Supporting Online Material for

Mcl-1 is Essential for Germinal Center Formation and B cell Memory

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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-xL) 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-1fl/fl, Aicda-Cre and Rosa26-CreER\textsuperscript{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\textsuperscript{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\textsuperscript{+/+} Aicda-Cre or Bcl2l1\textsuperscript{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\textsuperscript{+/+} Cre-ER, Mcl1\textsuperscript{fl/+} Cre-ER, or Mcl1\textsuperscript{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 Mcll 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\textsubscript{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\textsubscript{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\(^+\)IgG1\(^+\) 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-Bcl2I1 (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.
Fig. S1. Kinetics of the GC response in Bcl2l1 deficient mice. Flow cytometric analysis of splenocytes on days 7, 14 and 21 after intraperitoneal immunization with NP-KLH in alum. Isotype switched (IgM^+IgD^+Gr-1^−CD138^−B220^+) B cells were analysed for NP^+IgG_1^+ status and then sub-divided into GC (CD38^−) and memory (CD38^+) B cells. Numbers of each of the respective populations (total, GC and memory) are plotted. Data are the mean ± SEM of between three and six mice in each group and summarize two experiments for each time point. One experiment used mice reconstituted with Bcl2l1^fl/+Aicda-Cre or Bcl2l1^+/+Aicda-Cre bone marrow.
**Fig. S2.** The Bcl2l1 floxed allele is efficiently deleted in Bcl2l1<sup>fl/+</sup>-Aicda-Cre GC and memory B cells. PCR detecting the floxed and deleted Bcl2l1 alleles in DNA from sorted GC (NP<sup>+</sup>IgG<sub>1</sub><sup>+</sup>CD38<sup>-</sup>) and memory (NP<sup>+</sup>IgG<sub>1</sub><sup>+</sup>CD38<sup>+</sup>) B cells from two Bcl2l1<sup>fl/+</sup>-Aicda-Cre immunized mice, as well as in tail DNA from a Bcl2l1<sup>fl/+</sup>-Aicda-Cre mouse. Empty lanes from a single gel were removed from the image, rejoining indicated by the solid line. The data are representative of two independent experiments. Lower panel is a schematic representation of the Bcl2l1 alleles used in these experiments. The floxed allele, before and after Cre-mediated deletion, and the null allele are shown. The positions where PCR primers bind are also indicated.
**Fig. S3.** Expression of *Aicda* and Cre recombinase in GC B cells but not follicular B cells in *Bcl2l1fl/Aicda-Cre* mice.

**A)** Flow cytometric analysis of splenocytes 21 days after intraperitoneal immunization with NP-KLH in alum. Non-GC (IgD<sup>-</sup>CD19<sup>+</sup>PNA<sup>-</sup>) and GC (IgD<sup>-</sup>CD19<sup>+</sup>PNA<sup>+</sup>) B cells were analysed for levels of hCD2t indicating expression of *Aicda* and Cre recombinase. Numbers in dot plots and histograms are mean percentage ± SEM. **B)** Total cell numbers of the populations identified in (A) are plotted as the mean ± SEM of between four and six mice in each group, summarizing two experiments.
Fig. S4. *Bcl2l1* contributes to the survival of emigrating plasma cells and antibody titres.

(A) Frequencies of total and high affinity NP-specific IgG1-secreting antibody secreting cells in spleen and bone marrow 14 days after intraperitoneal immunization of *Bcl2l1* conditionally targeted mice with NP-KLH in alum. Data are the mean ± SEM of replicate wells with four to six mice in each group and summarize two experiments. (B) ELISA of total (NP13) and high affinity (NP2) anti-NP-IgG1 and total anti-NP-IgM in serum, measured on day 21 after immunization. Values from individual mice are shown, as are the means ± SEM. Significant differences in (A) and (B), determined by Student’s T-test, are shown.
**Supplementary figure 5**

**Fig. S5.** Conditional deletion of *Mcl1* abrogates the formation of NP-specific plasma cells. ELISA of total (NP13) and high affinity (NP2) anti-NP-IgG and total anti-NP-IgM in serum, measured on day 14 after intraperitoneal immunization with NP-KLH in alum. Data are the mean ± SEM of between five and eight mice in each group and summarize three experiments. Values from individual mice are shown, as are the means ± SEM. Significant differences, determined by Student’s T-test, are shown.
**Supplementary figure 6**

**Fig. S6.** Loss of *Mcl1* blocks appearance of NP⁺IgG₁⁺ B cells on day 5 of the immune response. 

(A) Frequency of NP-binding (left) and NP⁺IgG₁⁺ (right) B cells in inguinal lymph nodes (iLN) 5 days after subcutaneous immunization of mice with NP-KLH in alum. Status of the *Mcl1* locus and *Aicda-Cre* are indicated. All immunized mice show an expansion of NP-reactive B cells, but loss of *Mcl1* coincident with expression of AID prevents appearance of NP⁺IgG₁⁺ cells. B6 are non-immunized C57BL/6 controls. 

(B) Frozen sections of spleens from the indicated strains 5 days after immunization, stained with anti-IgM (green) and anti-CD3 (red). Foci of IgM ASC developed in the bridging channels in all strains, indicated by arrows. Original magnification x 20 with scale indicated. 

(C) ELISPOT analysis of NP-reactive ASC in spleens 5 days after immunization showing equal development of IgM ASC (right) but deficiency of IgG₁ ASC in strains capable of deleting *Mcl1*. Significant differences, calculated using Student’s T-test, are indicated. Data are from 3 mice per group.
Supplementary figure 7

Fig. S7. Loss of Mcl1 blocks appearance of NP⁺IgG₁⁺ B cells on day 7 of the immune response
(A) Absolute number of NP⁺IgG₁⁺ B cells in the spleens of mice immunized by intraperitoneal injection
of NP-KLH in alum 7 days previously. Status of the Mcl1 locus and Aicda-Cre are indicated. Significant
differences, calculated using Student’s T-test, are indicated. (B) Frozen sections of spleens from the indi-
cated strains 7 days after immunization, stained with anti-B220 (red) and anti-GL7 (green). GC are not
apparent in Mcl1flo/floAicda-Cre mice. Original magnification x 20 with scale indicated. (C) ELISPOT
analysis of NP-reactive ASC in spleens 7 days after immunization showing equal development of IgM
ASC but deficiency of IgG₁ ASC in mice capable of deleting Mcl1. Significant differences, calculated
using Student’s T-test, are indicated. Data are from 4 mice per group.
Fig. S8. Deletion of GC B cells expressing Aicda and Cre recombinase in Mcl1fl/flAicda-Cre mice. (A) Flow cytometric analysis of splenocytes 14 days after intraperitoneal immunization with NP-KLH in alum. Non-GC (IgD−CD19+PNA−) and GC (IgD−CD19+PNA+) B cells were analysed for hCD2 expression, indicating co-expression of Aicda and Cre recombinase. Numbers in plots and histograms are the mean percentages ± SEM. (B) Total cell numbers of the populations identified in (A) are graphed as the mean ± SEM of between four and eight mice in each group, summarizing three experiments. (C) Peyer’s patches, collected from the indicated groups of mice, were analysed for the representation of GC B cells (CD19+FAS+GL7+) amongst all B cells (left) and of hCD4+ GC B cells (right). Significant differences, calculated using Student’s T-test, are indicated. Data are from 3-4 mice per group.
Fig. S9. *Mcl1* gene-targeting construct
The construct designed to introduce LoxP sites flanking the *Mcl1* locus contains hCD4 such that upon *Mcl1* deletion, it is subjugated to the promoter of *Mcl1*. hCD4 is subsequently expressed on the cell surface serving as a reporter for both deletion of the *Mcl1* allele and *Mcl1* transcription.
Supplementary figure 10

Fig. S10. Mcl1 is efficiently deleted in GC and memory B cells as reported by hCD4 expression. (A) Flow cytometric analysis of splenocytes 14 days after intraperitoneal immunization with NP-KLH in alum. GC (IgD⁺B220⁺PNA⁺) B cells were analysed for hCD2, reporting co-expression of Aicda and Cre recombinase as well as amounts of hCD4, reporting Mcl1 deletion. Numbers in plots are the mean percentages ± SEM. Data are the means ± SEM of two or three mice in each group. (B) IgG1⁺ GC B cells (CD38⁻) and memory B cells (CD38⁺) in Mcl1fl/+ Aicda-Cre mice were assessed for hCD4 expression. Numbers in dotplots and histograms are mean percentages ± SEM of at least two mice in each group.
Fig. S11. Comparable fold reduction of Mcl1^fl/+ B cells amongst the GC and memory compartments that remain constant over time. 
Absolute numbers of NP^+IgG_1^+ GC (CD38^-) and memory B cells (CD38^+) arising from each genotype in Mcl1^fl/+ Aicda-Cre/Ly5.1 chimeras at 14, 33 and 54 days after intraperitoneal immunization with NP-KLH in alum are graphed as the mean ± SEM of between 2 and 3 mice for each time point.
**Fig. S12.** Affinity maturation amongst NP-reactive B cells is unaffected by loss of either Bcl2l1 or Mcl1. (A) Affinity maturation of NP-specific IgG₁ ASCs calculated as a ratio of NP2/NP13 for mice of the indicated genotypes on day 21 post immunization. Data are the mean ± SEM from 4 – 6 mice per group with tissues and configuration of the Bcl2l1 and Aicda-Cre alleles indicated. (B) Affinity maturation of NP-specific IgG₁ ASCs calculated as a ratio of NP2/NP13 for mice of the indicated genotypes on day 21 post immunization. Data are the mean ± SEM from 5 - 10 mice per group with tissues and configuration of the Mcl1 and Aicda-Cre alleles indicated. (C) Analysis of somatic hypermutation amongst NP⁺IgG₁⁺CD38hi memory B cells from mice of the indicated Bcl2l1 genotypes assessed by comparing point mutations in the VH186.2 genes of single antigen specific B cells sorted 21 days after immunization with those in controls. Average mutation indicates the average number of point mutations per VH186.2 gene segment determined by comparing the region encoding amino acids 10 to 96 with the germline sequence from 17 Bcl2l1 deleted cells with 14 controls. Position 33 (%) measures the frequency of the Trp to Leu exchange within CDR1 of VH186.2 that alone confers a 10-fold increase in affinity of binding NP.
Supplementary figure 13

Fig. S13. Induced deletion of Mcl1 leads to loss of NP<sup>+</sup>IgG<sub>1</sub><sup>+</sup> B cells during an immune response. (A) Irradiated C57BL/6-Ly5.1 mice were reconstituted with 80% B cell deficient (μMT) bone marrow plus 20% bone marrow from Mcl1<sup>+/+</sup>CreERT<sub>2</sub>, Mcl1<sup>fl/+</sup>CreERT<sub>2</sub>, or Mcl1<sup>fl/fl</sup>CreERT<sub>2</sub>. In the resultant chimeras, all B cells contained CreERT<sub>2</sub>, expressed from the Rosa26 locus, and were either resistant (Mcl1<sup>+/+</sup>) or sensitive to Mcl1 deletion on one (Mcl1<sup>fl/+</sup>) or both (Mcl1<sup>fl/fl</sup>) alleles. (B) Flow cytometric analysis of antigen-specific B cells persisting after induced deletion of Mcl1. Seven days after intraperitoneal immunization with NP-KLH in alum, mice were treated on two successive days with Tamoxifen to induce Cre activity and their spleens analysed for the frequency of isotype switched (IgM<sup>+</sup>IgD<sup>+</sup>Gr-1<sup>+</sup>CD138<sup>+</sup>)CD21<sup>hi</sup>)B cells that were NP<sup>+</sup>IgG<sub>1</sub><sup>+</sup>. The amount of hCD4<sup>+</sup> indicative of Mcl1 deletion--expressed on the remaining NP<sup>+</sup>IgG<sub>1</sub><sup>+</sup> cells was determined. (C) Total numbers of splenic B220<sup>+</sup> cells, CD23<sup>+</sup>IgM<sup>+</sup>CD21<sup>int</sup> transitional 1 (T1) cells, CD23<sup>+</sup>IgM<sup>hi</sup>CD21<sup>hi</sup> transitional 2 (T2) cells, CD23<sup>+</sup>IgM<sup>hi</sup>CD21<sup>hi</sup> follicular (Fo) cells and CD23<sup>+</sup>IgM<sup>+</sup>CD21<sup>hi</sup> marginal zone (MZ) cells in naive mice. Significant differences, calculated using Student’s T-test, are indicated. Data are from 3 mice per group.
Supplementary figure 14

**Fig. S14.** Induced deletion of *Mcl1* leads to reduction in IgM⁺ plasma blasts.
Irradiated C57BL/6-Ly5.1 mice were reconstituted with 80% B cell deficient (μMT) bone marrow plus 20% bone marrow from *Mcl1*⁺⁺*CreER*², *Mcl1*⁻⁻*CreER*², or *Mcl1*⁻⁻*CreER*². In the resultant chimeras, all B cells contained CreER², expressed from the Rosa26 locus, and were either resistant (*Mcl1*⁺⁺) or sensitive to *Mcl1* deletion on one (*Mcl1*⁻⁻⁺⁺) or both (*Mcl1*⁻⁻⁻⁻) alleles. Seven days after intraperitoneal immunization with NP-KLH in alum, mice were treated on two successive days with Tamoxifen to induce Cre activity. Frozen sections of spleens from the indicated strains were stained with anti-IgM (green) and anti-CD3 (red). IgM plasma blasts are indicated by dotted circles and arrows. Original magnification x 20 with scale indicated.
Online Methods References