Material and methods

Mice, antigens and immunisation. All mice were bred and maintained at the animal facilities of The Water and Eliza Hall Institute of Medical Research (Parkville, Australia) and were backcrossed to the C57BL/6 background for more than 10 generations. All procedures involving animal were approved by our institutional animal ethics committee. Mice with conditional (*1*) and null (*2*) alleles of *Bcl2l1* (*Bcl-x_L)* were kindly provided by Dr. L. Hennighausen (NIH, Bethesda, USA) and Dr. D.Y. Loh (Washington University School of Medicine, St. Louis, USA), respectively. *Mcl-1*$^{fl/fl}$, *Aicda*-Cre and *Rosa26*-CreER^{T2} (TaconicArtemis) mice have been described (*3-5*). Bone marrow reconstituted mice were generated using the following protocols. (A) Transfer of Ly5.1 wildtype and Ly5.2 *Mcl-1*$^{fl/+}$*Aicda*-Cre bone marrow into lethally irradiated (2 x 5.5 Gy, 3 hrs apart) Ly5.1 recipients at a ratio of 1:1. (B) Transfer of Ly5.2 *Bcl2l1*$^{+/+}$*Aicda*-Cre or *Bcl2l1*$^{fl/-}$*Aicda*-cre bone marrow cells into lethally irradiated Ly5.1 recipients. (C) Irradiated Ly5.1 mice were reconstituted with 80% B cell deficient (μMT) (*6*) plus 20% *Mcl1*$^{+/+}$ Cre-ER, *Mcl1*$^{fl/+}$ Cre-ER, or *Mcl1*$^{fl/fl}$ Cre-ER bone marrow. Immunisation comprised a single intraperitoneal injection of 100μg NP coupled to keyhole limpet hemocyanin (NP-KLH) at a ratio of 21:1 and precipitated onto alum, prepared as described previously (*7*). Estrogen receptor (ER) mediated deletion of LoxP *Mcl1* alleles was accomplished by oral gavage with Tamoxifen on day 7 and 8 after immunization as described (*8*). Spleens were isolated at day 9 after immunization.

Antibodies, flow cytometry, cell sorting and V_H gene sequencing. Single cell suspensions were stained as described(*9*) using antibodies to the following surface molecules: CD38 (NIMR-5), B220 (RA3-6B2), IgM (331.12), IgD (11-26C), Gr-1 (RB6-8C5), CD138 (281.2), IgG_1 (X56; BD Pharmingen), FcγR (2.4G2), PNA (FL-1071), CD19 (1D3; BD Pharmingen), CD45.2 (BD

Pharmingen), hCD2 (Lym-1) and hCD4 (OKT4). NP binding was detected as described (*9*). Stained, cells were analysed on an LSRII (BD Biosciences). B cells ($CD19^+PNA^-$), GC B cells ($CD19^+PNA^+$) or ($NP^+IgG1^+CD38^-$) and memory B cells ($NP^+IgG1^+CD38^+$) were sorted from splenocytes with a FACSAria (BD Biosciences) to more than 98% purity. Single $NP^+IgG_1^+$ cells and $NP^+IgG1^+CD38^+$ cells were sorted and processed for cDNA synthesis and V_H gene PCR amplification as described (*9*). Sequencing was done with ABI BigDye mix 3.1 with automated base calling.

Enzyme-linked immunospot (ELISPOT) assay and enzyme-linked immunosorbent assay (ELISA). The frequency of ASCs was determined as described (*9*). Cells were incubated O/N at 37°C on pre-coated 96-well MultiScreen-HA filter plates (Millipore). Spots were visualized with anti-IgM or anti-IgG_1-specific goat anti-mouse antibodies conjugated to horseradish peroxidase (Southern Biotechnology Associates; SBA) and colour was developed by addition of 3-amino-9-ethyl carbazole (Sigma-Aldrich). Plates were washed extensively and spots were counted with an AID ELIspot reader system (Autoimmune Diagnostika). Anti-NP ELISAs were performed as described (*9*).

Western blotting. Total cell lysates from sorted follicular ($CD19^+PNA^-$) or GC ($CD19^+PNA^+$) B cells were prepared, separated and blotted as described (*10*). Blots were blocked with 5% skim milk and 0.1% Tween20 in PBS and incubated overnight at 4°C with the following antibodies: rat anti-Mcl1 (clone 19C4-15), rat anti-Bcl2l1 (Bcl-x_L; clone 9C9 or polyclonal rabbit, BD Pharmingen), hamster anti-Bcl2 (clone 3F11), anti-Bim (rat monoclonal 3C5, ENZO Biosciences or polyclonal rabbit serum, Stressgen), anti-Bcl6 (7D1-10, rat IgG2a monoclonal raised against

aa. 261 – 386 of mouse BCL6 fused with GST) and anti-β-actin (AC40, Sigma). Bound primary antibodies were visualised by incubation with horseradish peroxidase conjugated to species-specific anti-IgG secondary antibody. Blots were developed with SuperSignal ECL reagent (Pierce). Band densities were measured by Optical densitometry using a GS-800 Calibrated Densitometer (Bio-Rad) with QuantityOne 4.6.1 software (Bio-Rad) and the intensity in GC B cells relative to follicular B cells is given for each protein.

Immunohistochemistry. Tissue samples were embedded, stored, sectioned and stained as described (*11*). Antibodies used were unlabeled GL7 and biotinylated anti-B220. GL7 was detected with Alexa555-conjugated goat anti-rat antibodies (Invitrogen) and biotinylated anti-B220 with streptavidin-Cy5 (SBA). Antibodies used for detecting IgM foci were unlabeled goat anti-IgM (SBA) and unlabeled rabbit anti-CD3 (SP7, supernatant), detected with Alexa488-conjugated donkey anti-goat antibodies (Invitrogen) and Alexa647-conjugated goat anti-rabbit antibodies (Invitrogen) in subsequent steps.

Statistical analysis. Statistical analysis was performed using GraphPad Prism software (GraphPad Software, San Diego, California, USA). A Student's *t*-test with two-tailed distributions for two samples with equal variance was used. Data are shown as mean ± SEM where applicable, with a $P<0.05$ considered significant and exact *P* values presented in the figures.

Online Methods References

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