Oncogene (2013), 1–12

www.nature.com/onc

ORIGINAL ARTICLE

Inducible expression of hyperactive Syk in B cells activates Blimp-1-dependent terminal differentiation

E Hug[1,2,3], E Hobeika[2,3], M Reth[1,2,3] and H Jumaa[1,2,3]

The non-receptor protein tyrosine kinase Syk (spleen tyrosine kinase) is an important mediator of signal transduction in B cells. By acting downstream of the B-cell antigen receptor, Syk promotes signaling pathways involved in proliferation, differentiation and survival of B cells. To study the oncogenic potential of Syk, we generated a mouse model for the inducible expression of the leukemia-derived TEL-Syk fusion protein exhibiting constitutive kinase activity. To achieve B-cell-specific expression of TEL-Syk in adult mice, we used a tamoxifen-inducible Cre mouse line. This study shows that inducible expression of TEL-Syk in B cells leads to transient proliferation and subsequent plasma cell differentiation. However, it does not lead to B-cell transformation. Instead, Syk activation induces the tumor suppressor B-lymphocyte-induced maturation protein-1 (Blimp-1), which interferes with the expression of the antiapoptotic protein Bcl-2. Combined induction of TEL-Syk with transgenic expression of Bcl-2 results in a severe phenotype and plasma cell expansion. Our results suggest that deregulated Syk activity by itself is not sufficient for the transformation of B cells, as downstream effectors, such as Blimp-1, limit the survival and expansion of the activated B cell.

Oncogene advance online publication, 19 August 2013; doi:10.1038/onc.2013.326

Keywords: B-cell transformation; Bcl-2; Blimp-1; hyperactive Syk; terminal differentiation

INTRODUCTION

Upon binding of a specific antigen to the B-cell antigen receptor (BCR), protein tyrosine kinases of the Src family and the spleen tyrosine kinase (Syk) become active and phosphorylate numerous downstream signaling substrates. Finally, different transcription factors are induced that control gene expression and determine the fate of the activated B cell by favoring proliferation, differentiation or cell death programs.[1–3]

Plasma cell development is the terminal stage of B-cell differentiation and is characterized by secretion of immunoglobulins (Igs). Plasma cell differentiation is dependent on the expression of B-lymphocyte-induced maturation protein-1 (Blimp-1), also called PR domain zinc-finger protein 1 (PRDM1).[4–6] Blimp-1 is a transcriptional repressor that regulates important genes in B cells, such as *Bcl6* and *Pax5*.[7] Its role as a tumor suppressor has been described in several studies showing that disruption of Blimp-1 function in activated B-cell-like diffuse large B-cell lymphoma (ABC-DLBCL) promotes lymphomagenesis by inhibiting terminal differentiation.[8,9]

In earlier studies, it has been shown that deregulated Syk activity leads to growth factor-independent proliferation and transformation of cell lines.[10,11] Elevated Syk activity has also been observed in different B-cell lymphomas.[12–16] Besides B cells, Syk is also expressed in many other cell types, such as immature T cells, mast cells, macrophages, platelets or epithelial cells, where it is involved in signal transduction and might also have a role in cancerous cell growth. Therefore, pharmacological Syk inhibition is a conceivable therapy in several pathologies and different Syk inhibitors are being tested in clinical trials.[17]

To study the role of deregulated Syk activity in different tissues, we generated a mouse model for the conditional expression of a constitutively active form of Syk, namely the TEL-Syk (TS) fusion protein. This chimeric protein was discovered in a patient with myelodysplastic syndrome.[18] It is derived from a chromosomal translocation, where the dimerization domain of the transcription factor TEL is fused to the C-terminal part of Syk containing the kinase domain, but lacking functional SH2 domains. Elevated activity of this fusion protein may be caused by dimerization and autophosphorylation events, which may result in permanent kinase activity and adaptor function of Syk.[10,11,19] In our previous study, we could show that the effect of hyperactive TS is comparable to the overexpression of wild-type Syk in pre-B-cell lines. In fact, TS was able to trigger B-cell receptor-associated signaling pathways that led to the transformation of B-cell lines *in vitro*.[11] The aim of the present study is to evaluate the oncogenic potential of Syk under physiological conditions. Using an inducible mouse system that allows B-cell-specific expression of our transgene in adult animals after the administration of tamoxifen, we show that expression of TS in peripheral B cells leads to their activation, initial proliferation and subsequent differentiation into short-lived plasma cells. We propose that the differentiation program induces apoptosis and that this is an essential mechanism to prevent the accumulation of B cells with elevated Syk activity. This suggests that Syk may act as an oncogene only if additional mutations are present that interfere with terminal differentiation.

RESULTS

Generation of mice for conditional expression of the TS fusion protein

Retroviral expression of a TS fusion construct, exhibiting deregulated kinase activity, leads to growth factor-independent proliferation of

[1]BIOSS Centre for Biological Signalling Studies, Albert-Ludwigs-Universität, Freiburg, Germany; [2]Department of Molecular Immunology, Faculty of Biology, Albert-Ludwigs-Universität, Freiburg, Germany and [3]Max-Planck-Institute of Immunobiology and Epigenetics, Freiburg, Germany. Correspondence: Dr H Jumaa, Max-Planck-Institute of Immunbiologie and Epigenetics, Stuebeweg 51, Freiburg 79108, Germany.
E-mail: jumaa@ie-freiburg.mpg.de
Received 18 February 2013; revised 4 July 2013; accepted 5 July 2013

pre-B-cell lines *in vitro*.[11,18] To further investigate the role of deregulated Syk activity in the development of different malignancies *in vivo*, we generated a mouse model for the expression of TS by inserting the sequence encoding for the TS fusion into the first intron of the ubiquitously expressed Rosa26 locus[20] (Supplementary Figure S1). The targeting construct contained a floxed stop cassette upstream of the TS coding sequence (Supplementary Figure S1). Cell-type-specific excision of the stop cassette and subsequent expression of the TS protein under the control of the locus-intrinsic promotor can be achieved by crossing Rosa26-TS (TS) mice with Cre mouse strains (Supplementary Figure S1).

Tamoxifen-inducible Cre-recombination system for B-cell-specific expression of transgenes

For B-cell-specific inducible expression of TS in adult animals, we used mb1-CreER^{T2} mice in which a tamoxifen-inducible Cre-recombinase (CreER^{T2})[21] was targeted into the *mb1* gene locus encoding Ig-α. For simplicity, mb1-CreER^{T2} mice are referred to as iCre (inducible Cre) mice in the text and figures. To determine the efficiency and specificity of the CreER^{T2} insertion, Rosa26-YFP reporter mice[22] were crossed with the iCre mice and the resulting YFP/iCre mice induced by a single tamoxifen treatment. The percentage of YFP-expressing B cells in different organs was monitored by FACS analysis. Induction of YFP expression was not detected in YFP/iCre mice without tamoxifen application (Figure 1a), but at day 7 after tamoxifen treatment, about 10–12% of CD19$^+$ B cells in blood, lymph nodes and spleen were identified as YFP$^+$ in FACS analysis (Figure 1b). Recombination occurred both in CD23$^+$CD21^{int} follicular (FO) cells and in CD23$^-$CD21^{high} marginal zone (MZ) B cells in the spleen (Supplementary Figure S2). In the peritoneal cavity and in the bone marrow, Cre-induced YFP expression was detected in about 20% and 3% of B cells, respectively (Figure 1b).

The advantage of this induction strategy is that we can turn on the expression of the transgene in only a small fraction of all B cells after single oral tamoxifen treatment. This allows us to monitor the fate of cells expressing an oncogene over time.

Activation and altered marker expression on TS-expressing B cells

We next crossed TS mice with iCre mice to achieve tamoxifen-inducible expression of TS in the resulting mice (hereafter referred to as TS/iCre mice). TS/iCre mice showed no abnormalities and did not develop any disease over 1 year of observation without the application of tamoxifen. After tamoxifen treatment of mice, spleens and lymph nodes of TS-expressing TS/iCre mice were enlarged at day 3 and more at day 7 postinduction as compared with controls, such as Cre-negative, iCre or untreated TS/iCre mice (Figure 2a). As all control animals showed a similar phenotype, only one representative negative control per experiment is shown in the following. The enlargement of the peripheral lymphoid organs of TS/iCre mice reflected the increase in total cell numbers, caused by an initial expansion of CD19$^+$ B cells (Supplementary Figure S3). The majority of peripheral B cells expressed the activation marker CD69 and downregulated the BCR (Figure 2b). The CD23$^-$CD21$^+$ phenotype of activated cells resembled MZ B cells (Figure 2b), which could be either due to the result of predominant proliferation of MZ cells or due to the down-regulation of the surface marker CD23 on FO B cells. To test this, we isolated splenic cells from mice 2 days after tamoxifen induction and sorted the cells into FO (CD23$^+$CD21^{int}) and MZ (CD23$^-$CD21^{high}) B-cell fractions. FACS analysis of these cells after 3 and 6 days of *in vitro* culture supports the latter scenario, as downregulation of CD23 was detected in the isolated FO B-cell population of TS/iCre compared with FO B cells from control mice (Figure 2c).

Apoptosis in B cells after initial enrichment of TS-expressing cells

B-cell-specific expression of TS was examined by western blot analysis using splenic B cells isolated from TS/iCre mice at different time points (Figure 3a). To identify directly TS-expressing cells, we performed intracellular FACS staining using an antibody, which

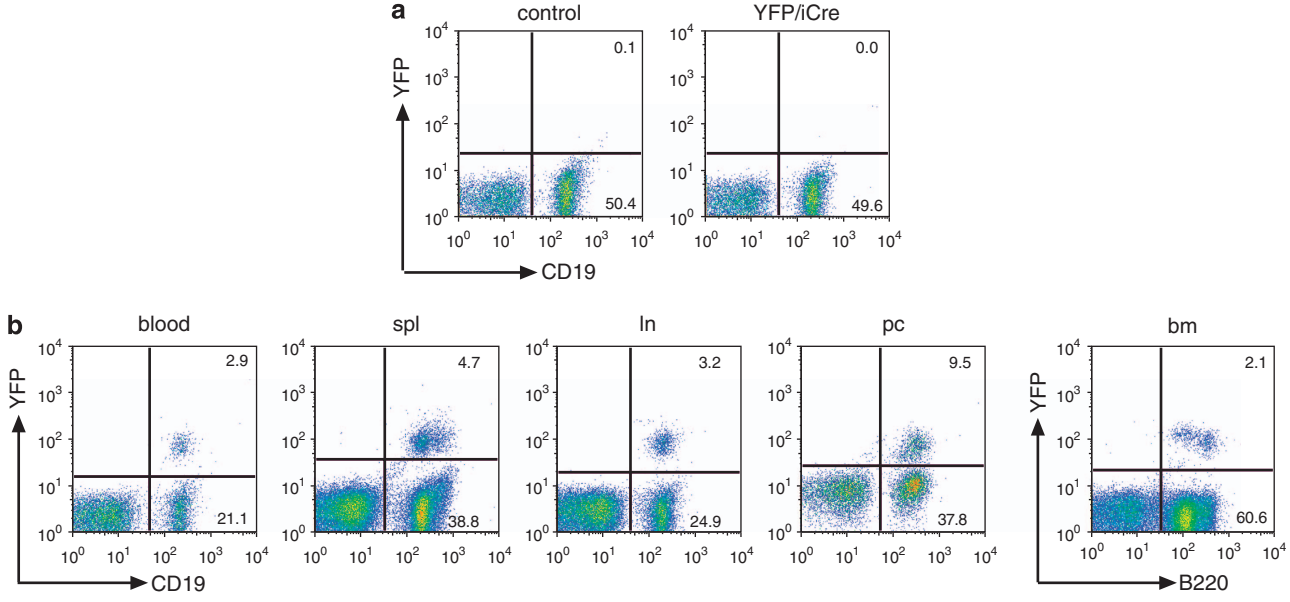

Figure 1. Testing tamoxifen-inducible transgene expression in B cells using Rosa26-YFP reporter mice. (**a**) FACS analysis of isolated peripheral blood cells from Rosa26-YFP/iCre (YFP/iCre) or iCre-negative control mice at day 0 before tamoxifen treatment. Cells were analyzed for the expression of YFP and the B-cell marker CD19. (**b**) FACS analysis of cells isolated from different lymphoid organs of YFP/iCre mice at day 7 after tamoxifen treatment. Cells were analyzed for the expression of YFP and the B-cell marker CD19 or B220. Spl, spleen; ln, lymph nodes; pc, peritoneal cavity; and bm, bone marrow. Numbers indicate the percentage of cells in each quadrant. Data are representative of at least two to three independent experiments.

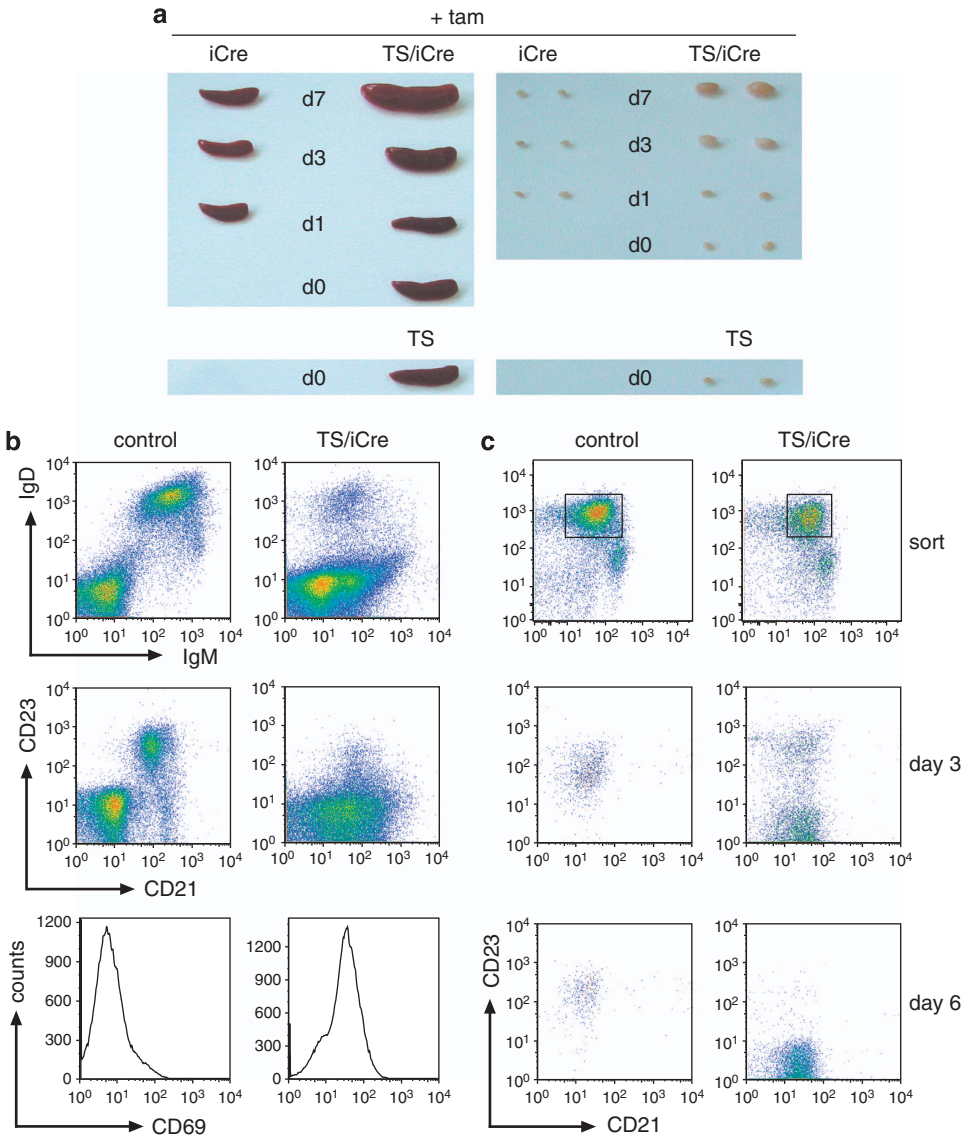

Figure 2. Activation and altered marker expression on TS-expressing B cell. (**a**) Representative picture of spleens and lymph nodes from TS/iCre and control mice at different time points after tamoxifen (+tam) treatment. Shown is one representative time-course experiment of at least three independent experiments. d, day. (**b**) CD69 up- and BCR downregulation on B cells and accumulation of $CD23^{-}CD21^{int/high}$ cells in the spleen of TS/iCre as compared with iCre-negative control mice at day 7 after induction. Histogram of CD69 staining shows all $CD19^{+}$ B cells in the spleen. (**c**) FACS analysis of sorted FO B cells ($CD23^{+}CD21^{int}$) isolated from iCre-negative control and TS/iCre mice at day 2 after tamoxifen induction (sort) and 3 and 6 days of *in vitro* culture. Data are representative of at least three independent experiments.

recognizes the human Syk protein and does not crossreact with murine Syk. Although no TS expression was detectable in Cre-negative or untreated control mice, we observed that TS-expressing B cells downregulated the expression of the B-cell-specific lineage markers CD19 (Figure 3b). By comparing the percentages of TS^{+} to total B cells, we monitored the kinetics of TS-expressing cells in the spleen. A growing accumulation of $TS^{+}CD19^{low}$ B cells up to 70% was observed between days 3 and 7 (Figure 3c). This increase suggests an initial proliferation phase of TS-expressing cells. To confirm this, we performed proliferation assays with isolated splenic B cells that were pretreated with 4-hydroxy-tamoxifen (4-OHT) for 1 day to induce TS expression *in vitro*. The cells were then labeled with the cell proliferation dye eFluor670, and 4 days later, the dilution of eFluor670 fluorescence in the B cells was analyzed by FACS. We detected proliferation of isolated B cells in TS/iCre cultures that were treated with 4-OHT, but not in untreated controls or in cultures of B cells isolated from Cre-negative control mice (Figure 3d). Of note, B-cell stimulation with lipopolyssacharide (LPS) induced a comparable proliferation rate similar to the induction of TS expression, whereas administration of the Syk-specific inhibitor R406 abolished proliferation in 4-OHT-treated TS/iCre-derived B-cell cultures (Figure 3d).

Interestingly, a decline in total B-cell numbers was detected in the spleen at day 7 (Supplementary Figure S3) and our results suggested that this was because of the induction of cell death, which affected mainly the $CD19^{+}$ B-cell compartment. Indeed, we found an up to 20-fold increase in $AnnexinV^{+}$ $7AAD^{-}$ apoptotic cells and an accumulation of $AnnexinV^{+}$ $7AAD^{+}$ dead cells in the spleen and lymph nodes of TS/iCre mice at day 7 postinduction as compared with control animals (Figure 3e). Surprisingly, we did not detect an accumulation of TS^{+} B cells

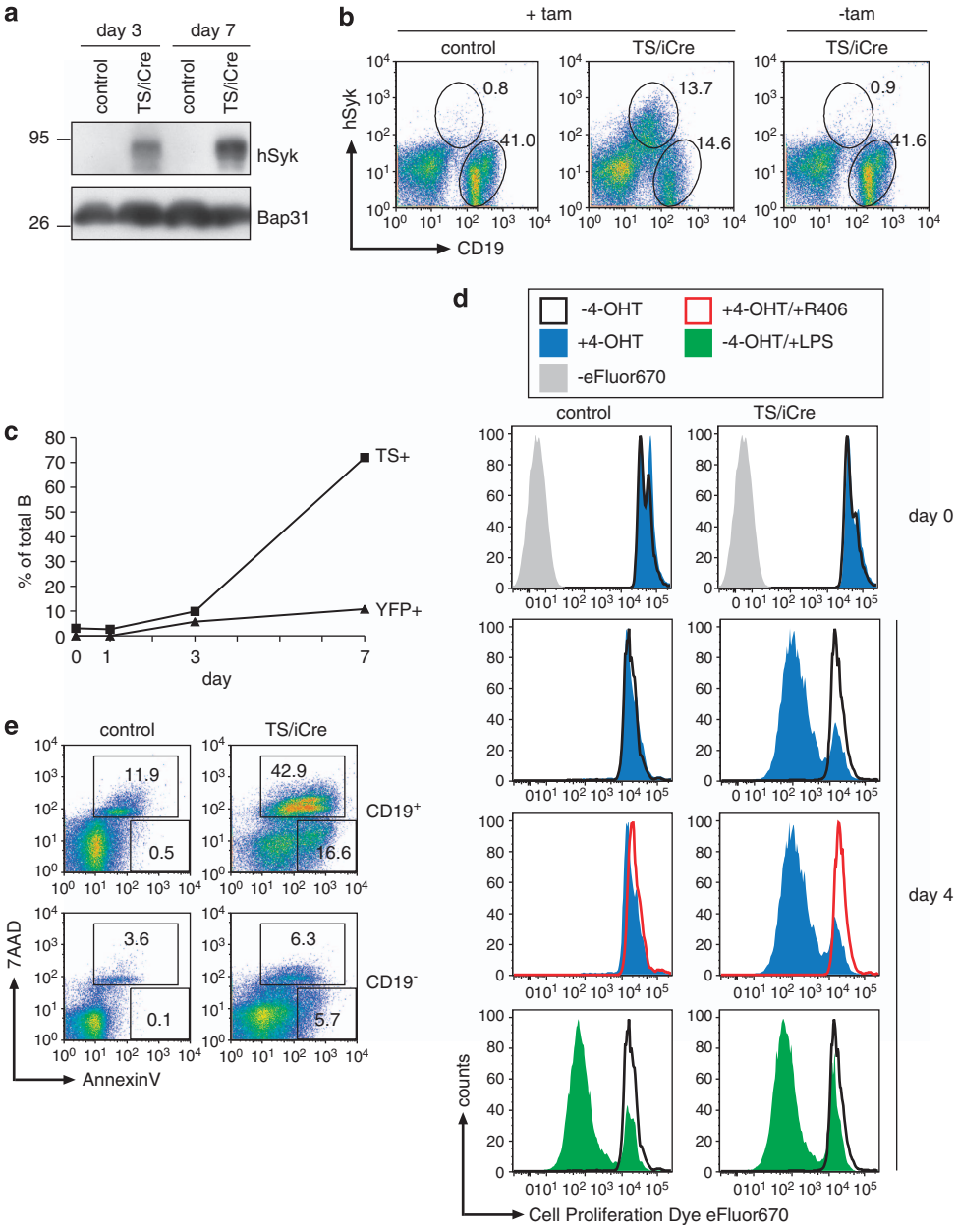

Figure 3. Inducible TS expression leads to initial expansion of TS-expressing B cells in peripheral lymphoid organs and to B-cell-specific induction of apoptosis. (**a** and **b**) TS induction in B cells was assessed (**a**) by western blot analysis with protein extracts of purified splenic B cells isolated at the indicated time points from iCre-negative control and TS/iCre mice, in which Bap31 served as a loading control, or (**b**) directly by intracellular FACS staining with an α-human Syk (hSyk) antibody in cells from TS/iCre mice after tamoxifen treatment (+tam). Shown is a representative experiment with lymph node cells isolated at day 7. Treated (+tam) iCre and untreated (−tam) TS/iCre mice are shown as negative controls. The percentage of $CD19^{low}TS^{+}$ and $CD19^{+}TS^{-}$ B cells in the indicated gates are shown. (**c**) Monitoring the enrichment of $CD19^{low}TS^{+}$ in the spleen as compared with total $CD19^{+/low}$ B cells at different time points. The kinetics of $CD19^{+}YFP^{+}$ as compared with total $CD19^{+}$ B cells in YFP/iCre reporter mice is shown as a Rosa26 locus recombination control. Shown is one representative time-course experiment of at least three independent experiments. (**d**) Isolated splenic B cells from iCre-negative control and TS/iCre mice were cultured with (+ 4-OHT, filled blue) or without 4-hydroxy-tamoxifen (− 4-OHT, black line) for 1 day and then labeled with the cell proliferation dye eFluor670. Histogram plots at day 0 show fluorescence of labeled cells (filled blue and black line) relative to unlabeled cells (filled gray) directly after labeling. FACS analysis at day 4 reveals the decay in cell fluorescence over time because of cell division. Shown are viable ($7AAD^{-}$) cells from different cultures (see legend). R406, Syk inhibitor. (**e**) FACS staining of spleen cells isolated from mice at day 7 after tamoxifen treatment and gated on $CD19^{+}$ B cells and $CD19^{-}$ non-B cells. Numbers indicate the percentages of apoptotic ($AnnexinV^{+}7AAD^{-}$) and dead cells ($7AAD^{+}$) in the parental population. Shown is a representative experiment of at least four independent experiments.

in the bone marrow or peritoneal cavity of TS/iCre mice postinduction (Figure 4).

At day 14 and beyond, no TS-expressing cells were detected in the spleen of TS/iCre mice and the spleen size was reduced to normal level (Figures 5a and b). An interesting consequence of the B-cell-specific apoptosis is that TS/iCre mice were depleted of B cells in the different lymphoid organs 7–14 days after tamoxifen treatment (Figures 4 and 5b, c and Supplementary Figure S4). The fact that TS induction is restricted to a small fraction of B cells in the different organs (Figure 1b and Supplementary Figure S4), but

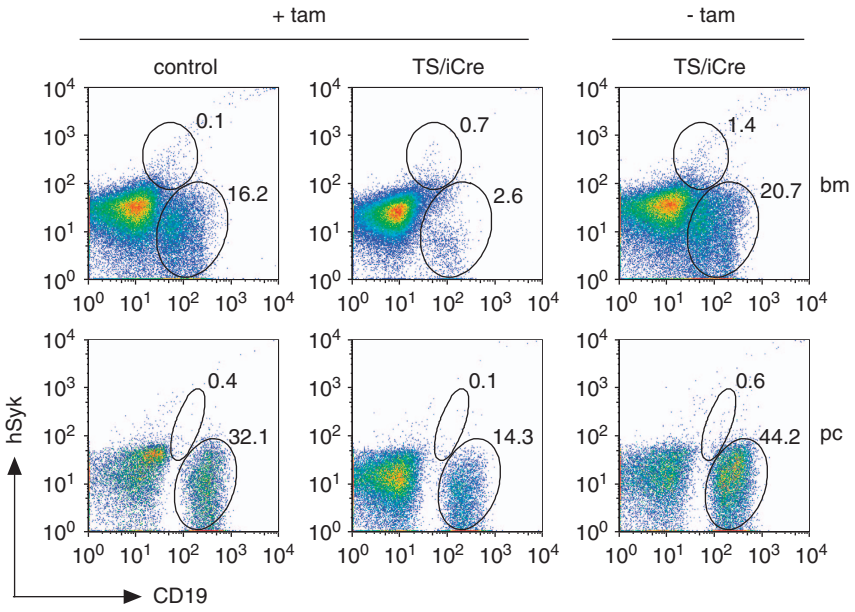

Figure 4. Absence of TS-expressing B cells in the bone marrow (bm) and peritoneal cavity (pc). FACS analysis of cells isolated from bm and pc at day 7 after tamoxifen (tam) treatment. TS expression was tested with intracellular FACS analysis using an α-human Syk (α-hSyk) antibody. Treated (+tam) iCre and untreated (−tam) TS/iCre mice are shown as negative controls. Numbers indicate the percentage of cells in the respective gates. Shown is a representative experiment of at least three independent experiments.

the majority of B cells react to TS expression, suggests that TS^- B cells in TS/iCre mice are also affected and are driven into apoptosis. This implies a trans effect of activated B cells on unrecombined TS^- B cells either by competition for growth factors or by the secretion of soluble mediators.

Indeed, we found different inflammatory cytokines, such as tumor necrosis factor (TNF), interferon γ (IFNγ), interleukin-6 (IL-6), interleukin-10 (IL-10) and the monocyte chemoattractant protein-1 (MCP-1), to be elevated in the serum of TS/iCre mice peaking at days 3–5 (Supplementary Figure S5A). Further *in vitro* experiments revealed that FO B cells are the primary source of some of these cytokines (tumor necrosis factor, interferon γ and interleukin-10) (Supplementary Figure S5B).

We suggest that FO B cells expressing constitutively active Syk undergo an initial proliferation phase and initiate the secretion of inflammatory cytokines, followed by increased induction of cell death in TS^- and TS^+ peripheral B cells. This scenario causes a subsequent immunodeficient state in the mice with drastically reduced B-cell numbers at days 7–14 postinduction and a recovery phase back to normal levels of about 2–3 months. Most importantly, TS/iCre mice did not develop any obvious disease in the following 8 months after initial tamoxifen treatment.

TS expression induces Blimp-1 and terminal differentiation of B cells into short-lived plasma cells

Accumulation of activated cells with an increased cell size was detected in the spleen and lymph nodes of TS/iCre mice (Figure 6a). Moreover, these cells showed a typical $B220^{low}$ $CD138^+$ plasma cell phenotype, whereas the respective population was not present in control animals (Figure 6a). To confirm plasma cell development, we measured Ig levels in the serum and observed more than 20-fold increased IgM titers in the serum of TS/iCre mice compared with control animals at day 7 after induction (Figure 6b). There was no obvious change in IgG antibody titers in the serum of TS-expressing mice and no class-switched TS^+ B cells were detected in the different lymphoid organs (Figure 6b). Plasma cell differentiation was independent of other cell types, as *in vitro* induction experiments with isolated splenic B cells using 4-OHT also resulted in $B220^{low}CD138^+IgM^+$ plasma cell differentiation after 6 days of culturing (Supplementary Figure S6).

To test whether the plasma cell differentiation factor Blimp-1 is involved in the TS-induced differentiation process, we performed intracellular FACS staining with spleen cells isolated at day 7. We detected Blimp-1 expression in the $B220^{low}$ B-cell population, as well as elevated IRF4 level, which has been linked to the initiation of plasma cell differentiation (Figure 6c). Furthermore, we observed induction of X-box-binding protein-1 (Xbp-1) and its spliced form, which is another reported downstream effector of Blimp-1 during plasma cell differentiation (Figure 6d).[23] As a consequence of Blimp-1-mediated repression, splenic B cells isolated at day 5 after tamoxifen treatment showed reduced Pax5 level and no upregulation of Bcl-6 (Figure 6e).

Our data suggest that TS expression in B cells leads to Blimp-1-dependent polyclonal terminal differentiation into IgM-secreting plasma cells with subsequent induction of apoptosis.

Blimp-1 induction depends on Syk activity

The data suggested that TS activity leads to Blimp-1 expression. To show that endogenous Syk activity induces Blimp-1 expression in normal B cells, we tested freshly isolated splenic B cells from mice that have the *Syk* gene deleted in peripheral B cells, for the ability to induce Blimp-1 expression after LPS stimulation. These mice were generated by crossing mice carrying two floxed *Syk* alleles ($Syk^{fl/fl}$)[24] with transgenic mice that express the Cre recombinase under the control of the CD21 promotor (CD21-Cre).[25] The resulting $Syk^{fl/fl}$/CD21-Cre mice possess an Syk-deficient mature B-cell population that shows a FO B-cell-like phenotype. By intracellular FACS analysis for mouse Syk, we were able to discriminate Syk-positive cells (Syk^+) from Syk-negative cells (Syk^-) (Figure 7a) and found that Syk^- mature B cells responded to *in vitro* LPS stimulation with the upregulation of the activation marker CD86 (Supplementary Figure S7), but were not able to induce robust Blimp-1 expression. In contrast, Syk^+ B cells or B cells from Cre-negative control mice induced Blimp-1 expression after LPS treatment (Figure 7a). Likewise, the inhibition of Syk activity in isolated wild-type splenic B cells after LPS treatment resulted in impaired Blimp-1 induction (Figure 7b).

From these results, we concluded that Syk is required for Blimp-1-dependent plasma cell differentiation.

Bcl-2 is downregulated in Blimp-1-expressing B cells

As the induction of Blimp-1 after TS expression led to the generation of obviously short-lived plasma cells, we hypothesized that Blimp-1 interferes with B-cell survival. Indeed, in T cells it has been reported that Blimp-1 is involved in the repression of the antiapoptotic protein Bcl-2.[26] To investigate whether Blimp-1 expression results in Bcl-2 downregulation in normal B cells, we analyzed Bcl-2 expression in splenic B cells after induction of Blimp-1 expression. Our experiments revealed that Blimp-1 induced by culturing the cells in the presence of LPS led to Bcl-2 downregulation (Figure 8a). Furthermore, we determined Bcl-2 expression levels in cells from TS/iCre mice and found reduced Bcl-2 protein levels as compared with cells from control mice. In contrast to this, expression levels of Bcl-XL, which is also a member of the prosurvival Bcl-2 family, were increased (Figure 8b). Further investigation revealed that other Bcl-2 family members, such as the antiapoptotic factor myeloid cell leukemia-1 (Mcl-1) and the proapoptotic factor Bim, were not altered after the induction of TS expression (Supplementary Figure S8).

Thus, Blimp-1-mediated downregulation of Bcl-2 during the differentiation phase may be a general mechanism to restrict the lifespan of terminally differentiated B cells and to maintain homeostasis.

Enforced expression of Bcl-2 prolongs the survival of TS-expressing plasma cells and causes a severe phenotype

The downregulation of Bcl-2 suggests that Bcl-2 might be critical for the survival of TS-expressing B cells. Therefore, we asked whether enforced Bcl-2 expression interferes with the induction of apoptosis and prolongs the survival of TS-expressing B cells. In fact, crossing TS/iCre with Bcl-2 transgenic (Bcl-2 tg) mice[27] and subsequent induction of TS caused a severe phenotype in the TS/iCre/Bcl-2 tg mice after 5–7 days, with drastically enlarged spleens and lymph nodes (Figure 8c). Indeed, we detected less apoptotic B cells in the spleens and lymph nodes of TS/iCre/Bcl-2 tg mice compared with TS/iCre mice at day 5 (Figure 8d) and an enrichment of $B220^{low}CD138^{+}$ plasma cells in the peripheral lymphoid organs and concomitant infiltration in the bone marrow (Supplementary Figure S9). Owing to the severity of the phenotype, all TS/iCre/Bcl-2 tg mice were killed 7 days after tamoxifen treatment, but *in vitro* culturing of spleen, lymph node and bone marrow cells revealed a prolonged survival (up to 4 weeks) of these $TS^{+}B220^{low}CD138^{+}$ plasma cells as compared with plasma cells isolated from TS/iCre mice (Figure 8e).

Taken together, our results show that deregulated Syk activation by itself does not induce uncontrolled proliferation, but leads to terminal differentiation of B cells into short-lived plasma cells in a process mediated by Blimp-1 expression. As apoptosis of TS^{+} plasma cells could be repressed by enforced expression of Bcl-2, downregulation of the prosurvival protein Bcl-2 seems to be the important event responsible for preventing uncontrolled B-cell expansion after receptor-induced Syk activation.

DISCUSSION

In this study, we present a mouse model for the inducible expression of the oncogenic TS fusion protein. Our findings

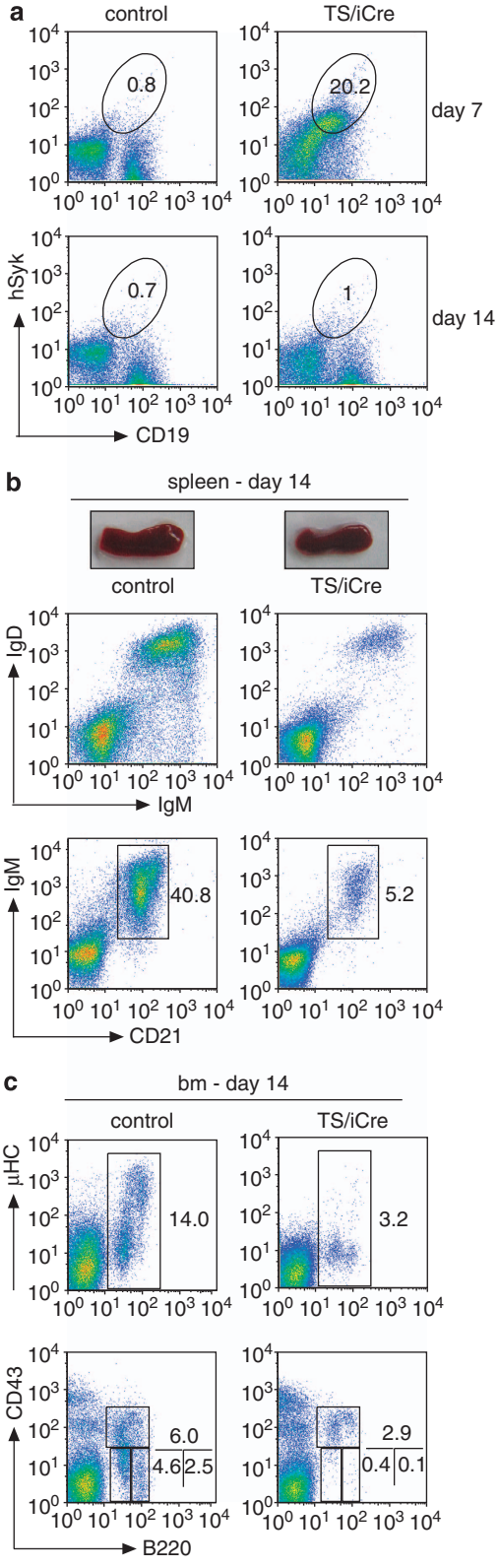

Figure 5. Loss of TS-expressing cells and general B-cell deficiency in mice 14 days after tamoxifen treatment. (**a**) Intracellular FACS staining with an α-hSyk antibody in cells isolated from iCre-negative control and TS/iCre mice. Shown is a representative experiment with spleen cells isolated at days 7 and 14 after tamoxifen treatment. (**b**) Picture of spleens at day 14 after tamoxifen treatment (top) and FACS staining of isolated cells with the indicated antibodies (bottom). (**c**) FACS staining of bone marrow (bm) cells isolated at day 14. Numbers indicate the percentage of cells in the respective gates; $B220^{low}CD43^{+}IgM^{-}$ (pro-B), $B220^{low}CD43^{-}IgM^{-}$ (pre-B), $B220^{low}CD43^{-}IgM^{+}$ (immature) and $B220^{high}IgM^{+}$ (recirculating). Data are representative of at least three independent experiments.

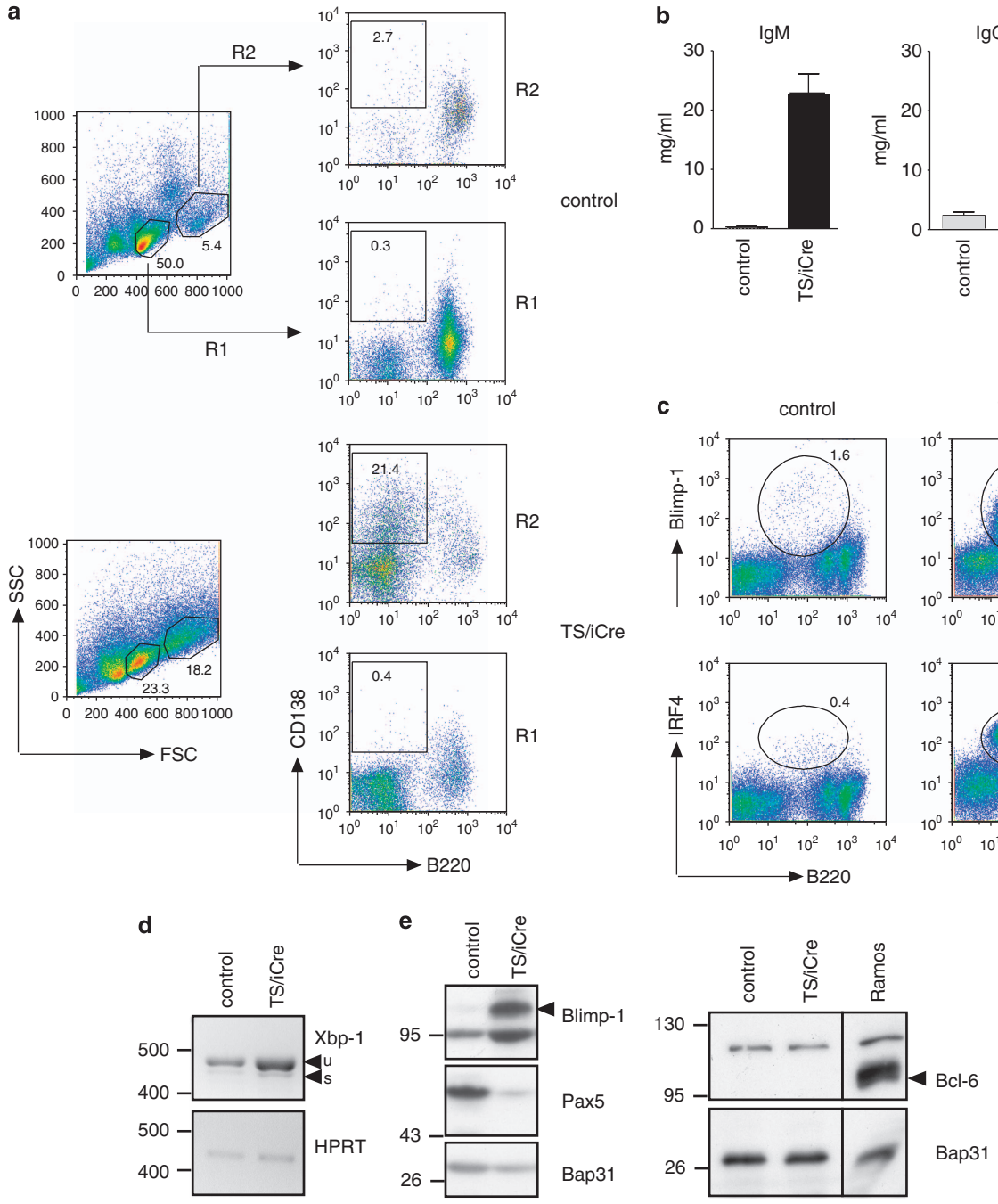

Figure 6. TS expression causes B-cell differentiation into short-lived plasma cells by inducing Blimp-1 expression. (**a**) Spleen cells isolated from iCre-negative control and TS/iCre mice were examined for cell size and granularity, gated for normal lymphocytes ($FSC^{int}SSC^{int}$) and enlarged activated cells ($FSC^{high}SSC^{high}$). Gated is the $B220^{low}CD138^{+}$ population (plasma cells). (**b**) Serum IgM (left) and IgG (right) titers of iCre-negative control and TS/iCre mice at day 7 as determined by enzyme-linked immunosorbent assay (ELISA). Data are means derived from four mice of each genotype ± s.d. (**c**) Spleen cells isolated at day 7 and stained with the indicated antibodies for the expression of IRF4 and Blimp-1. (**d**) Semiquantitative RT–PCR was used to measure Xbp-1 mRNA in isolated splenic B cells at day 5 after tamoxifen treatment. s, spliced Xbp-1; u, unspliced Xbp-1. HPRT served as a loading control. (**e**) Western blot analysis of purified splenic B cells isolated from iCre-negative control and TS/iCre mice at day 5. Immunoblots were incubated with the indicated antibodies. Ramos total cell lysate served as positive control for Bcl-6 expression. Bap31 served as a loading control. Data are representative of at least four independent experiments.

demonstrate that the expression of a constitutively active Syk kinase is not sufficient to induce transformation of B cells, as strong signaling via Syk in B cells favors the expression of the tumor suppressor Blimp-1 and terminal differentiation with subsequent induction of apoptotic cell death. This prevents aberrant proliferation of activated B cells.

The advantages of the B-cell-specific inducible mb1-$CreER^{T2}$ system are that B-cell development is not perturbed before induction of Cre activity by tamoxifen treatment and that the expression of the transgene is induced in only a fraction of cells in a 'wild-type' environment and this allows us to monitor the respective cells over time. After induction of TS expression

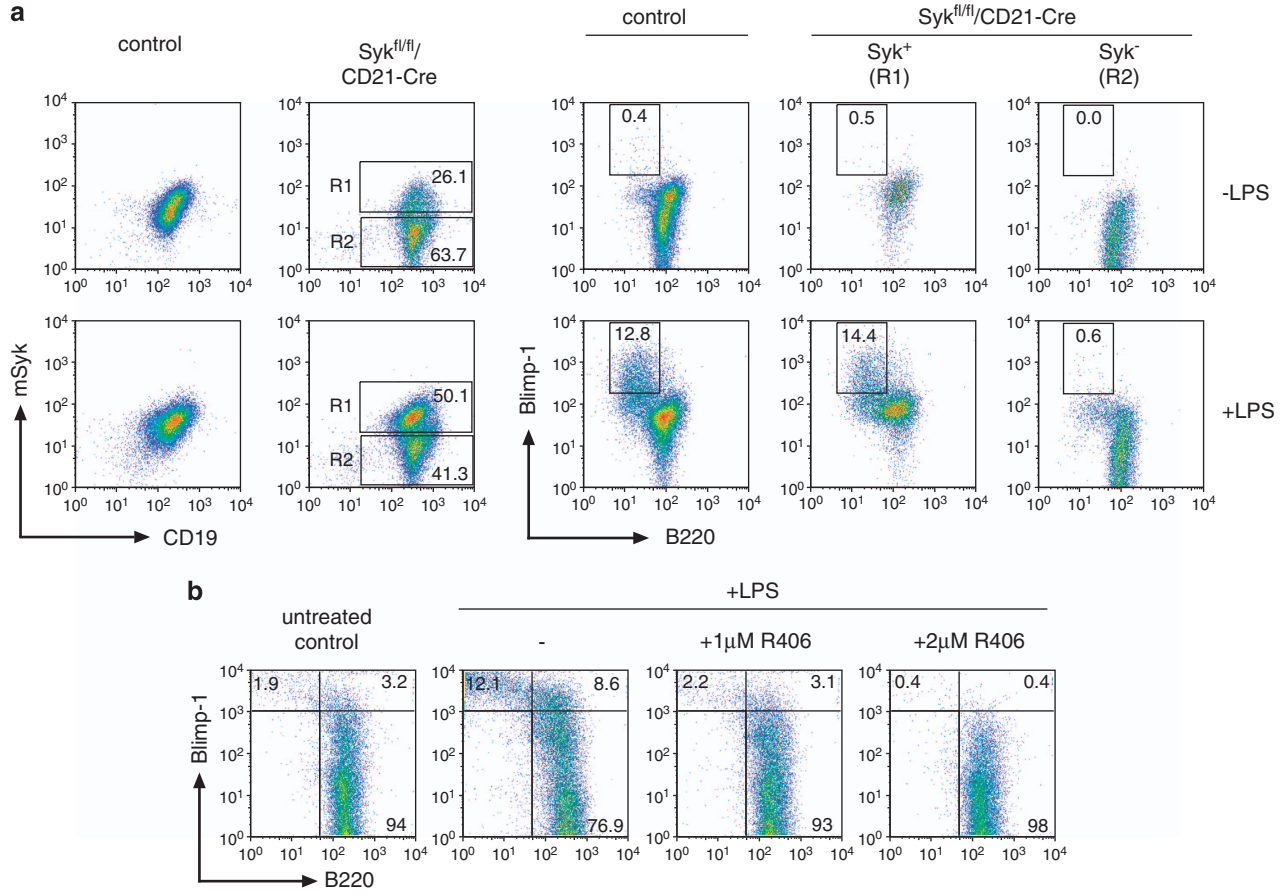

Figure 7. Blimp-1 induction is dependent on Syk activity. (**a**) Intracellular FACS analysis with isolated splenic B cells from Cre-negative control and Syk$^{fl/fl}$/CD21-Cre mice after *in vitro* stimulation with LPS for 3 days. Syk expression was determined by intracellular staining (left). Blimp-1 staining of B cells from control mice is shown, and for Syk$^{fl/fl}$/CD21-Cre mice, the Syk^{+} (R1) and Syk^{-} (R2) B-cell fraction are shown separately (right). Numbers indicate the percentages of plasma cells (B220^{low}Blimp-1^{+}) in the parental cell population. (**b**) Intracellular FACS analysis for Blimp-1 expression with purified wild-type splenic B cells after *in vitro* stimulation with LPS and concomitant treatment with the Syk-inhibitor R406 for 6 days. Data are representative of three and two independent experiments.

in a fraction of B cells, increased size and cell numbers were detected in spleens and lymph nodes between days 3 and 7 of treatment. As expected, the fraction of TS^{+} B cells initially showed a remarkable increase as compared with control cells and this was because of an initial phase of proliferation. However, induction of apoptosis and a rapid decrease in total B cells were observed in all lymphoid organs. Interestingly, TS^{-} B cells that have not undergone Cre-mediated recombination were also affected by this cell death, suggesting that they either have a disadvantage in competing with other cells for important growth factors, like the homeostatic B-cell-activating factor (BAFF), or that they are actively suppressed and driven into cell death by, yet unknown, mediators derived from TS^{+} B cells. The fact that TS^{+} B cells secrete different proinflammatory cytokines might indeed support this scenario. On the other hand, it cannot be excluded that TS expression in some B-cell fractions is below detection limit while being sufficient for induction of cell death or senescence. Although our results using the YFP reporter reveal no differences in promoter activity for transgene expression at different B-cell developmental stages, they cannot rule out differences in TS protein stability. For precursor B cells, it is also possible that the absence of TS^{+} cells in the bone marrow results from enhanced differentiation and exit from the bone marrow after the induction of TS expression. Taken together, the loss of B-cell precursors in the bone marrow together with the depletion of mature B cells in the periphery might contribute to the B-cell-deficient state in TS-expressing mice beyond day 7.

As *CD19* and *CD23* transcription is dependent on the B-cell lineage factor Pax5, reduced expression of these markers may be a direct consequence of low Pax5 level in TS^{+} B cells.[28,29] Thus, our results suggest that CD23^{+}CD21^{int} FO B cells that were the main responders to TS expression had downregulated CD23 expression in the course of Blimp-1 activation.

Terminal differentiation requires the expression of Blimp-1 and the suppression of *Pax5* as well as *Bcl-6*. Although Blimp-1 is the master regulator of plasma cell differentiation, Bcl-6 is required for germinal center B-cell differentiation. We observed plasma cell differentiation exclusively into IgM-secreting short-lived plasma cells, but no class-switched B cells. This is consistent with the elevated IgM serum levels, whereas total IgG levels in the serum were unaffected in TS/iCre mice. As Blimp-1 and Bcl-6 are able to repress one another,[5] the initial and robust induction of Blimp-1 expression in TS-expressing cells may hinder these B cells from inducing Bcl-6 and from undergoing germinal center reaction and subsequent class-switch recombination.

As it has been shown that NF-κB signaling is involved in the induction of terminal differentiation processes[2] and as this pathway is a major signaling cascade induced upon BCR engagement, it is conceivable that TS-induced signaling

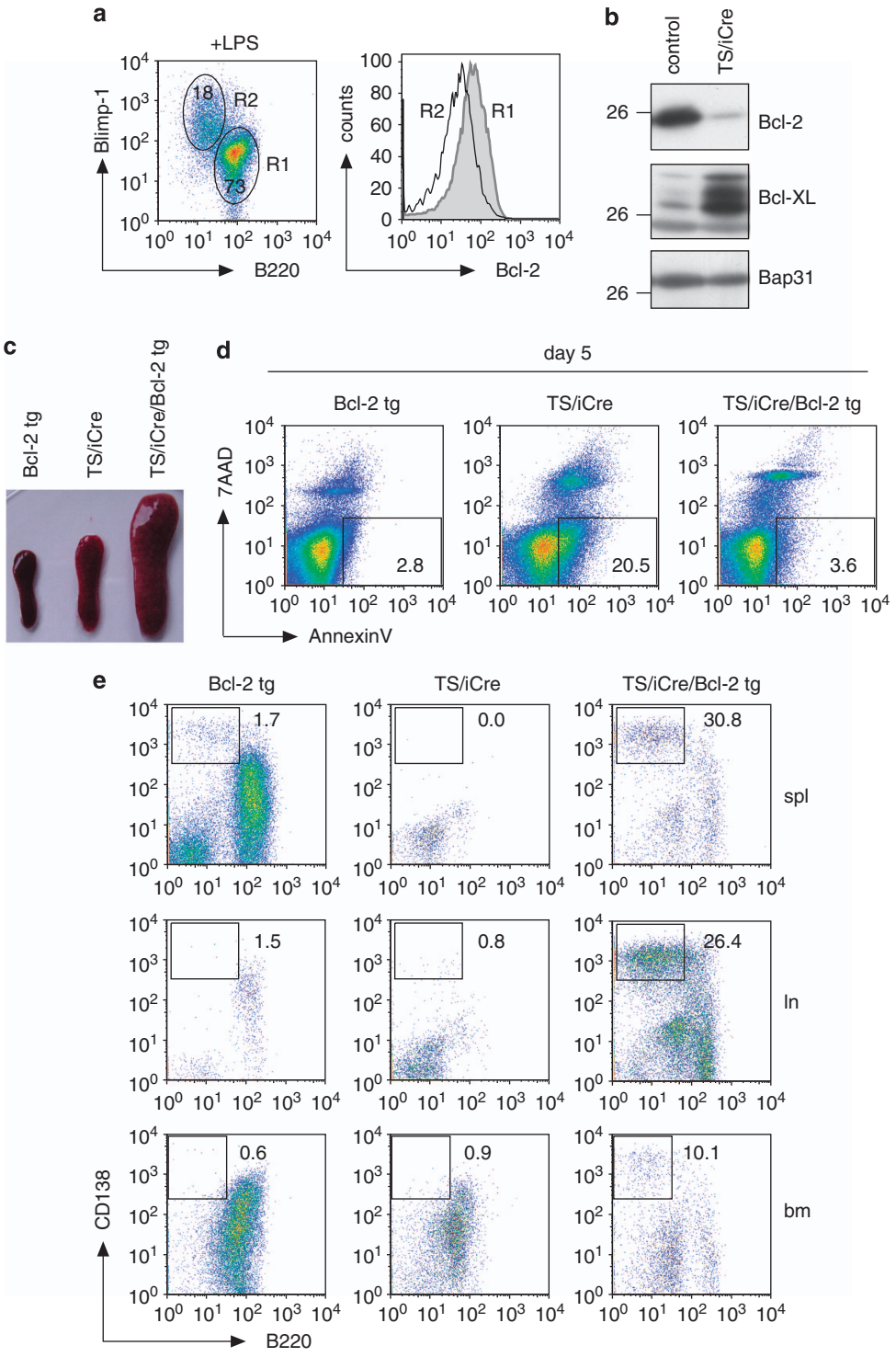

Figure 8. Bcl-2 is downregulated in Blimp-1$^+$ plasma cells and TS/Bcl-2 double-transgenic plasma cells show prolonged survival. (**a**) Intracellular FACS analysis for Blimp-1 and Bcl-2 expression with purified wild-type splenic B cells after *in vitro* stimulation with LPS for 3 days. The histogram compares Bcl-2 expression levels of cells in the respective gate. R1 (B220$^+$), B cells; R2 (B220^{low}Blimp-1$^+$), plasma cells. Shown is a representative experiment of at least two independent experiments. (**b**) Western blot analysis of purified splenic B cells isolated from iCre-negative control and TS/iCre mice at day 5. Immunoblots were incubated with the indicated antibodies. Bap31 served as a loading control. Data are representative of at least three independent experiments. (**c**–**e**) Tamoxifen induction experiments with mice of the indicated genotype. (**c**) Representative picture of spleens from mice isolated at day 5 after tamoxifen treatment. (**d**) FACS staining of spleen cells isolated from mice at day 5 after tamoxifen treatment and gated on CD19$^+$ B cells. Numbers indicate the percentages of apoptotic (AnnexinV$^+$ 7AAD$^-$) cells in the parental population. (**e**) FACS staining of cells isolated from the respective mice at day 7 after tamoxifen treatment and cultured *in vitro* for 11 days. Numbers indicate the portion of B220^{low}CD138$^+$ (plasma cells). Bcl-2 tg, Bcl-2 transgenic mice; spl, spleen; ln, lymph nodes; and bm, bone marrow. Data are representative of at least three independent experiments.

activates the NF-κB pathway, thereby leading to the induction of Blimp-1 expression. In this scenario, a failure to induce Blimp-1 might lead to uncontrolled proliferation because of the inability to undergo terminal differentiation. This is in agreement with reports showing that constitutive NF-κB signaling and disruption of Blimp-1 function strongly synergize in the development of B-cell malignancies.[8,9]

It is known that Bcl-2 is important for the development and survival of mature B cells and that naive B cells show elevated expression of this antiapoptotic factor.[30] Furthermore, Bcl-2 expression is also essential for the survival of newly generated plasma cells. For instance, a chemical compound (ABT-737) that targets Bcl-2, Bcl-XL and Bcl-w, but not Mcl-1, eliminates these cells, whereas it does not affect the survival of already established long-lived plasma cells in the bone marrow.[31,32] Although we do not detect elevated levels of the proapoptotic factor Bim during TS-induced plasma cell differentiation, we cannot exclude that other BH3-only proteins are upregulated and that the observed accumulation of Bcl-XL is involved in neutralizing higher amounts of such proapoptotic proteins. In this scenario, the drastically reduced levels of Bcl-2 may result in higher levels of free Bim and the consequent activation of Bax/Bak, thereby leading to apoptosis of the cells. This might explain why elevated Bcl-XL levels in TS-expressing plasma cells are not sufficient to compensate for Bcl-2 downregulation and prevent apoptosis.

Our findings suggest that Bcl-2 might be the critical survival protein for newly formed plasma cells and that its downregulation is an important mechanism to ensure the homeostasis of these cells and to prevent aberrant cell growth or survival. In contrast, it has been shown that long-lived bone marrow plasma cells critically depend on the Bcl-2 family member Mcl-1 and that its expression is maintained by survival factors, such as interleukin-6 or cytokines of the B-cell-activating factor family.[33] These survival factors are produced in high amounts by the bone marrow microenvironment and are available as soon as plasmablasts reach an appropriate niche and interact with bone marrow stroma cells.[34]

Interestingly, it has been reported that Blimp-1 limits Bcl-2 expression in mature effector regulatory T cells and that Blimp-1 deficiency leads to the accumulation of these cells.[26] Furthermore, Blimp-1 downregulates the Bcl-2 family member A1 during B- and T-cell differentiation.[5,35] Taken together, this suggests that Blimp-1 expression induced by TS or by treatment with LPS may directly lead to reduced Bcl-2 levels and subsequent apoptosis in the same manner as reported for short-lived plasma cells.[34] In line with this, we showed that enforced Bcl-2 expression in combination with TS led to aberrant accumulation of terminally differentiated B cells in peripheral organs and the bone marrow. The resulting plasma cell neoplasm was due to prolonged survival of TS^+Blimp^+ B cells.

Our data suggest that Blimp-1-dependent Bcl-2 repression restricts the survival of activated B cells and identify Syk as the central player in this process. In fact, Syk-deficient mature B cells are unable to induce Blimp-1 expression after LPS stimulation.

We propose that under physiological conditions this regulation is important for the termination of an immune response and for the homeostasis of activated B cells. Interestingly, Blimp-1 is important for T-cell homeostasis and T-cell-specific deletion of Blimp-1 leads to defective homeostasis of effector T cells and causes a lethal systemic inflammation reaction.[26,36]

Taken together, our data suggest a process for B-cell transformation, in which chronic B-cell signaling mediated by Syk fails to induce Blimp-1. Such scenarios may occur in ABC-DLBCL, where the B cells show signs of chronic BCR activation, whereas Blimp-1 expression is defective.[8,9] Thus, understanding how Syk activates Blimp-1 is important for conceiving the molecular mechanism underlying B-cell lymphoma development.

MATERIALS AND METHODS

Mice

To generate mice for the conditional expression of TS, we cloned the cDNA[11] into a Rosa26 targeting vector described previously.[37] 129Sv-derived W4 embryonic stem cells were transfected with the targeting vector and screened for homologous recombination by PCR and Southern blot analysis. For the latter, genomic DNA was digested with *Hind*III and hybridized using a 5′external probe as described earlier.[20] Blastocyst injection followed by chimera breeding resulted in Rosa26-TS mice. Offsprings were screened by PCR analysis (protocol available upon request). The tamoxifen-inducible mb1-CreER^{T2} mice were generated through a knock-in of the cDNA encoding the Cre recombinase and will be described elsewhere (E Hobeika, manuscript submitted). CD21-Cre, Rosa26-YFP, Bcl-2 tg mice and mice carrying floxed *Syk* were described previously.[22,24,25,27] Littermates were used in all described experiments. For tamoxifen induction experiments, 6 mg tamoxifen citrate was administered per mouse using the gavage technique. Clinoleic 20% (Baxter, Deerfield, IL, USA) was used as solvent. Mice were bred at the animal facility of the Max-Planck-Institute of Immunobiology and Epigenetics. Studies were conducted according to federal and institutional guidelines.

Cell culture and *in vitro* assays

Primary spleen cells were cultured in Iscove's medium (Biochrom AG, Berlin, Germany) containing 10% fetal calf serum (Vitromex GmbH, Geilenkirchen, Germany), 2 mM L-glutamine and 100 U/ml penicillin/streptomycin (Invitrogen, Carlsbad, CA, USA) and 50 μM 2-mercaptoethanol (Gibco by Life technology, Carlsbad, CA, USA). For *in vitro* induction experiments, purified splenic B cells were incubated with 2 μM 4-OHT (Merck Biosciences, Darmstadt, Germany) or ethanol as a solvent control. For inhibition of Syk, cells were cultured in medium supplemented with 1–2 μM R406 (Rigel Pharmaceuticals, South San Francisco, CA, USA). For *in vitro* stimulation of B cells, 2–10 μg/ml LPS (from Salmonella abortus equi, kindly provided by M Freudenberg (BIOSS Centre for Biological Signalling Studies, Freiburg, Germany)) was used.

For the *in vitro* cell proliferation assay, isolated B cells were pretreated with 2 μM 4-OHT for 24 h and then loaded with the cell proliferation dye eFluor670 (eBioscience, San Diego, CA, USA) according to the manufacturer's instructions. At day 5 of *in vitro* culturing, cells were stained with 7AAD (Immunostep, Salamanca, Spain). Viable cells were used for analysis.

Flow cytometry and cell sorting

Single-cell suspensions were prepared from different organs and stained with the following antibodies conjugated to fluorescein isothiocyanate (FITC), phycoerythrin (PE), peridin-chlorophyll (PerCP), allophycocyanin (APC), cyanin-5 (Cy5), phycoerythrin-cyanin-7 (PE-Cy7) or biotin: anti-μ heavy chain (μHC), anti-IgD and anti-B220 (RA3-6B2) from Southern Biotech (Birmingham, AL, USA); anti-CD19 (1D3), anti-CD5 (53-7.3), anti-CD23 (B3B4), anti-CD138 (281–2), anti-CD43 (S7), anti-CD21 (7G6) and anti-Bcl-2 (3F11) from BD Pharmingen (San Diego, CA, USA); anti-CD86 (GL1) and anti-IRF4 (3E4) from eBioscience; anti-human Syk (4D10) and anti-Blimp-1 (C-21) from Santa Cruz Biotechnology (Dallas, TX, USA); anti-mouse Syk (5F5) from BioLegend (San Diego, CA, USA); and streptavidin-Cy5/PerCP from Dianova/BD Bioscience (Hamburg, Germany). Anti-CD21 and anti-mouse Syk were labeled with the Lynx rapid APC antibody conjugation kit (AbD Serotec, Kidlington, UK) according to the manufacturer's instructions. For intracellular FACS analysis, fixation buffer (ADG; Bio Research GmbH, Kaumberg, Austria) was used according to the manufacturer's instructions. For apoptosis assays, AnnexinV (BD Pharmingen) and 7AAD (Immunostep) staining was performed in freshly prepared AnnexinV binding buffer (10 mM HEPES/NaOH (pH 7.4), 140 mM NaCl, 2.5 mM $CaCl_2$).

Acquisition was performed with FACSCalibur or LSRII (Becton Dickinson, Heidelberg, Germany) and results were analyzed with the FlowJo software (Tree Star Inc., Olten, Switzerland).

Splenic B cells were purified by depletion of $CD43^+$ cells with MACS beads (Miltenyi, Bergisch Gladbach, Germany) and cell sorting using a FACS DIVA (Becton Dickinson).

ELISA and inflammatory cytokine assay

Serum of mice was collected using serum collection tubes/Microtainer (Becton Dickinson). Antibodies for ELISA were purchased from Southern Biotech and 4-nitrophyl phosphate disodium salt hexahydrate

(Sigma-Aldrich, St Louis, MO, USA) was used as substrate for the developing reaction.

Serum and cell culture supernatants were tested for the concentrations of inflammatory cytokines with the FACS-based CBA 'Mouse Inflammation Kit' (BD Pharmingen) according to the manufacturer's instructions.

RT–PCR

RNA was isolated with the RNeasy Plus Mini Kit (Qiagen, Hilden, Germany) and subsequently RT reactions were performed using oligo-dT-primer. PCR amplification of Xbp-1 (spliced/unspliced) and HPRT on the synthesized cDNA was performed with the specific primers: HPRT fwd. (5′-CTTGCT CGAGATGTGATGAA-3′), HPRT rev. (5′-CAAATCCCTGAAGTATTCATT-3′), Xbp-1.3S (5′-AAACAGAGTAGCAGCGCAGACTGC-3′) and Xbp-1.12AS (5′-TCC TTCTGGGTAGACCTCTGGGAG-3′). PCR products were analyzed by electrophoresis in 2–3% agarose gel.

Immunoblot analysis

For total cell lysates, isolated cells were lysed in RIPA lysis buffer. Western blots were prepared as described previously[38] and probed with anti-Pax5 (1H9; Abcam, Cambridge, UK), anti-Bcl-2 (3F11; BD Pharmingen), anti-Bcl-X (E18; Epitomics), anti-Bim (Y36; Epitomics, Burlingame, CA, USA), anti-Blimp-1 (6D3; Santa Cruz Biotechnology), anti-human Syk (4D10; Santa Cruz Biotechnology), anti-Mcl-1 (S-19; Santa Cruz Biotechnology), anti-Bcl-6 (Cell Signaling, Cambridge, UK) and anti-Bap31 kindly provided by W. Schamel (Centre of Chronic Immunodeficiency, Freiburg, Germany).[39]

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

We thank A Nil for critical discussion and help with mouse analyses. We thank M Bach, C Eschbach, I Fiedler, C Sainz Rueda for technical assistance; U Zimber-Strobl for providing Rosa26-targeting construct; Benoit Kanzler and the transgenic mouse unit for ES cell injection; A Wuerch and S Hobitz for cell sorting; F Koehler and M Bach for proofreading the manuscript; S Srinivas for the Rosa26-YFP mice, A Strasser for the Bcl-2 tg mice, A Tarakhovsky for the $Syk^{fl/fl}$ mice and K Rajewsky for the CD21-Cre mice. This work was supported by the Deutsche Krebshilfe (project no. 108935), the Deutsche Forschungsgemeinschaft (SFB620) and the Excellence Initiative of the German Federal and State Governments (EXC294).

AUTHOR CONTRIBUTIONS

E Hug generated the Rosa26-TS mouse strain, designed and performed the experiments, analyzed the data and wrote the manuscript; E Hobeika and MR provided mb1-$CreER^{T2}$ mice; E Hobeika carried out the tamoxifen treatment of mice, provided materials and suggestions for experimental design; and HJ supervised the work, designed experiments and wrote the manuscript together with E Hug.

REFERENCES

1 Beitz LO, Fruman DA, Kurosaki T, Cantley LC, Scharenberg AM. SYK is upstream of phosphoinositide 3-kinase in B cell receptor signaling. *J Biol Chem* 1999; **274**: 32662–32666.
2 Marshall AJ, Niiro H, Yun TJ, Clark EA. Regulation of B-cell activation and differentiation by the phosphatidylinositol 3-kinase and phospholipase Cgamma pathway. *Immunol Rev* 2000; **176**: 30–46.
3 Werner M, Hobeika E, Jumaa H. Role of PI3K in the generation and survival of B cells. *Immunol Rev* 2010; **237**: 55–71.
4 Kallies A, Nutt SL. Terminal differentiation of lymphocytes depends on Blimp-1. *Curr Opin Immunol* 2007; **19**: 156–162.
5 Martins G, Calame K. Regulation and functions of Blimp-1 in T and B lymphocytes. *Annu Rev Immunol* 2008; **26**: 133–169.
6 Shapiro-Shelef M, Lin KI, McHeyzer-Williams LJ, Liao J, McHeyzer-Williams MG, Calame K. Blimp-1 is required for the formation of immunoglobulin secreting plasma cells and pre-plasma memory B cells. *Immunity* 2003; **19**: 607–620.
7 Shaffer AL, Lin KI, Kuo TC, Yu X, Hurt EM, Rosenwald A *et al*. Blimp-1 orchestrates plasma cell differentiation by extinguishing the mature B cell gene expression program. *Immunity* 2002; **17**: 51–62.
8 Calado DP, Zhang B, Srinivasan L, Sasaki Y, Seagal J, Unitt C *et al*. Constitutive canonical NF-kappaB activation cooperates with disruption of BLIMP1 in the pathogenesis of activated B cell-like diffuse large cell lymphoma. *Cancer Cell* 2010; **18**: 580–589.
9 Mandelbaum J, Bhagat G, Tang H, Mo T, Brahmachary M, Shen Q *et al*. BLIMP1 is a tumor suppressor gene frequently disrupted in activated B cell-like diffuse large B cell lymphoma. *Cancer Cell* 2010; **18**: 568–579.
10 Kanie T, Abe A, Matsuda T, Kuno Y, Towatari M, Yamamoto T *et al*. TEL-Syk fusion constitutively activates PI3-K/Akt, MAPK and JAK2-independent STAT5 signal pathways. *Leukemia* 2004; **18**: 548–555.
11 Wossning T, Herzog S, Kohler F, Meixlsperger S, Kulathu Y, Mittler G *et al*. Deregulated Syk inhibits differentiation and induces growth factor-independent proliferation of pre-B cells. *J Exp Med* 2006; **203**: 2829–2840.
12 Gobessi S, Laurenti L, Longo PG, Carsetti L, Berno V, Sica S *et al*. Inhibition of constitutive and BCR-induced Syk activation downregulates Mcl-1 and induces apoptosis in chronic lymphocytic leukemia B cells. *Leukemia* 2009; **23**: 686–697.
13 Leseux L, Hamdi SM, Al Saati T, Capilla F, Recher C, Laurent G *et al*. Syk-dependent mTOR activation in follicular lymphoma cells. *Blood* 2006; **108**: 4156–4162.
14 Rinaldi A, Kwee I, Taborelli M, Largo C, Uccella S, Martin V *et al*. Genomic and expression profiling identifies the B-cell associated tyrosine kinase Syk as a possible therapeutic target in mantle cell lymphoma. *Br J Haematol* 2006; **132**: 303–316.
15 Young RM, Hardy IR, Clarke RL, Lundy N, Pine P, Turner BC *et al*. Mouse models of non-Hodgkin lymphoma reveal Syk as an important therapeutic target. *Blood* 2009; **113**: 2508–2516.
16 Chen L, Monti S, Juszczynski P, Daley J, Chen W, Witzig TE *et al*. SYK-dependent tonic B-cell receptor signaling is a rational treatment target in diffuse large B-cell lymphoma. *Blood* 2008; **111**: 2230–2237.
17 Riccaboni M, Bianchi I, Petrillo P. Spleen tyrosine kinases: biology, therapeutic targets and drugs. *Drug Discov Today* 2010; **15**: 517–530.
18 Kuno Y, Abe A, Emi N, Iida M, Yokozawa T, Towatari M *et al*. Constitutive kinase activation of the TEL-Syk fusion gene in myelodysplastic syndrome with t(9;12)(q22;p12). *Blood* 2001; **97**: 1050–1055.
19 Kulathu Y, Hobeika E, Turchinovich G, Reth M. The kinase Syk as an adaptor controlling sustained calcium signalling and B-cell development. *EMBO J* 2008; **27**: 1333–1344.
20 Soriano P. Generalized lacZ expression with the ROSA26 Cre reporter strain. *Nat Genet* 1999; **21**: 70–71.
21 Feil R, Wagner J, Metzger D, Chambon P. Regulation of Cre recombinase activity by mutated estrogen receptor ligand-binding domains. *Biochem Biophys Res Commun* 1997; **237**: 752–757.
22 Srinivas S, Watanabe T, Lin CS, William CM, Tanabe Y, Jessell TM *et al*. Cre reporter strains produced by targeted insertion of EYFP and ECFP into the ROSA26 locus. *BMC Dev Biol* 2001; **1**: 4.
23 Shaffer AL, Shapiro-Shelef M, Iwakoshi NN, Lee AH, Qian SB, Zhao H *et al*. XBP1, downstream of Blimp-1, expands the secretory apparatus and other organelles, and increases protein synthesis in plasma cell differentiation. *Immunity* 2004; **21**: 81–93.
24 Saijo K, Schmedt C, Su IH, Karasuyama H, Lowell CA, Reth M *et al*. Essential role of Src-family protein tyrosine kinases in NF-kappaB activation during B cell development. *Nat Immunol* 2003; **4**: 274–279.
25 Kraus M, Alimzhanov MB, Rajewsky N, Rajewsky K. Survival of resting mature B lymphocytes depends on BCR signaling via the Igalpha/beta heterodimer. *Cell* 2004; **117**: 787–800.
26 Cretney E, Xin A, Shi W, Minnich M, Masson F, Miasari M *et al*. The transcription factors Blimp-1 and IRF4 jointly control the differentiation and function of effector regulatory T cells. *Nat Immunol* 2011; **12**: 304–311.
27 Strasser A, Whittingham S, Vaux DL, Bath ML, Adams JM, Cory S *et al*. Enforced BCL2 expression in B-lymphoid cells prolongs antibody responses and elicits autoimmune disease. *Proc Natl Acad Sci USA* 1991; **88**: 8661–8665.
28 Visan I, Goller M, Berberich I, Kneitz C, Tony HP. Pax-5 is a key regulator of the B cell-restricted expression of the CD23a isoform. *Eur J Immunol* 2003; **33**: 1163–1173.
29 Holmes ML, Pridans C, Nutt SL. The regulation of the B-cell gene expression programme by Pax5. *Immunol Cell Biol* 2008; **86**: 47–53.
30 Grossmann M, O'Reilly LA, Gugasyan R, Strasser A, Adams JM, Gerondakis S. The anti-apoptotic activities of Rel and RelA required during B-cell maturation involve the regulation of Bcl-2 expression. *EMBO J* 2000; **19**: 6351–6360.
31 Merino D, Khaw SL, Glaser SP, Anderson DJ, Belmont LD, Wong C *et al*. Bcl-2, Bcl-x(L), and Bcl-w are not equivalent targets of ABT-737 and navitoclax (ABT-263) in lymphoid and leukemic cells. *Blood* 2012; **119**: 5807–5816.
32 Carrington EM, Vikstrom IB, Light A, Sutherland RM, Londrigan SL, Mason KD *et al*. BH3 mimetics antagonizing restricted prosurvival Bcl-2 proteins represent another class of selective immune modulatory drugs. *Proc Natl Acad Sci USA* 2010; **107**: 10967–10971.

33 Peperzak V, Vikstrom I, Walker J, Glaser SP, LePage M, Coquery CM *et al.* Mcl-1 is essential for the survival of plasma cells. *Nat Immunol* 2013; **14**: 290–297.
34 De Vos J, Hose D, Reme T, Tarte K, Moreaux J, Mahtouk K *et al.* Microarray-based understanding of normal and malignant plasma cells. *Immunol Rev* 2006; **210**: 86–104.
35 Knodel M, Kuss AW, Lindemann D, Berberich I, Schimpl A. Reversal of Blimp-1-mediated apoptosis by A1, a member of the Bcl-2 family. *Eur J Immunol* 1999; **29**: 2988–2998.
36 Kallies A, Hawkins ED, Belz GT, Metcalf D, Hommel M, Corcoran LM *et al.* Transcriptional repressor Blimp-1 is essential for T cell homeostasis and self-tolerance. *Nat Immunol* 2006; **7**: 466–474.
37 Homig-Holzel C, Hojer C, Rastelli J, Casola S, Strobl LJ, Muller W *et al.* Constitutive CD40 signaling in B cells selectively activates the noncanonical NF-kappaB pathway and promotes lymphomagenesis. *J Exp Med* 2008; **205**: 1317–1329.
38 Herzog S, Hug E, Meixlsperger S, Paik JH, DePinho RA, Reth M *et al.* SLP-65 regulates immunoglobulin light chain gene recombination through the PI(3)K-PKB-Foxo pathway. *Nat Immunol* 2008; **9**: 623–631.
39 Adachi T, Schamel WW, Kim KM, Watanabe T, Becker B, Nielsen PJ *et al.* The specificity of association of the IgD molecule with the accessory proteins BAP31/BAP29 lies in the IgD transmembrane sequence. *EMBO J* 1996; **15**: 1534–1541.

Supplementary Information accompanies this paper on the Oncogene website (http://www.nature.com/onc)