Mice
Mice were used at 6–12 weeks of age, unless stated otherwise, age- and sex-matched within experiments and were handled in accordance with institutional and national guidelines. All animal experiments were performed after approval of our Institute’s animal ethics committee. C57BL/6 (B6), OT-1 and B6 Ly5.1 control mice were purchased from Charles River and kept as breeding colonies in our local animal facility. Noxa mice were a kind gift from Dr. A. Strasser (WEHI, Melbourne) and provided by Dr. M. Serrano (CNIO, Madrid). Noxa5.1 mice were generated by crossing Noxa− mice with B6 Ly5.1 mice from our in house colony. p53− tissues were a gift from Dr. M. Breuer (NKI, Amsterdam). All mice were either generated in B6 mice or backcrossed at least ten times on this background, apart from the p53− mice which were BALB/c.

Flow cytometry
Single-cell suspensions were obtained by mincing the specified organs through 40 μm cell strainers (Becton Dickinson). Erythrocytes were lysed with an ammonium chloride solution (155 mM NH4Cl, 10 mM KHCO3, and 1 mM EDTA) and cells were subsequently counted using an automated cell counter (SCHÄRFE SYSTEM). Cells (5 × 10^5 – 5 × 10^6) were collected in PBS with 0.5% bovine serum albumin (Sigma) and stained for 30 min at 4 C with antibodies in the presence of anti-CD16/CD32 (clone 2.4G2, anti-mouse FcγRII/RIII (a kind gift of Louis Boon, Bioceros, Utrecht, the Netherlands). The monoclonal antibodies against B220 (6B2), CD38 (90), GL7 (GL7), CD95 (15A7), CD5 (53-7.3), CD19 (eBio1D3), IgM (eB121-15F9) and IgD (11-26c) were purchased from eBioscience. The monoclonal antibodies against Ki67 (B56), CD21/35 (7G6), CD138 (281-2), IgG1 (×56) and CD43 (S7) were bought from BD Biosciences. Antigen-specific B cells were visualized by ex vivo labelling using PE (sigma) or NP-PE (Biosearch Technologies). FACS experiments were performed on a FACSCalibur or FACSCanto (Becton Dickinson) and analysed with FlowJo software (TriStar). In vitro cell viability, was assessed by staining with FITC conjugated AnnexinV (BD biosciences) followed by adding LIVE/DEAD far red dye (Invitrogen) shortly before measurement. Ex vivo cell viability was measured with the Vybrant® FAM caspase-3 and -7 assay kit (Invitrogen) according to manufacturers protocol. Intracellular stainings for Ki67 were performed using Foxp3 Fixation/Permeabilization Concentrate and Diluent (eBioscience), according to the manufactures protocol. Follicular (B220^IgM^IgD^CD21^bright^), marginal zone (B220^IgM^IgD^−CD21^bright^) and germinal center (B220^CD38^dim^GL7^bright^) B cells were sorted to >99% purity with a FACSaria (Becton Dickinson).

RT-MLPA analysis
Total RNA was extracted using the trizol isolation method (Invitrogen), mRNA levels were analyzed with the Apoptosis Mouse mRNA RT-MLPA kit (MRC-Holland) according to the manufacturer’s instructions. Samples were run through a Genescan and analyzed with GeneMapper (Applied Biosystems GmbH;) and subsequently Excell software (Microsoft). Normalization was performed by setting the sum of all peak data 100% to correct for fluctuations in total signal between samples, and individual peaks were calculated relative to the 100% value. Differential expression was calculated by fold induction relative to unstimulated cells at t=0 hrs, which were set at 1. The logarithm (base 2) of the resulting datasets were imported in the TIGR Multiexperiment viewer version 2.2 (www.tigr.org/software/tm4).
**Immunoblot, RT-PCR, and Immunohistochemistry**

For immunoblot cells were lysed in Laemmli lysis buffer at the indicated time points after stimulation. SDS-PAGE gel electrophoresis was performed using the Bio-Rad mini-PROTEAN electrophoresis system as described with primary antibodies against β-Actin (Santa Cruz Biotechnology), Bim (Stressgen), Bcl-XL (Transduction Laboratories) and Mcl-1 (Rockland). Binding was visualised using IRDye 680 or 800 labeled secondary antibodies and an Odyssey Imager (Li-Cor). Quantification of signal was performed using Odyssey 3.0 software. Total RNA was extracted using the Trizol isolation method (Invitrogen) and cDNA was generated using oligodeoxythymidine (oligo dT) and Superscript II Reverse Transcriptase (Invitrogen). RNA transcripts were amplified by polymerase chain reaction (PCR) using the following primers for Noxa (5'-CTCTCGAGCCCGGAGAAGGC-3' and 5'-GGGAATTCTCAGGTTACTAAA TTGAAGAGCT-3'), Mcl-1 (5'-GAGGAGGAAGAGGACCGTATACC-3' and 5'-AGTGTCTGCTATGGTTCGATGAAG-3') and Bim (5'-GAGTGTGACAAGTCAACACCAACC-3' and 5'-GAAGATAAGCGTAAACGGTGAAGATA-3'). 18S was used as a loading control (5'-TCAAGACGAAACATCGGAGG-3' and 5'-GGACATCTAAGGCGATCAC-3'). For immunohistochemistry, tissues were frozen in TissueTeq (Sakura Finetek). Sections were fixed with acetone, stained with home-made primary biotinylated antibodies against B220 (RA3-6B2), CD4 (GK1.5) or Cleaved Caspase-3 Asp175 (Cell Signaling). Germinal centers were stained with biotinylated PNA. For immunofluorescent staining, primary antibody staining was visualized with streptavidin labelled Alexa488 (BD Bioscience) or anti-Hamster labelled with Alexa647 (Invitrogen). To prepare tissue sections for light microscopic analysis, tissue sections were incubated with the indicated nonlabeled or biotinylated primary rat anti-mouse Abs and then with HRP- or alkaline phosphatase-conjugated anti-rat Abs or streptavidin (Jackson Immunoresearch Laboratories). After washing, tissue sections were developed with diaminobenzidine (Sigma-Aldrich) to visualize HRP conjugates and with 5-bromo-4-chloro-3-indolylphosphate-nitro blue tetrazolium (Sigma-Aldrich) to visualize AP conjugates. Tissue sections were mounted with Kaiser’s glycerin (Merck) and analyzed using an Olympus light microscope. Tissue sections were analyzed with a Leica 020-519.506 LB30S microscope and representative pictures were taken with a Leica DFC420 Camera. Leica Application Suite V3 software was used for digital analysis of the pictures.

**Statistical analysis**

Statistical analysis of the data was performed using the unpaired Student’s t-test, Wilcoxon rank-sum test or one-way ANOVA test where applicable. Asterisks denote significant differences (* p<0.05, ** p<0.005, *** p<0.0005).

**REFERENCES**

**Figure S1. In vitro differentiation of murine B cells**

Phenotypic analysis of B cells stimulated *in vitro* with anti-IgM and LPS in the (A) absence or (B) presence of IL-4 over time. Differentiation of Follicular B-type cells (B220⁺GL7⁻CD38⁺) into activated B cells (B220⁺GL7⁺CD38⁺), further differentiated B cells (B220⁺GL7⁺CD38⁻) and plasmacells (B220dimCD138⁺) is given. Shown is one of six independent experiments performed in triplo (n=3). (C) Expression profiling by RT-MLPA of purified WT and *trp53⁻/⁻* mouse B cells after *in vitro* stimulation. Cells were stimulated with anti-IgM and LPS. Gene induction of pro- and anti-apoptotic molecules is represented after log2 transformation of expression levels, relative to averaged values of unstimulated cells at day 0. Shown is pooled data from two independent experiments using 2 to 4 mice per experiment (WT=4, *Tp53⁻/⁻*=6).

**Figure S2. B cells in Noxa⁻/⁻ mice are unaltered under homeostatic conditions**

(A) B cells were purified from mouse spleens and stimulated *in vitro* with anti-IgM/LPS alone (−IL4) or in combination with IL4 (+IL4). CFSE dilution of B220⁺CD38⁺ cells after two days of stimulation with anti-IgM/LPS and IL4. Representative results from one of at least four independent experiments are shown. Gated is for B220⁺ cells (B) Wild Type (black bars) and Noxa⁻/⁻ (white bars) mice were maintained under homeostatic conditions for 12 weeks and analyzed by flow cytometry. Absolute number of (top left) total B cells (B220⁺) in peripheral organs, (top middle) B-cell precursors in the Bone Marrow (defined by expression of B220, CD43 and IgM), (bottom left) B1 cells (B220⁺CD5⁺) in peripheral organs, (bottom middle) B-cell subsets in the spleen (defined by expression of B220, IgM, IgD and CD21/35), (top right) Average expression levels of IgM within splenic Follicular B cells (FB cells) and Marginal Zone B cells (MZB cells). (bottom right) Average expression levels of IgD within Follicular B cells in spleen and peripheral lymph nodes (n=3 per genotype; One of four experiments with similar results is shown). (C) Spleen sections of WT and Noxa⁻/⁻ mice (400× enlarged) were immunostained for B220 (green) and CD4 (red) representative stainings of several mouse organs (n=3 per genotype) is shown.

**Figure S3. B cells in Noxa⁻/⁻ mice show increased survival after activation**

Mice were infected intranasal with Influenza A and analyzed at various time points after infection. (A) Representative pictures (100× enlarged) of immunostained sections from spleens and mediastinal lymph nodes 12 days after Influenza infection. (yellow) B220⁺ and (purple) PNA⁻ cells were stained. Controls represent sections of non-infected WT mice. (B) Absolute numbers of Germinal Center B cells in the spleens of mice were determined before (day 0) and at various timepoints after infection (n=3 per genotype per timepoint). (C) Histograms of follicular (FB) and Germinal Center (GC) B cells stained intracellular with Ki67. (D) Quantification of Germinal Center (GC) B cells in spleen and mLN staining positive for Ki67 (top panel) 10 days after influenza infection and 2 hours after BrdU injection (WT n=4, Noxa⁻/⁻ n=5). * p<0,05 (student t test).

**Figure S4. Reduced antibody affinity in Noxa⁻/⁻ mice after PE-immunization**

Mice were immunized with PE/Alum and 12 days after immunization responses were analyzed (n=5 per genotype). (A) FACS plots after *ex vivo* staining with 1 μg/ml PE. Gated is on Germinal Center B cells (B220⁺CD38dimGL7⁺) or Follicular B cells (B220⁺CD38⁺GL7⁺). (B) Average MFI of IgG1-staining of GC B cells (B220⁺CD38dimGL7⁺). (C) FACS plots of IgG1 positive B cells in the germinal center, stained with PE. Gated is for B220⁺CD38dimGL7⁺IgG1⁺ cells. (D) Ratio
of PE binding relative to the expression levels of IgG1. Quantification of the ratio of PE fluorescence intensity divided by fluorescence intensity of IgG1 staining of GC B cells in the spleen (left panel) and mLN (right panel).

Figure S5. Reduced antibody affinity in Noxa<sup>−/−</sup> mice after antigen encounter
(A) Mice were immunized with TNP-KLH/Alum and 14 days after immunization responses were analyzed. Expression of MHC-II on follicular B cells (FB) and Germinal Center (GC) B cells is shown (n=5 per genotype). (B) Mice were immunized with PE/Alum and PE-specific antibody responses were assessed by ELISA under limiting serum dilutions with fixed PE coating (5ug/ml). Shown are IgM (left) and IgG1 (right) responses (n=5 per genotype). X-axis represents the percentage of antiserum in the dilution buffer used for detection of antibodies. (C) Mice were immunized with TNP-KLH/Alum and 14 days after immunization responses were analyzed. TNP-specific antibody responses were assessed by ELISA under limiting dilutions of coated antigen with a fixed concentration of antiserum. Shown are responses for IgM and IgG1, plotted as a percentage of the maximum signal at plateau levels (n=5 per genotype). (D) Levels of TNP-specific IgG1 producing plasmacells were determined in the spleen, measured by ELISpOT (n=5 per genotype). (E) Mice were infected with Influenza A and virus-specific IgM and IgG1 antibody responses were assessed by ELISA under limiting serum dilutions after 14 days with fixed antigen-coating (n=8 per genotype). * p<0.05, ** p<0.005 (student t test). p.i.-serum = Pre-Immune serum (sera isolated from mice before immunization).

Figure S6. Absolute number of Noxa<sup>−/−</sup> cells in bone marrow chimeric mice after immunization
WT (CD45.2) recipients (n=10) were lethally irradiated and transferred with mixed WT (CD45.2): Noxa<sup>−/−</sup> (CD45.1) bone marrow in a 4:1 ratio. Two months after transfer, 8 mice were immunized with PE and after twelve days B-cell responses were assessed. (A) Absolute number of WT and Noxa<sup>−/−</sup> Follicular B cells (FB; B220<sup>−</sup>CD38<sup>−</sup>GL7<sup>−</sup>) in the spleen. (B) Absolute number of WT and Noxa<sup>−/−</sup> PE<sup>+</sup> Germinal Center B cells (B220<sup>−</sup>PE<sup>+</sup>CD38<sup>dim</sup>) in the spleen. (C) Absolute number of WT and Noxa<sup>−/−</sup> Follicular B cells (FB; CD38<sup>−</sup>GL7<sup>−</sup>) in the mediastinal lymph node. (D) Absolute number of WT and Noxa<sup>−/−</sup> PE<sup>+</sup> Germinal Center B cells (PE<sup>+</sup>CD38<sup>dim</sup>) in the mediastinal lymph node. (E) FACS plot of GC B cells (B220<sup>−</sup>CD38<sup>dim</sup>), stained for CD45.1 and PE. Arbitrary gating shows a reduced fraction of PE<sup>bright</sup> cells within the CD45.1<sup>+</sup> (Noxa<sup>−/−</sup>) population. (F) Quantification of the relative number of PE<sup>bright</sup> cells within the WT (CD45.1<sup>+</sup>) and Noxa<sup>−/−</sup> (CD45.1<sup>−</sup>) GC B-cell pools. * p<0.05, ** p<0.005, ***P<0.0001 (student t test).

Figure S7. Noxa ablation does not influence hypermutation rates
Mice were immunized with NP-CGG and boosted after 28 days. Seven days after boost, NP-specific B cells (Dump<sup>−</sup>B220<sup>−</sup>IgG1<sup>+</sup>NP+) were sorted, V<sub>H</sub>186.2-C<sub>γ</sub>1 fragments were PCR-amplified and their sequences analyzed (n=2 per genotype). (A) Summary of V<sub>H</sub>186.2 sequences from NP-specific IgG1<sup>+</sup> B cells (B) Average number of hypermutations (CDR1+CDR2 region) per retrieved sequence. (C) Average number of GC-seeding clones (based on non-identical CDR3 regions) per mouse.
Figure S8. Activated Noxa−/− B cells show reduced clonal selection
Mice were immunized with PE and 12 days after immunization splenic B cells were analyzed (n=3 per genotype). (A–B) FB cells (B220+CD38−GL7−) and PE-specific GC B cells (B220+CD38−GL7+PE+) were sorted and (A) V_{H}186.2-C_{\mu} and (B) V_{H}186.2-C_{\gamma1} fragments were PCR-amplified and analyzed via spectratyping. (C) Sequence alignment of PE+ B cells of mice shown in figure 5a. Colors represent physio-chemical properties of amino acids (Red: Small, Blue: Acidic, Magenta: Basic, Green: Hydroxyl/Amine/Basic). Numbers indicate CDR3 length. "*" Indicate residues in that column which are identical in all sequences in the alignment.

Figure S9. Noxa ablation leads to impaired memory B-cell responses
Mice were immunized with TNP-KLH and boosted after 28 days. Seven days after boost TNP-specific antibody responses were assessed by ELISA, using saturating serum concentrations (n=5 per genotype). Shown are (left) IgM, (middle) IgG1 and (right) IgG2b responses. * p<0,05, ** p<0,005, ***P<0,0001 (student t test)

Figure S10. Working model for early B-cell selection
Upon antigen encounter, activated B cells will start proliferating. Alterations in their transcriptional profile (e.g. Noxa induction), as well as competition with other activated B cells for antigen, nutrients and cytokines will terminate B cells that receive minimal signal strength in a Noxa and possibly Bim dependent fashion. Subsequently, in a T-cell–dependent B-cell response, the remaining clones will seed the germinal center and increase affinity and diversity via hypermutation, in a selection process mediated by Bim and FAS. In a T-cell–independent response (not shown), the last phase will not occur.
Figure S1

(a) B-cell differentiation -IL4

(b) B-cell differentiation +IL-4

(c) LPS stimulated B cells
Figure S2

a

Day 0  Day 2  Day 6

-IL4 (B220-CD38+)

+IL4 (B220-CD38+)

% of Max.

CFSE

Wild Type  Noxa−

b

B cells

B cell precursors BM

IgM expression Spleen

Cells (×10^6)

Spleen  pLN  BM

Pe-ProB-cells  ProB-cells  Mature B cells

Cells (×10^6)

Wild Type  Noxa−

IgD expression

Cells (×10^6)

Spleen  pLN

MZB  T1  T2  FBC

IgD expression

Wild Type  Noxa−

B220

CD4

Wild Type  Noxa−
**Figure S3**

**a**

<table>
<thead>
<tr>
<th>Spleen</th>
<th>Mediastinal Lymph node</th>
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<tr>
<td><strong>Wild Type</strong></td>
<td><strong>Noxa</strong>^-/-^</td>
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<tr>
<td><img src="image5.png" alt="Image" /></td>
<td><img src="image6.png" alt="Image" /></td>
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</tbody>
</table>

**b**

**Germinal Center B cells Spleen**

![Bar graph](image7.png)

- Wild Type
- Noxa^-/-^  

*Day 0, Day 8, Day 10, Day 17*

**c**

**Ki67^+ cells in the GC**

![Graph](image8.png)

- Spleen
- mLN

**d**

**Counts (% of Max.):**

- GC B cells
  - B220^+^CD38^+^GL7^+^  
  - B220^+^CD38^dim^GL7^-^  

- FB cells
  - B220^+^CD38^+^GL7^-^  

**Ki67**

- Wild Type
- Noxa^-/-^  

**Isotype**

- Ki67
- Isotype
Figure S4

a) Flow cytometry plots showing the percentage of GC B cells and FB cells in Wild Type and Noxa^{-/-} mice.

b) Bar graph showing the average IgG1 expression of GC B cells in Spleen and mLN for Wild Type and Noxa^{-/-} mice.

c) Flow cytometry plots showing the IgG1 expression levels in Wild Type and Noxa^{-/-} mice.

d) Box plots showing the IgG1 to PE ratio of GC B cells in Spleen and mLN for Wild Type and Noxa^{-/-} mice.

The IgG1 to PE ratio of GC B cells in mLN is significantly higher in Wild Type compared to Noxa^{-/-} mice (p < 0.05).
Figure S5

a) MHC-II expression

b) IgM and IgG1

- Wild Type
- Noxa^-/
- Control

Serum concentration (%)

OD 415 nm

IgM

Serum concentration (%)

OD 415 nm

IgG1

Serum concentration (%)

OD 415 nm

c) IgM and IgG1

- Wild Type
- Noxa^-/

% of Max.

Antigen (µg/ml)

% of Max.

Antigen (µg/ml)

d) TNP-specific IgG1* plasmacells

- Wild Type
- Noxa^-/

Plasmacells / Spleen (10^3)

Control

Immunized

e) IgM and IgG1

- Wild Type
- Noxa^-/

OD 415

OD 415

Serum Concentration (%)

Serum Concentration (%)

* p.i.-Serum
Figure S6

(a) Absolute contribution of cells in the spleen

(b) Absolute contribution of cells in the spleen

(c) Absolute contribution of cells in the mLN

(d) Absolute contribution of cells in the mLN

(e) Relative number of PE<sup>bright</sup> cells

(f) Relative number of PE<sup>bright</sup> cells
Figure S7

<table>
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<th>WT</th>
<th>Noxa&lt;sup&gt;−/−&lt;/sup&gt;</th>
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<tr>
<td>Total sequences</td>
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<td>97</td>
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<tr>
<td>Unique V&lt;sub&gt;H&lt;/sub&gt;186.2 Sequences</td>
<td>65</td>
<td>74</td>
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<td>Sequences with out of frame mutations</td>
<td>1</td>
<td>3</td>
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<tr>
<td>Sequences with 0 mutations</td>
<td>3,1 %</td>
<td>12,7 %</td>
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<tr>
<td>Silent mutations (SD)</td>
<td>25,5 %</td>
<td>9,6 %</td>
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<tr>
<td>Average nr. of mutations per V&lt;sub&gt;H&lt;/sub&gt;186.2 (SD)</td>
<td>7,9 (1,0)</td>
<td>6,8 (3,5)</td>
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<tr>
<td>W33L substitutions (%)</td>
<td>44,1 %</td>
<td>53,1 %</td>
</tr>
</tbody>
</table>

**b**

**Average number of mutations per sequence**

![Bar chart showing the average number of mutations per sequence](image)

- **Wild Type**
- **Noxa<sup>−/−</sup>**

**c**

**Average number of clones per mouse**

![Bar chart showing the average number of clones per mouse](image)

- **Wild Type**
- **Noxa<sup>−/−</sup>**
Figure S8

(a) Wild Type  

(b) Noxa^{-/-}  

V_{H}^{186.2} - C_{H}

Sequence Length

(c) Wild Type

(c') Noxa^{-/-}

Sequence: 

Wild Type

Noxa^{-/-}

***
Figure S9

**IgM**

- Wild Type
- Noxa/−

**IgG1**

- Wild Type
- Noxa/−

**IgG2b**

- Wild Type
- Noxa/−

**Time (days)**

**µg/ml**
Figure S10

Activation & Proliferation → Competition for survival factors → Hypermution (T cell dependent)

Diversity

Affinity

Non-Responder  High-affinity B Cell  Low-affinity B Cell