SUPPLEMENTARY APPENDIX

c-Myc dysregulation is a co-transforming event for nuclear factor-κB activated B cells

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 doi:10.3324/haematol.2016.156281

Received: September 8, 2016.

Accepted: February 21, 2017.

Pre-published: February 23, 2017.

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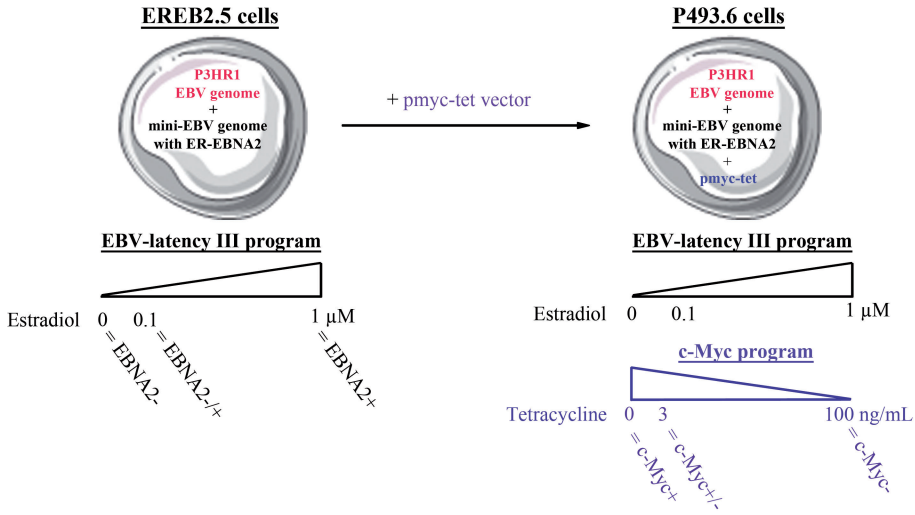
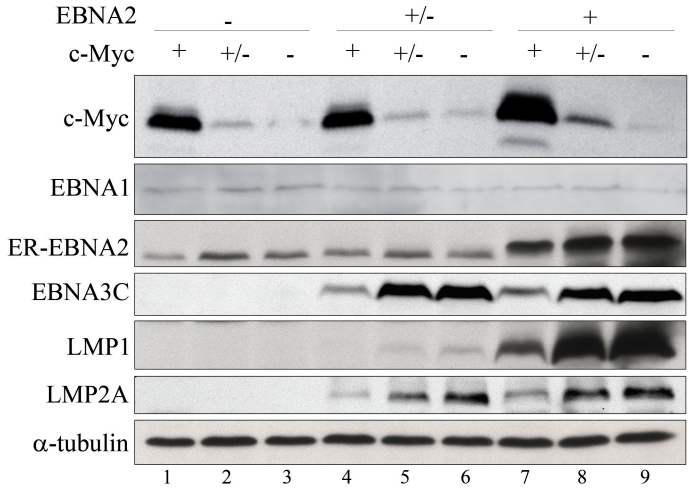
Figure S1

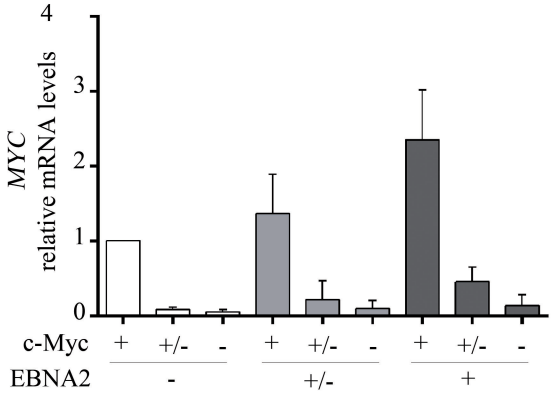
a

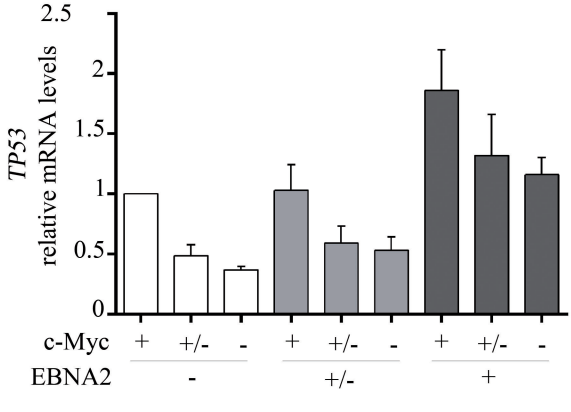


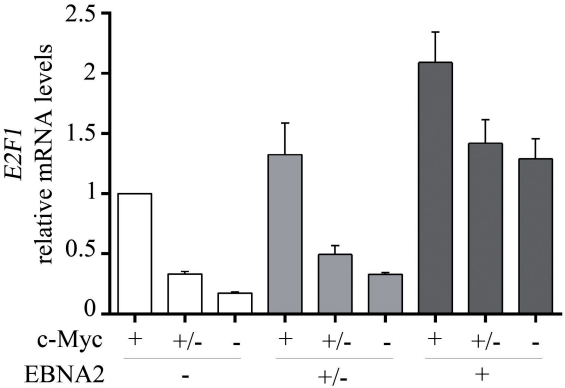
b



c







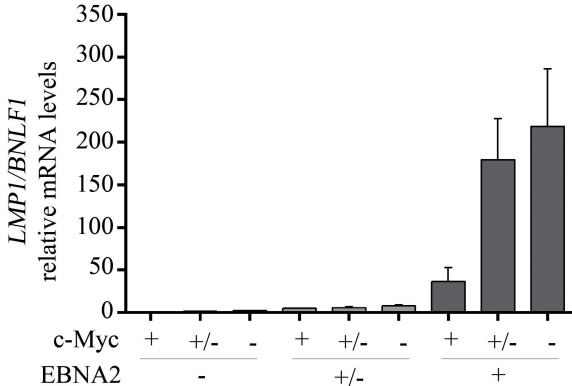


Figure S1. C-Myc and EBV proteins expression in the P493.6 cell model. (**a**) Scheme showing P493.6 cells derived from EREB2.5 cell line. The EBV-latency III program (*i.e.* LMP1, LMP2a, EBNA1, EBNA2, EBNA3A/B/C) is induced by estradiol addition, and c-Myc is down-regulated by tetracycline addition in a dose dependent manner. (**b**) P493.6 cells were treated or not with two doses of estradiol (0=EBNA2-, 0.1=EBNA2+/- and 1 µM=EBNA2+) and/or with tetracycline (0= c-Myc+, 3 µg/mL=c-Myc+/- and 100 µg/mL=c-Myc-) for 48 hours. Expression of c-Myc, EBNA1, ER-EBNA2, EBNA3C, LMP1 and LMP2a was assessed by Western-blot. Detection of α-tubulin was used as a loading control. One representative of 3 independent experiments is shown. (**c**) For each cell condition, mRNA expression of *MYC*, *TP53*, *E2F1*, and *LMP1/BNLF1* genes was quantified using TaqMan® Gene Expression Assays. Relative mRNA fold changes were calculated as the ratio of control cells to P493.6 cells without estradiol or tetracycline (*i.e.* cells under c-Myc alone). Data are shown as mean ± standard deviation (n=2-3 independent experiments).

Comment:

P493.6 cell line, in which c-Myc over-expression is negatively regulated by addition of tetracycline (*Tet-off* system), is derived from EREB2-5 cells co-infected by the EBNA2-deficient P3HR1 EBV strain and a mini-EBV vector harboring the open reading frame sequence coding for the estradiol regulatable Estrogen Receptor-EBNA2 fusion protein (ER-EBNA2) [1–3] (Figure S1a). Without estrogen or tetracycline, P493.6 cells mimicked EBV features of Burkitt's Lymphoma with an EBV-latency I pattern of viral proteins [2], *i.e.* EBNA1 expression and absence of EBNA3C, LMP1 and LMP2A expression (Figure S1b, lane 1). Residual expression of inactive ER-EBNA2 corresponded to Wp promoter activity. After tetracycline addition, c-Myc expression was turned-off at both protein (Figure S1b, lanes 2 and 3), and mRNA levels (Figure S1c). Expression of *TP53* and *E2F1* genes, both well-

known c-Myc target genes, was also inhibited by c-Myc repression (Figure S1c). The EBV-latency III program was turned on by estradiol addition, as assessed by increased ER-EBNA2 expression and induction of EBNA3C, LMP1 and LMP2A (Figure S1b, lanes 4 and 7). When the EBV-latency III program was induced, c-Myc down-regulation led to a significant increase in EBNA2, EBNA3C, LMP1, and LMP2A expression in a dose-dependent manner (Figure S1b, lanes 5, 6, 8, and 9; and Figure S1c for LMP1 mRNA expression). Highest levels of EBV-latency III proteins were observed when c-Myc over-expression was totally repressed (Figure S1b, lane 9). Repression exerted by c-Myc on EBV-latency III was likely to be due to repression of EBNA2 expression as previously suggested [4–6].

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Figure S2

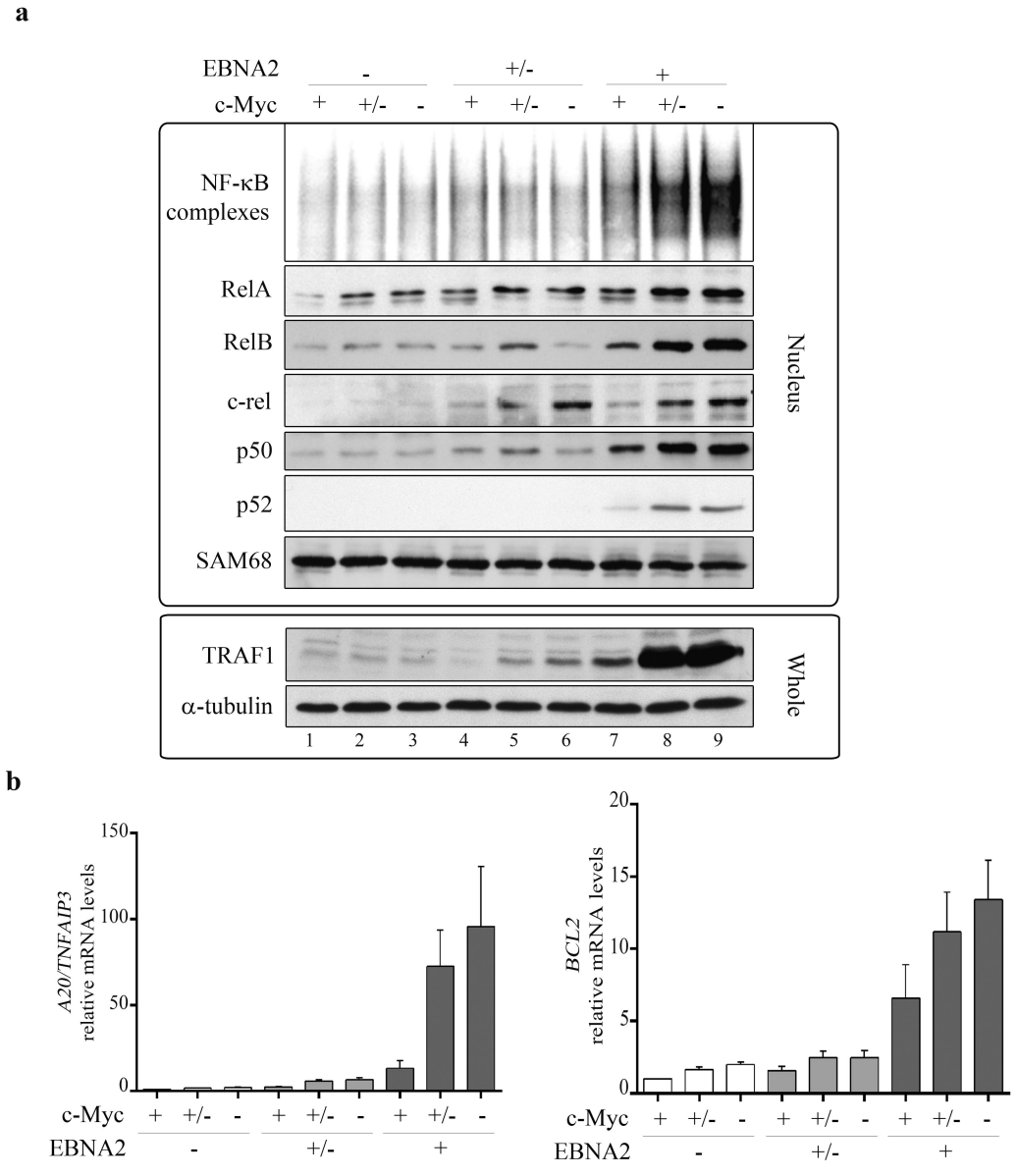

a
EBNA2
-
+/-
+
c-Myc
+ +/- - + +/- - + +/- -
NF-κB complexes
RelA
RelB
c-rel
p50
p52
SAM68
Nucleus
TRAF1
α-tubulin
Whole
1 2 3 4 5 6 7 8 9
b
A20/TNFAIP3 relative mRNA levels
150
100
50
0
c-Myc + +/- - + +/- - + +/- -
EBNA2 - +/- +
BCL2 relative mRNA levels
20
15
10
5
0
c-Myc + +/- - + +/- - + +/- -
EBNA2 - +/- +

Figure S2. Characterization of NF-κB in P493.6 cells after induction of EBV-latency III and/or c-Myc. P493.6 cells were treated or not with increasing doses of estradiol (0=EBNA2-, 0.1=EBNA2+/- and 1 μM=EBNA2+) and/or tetracycline (0= c-Myc+, 3=c-Myc+/- and 100 μg/mL=c-Myc-) for 48 hours. (**a**) Upper panel: nuclear NF-κB DNA binding assessed by EMSA. Middle panel: Western-blot detection of nuclear RelA, RelB, c-rel, p50, and p52. Detection of SAM68 was used as a loading control. Lower panel: Western-blot detection of TRAF1 and α-tubulin (loading control) from whole cell extracts. One representative of 3 independent experiments is shown. (**b**) For each cell condition, mRNA expression of *A20/TNFAIP3*, and *BCL2* genes was quantified using TaqMan® Gene Expression Assays. Relative mRNA fold changes were calculated as the ratio of control cells to P493.6 cells without estradiol nor tetracycline (*i.e.* cells under c-Myc alone). Each experiment was performed three times.

Figure S3

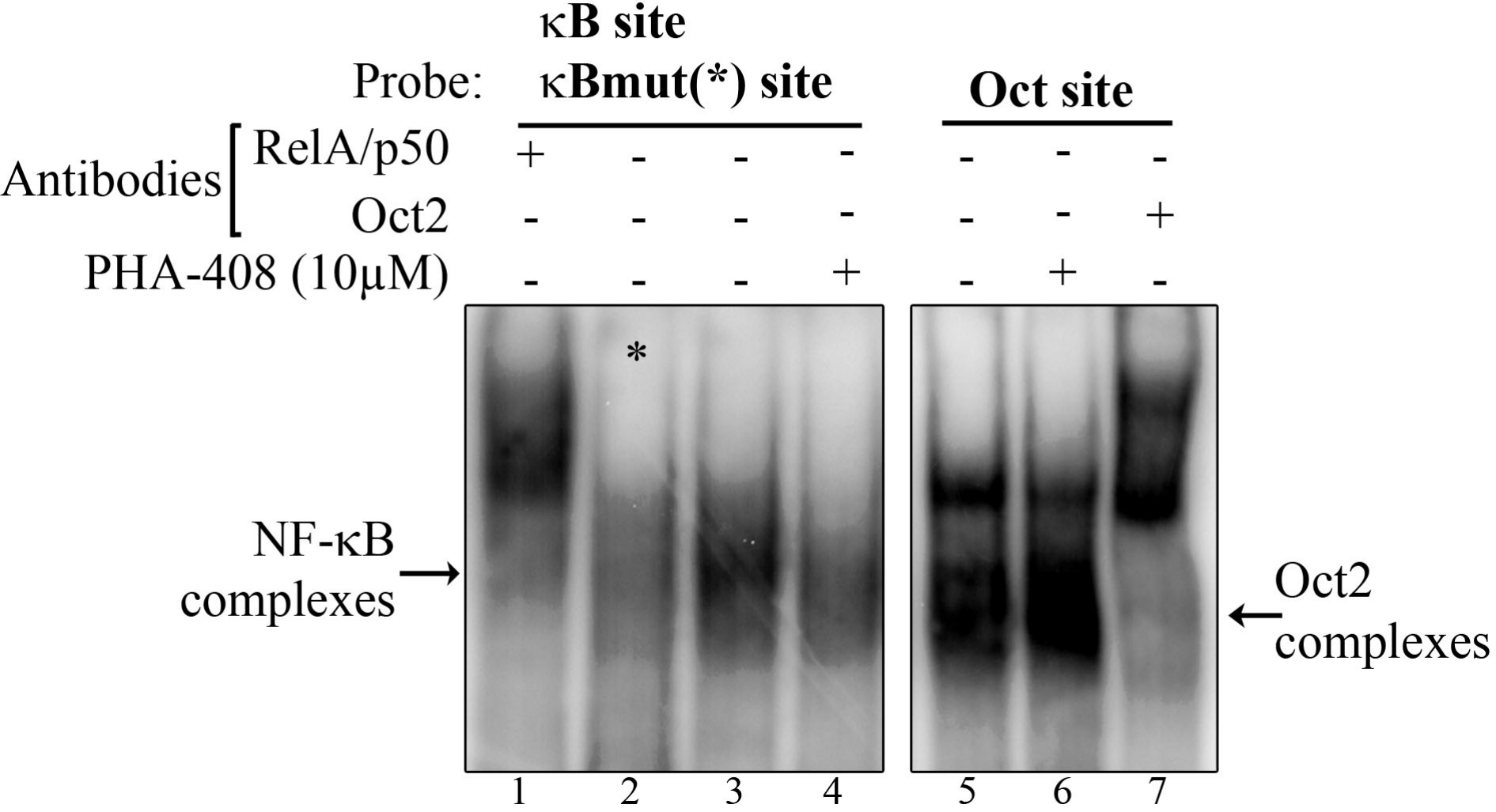

κB site
Probe:
κBmut(*) site
Oct site
Antibodies
RelA/p50
Oct2
PHA-408 (10μM)
+ - - - - - -
- - - - - - +
- - - + - + -
*
NF-κB complexes
Oct2 complexes
1 2 3 4 5 6 7

Figure S3. Effect of PHA-408 on NF-κB and Oct2 DNA binding activities in P493.6 cells after induction of EBV-latency III. P493.6 cells induced for EBV-latency III program (*i.e.* with 1 μM estradiol plus 100 ng/mL tetracycline) were treated (+) or not (-) with 10 μM PHA-408 for 48 hours. DNA binding by EMSA was assessed using a probe containing κB (PRE), κBmut(*), or Oct binding sites. Super-shift experiments were done using antibodies against RelA, p50, and Oct2.

Figure S4

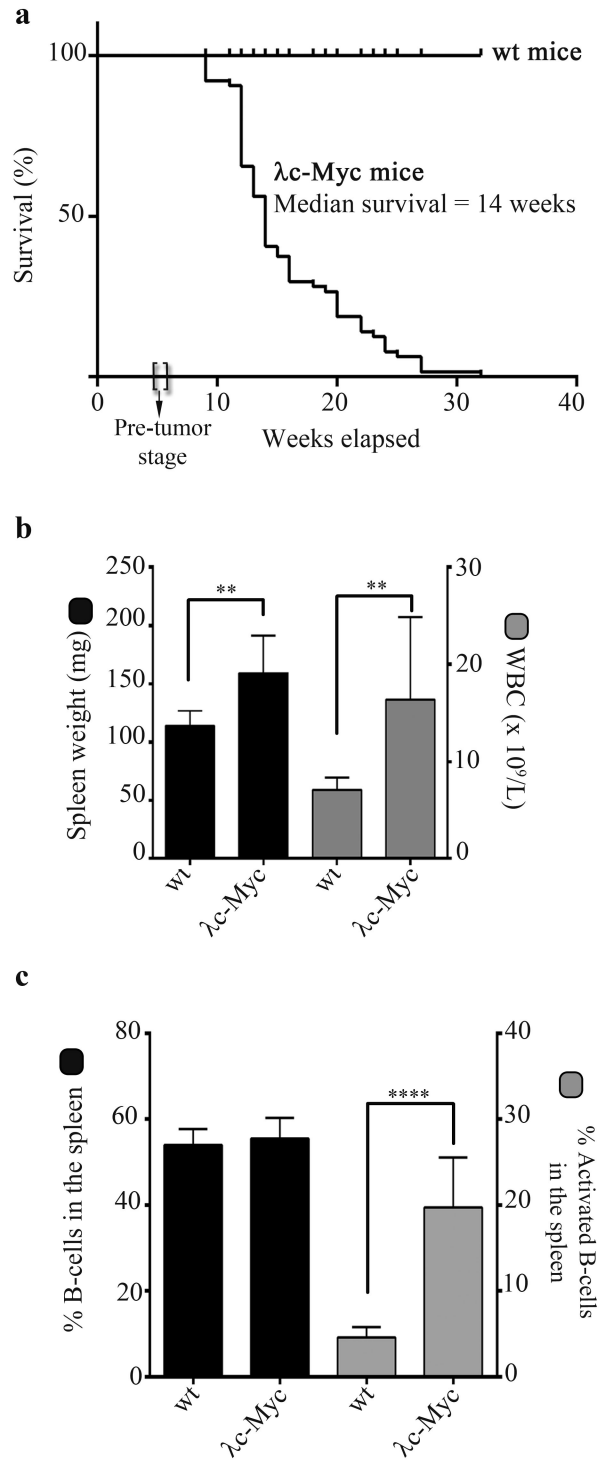

a
100
50
Survival (%)
wt mice
λc-Myc mice
Median survival = 14 weeks
0
10
20
30
40
Pre-tumor stage
Weeks elapsed
b
250
200
150
100
50
0
Spleen weight (mg)
**
**
30
20
10
0
WBC (x 10⁹/L)
wt
λc-Myc
wt
λc-Myc
c
80
60
40
20
0
% B-cells in the spleen
****
40
30
20
10
0
% Activated B-cells in the spleen
wt
λc-Myc
wt
λc-Myc

Figure S4. Characterization of pre-tumor λc-Myc mice. (**a**) Kaplan-Meier survival plot of wild-type (wt) and λc-Myc mice. Each group of mice contained 66 subjects. Median survival for λc-Myc mice is indicated. The pre-tumor stage at 5-6 weeks old is indicated. (**b**, and **c**) Transgenic λc-Myc mice at 5-6 weeks old. (**b**) Spleen weight (mg; black histograms) and white blood cell counts (x 10^9/L; grey histograms). (**c**) Percentages of B-cells ($B220^+$ cells among TO-PRO®3^- cells; black histograms) and activated B-cells ($CD86^+$ cells among $B220^+$ B-cells) in the spleen (grey histograms). Data are shown as mean ± standard deviation (n=8 mice in each group of experiment). Statistical significance was determined by Student's t test (**, $P<0.01$; **** $P<0.0001$).

Figure S5

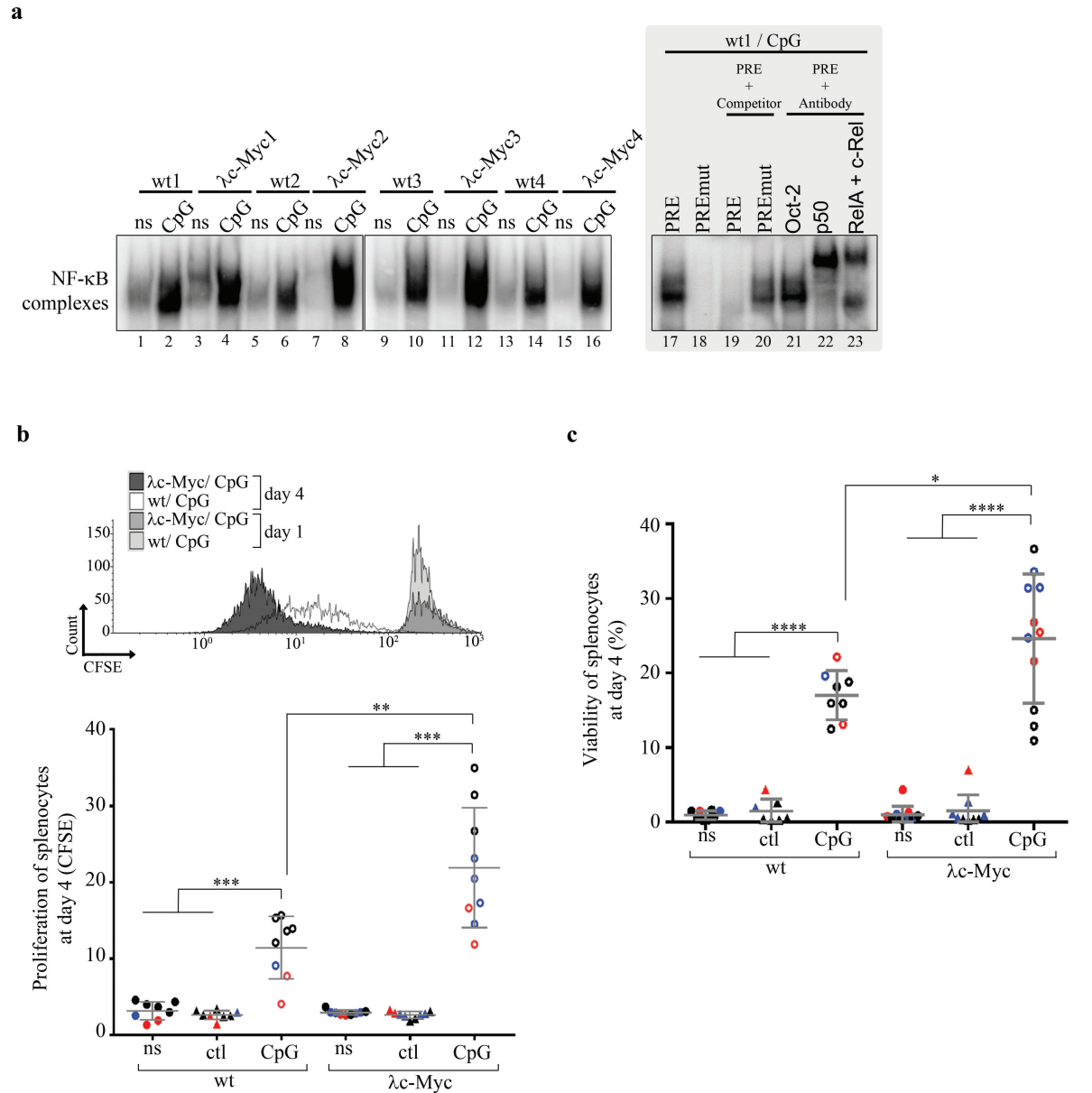
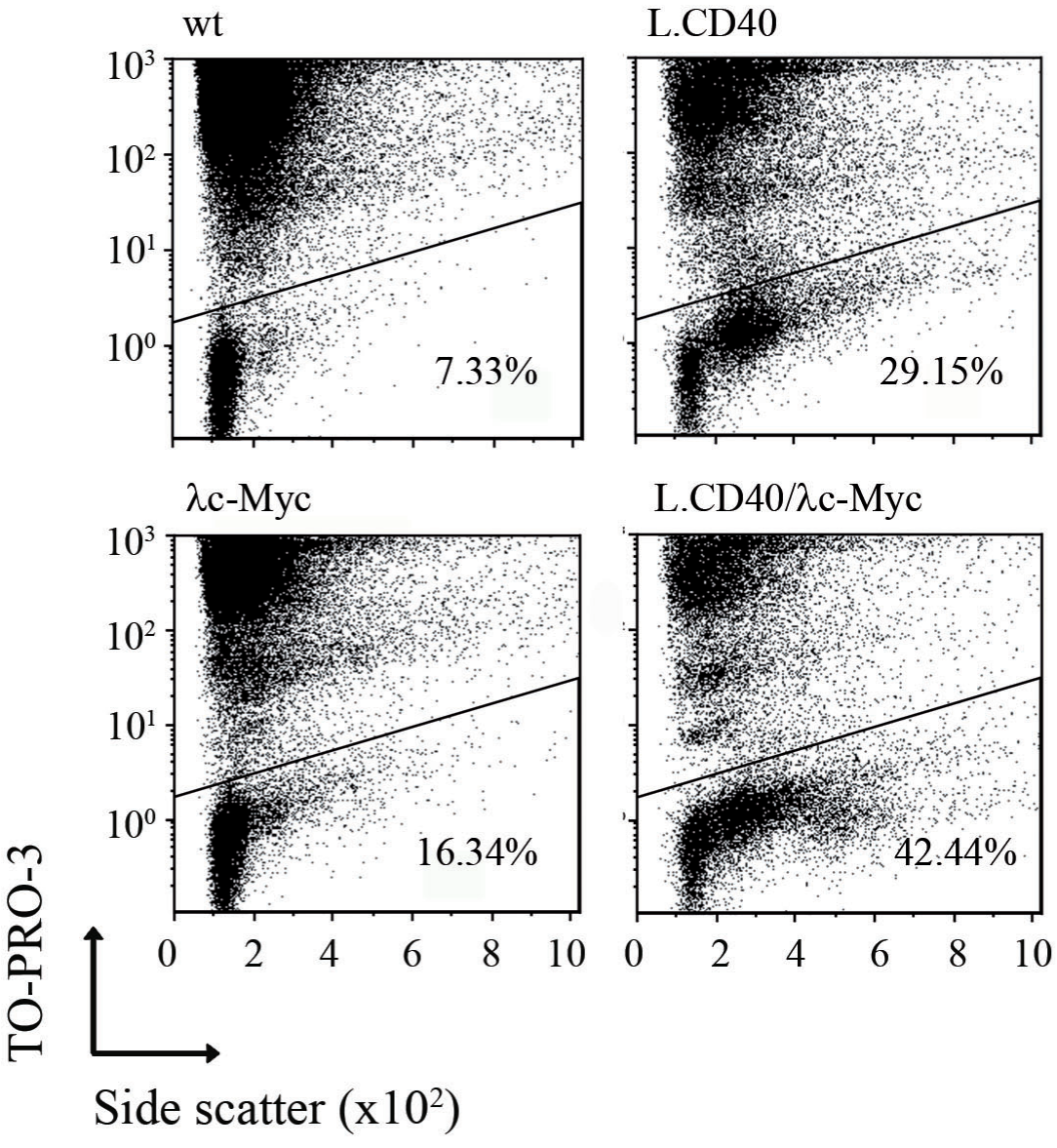

a
wt1
λc-Myc1
wt2
λc-Myc2
wt3
λc-Myc3
wt4
λc-Myc4
ns CpG ns CpG ns CpG ns CpG ns CpG ns CpG ns CpG ns CpG
NF-κB complexes
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
wt1 / CpG
PRE + Competitor
PRE + Antibody
PRE
PREmut
PRE
PREmut
Oct-2
p50
RelA + c-Rel
17 18 19 20 21 22 23
b
λc-Myc/ CpG
wt/ CpG
day 4
λc-Myc/ CpG
wt/ CpG
day 1
Count
CFSE
Proliferation of splenocytes at day 4 (CFSE)
ns ctl CpG ns ctl CpG
wt
λc-Myc
c
Viability of splenocytes at day 4 (%)
ns ctl CpG ns ctl CpG
wt
λc-Myc

Figure S5. TLR9 stimulation of splenocytes from pre-tumor λc-Myc mice. Total splenocytes from 5-6 week old wild type (wt1-4), and λc-Myc (1-4) mice were stimulated (CpG) or not (ns) with CpG containing oligodeoxynucleotides for 4 days. (**a**) NF-κB DNA binding activity by EMSA from wt1-4 and pre-tumor λc-Myc1-4 nuclear extracts. Lanes 1, 5, 9, and 13: unstimulated wt cells. Lanes 2, 6, 10, and 14: wt cells treated with CpG. Lanes 3, 7, 11, and 15: unstimulated λc-Myc cells. Lanes 4, 8, 12, and 16: λc-Myc cells treated with CpG. Lanes 17 to 23: NF-κB DNA binding activity specificity from CpG stimulated wt1 nuclear extracts. Lanes 17 and 18, extracts were incubated with radiolabelled wild type PRE (containing two κB sites) or PREmut (containing mutated κB sites) probes respectively. Lanes 19 and 20, competition experiments with PRE probe with a 50-fold molar excess of cold PRE, or PREmut probe, respectively. Lanes 21 to 23, super-shift experiments using anti-Oct2 (irrelevant control), anti-p50, or anti-RelA plus anti-cRel antibodies, respectively. (**b**) Upper panel: CFSE labelling of TLR9 stimulated splenocytes at day 1 and 4. Lower panel: Proliferation rate at day 4 assessed by CFSE labelling of splenocytes. Mice from the same experiment are indicated by the same colour code. Data are shown as mean ± standard deviation (n=8-9 mice in each group of experiment). Statistical significance was determined by Student's t test (**, $P<0.01$; *** $P<0.001$). (**c**) Splenocytes viability at day 4: percentage of TO-PRO®3 negative cells is shown. Mice from the same experiment are indicated by the same colour code. Data are shown as mean ± standard deviation (n=8-11 mice in each group of experiment). Statistical significance was determined by Student's t test (*, $P<0.05$; **** $P<0.0001$).

Figure S6


wt
L.CD40
λc-Myc
L.CD40/λc-Myc
7.33%
29.15%
16.34%
42.44%
TO-PRO-3
Side scatter (x10²)

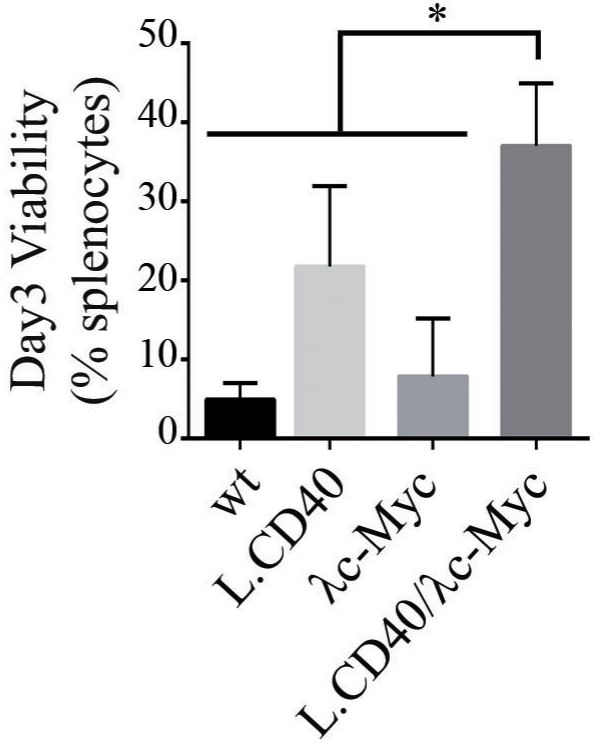

Day3 Viability
(% splenocytes)
*
wt
L.CD40
λc-Myc
L.CD40/λc-Myc

Figure S6. *Ex vivo* viability of splenocytes from L.CD40/λc-Myc mice. Splenocytes were isolated from wt, L.CD40, λc-Myc and L.CD40/λc-Myc mice. Cells were seeded at 2.5 10^6/mL in complete medium and spontaneous viability was determined at day 3 using TO-PRO-3 staining. Left panel, representative biparametric TO-PRO-3 versus Side scatter cytograms. Viability percentages were indicated on each cytogram. Right panel, histograms corresponding to viability percentages among splenocytes from wt, L.CD40, λc-Myc and L.CD40/λc-Myc mice. Statistical significance was determined by Student's t test (*, $P<0.05$).

Table S1. Five clusters of probes corresponding to deregulated genes in P493.6 cells after induction of EBV-latency III and/or c-Myc. We selected 1 648 probes according to their fold change variation of at least two in one of the four cell conditions of P493.6 cells (c-Myc on or off and/or EBV-latency III on or off) when compared to the median of each probe. Probes were grouped into five clusters. For each cluster and cell condition, centered means of gene expressions values with standard error (±SE) were calculated. Gene expressions values refer to log2-values were reported in the table for each sample condition. Each cell condition was repeated once.

Table S2. Thirteen clusters of probes corresponding to deregulated genes in tumor lymph nodes from λc-Myc/L.CD40 mice. Supervised analysis led to selection of 2 437 significant probes which were partitioned in 13 clusters (Cluster 1 to C13). Gene expression values (log2-values) centered to the wild type condition, were reported in the table for each clusters. For each sample conditions, fold change value compared to wild type samples were calculated from the mean of centered gene expression values.

Supplementary Methods

Cells, Treatment and Transfection

EREB2.5 cells are a non-classical LCL with an estradiol inducible EBV-latency III proliferation program due to an estrogen receptor fused to the EBNA2 viral protein [1]. The P493.6 cell line is an EREB2.5 derivative transfected with a Tet-off inducible c-Myc expressing vector [2]. Cell culture conditions have been described elsewhere [3]. For transient transfections, P493.6 cells expressing both EBV-latency III and c-Myc programs were transfected with pcDNA3 vector using the Amaxa O.017 program (Amaxa Biosystems, Basel, Switzerland).

Human B-cell lines were TLR9 stimulated with 0.5 μM ODN-ctl or ODN-CpG (Oligodeoxynucleotides human and mouse CpG-B DNA, prototype ODN 2006 ; Oligodeoxyncleotides non-CpG DNA; Hycult Biotech, Uden, The Netherlands) for 48 hours.

NF-κB inhibition was performed with 5 μM and 10 μM PHA-408 [8-(5-chloro-2-(4-methylpiperazin-1-yl)isonicotinamido)-1-(4-fluorophenyl)-4,5-dihydro-1H-benzo[g]indazole-3-carboxamide] (Axon Medchem, Groningen, The Netherlands) for 48 hours.

Plasmid Constructs

Complementary DNA for IκBα$_{S32,36A}$ (super-repressor form of IκBα) was cloned into the pcDNA3.1 vector (Life Technologies, Carlsbad, USA) [4].

Western-Blotting

Western-blots were done as previously described [5]. Antibodies used were anti-EBNA1 (mAb 1H4 [6]; at 1/100, anti-EBNA2 (mAb PE2; Dako, Les Ulis, France) at 1/200, anti-EBNA3C (mAb E3cA10) [7] at 1/500, anti-LMP2A (mAb MD-14-1221; Clinisciences, Nanterre, France) at 1/500, anti-LMP1 (Hybridoma S12) at 1/100, anti-c-Myc (mAb 9E10; Santa Cruz

Biotechnology) at 1/200, anti-p65 (pAb 3034; Cell Signaling Technology) at 1/1000, anti-p100/p52 (pAb 4882; Cell Signaling Technology) at 1/700, anti-p100/p50(NLS) (pAb sc-114; Santa Cruz Biotechnology) at 1/2000, anti-RelB(C-19) (pAb sc-226; Santa Cruz Biotechnology) at 1/2000, anti-c-Rel(N) (pAb sc-70; Santa Cruz Biotechnology) at 1/2000, anti-TRAF1(G-20) (pAb sc-983; Santa Cruz Biotechnology) at 1/1000, anti-IκBα(FL) (pAb sc-847; Santa Cruz Biotechnology) at 1/200, anti-α-tubulin (mAb B-5-1-2; Santa Cruz Biotechnology) at 1/200. Quantification of western-blots was done using ImageJ software.

Real Time Quantitative Reverse-Transcription PCR

Complementary DNAs were reverse transcribed from total RNA samples using the High Capacity cDNA Archive Kit (Life Technologies). PCR products were amplified from 100 ng of each cDNA using the TaqMan® Universal PCR Master Mix and TaqMan® Gene Gene Expression Assays were *c-Myc*: Hs00153408_m1; *TP53* gene: Hs00153340_m1; *e2f1*: Hs00153451_m1; *Bcl2*: Hs00153350_m1; and *CD19*: Hs00174333_m1) on an ABI PRISM 7000 automat (Life Technologies). LMP1 mRNAs were quantified using the following probe and primers: primer F-LMP1 5'CATGGACAACGACACAGTGATGA3', primer R-LMP1 5'TTGGAGTTAGAGTCAGATTCATGGC3', and probe 5'(6-Fam) CACCACCACGATGACTCCCTCCCGC3'(Tamra) (Sigma-Proligo, TX, USA). The *CD19* gene was used as a reference gene. The calculated relative gene expression level was equal to 2^{-DDCT}, where DDCT is the delta delta cycle threshold, as previously described [5]. Gene expression fold changes were calculated as the ratio of the test condition to its control.

Gene Expression Profiling

Amplification of RNAs and hybridization onto microarrays were performed on an Affymetrix Gene Atlas system® with: Affymetrix® Human Genome U219 Array Strip, and Affymetrix®

Mouse Gene 2.1 ST Array Strip as previously described [5]. Supervised analysis was performed with the BRB-array tools package, version 4.4, with the "class comparison between group of array" tool (http://brb.nci.nih.gov/BRB-ArrayTools/ from the NIH institute) [8]. Hierarchical and K-mean clustering were performed as described [9] with Cluster 3 software (http://bonsai.hgc.jp/~mdehoon/software/cluster/software.htm) [10]. Heat-maps were done with Java Treeview (http://jtreeview.sourceforge.net/). Biological functions of genes were studied using "Gene Set Enrichment Analysis" (GSEA) (www.broadinstitute.org/gsea/index.jsp) and "DAVID Bioinformatics Database" (www.david.ncifcrf.gov/home.jsp).

Preparation of Nuclear Extracts and Electromobility Shift Assays (EMSA)

Methods for nuclear extracts and EMSA are described elsewhere [5]. The nucleotide sequence of the probe with the Oct binding site was chosen according to Klapproth *et al* [11]: 5'- ACC TGG GTAA TTT GCA TTT CTA AAA T -3'.

Mouse Models

λc-Myc mice on a C57BL/6 background [12] were kindly provided by Pr. Georg Bornkamm (Helmholtz Center, Munich, Germany). The conditional transgenic mouse model overexpressing LMP1.CD40 fusion transgene in B lymphocytes on a BALB/c background has been described elsewhere [13]. All procedures were conducted under an approved protocol according to European guidelines for animal experimentation (French national authorization number: 87-022 and French ethics committee registration number "CREEAL": 09-07-2012).

Cell Labeling and Proliferation

After lysis of red-blood cells using RBC Lysis buffer (eBioscience, San Diego, USA), total mouse splenocytes were immunostained and proliferation was assessed using CFSE

(Carboxyfluorescein Diacetate Succinimidyl Ester; Life Technologies) staining and EdU (5-ethynyl-2'-deoxyuridine; Life Technologies) incorporation.

To assess *in vitro* proliferation of cultured B-cells, BrdU (5-bromo-2'-deoxyuridine; Sigma-Aldrich, Saint-Louis, USA) incorporation was performed as previously described [9]. Briefly, cells were incubated in 18 µg/mL BrdU over 2 and 4 hours for P493.6 cell line and primary B-cells respectively.

Ki-67 labelling was also used to follow proliferation using imaging flow cytometry with the ImageStream 100 apparatus (Amnis*; Merck, Darmstadt, Germany).

In details, After lysis of red-blood cells using RBC Lysis buffer (eBioscience, San Diego, USA), total splenocytes were immunostained with anti-B220-FITC (mAb RA3-6B2; Biolegend, San Diego, USA) at 1/200, anti-B220-PerCP (mAb RA3-6B2; Biolegend) at 1/50, anti-CD80-APC (16-10A1; Biolegend) at 1/1200, and anti-CD86-FITC (mAb GL-1; Biolegend) at 1/300. Cell viability was estimated with 2 µM TO-PRO®-3 (Life Technologies). To analyze B-cell sub-populations, splenocytes were immunostained with anti-B220-BV510 (mAb RA3-6B2; BD Biosciences, Le Pont de Claix, France) at 1/100, anti-CD19-APC-H7 (1D3; BD Biosciences) at 1/100, anti-CD21-BV605 (7G6; BD Biosciences) at 1/100, and anti-CD23- APC-Cy7 (EBVCS-5; Biolegend) at 1/100.

To assess *ex vivo* proliferation of splenocytes, cells were labeled with CellTrace CFSE (Life Technologies), seeded on 96-well plates at 2.5x 10^6 /mL, and stimulated with: 2 µg/mL ODN-Control (ODN 1826 control; InvivoGen, Toulouse, France), and 2 µg/mL ODN-CpG (ODN 1826, InvivoGen). At days 1 and 4, 2 µM TO-PRO®-3 was added, and CFSE mean fluorescent intensity (MFI) was determined on living cells. Proliferation rate at day 4 was

calculated: ratio of MFI at day 1 to MFI at day 4. Proliferation was also evaluated by 50 µM EdU (5-ethynyl-2'-deoxyuridine) incorporation for 14 hours. Then, cells were stained with anti-B220-FITC (mAb RA3-6B2; Biologend) and EdU was detected using reagents in the Click-iT® EdU Alexa Fluor® 647 Imaging Kit (Life Technologies).

Cell counts were performed with the CELL-DYN Emerald cell counter (Abbott, Rungis, France). Flow cytometry data were acquired on a FACS Calibur cytometer (BD Biosciences) or LSR-FORTESSA (BD Biosciences) and analyzed with the Kaluza® Flow Analysis software (Beckman Coulter, Roissy, France).

Follicular (FO, CD23-high, CD21-low), marginal zone (MZ, CD23-low, CD21-high), and CD21/CD23 negatives B-cells were sorted after immunostaining with anti-B220-APC (mAb RA3-6B2; Biolegend) at 1/100, anti-CD21-FITC (7G6; BD Biosciences) at 1/100, and anti-CD23-PerCP (EBVCS-5; Biolegend) at 1/100, using the FACS-ARIA.III cell sorter (BD Biosciences). Then, B-cells were seeded at 5x 10^5 /mL, and stimulated with: 2 µg/mL ODN-Control and ODN-CpG. At day 4, cells were counted using Trypan-blue exclusion and cytospins were performed after BrdU (5-bromo-2'-deoxyuridine; Sigma-Aldrich) incorporation at 18 µg/mL for 4 hours. BrdU positive cells were immunostained using BrdU *In-Situ* Detection Kit (BD Biosciences).

In vivo TLR9 stimulation was done through 10 intraperitoneal injections with 50 µg ODN-CpG or control (ODN-ctl) (ODN 1826, InvivoGen). To follow *in vivo* B-cell proliferation, mice were intraperitonealy injected with 2 mg BrdU about 24 hours before their sacrifice. BrdU positives B-cells were immunostained using anti-BrdU-FITC (3D4; BD Pharmingen) and 1/100 anti-B220-BV421 (mAb RA3-6B2; Biolegend) antibodies.

Imaging Flow Cytometry for Ki67 Labeling (Amnis)

Cells were fixed and permeabilized using the PerFix-nc Kit (Beckman Coulter). Labeling was performed with anti-Human Ki-67 at 1/100 (mAb MIB-1, Dako), and the antibody Alexa Fluor® 488 Goat Anti-Mouse IgG (H+L) at 1/200 (Life Technologies) was used as secondary antibody. Isotypic control was Negative Control Mouse IgG1 (X0931, Dako). Then, DRAQ5® (Cell Signaling) was added at 1/1000, and cells were analyzed with ImageStream 100 apparatus (Amnis*; Merck).

IDEAS® image analysis software (Amnis*) was used to quantify nuclear events by automatically correlating the images of Ki-67 labeling and the nucleus (DRAQ5®), using the Similarity score (pixel-by-pixel correlation of the Ki-67 and DRAQ5® image pair within the nuclear morphology mask). Among cells with nuclear Ki-67 staining, percentage of cells with high Ki-67 levels was defined.

Apoptosis was assessed from Ki-67 and DRAQ5® non-colocalized cells. Nuclear morphometric parameters were then used to define the percentage of apoptotic cells using analysis software.

Immunohistochemistry

Formalin-fixed paraffin-embedded murine samples were sliced at 5 mm. Sections were stained with hematoxylin and eosin (H&E) and antibodies against Ki-67 (LifeSpan BioSciences). Revelation was performed with Zytochem-Plus HRP kit (Zymoted Systems).

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