice. n=6. **(K, L)** Representative flow-cytometry staining and summarized data for B1a ($CD19^+CD11b^+CD5^+$), B1b ($CD19^+CD11b^+CD5^-$), and B2 ($CD19^+CD11b^-CD5^-$) cells in the peritoneal cavity. Values shown as mean ± SD. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, Student's t-test. n=10.

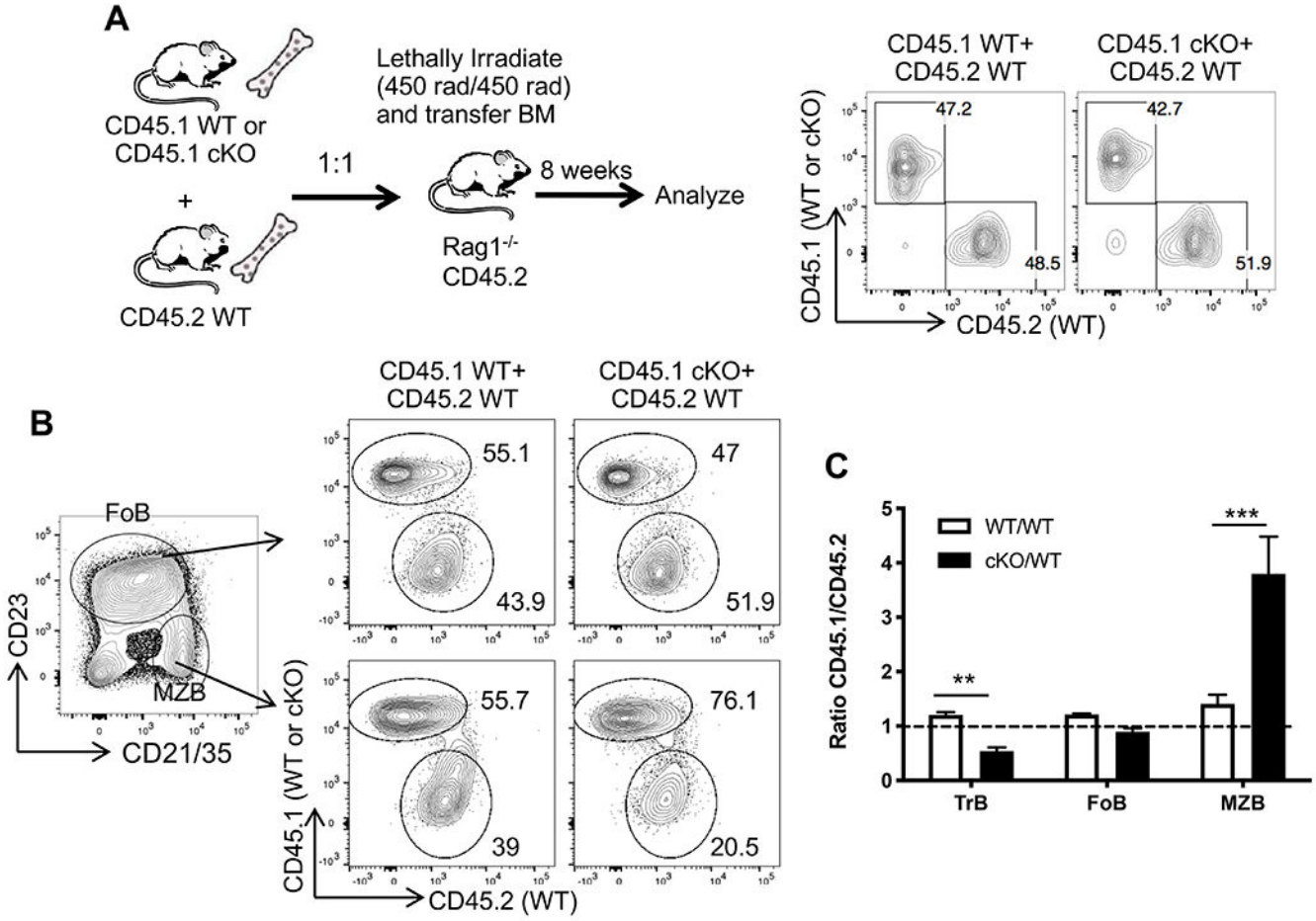

Figure 3. Enhanced MZB Cell Differentiation in CK2α-cKO Mice is B-cell-intrinsic.

(A) Schematic of experimental design. To generate mixed bone-marrow chimeric mice, 5–10 × 10^6 WT or CK2α-cKO (CD45.1) BM cells and 5–10 × 10^6 WT (CD45.2) BM cells were co-transferred into lethally irradiated $Rag1^{-/-}$ mice for 8 weeks before analysis. **(B)** Flow-cytometry staining of spleens from chimeric mice to evaluate the ratio between CD45.2 WT and CD45.1 WT or CK2α-cKO cells within the FoB cell and MZB cell populations. Numbers adjacent to gates represent percentages of cells within the gates. (**C**) Ratios of WT/WT and WT/CK2α-cKO frequencies of MZB, FoB and TrB cell populations per mouse. ** $p<0.01$, *** $p<0.001$, n=5.

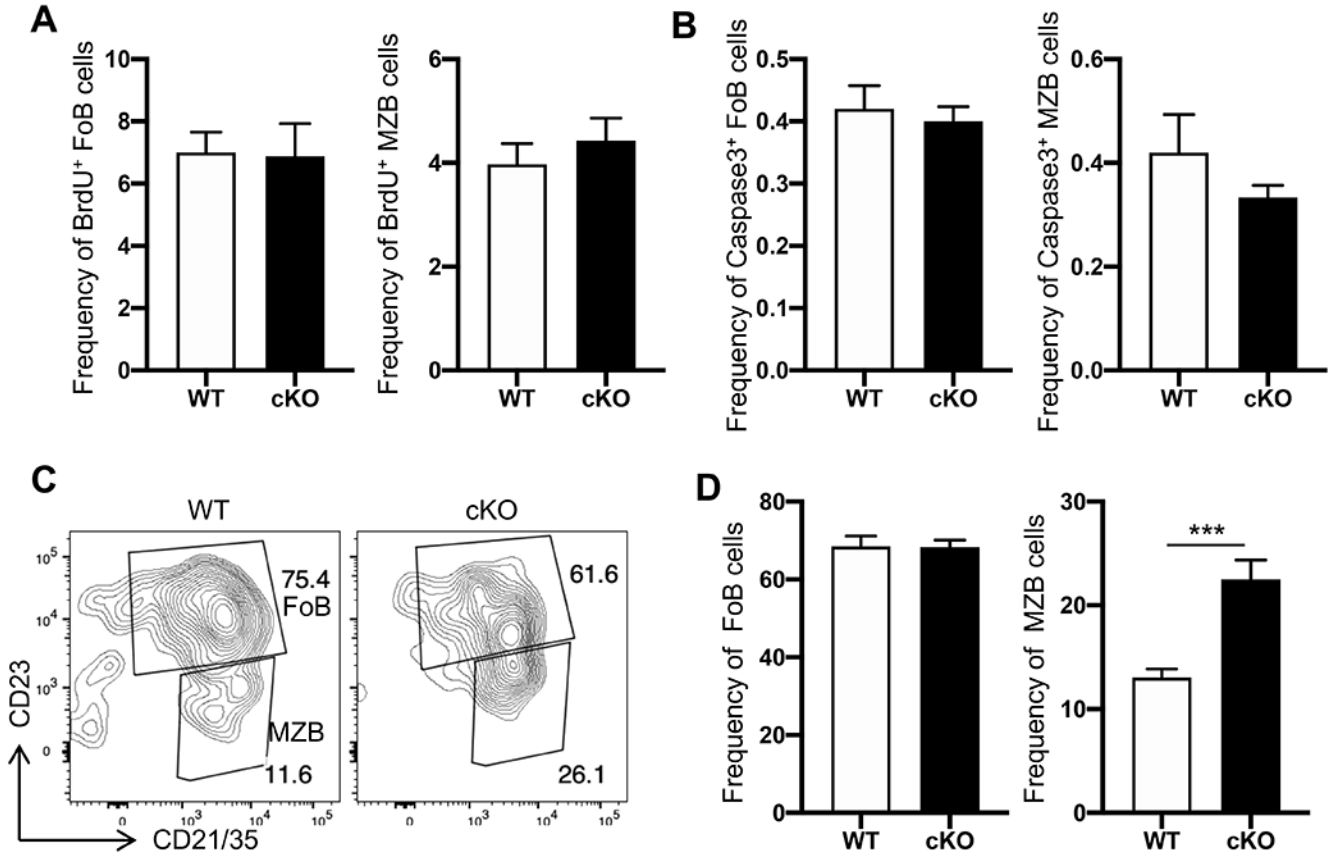

Figure 4. CK2α Restricts TrB Cell Differentiation into MZB Cells.

(A) WT or CK2α-cKO mice were injected with 1 mg BrdU i.v. 22 h before sacrifice. Frequency of $BrdU^+$ cells in splenic B-cell subsets in WT or CK2α-cKO mice. **(B)** Frequency of Caspase 3^+ splenic B-cell subsets in WT or CK2α-cKO mice. **(C)** Flow-cytometry staining of spleens for the presence of $CD21/35^{hi}CD23^{lo}$ MZB cells and $CD21/35^{low}CD23^{hi}$ FoB cells in $Rag1^{-/-}$ recipients (CD45.2) adoptively transferred with 1×10^6 $CD93^+$ TrB cells (CD45.1) from WT or CK2α-cKO mice mixed with 10×10^6 CD45.2 splenocytes. Numbers adjacent to outlined areas indicate the percentage of $CD21/35^{hi}CD23^{lo}$ MZB cells and $CD21/35^{lo}CD23^{hi}$ FoB cells after adoptive transfer of TrB cells. **(D)** Quantification of MZB cells and FoB cells formed in $Rag1^{-/-}$ recipients adoptively transferred with $CD93^+$ TrB cells from WT or CK2α-cKO mice. Values shown as mean ± SD. *** $p<0.001$. n=6.

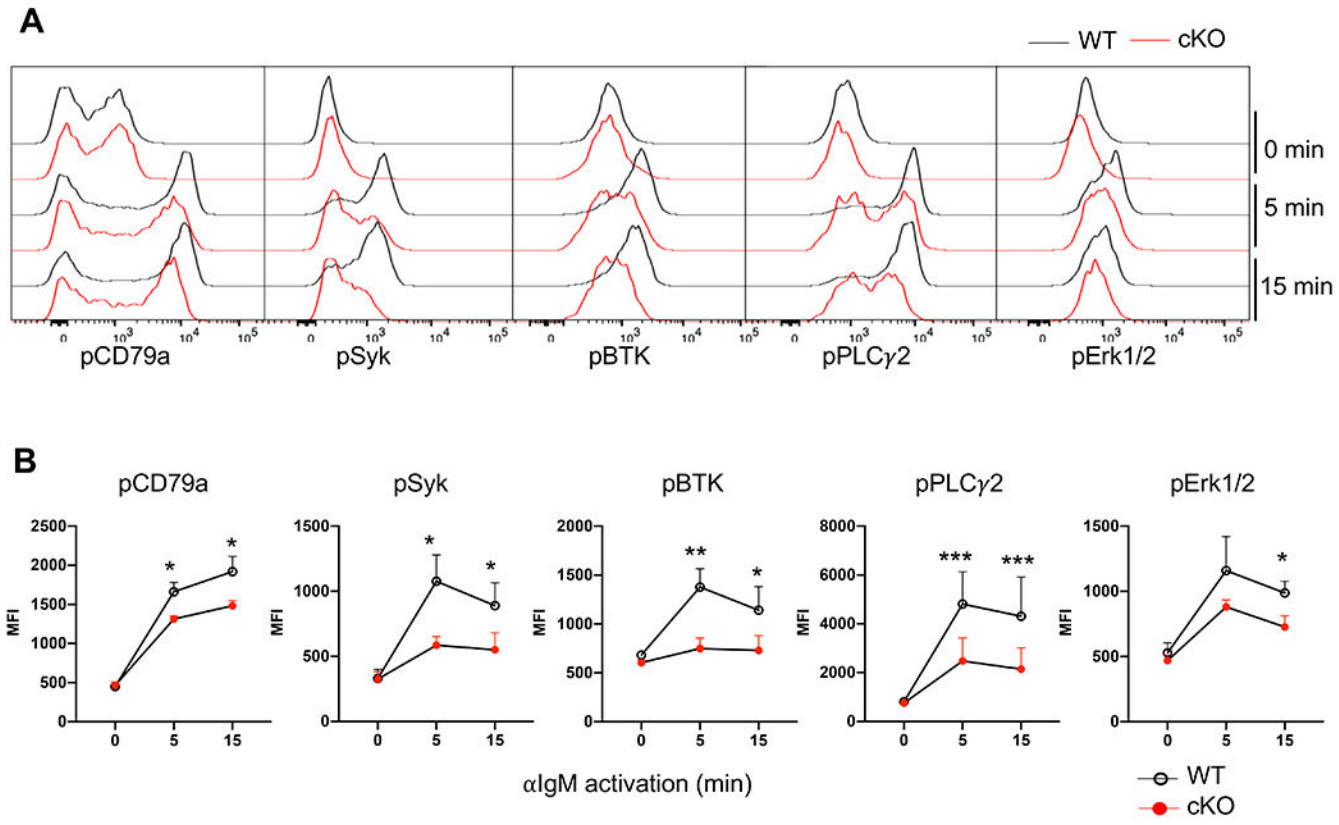

Figure 5. Reduced B-Cell Receptor (BCR) Signaling in CK2α-deficient TrB Cells.

(A) Representative flow cytometry analyzing phosphorylation of CD79a (pY182), Syk (pY352), BTK (pY223), PLCγ2 (pY759) and Erk1/2 (pT202/pY204) in WT and CK2α-cKO TrB cells stimulated with anti-IgM (10 μg/ml) for the indicated time points. **(B)** Summarized data show phospho-specific antibody binding to indicated intracellular signaling substrates downstream of the BCR, after stimulation with anti-IgM. Values shown as mean ± SD. * $p<0.05$, ** $p<0.01$, *** $p<0.001$. n=5.

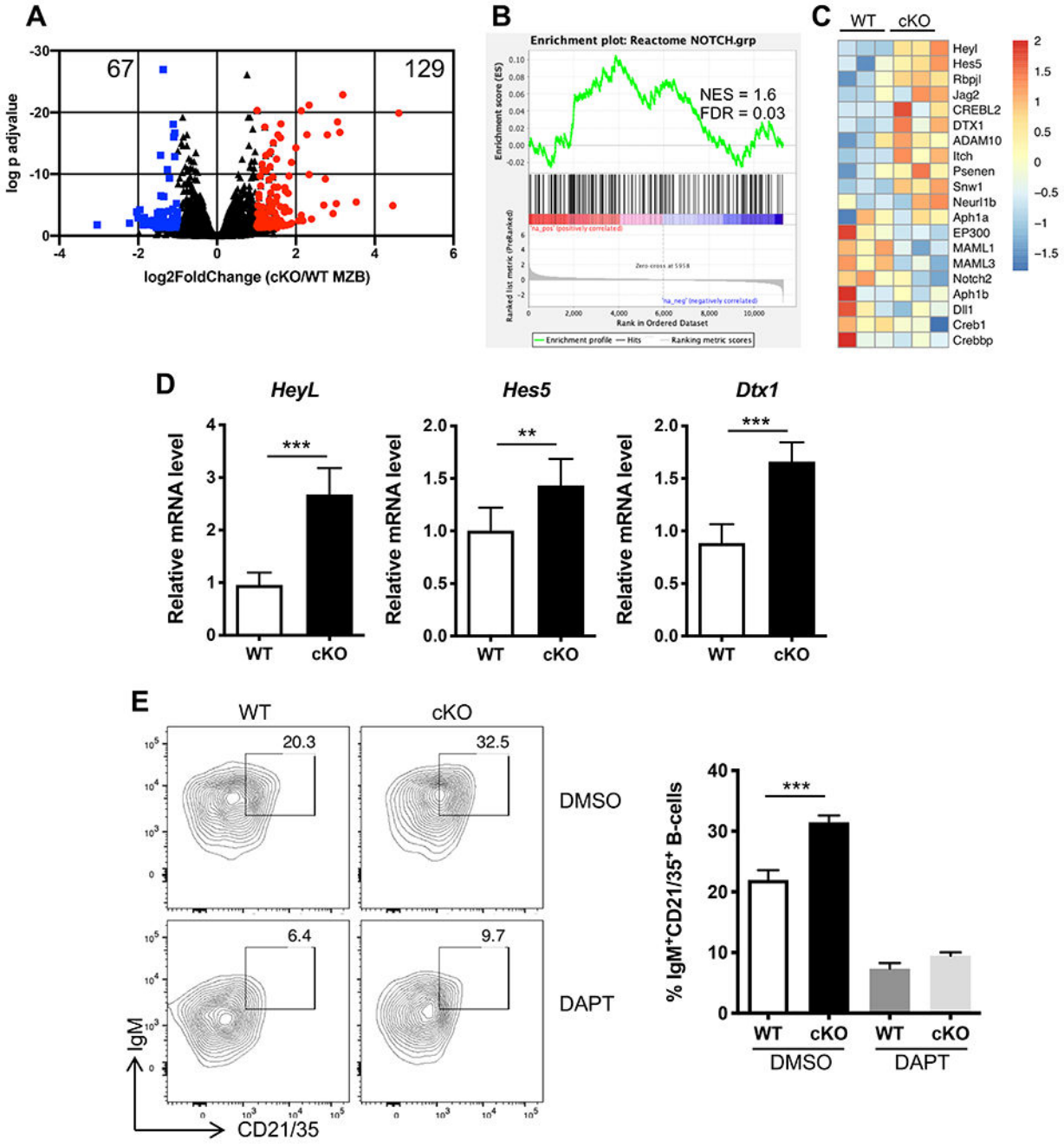

Figure 6. CK2α Deficiency Enhances Notch2 Activation in TrB Cells.

(A) RNA sequencing of MZB cells from spleens of WT and CK2α-cKO mice. Summary of genes differentially regulated by CK2α using the following cutoffs are shown: $p < 0.05$, fold change >1.5. n=3. **(B)** GSEA plot shows enrichment of RNA-sequencing data compared with Reactome Notch-pathway dataset. n=3. **(C)** Heat map shows Notch signaling relative gene expression in MZB cells. (**D**) Quantification of Notch target-gene expression in $CD93^{+}$ TrB cells from WT and CK2α-cKO mice. n=4. (**E**) Flow-cytometry analysis for induction of CD21/35 and IgM expression on TrB cells cultured on OP9-Dll1 cells for 3 days in the

absence or presence of the Notch inhibitor DAPT (25 μM). The fluorescence minus one (FMO) control was used for gating. ** $p<0.01$, *** $p<0.001$. n=3.

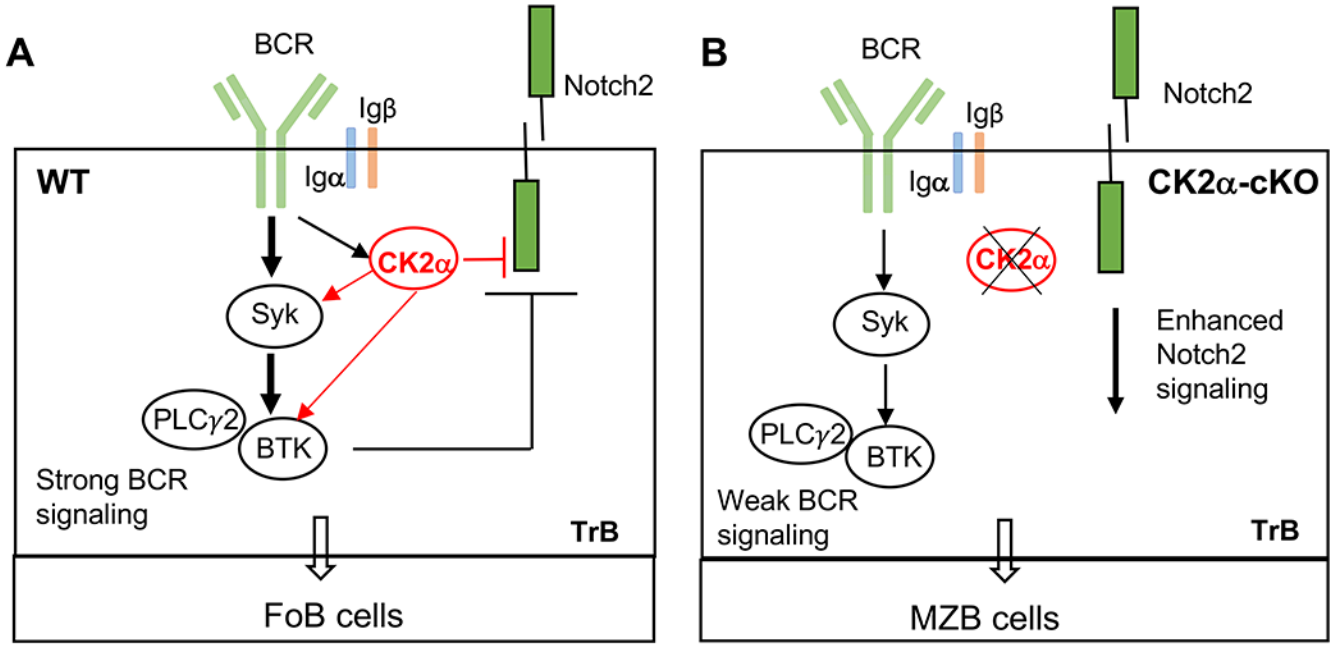

Figure 7. Model Depicting Involvement of CK2α in Mature B-cell Differentiation.

(A) In the presence of CK2α, B-cell activation induces CK2 expression and kinase activity, which increases the magnitude of BCR signaling and suppresses Notch2 signaling. Strong BCR signaling and suppression of Notch2 signaling instructs B-cell development into FoB cells. **(B)** In the absence of CK2α, weak BCR signaling enhanced Notch2 signaling drives B-cell differentiation into MZB cells.