Supplemental data

IKKα-mediated signaling circuitry regulates early B lymphopoiesis during hematopoiesis

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Methods

Construction of *Pax5* and *Irf4* promoter reporter vectors

pMSCV-Pax5, pBSK5-Pax5 and pcDNA3 Flag-mIRF-4 were generous gifts from Dr. Manabu Sugai (Kyoto University Hospital, Japan) and Dr. Kiri Honma (Division of Immunology, Nagasaki University, Japan). IKKα and IKKα-KA expression plasmid vectors were previously described.[1] The luciferase reporter plasmid vector containing a 1.8-kb promoter fragment of the mouse-*Pax5* promoter reporter was a generous gift from Dr. Rudolf Grosschedl (Max Planck Institute of Immunology, Freiburg, Germany). The 2.3-kb mouse *Irf4* (GenBank accession NM_013674 and promoter database accession 54173) promoter luciferase reporter plasmid vector was constructed through polymerase chain reaction (PCR) amplification of mouse genomic DNA (636402, Clontech) using primers 5′-CCGCTCGAGCGGAAATGTGTGTCTCA-3′ and 5′-GAAGATCTTCGATGTTCTGGAACTCC-3′. The PCR product was inserted into a pGEM-T easy vector (A1360, Promega) and confirmed with sequencing. The *Irf4* promoter was re-cloned into the pGL3b luciferase reporter vector (e1751, Promega). The mutations in the κB1 and κB2 sites within the pGL3-*Irf4* and -*Pax5* luciferase reporter were generated using the Quick Change Site-Directed Mutagenesis Kit (200524, Stratagene). The primer oligos used for generating mutations included *Irf4* κB1 site: 5′-CTATCACTTCTGAAGGATACACACAA-3′ and 5′-CTCGCTCGTTGTGTGTATCCTTCAGA-3′; *Irf4* κB2 site: 5′-CTACCTGAGTACTCAAAGATCCCCCA-3′ and 5′-CATAAAAGAGATGGGGGATCTTTGAG-3′; *Pax5* κB1 site: 5′-

CTTGGTGGGGGTGGAAGACTCCCGGGAATCCACAG-3′ and 5′-CTGTGGATTCCCGGGAGTCTTCCACCCCCACCAAG-3′; and *Pax5* κB2 site: 5′-GTAGTTAAGAACCCCGAGGGCG-3′ and 5′-CGCCCTCGGGGTTCTTAACTAC-3′.

Preparation of cytoplasmic and nuclear extracts from B cells

The cytoplasmic extracts from 10^7 B220$^+$-enriched B cells were prepared in a buffer containing 20 mM Tris (pH 8), 10 mM NaCl, 3 mM $MgCl_2$, 20% glycerol, 0.2 mM ethylenediaminetetraacetic acid (EDTA), 1 mM dithiothreitol (DTT), and 0.1% Nonidet P-40 (NP-40). Nuclear extracts from 10^7 B220$^+$-enriched B cells were prepared in a buffer containing 20 mM Tris (pH 8), 400 mM NaCl, 20% glycerol, 0.2 mM EDTA, 1 mM DTT, and a protease/phosphatase inhibitor cocktail (Sigma-Aldrich). Raji (CCL-86, ATCC) cells were maintained in Dulbecco's Modified Eagle's medium with 10% fetal bovine serum (FBS).

Transfections, <u>immunoprecipitation</u>, and immunoblot analysis

The mouse B cell nucleofector kit (VPA-1010, program Z-01) and cell line kit (VCA-1003, program M13) were purchased from Lonza to transfect vector plasmid DNA into primary B cells and the Raji cell line using the manufacturer's recommended protocol. Transfection efficiency achieved in B cells was above 50%. Empty vector plasmid DNA was used to keep the total amount of transfected DNA equal in each experimental condition. Samples were lysed in a radioimmunoprecipitation assay (RIPA) lysis buffer (20 mM Tris [pH 7.9]), 50 mM NaCl, 5 mM EDTA, 0.1% NP-40, 10% glycerol, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 0.2 mM of a protease inhibitor mixture and phosphate inhibitor cocktail I and II (Sigma-

Aldrich). Standard protocol was followed for co-immunoprecipitation assays. Protein A/G agarose (sc-2003) was purchased from Santa Cruz Biotechnology.

For sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel analyses, 40−50 μg of the protein was resolved on 8% or 10% polyacrylamide gels, and then transferred to nitrocellulose or polyvinylidene fluoride (PVDF) membranes (Millipore). Membranes were blocked for 1 hour at room temperature in Tris buffered saline (TBST) containing 5% dry skim milk powder, and then incubated overnight with the respective antibodies. The membranes were subsequently incubated for 1 hour at room temperature with horseradish peroxidase (HRP)-conjugated anti-mouse or anti-rabbit IgG antibody (NA9310 and NA9340, GE Healthcare Biosciences), and immuno-detection was performed with ECL reagents (Amersham Biosciences), followed by autoradiography on HyBlot CL film (Denville Scientific). Membranes were stripped using a stripping solution (21059, Pierce, Thermo Fisher Scientific). We used the following antibodies: RelA-p65 (AF5078, R&D Systems), TER119 (116214, Biolegend), Sp1 (17-601, Millipore), Phospho-Stat3 (9131, Cell Signaling Technology), IKKα (IMG-136A, Imgenex), and NF-κB1 p105 (4717, Cell Signaling Technology). p52 (sc-7386), RelB (sc-226), IRF4 (sc-28696), Pax5 (sc-13146), EBF (sc-137065), Ikaros (sc-13039), and Lamin-B (sc-6216) were purchased from Santa Cruz Biotechnology.

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was prepared from 5 x 10^6 $B220^+$ cells using the Trizol Reagent (Gibco-BRL, Life Technologies). RT-PCR was performed using the one-step RT-PCR kit (210212, Qiagen), following the manufacturer's protocol. The PCR products were separated on agarose gels and

visualized using ethidium bromide staining on a gel documentation system (UltraLum). PCR primers for generating cDNA fragments are listed in Table 2.

Table 2. The Primer Sequences Used for RT-PCR Analysis

IRF4-F*	GCCCAACAAGCTAGAAAG
IRF4-R#	TCTCTGAGGGTCTGGAAACT
Pax5-F	CTGAAAAATCAAAATGGATTTAGAG
Pax5-R	TGG CTGCTGTACTTTTGTCC
β-Actin-F	CATTGGCAATGAGCGGTTGG
β-Actin-R	AGTGATCTCCTTCTGCATCC
Tnfsf11-F	CCCGGCCTCTTCCCCTACCA
Tnfsf11-R	TGTTGCGTTCCCGCCGTACC
GATA1-F	GCTGGGGACTGCACTGCCTG
GATA1-R	CCAGCTGGTCCTTCAGCCGC

* F, Forward primers for PCR; #R, Reverse primers for PCR

B220$^+$ cell culture and growth factors

B220$^+$ B cells were isolated from the tibias and femurs of mice, and red blood cells were lysed in ACK lysing buffer (A10492, Invitrogen). After thorough washing, cells were suspended in MACS buffer and incubated with mouse CD45R (B220) microbeads (130-049-501, Miltenyi Biotec GmbH) for 30 minutes. B220$^+$ cells were enriched through a positive selection method using LS columns (130-042-401, Miltenyi Biotec GmbH) on an MACS separation unit. A total of 1.0 x 10^7 cells were cultured in 12-well plates (Corning). Cells were cultured in either Iscove's Modified Dulbecco's Media (IMDM; 12200-036, Invitrogen) supplemented with 10% FBS or in stromal cell ST-2 co-culture in IMDM supplemented with 10% FBS and recombinant mouse cytokines, including IL-7 (407-ML), IL3 (403-ML), M-SCF (416-ML), Flt-3 ligand (427-FL),

and SCF (455-MC), which were purchased from R&D Systems and used at a final concentration of 25 ng ml^{-1}.

Whole-mount immunohistochemical staining of Peyer's patches

Mouse intestines were rinsed in phosphate-buffered saline (PBS), fixed in 2% paraformaldehyde overnight, washed with PBS three times, and dehydrated with 50%, 70%, 80%, 90%, 95%, and 100% methanol (each for 10 minutes). The intestines were treated with H_2O_2 (67 μl H_2O_2 in 20 ml methanol), rehydrated in PBS for 10 minutes, treated with PBSMT (2% milk, 0.3% Triton X-100) at room temperature for 2 hours, stained with anti-VCAM-1 antibody (550547, BD Biosciences) overnight at 4°C, washed with PBSMT three times, incubated with goat anti-rat IgG-HRP (sc-3823, Santa Cruz Biotechnology) with PBSMT at room temperature for 1 hour, and washed three times with PBS containing 0.3% Triton X-100. The stained color on the intestines was developed using the DAB kit (SK-4100, Vector Laboratories), following the manufacturer's instructions.

Histopathology, immunohistochemistry, and microarray analyses

Organs were fixed in 10% buffered formalin (23-245-685, Thermo Fisher Scientific), and the next day they were transferred into 70% ethanol. Tissue sections were processed for hematoxylin and eosin staining at Histoserv, Inc. The unstained frozen tissue sections underwent immunohistochemical analysis at the NCI-Frederick Pathology/Histotechnology core laboratory (http://web.ncifcrf.gov/rtp/lasp/phl/immuno/). Microarray analysis was performed on RNA isolated from the BM of $B220^+$ cells from 4-week-old wild-type (WT) and KA/KA mice using the Affymetrix mouse 430 2.0 array chip, containing 45,000 genes, at the Laboratory of

Molecular Technology, SAIC-Frederick. Data were normalized and log2 transformations were generated using Partek software (St. Louis, MO, USA).

Bone marrow (BM) and fetal liver (FL) cell transplants

FVB WT and KA/KA mice at 4 to 5 weeks old were γ-irradiated with 9 Gy using the ^{137}Cs irradiator. After irradiation, mice were maintained on antibiotics by supplementing the water with amoxicillin according to standard experimental protocols.[2] The irradiated mice were intravenously injected with 1.5 x 10^6 whole BM cells derived from KA/KA and WT mice on FVB background. Single-cell suspensions were prepared using 70-µm and 25-µm cell strainers, followed by fluorescence activated cell sorter (FACS) analysis. Eight weeks after BM transfer, BM cells from reconstituted mice were analyzed using flow cytometry with the indicated antibodies. For mixed BM transfer, WT BM cells expressing the LY5.2 (CD45.1) cell surface marker were mixed in equal ratio with KA/KA BM cells expressing CD45.2 (1.5 x 10^6 in total), which were intravenously injected into irradiated recipients as described above. Eight weeks post-transfer, BM was taken and analyzed using flow cytometry. Single FL cells isolated from *Ikkα*$^{-/-}$, KA/KA, and WT embryos (each 1 x 10^6 cells) were intravenously injected into γ-irradiated (3 Gy) *Rag1*$^{-/-}$ mice. BM and spleens were collected from recipient mice 8 weeks post-transplant and were analyzed using flow cytometry.

References

1. Liu B, Xia X, Zhu F, et al. IKKalpha is required to maintain skin homeostasis and prevent skin cancer. *Cancer Cell*. 2008;14(3):212-225.
2. Sanyal M, Fernandez R, Levy S. Enhanced B cell activation in the absence of CD81. *Int Immunol*. 2009;21(11):1225-1237.

Supplemental Figures

Figure S1

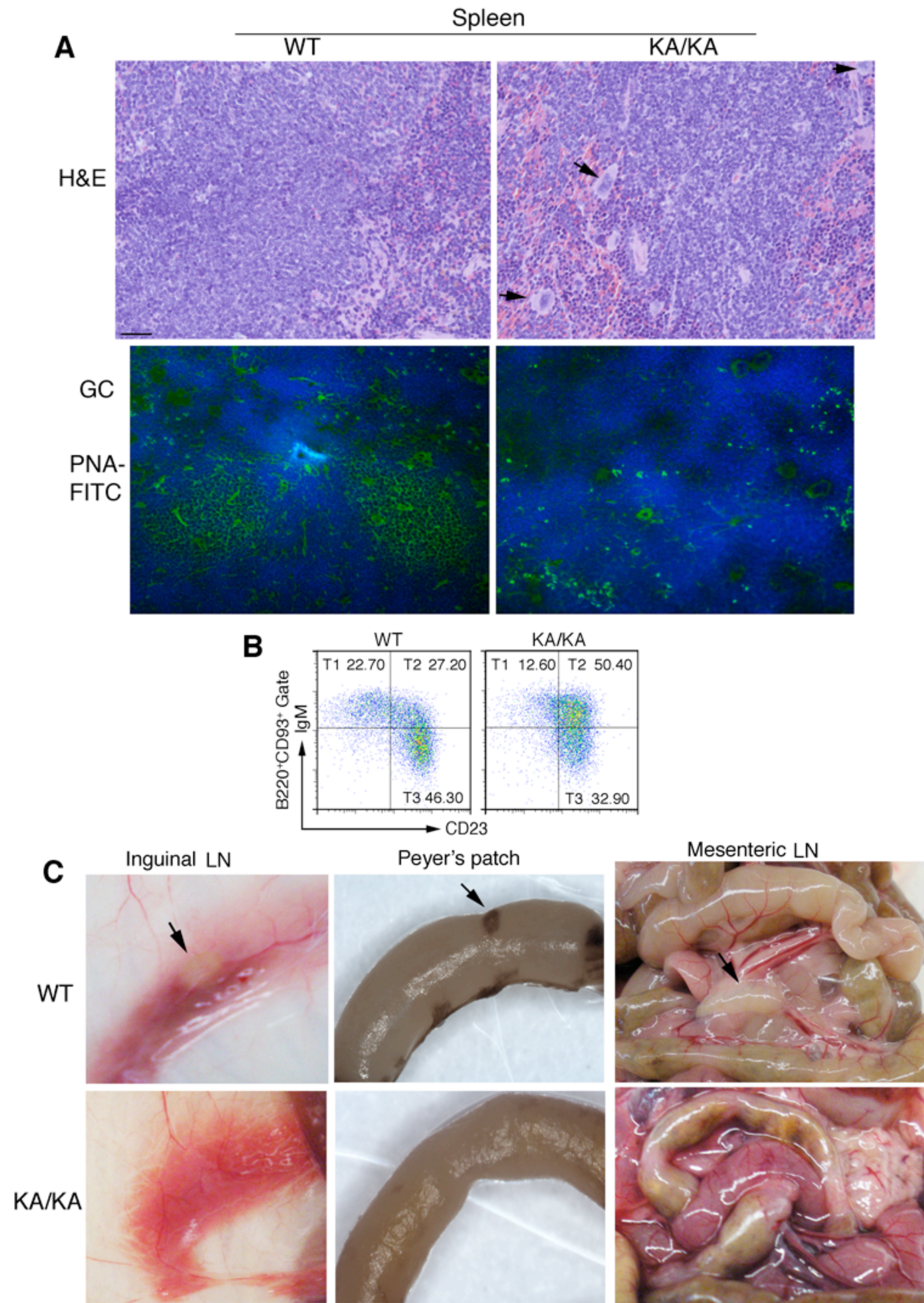

Figure S1. Examination of spleens and lymph nodes of WT and KA/KA mice. (A) Histology of the spleen of 2-week-old WT and KA/KA mice. The top panel shows hematoxylin and eosin–stained spleen sections. Arrows indicate megakaryocyte cells. The bottom panel shows peanut agglutinin (PNA)–fluorescein isothiocyanate (FITC) immunofluorescent-stained spleens. GC, germinal center; green, FITC staining; blue, 4,6 diamidino-2-phenylindole (DAPI) nuclear staining. Scale bar, 50 μm. (B) Analysis of the transitional type 1 (T1), T2, and T3 splenic B cells of WT and KA/KA mice at 6 weeks of age using flow cytometry with indicated B cell surface markers CD23 and IgM gated on $B220^{+}CD93^{+}$. Numbers, the percentage of cell population. (C) Arrows denote inguinal lymph node (LN), Peyer's patch, and mesenteric LN from WT mice. No LNs were detected in KA/KA mice. LNs were obtained from mice at 3 months of age. Peyer's patches from 4-day-old mice were stained with anti-VCAM-1 antibody.

Figure S2

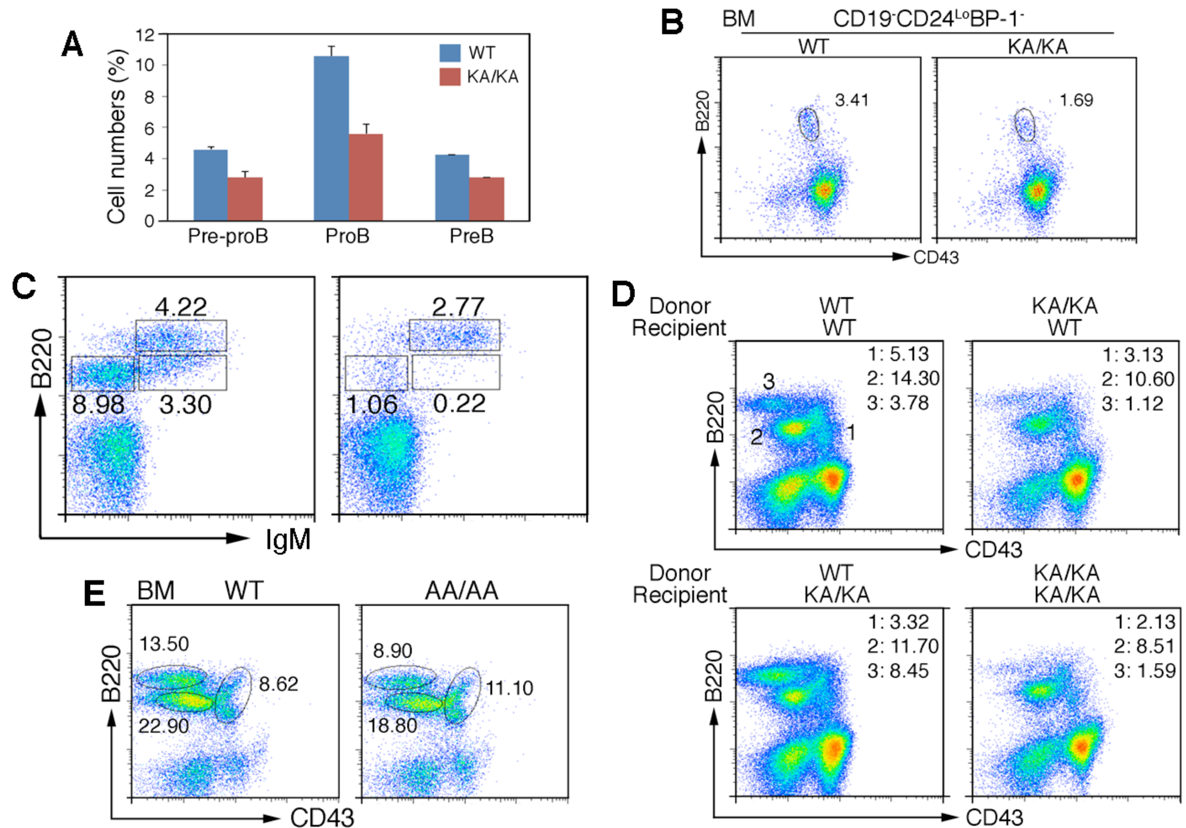

Figure S2. Examination of BM B cell profiles in WT, KA/KA, and *Ikkα*^{AA} (AA/AA) mice. (A) Statistical representation of WT and KA/KA BM pre-pro-B, pro-B, and pre-B cell profiles using flow cytometry with B220$^+$CD43$^+$ markers (data represents mean ± standard deviation calculated from three independent experiments; a representative experiment can be seen at the top panel of Figure 2B). (B) Comparison of WT and KA/KA BM pre-pro-B cells gated on CD19$^-$CD24^{Lo}BP-1$^-$ cells. Numbers represent the percentage of cell populations. (C) Comparison of WT and KA/KA BM pro-B and pre-B cells (B220$^+$IgM$^-$), immature B cells (B220^{lo}IgM$^+$) and mature recirculating B cells (B220^{hi}IgM$^+$). The results show reduced KA/KA B220$^+$IgM$^-$, B220^{lo}IgM$^+$, and B220^{hi}IgM$^+$ cells compared to WT. (D) Profiles of pre-pro-B (1), pro-B (2), and pre-B (3) cells of the

BM transplant experiment. Numbers represent the percentage of each population. This represents one independent experiment (seen in Figure 3B). (E) Flow cytometry analysis of BM B cell profiles of WT and *Ikkα*$^{AA/AA}$ (AA/AA) mice with B220 and CD43 markers.

Figure S3

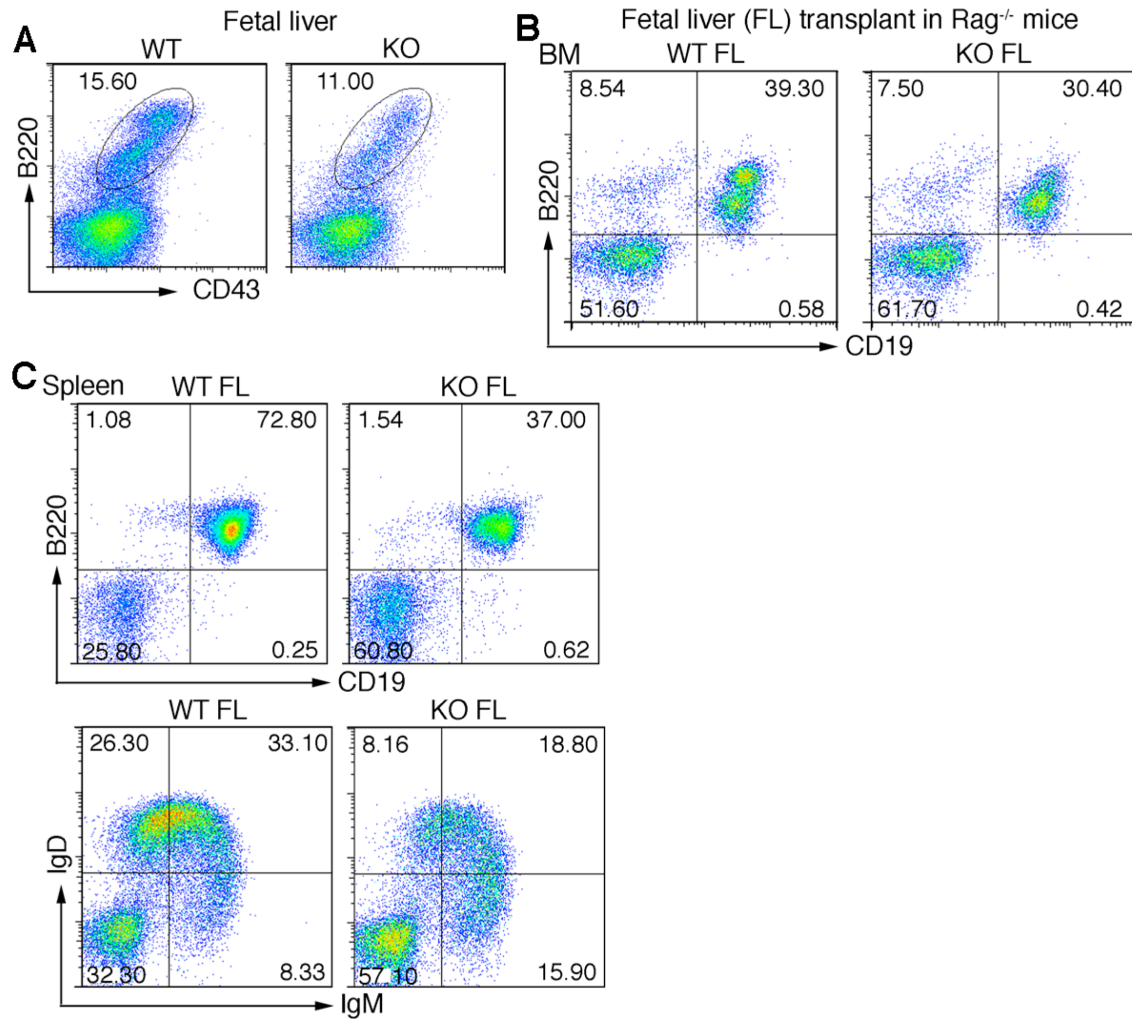

Figure S3. *Ikkα*$^{-/-}$ (KO) fetal liver (FL) cells show a defect in B cells and generate defects in BM and splenic B cells in irradiated *Rag*$^{-/-}$ mice. (A) Flow cytometry analysis shows reduced B220^{+}CD43^{+} cells in the FL cells of E12.5 KO embryos compared to E12.5

WT embryos. (B and C) Flow cytometry analysis shows BM and splenic B cell profiles of B220^{+}CD19^{+} and IgD^{+}IgM^{+} populations in irradiated *Rag*$^{-/-}$ mouse recipients.

Figure S4

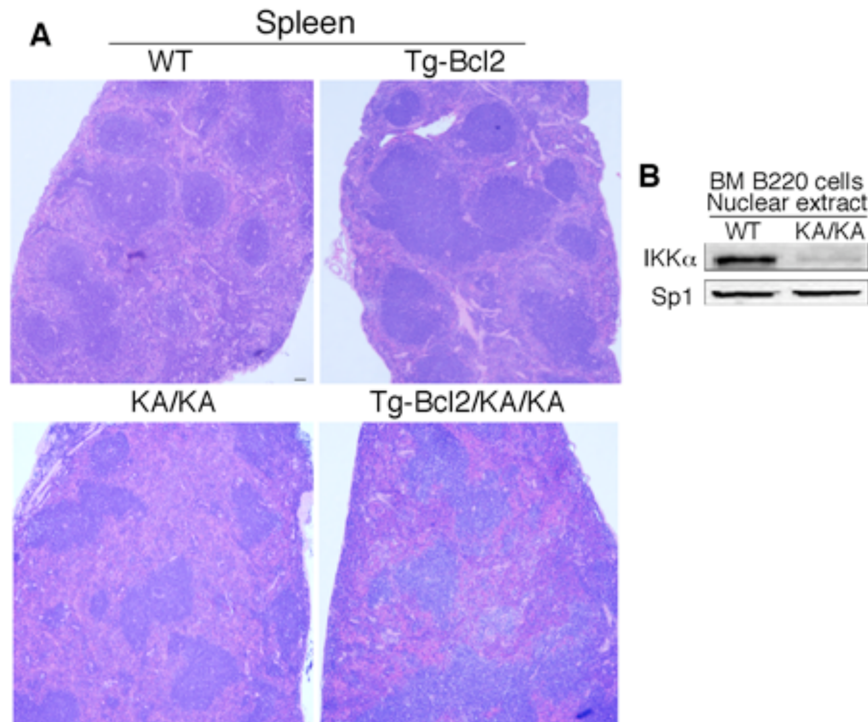

Figure S4. B cell–specific Bcl2 transgene does not rescue the defect in B cell differentiation in KA/KA spleen. (A) Histopathology of hematoxylin and eosin–stained spleens from WT, Tg-Bcl2, KA/KA, and Tg-Bcl2/KA/KA mice. Scale bar, 50 μm. (B) Western blot detects nuclear IKKα levels in BM B220^{+} cell lysate. Sp1 was used as a protein-loading control and to show the purity of the nuclear extract preparation.

Figure S5

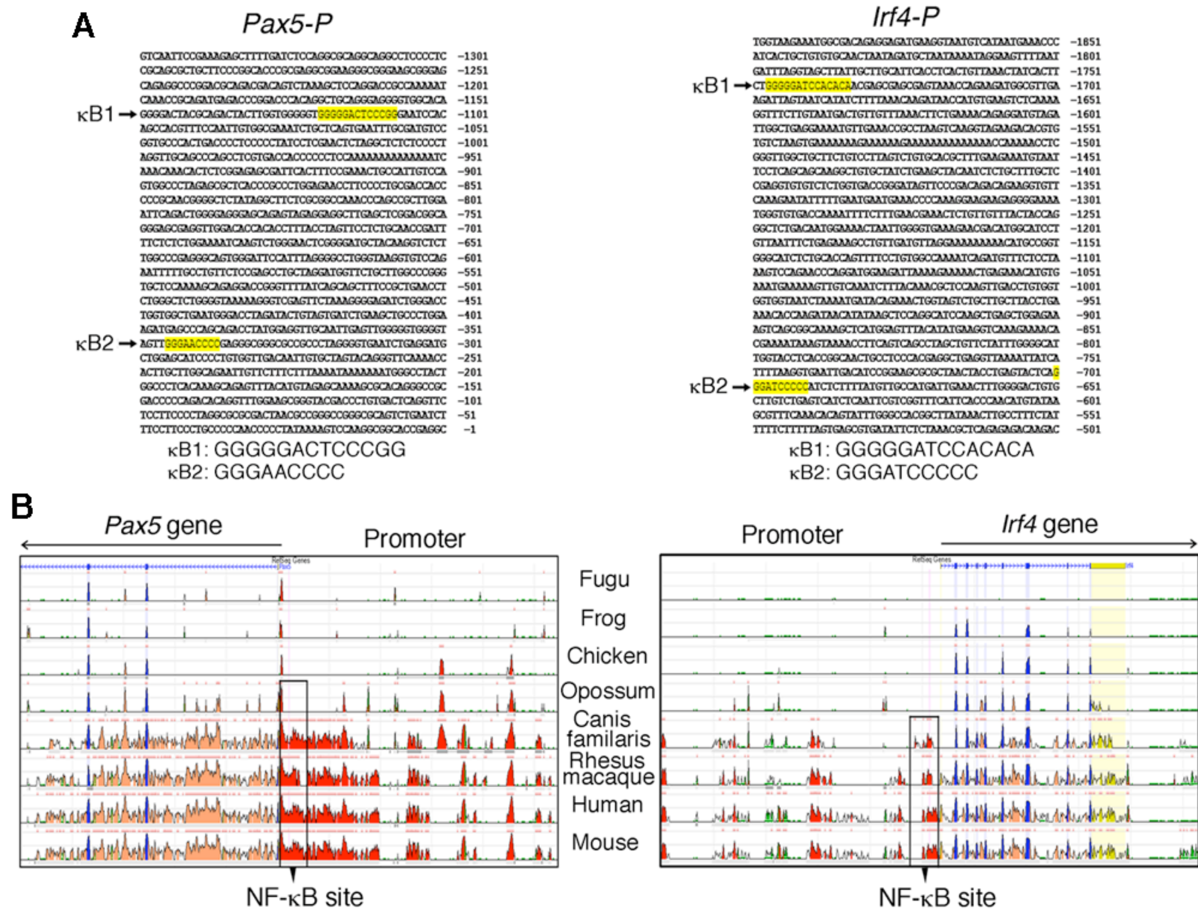

Figure S5. Analysis of NF-κB-binding sites in the *Pax5* and *Irf4* promoters. (A) Promoter sequences of the *Irf4* and *Pax5* genes. Mouse *Irf4* and *Pax5* basal promoters cloned into pGL3b luciferase reporter constructs. The Rel-NF-κB putative transcription-binding sites, κB1 and κB2, are shown with arrows and highlighted. The nucleotide sequences of κB1 and κB2 are shown at the bottom. *P*, promoter. (B) Evolutionary-conserved promoter regions of *Pax5* and *Irf4* containing NF-κB sites. Regions of the 2.1-kb- and 1.9-kb-long genomic locus of mouse chromosome 13 (*Irf4*, NC_000079.5) and chromosome 4 (*Pax5*, NC_000070.5) containing the evolutionary-conserved NF-κB1 and NF-κB2 sites are shown in boxes. The publicly available database,

http://ecrbrowser.dcode.org, and rVISTA 2.0 were used to align genomic regions to determine whether the regulatory sequences were evolutionary conserved. Arrows point to the red peaks that indicate the noncoding genomic region, which contains conserved NF-κB-binding sites. The *Irf4* and *Pax5* genes are depicted as a horizontal blue line above the graph, with strand/transcriptional orientation indicated with arrows. Blue boxes along the line correspond to the positions of coding exons, and yellow boxes correspond to UTRs. Peaks that correspond to the exons are colored in blue or salmon if they lie within an intron. Regions colored in green correspond to transposable elements and simple repeats.

Figure S6

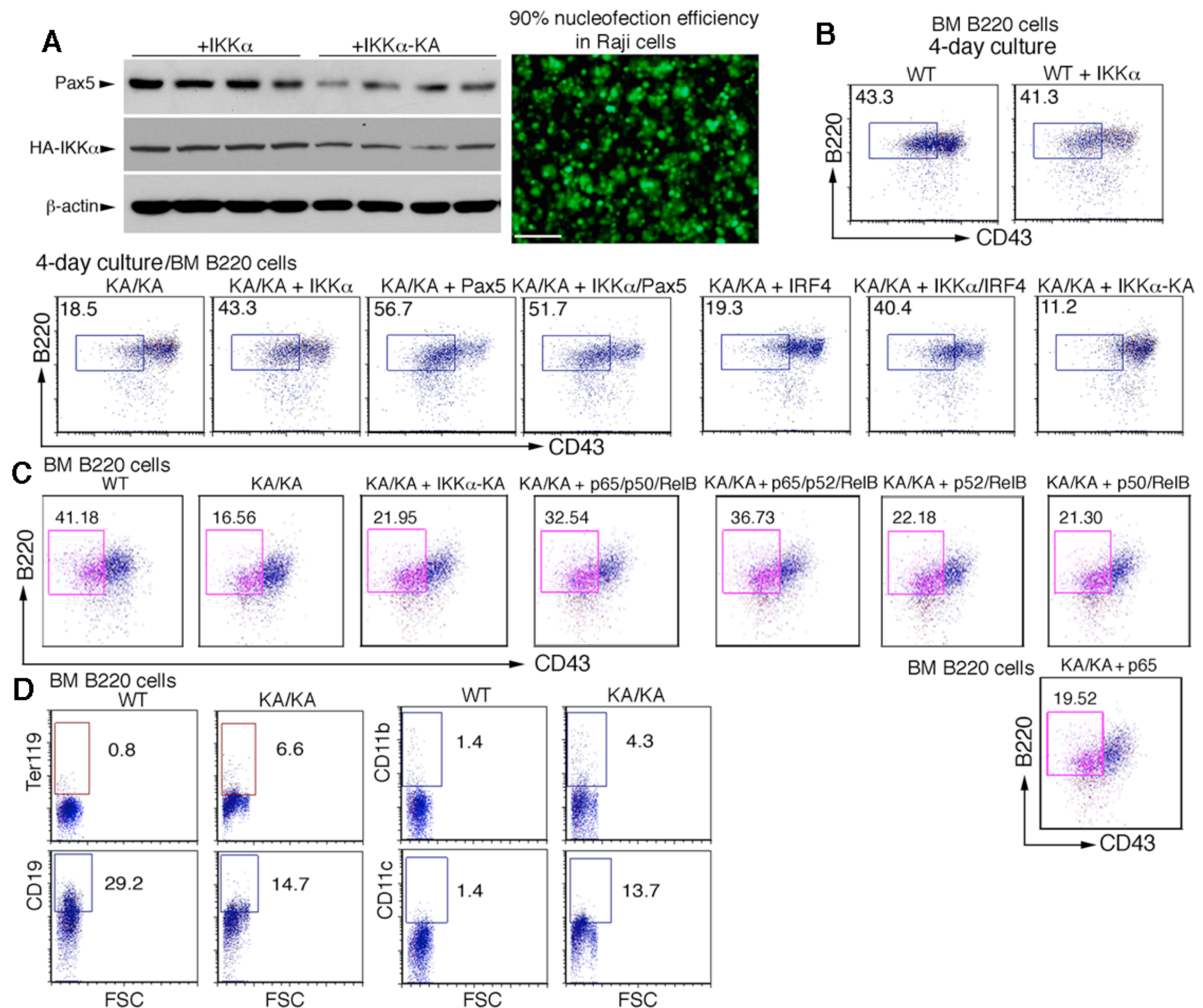

Figure S6. IKKα, Pax5, and combined NF-κB components regulate BM B cell differentiation. (A) IKKα induces the expression of Pax5 in Raji cells. Western blot shows levels of Pax5 in Raji cell lysate 24 hours after transfection with the luciferase reporter pGL3b-Pax5 plasmid, IKKα, and/or IKKα-KA expression plasmids (Figure 5B). β-actin, protein-loading control. The right panel shows the transfection efficiency of the green fluorescent protein (GFP) vector in Raji cells using the AMAXA nucleofection reagent. Scale bar, 50 μm. (B and C) Profiles of BM B220^{+} cells from WT and KA/KA

after AMAXA nucleofections of indicated expression plasmids cultured for 4 days. (D) Flow cytometry analysis of WT and KA/KA BM B cells cultured for 3 days with cell markers CD19 (B cells), TER119 (erythroid cells), CD11b, and CD11c (myeloid cells).

Tables S1 and S2. **Lists of some of the deregulated genes relevant to B cell development that have been identified through microarray analysis.**