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Generation of precursor, immature, and mature murine B1-cell lines from c-myc/bcl-xL-overexpressing pre-BI cells

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Deregulated expression of c-myc and bcl-xL is long known to generate transformed B cells in humans and mice. We overexpressed these genes to induce in vitro and in vivo differentiation of fetal liver-derived mouse pre-BI cells to B1-lineage pre-BII-like, immature and mature B-cell lines, and to Ig-secreting cells. In vitro, doxycycline-controlled c-myc/bcl-xL-overexpressing $CD19^+CD93^+c\text{-}kit^+IgM^-$ pre-BI cells differentiate to and survive as $CD19^+CD93^+c\text{-}kit^-IgM^+$ immature B1 cells. Timed CpG stimulation of these oncogene-overexpressing pre-B or immature B1 cells generates either $CD19^+CD93^{low}$ $c\text{-}kit^-IgM^-SLC^-$ pre-BII-like or $IgM^+MHCII^+CD73^+CD80^+CD40^+$ mature B1-cell lines and IgM-secreting B1 cells in vitro and fixes their state of differentiation. All cell lines are clonable, but a majority of immature and mature B1-cell clones eventually reach a nonproliferating, surviving G_0-state. Transplanted in vivo, c-myc/bcl-xL-overexpressing pre-B cells expand to mature B1 cells, and to IgM- and IgA-secreting plasmablasts and plasma cells. Within 2 months, plasmablasts have expanded most prominently in BM and spleen, indicating that the host selectively expanded development of these transformed plasma cells. The $sIgM^+$ B1-cell lines and clones offer the possibility to study their roles in the development of B1-Ab repertoires, of B1-cell-mediated autoimmune diseases and of B1-cell malignancies.

Keywords: B1-cell differentiation · c-myc/bcl-xL · CpG stimulation · Pre-BI cell · Proliferation

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Introduction

Deregulated expression of c-myc is frequently associated with B-cell transformation in mice and humans, leading to deregulated cell cycle progression and proliferation, and also to apoptotic cell death [1]. Co-overexpression of the antiapoptotic gene bcl-xL, which inhibits the proapoptotic action of c-myc, is sufficient to induce fully transformed states of B-lymphocyte lineage cells [2].

In normal murine B-cell development c-myc is highly expressed in proliferating B-lineage cells, such as pre-BI, large pre-BII, and stimulated mature B cells, and is downregulated in nonactivated, resting immature, and naïve mature B cells and plasma cells [3–5]. c-myc regulates a wide variety of cellular processes [6]. By direct or indirect activation of cell cycle activating genes including cyclins and cyclin-dependent kinases, or by antagonizing cell cycle inhibitors, c-myc regulates cell cycle progression and proliferation [7]. In parallel, by inhibiting the ARF-MDM2-p53 tumor suppressor pathway, c-myc further favors the initiation of apoptosis [8].

bcl-xL, like bcl-2, is thought to protect the mitochondrial membrane from releasing cytochrome c after apoptotic

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stimulation [2], thereby inhibiting apoptosis. bcl-xL is expressed throughout the early mouse B-cell development and becomes undetectable in mature B cells [9–11]. Furthermore, bcl-xL, but not bcl-2, is rapidly induced in peripheral B cells upon surface IgM cross-linking, CD40 signaling, or LPS stimulation [10, 11].

bcl-xL transgenic immature B cells that express self-reactive BCR, escape central tolerance [12]. bcl-xL has been found overexpressed in several types of hematopoietic tumors, including acute lymphoblastic leukemias [1], non-Hodgkin's, Hodgkin's, and HIV-associated lymphomas, and in acute myelogenous leukemia and myeloma [13]. B-lineage cell stage-specific, promoter-controlled targeting of oncogene-overexpression to early or late stages of B-cell development influences the preferential development of B-lineage tumors of early, for example, acute lymphoblastic leukemias, or of late, for example, plasmacytomas and multiple myelomas [1, 14] in vivo.

Here, we overexpress the cooperating, malignancy-inducing oncogenes, c-myc and bcl-xL (c-myc/bcl-xL), introduced by doxycycline-induced retroviral vectors and continuously overexpress these oncogenes from fetal liver-derived mouse pre-BI cells through all stages of B1-cell development [15]. We follow the effects of doxycycline-reversible c-myc/bcl-xL-overexpression on the differentiation of pre-BI cells to B cells and plasma cells in vitro and, after transplantation into immunodeficient recipient mice in vivo. We find that under differentiating culture conditions c-myc/bcl-xL-overexpressing pre-BI cells terminate proliferation, survive for long periods of time, and increasingly develop within 18 days to immature B1 cells.

We then stimulate in vitro differentiating, c-myc/bcl-xL-overexpressing B1-lineage cells at different times of differentiation with unmethylated CpG oligodeoxynucleotides (CpG ODN1826) [16] and with LPSs [17]. Surprisingly, only CpG, but not LPS stimulates proliferation of early differentiating pre-B cells, and of in vitro generated immature sIgM$^+$ B cells. Furthermore, CpG-stimulated CD19$^+$CD93$^+$c-kit$^+$SLC$^+$sIgM$^-$ pre-BI cells differentiate to CD19$^+$CD93^{low}c-kit$^-$SLC$^-$sIgM$^-$ pre-BII-like cells, while CpG-stimulated, in vitro generated immature sIgM$^+$ B cells become sIgM$^+$CD93$^{low/-}$MHCII$^+$CD73$^+$CD80$^+$CD40$^+$ mature B cells. Our findings indicate that CpG stimulation has differentiation-inducing effects on c-myc/bcl-xL-overexpressing B-lineage cells. Additionally, immature sIgM$^+$ B cells that have differentiated in vitro for longer than a month can be stimulated by CpG to secrete IgM in vitro.

When c-myc/bcl-xL-overexpressing pre-BI cells are transplanted into RAG$^{-/-}$ mice, expanding pre-B cells, mature B1-cells, and IgM- and IgA-secreting plasmablasts and plasma cells develop within 2 months in BM and spleen, unless oncogene expression is terminated by omitting doxycycline from the drinking water. Hence, these cell lines offer new experimental opportunities to study the molecular models and cellular stages of B1-cell development in vitro and in vivo, their repertoire development [18], their contributions to autoimmune diseases [19], and their development to B1-cell leukemias [20].

Results

Induction of longevity and differentiation of c-myc/bcl-xL-overexpressing B-lineage cells in vitro

Fetal liver-derived pre-BI cells constitutively expressing GFP and high levels of reverse transactivator (rtTA) (GFP-rtTA-pre-BI) were constructed as previously described in more detail [21, 22]. In the presence of doxycycline, rtTA activates a tetracycline-inducible promoter (tetON) [23], that controls expression of c-myc or bcl-xL, or c-myc and bcl-xL together (c-myc/bcl-xL), joined by 2A peptide, in self-inactivating retroviral vectors [21] (Supporting Information 1). These doxycycline-inducible vectors were transduced into the GFP-rtTA-pre-BI cells to generate single- and double-oncogene-transduced cell lines, which could be selected for successful retroviral transduction by addition of antibiotics in the presence of stromal cells (OP9) and IL7. The detailed construction of these cell lines, and the tests for oncogene expression and for functional changes in cell cycle entry and apoptosis are described in Supporting Information 1 and show that the synergistic overexpression of both oncogenes, c-myc and bcl-xL, has no effects on in vitro cultured, proliferating pre-BI cells.

After induction of oncogene-overexpression, differentiation was induced by removal of OP9 stromal cells and IL7 (Fig. 1A). Almost all c-myc or bcl-xL single-expressing, as well as c-myc/bcl-xL double-transduced, nonexpressing pre-BI cells, died within 5–9 of days, as pre-BI cells normally do [24] (Fig. 1B, type-1 and Supporting Information Fig. 1H). By contrast, c-myc/bcl-xL double-expressing pre-BI cells survived for more than a month (Fig. 1B, type-2). The termination of c-myc/bcl-xL-overexpression by removal of doxycycline induced rapid apoptosis of all stages of in vitro differentiation (Fig. 1B).

When c-myc/bcl-xL-overexpressing cells were labeled with eF670—a cell surface dye that is diluted out as cells divide—a large fraction remained eF670$^+$ (Fig. 1C), thus did not divide for 6 days of differentiation. On day 4 of differentiation, only a small fraction (~5%) of the surviving cells was found to proliferate, that is, expressed Ki-67 and lost eF670 label (Fig. 1C and D, type-2 d4). After 18 days, the majority of the differentiating cells were Ki-67$^+$ and eF670$^-$ (Fig. 1C and D, type-2 d18). An extrapolation to day 0 of the exponential part of the growth curve (Fig. 1B, dashed gray line) indicates that a small part of all cells proliferated (around 1×10^3 in 1×10^6 cells). The growth curves indicate that initially, the majority of retrovirally transduced cells are Ki-67$^-$ nondividing. The proliferating cells expanded 100-fold within 7 days, suggesting that these proliferating Ki-67$^+$ cells outgrew any resting, surviving cells, and that the average cell cycle time was around 40 h, that is, longer than the 18 h of proliferating pre-BI cells (Supporting Information Fig. 1F). Hence, all subsequent analyses of pools of the in vitro differentiated cells represent, to a considerable degree, the properties of these proliferating cells.

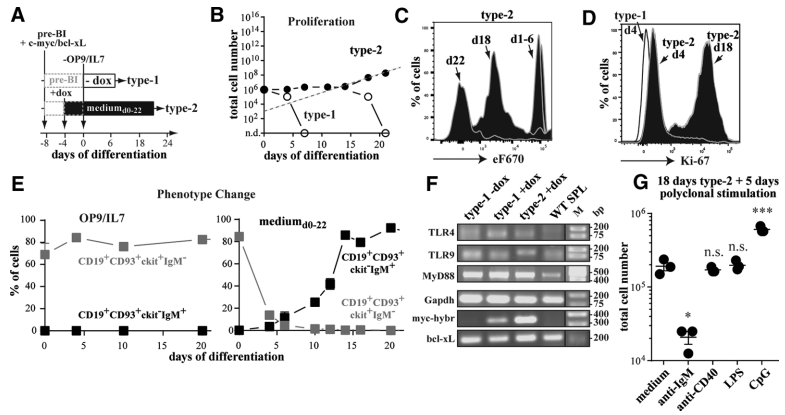

Figure 1. Joint c-myc/bcl-xL-overexpression induces longevity and differentiation of pre-BI to immature B cells in vitro. (A) Experimental schedule illustrating in vitro differentiation experiments of c-myc/bcl-xL-overexpressing fetal liver-derived pre-BI cells from C57BL/6 mice. The cells were cultured on OP9 stromal cells and IL7 (OP9/IL7) (gray dashed symbols) in the presence (closed symbols) or absence (open symbols) of doxycycline (dox). Their differentiation was induced on day 0 by removal of OP9/IL7 (-OP9/IL7), generating type-2 (+dox, $medium_{d0\text{-}22}$). Type-1 cells represent the survival of transduced, but doxycycline-noninduced (-dox) pre-BI cells. (B) Absolute numbers of c-myc/bcl-xL-overexpressing type-1 and type-2 cells were determined by counting using a hemacytometer and Trypan blue exclusion. Dashed line extrapolates exponentially growing type-2 cells to the initiation of differentiation (day 0). As control, differentiating cells were also cultured in the absence of doxycycline from day 0 or 12 of differentiation (open symbols). (C) Proliferation of day 18–22 differentiating type-2 was measured by labeling the cells with 5 μM cell eFlour670. On days 1, 4, and 6 (d1–6) after labeling, cell division was monitored by flow cytometry. (D) Ki-67 expression of type-1 and 2 c-myc/bcl-xL-overexpressing cells cultured with or without doxycycline for 4 (d4) and 18 days (d18) in the absence of OP9/IL7 was measured by flow cytometry. (E) Differentiation of c-myc/bcl-xL-overexpressing $CD19^+CD93^+$c-kit^+IgM^- pre-BI-cell cultures (gray rectangles) to $CD19^+CD93^+$c-kit^-IgM^+ immature-like B cells (black rectangles) was determined by FACS. (F) RT-PCR of genes expressed during B-cell development in c-myc/bcl-xL-overexpressing pre-BI cells cultured with (type-1) or without (type-2) OP9/IL7 with (+) or without (–) doxycycline (dox). WT SPL = WT spleen control, M = 1 kb plus size marker. (G) Polyclonal activation of c-myc/bcl-xL-overexpressing, type-2 cells 5 days after stimulation. Significance was determined comparing polyclonal stimulated and nonstimulated (medium) immature-like B cells by two-tailed unpaired *t*-test; $^*p < 0.05$, $^{***}p < 0.0005$, n.s = not significant. (B, E, and G) Data are shown as mean ± SEM and are from a single experiment representative of three to six (B–G) independent experiments with three samples per experiment.

We have previously found that the co-culture of LPS-activated splenic B cells with rat thymus cells increased the cloning efficiency of proliferating B-cell blasts more than 1000-fold to one in three blast cells [25]. We employed this cloning protocol to the c-myc/bcl-xL-overexpressing B1-lineage cell lines to study the further development of these different cell stages on a clonal level. Upon single cell sorting on rat thymus cells, one in four of these type-2 cells were found to form clones (Supporting Information 2, type-2). Some of them continued proliferation; others reverted to long-term survival after 14 days in culture.

After induction of differentiation, c-myc/bcl-xL-overexpressing $CD19^+CD93^+$c-kit^+sIgM^- pre-BI cells (type-2) downregulated c-kit expression, as differentiating nontransduced cells do [24] (Fig. 1E, detailed gating strategy see Supporting Information Fig. 3), while sIgM expression became detectable. Between 18 and 22 days, and at least until day 32 of differentiation, the majority of the c-myc/bcl-xL-overexpressing type-2 cells were $CD19^+CD93^+$c-kit^-sIgM^+ (Fig. 1E), a phenotype characteristic for immature B cells [26]. This $CD19^+CD93^+$c-kit^-sIgM^+ immature B-cell phenotype was monitored on all clones, whether they continued to proliferate for 32 days, or whether they ceased to proliferate after additional 14 days (Supporting Information 4).

In summary, double transduction of pre-BI cells with c-myc and bcl-xL, and their subsequent differentiation, established long-term surviving immature B-cell-like cell lines, of which a majority is resting, nonproliferating, and a minority proliferating.

Polyclonal stimulation of in vitro differentiated immature B cells

Previous reports have shown that normal, untransduced immature B cells can functionally be distinguished from mature B cells by their differential responsiveness to polyclonal activators [26]. When stimulated by IgM-specific antibodies, immature B cells die by apoptosis, while mature $CD19^+CD93^-$sIgM^+ B cells are induced to proliferate. Five days after exposure to IgM-specific antibodies, 18 days differentiated $CD19^+CD93^+$c-kit^-IgM^+ c-myc/bcl-xL-overexpressing B cells were found reduced in numbers, when compared to nonstimulated controls (Fig. 1G). These results suggest that IgM-specific antibodies convey inhibitory effects on in vitro differentiated c-myc/bcl-xL-overexpressing immature-like B cells. By contrast, other polyclonal B-cell activators, such as LPS- or CD40-specific antibodies did neither inhibit proliferation nor induced death of nontransduced c-myc/bcl-xL immature B cells, as it was also shown for WT nontransduced immature B cells [26] (not shown). Thus, in vitro differentiated c-myc/bcl-xL-overexpressing immature-like B cells display some of functional properties of immature B cells.

CpG stimulation of c-myc/bcl-xL-overexpressing pre-B cells generates pre-BII-like cells in vitro

RT-PCR analyses detected the expression of TLR4, TLR9, and MyD88 [27] in c-myc/bcl-xL-overexpressing pre-BI cells

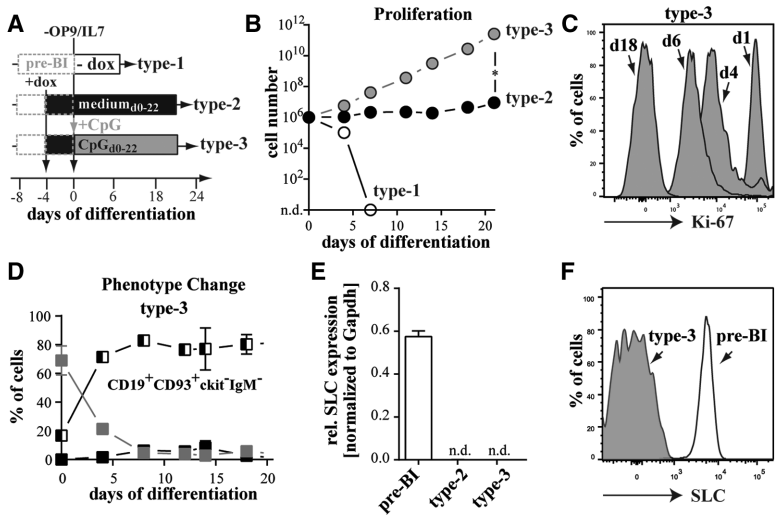

Figure 2. CpG-stimulated c-myc/bcl-xL-overexpressing pre-BI cells expand by proliferation. (A) Experimental schedule illustrating in vitro differentiation experiments (see Fig. 1A). Here, c-myc/bcl-xL double-expressing pre-BI cells were additionally stimulated with CpG from day 0 of differentiation. (B) Growth curves of c-myc/bcl-xL-overexpressing cells differentiating without OP9/IL7, in the presence (closed circles) or absence (open circles) of doxycycline in medium alone (black circles, type-2) or in the presence of CpG (gray circles, type-3) from day 0 of differentiation. Live cells were counted using a hemacytometer and Trypan blue exclusion. (C) Percent of proliferating Ki-67+ eF670-labeled type-3 cells on days (d) 1, 4, 6, and 18 of differentiation was determined by flow cytometry. (D) Summary of FACS analyses showing surface marker expression changes of c-myc/bcl-xL-overexpressing $CD19^+CD93^+$c-kit^+IgM^- pre-BI cells (gray boxes) to $CD19^+CD93^{low}$c-kit^-IgM^- pre-BII-like cells (black/white boxes). $CD19^+CD93^{low/-}$c-kit^-IgM^+ immature B cells (black boxes) remained undetectable. (E) Quantitative RT-PCR of surrogate light chain (SLC) component λ5 expression in c-myc/bcl-xL-overexpressing pre-BI cells or in c-myc/bcl-xL-overexpressing cell lines differentiating either without (type-2) or with CpG (type-3), respectively. N.d. = expressed SLC mRNA levels not detected. (F) Intracellular FACS analysis of SLC expression in c-myc/bcl-xL type-3 cells. White histogram = pre-BI-cell control (positive control). (B, D, and E) Data are shown as mean ± SEM and are from a single experiment representative of three to six (B–F) independent experiments with three samples per experiment. Significance was determined in B by two-tailed unpaired *t*-test, $^*p < 0.05$.

(type-1) and immature B cells (Fig. 1F, type-2). Therefore, we next stimulated both types of cells either with LPS or CpG (Fig. 1G). Interestingly, CpG, but not LPS, stimulated increased proliferation in 95–98% of the double oncogene-overexpressing cells (Fig. 1G) either differentiated for less than a day (d0) (Fig. 2A) or for 18 days (d18) (Fig. 3A), shortening the average cell cycle time from 40 to 18 h, losing eF670-label (Fig. 2B and C), and expressing high levels of Ki-67 (Fig. 3C). Cells stimulated at day 0 of induction of differentiation by removal of IL7 and OP9 stromal cells with CpG, downregulated c-kit expression levels within 4 days of CpG stimulation, while sIgM expression remained undetectable, either on the cell surface, or in the cytoplasm. Within 9 days of differentiation the d0-CpG-stimulated, c-myc/bcl-xL-overexpressing pre-BI cells became $CD19^+CD93^{low}$c-kit^-IgM^- (Fig. 2D). The surrogate light chain (SLC) expression was downregulated on mRNA and on protein levels (Fig. 2E and F). These results suggest that the d0-CpG-stimulated pre-BI cells (type-3) became and remained arrested at a small, D_H-J_H-rearranged (data not shown) pre-BII-like stage of B1-cell development.

Type-3 cells were cloned in cultures containing CpG and rat thymus cells (Supporting Information Fig. 2, type-3). At least two of three cells yielded clones, all of which continued to proliferate beyond 2 weeks with average cell cycle times of 18 h. They retained their $CD19^+CD93^+$c-kit^-Ki-$67^+SLC^-sIgM^-$ phenotypes resembling pre-BII-like cells for at least additional 28 days in cell culture (Supporting Information Fig. 4). This pre-BII-cell-like phenotype has previously not been detected in normal differentiation of pre-BI cells in vitro [28].

CpG stimulation of c-myc/bcl-xL-overexpressing immature B cells generates mature B cells in vitro

CpG stimulation of the day 18 differentiated $CD19^+CD93^+$ c-$kit^-SLC^-sIgM^+$ immature B cells (type-2) (Fig. 3A) induced increased proliferation with average cell cycle times changing from 40 h to 18 h. It generated a new cell type (type-4) expressing high levels Ki-67 (Fig. 3B and C) with downregulated CD93 expression and upregulated expression of costimulatory molecules CD40, CD73, CD80, and MHCII [29] (Fig. 3D and E, Supporting Information Fig. 4). Like mature B cells [26], these CpG-stimulated cells lost the sensitivity to be inhibited by IgM-specific antibodies (Fig. 3F). These results indicate that CpG stimulation had induced immature B cells to become mature B cells.

After single cell sorting on rat thymus cells, one in six type-4 cells were capable to establish large CpG-stimulated clones with at least 10^4 cells/clone in 14 days (Supporting Information 2, type-4). Clones with smaller numbers of cells (0.5×10^4 and less) were also detectable (approximately one in 2–3), but they were not investigated further. Approximately 90% of the CpG-stimulated mature B-cell clones terminated proliferation shortly after collection and survived as Ki-67^- cells (Supporting Information Fig. 2B

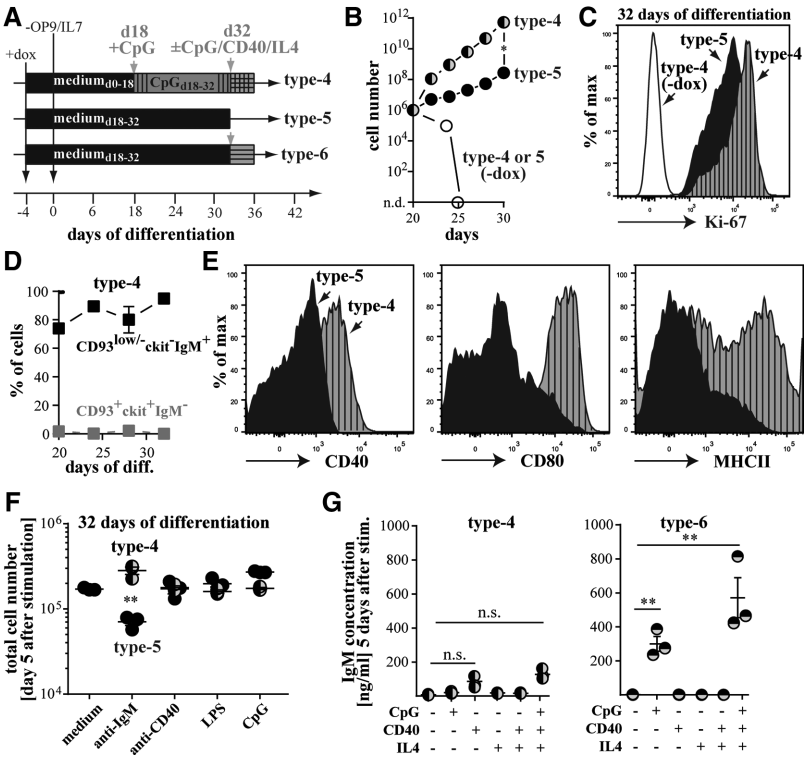

Figure 3. c-myc/bcl-xL-overexpressing immature B cells generated in vitro for 32 days differentiate to IgM-secreting cells upon stimulation with CpG or with CpG and CD40/IL-4. (A) Experimental schedule (see also Fig. 1A). c-myc/bcl-xL-overexpressing pre-BI cells were cultured with CpG (gray filled symbols) either from day 18 or day 32 of differentiation, generating type-4, type-5, or type-6 cell lines, respectively. (B) Proliferation of c-myc/bcl-xL-overexpressing B cells cultured in the presence (closed circles) or absence (open circles) of doxycycline in medium alone (black circles, type-5) or in the presence of CpG from day 18 of differentiation (black/gray circles, type-4) was determined by flow cytometry. (C) Ki-67 staining of type-4 and type-5 c-myc/bcl-xL cells cultured for 32 day with or without doxycycline was assessed by flow cytometry. (D) Summary of FACS analysis illustrating the surface marker expression of $CD19^+CD93^+c\text{-}kit^+IgM^-$ pre-BI cells (gray box) and $CD19^+CD93^{low/-}c\text{-}kit^-IgM^+$ B-lineage cells (black box). (E) CD40, CD80, and MHCII surface marker expression of type-4 and type-5 B cells. Representative flow histograms are shown. (F) Mitogen response 5 days after polyclonal stimulation of type-4 or type-5 cells. Significance was determined comparing CpG-stimulated type-4 to type-5 B cells by two-tailed unpaired *t*-test; $^{**}p < 0.005$. (G) ELISA analysis of secreted IgM in supernatants of 1×10^4 type-4 or type-6 bulk cell populations stimulated for 5 days either with (+) or without (−) CpG and/or CD40 and/or IL-4. Significance was determined comparing nonstimulated (medium) to stimulated cells by two-tailed unpaired *t*-test; $^{**}p < 0.005$, n.s. = not significant. (B, D, F, and G) Data are shown as mean ± SEM and are from a single experiment representative of three to six independent experiments with three samples per experiment.

and C). Ten percent of clones continued CpG-stimulated proliferation with average cell cycle times of 18 h. All surviving and proliferating clones were $CD19^+CD93^{low/-}$, that is, displayed a mature B-cell phenotype. The clones retained this phenotype for additional 32 days of in vitro culture (Supporting Information Fig. 4, type-4). These results surprisingly suggest that a large part of CpG-stimulated mature B cells are no longer capable to proliferate in response to CpG. They appeared to reach a nonproliferating G_0-like cell cycle state that only becomes detectable because clones of these cells gain long-term survival capacity through the overexpression of c-myc and bcl-xL.

CpG stimulation of c-myc/bcl-xL-overexpressing, immature B cells generates IgM secreting cells in vitro

The CpG-dependent development of mature B-cell markers prompted an analysis of their capacity to secrete Ig. Therefore, after 18 days of differentiation, we cultured the differentiated B-lineage cells either in the presence or absence of CpG for another 14 days. Thereafter, CpG, CD40-specific Ab, and/or IL-4 were added for 5 days, generating conditions that have been shown to induce polyclonal mature B cells proliferate and to switch the IgM isotypes to IgG1 and IgE (Fig. 3A) [30]. Five days after stimulation, secreted Ig was monitored in the supernatant medium by ELISA (Fig. 3G).

Immature B cells (type-2) differentiated for 18 days did not secrete significant amounts of Ig under any of the chosen stimulation conditions. Mature B cells (type-4) that differentiated for 18 days without and for 14 days with CpG did not secrete detectable amounts of IgM, IgG, IgE, or IgA nor did they do so after stimulation with CD40-specific antibodies and IL-4. However, surprisingly, 1×10^4 immature B cells that had differentiated for more than 32 days without CpG (type-5), and which were then stimulated for 5 days with CpG, generating a type-6 cell type, were found to secrete 240–380 ng/mL IgM, while IgG or IgE secretion remained undetectable. Furthermore, the combined stimulation by CpG, CD40-specific antibodies, and IL-4 increased the levels of IgM to 440–800 ng/mL. IgG, IgA, and IgE secretion remained undetectable.

We conclude that the differentiation of c-myc/bcl-xL-overexpressing immature B cells continues for several weeks in vitro in the absence of CpG, allowing the development of cells that can be induced by CpG or by combining CpG and CD40-specific antibodies and IL-4 to secrete IgM. Addition of CpG at earlier times, for example, at 18 days of differentiation, appeared to inhibit this capacity to be stimulable by CpG, CD40-specific antibodies, and IL-4 to secrete IgM. Our results point to a surprising capacity of B1 cells stimulated for extended periods of time to develop capacities to secrete antibodies. The cell lines, which we have generated, should be useful tools to study the molecular modes, by which CpG-induced TLR9 signaling develops this capacity of B1 cells to secrete.

c-myc/bcl-xL pre-BI-cell transplantation into immunodeficient RAG$^{-/-}$ mice

When normal fetal liver-derived pre-BI cells are transplanted into RAG$^{-/-}$ mice, they populate the spleen as CD5^{-} and the peritoneum as CD5^{+} B1-cells, but not the BM of the host [31, 32]. Transplantations of c-myc- or bcl-xL-single-expressing pre-BI cells into doxycycline-fed RAG$^{-/-}$ mice do not alter this transplantation behavior [21, 22].

However, when the GFP^{+} c-myc/bcl-xL double-oncogene-expressing pre-BI cells were transplanted here into RAG$^{-/-}$ mice (Fig. 4A), GFP^{+} cells were detected not only in the spleen, but also in the BM of doxycycline-fed mice (Fig. 4B). At least ten- to 15-fold increased numbers of GFP^{+} were detected within 4 weeks, and 100- to 1000-fold higher numbers after 8 weeks of transplantation, when compared to control mice, which received no doxycycline in their drinking water. Most of these GFP^{+} cells were Ki-67^{+}, a sign that the transplanted cells expanded, causing splenomegaly (Fig. 4C and D).

The phenotypes of expanded GFP^{+} c-myc/bcl-xL cells were analyzed by FACS analyses (Fig. 4E). Pre-BI cells, immature B cells, mature B1 cells, and cells with phenotypes resembling plasmablasts and terminally differentiated plasma cells were detected in BM and spleen (Fig. 4E). Notable was the selective, up to 10^4-fold increase of plasmablast- and plasma cell-like cells 8 weeks after transplantation. Consistent with the detection of elevated numbers of plasmablasts and plasma cells, slightly higher than normal levels of IgM (~350 ng/mL) and nearly normal levels of IgA (~100 ng/mL), but only low levels of IgG (~80 ng/mL) were detected (Fig. 4F). Hence, in contrast to the in vitro differentiation of Ig-secreting cells, these c-myc/bcl-xL-overexpressing cells also secreted IgA, but no IgG in vivo. This is in line with previous observations that normal fetal liver-derived pre-BI cells restore normal levels of IgA but no IgG, when transplanted into immunodeficient mice [24].

When the in vivo oncogene expression was terminated 8 weeks after transplantation by removing doxycycline from the drinking water, the expansion of GFP^{+} cells could be reversed within the next 4 weeks (Fig. 4B and E). Hence, the in vivo expanded double oncogene-transduced plasmablast- and plasma cell-like populations were reduced as much as 10^3-fold to low residual levels, that is, below 10^4 cells each in BM and spleen. Thus, proliferation, differentiation, and survival of a large majority of c-myc/bcl-xL-transformed cells depended on oncogene-overexpression. In line with the reduced cell numbers of plasmablast and plasma cells, the levels of secreted IgM and IgA were 100-fold reduced 4 weeks after termination of oncogene-overexpression.

These results suggest that the in vivo environment of the host allows not only the differentiation of oncogene-overexpressing pre-BI cells to differentiated B-cell stages seen in the in vitro development, but also promotes their proliferative expansion and differentiation to Ab-secreting plasmablasts and plasma cells.

Discussion

Fetal liver-derived mouse pre-BI-cell lines as a source of B1-type B-lineage cells [15] are excellent targets for in vitro transductions by retroviral vectors, because they proliferate in the presence of IL7 and on stroma cells [24] or on plate-immobilized recombinant Kit-ligand [33] for long periods of time and do not differentiate. Upon removal of growth-supporting factors, these pre-BI cells terminate proliferation and start to differentiate to immature B cells, which, however, die within a couple of days by apoptosis.

The combined transgenic overexpression of c-myc and bcl-2 was first shown by Vaux et al. [34] to transform mouse pre-B cells, and these studies were later extended to an analysis of multiple stages of B-cell development, suggesting, that synergy of bcl-2 and c-myc effects hyperproliferation of pre-B and B cells and accelerated development of B-lineage tumors, which, in the case of bcl-2/c-myc double transgenic mice might derive from a hematopoietic progenitor cell, maybe a lymphoid-committed progenitor. Our studies suggest that pre-B cells can be the origins of long-term proliferating cell lines, which, upon transplantation are supported by the host environment to develop into plasma cell tumors. The role of bcl-xL instead of bcl-2 was first investigated by Chao et al. [35], who first found that bcl-xL and bcl-2 repress a common pathway of cell death in B-lineage cells.

From Eμ-bcl2-myc transgenic mice Strasser et al. [36, 37] generated cell lines with lymphomyeloid differentiation potential, which need the support of stromal cells to survive in culture. Our cell lines differ from these lines, as they are generated by bcl-xL-myc transduction of more restricted B-lymphoid differentiation potential that, upon differentiation, survive in culture without the help of stromal cells or cytokines as immature, eventually IgM^{+} B cells. Hence, as suggested by Strasser et al., earlier bipotential lymphomyeloid progenitors need additional factors to survive and differentiate in culture, while our experiments show that B-lymphoid-committed cells do not need these factors any longer to survive.

The cell lines, which we have generated by combined overexpression of c-myc and bcl-xL are long lived, but may be not immortal, since they might shorten their telomeres with continued proliferation. In fact, we have previously found that in vitro propagated Pax5-deficient as well as WT pre-B cells shorten their

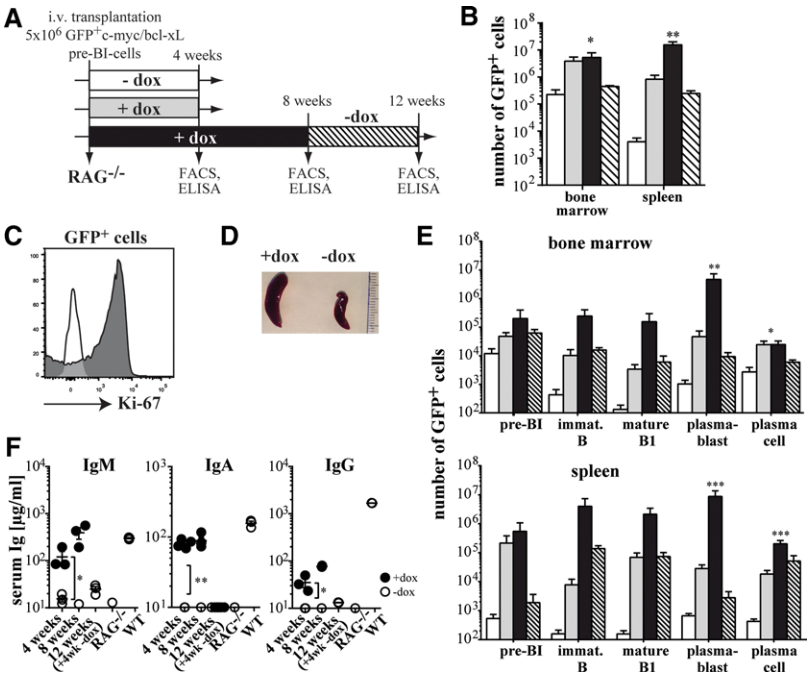

Figure 4. Expansion and differentiation of fetal liver-derived c-myc/bcl-xL-overexpressing pre-BI cells in vivo. (A) Transplantation schedule of GFP$^+$ c-myc/bcl-xL pre-BI cells, cultured on OP9 cells and IL7 in vitro, into doxycycline-fed (+dox, closed bars) and nonfed (-dox, open bars), sublethally irradiated RAG$^{-/-}$ mice. Eight weeks after transplantation, doxycycline was removed from the drinking water of one group of these mice (black/white shaded bars). Four (gray bars), 8 (black bars), and 12 (black/white shaded bars) weeks after transplantation, the recipients were sacrificed, and GFP$^+$ cells from BM (one hind leg = tibia and femur) and spleen were analyzed. (B) Detection of GFP$^+$ c-myc/bcl-xL B-lineage cells from the designated organs at defined time points was performed by FACS. (C) Ki-67 staining of BM-derived GFP$^+$ cells from doxycycline-fed (closed histogram) and nonfed (open histogram) recipients was assessed by flow cytometry 4 weeks after transplantation. (D) Spleens from doxycycline-fed and nonfed recipients, 4 weeks after injection of GFP$^+$ c-myc/bcl-xL pre-BI cells. (E) Cell surface expression analysis of BM- and spleen-derived GFP$^+$ cells: gating for CD19$^+$CD93$^+$c-kit$^+$sIgM$^-$ pre-BI cells, CD19$^+$CD93$^+$c-kit$^-$sIgM$^+$ immature B-, CD19$^+$CD93$^-$c-kit$^-$sIgM$^+$ mature B1 cells, CD19$^{+/low}$CD138$^+$sIgM$^+$IgκL$^+$MHCII$^+$TACI$^-$ plasmablast, and CD19$^{low/-}$CD138$^+$sIgM^{low}/$^-$IgκL$^{low/-}$MHCII$^-$TACI$^+$ plasma cells. (F) ELISA analysis of IgM, IgA, and total IgG detected in sera of transplanted mice at the designated time points after transplantation. Sera from RAG$^{-/-}$ and WT C57BL/6 were used as controls. (B, E, and F) Data are shown as mean ± SEM and are representative of three independent experiments with $n = 3$ mice per experiment. Statistical significance was determined by one-way ANOVA (B, E) and two-tailed unpaired t-test (F); $^*p < 0.05$, $^{**}p < 0.005$, $^{***}p < 0.0005$.

telomeres by 70–90 bp per division, while they do not express sufficient telomerase activity to make up for this loss [38]. Since mouse telomeres are 60 kb long, we have previously estimated that such cell lines could divide 600 times, that is, at division times of 18 h for more than a year, before they lose their capacity to proliferate.

It is interesting to note that our fetal liver pre-B-cell-derived immature sIgM$^+$ B-cell lines have kappa-L- and lambda L-Ig gene loci rearranged in expected frequencies (around 90%:10%), but do not continue in culture to rearrange in high frequencies kappa-L- and lambda-L-Ig gene loci in secondary rearrangements, as it has been found with bcl-2 transgenic immature B cells [39], although they continue to express lower, but detectable levels of RAG1 and 2 (unpublished). This differential behavior of immature B cells could be due to the difference in oncogene expression (bcl-xL versus bcl-2), a difference of B-lineage (B1 versus B2), or a difference of in vitro (this study) versus in vivo (BM derived) isolated immature B cells [39]. Future studies will have to investigate this differential behavior by comparison of bcl-2 with bcl-xL single, or with bcl-2 or bcl-xL-myc double transgenic immature B cells from fetal liver and BM ex vivo/in vitro from the appropriate transgenic mice, or with immature B cells generated from fetal liver-derived or BM-derived double transgenic pre-B-cell lines, once they become available.

By overexpressing c-myc and bcl-xL in differentiating pre-BI-cell lines, and timed stimulation with CpG, we here present a new method that has allowed us to establish stable, long-term surviving and proliferating immature B (type-2), pre-BII-like (type-3), mature B cell (type-4) cell lines, and IgM-secreting cell s (type-6) from fetal liver-derived B1-lineage pre-BI cells (Fig. 5). Thus, our study provides a unique protocol to generate cell lines representing various stages of B1-cell development, which offer a system to study the ontogeny of these cells.

We have recently been able to establish pre-BI-cell lines from the BM as possible source for conventional B2-type B cells [33]. This oncogene-overexpression will allow us in the future to compare the developments of oncogene-transduced B1- and B2-type B cells, their repertoire selection, contribution to autoimmunity and autoimmune diseases, and to the development of different B-cell leukemias.

CpG stimulation was previously found to develop IgM-secreting B cells from ex vivo cultured spleen- or blood-derived transitional, naïve mature, or memory conventional B2-type B cells [40–42].

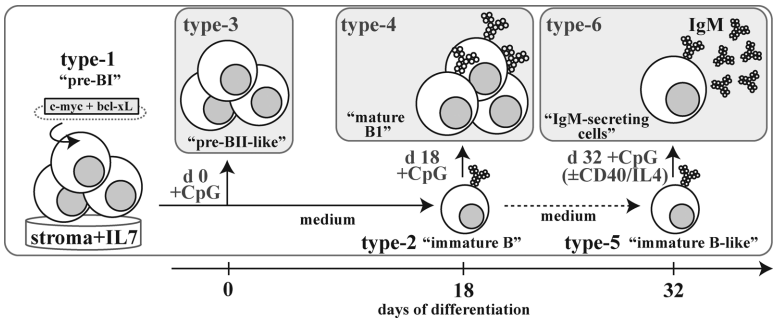

Figure 5. Generation of different cell lines from in vitro differentiating c-myc/bcl-xL-overexpressing fetal liver-derived B1-type pre-BI cells. After initial stimulation, the pre-BII-like cells (type-3), mature B1-cells (type-4), and IgM-secreting B1-cells (type-6) were continuously cultured in the presence of CpG.

Our studies expand the spectrum of CpG-reactive B-lineage cells to B1-lineage pre-B cells, mature and immature B cells with capacities to differentiate to Ig-secreting B cells in vitro and in vivo. Future experiments should attempt to overexpress the two oncogenes in precursors B cells of marginal zone and conventional B2-type B cells to evaluate possible functional differences of pre-BII-like, mature and immature B and Ig-secreting cells developed from them.

Normal B-cell development from pre-BI cells to Ig-secreting plasma cells occurs, in considerable parts, in resting cells without proliferation [28]. In pre-B cells, functional Ig μ heavy chains assemble with SLC as pre-B-cell receptors (preBcR), which are deposited on the cell surface [43]. PreBcRs stimulate a proliferative burst of large pre-BII-cells, which comes to rest, as SLC expression is turned off, and preBcR expression is terminated. This proliferative burst does not occur, when pre-BI cells are induced in vitro to differentiate. In fact, these differentiating cells die rapidly by apoptosis. Furthermore, immature and naïve mature B cells have short half-lives even in vivo [44]. We find that co-overexpression of c-myc and bcl-xL rescues differentiating B-lineage cells from this apoptosis in vitro. Most of these c-myc/bcl-xL-overexpressing, differentiating cells remain resting in vitro for at least a week. A small subpopulation of the immature B-cell pool begins to expand by proliferation in vitro. The small numbers of proliferating immature B cells might carry retroviral inserts at sites in the genome that control proliferation and that are activated by the inserts. Since we have been able to separate these proliferating, eF670-dye-diluting cells from these nonproliferating, eF670-dye-retaining cells, future experiments should be able to identify such retroviral inserts in the proliferating immature B-cell clones and determine the nature of affected genes and their functions.

Immature B cells react to autoantigenic stimulation by apoptosis [28]. In this process of negative repertoire selection, the autoreactive B cells are deleted, establishing central tolerance. We have previously seen that IgM^+ immature B cells are induced to apoptosis by IgM-specific antibodies [26]. Here we show that c-myc/bcl-xL-overexpressing $sIgM^+$ immature B cells are inhibited from further expansion by IgM-specific antibodies in vitro. It suggests that they perceive sIgM-mediated stimulation as negative signals. Since we have been able to clone individual, that is, mAb-producing B-lineage cells, future experiments with a collection of such clones could determine their recognition of foreign Ags and autoantigens. This would allow testing a possible positive or negative reaction to foreign or autoantigens to individual B-cell clones in vitro and in vivo and the influence of IgM B-cell receptor (BCR) on the in vivo development of plasma cell tumors after transplantation. Such experiments might be able to shed light on the role of the BCR expressed in B1 cells in development of Ab repertoires, of B-cell- and Ab-mediated autoimmune disease, and the development of B-cell malignancies.

Materials and methods

Cell culture

Pre-BI cells were isolated from fetal liver of C57BL/6 mice on day 18 of gestation as previously described [21]. The rtTA-transduced pre-BI cells, OP9 stroma cells, and Plat-E packaging cell line (Life Technologies) were cultured, transfected, transduced, and differentiated as previously described [21, 45]. Differentiation of pre-BI cells was induced upon removal of OP9 stroma cells and IL7. Stimulation of 0.5×10^6 cells with 0.1 μM CpG ODN 1826 (Miltenyi Biotec), 2% recombinant IL-4 supernatant (in house [46]), LPS, CD40- (clone FGK), or IgM-specific Ab was performed as described previously [21]. For proliferation assay, the cells were labeled with eF670 proliferation dye as recommended by the manufacturer (eBioscience).The Ki-67 staining was performed as recommended by the manufacturer (eBioscience).

Cell cycle analysis

After 2 days differentiation, 1×10^6 cells were fixed in 70% ice-cold ethanol at −20°C and stained with 10 μg/mL DAPI (Roth) in PBS. DAPI was recorded in linear mode by FACS and cells in S/G2/M phases were gated manually.

AnnexinV staining

On day 2 of differentiation, doxycycline-treated and nontreated cells were stained with AnnexinV-Cy5 (BD Pharmingen) according manufacturer's protocol.

Vectors

Doxycycline-inducible, "TreTight"-TetOn-controlled [23] SIN retroviral vectors encoding c-myc and/or bcl-xL were constructed as reported [21, 22] (Supporting Information 1).

Western blot and flow cytometry

Western blot and flow cytometry were performed as previously described [21, 22]. For c-Myc detection, the membranes were probed with mouse anti-c-Myc (clone 9E10, Santa Cruz Biotech), while bcl-xL detection was performed with rabbit anti-bcl-xL (polyclonal, Cell Signaling).

Semiquantitative and quantitative real-time RT-PCR

Total RNA was purified using TRIzol® Reagent (Life Technologies). RNA was reverse transcribed using SuperScript III reverse transcriptase (Life Technologies). RT-PCR was performed using Taq DNA Polymerase (Thermo Fisher Scientific) according to manufacturer's protocol. Quantitative RT-PCR was conducted with QuantiTect SYBR Green PCR Kit (Qiagen). Amplification was performed using following primers:

Myc-hybr (Supporting Information 1) 5′-CGGATTCTCTGCTC TCCTCGAC-3′ and 5′-CGTCGAGGAGAGCAGAGAATCC-3′,
Gapdh 5′-TTGAGGTCAATGAAGGGGTC-3′ and 5′-TCGTCCCG TAGACAAAATGG-3′,
MyD88 5′-CACTCGCAGTTTGTTGGATG-3′ and 5′-CACTCGCAG TTTGTTGGATG-3′,
TLR4 5′-CAAGGGGTTGAAGCTCAGAT-3′ and 5′-TTCTTCTCC TGCCTGACACC-3′,
TLR9 5′-GGCTTCAGCTCACAGGGTAG-3′ and 5′-GAATCCTCC ATCTCCCAACA-3′,
SLC 5′-CCGTGGGATGATCTGGAACA-3′ and 5′-TCAGCAGAA AGGAGCAGAGC-3′

Transplantation of pre-BI and differentiated cells into RAG$^{-/-}$ recipients

All animal procedures were conducted in compliance with the German animal protection laws with the protocol approved by the Landesamt fur Gesundheit und Soziales, Berlin (G0140-11) as previously described [22].

ELISA

Ig levels of cell culture supernatants and serum samples, compared with standard Ig class samples, were determined with Maxisorp ELISA 96-well plates coated with goat antimouse IgM or total IgG as prescribed by Southern Biotech, Birmingham AL, USA. Using AP-labeled goat antimouse IgM-AP, or total IgG-AP Ab (Southern Biotec) the serum Igs were quantified.

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Abbreviations: **preBcR**: pre-B-cell receptor · **rtTA**: reverse transactivator · **sIgM**: surface IgM · **SLC**: surrogate light chain

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