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Absence of the *Shb* gene in mixed-lineage leukemia MLL-AF9 cells increases latency in mice despite higher proliferation rates *in vitro*

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A R T I C L E I N F O

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Mixed lineage leukemia (MLL) arises from several *KMT2A*-gene chromosomal translocations. *Shb* gene deficiency has been found to exhibit pleiotropic effects in different models of leukemia, and consequently, this study aimed to investigate MLL-AF9-induced leukemia in *Shb* deficiency. Bone marrow cells from wild type and *Shb* knockout (KO) mice were transduced with the *MLL-AF9* gene. *Shb* KO MLL-AF9 cells proliferated at an increased rate, exhibited altered expression of certain cytokine genes (*Kitl*, *Csf3, IL6, IL1b*) and higher expression of cell cycle genes (*Ccnd2*, *Ccne1*). Mice receiving *Shb* KO MLL-AF9 cells showed longer latency without displaying any difference in rates of leukemic cell proliferation, indicating a dichotomy between the *in vitro* and *in vivo* phenotypes. The mice with *Shb* deficient MLL-AF9 cells had a lower content of leukemic bone marrow cells allowing elevated normal hematopoiesis, explaining the longer latency. Finally, *Shb* knockout GFP-positive bone marrow cells showed a higher percentage of cells expressing myeloid markers. The result suggests a role of *Shb* in the progression of leukemia and that the relevance of the *Shb* gene is context-dependent as inferred from the differences between the *in vivo* and *in vitro* responses. These findings help to obtain an increased understanding of human MLL-AF9 leukemia.

1. Introduction

The Mixed Lineage Leukemia (MLL) product is a multi-domain protein expressed in hematopoietic stem and progenitor cells and coded for by the *KMT2A* gene [1–4]. The *KMT2A* gene at chromosome 11q23 is a frequent target for chromosomal translocations found in human acute leukemias. Chromosomal rearrangements involving the *MLL (KMT2A)* gene cause approximately 70% of infant leukemias with either acute myeloid leukemia (AML) or acute lymphoid leukemia (ALL) phenotypes and approximately 10% of AMLs in adults [5,6]. One of the most frequent *MLL* rearrangements is [9,11] (p22; q23) or *MLL–AF9*. The *MLL* gene encodes a DNA-binding protein which methylates histone H3 lysine 4 (H3K4) [7]. The inter-chromosomal translocation results in the fusion of the MLL N-terminus to the C-terminus of several partners, deleting the *KMT2A* gene of its H3K4 methyltransferase activity. These novel fusion genes will have consequences for chromatin structure [7] and the altered epigenetic programming that ensues causes aberrant gene expression [8] and aggressive tumor development [4,9].

The Src homology-2 domain containing protein B (SHB) encodes an adaptor protein that operates downstream of several tyrosine kinase receptors. SHB has pleiotropic effects on the cell, and regulates numerous responses such as apoptosis, proliferation, differentiation and the cytoskeleton [10–13]. In a previous study, we investigated *Shb* deficiency in p210 BCR-*ABL* leukemia and observed that it resulted in accelerated development of disease with high counts of neutrophils in blood. The phenotypic response was thought to be due to elevated interleukin-6 (IL-6) and granulocyte colony stimulating factor (G-CSF) gene expression compared to wild type control [14]. In another study, we showed that *Shb* deficiency in *CSF3R*^{T618I}-induced leukemia caused shorter latency and an elevated level of myeloid cells in peripheral blood which suggested a more aggressive disease in the absence of *Shb* [15]. Thus, in two models of myeloid leukemia, absence of *Shb* increases the output of myeloid cells in blood. However, investigating the role of *Shb* in the p190-BCR-*ABL* B-cell ALL model revealed no difference in latency

Abbreviations: MLL, Mixed lineage leukemia; AML, Acute myeloid leukemia; ALL, Acute lymphoid leukemia; Shb, Src homology-2 domain containing protein B; KO, Knockout; IL, Interleukin; G-CSF, Granulocyte colony-stimulation factor; FACS, Fluorescence-activated cell sorting; CML, Chronic myeloid leukemia; TNF, Tumor necrosis factor; SCF, Stem cell factor; CXCL, Chemokine (C-X-C motif) ligand; GFP, Green fluorescent protein; BCR, Breakpoint cluster region; H3K4, Histone H3 lysine 4; CD, Cluster of differentiation; RBC, Red blood cells; WBC, White blood cells.

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in the absence of *Shb*. Reduced expression of IL-7 and Chemokine (C-X-C motif) ligand-12 (CXCL-12) in leukemic *Shb* knockout bone marrow were accompanied by a reduction of leukemic blood cell numbers, and the peripheral blood cell counts showed a preponderance towards neutrophilia [15]. In an experimental murine T-ALL model caused by transplantation of bone marrow from mice carrying active *Kras*^{G12D} to wild type recipients, *Shb* deficiency increased the level of less mature CD8/CD4 double positive cells, resulting in an expansion of thymus size [15]. The signaling characteristics of *Shb* deficient leukemic cells have not revealed any consistent differences, suggesting that there is no consensus with respect to how absence of *Shb* influences the signaling signature in different leukemic models [14,15]. Based on our findings demonstrating different phenotypic responses to *Shb* deficiency in different experimental models of leukemia, we decided to investigate a role of *Shb* in MLL-AF9 induced leukemia.

2. Methods

2.1. Mice

Shb wild type or knockout mice on a Balb/c background were used for induction of MLL-AF9 leukemia. The generation of *Shb* knockout mice was described previously [16]. The experiments were ethically approved by the local animal ethics committee at Uppsala University (approval number C104/16).

2.2. Transduction of bone marrow and transplantation

Bone marrow cells from wild type and *Shb*-knockout mice were isolated. For depletion of LIN-positive cells, cells were stained with a lineage excluding cocktail consisting of purified rat anti-mouse antibodies for CD3, CD8, CD4, CD19, B220, Gr-1, and CD11b (Suppl. Table 1). The stained samples were thereafter incubated with Biotin goat anti-rat IgG secondary antibody (Suppl. Table 1), followed by incubating with anti-Biotin microbeads (Suppl. Table 1), based on the instructions provided by manufacturer. The flow-through cells were then incubated with anti-c-Kit labeled magnetic microbeads (Suppl. Table 1) to enrich c-Kit^{+}LIN^{-} cells. Cells were cultured at 37 °C in the presence of 5% CO2 in RPMI-1640 (Sigma Aldrich, St Louis, MO), supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Sigma Aldrich), 2 mM L-glutamine, streptomycin (0.1 mg/ml), penicillin (100 U/ml)(Gibco, Paisley, UK), interleukin 3 (IL-3) (6 ng/ml), stem cell factor (SCF) (10 ng/ml) and interleukin-6 (IL-6) (10 ng/ml) (all cytokines were purchased from PeproTech, Rocky Hill, NJ).

Production of retroviruses expressing MLL-AF9-GFP was done by using the MLL-AF9-MIG vector [17]. In order to increase the infection efficiency, spin infection was done on Recombinant Human Fibronectin Fragment (RetroNectin®)-coated plates (4 μg/cm^2) (Takara Bio, Japan). After spin infection, the cells were expanded in RPMI-1640 supplemented with 10 ng/ml SCF and 6 ng/ml IL-3. At this point, the cells were FACS-sorted (BD FACSariaIII) for GFP-positivity and 1.5 million GFP-positive cells were expanded in culture for four days to 25 million cells before cryopreservation and storage at −150 °C. After thawing, the GFP-positive cells were expanded three passages in culture before experimentation ("*in vitro*") or transplantation into recipient mice ("*in vivo*"). Wild type recipient mice were sub-lethally irradiated with a dose of 4.5 Gy in XRad225^{IR} PRECISION irradiator. Shortly thereafter, 5 million GFP-positive cells were transplanted to the recipient mice by tail vein injection.

2.3. Proliferation assay

MLL-AF9 cells were seeded at 20,000 cells/ml per well in a 24-well plate in RPMI 1640 supplemented with SCF and IL-3 as above. The cells were counted in a Bürker chamber day 1 to day 3 for the determination of proliferation rates.

2.4. Cytostatic and apoptosis assay

MLL-AF9 cells were seeded in the amount of 50,000 cells/ml per well in a 24-well plate. Cytarabine and etoposide at the concentrations of 0, 1 and 5 μM and daunorubicin hydrochloride at the concentrations of 0, 0,001 and 0,01 μM (all cytostatics were purchased from Tocris Bioscience, Abingdon, UK), were added to the cells. The cells were collected after 24h and counted and stained, using Alexa Fluor® 488 Annexin V/Dead Cell Apoptosis Kit (Thermo Fisher Scientific, Massachusetts, USA). The cells were subsequently analyzed on a BD Accuri™ C6 Plus Flow Cytometer (BD Bioscience, Erembodegem, Belgium). The values were normalized by subtracting unstained cell values.

2.5. Gene expression by qPCR

RNA of MLL-AF9 cells was extracted using RNeasy Mini Kit (Quiagen, Hilden, Germany). One-step quantitative real-time RT-PCR was performed with QuantiTect™ SYBR®Green RT-PCR-kit (Qiagen) on a LightCycler™ real-time PCR machine (lightcycler 2.0; Roche, Mannheim, Germany). PCR was performed according to the following program; reverse transcription at 50 °C for 20 min, inactivation at 95 °C for 15 min, 45 cycles of denaturation at 94 °C for 15 s, annealing for 25 s at 60 °C, and extension at 72 °C for 15 s (Sequences of primers used in RT-PCR is provided in Suppl. table 2). Cycle threshold (Ct) values were determined with the LightCycler Software v3.5 (Roche). Gene expression was normalized for differences in RNA by subtracting the corresponding beta-actin Ct-value. Statistical comparisons were made on normalized Ct-values.

2.6. Blood profile

Peripheral blood from moribund mice was collected in Microtainer K2E tubes (BD, Franklin Lakes, USA). The blood was subsequently analyzed using a Sysmex XP-300™ Automated Hematology Analyzer (Sysmex, USA). Peripheral blood smears were stained with May–-Grunwald Giemsa (Merck Millipore, Germany) based on the manufacturer's protocol.

2.7. Colony forming assays

The MLL-AF9 cells were mixed with methylcellulose medium M3434 containing SCF, IL-3, IL-6 and erythropoietin (Stem Cell Technologies, Vancouver, BC) at a density of 1x10^3 cells per dish and were plated in 3 cm dishes. Scoring of colonies was done on day 3 and 6 post culture.

2.8. Flow cytometry by fluorescent activated cell-sorting (FACS) analysis

In order to identify B-lymphocyte, T-lymphocyte and myeloid cell population, paraformaldehyde fixed peripheral blood, bone marrow and spleen cells were stained with antibodies recognizing lineage markers, such as B220 and CD19 for B-cells, CD3, CD4 and CD8 for T-cells or Gr-1 and CD11b for myeloid cells. The cells were thereafter incubated with biotin goat anti-rat IgG secondary antibody when staining for the B-cell and myeloid markers, followed by incubation with PerCP/Cy5.5 Streptavidin (Supple table 1) in all stains. Cell cycle was determined by staining for Hoechst (*in vivo*) or Hoechst plus Ki-67 (*in vitro*) [14].

All flow cytometric experiments were performed on BD Accuri™ C6 Plus Flow Cytometer and the data were analyzed with FlowJo (TreeStar, Ashland, OR). Gating strategies are shown in Supplemental Fig. 1 and are based on blank versus positive staining signals.

2.9. Statistical analysis

The values are presented as the mean ± standard error of the mean (SEM). One separate experiment indicates an experiment performed at a separate occasion and observations in tumor experiments indicates

individual mice. Comparing survival between wild type and *Shb* knockout was according to Kaplan-Meier plots. Comparison of two groups was confirmed by Student's t-test. $P<0.05$ was considered as statistically significant.

3. Results

3.1. Cell proliferation in vitro

The MLL-AF9 model of murine leukemia was employed to increase our knowledge of the role of the *Shb* gene for leukemia. MLL-AF9 cells were obtained by transfection of *Shb* knockout and wild type c-Kit^{+}LIN^{-} bone marrow cells with MLL-AF9 retroviruses after which GFP-positive cells were sorted and expanded equally for the two genotypes for approximately six passages during which the cells were kept in culture in the presence of SCF and IL-3. Cells deficient in *Shb* exhibited higher proliferation rates in comparison with wild type MLL-AF9 cells (Fig. 1a). On day 1, the average cell number for wild type was 72000 cells and that of *Shb* knockout was 90000 ($p< 0.05$). The *Shb* knockout cells also showed a lower percentage of cells in the G0/G1 phase of the cell cycle and a higher percentage of cells in the S phase of the cell cycle (Fig. 1b) when cell cycle analysis was performed by flow cytometry by staining for Ki-67 and Hoechst.

The cell proliferation and cell cycle experiments were supplemented with a colony formation assay in methylcellulose (M3434) to identify the presence of multi-potential progenitors and lineage restricted progenitors of the granulocytic and monocyte-macrophage lineages in the MLL-AF9 cells. Both wild type and *Shb* knockout MLL-AF9 cells readily formed colonies and the *Shb* knockout cells produced more colonies in total compared to wild type bone marrow cells (116 ± 2.6% relative wild type control in four separate experiments, $p< 0.01$). This result was in line with the higher *in vitro* proliferation rate of the *Shb* null cells. Moreover, *Shb* knockout cells formed a significantly higher number of colony-forming unit - granulocyte monocyte (CFU-GM) in comparison with wild type (Fig. 2, Suppl. figure 1).

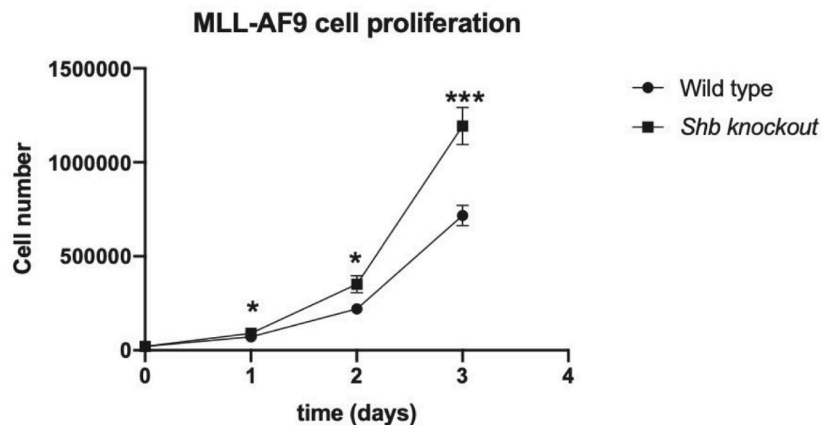
3.2. Susceptibility to cytostatic compounds

Since cell susceptibility to exposure to cytostatic agents is of relevance for treatment of leukemia in patients, we decided to investigated how *Shb* deficiency influenced the response of the leukemic cells to cytostatic compounds commonly used in myeloid leukemia. *Shb* knockout MLL-AF9 cell numbers were significantly decreased in response to a low concentration of etoposide from 10940 cells per well to 4460 cells per well. No significant differences in response to daunorubicin and cytarabine were noted (Fig. 3). No significant effects of *Shb* deficiency, as determined by a cell death assay, were noted in response to etoposide, cytarabine or daunorubicin (results not shown).

3.3. Gene expression

Cytokine production by leukemic cells and other cell types in the bone marrow environment is of relevance for progression of disease. To explore the expression profile of cytokines in the *in vitro* MLL-AF9 model, the expression levels of a number of genes coding for cytokines and cell cycle proteins [18–23], related to regulation of leukemogenesis and hematopoietic cell proliferation and differentiation, were determined. As a result, *Ccnd2* (Cyclin D2), *Ccne1* (Cyclin E), *Kitl* (SCF) and *Csf3* (G-CSF) were expressed at significantly higher levels in the absence of *Shb*. The expression of *Il-1b* (IL-1b) and *Il-6* (IL-6) were on the other

a



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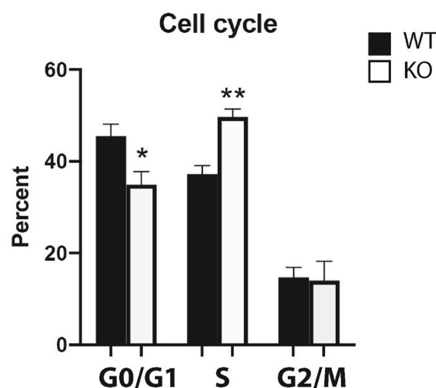

Fig. 1. Effects of *Shb* deficiency on MLL-AF9 cell proliferation rates when cells were cultured *in vitro* (a). Day 0 started with $2x10^4$ cells/ml and cells were counted on daily basis from day 1 to day 3. Data are means ± SEM based on 3 separate experiments. * and *** indicate $p < 0.05$ and $p < 0.001$ respectively using Student's *t*-test. Cell cycle analysis after staining for Ki-67 and Hoechst (b). Values are means ± SEM for 4 separate experiments. * and ** indicate $p<0.05$ and 0.01, respectively, when compared with wild type.

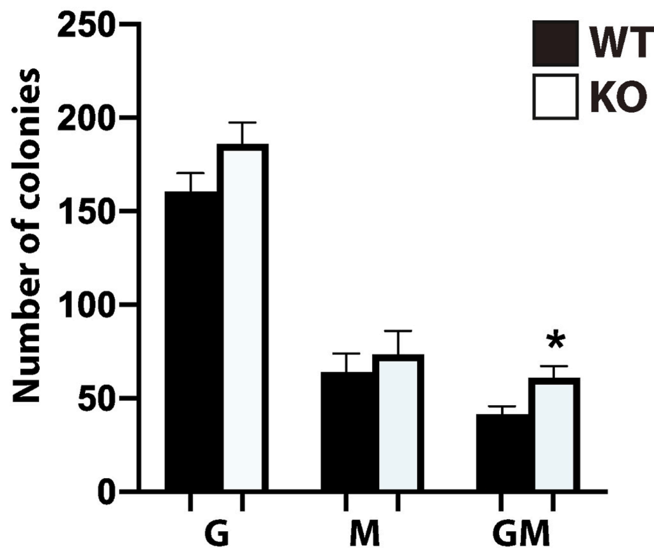

Fig. 2. Effects of *Shb* deficiency in colony formation. *In vitro* MLL-AF9 cells were plated on M3434 semisolid medium. The number and types of colonies were determined on day 6 (colony identity in total numbers) of culture. [Granulocyte Monocyte (GM), Monocyte (M), Granulocyte (G)]. Data are means ± SEM based on 3 separate experiments. * represents $p < 0.05$ a as determined by Student's *t*-test.

hand significantly decreased in the absence *of Shb* (Fig. 4).

Other genes coding for cytokines or cell cycle proteins that play a role in leukemia are *Cdkn1a*, *Cdkn1b*, *Ccnd1*, *Ccnd3*, *Csf2*, *Angpt1*, *Angpt2*, *Vegfa*, *Pdgfa*, *Il3*, *Tnf*, *Cxcl12*, *Ccl3* and *Ccl4* [24,25]. The transcript levels of these factors were therefore determined in *in vitro* MLL-AF9 bone marrow cells. No significant differences were detected between wild type and *Shb* deficient samples for any of these genes (Suppl. Table 3).

3.4. Leukemia in vivo

HSCs with deficient *Shb* are less proliferative whereas *Shb* deficient p210 BCR-ABL leukemic cells exhibit increased proliferation *in vivo* and shorter latency [14,26]. Since we currently observe increased proliferation *in vitro* as a consequence of absence of *Shb* we decided to investigate the effect of *Shb* deletion on the development of MLL-AF9 induced leukemia. Mice transplanted with *Shb* knockout MLL-AF9 cells that developed leukemia became moribund at later time points compared to recipients of wild type MLL-AF9 cells (Fig. 5a). Three mice receiving *Shb* null and two wild type MLL-AF9 cells died from anemia without any signs of leukemia and these were excluded from the study. Peripheral blood of moribund mice exhibited high white blood cell (WBC) counts and low red blood cell (RBC) and platelet counts. No differences between wild type and *Shb* knockout mice were noted (Fig. 5b and c, see Suppl. Figure 1 for typical peripheral blood smear stains).

End stage leukemia shows weight loss as well as splenomegaly and hepatomegaly, the latter two due to the infiltration of leukemic cells from bone marrow to spleen and liver [27,28]. Investigating weight loss and liver size showed no significant differences between wild type and

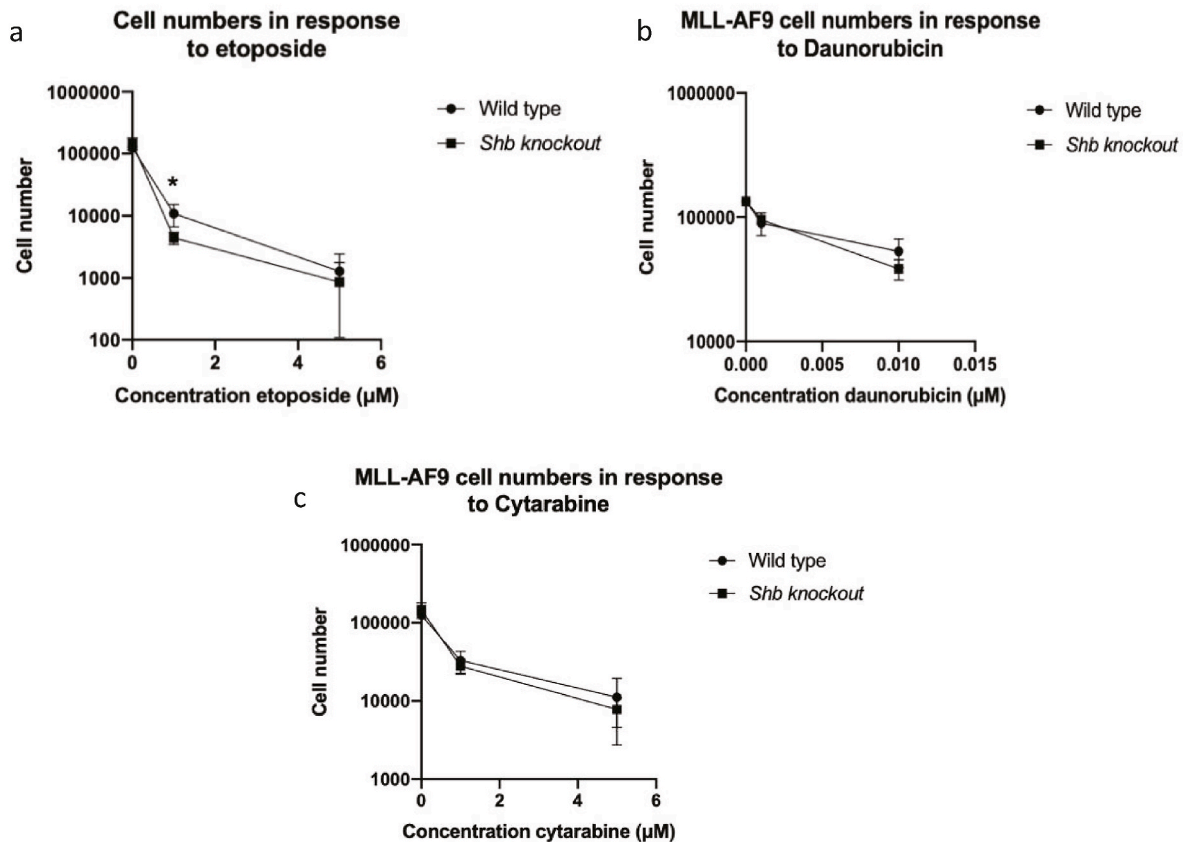

Fig. 3. Effects of cytostatic compounds on MLL-AF9 cell numbers. The cells were seeded at a concentration of $5x10^4$ cells/ml. a) Cells were treated with 0, 1 and 5 μM etoposide and counted after 24 h. b) Cells were treated with 0, 0,001 and 0,01 μM daunorubicin hydrochloride and counted after 24 h. c) Cells were treated with 0, 1 and 5 μM cytarabine and counted after 24 h. Data are means ± SEM based on 3 separate experiments. * denotes $p < 0.05$ by Student's *t*-test. Please note the exponential Y-axes.

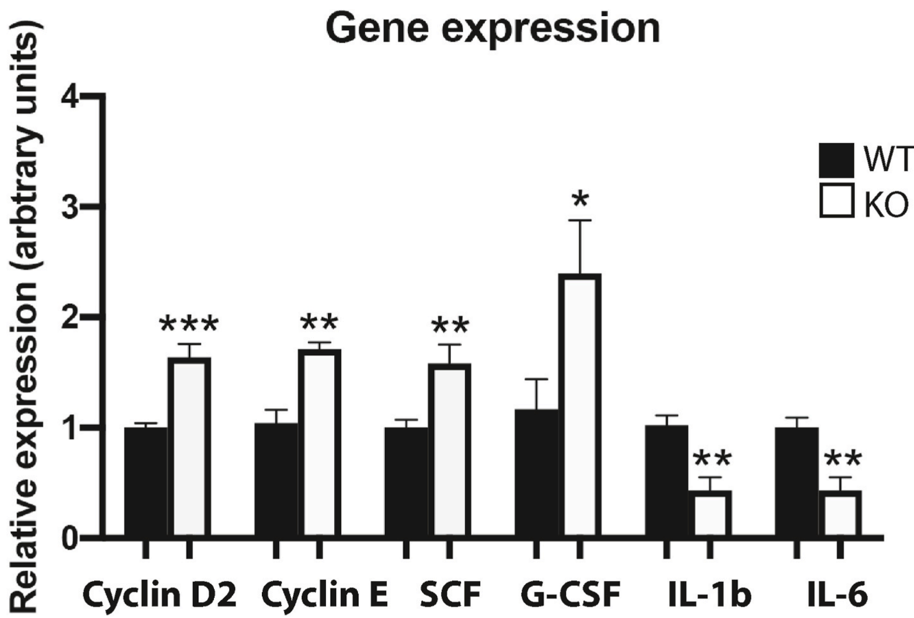

Fig. 4. Gene expression in MLL-AF9 cells. The expression levels of various hematopoietic cytokine/cell cycle genes were determined by semi-quantitative real-time RT-PCR in the samples isolated from *in vitro* MLL-AF9 cells. All Ct values were normalized to β-actin and *Shb* knockout samples were related to the corresponding wild type values. Means are presented as $2^{-\Delta\Delta Ct}$ ± SEM to demonstrate fold change in mRNA content. Data are means ± SEM based on 6–9 observations based on 2-3 separate experiments. *, ** and *** denote $p < 0.05$, $p < 0.01$ and $p < 0.001$ respectively as determined by Student's *t*-test.

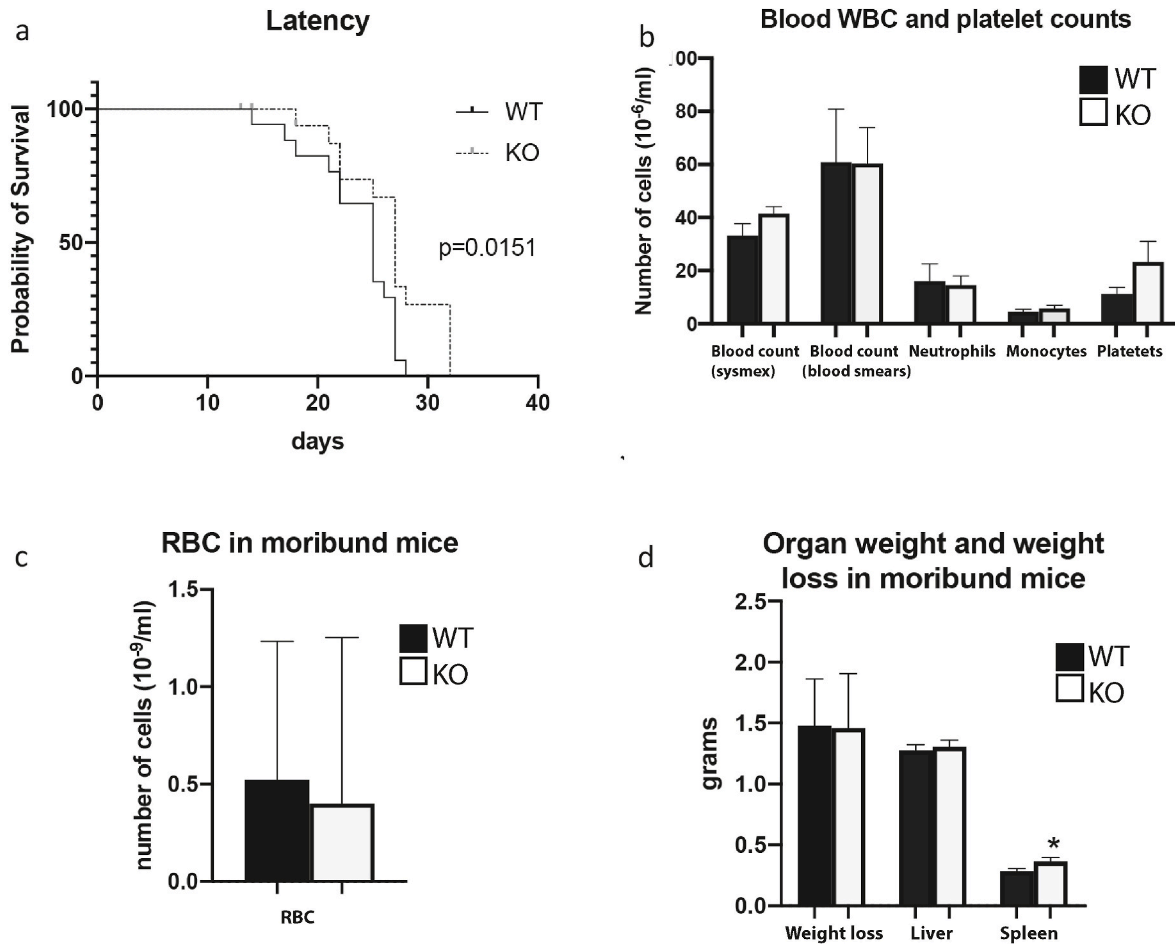

Fig. 5. MLL-AF9 induced leukemia. (a) Kaplan-Meier curve demonstrating survival of mice receiving either wild type or *Shb* knockout MLL-AF9 *in vitro* bone marrow cells after irradiated sub-lethally with 4.5 Gy. (b, c) Peripheral blood cell counts by Sysmex Hematology Analyzer and alternatively by staining with May GrÜnwald-Giemsa and counting in the light microscope. For WBC and platelets the units are 10^6/ml and for RBC 10^9/ml. (d) Analysis of disease parameters including weight loss at the end-stage of the disease as well as liver and spleen weight. *denotes $p < 0.05$ as determined by Student's t-test.

Shb knockout counterparts; however, the spleen size was increased in the absence of *Shb* (Fig. 5d).

No difference in the total bone marrow cell number was observed (results not shown). Flow cytometric analysis of cell cycle marker Hoechst (Supplemental Fig. 2a), myeloid lineage markers Gr-1 and Mac-1(Supplemental Fig. 2b), T-cell markers CD3, CD4 and CD8 (Supplemental Fig. 2c), B-cell markers B220 and CD19 (Supplemental Fig. 2d) failed to show any differences between wild type and *Shb* null bone marrows, spleens and blood. Myeloid cells comprised the majority of cells in bone marrow and blood.

Flow cytometric analysis of bone marrow, spleen and blood for the level of GFP-positive cells, which represents leukemic cells, revealed a lower percentage of GFP-positive cells in *Shb* null bone marrows (Fig. 6a) indicating that non-leukemic hematopoiesis proceeds at a higher rate in these mice than in the wild type MLL-AF9 mice. *Shb* knockout GFP-positive cells (leukemic cells) exhibited a higher number of cells that expressed myeloid markers in the bone marrow (Fig. 6b), suggesting a propensity for *Shb* deficiency to promote myeloid differentiation as has been noted previously [14,15]. GFP-positive *Shb* knockout cells in blood were, on the other hand, expressing fewer B cells (Fig. 6c). GFP-positive T cells were rare are thus not shown.

Assessing *in vivo* cytokine gene expression in bone marrow cells from moribund mice revealed no significant differences in expression of the genes that were found to be altered *in vitro* (Supplemental Fig. 3). It should be noted that the proportion leukemic (GFP-positive) cells in the *Shb* knockout situation was less than 30% which would obfuscate the contribution of gene expression changes that could be derived from MLL-AF9 cells.

4. Discussion

In this study we have explored the effects of *Shb* deficiency on MLL-AF9-induced leukemia. We observe that *Shb* deficiency caused an increased proliferation rate *in vitro*, whereas mice that received *Shb* deficient MLL-AF9 cells showed a milder disease phenotype with longer survival compare to wild type mice. A tentative explanation for this discrepancy is that the SHB protein operates in a context-dependent manner and that the *in vivo* conditions were such that the absence of the SHB protein failed to increase leukemic cell proliferation. The increased proliferation observed *in vitro* was detected both in culture in the presence of SCF and IL-3, and in a colony-forming semisolid medium containing the same cytokines. We also demonstrated that the levels of

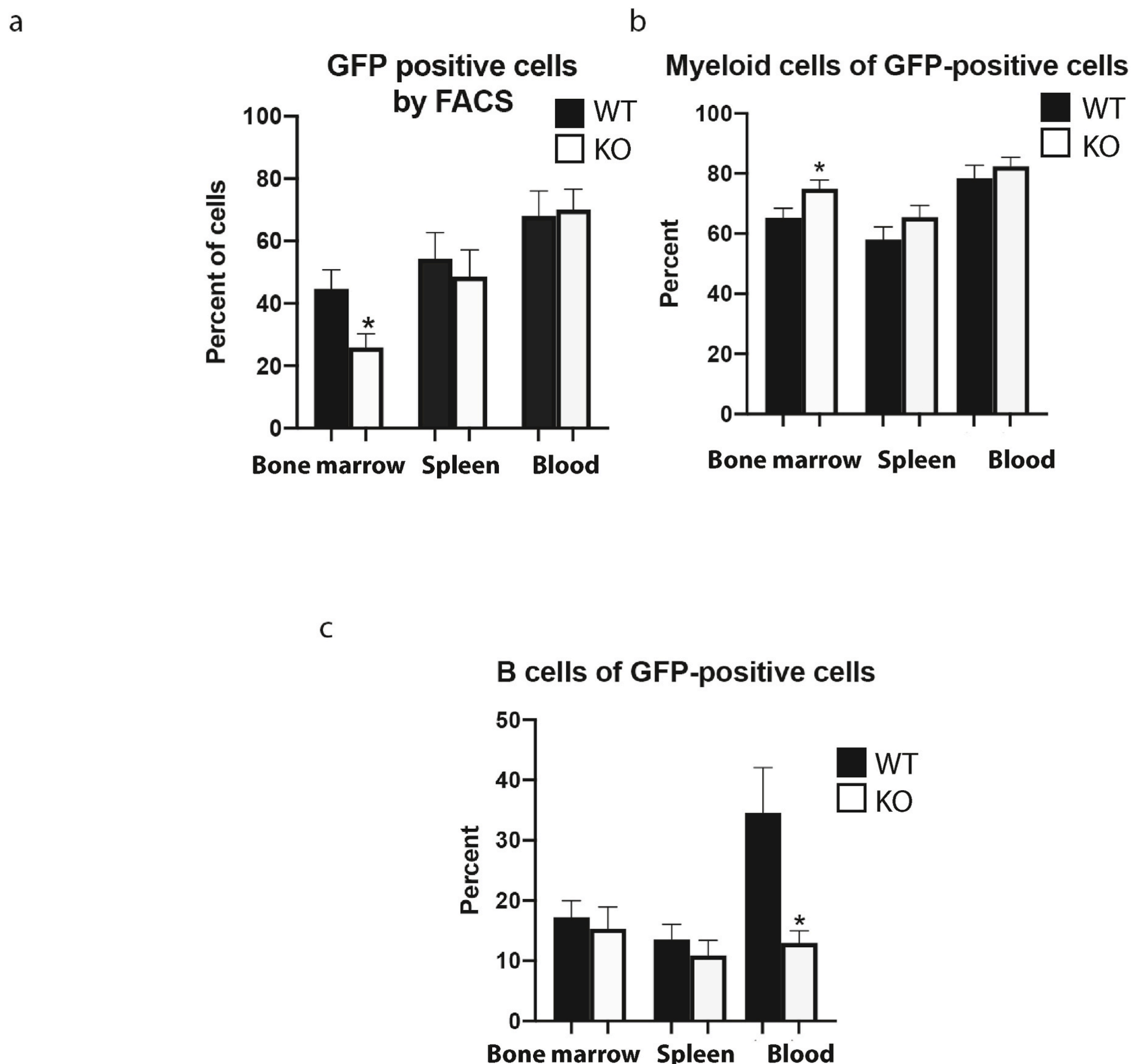

Fig. 6. GFP-positive (leukemic) cells and their contribution of myeloid and B cells in peripheral blood, bone marrow and spleen. Wild type and *Shb* knockout GFP-positive cells (a) were first gated followed by gating for myeloid (Gr1 + Mac1) (b) or B cell (B220 + CD16) (c) markers. GFP-positive T cell values were very low and thus not shown. Values are percent of parental population for 17 mice ±SEM. * indicates $p< 0.05$ when compared with wild type control.

cell cycle regulators Cyclin D2 and Cyclin E were upregulated in *Shb* null MLL-AF9 cells, further reinforcing the cell proliferative phenotype. Although there were no major differences in the susceptibility to the cytostatic compounds studied, the slightly enhanced response to a low concentration of etoposide was likely to reflect the higher proliferation rate.

Dysregulation of cytokine expression supports the progress of hematological malignancies [29]. SCF is known to promote proliferation of leukemic cells [30] and G-CSF is secreted by primitive leukemic cells, thus becoming stimulated by an autocrine mechanism [31]. Of possible interest is that in a p210 BCR-*ABL* model of myeloid leukemia, we demonstrated increased expression of G-CSF which was paralleled by neutrophilia [14].

In contrast to the *in vitro* proliferation data, mice receiving *Shb* knockout MLL-AF9 cells survived longer than controls. In addition, their relative contents of bone marrow GFP-positive cells were lower, indicating that they exhibit relatively higher rates of non-leukemic hematopoiesis. This provides a plausible explanation for the longer latency since anemia and thrombocytopenia develop later in mice receiving *Shb* knockout MLL-AF9 cells as indicated by the blood RBC and platelet counts when the mice were moribund.

IL-6 is a pleiotropic cytokine acting both as an inflammatory cytokine and regulator of hematopoiesis, myeloid lineage output and leukemic blast formation in cooperation with other cytokines whereas IL-1 has been shown to be a major pro-inflammatory cytokine in the development of inflammatory diseases [24,29,32–36]. IL-1b production is a frequent event in hematological malignancies and is a biomarker for poor prognosis in AML patients [37,38]. In general, IL-1b is known to assist survival and proliferation of AML cells [39,40]. It has recently been shown that IL-6 interferes with red blood cell differentiation and that treatment with IL-6 blocking antibodies improves overall survival of AML patients [41], in line with other observations showing that IL-1b and IL-6 tend to increase AML aggressiveness [29]. Despite increased *in vitro* proliferation rates, the MLL-AF9 *Shb* knockout cells will display reduced aggressiveness when transplanted *in vivo*. Therefore, these findings are compatible with the notion that *Shb* deficiency results in longer survival in the MLL-AF9 leukemia model due to the lower expression levels of these cytokines *in vitro* prior to transplantation, allowing sustenance of a higher rate of non-leukemic hematopoiesis *in vivo* shortly after transplantation. It should be noted that only a minority of the bone marrow cells were leukemic in moribund mice that received *Shb* knockout cells and thus the gene expression values do not reflect this cell population. In addition, the knockout cells will probably have undergone selection *in vivo* and will at that point have restored expression of these cytokines (IL-1b and IL-6) to comparable levels as those of the wild type control cells.

Absence of *Shb* will have disparate effects on leukemia progression, in agreement with the notion that the effects of *Shb* are context dependent. The currently observed discrepancy between the *in vitro* and *in vivo* responses is in line with this concept. Since the *MLL-AF9* gene product alters epigenetic programming with different gene expression as a consequence, it is easily conceived that differences in the local milieu (cytokines or cell-cell interactions) will have a major impact on the phenotypic responses to *Shb* deficiency. This concept is of relevance for understanding human disease.

5. Conclusions

SHB has an impact on the MLL-AF9 phenotype that is dependent on the surrounding milieu. Despite promoting cell proliferation *in vitro*, absence of SHB will delay the development of leukemia *in vivo*, possibly due to allowing higher rates of non-leukemic hematopoiesis. Such pleiotropic effects are of relevance for understanding human disease.

CRediT authorship contribution statement

Maria Jamalpour: did the experiments, Conceptualization, Formal analysis, Writing - original draft. **Eric Bergquist:** did the experiments. **Michael Welsh:** did the experiments, Conceptualization, Formal analysis, Writing - original draft.

Declaration of competing interest

The authors have no conflicts of interest to report.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.yexcr.2020.112368.

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