SUPPLEMENTAL INFORMATION

Requirement for Rictor in homeostasis and function of mature B lymphoid cells

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Detailed Methods

Mice. *Rictor*$^{fl/fl}$ mice (backcrossed at least eight generations to C57BL/6 (B6) were crossed with B6 transgenic mice expressing Cre recombinase under control of the *Vav1* promoter (*Vav*-Cre)[1] or mice containing the 4-hydroxytamoxifen-activated Cre-ER^{T2} cassette inserted into the *ROSA26* locus (*ROSA26*-Cre-ER^{T2})[2] [generous gift of T. Ludwig]. No abnormality of lymphocyte frequencies or numbers was associated with either Cre transgene, and direct analyses of each type of WT mouse (*Vav*-Cre^{+}, *Rictor*$^{+/+}$ and Cre^{-}, *Rictor*$^{fl/fl}$) were compared to each other or

matched cKO (*Vav*-Cre^{+}, *Rictor*$^{fl/fl}$). Tamoxifen was administered intraperitoneally (i.p.) in safflower oil (3 mg/dose for three doses at 2 d intervals). All mice were housed in Specified Pathogen Free conditions and used as monitored by the Vanderbilt University Office of Animal Welfare Assurance after approval of experimental protocols by the Institutional Animal Care and Use Committee.

Bone marrow chimeras and adoptive transfer. Mixed competitive chimeras, for which bone marrow cells were obtained from B6 (CD45.2) WT and *Vav*-Cre *Rictor*$^{fl/fl}$ as well as B6-CD45.1 mice, were generated essentially as described.[3] After depletion of B and T lineage cells using anti-Thy1- and anti-CD19-conjugated microbeads, 4 x 10^6 marrow cells were mixed with equal number of CD45.1 marrow cells, and injected intravenously (i.v.) into lethally irradiated (10 Gy in two divided doses) B6 CD45.1 recipients. For chimeras to analyze homeostasis of mature B cells, 5 x 10^6 LN cells of B6 WT or Cre-ER^{T2} *Rictor*$^{fl/fl}$ were mixed with an equal number of CD45.1 LN cells and transferred i.v. into B6 *Rag2* $^{-/-}$ (RAG2^0) mice. Lymphoid cells were analyzed (enumeration and flow cytometry) 4 wk (lymphocyte transfers) or 12 wk (bone marrow transfers) after transfer and treatment of the recipients with tamoxifen (3 mg x 3 doses). Analyses of division rates for CFDA-SE labeled cells transferred into RAG2^0 recipients were performed as described.[4]

Immunization, ELISPOT assays, and ELISA. Mice were immunized by i.p. injection with a depot of 100 µg NP-KLH or NP-ovalbumin precipitated in alum (Imject, Pierce) as described,[4] and boosted with the same after 2 wk. Ag levels in sera collected before and after the booster immunization were quantitated by ELISA using NP-BSA-coated plates and a set of Ig isotype-specific secondary Ab (Southern Biotech). The frequency of Ab-secreting cells in marrow and spleen were determined by ELISPOT assays. Spleen or bone marrow cells were plated on a 96-well MultiScreen^{TM} filter plates (Millipore) coated with 10 µg/ml NP_{26}-BSA and incubated for 24 hr. Anti-NP IgM or IgG1 was detected using goat anti-mouse Ig isotype-specific HRP- conjugated secondary Abs (Southern Biotech) and visualized with substrate 3-amino-9-ethylcarbazole

(Sigma). Spots were counted using an automated reader (CTL-ImmunoSpot, Cellular technology).#

Immunohistochemistry. Spleens were snap-frozen after harvesting from WT and Rictor-deficient mice that had undergone primary immunization 3 wk before harvest, followed by boosting after 2 wk (each with NP-ovalbumin as described in the preceding section). Immunohistochemistry was performed on frozen sections of these samples as described.[5] Sections were prepared on glass slides, fixed with acetone, blocked with 5% fetal bovine serum in PBS and stained with anti-moma1-biotin/ Streptavidin-Cy3 and anti-IgM-APC. Slides were mounted with with Fluoro-Gel (Electron Microscopy Sciences, Hatfield) prior to imaging with a Zeiss Axio ImagerM1 (Carl Zeiss MicroImaging, Inc., Thornwood), using Slidebook software (Intelligent Imaging innovations, Denver). A Hamamatsu ORCA-ER digital camera (Hamamatsu Photonics, Japan) and EC Plan-Neofluar 40x/0,75 and 10x/0,3 objective lenses (Zeiss) were used.

B cell isolation, and flow cytometry. Direct and indirect immunofluorescent staining of single cell suspensions prepared from bone marrow, spleens and LNs were conducted as described.[4] Fluorophore-conjugated antibodies were purchased from BD Pharmingen or eBioscience. Data were acquired using a BD FACS Calibur or LSR II, and analyzed using FlowJo (TreeStar). Mature B cells were purified from pooled spleen and LN by depleting $AA4.1^+$ and $Thy1.2^+$ cells using biotinylated antibodies and IMag streptavidin particles (BD). B lineage cells were isolated from bone marrow suspensions using anti-B220 microbeads and magnetic cell sorting (MACS) (Miltenyi).

Cell culture, and BrdU incorporation assay. Cells were cultured in complete IMDM containing 10% FBS and supplemented as described.[4] Splenocytes or B cells (4×10^6 cells/ml) were stimulated with 2.5 µg/ml anti-IgM, 100 ng/ml BAFF (R&D Systems), or 2 µg/ml LPS. To assay

proliferation, cells were activated, cultured for 48 h, followed by 18 hr with 10 μM BrdU added to the medium. S phase entry rates were analyzed by anti-BrdU staining and flow cytometry, as described.[3]

Annexin-V staining. Splenocytes or cultured B cells were stained using PE-conjugated Annexin-V (BD) in 10 mM HEPES (pH 7.4), 140 mM NaCl and 2.5 mM $CaCl_2$ along with surface markers at room temperature for 20 min. Incidence of Annexin-V^+ apoptotic B cells was analyzed using flow cytometry.

Western blotting and measurements of RNA. Cell lysates were analyzed by immunoblotting using primary antibodies followed by IR dye-conjugated secondary antibodies after which data were imaged and quantitated with the Odyssey Infrared Imaging System (LI-COR). All primary Abs were purchased from Cell Signaling Tech except Rictor (Bethyl Laboratories), S6K (Santa Cruz), PARP1 (Santa Cruz), NDRG1 (Sigma), and β-tubulin (Sigma). Using primer pairs tabulated (Supplementary Table I), RNA levels were measured using purified mature B cells or cultured B cells in the presence of anti-IgM or BAFF by qRT^2-PCR as described.[3]

Electrophoretic mobility shift assay (EMSA). Nuclear extracts from B cells were prepared from purified mature B cells ($B220^+$ $AA4.1^-$) or cultured B cells in the presence of anti-IgM or BAFF as described.[3] The NF-κB consensus oligonucleotides (Santa Cruz) were ^{32}P end-labeled using polynucleotide kinase and γ-[^{32}P]-ATP and incubated with 5 μg of nuclear extract protein at room temperature for 20 min in a binding buffer (10 mM HEPES pH 7.9, 65 mM NaCl, 1 mM DTT, 0.2 mM EDTA, 0.02% NP-40, 50 μg/ml poly(dI•dC) and 8% glycerol). Specificity assays (Ab blocking / supershift) were performed by pre-incubating with Abs against RelB (Santa Cruz) or controls (non-specific serum or IgG, as indicated) on ice for 2 h. Complexes were resolved by non-denaturing 4% PAGE.

In vitro differentiation of B lymphoblasts. Purified B cells were activated with 10 μg/ml LPS in the presence of 50 ng/ml BAFF, and cultured with 10 ng/ml IL-4 or 100 ng/ml IFN-γ for 5 days. B cells expressing IgG1 or IgG2a were analyzed by flow cytometry.

Supplemental Figure Legends

Figure S1. Reduced number of mature B cells in lymphoid and non-lymphoid organs, and impaired generation of B1 B cells in peritoneal cavity. (a-c) lymphoid organs of 10-12 wk-old *Vav*-Cre *Rictor*$^{fl/fl}$ (cKO) or control mice (WT) were analyzed as in Fig. 1. (n = 8 WT *vs.* 8 *Vav*-Cre *Rictor*$^{fl/fl}$). (a) Shown are representative FACS profiles of BM cells with frequencies of IgD^{hi} IgM^{+} cells in the B220^{+} gates (left). Mean (± SEM) numbers of IgD^{hi} IgM^{+} recirculating mature B cells in the BM were calculated from flow analyses and cell counts (right): n = 8 WT *vs.* 8 *Vav*-Cre cKO. (b) Fluorescent imaging of B cells in frozen sections of spleen (upper panels, 100X; bottom panels, 400X): IgM, blue; Moma-1, Red. Arrow indicates B (IgM^{+}) cells in the marginal zone beyond the Moma-1^{+} margin. Data are representative of four WT and four cKO mice. (c) Shown are mean (± SEM) numbers of B220^{+} cells in the lymph nodes. (d) Lymphocytes were fractionated from liver and lung dispersions using Percoll, counted, and analyzed by flow cytometry. Shown are mean (±SEM) cell numbers of 7-AAD^{-} B220^{+} in each organ (n = 5 WT vs. 5 *Vav*-Cre *Rictor*$^{fl/fl}$). (e) Rictor is required for generation of B1a B cells. Cells were isolated from peritoneal cavity and analyzed by flow cytometry. Shown are the representative FACS profiles of CD5 and CD11b in the B220^{+} CD23^{-} gates, and mean (± SEM) cell numbers of B1a (CD5^{+} B220^{+} CD23^{-}) and B1b (CD5^{-} B220^{+} CD23^{-}) subsets. (n = 6 WT vs. 6 *Vav*-Cre *Rictor*$^{fl/fl}$).

Figure S2. Increased apoptosis of *Rictor*-deficient B cells. (a-c) Spleen cells were isolated from Cre-ER^{T2} *Rictor*$^{fl/fl}$ or control mice pretreated with tamoxifen (4 wks), and cultured in the presence or absence of BAFF for 20 h. Shown are representative histograms (a) along with (b) mean

(±SEM) frequencies of Annexin-V$^+$ cells in the transitional (B220$^+$ AA4.1$^+$) and mature (B220$^+$ AA4.1$^-$) B cell gates for five WT *vs.* five Cre-ER^{T2} cKO samples in two independent experiments. (c) Purified mature B cells were cultured with BAFF for 6 h, and Western blotting was performed using anti-PARP1 Ab, with arrows designating the full-length and cleaved forms. A representative result from two independent experiments with similar data is shown. (d) Splenocytes from *Vav*-Cre *Rictor*$^{fl/fl}$ mice and WT controls were cultured as in (a), stained for surface markers, fixed, permeabilized, and the stained with fluorophore-conjugated Ab specific for cleaved, activated caspase-3. Shown are flow histograms from the indicated gates for immature (AA4.1$^+$) and mature (AA4.1$^-$) B cells, with inset numbers denoting the fraction (%) of events in the gate positive for the activated executioner caspase. Data representative of two independent replicates.

Figure S3. Induction of NF-κB pathway and NF-κB-dependent expression of survival genes depend on Rictor. (a) Nuclear extracts prepared from mature B cells were preincubated with Abs specific for RelB or controls [non-immune ("Rb sera") and IgG], and resolved by EMSA using a consensus NF-κB probe as in Fig. 5. Shown is the autoradiograph of results from one replicate representative of two independent experiments: arrowhead indicates complexes formed with NF-κB proteins, and line indicates free probe bands. (b) Conceptual schema of mTORC2-dependent regulation of NF-κB pathway.[6-9] (c and d) Induction of Mcl-1 and Bcl-xL depends on Rictor. B cells were purified from Cre-ER^{T2} *Rictor*$^{fl/fl}$ or control mice pretreated with tamoxifen, and cultured in the presence or absence of anti-IgM or BAFF for 24 h. Cells were stained for surface markers, fixed, permeablized, and incubated with antibodies to Mcl-1, Bcl-xL, or control IgG. After stained with Alexa Fluor 647-conjugated secondary Ab, cells were analyzed by flow cytometry. Shown are representative histograms in 7-AAD$^-$ B220$^+$ gates: shaded, control IgG; black line, media alone; blue line, IgM; red line, BAFF. ΔMFI values, shown as means (± SEM), were calculated by subtracting the MFI for control IgG from that of Mcl-1 or Bcl-xL for each individual sample (n = 4 WT *vs.* 4 Cre-ER^{T2} *Rictor*$^{fl/fl}$).

Figure S4. Effect of Rictor deletion on Ab responses. (a) *Vav*-Cre *Rictor*$^{fl/fl}$ or control mice were immunized with NP-KLH in Alum and boosted 14 d after primary immunization. Sera were collected 7 d after boost immunization and analyzed by ELISA after screening analyses to establish dilutions that yield absorbances (OD) in the linear range of the assay. Shown are mean (±SEM) OD values detecting NP-specific Ig isotypes (NP_{26}), (4-hydroxy-5-iodo-3-nitrophenyl)Acetyl (NIP)-specific IgG1, or NP-specific high affinity (NP_2) immunoglobulins (n = 6 WT *vs*. 6 *Vav*-Cre *Rictor*$^{fl/fl}$). (b) Mice were immunized and boosted with TNP-Ficoll in PBS as in (a), and sera were analyzed by TNP-specific ELISA (n = 5 WT *vs.* 5 *Vav*-Cre *Rictor*$^{fl/fl}$) (c) Spleen sections were analyzed using immunohistochemistry: PNA, red; B220, green; CD3, blue. Shown is representative of 3 WT *vs*. 3 *Vav*-Cre *Rictor*$^{fl/fl}$.

Figure S5. B cell-intrinsic role of mTORC2 in generating Ab secreting cells. B cells of Cre-ER^{T2} *Rictor*$^{fl/fl}$ or control mice pretreated with tamoxifen were transferred into *Rag2* -/- recipient along with WT CD4 T cells, and immunized with NP-ovalbumin as in Fig. 7. Ab secreting cells in spleen and bone marrow were evaluated by ELISPOT. Shown are mean (± SEM) spot numbers of cells secreting NP-specific IgM (a) and IgG1 (b) (3 *vs*. 3).

Figure S6. Effect of Rictor depletion on IgG1- and IgG2a-class switching of cultured B lymphoblasts driven by TLR activation. Purified B cells were activated with LPS and cultured in the presence or absence of IL-4 or IFNγ for 5 d. (a) Cell were stained for immunoglobulins along with surface markers, and analyzed by flow cytometry. Shown are representative FACS profiles in the B220$^+$ 7-AAD$^-$ gates with frequencies of IgM^{lo} IgG1$^+$ or IgM^{lo} IgG2a$^+$ cells: n = three independent experiments.

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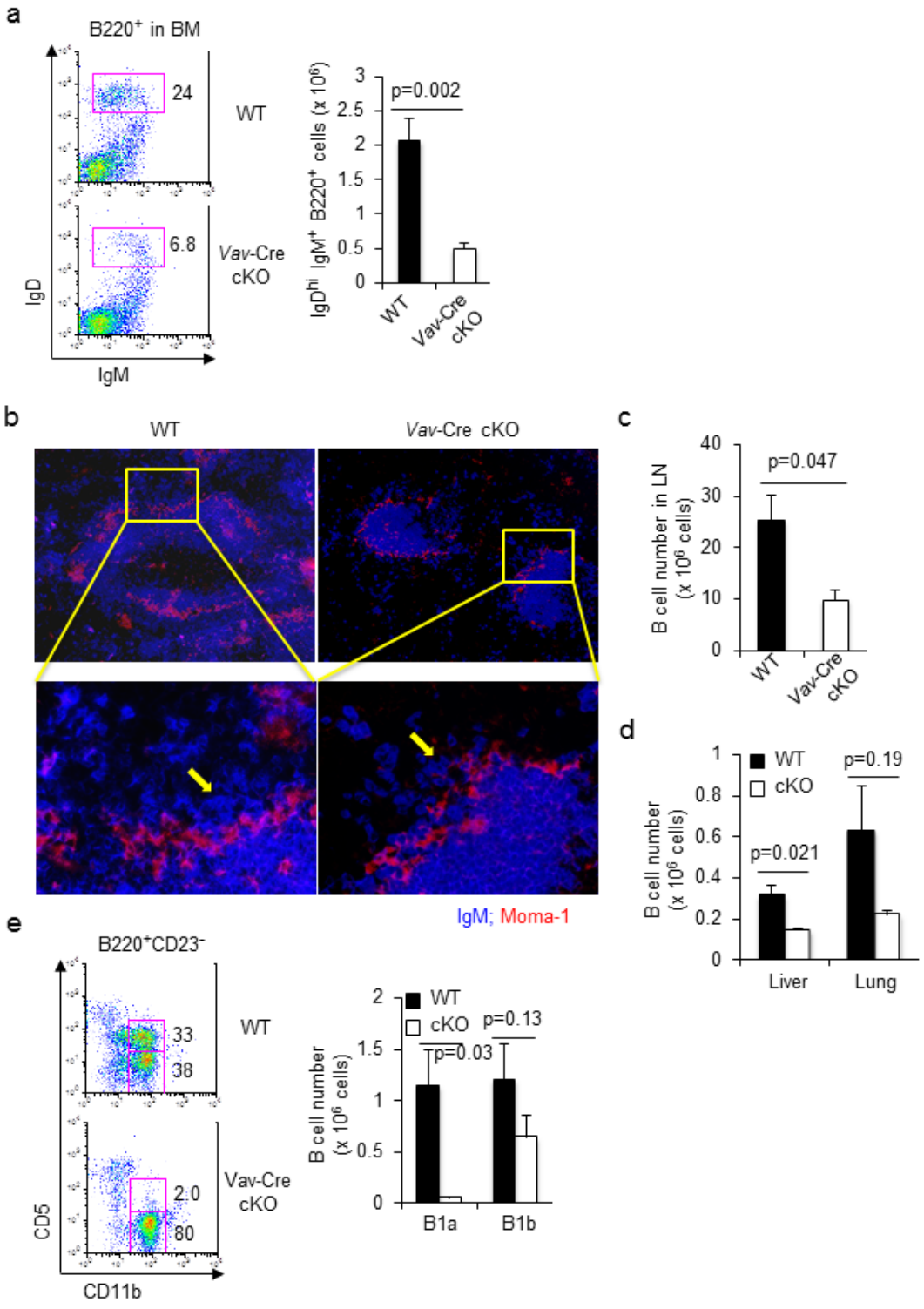
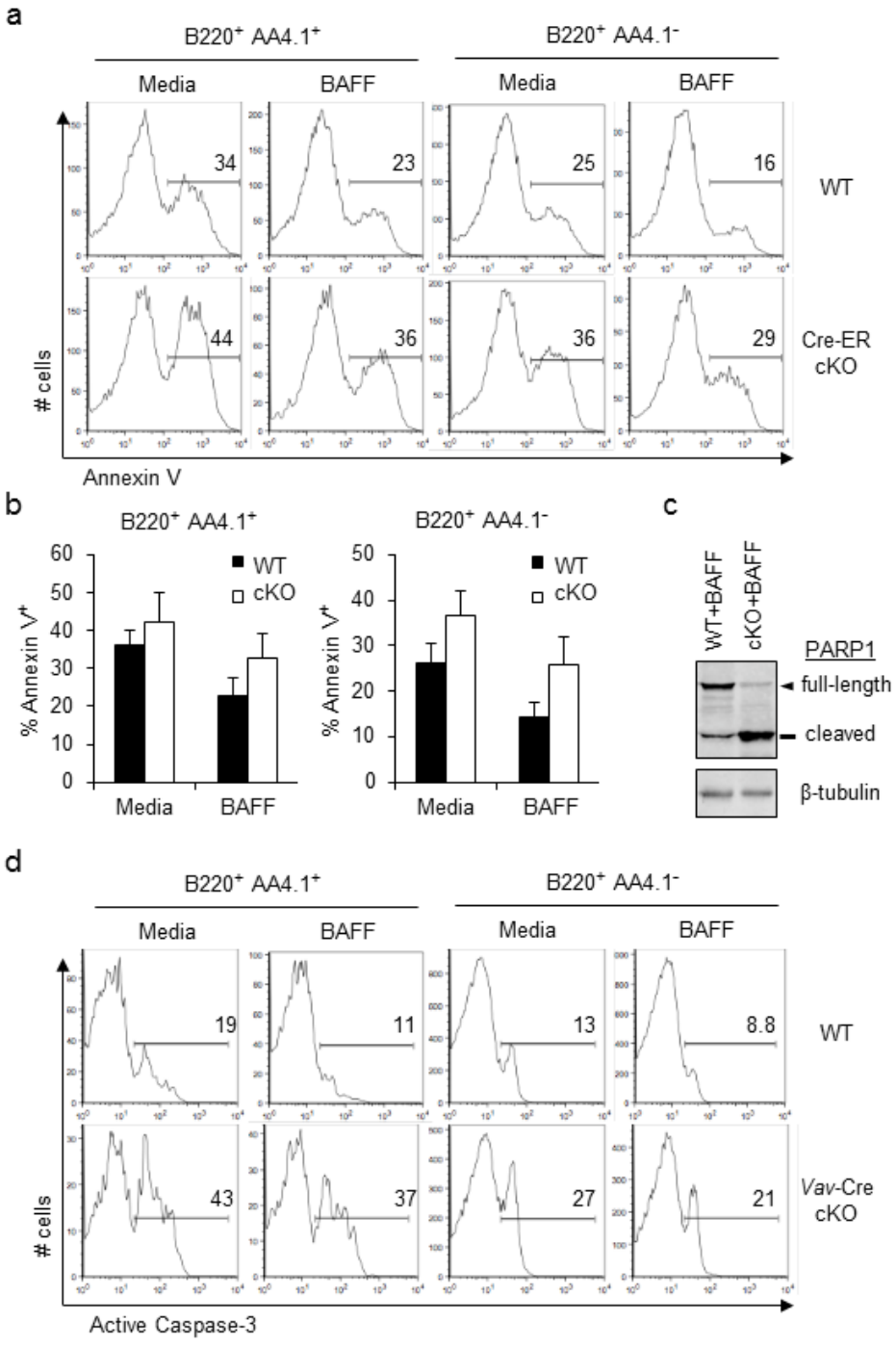
Supplemental Table S1. Primer sequences used in the qRT-PCR

Gene	Sequence (5' to 3')	Product size (bp)
Actb	GGC ACC ACA CCT TCT ACA ATC GGG GTG TTG AAG GTC TCA AAC	149
Bcl2	ACG GAG GCT GGG ATG CCT TT GAG TGA TGC AGG CCC CGA CC	120
Bcl2l1	GCT GGG ACA CTT TTG TGG AT TGT CTG GTC ACT TCC GAC TG	150
Mcl1	TCA AAG ATG GCG TAA CAA ACT GG CCC GTT TCG TCC TTA CAA GAA C	150
Pim2	GTA CCA TGC CCT GCC AGC GA GGG CAC AGC AAT CTG GGG AGA	146
Nfkb2	GCC TGG ATG GCA TCC CCG AA CTT CTC ACT GGA GGC ACC TG	194
Bcl2l11	TCT GCG CCC GGA GAT ACG GA TCA TTT GCA AAC ACC CTC CTT GTG T	90
Bbc3	CCC GGA CGG TCC TCA GCC CT CAG CTT GGGTGG GGC CTC C	101


a
B220+ in BM
24
WT
6.8
Vav-Cre cKO
IgD
IgM
IgDhi IgM+ B220+ cells (x 10^6)
p=0.002
WT
Vav-Cre cKO
b
WT
Vav-Cre cKO
IgM; Moma-1
c
B cell number in LN (x 10^6 cells)
p=0.047
WT
Vav-Cre cKO
d
WT
cKO
p=0.19
p=0.021
B cell number (x 10^6 cells)
Liver
Lung
e
B220+CD23-
33
38
WT
2.0
80
Vav-Cre cKO
CD5
CD11b
B cell number (x 10^6 cells)
WT
cKO
p=0.13
p=0.03
B1a
B1b


a
B220+ AA4.1+
B220+ AA4.1-
Media
BAFF
Media
BAFF
34
23
25
16
WT
44
36
36
29
Cre-ER cKO
cells
Annexin V
b
B220+ AA4.1+
B220+ AA4.1-
WT
cKO
% Annexin V+
Media
BAFF
c
WT+BAFF
cKO+BAFF
PARP1
full-length
cleaved
β-tubulin
d
B220+ AA4.1+
B220+ AA4.1-
Media
BAFF
Media
BAFF
19
11
13
8.8
WT
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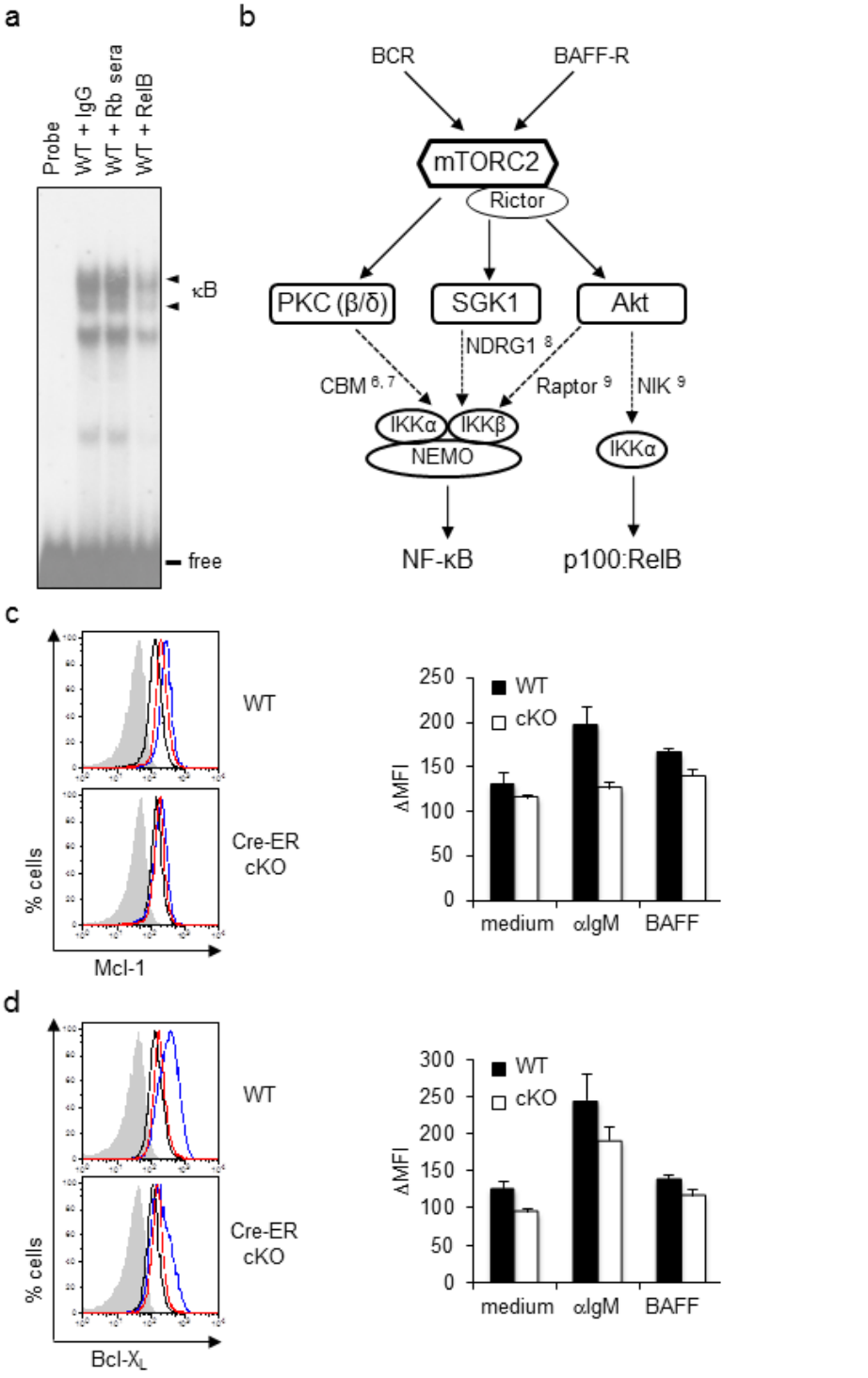
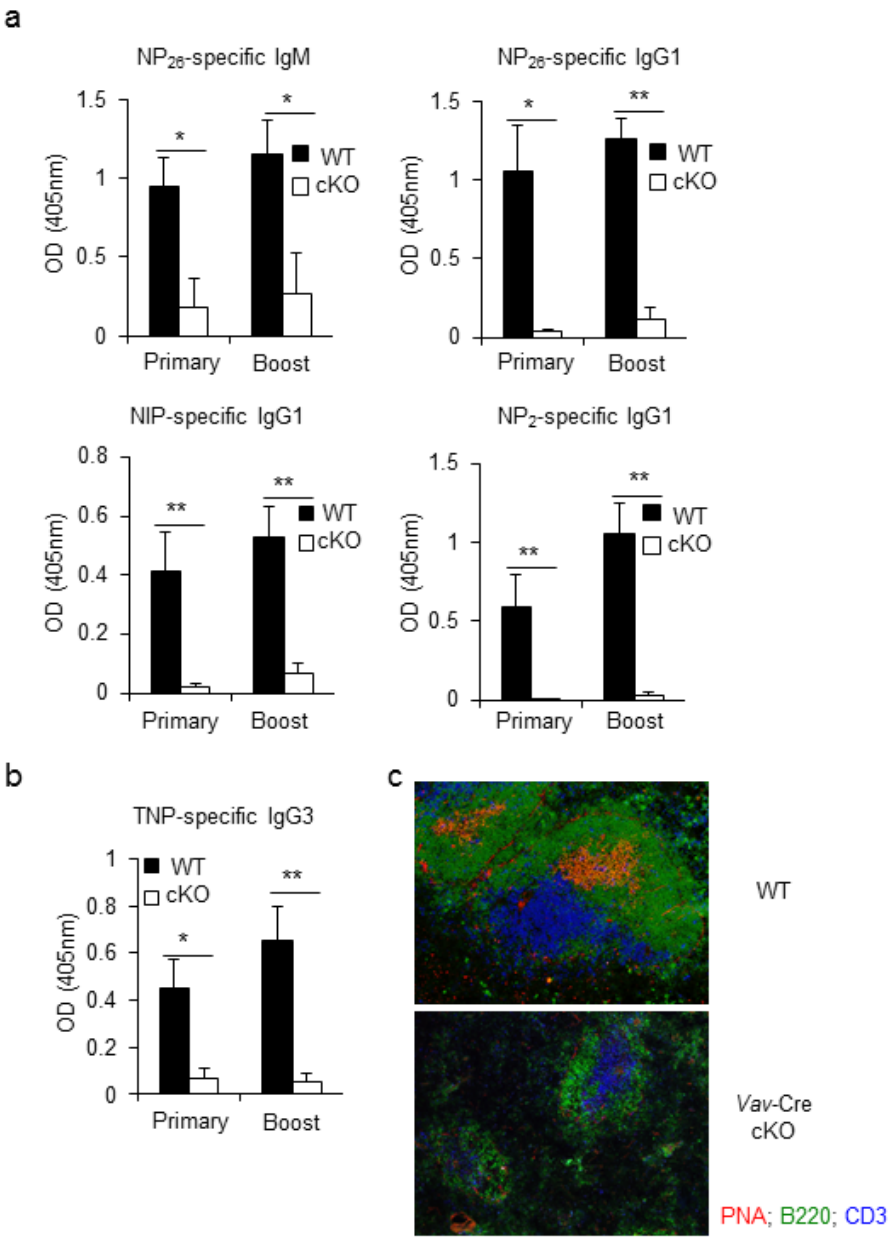
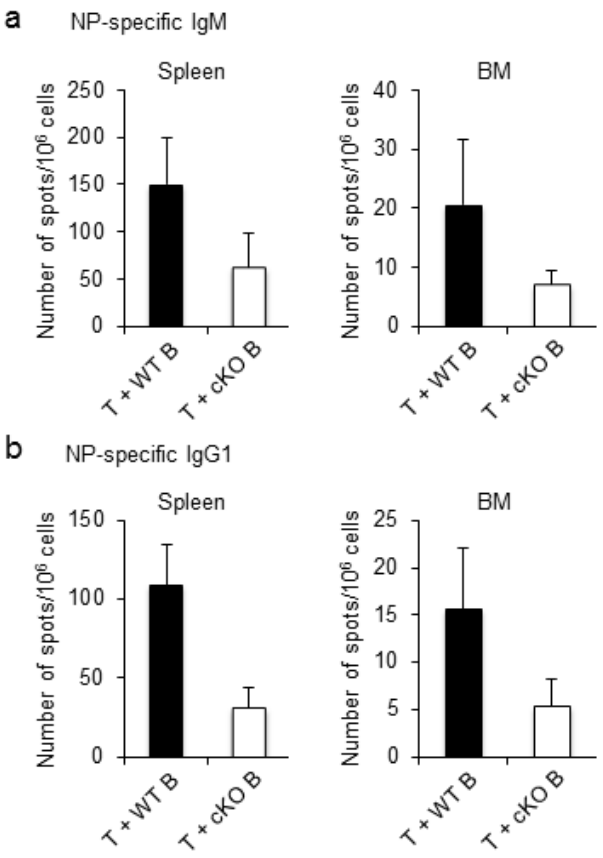
21
Vav-Cre cKO
cells
Active Caspase-3


a
Probe
WT + IgG
WT + Rb sera
WT + RelB
κB
free
b
BCR
BAFF-R
mTORC2
Rictor
PKC (β/δ)
SGK1
Akt
NDRG1 8
CBM 6, 7
Raptor 9
NIK 9
IKKα
IKKβ
NEMO
IKKα
NF-κB
p100:RelB
c
WT
Cre-ER cKO
% cells
Mcl-1
250
200
150
100
50
0
WT
cKO
ΔMFI
medium
αIgM
BAFF
d
WT
Cre-ER cKO
% cells
Bcl-XL
300
250
200
150
100
50
0
WT
cKO
ΔMFI
medium
αIgM
BAFF


a
NP26-specific IgM
NP26-specific IgG1
NIP-specific IgG1
NP2-specific IgG1
OD (405nm)
WT
cKO
Primary
Boost
b
TNP-specific IgG3
c
WT
Vav-Cre cKO
PNA; B220; CD3


a
NP-specific IgM
Spleen
BM
Number of spots/10^6 cells
250
200
150
100
50
0
40
30
20
10
0
T + WT B
T + cKO B
b
NP-specific IgG1
Spleen
BM
150
100
50
0
25
20
15
10
5
0

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