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Combined loss of PUMA and p21 accelerates c-MYC-driven lymphoma development considerably less than loss of one allele of *p53*

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Running title: Combined role of PUMA and p21 in lymphoma suppression

Abstract

The tumor suppressor p53 is mutated in ~50% of human cancers. P53 is activated by a range of stimuli and regulates several cellular processes, including apoptotic cell death, cell cycle arrest, senescence and DNA repair. P53 induces apoptosis via transcriptional induction of the BH3-only proteins PUMA and NOXA and cell cycle arrest via p21. Induction of these processes was proposed to be critical for p53-mediated tumor suppression. It is therefore surprising that mice lacking PUMA, NOXA and p21 as well as mice bearing mutations in p53 that impair the transcriptional activation of these genes, are not tumor prone, unlike mice lacking *p53* function, which spontaneously develop tumors with 100% incidence. These p53 target genes and the processes they regulate may, however, impact differently on tumor development depending on the oncogenic drivers. For example, loss of PUMA enhances c-MYC-driven lymphoma development in mice but, interestingly, the acceleration was less impressive compared to that caused by the loss of even a single *p53* allele. Different studies have reported that loss of p21 can either accelerate, delay or have no impact on tumorigenesis. In an attempt to resolve this controversy we examined whether loss of p21-mediated cell cycle arrest cooperates with PUMA deficiency in accelerating lymphoma development in *Eμ-Myc* mice (over-expressing c-MYC in B lymphoid cells). We found that *Eμ-Myc* mice lacking both p21 and PUMA (*Eμ-Myc;Puma*$^{-/-}$*;p21*$^{-/-}$) developed lymphoma at a rate comparable to *Eμ-Myc;Puma*$^{-/-}$ animals, notably with considerably longer latency than *Eμ-Myc;p53*$^{+/-}$ mice. Loss of p21 had no impact on the numbers, cycling or survival of pre-leukemic *Eμ-Myc* B lymphoid cells, even when PUMA was lost concomitantly. These results demonstrate that even in the context of deregulated c-MYC expression, p53 must suppress tumor development by activating processes apart from, or in addition to PUMA-mediated apoptosis and p21-induced cell cycle arrest.

Introduction

The tumor suppressor p53 and the effector processes that are activated by this transcription factor impose a critical barrier to tumor formation [1]. p53's essential role in blocking the progression of nascent neoplastic cells to malignancy is evidenced by the fact that ~50% of spontaneous human cancers bear mutations in p53, the majority residing in its DNA binding domain. Moreover, another ~40% of human cancers contain other abnormalities that impair the p53 signaling pathway, for example over-expression of negative regulators of p53, such as MDM2 (Mouse Double Minute 2, called HDM2 in humans), or mutation or epigenetic silencing of downstream target genes, such as *Puma* [2-4]. Accordingly, mice deficient for p53 function develop thymic lymphoma or more rarely sarcoma with 100% incidence by ~280 days of age [5, 6].

In nascent neoplastic cells, p53 is activated in response to oncogene activation, such as deregulated expression of c-MYC or mutant RAS [7]. Activation of these onco-proteins triggers expression of the tumor suppressor p14ARF (Alternative Reading Frame; human)/p19ARF (mouse), which disrupt the p53-MDM2 interaction. This prevents ubiquitination and proteasomal degradation of p53, leading to p53 accumulation with consequent effector pathway activation [8-10].

p53 plays a critical tumor suppressive role in the development of cancers driven by deregulated expression of c-MYC*,* which promotes aberrant cell proliferation. For example, loss of a single allele of *p53* substantially accelerates lymphoma development in *Eμ-Myc* mice [11, 12], which over-express c-MYC in their B lymphoid cells [13]. Moreover, defects in the ARF-MDM2-p53 pathway are selected for in ~20% of lymphomas that arise spontaneously in *Eμ-Myc* mice [14].

Loss of the BH3-only protein, PUMA (*p53*-upregulated modulator of apoptosis), a direct transcriptional target of p53 that is critical for its ability to kill cells [15, 16], substantially accelerates c-MYC-driven lymphoma development [11, 12]. However, lymphoma onset in *Eμ-Myc* mice lacking PUMA and even in those lacking both PUMA and NOXA, another pro-apoptotic BH3-only protein that is a direct transcriptional target of p53, is still considerably slower compared to *Eμ-Myc;p53*$^{+/-}$ mice [11]. This suggests that processes in addition to PUMA- and NOXA-mediated apoptosis must contribute to p53-dependent suppression of *Eμ-Myc* induced lymphoma development.

Unrestrained cellular proliferation is a hallmark of cancer [17]. The cyclin-dependent kinase (CDK)-inhibitor p21 (protein 21 KDa, also called *CIP1* or *WAF1*) is a downstream target of p53 that is critical for its ability to induce G1/S boundary cell cycle arrest and

senescence (although other p53 target genes are also critical for the latter, e.g. *PML*, *PAI-1*) [18]. Surprisingly, despite p21's critical role in restraining cellular proliferation, p21-deficient mice are not tumor prone [18, 19] and mutations in the *p21* gene are only rarely found in human cancers [20-22]. The role of p21 in tumorigenesis therefore remains controversial. Various studies using carcinogens or transgenic mice expressing different oncogenes reached discordant conclusions, namely that p21 can either suppress or promote tumor development, or in some cases it had no discernible impact. For example, in chemical carcinogen-induced models [23, 24], the MMTV-Ras–driven mammary adenocarcinoma mouse model [25] and in the mutant APC–driven model of intestinal cancer (*APC1638*$^{+/-}$ mice) [26] loss of p21 was reported to accelerate tumorigenesis. This indicates that at least in these contexts p21 functions as a tumor suppressor. In contrast, loss of p21 was found to delay thymic lymphoma development caused by loss of the DNA-damage response activator ATM [27], or by low dose γ-radiation [28]. Similarly, loss of p21 delayed tumor development in the murine MMTV-MYC mammary cancer model [29]. Thus, in these settings p21-mediated cell cycle arrest may promote tumorigenesis. Finally, in a murine leukemia virus-induced T cell lymphoma model [30] and in c-MYC-driven pre-B/B lymphomagenesis loss of p21 had no impact on the rate of tumor development [19].

Studies using gene-targeted mice revealed that cooperation between p21-induced cell cycle arrest and induction of apoptosis is critical for p53-mediated tumor suppression [31]. It was also suggested that expression of p21 and consequent induction of cell senescence is required to suppress c-MYC-driven tumorigenesis when p53-mediated apoptosis is impaired [32]. However, mice lacking p21 plus the essential mediators of p53-mediated apoptosis, PUMA and NOXA (*Puma*$^{-/-}$*;Noxa*$^{-/-}$*;p21*$^{-/-}$) [33] and mice bearing mutations in p53 that impair its ability to induce these target genes [34] were not abnormally tumor prone, the latter animals even in context of expression of oncogenes, such as mutant *Ras*. In order to directly test the overlapping roles between p21–mediated cell cycle arrest/senescence and PUMA-mediated apoptosis in the suppression of c-MYC-driven lymphomagenesis we generated *Eμ-Myc;Puma*$^{-/-}$*;p21*$^{-/-}$ mice. The analysis of these animals revealed that loss of p21 had no impact on the pre-leukemic state, rate of lymphoma development and disease severity imparted by deregulated c-MYC expression even when PUMA was also absent. This demonstrates that even in the context of the potent oncogenic driver, c-MYC, p53 must suppress

tumorigenesis through processes in addition to induction of apoptosis and cell cycle arrest/senescence.

Results

Expression of p21 is increased upon DNA damage in pre-leukemic Eμ-Myc B lymphoid cells

The *Eμ-Myc* mice used in this study overexpress c-MYC in B lymphoid cells and have been employed extensively to study the impact of defects in the p53-pathway in tumorigenesis [11, 12, 14]. Since there is considerable controversy on whether de-regulated c-MYC expression is able to repress the induction of the cell cycle inhibitor p21, we investigated the impact of c-MYC overexpression on p21 protein expression in pre-leukemic B lymphoid ($CD19^+$) cells. These cells were isolated from the bone marrow of 4-week old *Eμ-Myc* mice (before they present with malignant lymphoma) and compared to B lymphoid cells from wt animals. Western blot analysis revealed that after DNA damage elicited by treatment with etoposide, p21 was induced in the *Eμ-Myc* B lymphoid cells at least to the same extent as in wt B lymphoid cells (Figure 1). PUMA protein could be readily detected in etoposide treated B lymphoid cells from both *Eμ-Myc* and wt B lymphoid cells but, as previously reported [11], PUMA levels were already high in B lymphoid cells from *Eμ-Myc* mice (Figure 1). These data show that deregulated c-MYC expression does not prevent the DNA damage induced increase in p21 expression in B lymphoid cells. They also show that deregulated c-MYC expression causes an increase in PUMA but not p21 expression in pre-leukemic B lymphoid cells.

Loss of p21 does not further accelerate the onset of lymphoma in Eμ-Myc mice beyond the impact of loss of PUMA

MYC-driven lymphoma development is dramatically accelerated by loss of p53 [11, 14, 35]. Loss of PUMA, the major mediator of p53-induced apoptosis, accelerates lymphoma development in *Eμ-Myc* mice to a considerably lesser extent than loss of even a single allele of *p53*. We therefore examined the combined impact of loss of the cyclin dependent kinase inhibitor, p21, and the critical p53 activated apoptosis initiator, PUMA, on lymphoma development by generating *Eμ-Myc;Puma*$^{-/-}$*;p21*$^{-/-}$ mice. Consistent with previous reports [11, 12], loss of PUMA significantly accelerated lymphoma development in *Eμ-Myc* mice (median survival, *Eμ-Myc*: 163 days, *Eμ-Myc;Puma*$^{-/-}$: 96 day p=0.007; Figure 1). In contrast, and consistent with a previous publication [19], loss of p21 did not

significantly alter the rate of lymphoma development in *Eμ-Myc* mice (median survival: 274 days, p=0.33 compared to *Eμ-Myc* mice; Figure 2). Interestingly, the median lymphoma-free survival of *Eμ-Myc;Puma$^{-/-}$;p21$^{-/-}$* (93 days) was comparable to that of *Eμ-Myc;Puma$^{-/-}$* mice (96 days, p=0.73; Figure 2). These results demonstrate that loss of p21 does not cooperate with loss of PUMA to accelerate MYC-driven lymphoma development.

Impact of combined loss of p21 and PUMA on lymphoma burden in Eμ-Myc mice

We next investigated the impact of combined loss of p21 and PUMA on lymphoma burden in sick *Eμ-Myc* mice by measuring spleen weights and lymphocyte counts in peripheral blood. Spleen weights and peripheral lymphocyte counts in sick *Eμ-Myc;Puma$^{-/-}$;p21$^{-/-}$* mice did not differ significantly from those observed in lymphoma-burdened *Eμ-Myc;Puma$^{-/-}$* mice (p=0.11 and p=0.06, respectively) (Figure 3A). Immunophenotyping of lymphomas from sick *Eμ-Myc;Puma$^{-/-}$* and *Eμ-Myc;Puma$^{-/-}$;p21$^{-/-}$* animals showed similar percentages of surface immunoglobulin sIg^{-} pre-B and sIg^{+} B cell lymphomas (Figure 3B).

Impact of combined loss of p21 and PUMA on pre-leukemic pre-B cells and sIg^{+} B cells in Eμ-Myc mice

In *Eμ-Myc* mice lymphomas arise from abnormally cycling pre-B cells [36, 37]. Loss of PUMA enhances the survival of these pre-leukemic cells, increasing their risk of acquiring collaborating oncogenic lesions, and thereby accelerates c-MYC-driven lymphomagenesis [11, 12]. We investigated the impact of combined loss of p21 and PUMA on the pre-leukemic B lymphoid cells in *Eμ-Myc* mice. The numbers of pre-leukemic pre-B cells and sIg^{+} B cells in the bone marrow of young (~4 week-old; i.e. before they present with malignant, clonal lymphoma) *Eμ-Myc;Puma$^{-/-}$;p21$^{-/-}$* mice were not significantly different from those of *Eμ-Myc;Puma$^{-/-}$* mice ($p_{pre\text{-}B}$=0.33; $p_{sIg\text{-}B}$=0.99; Figure 4A). The pre-leukemic pre-B cells and sIg^{+} B cells from *Eμ-Myc* mice die more rapidly in culture compared to the corresponding cells from wt mice [38] and this cell death can be greatly delayed by loss of PUMA [11] or over-expression of pro-survival BCL-2 [38, 39]. The pre-leukemic pre-B cells and sIg^{+} B cells from young *Eμ-Myc;Puma$^{-/-}$;p21$^{-/-}$* mice did not survive significantly better at 24 and 48 h in simple tissue culture medium (no added cytokines) when compared to the corresponding cells from pre-leukemic *Eμ-Myc;Puma$^{-/-}$* mice (p_{24h}=0.29; p_{48h}=0.45; Figure 4B-C).

The CDK inhibitor p21 inhibits cell cycle progression [18]. We therefore examined the impact of loss of p21 alone or in combination with loss of PUMA on the distribution of pre-leukemic *Eμ-Myc* pre-B and sIg^+ B cells in the G1, S and G2/M phases of the cell cycle. Young *Eμ-Myc* mice of all genotypes tested presented with more pre-leukemic pre-B cells and sIg^+ B cells in the S and G2/M phases of the cell cycle compared to wt mice (Figure 4D). Notably, there was no significant difference in cell cycle distribution between pre-B cells and sIg^+ B cells from *Eμ-Myc, Eμ-Myc;p21*$^{-/-}$, *Eμ-Myc;Puma*$^{-/-}$ and *Eμ-Myc;Puma*$^{-/-}$*;p21*$^{-/-}$ mice (Figure 4D). These results demonstrate that loss of p21 does not alter the cycling of pre-leukemic B lymphoid cells in *Eμ-Myc* mice on its own or even when PUMA is also lost.

DISCUSSION

Over-expression of c-MYC is observed in ~70% of all human cancers; therefore understanding the tumor suppressive mechanisms that curtail c-MYC-driven tumorigenesis may provide insight for improved treatment of such cancers. p53 potently suppresses c-MYC-induced lymphoma development [11, 14, 35].

p53 activates several tumor suppressive processes, including apoptosis, cell cycle arrest, senescence and DNA repair [4]. The importance of p53-mediated apoptosis in suppressing lymphoma development in *Eμ-Myc* mice is well established. Loss of PUMA or combined loss of PUMA and NOXA, the essential mediators of p53-induced apoptosis [15, 16], accelerate lymphoma development in *Eμ-Myc* mice, albeit to a substantially lesser extent than loss of even a single allele of *p53* [11, 12]. Hence loss of other p53-related processes beyond loss of apoptosis induction by PUMA (and NOXA) must be involved to achieve this substantial acceleration in c-MYC-driven lymphomagenesis caused by loss of only one allele of p53. The p53 target p21 induces G1/S boundary cell cycle arrest and/or senescence upon stress signals, such as DNA damage. Much controversy exists in regards to whether p21 functions as a tumor suppressor or a tumor promoter (or has no impact) in general and in the context of deregulated c-MYC expression. We found that upon DNA damage the levels of p21 were markedly increased in pre-leukemic B lymphoid cells from *Eμ-Myc* mice, demonstrating that deregulated c-MYC expression does not prevent the induction of this cell cycle inhibitor. Interestingly, deregulated c-MYC expression on its own caused an increase in PUMA but not p21. Since the *Puma* and *p21* genes are both direct p53 targets [40], this finding suggests that their expression must also be affected by regulators in addition to p53.

Consistent with a previous report we found that loss of p21 on its own had no impact on lymphoma development in *Eμ-Myc* mice [19]. Moreover, we found that combined loss of p21 and PUMA accelerated c-MYC-induced lymphomagenesis no more than loss of PUMA alone. Remarkably, lymphoma development in *Eμ-Myc;Puma*$^{-/-}$*;p21*$^{-/-}$ mice was much slower compared to *Eμ-Myc;p53*$^{+/-}$ mice. In a similar vein, it was found that impaired induction of both PUMA and p21 (due to a mutation in *p53*, *p53*^{25,26}) accelerated c-MYC-driven lymphoma development considerably less than loss of one allele of *p53* [41]. The inability of loss of p21 to cooperate with PUMA-deficiency in accelerating c-MYC-driven lymphomagenesis may be explained by the finding that c-MYC over-expression down-regulates p21 levels [42]. However, we found that p21 can still be induced in cells with deregulatred c-MYC expression, at least in the context of DNA damage (see above). Moreover, it was previously shown that *Eμ-Myc* mice bearing the R172P mutant p53, which is defective in *Puma* induction but could still transcriptionally activate p21, exhibited slower lymphoma onset than *Eμ-Myc;p53*$^{+/-}$ mice (although still faster than *Eμ-Myc* controls). The lymphomas in the *Eμ-Myc;p53* (R172P mutant) mice exhibited increased p21 expression and bore markers of senescence, suggesting that c-MYC-overexpression is unable to block p21 induction in these cells [32].

So, the question remains, which processes in addition to PUMA-induced apoptosis contribute to p53-mediated tumor suppression in *Eμ-Myc* mice? p53 has been shown to also regulate DNA repair, cellular metabolism and cell division through mechanisms not involving p21 [43, 44]. It appears likely that some of these processes cooperate with p53-induced apoptosis in tumor suppression in the context of deregulated c-MYC expression and possibly also in the context of other drivers of neoplastic transformation. In conclusion, this work and previous studies on spontaneous tumor development [33, 34, 45] demonstrate that p53 must suppress tumorigenesis by activating a broad range of cellular processes, where the relative contributions of apoptosis, cell cycle arrest, senescence and other processes may vary depending on the nature of the oncogenic driver and cell type.

Authorship contributions

LV, SG, AJ and AS conceived the study, planned experiments, interpreted results and prepared the manuscript. LV, AJ, CV and SG conducted the majority of experiments.

Declaration of Conflict of Interest

The authors declare no conflict of interest.

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Figures

Figure 1. ***Expression of p21 is increased upon DNA damage in pre-leukemic Eμ-Myc B lymphoid cells***

Western blot analysis of p21, PUMA and β-actin (loading control) on extracts from pre-leukemic B lymphoid ($CD19^+$) cells from the bone marrow of *Eμ-Myc* mice and control B lymphoid cells from wt mice, that had been left untreated (-) or (+) treated for 6 h with etoposide.

$CD19^+$ B cells were isolated from the bone marrow of two wt and two *Eμ-Myc* mice (all 4 week-old) by MACS cell separation (Miltenyi Biotech, Bergisch Gladbach, Germany) using CD19 MicroBeads and LS columns according to the manufacturer's protocols. Cells were cultured at 1.5 $x10^6$/mL for 6 h with 50 μM Q-VD-OPH (MP Biomedicals; caspase inhibitor to prevent cell degradation) alone or plus 1 μM etoposide (Sigma).

Protein extracts were prepared by lysis in RIPA buffer (300 mM NaCl, 2% IGEPAL CA-630, 1% deoxycholic acid, 0.2% SDS, 100 mM Tris-HCl pH 8.0) containing complete ULTRA protease inhibitors (Roche, Basel, Switzerland) and 1 mM PMSF (Sigma). Western blotting was carried out using 25 µg total protein per sample run on NuPAGE Bis-Tris gels (Life Technologies). Proteins were transferred onto nitrocellulose membranes with an iBlot (Life Technologies) according to the manufacturer's protocols. Blots were probed with antibodies against p21 (rabbit polyclonal ab7960, Abcam), PUMA (rabbit polyclonal ab27669, Abcam) and β-actin (mouse monoclonal AC-74, Sigma-Aldrich; loading control).

Figure 2. ***Loss of p21 does not further accelerate lymphoma development in Eμ-Myc;Puma$^{-/-}$ mice.***

Kaplan-Meier survival curves showing rate of lymphoma development in *Eμ-Myc* transgenic mice of the indicated genotypes. Median survival *Eμ-Myc* = 163 days; *Eμ-Myc;p53*$^{+/-}$= 32 days (p<0.0001 *vs Eμ-Myc*); *Eμ-Myc;Puma*$^{-/-}$ = 96 days (p=0.007 *vs Eμ-Myc*); *Eμ-Myc;p21*$^{-/-}$ = 274 days (p=0.33 *vs Eμ-Myc*), *Eμ-Myc;Puma*$^{-/-}$*;p21*$^{-/-}$ = 93 days, p=0.0002 *vs Eμ-Myc*). There was no significant difference in lymphoma onset between *Eμ-Myc;Puma*$^{-/-}$ and *Eμ-Myc;Puma*$^{-/-}$*;p21*$^{-/-}$ mice (p=0.73). The numbers of *Eμ-Myc, Eμ-Myc;p21*$^{-/-}$, *Eμ-Myc;Puma*$^{-/-,}$ *Eμ-Myc;Puma*$^{-/-}$*;p21*$^{-/-}$ and *Eμ-Myc;p53*$^{+/-}$ mice followed were 28, 26, 16, 30 and 7, respectively.

Experiments with mice were conducted according to the guidelines of the Walter and Eliza Hall Institute of Medical Research Animal Ethics Committee. The *Eμ-Myc* [13], *Puma*$^{-/-}$ [15], *p21*$^{-/-}$ [46] and *p53*$^{-/-}$ [6] mice, females and males, were all maintained on a C57BL/6 background. Protocols for PCR-based genotyping of these mice will be provided upon request. Animal survival data were plotted using Kaplan-Meier curves and compared using Log-Rank Mantel-Cox test. P values of <0.05 were considered significant.

Figure 3. ***Lymphoma burden in sick Eμ-Myc mice of the different genotypes.***

(A) Spleen weights and lymphocyte counts in the blood of sick *Eμ-Myc* transgenic mice of the indicated genotypes. Lymphocyte counts were determined by using the Advia automated hematology system (Bayer). Data represent mean cell counts±SEM. Each circle represents the lymphocyte count or spleen weight from a single mouse. No significant differences were observed in blood lymphocyte counts or spleen weights between sick *Eμ-Myc;Puma*$^{-/-}$*;p21*$^{-/-}$ and *Eμ-Myc;Puma*$^{-/-}$ mice (p=0.11 and p=0.06,

respectively; as determined by unpaired t-test analysis). (B) The proportions of sIg^- pre-B and sIg^+ B cell lymphomas in sick *Eμ-Myc* mice of the indicated genotypes. The numbers of *Eμ-Myc, Eμ-Myc;p21*$^{-/-}$, *Eμ-Myc;Puma*$^{-/-}$ and *Eμ-Myc;Puma*$^{-/-}$*;p21*$^{-/-}$ lymphomas analyzed were 10, 5, 7 and 10, respectively.

Lymphoid organs from lymphoma-burdened mice were harvested, and single-cell suspensions prepared using 100 μM sieves (BD BioSciences, San Jose, CA). Cells (5×10^4) were stained for surface markers using fluorochrome–conjugated (fluorescein isothiocyanate, APC, R-phycoerythrin; Life technologies, Mulgrave, VIC, Australia) monoclonal antibodies to B220 (RA3-6B2), IgM (5.1), IgD (11-26C) and C-KIT (ACK4) for 30 min in balanced salt solution supplemented with 2% fetal calf serum (Life Technologies, Mulgrave, VIC, Australia). Cells were analyzed in a FACS-Calibur (BD BioSciences, San Jose, CA). Cell counts were determined using a CASY counter (BD BioSciences, San Jose, CA).

Figure 4*. Impact of combined loss of PUMA and p21 on the accumulation, survival and cycling of pre-leukemic Eμ-Myc pre-B cells*

Pre-leukemic analysis was conducted on 4-5 week old mice, females or males, of the indicated genotypes. The numbers of *Eμ-Myc, Eμ-Myc;p21*$^{-/-}$, *Eμ-Myc;Puma*$^{-/-}$*, ,Eμ-Myc;Puma*$^{-/-}$*;p21*$^{-/-}$*, and* wt mice analyzed were 3, 3, 3, 4 and 4, respectively, and the mean ±SEM are indicated. **(A)** Enumeration of pre-B cells and sIg^+ B cells in the bone marrow (data presented per femur) of mice of the indicated genotypes. **(B)** *In vitro* survival assay of FACS sorted pre-B cells ($B220^+C\text{-}KIT^-sIg^-$) and sIg^+ B cells ($B220^+C\text{-}KIT^-IgM/IgD^+$) in normal medium without addition of cytokines. Cell survival was measured at 0, 24 and 48 h by PI staining and flow cytometric analysis. **(C)** *Eμ-Myc;Puma*$^{-/-}$*;p21*$^{-/-}$ pre-B cells and sIg^+ B cells did not survive significantly better compared to *Eμ-Myc;Puma*$^{-/-}$ pre-B or sIg^+ B cells (pre-B cells: p_{24h}=0.22; p_{48h}=0.94; sIg^+ B cells: p_{24h}=0.29; p_{48h}=0.45; as determined by unpaired t-test analysis). Flow cytometric analysis was performed as indicated in Figure 2. For cell sorting and cell survival assays, bone marrow cells were extracted by flushing both femurs with PBS containing 10% FCS. Red blood cells were depleted prior to staining with surface marker specific antibodies (see above) and cell sorting was performed on a BD FACSAriaIII (BD BioSciences, San Jose, CA). Cells were plated at $5x10^5$ cells/well in DMEM containing 10% FCS and 50 μM β-mercaptoethanol without addition of cytokines. Cell viability was assessed by staining with propidium iodide (2 μg/mL) and analysis on a FACS-Calibur

(BD BioSciences, San Jose, CA). **(D)** Cell cycle analysis of pre-leukemic pre-B cells and sIg^+ B cells from 3-5 week-old mice of the indicated genotypes. No significant differences in cell cycle distribution were observed (as determined by unpaired t-test analysis). Pre-B cells from *Eμ-Myc* (G1=43%; S=48%; G2/M=8%)*; Eμ-Myc;Puma*$^{-/-}$ (G1=51%; S=42%; G2/M=5%); *Eμ-Myc;p21*$^{-/-}$ (G1=44%; S=47%; G2/M=7%) and *Eμ-Myc;Puma*$^{-/-}$*;p21*$^{-/-}$ (G1=53%; S=39%; G2/M=6%) compared to wild-type cells (G1=69%; S=23%, G2/M=4%). sIg^+ B cells from *Eμ-Myc* (G1=50%; S=40%; G2/M=7%)*; Eμ-Myc;Puma*$^{-/-}$ (G1=52%; S=40%; G2/M=5%); *Eμ-Myc;p21*$^{-/-}$ (G1=56%; S=36%; G2/M=6%) and *Eμ-Myc;Puma*$^{-/-}$*;p21*$^{-/-}$ (G1=57%; S=34%; G2/M=6%) mice compared to wt mice (G1=88%; S=7%; G2/M=3%).

For cell cycle analysis, bone marrow cell suspensions were surface stained with antibodies to B220, IgM and IgD (see above), fixed and permeabilized with the Transcription Factor staining Buffer Set (eBioscience, San Diego, CA, USA), then stained with Propidium Iodide/RNase staining solution (Cell Signaling Technology, Danvers, MA, USA) for 30 min prior to analysis on an LSR II flow cytometer (BD Biosciences). Cell cycle distribution was determined using FlowJo employing the Watson pragmatic model (FlowJo, Ashland, US).

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Figure 1 **Valente et al**

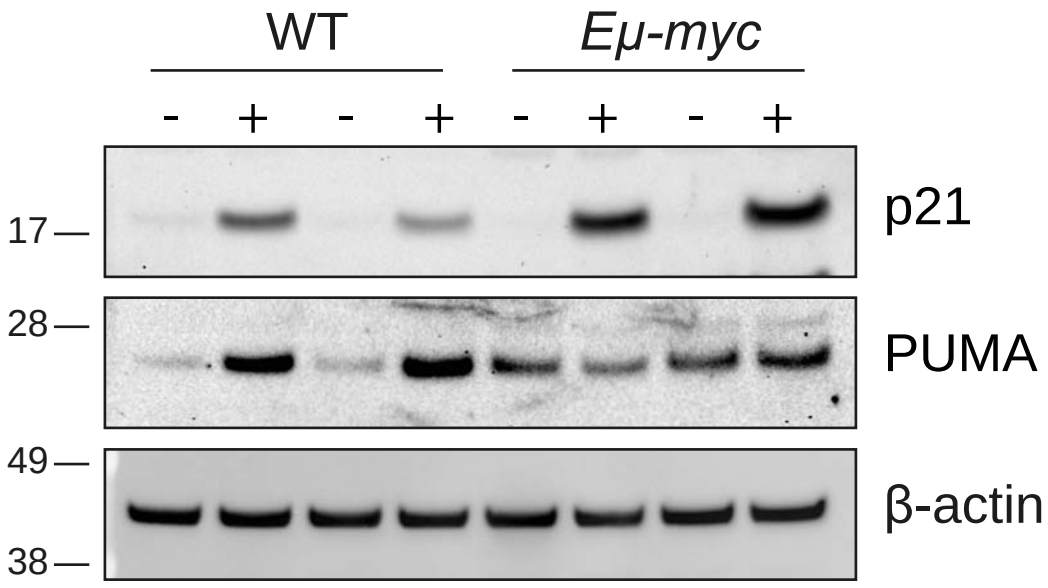


Figure 2

Valente et al

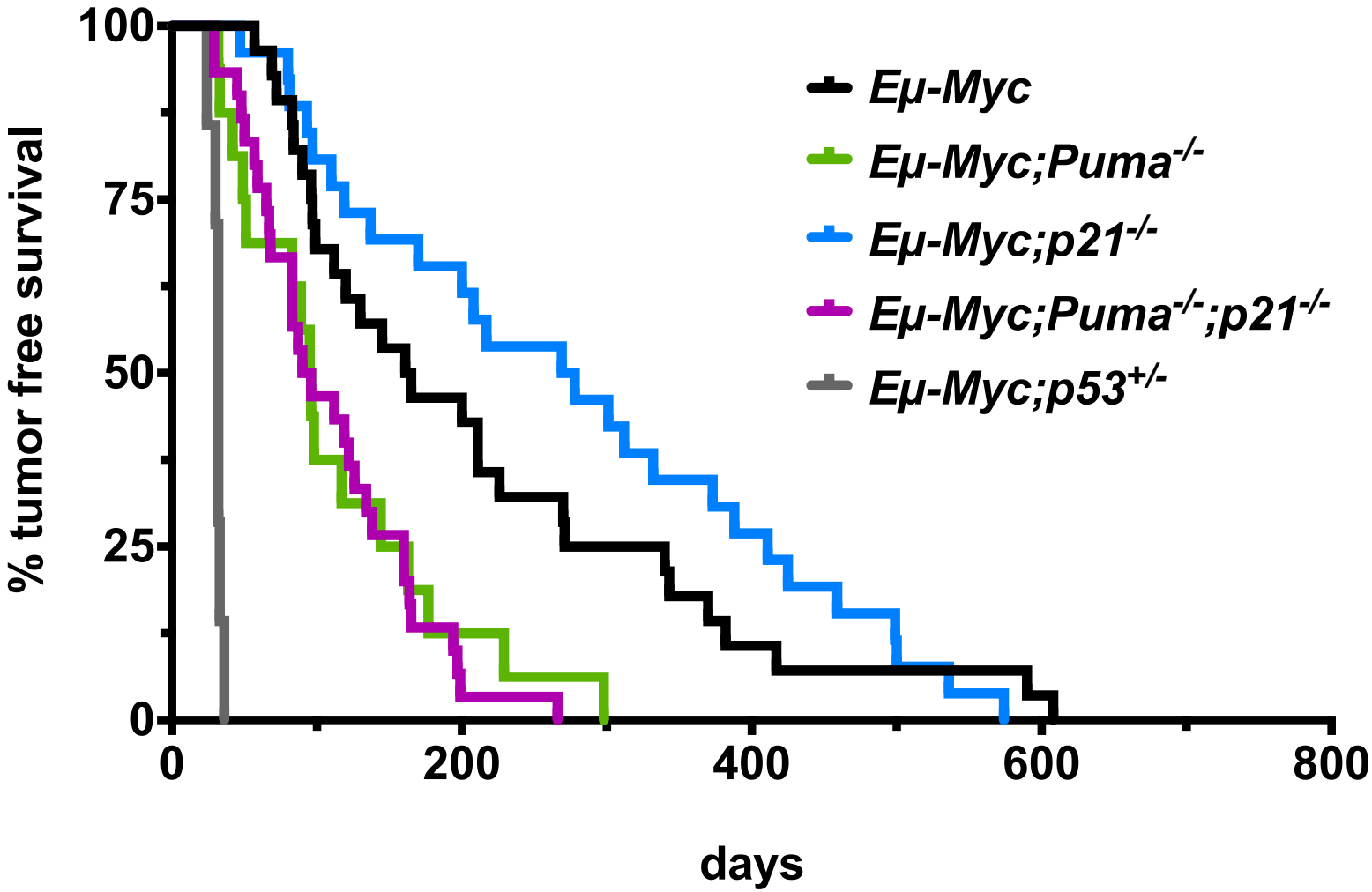
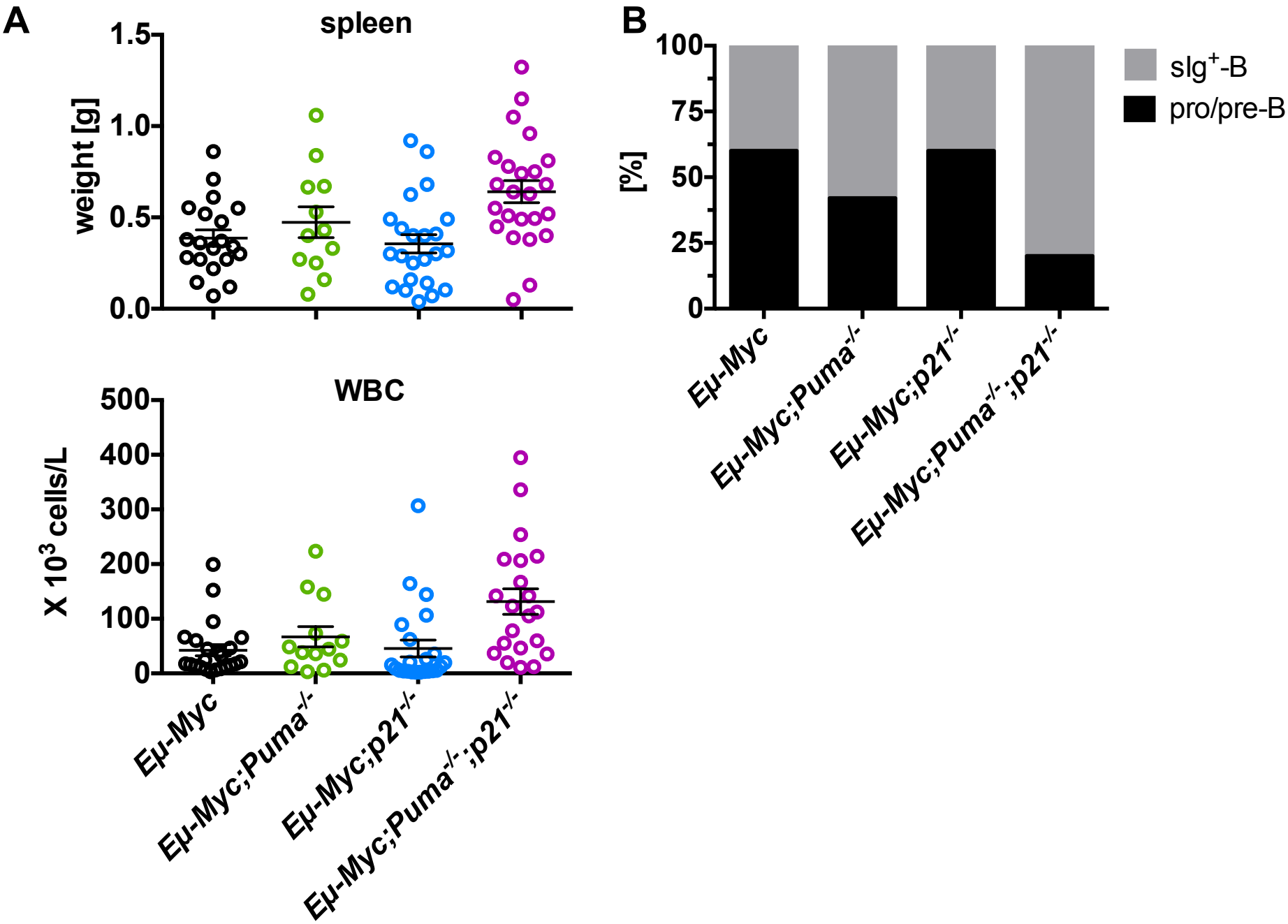
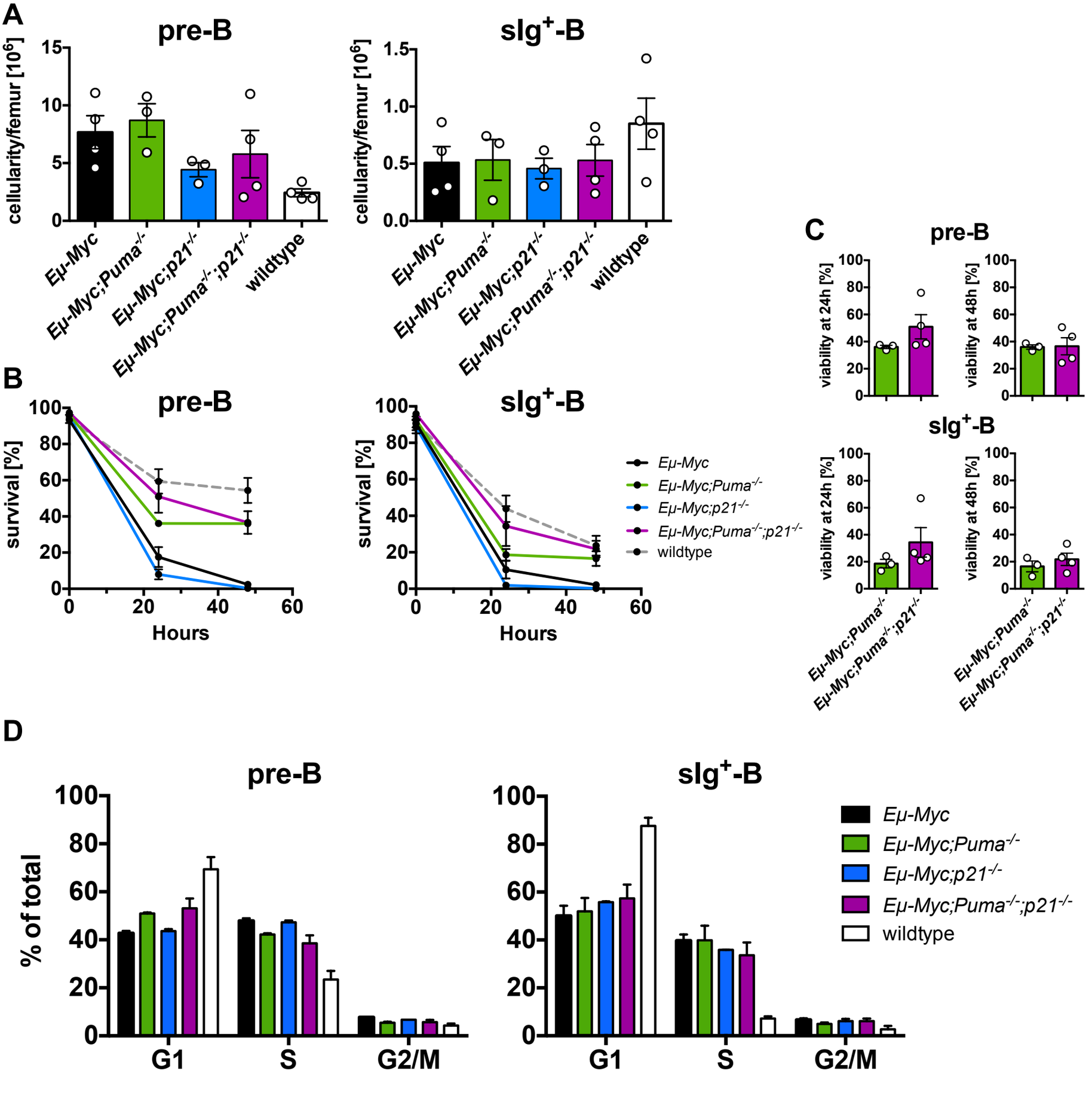


Figure 3

Valente et al




A
pre-B
sIg+-B
cellularity/femur [10^6]
Eμ-Myc
Eμ-Myc;Puma-/-
Eμ-Myc;p21-/-
Eμ-Myc;Puma-/-;p21-/-
wildtype
B
pre-B
sIg+-B
survival [%]
Hours
Eμ-Myc
Eμ-Myc;Puma-/-
Eμ-Myc;p21-/-
Eμ-Myc;Puma-/-;p21-/-
wildtype
C
pre-B
sIg+-B
viability at 24h [%]
viability at 48h [%]
Eμ-Myc;Puma-/-
Eμ-Myc;Puma-/-;p21-/-
D
pre-B
sIg+-B
% of total
G1
S
G2/M
Eμ-Myc
Eμ-Myc;Puma-/-
Eμ-Myc;p21-/-
Eμ-Myc;Puma-/-;p21-/-
wildtype