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Title: Modeling BCR-ABL and MLL-AF9 leukemia in a human bone marrow-like scaffold based xenograft model

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Abstract

While NOD-SCID IL2R $\gamma^{-/-}$ (NSG) xenograft mice are currently the most frequently used model to study human leukemia *in vivo*, the absence of a human niche severely hampers faithful recapitulation of the disease. We used NSG mice in which ceramic scaffolds seeded with human mesenchymal stromal cells were implanted to generate a human bone marrow (huBM-sc)-like niche. We observed that, in contrast to the murine bone marrow (mBM) niche, expression of BCR-ABL or MLL-AF9 was sufficient to induce both primary AML and ALL. Stemness was preserved within the human niches as demonstrated by serial transplantation assays. Efficient engraftment of AML MLL-AF9 and blast-crisis CML patient cells was also observed, whereby the immature blast-like phenotype was maintained in the huBM-sc niche, but to a much lesser extent in mBM niches. We compared transcriptomes of leukemias derived from mBM niches versus leukemias from huBM-like scaffold-based niches, which revealed striking differences in expression of genes associated with hypoxia, mitochondria and metabolism. Finally, we utilized the huBM-sc MLL-AF9 B-ALL model to evaluate the efficacy of the I-BET151 inhibitor *in vivo*. In conclusion, we have established human niche models in which the myeloid and lymphoid features of BCR-ABL⁺ and MLL-AF9⁺ leukemias can be studied in detail.

Introduction

Establishing xenograft models is essential to study the molecular mechanisms involved in human leukemias aimed at improving treatment options. Currently, NOD-SCID IL2R γ ^{-/-} (NSG) xenograft models are considered as the gold standard for evaluating the engraftment of human hematological malignancies. However, despite being the most immune compromised mouse strain available allowing a more efficient and faster disease development compared to other NOD/SCID-related strains,¹⁻³ also NSG mice are suboptimal since 30-40% of primary AML samples failed to engraft.⁴ Obviously, a human bone marrow (huBM) environment is lacking in these animals, and such a humanized niche might be essential to provide the optimum concentration of endogenous cytokines, chemokines and adhesion factors to allow efficient engraftment and maintenance of different leukemia clones. Indeed, engraftment of normal human CD34⁺ cells in NSG mice is lymphoid biased. Using retro/lentiviral systems to model human leukemias we frequently observe that while *in vitro* both myeloid and lymphoid transformation can be achieved upon expression of oncogenes such as MLL-AF9 or BCR-ABL/BMI1, *in vivo* transformation is strongly biased towards ALL.^{5,6} In patients the BCR-ABL p210 oncoprotein can give rise to both myeloid and lymphoid leukemias, and the same is true for MLL-AF9 in pediatric leukemia patients. Together, these observations suggest that a specific human microenvironment might be necessary for engraftment and maintenance of self-renewal of myeloid leukemic stem cells (LSCs). Since certain myeloid growth factors are often species-specific, the mouse niche does not suffice to provide the appropriate environment for human LSCs.

To develop *in vivo* models that more faithfully recapitulate human disease driven by the BCR-ABL p210 translocation and by MLL-AF9 we made use of a humanized niche NSG xenograft model in which ceramic scaffolds coated with human mesenchymal stromal cells (MSCs) were implanted in NSG mice where they developed into structures mimicking a human bone marrow microenvironment.^{7,8} Next, we studied the engraftment and initiation of CML by cord blood (CB) CD34⁺ cells transduced with BCR-ABL with or without BMI1 (where indicated) or with MLL-AF9, as well as engraftment of primary CML and AML patient cells. Our data indicate that serially transplantable ALL and primary AML could be established in the humanized scaffold xenograft model. Differences between transcriptome profiles of BCR-ABL cells harvested from murine and human niches were analyzed, and thus we identified niche-specific BCR-ABL signatures. Ultimately, we also took advantage of the huBM-sc MLL-AF9 B-ALL model to evaluate the efficacy of I-BET151 inhibitor *in vivo*.

Materials and Methods

Establishment of the humanized scaffold niche xenograft model

The ectopic bone model was established as described previously.^{7,8} Briefly, four hybrid scaffolds consisting of three 2–3mm biphasic calcium phosphate (BCP) particles loaded with human MSCs were implanted subcutaneously into 8 week old female NOD.Cγ-Prkdcscid Il2rytm1Wjl/SzJ (NSG) mice. Neonatal CB samples were obtained after informed consent from healthy full-term pregnancies from the obstetrics departments of the University Medical Center in Groningen (UMCG) and Martini Hospital Groningen.

CB CD34⁺ cells were isolated as described previously.^{6,9} 500,000 non-sorted cells were directly injected into the scaffolds or i.v. as indicated during primary as well as secondary transplantations. CD45 engraftment was analyzed by timely bleeding procedures.

Retroviral and lentiviral transductions

The murine stem cell virus (MSCV)-BMI1-internal ribosomal entry site (IRES2)-enhanced green fluorescent protein (EGFP) and MSCV-BCR-ABL-IRES2-truncated nerve growth factor receptor (tNGFR) retroviral vectors were cloned into MiGR1 and MINR1 as previously described.⁶ MLL-AF9 vectors were described previously.^{5,10} Transductions of CB CD34⁺ cells were performed as described previously.^{6,9}

LTC-IC

For the LTC-IC assays, newly transduced cells were sorted on MS5 stromal cells in limiting dilutions from 10 to 500 cells per well in 96-well plates as described previously.⁹ After 5 weeks of culture, the wells containing cobblestone areas were scored, after which the medium from the wells was aspirated and replaced with methylcellulose-containing cytokines. After an additional 2 weeks of culture, wells were scored as positive or negative to yield the LTC-IC frequency. Stem cell frequencies were calculated using L-Calc software (Stem Cell Technologies).

Further Materials and methods are provided as supplemental information.

Results

Murine versus human extrinsic cues dictate transformation potential in BCR-ABL/BMI1 and MLL-AF9-induced leukemia in humanized xenograft models.

We have evaluated human BCR-ABL, BCR-ABL/BMI1 and MLL-AF9-driven leukemogenesis in the murine BM niche using classical NSG xenotransplantation models, and compared that to leukemogenesis in the presence of a humanized bone marrow microenvironment. In line with what we observed previously,⁶ only cells expressing both BCR-ABL and BMI1 were able to generate CD34⁺/CD19⁺ B-ALL in the mouse bone marrow environment when injected intravenously (i.v.) into sublethally irradiated NSG mice (Figure 1a, c, d and Supplementary Table 1). Infiltration of double positive ALL cells was seen in spleen and liver (Supplementary Figure S1a-b) and no myeloid or erythroid cells expressing CD33, CD11b, CD15 or GPA were observed in leukemic mice.

Next, we injected non-sorted CB CD34⁺ cells transduced with BCR-ABL and BMI1 directly into a humanized model that is based on subcutaneous implantation of human BM-like scaffolds (huBM-sc), as described previously.^{7,8} In 11-21 weeks, 11 out of 13 mice developed palpable tumors at the site of injection and data for all these 11 mice is summarized in Figure 1a, Supplementary Table 1, and Supplementary Figure S1c. In contrast to i.v. injected mice that all developed CD19⁺ B-ALL, in transcutaneous injected huBM-sc mice both CD19⁺ B-ALL phenotypes and CD19⁻ AML phenotypes were observed (Figure 1c, 1d, Supplementary Table 1). When transduced cells were injected into empty scaffolds or scaffolds coated with MSCs that were irradiated with 30 Gy prior to implantation leukemia development was never observed. Migration of cells to the

murine BM was observed in 3 out of 11 huBM-sc mice, typically with a lineage phenotype resembling that of the leukemic cells in the huBM scaffolds (Supplementary Table 1).

When freshly transduced CB MLL-AF9 cells were transplanted i.v. into NSG mice, a CD19⁺ lymphoid leukemia could be generated within 11-25 weeks, and again myeloid engraftment was difficult to achieve. In only about 10% of the cases, mixed-lineage leukemias were observed in which both CD19⁺ B-ALL and CD33⁺/CD19⁻ myeloid clones were only observed, in line with previous observations.^{5,11} Upon direct injection of CB MLL-AF9 into huBM-sc mice, all animals developed a fatal leukemia with a latency of 16-32 weeks. Although leukemia development was still biased towards CD19⁺ B-ALL, more mixed leukemias with both B-ALL and AML clones were observed within the humanized niche, and in one case AML without B-ALL clones was observed (Figure 1c-d, Supplementary Table 1). Overall, leukemia development was slightly slower in the huBM-sc model compared to the muBM i.v. model (although the difference did not reach significance, Mantel-Cox test p=0.0678), possibly related to the observation that myeloid leukemias developed less fast compared to lymphoid B-ALLs.

BCR-ABL can induce lymphoid and myeloid leukemia in a human niche xenotransplant model without exogenous BMI1.

Within 11-21 weeks, 11 out of 13 mice developed tumors at the site of injection of non-sorted BCR-ABL/BMI1-transduced cells in huBM-sc mice. Interestingly, 14 out of 18 leukemic scaffolds contained BCR-ABL-single positive leukemias, which was never observed in the mouse BM niche in i.v. injected mice, while 4 out of 18 huBM-sc derived

176 leukemias both BCR-ABL single positive as well as BCR-ABL and BMI1 double positive
177 clones were detected (Figure 1d, Supplementary Table 1). Apparently, in a humanized
178 microenvironment co-expression of exogenous BMI1 was not required to induce BCR-
179 ABL-positive leukemia.

180 Furthermore, the huBM-sc allowed for the development of a variety of phenotypes
181 ranging from B-ALL with aberrant expression of myeloid markers, to more erythroid- and
182 myeloid-biased leukemias, regardless of whether these were BCR-ABL single positive
183 or BCR-ABL and BMI1 double positive clones. For example, huBM scaffold 3 of mouse
184 m5 (Figure 2a-e) developed tumors that were white of color, were BCR-ABL single
185 positive and expressed CD19 and CD38, while CD34, GPA and CD11b were not
186 expressed (Figure 2a). CD33, CD36 and CD15 expression was also detected (Figure
187 2a). Immunohistochemical analysis confirmed human bone formation in the scaffolds
188 (Figure 2c, d, Supplementary Figure S2a). Infiltration of BCR-ABL single cells was
189 observed in different organs like BM, spleen and liver and these cells showed a similar
190 marker expression as the cells engrafted in the huBM-sc (Supplementary Figure 2b-d).
191 MGG staining confirmed a lymphoblastic phenotype of these cells and hence we
192 concluded that mice developed B-ALL with aberrant expression of certain myeloid
193 antigens (Figure 2e). Similar results were obtained for other B-ALLs in mouse m5 huBM
194 scaffold 2 and mouse m9 huBM scaffold 2 (Supplementary Table 1).

195 In the huBM-sc mouse m1 the developing tumors were more compact and red (huBM
196 scaffold 3) or green (huBM scaffolds 1 and 4) indicative of erythroid development and
197 myeloperoxidase expression, respectively. These were CD19^{neg} but did express
198 intermediate to high levels of GPA and CD33, with the highest expression of GPA in

huBM scaffold 3 (Figure 2f). In addition, mice with both BCR-ABL single as well as BCR-ABL/BMI1 double positive leukemic clones were obtained (m10, Figure S2e). This mouse displayed an immature tumor phenotype as indicated by high expression of C-KIT (CD117) and low expression of other myeloid markers. No differences in FACS phenotype were observed between BCR-ABL single and BCR-ABL/BMI1 double positive clones (Figure S2e).

Increased incidence of myeloid leukemia observed in huBM-Sc MLL-AF9-induced models.

Three out of the 4 scaffolds were injected with 3×10^5 unsorted CB CD34⁺ cells transduced with MLL-AF9 in a total of 8 huBM-scaffold mice. Palpable tumors developed in 19/24 injected scaffolds and also in 5 out of 8 of the non-injected scaffolds. Clearly, huBM-sc were sufficiently vascularized to allow migration of tumor cells from injected scaffolds to non-injected scaffolds, as well as to mouse compartments. LM-PCRs were performed to study clonality (Supplementary Figure S3b-d). We observed that multiple co-existing clones and integration sites could be identified that were different in independent experiments. Furthermore, in mice in which one scaffold was the most dominant growing one (e.g. Sc2 in mouse 5, Supplementary Figure S3b) similar integrations sites were found in the human scaffold and the murine BM (e.g. the red integration site), but also that slight changes in clonality were observed as well (e.g. the yellow integrations site in BM and Sc2). In other cases where multiple scaffolds generated leukemias, different unique clones could engraft in individually injected scaffolds (Sc1 versus Sc3, indicated by different colors), whereby one clone that was

seen in the dominant clone in bone marrow (red clone) was also seen in the scaffolds.
And finally, in a secondary transplantation experiment most but not all primary clones
were seen in secondary leukemias grown in the scaffolds or BM (Supplementary Figure
S3d, Sc2 in mouse 1 versus 2nd Sc1-3 and BM, red integration site), although some
integration sites were only seen in one scaffold (Sc3, green integration site), and
additional clones appeared upon 2nd transplantation as well (sc2-3, yellow integration
site), which were most likely quiescent in the primary transplant.

In 3/8 huBM-sc mice (m1-m3), exclusively CD19⁺ B-ALL was observed in the scaffolds,
with phenotypes resembling those we previously observed in i.v. injected NSG mice
without human niches (^{5,6} and Figure 3a-d, Supplementary Table 1). Within these mice,
infiltration to mouse organs was observed as well, displaying phenotypes similar to
those in the humanized scaffolds. Some mice displayed a somewhat more mature
leukemic B-ALL phenotype with the presence of CD19⁺/CD20⁺ clones (data not shown).
In 4 out of 8 huBM-sc mice (m4-m7) both B-ALL as well as myeloid CD19⁻/CD33⁺
clones were detected (Figure 3e, Supplementary Table 1, Supplementary Figure 3), and
in one mouse (m8) one scaffold was completely lymphoid while another was completely
myeloid. Within this myeloid clone, some co-expression of myeloid markers such as
CD11b, CD15 and CD14 with CD33 was also detected, resembling the phenotype we
observe in *in vitro* myeloid cultures as well as in patients, and the lymphoid and myeloid
phenotypes were confirmed by MGG staining (Figure 3f-g).

In total, and in contrast with murine niches, out of 24 engrafted scaffolds we observed
co-existing myeloid and lymphoid clones in 9 cases (37.5%), and a complete myeloid
clone in 1 case (4.1%) although this difference did not reach statistical significance

(p=0.0804). Taken together these data suggest that the ectopic human BM microenvironment provides a more permissive microenvironment to drive LSCs towards myeloid differentiation.

Self-renewal is maintained within human niches as shown by serial transplantation, but remains lymphoid biased.

In order to evaluate the self-renewal potential of BCR-ABL single positive leukemic clones we performed secondary transplantations. Cells harvested from huBM scaffold 3 of mouse m5 were transplanted into 3 sub-lethally irradiated NSG mice carrying huBM scaffolds, such that 3 scaffolds were inoculated with 0.16×10^6 cells per scaffold, while 1 scaffold was left uninjected (Figure S4a). Large tumors were observed in all 3 secondary transplanted mice and within 6 weeks mice presented with overt leukemia (Figure 1a, d, S4b). All injected huBM scaffolds of all 3 secondary transplanted mice contained BCR-ABL single-positive cells. FACS analyses indicated that different clones had engrafted into individual scaffolds. Some scaffolds contained CD19, CD38 and CD33 positive cells comparable to their phenotype in primary mice, although expression of CD15 was lost (Figure S4c-e). huBM scaffold 2 from mouse m5.1 displayed multiple clones that varied in tNGFR expression, suggesting different levels of BCR-ABL expression in these clones. Clones with intermediate levels of BCR-ABL expression were positive for GPA while clones with the highest expression of BCR-ABL did not express GPA but in contrast expressed high levels of CD19 (Supplementary Figure S4f and data not shown). Infiltration of BCR-ABL single positive cells was also observed to

some extend in BM, spleen and liver, although chimerism levels were very low (Supplementary Table 1).

To obtain further insight into the clonality of these different engrafting populations, we performed LM-PCR on cells harvested from primary as well as secondary huBM scaffolds. As shown in Figure S4g, multiple bands were observed suggesting the coexistence of multiple clones. The clone indicated by the red asterisk was detected in both the primary and all secondary transplanted animals. Furthermore, multiple different other integration sites were observed in secondary leukemic mice, indicative for oligoclonal disease. Since a number of these clones were not detected in the primary mouse these data suggest the presence of quiescent clones in the primary leukemic cell population that were activated upon serial transplantation.

In order to assess the self renewal potential of CB MLL-AF9 leukemic cells generated in huBM-sc of primary recipients, we also performed secondary transplantation. CD19⁺/CD15⁺ lymphoid leukemic cells harvested from mouse m1 huBM-sc2 were transplanted into humanized NSG mice. Tumor formation was observed in all the injected scaffolds and FACS analyses displayed the same phenotype observed in the primary recipient, although expression of CD15⁺ was lost (Figure S4h). Infiltration of CB MLL-AF9 positive cells was also observed in BM, spleen and liver albeit the chimerism levels were lower in comparison to primary recipients (Supplementary Table 1). Furthermore, we performed a secondary transplantation using cells from m6 huBM-sc2 that contained two distinct clones of CD33⁺ (78.3%) and CD19⁺ (~3%). Due to the small number of cells available, only a single secondary huBM-sc could be injected with 1x10⁵ unsorted cells. Tumor formation was observed in the injected scaffold and the mouse

was sacrificed upon signs of illness after 15 weeks. Despite the fact that the vast majority of injected cells were of myeloid origin, a CD19⁺/CD33⁻ B-ALL developed in the scaffold, most likely arising from the small population of CD19⁺ cells in the primary recipient (Figure S4i), a feature we have observed more frequently. These results indicate that despite the presence of a human niche microenvironment, MLL-AF9-induced transformation of neonatal CB CD34⁺ cells remain biased towards B-ALL.

Molecular characterization of cells derived from the murine niche versus the human niche.

Illumina microarray analysis was performed to study transcriptome differences between murine niche-derived and human niche-derived BCR-ABL positive cells. BCR-ABL cells harvested from scaffolds from primary mice (1st huBM-sc), BCR-ABL cells harvested from scaffolds from secondary transplanted mice (2nd huBM-sc) as well as primary BCR-ABL/BMI1 cells harvested from murine BM and spleen (1st mBM/Spleen) were compared. 577 probesets were significantly upregulated in the huBM-sc derived leukemic cells compared to the mBM/Spleen-derived cells, while 458 probesets were downregulated (corrected p value<0.01 with Benjamini-Hochberg multiple testing correction, >3 fold differentially expressed, Supplementary Table 2). These data indicate that significant differences existed in transcriptome signatures between leukemias that developed in the mBM versus huBM scaffold niches. The top differentially expressed genes are shown in the supervised cluster analysis in Figure 4a, indicating that the 1st huBM-sc sample clustered tightly together with the eight 2nd huBM-sc samples, while all three 1st mBM/Spleen samples clustered together, away from all huBM-sc samples.

Gene ontology (GO) analyses indicated that the huBM-sc overexpressed genes were significantly (FDR<1) enriched for processes such as apoptosis and mitochondrion. Genes upregulated in mBM-derived leukemias were significantly (FDR<1) enriched for GO terms methylation and histone core related genes (Figure 4b). GSEA analysis indicated that huBM scaffold-derived leukemias were enriched for MYC targets and hypoxia signatures, suggesting that BCR-ABL-positive cells derived from the humanized scaffold display differences in MYC dependency and hypoxic state as compared to mBM-derived leukemias (Figure 4c). A number of selected GO terms for genes upregulated in huBM scaffold-derived leukemias are shown in Figure 5d and Supplementary Figure S5. Interestingly, p57, and MYC were some of the significantly overexpressed secreted proteins in huBM scaffold-derived leukemias. Another interesting observation was that endogenous BMI1 was overexpressed in BCR-ABL positive cells derived from humanized scaffolds, possibly explaining why exogenously overexpressed BMI1 is not required as is the case for leukemias that develop in the murine BM niche. Illumina data were validated by independent Q-PCR analyses (Figure 4e).

Primary Blast Crisis (BC)-CML and AML-MLL-AF9 cells can induce a fatal myeloid leukemia within 16 weeks post transplantation in the huBM scaffold mouse model

Next, cells from a blast crisis-chronic myeloid leukemia (BC-CML) primary patient were injected i.sc. in huBM scaffold mice (Figure 5a). We observed that all three injected mice developed tumors within 16 weeks post-transplantation. Tumors were well vascularized and greenish of color (Figure 5b), and were positive for huCD45 as well as

for myeloid markers like CD33, CD11b, CD14, and CD38 (Figure 5c). MGG staining clearly revealed the immature phenotype of scaffold-harvested cells as compared to murine BM-harvested cells (Figure 5d). Infiltration of myeloid cells was observed in mouse BM, spleen and liver (Supplementary Figure S6a). Cells in the mBM niche displayed a more differentiated phenotype in terms of a higher proportion of cells expressing the markers CD11b, CD14 and CD15 while a lower proportion of cells expressed CD34 (Figure 5c, S6a-c). In contrast, leukemic cells derived from the huBM scaffold more closely resembled the original patient and in particular CD34 percentages were quite comparable (Figure 5c, S6a-c). These FACS analyses were confirmed by immunohistochemistry (Supplementary Figure S6b).

The self-renewing potential of cells harvested from the murine BM niche and those retrieved from the human niche in the huBM scaffolds was functionally studied in LTC-IC assays. CD34⁺ and CD34⁻ cells were sorted and plated in limiting dilution and were scored after 5 weeks. No cobblestone area forming cells (CAFCs) were detected at week 5 in CD34⁺ or CD34⁻ isolated from murine BM harvested cells (Figure S6d), and only differentiated macrophages could be observed (data not shown). In contrast, CAFCs could readily be detected in cultures initiated with CD34⁺ cells isolated from the huBM scaffold harvested cells, with an LTC-IC frequency of 1 in 323 +/-115 (Figure S6d). Thus, these results suggest that self-renewal of LT BC-CML LSCs was maintained in the context of a humanized BM niche. Maintenance of self-renewal was also demonstrated *in vivo* by serial transplantation assays (Antonelli, Groen and Schuringa, to be submitted).

Finally, Q-PCR analyses was performed on BC-CML samples harvested from the mouse BM and human scaffold niches, and similar as observed in our BCR-ABL retroviral overexpression models, a significantly increased level of endogenous BMI1 was observed in cells derived from human scaffolds (Supplementary Figure S6e). Lastly, we also injected primary MLL-AF9 AML patient material into huBM-sc of a NSG mouse. Two scaffolds were injected with 1×10^6 cells each. While we did not detect any CD45⁺ chimerism in the blood during the course of the experiment, we sacrificed the mouse 20 weeks after transplantation upon palpation of tumor growth on one of the scaffolds (Figure 5e). Cells retrieved from the huBM-sc3 displayed a myeloid phenotype with expression of CD33, CD11b and CD15 (Figure 5f), which was further confirmed by MGG staining of cytopins (Figure 5g). No human cells could be detected in mouse organs such as spleen, liver and or BM by FACS analyses (Figure 5f), and the spleen size was not enlarged (weight: 0.12g).

Intrascaffold *in vivo* treatment with IBET151

We next evaluated whether the huBM-sc model could also be used for drug testing. In our *in vitro* CB MLL-AF9 myeloid and lymphoid MS5 co-culture system we observed that, upon inhibition of BRD3/4 binding to acetylated histones with the small molecule I-BET151^{12,13} proliferation was severely impaired (data not shown). Here, we assessed the therapeutic potential of I-BET151 *in vivo* in our huBM-sc B-ALL CB MLL-AF9 xenograft model. 5×10^5 cells harvested from a primary B-ALL MLL-AF9 generated in huBM-sc mice were injected into 3 huBM scaffolds of secondary recipients, while the fourth scaffold was left uninjected. Four weeks after injection of the cells, we performed

daily i.sc. injections of the inhibitor for a total of 23 days. One control group (n=5) was injected with a solution of PBS and DMSO 1%, one group (n=3) with I-BET151 500 μ M and another group (n=5) with I-BET151 5mM. Tumor volume size of each scaffold was measured during the experiment every 3 days, allowing us to monitor the progression of the disease in time. All mice were sacrificed at the same time at day 54 after injection. Mice treated with 5 mM I-BET151 displayed a significant reduction in tumor volume (~2 fold, $p < 0.0001$). Accordingly, also the cell number of the cells harvested from the tumors was significantly different (for 500 μ M group $p = 0.04$, for 5 mM group $p = 0.01$) (Figure 6a-d). No significant differences were found in spleen weight or blood and BM chimerism. Together, these data indicate that I-BET151 treatment significantly delays lymphoid leukemia progression *in vivo*, but is not sufficient for a complete eradication of the leukemic cells, at least not in this experimental setting.

Discussion

Despite many advantages of various xenotransplantation mouse models for human leukemias that have been developed till date, faithful recapitulation of myeloid phenotypes and efficient maintenance of long-term self-renewal of LSCs has remained challenging. One of the fundamental drawbacks of currently available mouse models to study human leukemias is the absence of a human microenvironment that would potentially provide species-specific cytokines, growth factors and adhesion molecules that are essential for self-renewal and myelopoiesis. Here we used our previously developed humanized mouse model that provides a human BM-like scaffold-based

(huBM-sc) microenvironment^{7,8} in which human myeloid and lymphoid leukemias can be studied in detail.

Various retro/lentiviral models have been established to model human myeloid and lymphoid leukemias, and while *in vitro* BCR-ABL or MLL-AF9 oncogenes (either alone or in combination with other hits) could induce transformation along myeloid or lymphoid lineages, *in vivo* transformation was always heavily lymphoid biased and *in vivo* myeloid transformation has been much more difficult to achieve in NOD-SCID based xenograft models, including the NSG mice.^{5,6,11,14} Furthermore, a significant number of primary AML samples does not engraft in the NSG mouse model⁴, again pinpointing to the notion that a niche that allows self-renewal of myeloid (malignant) clones is not provided for in the NSG mouse. Over the past years, xenograft leukemia models have already been significantly improved, and advantages and disadvantages of various humanized mouse models have recently been nicely reviewed.^{3,15} NOD/SCID-3/GM/SF mice engineered to produce human IL3, GM-CSF and SF^{16,17} and NOD-SCID IL2R γ ^{-/-} (NSG) mice expressing human IL3, GM-CSF and SF (NS-SGM3)¹⁸ have been generated which enhanced engraftability of primary human AML samples, as well as others¹⁹⁻²³ including MISTRG mice expressing M-CSF, IL3, GM-CSF, TPO and SIRP α .¹⁹ Even MDS patient samples or MDS-derived cell lines were reported to engraft making use of human cytokine mice.^{24,25} These models are clearly useful tools, although other human niche-specific factors might still be missing.

We made use of a humanized mouse xenograft model that harbors a human BM-like niche in which we could recapitulate both the myelomonocytic and B-ALL phenotypes that are observed in MLL-AF9 pediatric patients. Compared to the murine environment

of NOD-SCID/NSG mice, a higher percentage of myeloid clones could be generated in the huBM-sc model. This observation reinforces the notion that the lineage fate of MLL-AF9 LSCs is dictated primarily by microenvironmental cues and cell of origin rather than an instructive role of the fusion partner gene. While we could easily serially transplant huBM-sc derived B-ALL clones, 2nd engraftment of myeloid clones was more difficult to achieve. Possibly, the level of myeloid growth factors such as IL3¹⁴, an important and species-specific growth factor for myeloid leukemias driven by MLL-AF9, are still too low in our huBM-sc model. Ongoing experiments include further improvement of the huBM-sc model using MSCs that we have genetically engineered to express cytokines such as IL3 and TPO. On the other hand, Wei and colleagues reported that upon injection of CB CD34⁺ cells transduced with MLL-AF9 into NS-SGM3 mice only AML but not B-ALL developed, suggesting that these mice are now strongly biased towards myelopoiesis. Besides lentiviral models for MLL-AF9, we also showed that primary AML MLL-AF9 derived from an adult patient could engraft efficiently in the huBM-sc, and it will be interesting to compare the human cytokine mice with our humanized niche scaffold model side-by-side in future experiments.

We also find that primary CD34⁺ CML patient cells can efficiently engraft in this xenograft model, whereby stemness is much better preserved in the human environment as compared to the mouse environment. Furthermore, we describe that this humanized niche model also allows for BCR-ABL-driven myeloid and lymphoid disease, in contrast to the NOD-SCID/NSG mouse in which exclusively CD19⁺ B-ALL was observed upon transplantation of transduced CB CD34⁺ cells (current study and ¹⁶). Our previous studies in NSG mice showed that BCR-ABL as single hit was never

sufficient to induce leukemia in the context of a murine niche⁶, in line with data published by others.²⁶ Only when additional hits were provided, such as co-expression of BMI1, a serially transplantable CD19⁺ B-ALL could be induced.⁶ Possibly, BMI1 was required to bypass (oncogene-induced) senescence, and also in mouse models BMI1 was shown to reprogram BCR-ABL⁺ lymphoid progenitors into B-ALL-initiating cells.²⁷ In the huBM-sc xenograft model that we present here, transformation was achieved with both BCR-ABL-only expressing cells as well as with cells that expressed both BCR-ABL and BMI1. Apparently, exogenous BMI1 is not an absolute requirement to allow transformation of human CB CD34⁺ cells transduced with only BCR-ABL. Finally, striking differences were observed between transcriptomes of leukemic cells derived from murine BM niches versus leukemic cells derived from the huBM scaffold niches, including in genes associated with hypoxia signaling, mitochondria and metabolism. Within the top differentially expressed genes there was enrichment for cell cycle inhibitors, like CDKN1C/p57, and hypoxic signature-related genes in the humanized microenvironment, genes that have been shown to contribute to stemness of HSCs/LSCs.²⁸⁻³² Also, we find that endogenous BMI1 was strongly upregulated in the human BM niche. Apparently, the human BM niche can provide for factors that induce BMI1 expression, possibly through activation of SALL4 or the Sonic Hedgehog pathway, which both have been shown to be able to induce BMI1 expression.^{33,34} In conclusion, we describe here a huBM-sc xenograft model in which the myeloid and lymphoid features of MLL-AF9 and BCR-ABL⁺ leukemias can be studied in detail, both using lenti/retroviral model systems as well as patient samples, in which the efficacy of novel drugs can be evaluated.

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482 **Authorship**

483 PS and MC designed and conducted experiments, performed analysis and wrote the
484 manuscript; JJ, AZBV, LLA, RWJG and JJS conducted experiments and analyzed data;
485 HY and JDB contributed scaffold materials; ACMM, RWJG and EV analyzed data and
486 participated in writing the manuscript; JJS designed experiments, performed analysis,
487 wrote the manuscript and supervised the project.

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489 **Conflict of Interest**

490 The authors have declared that no competing interests exist.

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Figure legends

Figure 1. Mouse versus human extrinsic cues dictate transformation potential in BCR-ABL/BMI1 and MLL-AF9-induced leukemia in humanized xenograft models.

(a) Kaplan-Meier survival curves of BCR-ABL/BMI injected primary and secondary mice from mBM i.v. and huBM i.sc. models displaying the differences in kinetics of leukemia development. **(b)** Kaplan-Meier survival curves of CB MLL-AF9 injected primary and secondary mice from mBM i.v. and huBM i.sc. models displaying the differences in kinetics of leukemia development. **(c-d)** A summary of phenotypes observed in mBM i.v and huBM i.sc models for BCR-ABL and MLL-AF9.

Figure 2. BCR-ABL can induce lymphoid and myeloid leukemia in a human niche xenotransplant model without exogenous BMI1.

(a) Detailed FACS analyses of BCR-ABL positive cells from Scaffold 3 of Mouse 5 indicating positive surface marker expression for CD19, CD38 and also for CD33, CD15. **(b)** 16 weeks post-transplantation a tumor was detected at the site of injection. **(c)** HE staining of B-ALL tumor scaffold demonstrating efficient bone formation. **(d)** B-ALL tumor scaffold section stained by immunohistochemistry for CD45 demonstrated engraftment of CD45⁺ human cells. CD45⁻ cells were also observed suggesting the presence of endothelial cells forming a vascularized niche. **(e)** MGG staining confirmed the lymphoblastic phenotype of BCR-ABL single positive cells. **(f)** FACS analyses of BCR-ABL positive cells from Scaffold 1 and 3 of Mouse 1 indicating expression of GPA, CD71 and CD33 markers. The more reddish Scaffold 3 was also the most positive for GPA.

Figure 3. Increased incidence of myeloid leukemia on MLL-AF9 induction observed in huBM-sc as compared to NSG. (a) FACS analyses of MLL-AF9 cells from BM, Sc1 and Sc2 of ISC mouse 1. **(b)** Picture showing tumor initiated on the scaffolds for mouse 1. **(c)** B-ALL tumor scaffold 1 section stained by immunohistochemistry for CD45 demonstrated engraftment of CD45⁺ human cells. **(d)** HE staining of B-ALL tumor scaffold 1 demonstrating efficient bone formation. **(e)** FACS analyses of MLL-AF9 cells from BM, Sc1 and Sc2 of ISC mouse 6. **(f-g)** MGG staining of BM and Sc1 of ISC mouse 6 confirming the lymphoblastic phenotype **(h)** MGG staining of Sc2 of mouse 6 confirming the presence of lymphoid and myeloid cells.

Figure 4. Molecular characterization of murine niche versus human niche derived cells. (a) Gene expression profile of BCR-ABL positive cells harvested from a primary huBM-sc m5 (Scaffold 3), 8 secondary huBM-sc scaffolds (from m5.1, m5.2 and m5.3) as well as BCR-ABL/BMI1 positive cells harvested from BM and spleen (2x) from primary i.v. injected mice. A supervised hierarchical clustering analyses of differentially expressed genes is shown (differentially expressed genes were determined by using a corrected p value<0.01 with Benjamini-Hochberg multiple testing correction, and >3 fold difference in expression levels, Supplementary Table S1). Primary and secondary huBM-sc. samples clustered together, away from all 3 primary i.v. injected samples. BMI1, CDKN1C/p57, and MYC were some of the interesting candidate genes upregulated in huBM-sc 1st and 2nd scaffold derived samples. **(b)** Gene Ontology (GO) analyses on genes up- or downregulated in huBM-sc (human) niche derived cells versus i.v. injected murine niche-derived samples (>3 fold in i.sc. versus i.v. samples, p-

value of ≤ 0.01). (c) GSEA analyses on i.sc. versus i.v. samples. (d) GO terms of up regulated genes in huBM-sc 1st and 2nd scaffold derived samples. (e) Validation of Illumina data by real time PCR indicating expression of endogenous BMI1, p57 and MYC relative to the house-keeping gene RPL27. Experiments were performed in triplicate.

Figure 5. Primary Blast Crisis (BC)-CML and AML MLL-AF9 cells can engraft in the huBM scaffold mouse model and induce a fatal myeloid leukemia within 16 weeks post transplantation. (a) A myeloid BC-CML patient sample was injected in 3 scaffolds and 1 scaffold was left uninjected in 3 different humanized mice. 1.5×10^5 50,000 cells per scaffold were injected. (b) All 3 injected mice developed enlarged greenish tumors within 16 weeks post-transplantation. (c) Detailed FACS analyses of CD45⁺ engrafted cells derived from humanized scaffolds as well as from the murine BM. CD34 was higher expressed in cells retrieved from the human scaffold while CD15, CD11b and CD14 were higher expressed in cells derived from the murine BM. (d) MGG staining indicated a more immature phenotype of scaffold-harvested cells as compared to more differentiated cells from the murine BM (63X magnification). (e). Tumor growth of a scaffold injected with 1×10^6 primary MLL-AF9 AML patient cells. (f). FACS phenotype of cells harvested from the scaffold displayed in g and from mouse compartments (PB, BM, Liver and Spleen). (g) MGG staining of cytopins of cells harvested from the scaffold displayed in e.

Figure 6. I-BET1 efficacy using MLL-AF9 huBM-sc model.

(a) Schematic representation of the experimental design. Control mice were injected with normal saline containing 1% (v/v) DMSO. Treated mice were injected with 500μM and 5mM I-BET151 (b) Tumor volumes measured by caliper during the course of the experiment. (c) Pictures depicting tumors originated on huBM-sc. Comparison between control group and 5mM I-BET151 treated group. (d) Total cell number of tumors tumors originated on huBM-sc in the three groups.

Figures

Supplementary Files associated with this manuscript

Supplementary Materials and methods, and Supplementary figures:

- **Figure S1. mBM (i.v.) versus huBM (i.sc.) human bone-marrow scaffold-based xenograft mouse model.**
- **Figure S2. Scaffold initiated BCR-ABL single positive cells induced infiltrative ALL and AML.**
- **Figure S3. Scaffold initiated MLL-AF9 cells induced ALL and AML clones in an individual mouse.**
- **Figure S4. BCR-ABL single expressing cells and MLL-AF9 cells can induce serially transplantable ALL.**
- **Figure S5. Gene Ontology analyses on differentially expressed genes (>3 fold) in cells retrieved from huBM (i.sc.) versus muBM (i.v.) tumors.**
- **Figure S6. Self-renewal of a BC-CML patient sample can be better maintained in the humanized xenograft model.**

Supplementary Table 1. Overview of engraftment studies.

Supplementary Table 2. Transcriptome data.