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

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39 **Abstract**

40 While NOD-SCID IL2Rγ$^{-/-}$ (NSG) xenograft mice are currently the most frequently used
41 model to study human leukemia *in vivo*, the absence of a human niche severely
42 hampers faithful recapitulation of the disease. We used NSG mice in which ceramic
43 scaffolds seeded with human mesenchymal stromal cells were implanted to generate a
44 human bone marrow (huBM-sc)-like niche. We observed that, in contrast to the murine
45 bone marrow (mBM) niche, expression of BCR-ABL or MLL-AF9 was sufficient to
46 induce both primary AML and ALL. Stemness was preserved within the human niches
47 as demonstrated by serial transplantation assays. Efficient engraftment of AML MLL-
48 AF9 and blast-crisis CML patient cells was also observed, whereby the immature blast-
49 like phenotype was maintained in the huBM-sc niche, but to a much lesser extent in
50 mBM niches. We compared transcriptomes of leukemias derived from mBM niches
51 versus leukemias from huBM-like scaffold-based niches, which revealed striking
52 differences in expression of genes associated with hypoxia, mitochondria and
53 metabolism. Finally, we utilized the huBM-sc MLL-AF9 B-ALL model to evaluate the
54 efficacy of the I-BET151 inhibitor in *vivo*. In conclusion, we have established human
55 niche models in which the myeloid and lymphoid features of BCR-ABL^{+} and MLL-AF9^{+}
56 leukemias can be studied in detail.

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62 **Introduction**

63 Establishing xenograft models is essential to study the molecular mechanisms involved
64 in human leukemias aimed at improving treatment options. Currently, NOD-SCID IL2Rγ^{-}
65 $^{/-}$ (NSG) xenograft models are considered as the gold standard for evaluating the
66 engraftment of human hematological malignancies. However, despite being the most
67 immune compromised mouse strain available allowing a more efficient and faster
68 disease development compared to other NOD/SCID-related strains,[1-3] also NSG mice
69 are suboptimal since 30-40% of primary AML samples failed to engraft.[4] Obviously, a
70 human bone marrow (huBM) environment is lacking in these animals, and such a
71 humanized niche might be essential to provide the optimum concentration of
72 endogenous cytokines, chemokines and adhesion factors to allow efficient engraftment
73 and maintenance of different leukemia clones. Indeed, engraftment of normal human
74 $CD34^+$ cells in NSG mice is lymphoid biased. Using retro/lentiviral systems to model
75 human leukemias we frequently observe that while *in vitro* both myeloid and lymphoid
76 transformation can be achieved upon expression of oncogenes such as MLL-AF9 or
77 BCR-ABL/BMI1, *in vivo* transformation is strongly biased towards ALL.[5,6] In patients the
78 BCR-ABL p210 oncoprotein can give rise to both myeloid and lymphoid leukemias, and
79 the same is true for MLL-AF9 in pediatric leukemia patients. Together, these
80 observations suggest that a specific human microenvironment might be necessary for
81 engraftment and maintenance of self-renewal of myeloid leukemic stem cells (LSCs).
82 Since certain myeloid growth factors are often species-specific, the mouse niche does
83 not suffice to provide the appropriate environment for human LSCs.

84 To develop *in vivo* models that more faithfully recapitulate human disease driven by the
85 BCR-ABL p210 translocation and by MLL-AF9 we made use of a humanized niche NSG
86 xenograft model in which ceramic scaffolds coated with human mesenchymal stromal
87 cells (MSCs) were implanted in NSG mice where they developed into structures
88 mimicking a human bone marrow microenvironment.[7,8] Next, we studied the
89 engraftment and initiation of CML by cord blood (CB) CD34$^+$ cells transduced with BCR-
90 ABL with or without BMI1 (where indicated) or with MLL-AF9, as well as engraftment of
91 primary CML and AML patient cells. Our data indicate that serially transplantable ALL
92 and primary AML could be established in the humanized scaffold xenograft model.
93 Differences between transcriptome profiles of BCR-ABL cells harvested from murine
94 and human niches were analyzed, and thus we identified niche-specific BCR-ABL
95 signatures. Ultimately, we also took advantage of the huBM-sc MLL-AF9 B-ALL model
96 to evaluate the efficacy of I-BET151 inhibitor *in vivo*.
97
98 **Materials and Methods**
99
100 **Establishment of the humanized scaffold niche xenograft model**
101 The ectopic bone model was established as described previously.[7,8] Briefly, four hybrid
102 scaffolds consisting of three 2–3mm biphasic calcium phosphate (BCP) particles loaded
103 with human MSCs were implanted subcutaneously into 8 week old female NOD.Cγ-
104 Prkdcscid Il2rγtm1Wjl/SzJ (NSG) mice. Neonatal CB samples were obtained after
105 informed consent from healthy full-term pregnancies from the obstetrics departments of
106 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.

130 **Results**

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

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

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

153 murine BM was observed in 3 out of 11 huBM-sc mice, typically with a lineage
154 phenotype resembling that of the leukemic cells in the huBM scaffolds (Supplementary
155 Table 1).
156 When freshly transduced CB MLL-AF9 cells were transplanted i.v. into NSG mice, a
157 CD19^{+} lymphoid leukemia could be generated within 11-25 weeks, and again myeloid
158 engraftment was difficult to achieve. In only about 10% of the cases, mixed-lineage
159 leukemias were observed in which both CD19^{+} B-ALL and CD33^{+}/CD19^{-} myeloid clones
160 were only observed, in line with previous observations.[5,11] Upon direct injection of CB
161 MLL-AF9 into huBM-sc mice, all animals developed a fatal leukemia with a latency of
162 16-32 weeks. Although leukemia development was still biased towards CD19^{+} B-ALL,
163 more mixed leukemias with both B-ALL and AML clones were observed within the
164 humanized niche, and in one case AML without B-ALL clones was observed (Figure 1c-
165 d, Supplementary Table 1). Overall, leukemia development was slightly slower in the
166 huBM-sc model compared to the muBM i.v. model (although the difference did not
167 reach significance, Mantel-Cox test p=0.0678), possibly related to the observation that
168 myeloid leukemias developed less fast compared to lymphoid B-ALLs.
169

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

172 Within 11-21 weeks, 11 out of 13 mice developed tumors at the site of injection of non-
173 sorted BCR-ABL/BMI1-transduced cells in huBM-sc mice. Interestingly, 14 out of 18
174 leukemic scaffolds contained BCR-ABL-single positive leukemias, which was never
175 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

199 huBM scaffold 3 (Figure 2f). In addition, mice with both BCR-ABL single as well as
200 BCR-ABL/BMI1 double positive leukemic clones were obtained (m10, Figure S2e). This
201 mouse displayed an immature tumor phenotype as indicated by high expression of C-
202 KIT (CD117) and low expression of other myeloid markers. No differences in FACS
203 phenotype were observed between BCR-ABL single and BCR-ABL/BMI1 double
204 positive clones (Figure S2e).
205

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

208 Three out of the 4 scaffolds were injected with $3x10^5$ unsorted CB $CD34^+$ cells
209 transduced with MLL-AF9 in a total of 8 huBM-scaffold mice. Palpable tumors
210 developed in 19/24 injected scaffolds and also in 5 out of 8 of the non-injected scaffolds.
211 Clearly, huBM-sc were sufficiently vascularized to allow migration of tumor cells from
212 injected scaffolds to non-injected scaffolds, as well as to mouse compartments. LM-
213 PCRs were performed to study clonality (Supplementary Figure S3b-d). We observed
214 that multiple co-existing clones and integration sites could be identified that were
215 different in independent experiments. Furthermore, in mice in which one scaffold was
216 the most dominant growing one (e.g. Sc2 in mouse 5, Supplementary Figure S3b)
217 similar integrations sites were found in the human scaffold and the murine BM (e.g. the
218 red integration site), but also that slight changes in clonality were observed as well (e.g.
219 the yellow integrations site in BM and Sc2). In other cases where multiple scaffolds
220 generated leukemias, different unique clones could engraft in individually injected
221 scaffolds (Sc1 versus Sc3, indicated by different colors), whereby one clone that was

222 seen in the dominant clone in bone marrow (red clone) was also seen in the scaffolds.
223 And finally, in a secondary transplantation experiment most but not all primary clones
224 were seen in secondary leukemias grown in the scaffolds or BM (Supplementary Figure
225 S3d, Sc2 in mouse 1 versus 2nd Sc1-3 and BM, red integration site), although some
226 integration sites were only seen in one scaffold (Sc3, green integration site), and
227 additional clones appeared upon 2nd transplantation as well (sc2-3, yellow integration
228 site), which were most likely quiescent in the primary transplant.
229 In 3/8 huBM-sc mice (m1-m3), exclusively $CD19^+$ B-ALL was observed in the scaffolds,
230 with phenotypes resembling those we previously observed in i.v. injected NSG mice
231 without human niches ([5,6] and Figure 3a-d, Supplementary Table 1). Within these mice,
232 infiltration to mouse organs was observed as well, displaying phenotypes similar to
233 those in the humanized scaffolds. Some mice displayed a somewhat more mature
234 leukemic B-ALL phenotype with the presence of $CD19^+$/$CD20^+$ clones (data not shown).
235 In 4 out of 8 huBM-sc mice (m4-m7) both B-ALL as well as myeloid $CD19^-$/$CD33^+$
236 clones were detected (Figure 3e, Supplementary Table 1, Supplementary Figure 3), and
237 in one mouse (m8) one scaffold was completely lymphoid while another was completely
238 myeloid. Within this myeloid clone, some co-expression of myeloid markers such as
239 CD11b, CD15 and CD14 with CD33 was also detected, resembling the phenotype we
240 observe in *in vitro* myeloid cultures as well as in patients, and the lymphoid and myeloid
241 phenotypes were confirmed by MGG staining (Figure 3f-g).
242 In total, and in contrast with murine niches, out of 24 engrafted scaffolds we observed
243 co-existing myeloid and lymphoid clones in 9 cases (37.5%), and a complete myeloid
244 clone in 1 case (4.1%) although this difference did not reach statistical significance

245 (p=0.0804). Taken together these data suggest that the ectopic human BM
246 microenvironment provides a more permissive microenvironment to drive LSCs towards
247 myeloid differentiation.
248

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

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

267 some extend in BM, spleen and liver, although chimerism levels were very low
268 (Supplementary Table 1).
269 To obtain further insight into the clonality of these different engrafting populations, we
270 performed LM-PCR on cells harvested from primary as well as secondary huBM
271 scaffolds. As shown in Figure S4g, multiple bands were observed suggesting the
272 coexistence of multiple clones. The clone indicated by the red asterisk was detected in
273 both the primary and all secondary transplanted animals. Furthermore, multiple different
274 other integration sites were observed in secondary leukemic mice, indicative for
275 oligoclonal disease. Since a number of these clones were not detected in the primary
276 mouse these data suggest the presence of quiescent clones in the primary leukemic cell
277 population that were activated upon serial transplantation.
278 In order to assess the self renewal potential of CB MLL-AF9 leukemic cells generated in
279 huBM-sc of primary recipients, we also performed secondary transplantation.
280 $CD19^+/CD15^+$ lymphoid leukemic cells harvested from mouse m1 huBM-sc2 were
281 transplanted into humanized NSG mice. Tumor formation was observed in all the
282 injected scaffolds and FACS analyses displayed the same phenotype observed in the
283 primary recipient, although expression of $CD15^+$ was lost (Figure S4h). Infiltration of CB
284 MLL-AF9 positive cells was also observed in BM, spleen and liver albeit the chimerism
285 levels were lower in comparison to primary recipients (Supplementary Table 1).
286 Furthermore, we performed a secondary transplantation using cells from m6 huBM-sc2
287 that contained two distinct clones of $CD33^+$ (78.3%) and $CD19^+$ (~3%). Due to the small
288 number of cells available, only a single secondary huBM-sc could be injected with $1x10^5$
289 unsorted cells. Tumor formation was observed in the injected scaffold and the mouse

290 was sacrificed upon signs of illness after 15 weeks. Despite the fact that the vast
291 majority of injected cells were of myeloid origin, a $CD19^{+}/CD33^{-}$ B-ALL developed in the
292 scaffold, most likely arising from the small population of $CD19^{+}$ cells in the primary
293 recipient (Figure S4i), a feature we have observed more frequently. These results
294 indicate that despite the presence of a human niche microenvironment, MLL-AF9-
295 induced transformation of neonatal CB $CD34^{+}$ cells remain biased towards B-ALL.
296

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

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

313 Gene ontology (GO) analyses indicated that the huBM-sc overexpressed genes were
314 significantly (FDR<1) enriched for processes such as apoptosis and mitochondrion.
315 Genes upregulated in mBM-derived leukemias were significantly (FDR<1) enriched for
316 GO terms methylation and histone core related genes (Figure 4b). GSEA analysis
317 indicated that huBM scaffold-derived leukemias were enriched for MYC targets and
318 hypoxia signatures, suggesting that BCR-ABL-positive cells derived from the humanized
319 scaffold display differences in MYC dependency and hypoxic state as compared to
320 mBM-derived leukemias (Figure 4c). A number of selected GO terms for genes
321 upregulated in huBM scaffold-derived leukemias are shown in Figure 5d and
322 Supplementary Figure S5. Interestingly, p57, and MYC were some of the significantly
323 overexpressed secreted proteins in huBM scaffold-derived leukemias. Another
324 interesting observation was that endogenous BMI1 was overexpressed in BCR-ABL
325 positive cells derived from humanized scaffolds, possibly explaining why exogenously
326 overexpressed BMI1 is not required as is the case for leukemias that develop in the
327 murine BM niche. Illumina data were validated by independent Q-PCR analyses (Figure
328 4e).
329

330 **Primary Blast Crisis (BC)-CML and AML-MLL-AF9 cells can induce a fatal myeloid**
331 **leukemia within 16 weeks post transplantation in the huBM scaffold mouse model**
332 Next, cells from a blast crisis-chronic myeloid leukemia (BC-CML) primary patient were
333 injected i.sc. in huBM scaffold mice (Figure 5a). We observed that all three injected
334 mice developed tumors within 16 weeks post-transplantation. Tumors were well
335 vascularized and greenish of color (Figure 5b), and were positive for huCD45 as well as

336 for myeloid markers like CD33, CD11b, CD14, and CD38 (Figure 5c). MGG staining
337 clearly revealed the immature phenotype of scaffold-harvested cells as compared to
338 murine BM-harvested cells (Figure 5d). Infiltration of myeloid cells was observed in
339 mouse BM, spleen and liver (Supplementary Figure S6a). Cells in the mBM niche
340 displayed a more differentiated phenotype in terms of a higher proportion of cells
341 expressing the markers CD11b, CD14 and CD15 while a lower proportion of cells
342 expressed CD34 (Figure 5c, S6a-c). In contrast, leukemic cells derived from the huBM
343 scaffold more closely resembled the original patient and in particular CD34 percentages
344 were quite comparable (Figure 5c, S6a-c). These FACS analyses were confirmed by
345 immunohistochemistry (Supplementary Figure S6b).
346 The self-renewing potential of cells harvested from the murine BM niche and those
347 retrieved from the human niche in the huBM scaffolds was functionally studied in LTC-
348 IC assays. $CD34^+$ and $CD34^-$ cells were sorted and plated in limiting dilution and were
349 scored after 5 weeks. No cobblestone area forming cells (CAFCs) were detected at
350 week 5 in $CD34^+$ or $CD34^-$ isolated from murine BM harvested cells (Figure S6d), and
351 only differentiated macrophages could be observed (data not shown). In contrast,
352 CAFCs could readily be detected in cultures initiated with $CD34^+$ cells isolated from the
353 huBM scaffold harvested cells, with an LTC-IC frequency of 1 in 323 +/-115 (Figure
354 S6d). Thus, these results suggest that self-renewal of LT BC-CML LSCs was
355 maintained in the context of a humanized BM niche. Maintenance of self-renewal was
356 also demonstrated *in vivo* by serial transplantation assays (Antonelli, Groen and
357 Schuringa, to be submitted).

358 Finally, Q-PCR analyses was performed on BC-CML samples harvested from the
359 mouse BM and human scaffold niches, and similar as observed in our BCR-ABL
360 retroviral overexpression models, a significantly increased level of endogenous BMI1
361 was observed in cells derived from human scaffolds (Supplementary Figure S6e).
362 Lastly, we also injected primary MLL-AF9 AML patient material into huBM-sc of a NSG
363 mouse. Two scaffolds were injected with 1x10^{6} cells each. While we did not detect any
364 CD45^{+} chimerism in the blood during the course of the experiment, we sacrificed the
365 mouse 20 weeks after transplantation upon palpation of tumor growth on one of the
366 scaffolds (Figure 5e). Cells retrieved from the huBM-sc3 displayed a myeloid phenotype
367 with expression of CD33, CD11b and CD15 (Figure 5f), which was further confirmed by
368 MGG staining of cytospins (Figure 5g). No human cells could be detected in mouse
369 organs such as spleen, liver and or BM by FACS analyses (Figure 5f), and the spleen
370 size was not enlarged (weight: 0.12g).
371
372 **Intrascaffold *in vivo* treatment with IBET151**
373 We next evaluated whether the huBM-sc model could also be used for drug testing. In
374 our *in vitro* CB MLL-AF9 myeloid and lymphoid MS5 co-culture system we observed
375 that, upon inhibition of BRD3/4 binding to acetylated histones with the small molecule I-
376 BET151[12,13] proliferation was severely impaired (data not shown). Here, we assessed
377 the therapeutic potential of I-BET151 *in vivo* in our huBM-sc B-ALL CB MLL-AF9
378 xenograft model. 5x10^{5} cells harvested from a primary B-ALL MLL-AF9 generated in
379 huBM-sc mice were injected into 3 huBM scaffolds of secondary recipients, while the
380 fourth scaffold was left uninjected. Four weeks after injection of the cells, we performed

381 daily i.sc. injections of the inhibitor for a total of 23 days. One control group (n=5) was
382 injected with a solution of PBS and DMSO 1%, one group (n=3) with I-BET151 500 μM
383 and another group (n=5) with I-BET151 5mM. Tumor volume size of each scaffold was
384 measured during the experiment every 3 days, allowing us to monitor the progression of
385 the disease in time. <u>All mice were sacrificed at the same time at day 54 after injection.</u>
386 Mice treated with 5 mM I-BET151 displayed a significant reduction in tumor volume (~2
387 fold, $p<0.0001$). Accordingly, also the cell number of the cells harvested from the tumors
388 was significantly different (for 500 μM group $p=0.04$, for 5 mM group $p=0.01$) (Figure 6a-
389 d). No significant differences were found in spleen weight or blood and BM chimerism.
390 Together, these data indicate that I-BET151 treatment significantly delays lymphoid
391 leukemia progression *in vivo*, but is not sufficient for a complete eradication of the
392 leukemic cells, at least not in this experimental setting.
393

394 **Discussion**

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

403 (huBM-sc) microenvironment[7,8] in which human myeloid and lymphoid leukemias can be
404 studied in detail.

405 Various retro/lentiviral models have been established to model human myeloid and
406 lymphoid leukemias, and while *in vitro* BCR-ABL or MLL-AF9 oncogenes (either alone
407 or in combination with other hits) could induce transformation along myeloid or lymphoid
408 lineages, *in vivo* transformation was always heavily lymphoid biased and *in vivo* myeloid
409 transformation has been much more difficult to achieve in NOD-SCID based xenograft
410 models, including the NSG mice.[5,6,11,14] Furthermore, a significant number of primary
411 AML samples does not engraft in the NSG mouse model[4], again pinpointing to the
412 notion that a niche that allows self-renewal of myeloid (malignant) clones is not provided
413 for in the NSG mouse. Over the past years, xenograft leukemia models have already
414 been significantly improved, and advantages and disadvantages of various humanized
415 mouse models have recently been nicely reviewed.[3,15] NOD/SCID-3/GM/SF mice
416 engineered to produce human IL3, GM-CSF and SF[16,17] and NOD-SCID IL2R$\gamma^{-/-}$ (NSG)
417 mice expressing human IL3, GM-CSF and SF (NS-SGM3)[18] have been generated
418 which enhanced engraftability of primary human AML samples, as well as others[19-23]
419 including MISTRG mice expressing M-CSF, IL3, GM-CSF, TPO and SIRPα.[19] Even
420 MDS patient samples or MDS-derived cell lines were reported to engraft making use of
421 human cytokine mice.[24,25] These models are clearly useful tools, although other human
422 niche-specific factors might still be missing.

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

426 of NOD-SCID/NSG mice, a higher percentage of myeloid clones could be generated in
427 the huBM-sc model. This observation reinforces the notion that the lineage fate of MLL-
428 AF9 LSCs is dictated primarily by microenvironmental cues and cell of origin rather than
429 an instructive role of the fusion partner gene. While we could easily serially transplant
430 huBM-sc derived B-ALL clones, 2nd engraftment of myeloid clones was more difficult to
431 achieve. Possibly, the level of myeloid growth factors such as IL3[14], an important and
432 species-specific growth factor for myeloid leukemias driven by MLL-AF9, are still too low
433 in our huBM-sc model. Ongoing experiments include further improvement of the huBM-
434 sc model using MSCs that we have genetically engineered to express cytokines such as
435 IL3 and TPO. On the other hand, Wei and colleagues reported that upon injection of CB
436 CD34$^+$ cells transduced with MLL-AF9 into NS-SGM3 mice only AML but not B-ALL
437 developed, suggesting that these mice are now strongly biased towards myelopoiesis.
438 Besides lentiviral models for MLL-AF9, we also showed that primary AML MLL-AF9
439 derived from an adult patient could engraft efficiently in the huBM-sc, and it will be
440 interesting to compare the human cytokine mice with our humanized niche scaffold
441 model side-by-side in future experiments.
442 We also find that primary CD34$^+$ CML patient cells can efficiently engraft in this
443 xenograft model, whereby stemness is much better preserved in the human
444 environment as compared to the mouse environment. Furthermore, we describe that
445 this humanized niche model also allows for BCR-ABL-driven myeloid and lymphoid
446 disease, in contrast to the NOD-SCID/NSG mouse in which exclusively CD19$^+$ B-ALL
447 was observed upon transplantation of transduced CB CD34$^+$ cells (current study and [16]).
448 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 niche[6], 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.

472

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481

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.

488

489 **Conflict of Interest**

490 The authors have declared that no competing interests exist.

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597

598 **Figure legends**

599 **Figure 1. Mouse versus human extrinsic cues dictate transformation potential in**
600 **BCR-ABL/BMI1 and MLL-AF9-induced leukemia in humanized xenograft models.**
601 **(a)** Kaplan-Meier survival curves of BCR-ABL/BMI injected primary and secondary mice
602 from mBM i.v. and huBM i.sc. models displaying the differences in kinetics of leukemia
603 development. (**b)** Kaplan-Meier survival curves of CB MLL-AF9 injected primary and
604 secondary mice from mBM i.v. and huBM i.sc. models displaying the differences in
605 kinetics of leukemia development. (**c-d**) A summary of phenotypes observed in mBM i.v
606 and huBM i.sc models for BCR-ABL and MLL-AF9.

607

608 **Figure 2. BCR-ABL can induce lymphoid and myeloid leukemia in a human niche**
609 **xenotransplant model without exogenous BMI1. (a)** Detailed FACS analyses of
610 BCR-ABL positive cells from Scaffold 3 of Mouse 5 indicating positive surface marker
611 expression for CD19, CD38 and also for CD33, CD15. (**b**) 16 weeks post-
612 transplantation a tumor was detected at the site of injection. (**c**) HE staining of B-ALL
613 tumor scaffold demonstrating efficient bone formation. (**d**) B-ALL tumor scaffold section
614 stained by immunohistochemistry for CD45 demonstrated engraftment of $CD45^+$ human
615 cells. $CD45^-$ cells were also observed suggesting the presence of endothelial cells
616 forming a vascularized niche. (**e**) MGG staining confirmed the lymphoblastic phenotype
617 of BCR-ABL single positive cells. (**f**) FACS analyses of BCR-ABL positive cells from
618 Scaffold 1 and 3 of Mouse 1 indicating expression of GPA, CD71 and CD33 markers.
619 The more reddish Scaffold 3 was also the most positive for GPA.

620

621 **Figure 3. Increased incidence of myeloid leukemia on MLL-AF9 induction**
622 **observed in huBM-sc as compared to NSG. (a)** FACS analyses of MLL-AF9 cells
623 from BM, Sc1 and Sc2 of ISC mouse 1. **(b)** Picture showing tumor initiated on the
624 scaffolds for mouse 1. (**c**) B-ALL tumor scaffold 1 section stained by
625 immunohistochemistry for CD45 demonstrated engraftment of $CD45^+$ human cells. (**d**)
626 HE staining of B-ALL tumor scaffold 1 demonstrating efficient bone formation. (**e**) FACS
627 analyses of MLL-AF9 cells from BM, Sc1 and Sc2 of ISC mouse 6. **(f-g)** MGG staining
628 of BM and Sc1 of ISC mouse 6 confirming the lymphoblastic phenotype **(h)** MGG
629 staining of Sc2 of mouse 6 confirming the presence of lymphoid and myeloid cells.
630

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

644 value of ≤ 0.01). (**c**) GSEA analyses on i.sc. versus i.v. samples. (**d**) GO terms of up
645 regulated genes in huBM-sc 1st and 2nd scaffold derived samples. (**e**) Validation of
646 Illumina data by real time PCR indicating expression of endogenous BMI1, p57 and
647 MYC relative to the house-keeping gene RPL27. Experiments were performed in
648 triplicate.
649
650 **Figure 5. Primary Blast Crisis (BC)-CML and AML MLL-AF9 cells can engraft in**
651 **the huBM scaffold mouse model and induce a fatal myeloid leukemia within 16**
652 **weeks post transplantation.** (**a**) A myeloid BC-CML patient sample was injected in 3
653 scaffolds and 1 scaffold was left uninjected in 3 different humanized mice. $1.5x10^5$
654 50,000 cells per scaffold were injected. (**b**) All 3 injected mice developed enlarged
655 greenish tumors within 16 weeks post-transplantation. (**c**) Detailed FACS analyses of
656 $CD45^+$ engrafted cells derived from humanized scaffolds as well as from the murine BM.
657 CD34 was higher expressed in cells retrieved from the human scaffold while CD15,
658 CD11b and CD14 were higher expressed in cells derived from the murine BM. (**d**) MGG
659 staining indicated a more immature phenotype of scaffold-harvested cells as compared
660 to more differentiated cells from the murine BM (63X magnification). (**e**). Tumor growth
661 of a scaffold injected with 1×10^6 primary MLL-AF9 AML patient cells. (**f**). FACS
662 phenotype of cells harvested from the scaffold displayed in g and from mouse
663 compartments (PB, BM, Liver and Spleen). (**g**) MGG staining of cytospins of cells
664 harvested from the scaffold displayed in e.
665
666 **Figure 6. I-BET1 efficacy using MLL-AF9 huBM-sc model.**

667 (**a**) Schematic representation of the experimental design. Control mice were injected
668 with normal saline containing 1% (v/v) DMSO. Treated mice were injected with 500μM
669 and 5mM I-BET151 (**b**) Tumor volumes measured by caliper during the course of the
670 experiment. (**c**) Pictures depicting tumors originated on huBM-sc. Comparison between
671 control group and 5mM I-BET151 treated group. (**d**) Total cell number of tumors tumors
672 originated on huBM-sc in the three groups.
673
674

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.