Recipient sex and donor leukemic cell characteristics determine leukemogenesis in patient-derived models

Anna M.P. Stanger,[1*] Marlon Arnone,[1*] Pauline Hanns,[2*] Lucca M. Kimmich,[1] Jessica Kübler,[1] Sarah Gekeler,[1] Elsa S. Görsch,[1] Lea Kramer,[1] Marcelle Baer,[2] Jan C. Schroeder,[1] Taylor S. Mills,[1] Martina Konantz,[2] Saskia S. Rudat[1,3] and Claudia Lengerke[1,3]

[1]University Clinic Tübingen, Department for Internal Medicine II, University of Tübingen, Tübingen, Germany; [2]University of Basel and University Hospital Basel, Department Biomedicine, Basel, Switzerland and [3]German Cancer Consortium (DKTK), partner site Tübingen, a partnership between DKFZ and University Hospital Tübingen, Germany

AMPS, MA and PH contributed equally as first authors.

Correspondence: C. Lengerke
claudia.lengerke@med.uni-tuebingen.de

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Abstract

In acute myeloid leukemia (AML), leukemogenesis depends on cell-intrinsic genetic aberrations and, therefore, studies on AML require investigations in an *in vivo* setting as provided by patient-derived xenograft (PDX) models. Here we report that, next to leukemic cell characteristics, recipient sex strongly influences the outgrowth of AML cells in PDX models, with females being much better repopulated than males in primary as well as secondary transplantation assays. Testosterone may be the more important player since, strikingly, better engraftment was seen in castrated male recipients than in control ones, while ovariectomy did not significantly impair engraftment in females. Shorter time to engraftment and mouse survival were observed in cases with adverse molecular risk, and respectively with a high ratio of *FLT3*-ITD mutated AML cells. Furthermore, cases of adverse-risk AML showed higher percentages of phenotypic leukemic stem cells, suggesting impaired differentiation capacity in these AML subtypes. Overall, we achieved successful repopulation with 14/23 (61%) favorable-risk, 18/30 (60%) intermediate-risk and 4/8 (50%) adverse-risk AML cases in female recipient PDX models. Our data identify recipient sex as an important experimental confounder in leukemia PDX models, and the contribution of the sex hormones to leukemogenesis as an intriguing, underexplored area for research.

Introduction

Acute myeloid leukemia (AML) is known to develop from hematopoietic stem and progenitor cells upon acquisition of various genetic aberrations.[1] To guide therapy selection, molecular criteria that stratify AML cases into favorable-, intermediate- and adverse-risk groups were established based on analyses of patients' outcomes over the past decades.[2]

AML cells harbor cellular subpopulations of so-called leukemic stem cells (LSC) that are responsible for disease initiation, as well as for the fatal disease relapses commonly occurring even in patients reported to have achieved complete remission.[3,4] Similar to healthy hematopoietic stem and progenitor cells as their cell of origin, AML LSC rely on microenvironmental influences that are challenging to reproduce in a cell culture dish.[5-7] Patient-derived xenograft (PDX) models in which human AML cells are transplanted to immune-suppressed mice are instead commonly used to study human AML and LSC *in vivo*. Given their ability to mimic disease onset, evolution, heterogeneity and the interactions between AML cells and the microenvironment, PDX models are powerful tools to investigate AML pathogenesis, drug response, and LSC biology. However, PDX models have been mainly used to study AML with adverse molecular risk, characterized by robust leukemic repopulation capacity, while other AML cases, such as favorable-risk AML, remain understudied because they hardly engraft animals in standard assays.[8-10]

Here we show that, next to molecular characteristics, recipient sex has a strong impact on leukemogenesis in PDX models. Transplantation into female recipients promotes leukemic engraftment across various genetic subtypes, allowing robust repopulation with subtypes that were previously reported to be difficult to engraft, such as favorable-risk AML. To further study the impact of sex hormones on leukemogenesis, we compared the use of castrated males and ovariectomized female mice as recipients. Our results demonstrate that experiments with PDX models need to consider recipient sex to yield

reliable results. Furthermore, they indicate the impact of sex hormones and the female environment on leukemia development and therapy response as a novel intriguing area of research.

Methods

Patients' samples

Peripheral blood (PB) samples (*Online Supplementary Tables S1* and *S2*) and clinical data were collected following approval by the Ethics Review Board of the University Hospitals of Basel (EKNZ 2015-335) and Tübingen (953/2021B02) from patients with AML at first diagnosis. PB mononuclear cells were enriched by density gradient centrifugation (Sepmate, StemCell and Pancoll, Pan-Biotech) and viably frozen in RPMI1640 medium (Thermo Fisher) supplemented with 20% fetal bovine serum (Gibco) and 10% dimethylsulfoxide (AppliChem). Patients' data were collected from the routine clinical database. Complete remission is defined as <5% blasts in bone marrow (BM) punctures performed in hematologic regeneration after treatment, and residual disease as ≥5% blasts in post-treatment BM samples.

Mice, xenotransplantation and homing assay

NOD.Cg-*Prkdcscid IL2rgtmWjl*/Sz (NSG) mice purchased from Jackson Laboratory (Bar Harbor, ME, USA) were bred in-house under pathogen-free conditions according to German and Swiss federal and state regulations. PB mononuclear cells were freshly thawed before each experiment. For samples with a CD33 blast count <95%, magnetic cell separation was performed to deplete $CD19^+$ and $CD3^+$ cells (Miltenyi Biotec). For the comparisons of leukemic engraftment between male and female NSG mice, $1x10^6$ cells were injected intravenously (i.v). For the subsequent kinetic engraftment observations, $0.5x10^6$ cells were injected intra-femorally in 6- to 10-week-old female NSG mice. The transplantation procedure and monitoring were performed as previously described[11] and detailed in the *Online Supplementary Information*. Of note, within one experiment all female and male mice had the same age at transplantation, but between experiments the age ranged from 6 to 10 weeks. All animals were pre-conditioned with sublethal irradiation (100 Gy/kg) 24 hours prior to transplantation and each sample was injected into age-matched animals. Mice were considered engrafted upon detection of >0.1% human leukemic cells among murine cells of PB, BM or other analyzed tissues. The experiment was terminated at detection of a high leukemic burden (defined as >60% of leukemic cells among mouse cells in the murine PB or BM), or a sickness score exceeding 3, or at a follow-up 1 year after the transplant.

For secondary transplant assays, BM cells from mice engrafted with one primary AML were pooled, enriched for human CD33 expression as described[11] and then injected i.v. at equal numbers ($1x10^6$) as described above.

For homing experiments, $1x10^6$ primary AML cells were labeled with carboxyfluorescein succinimidyl ester (CFSE) and injected i.v. into non-irradiated male NSG mice. Sixteen hours after injection, BM and PB were sampled and analyzed for $CFSE^+$ AML cells using flow cytometry.

Flow cytometry analysis

Single-cell suspensions from patients or mouse BM, PB, spleen and liver were stained with fluorescently labeled antibodies against human CD33, CD34, CD117, CD45RA, CD38, CD47 (BD Biosciences), NKG2DL (R&D), CD123 (Miltenyi), GPR56, CD3 and CD19 (Biolegend). Dead cells were excluded using the fixable aqua dead cell stain kit (Thermo Fisher). All marker expression values derive from live $CD33^+$ cells.

Statistical analysis

Data are expressed as mean ± standard deviation. Comparisons were performed after analysis of normal distribution using (un)paired Student *t* tests, the Mann-Whitney U test or one-way analysis of variance. Survival analyses were performed using the Kaplan-Meier method. All analyses were conducted with GraphPad Prism v9.4.1 and statistical significance is defined as a *P* value <0.05.

Results

Recipient sex influences leukemogenesis in acute myeloid leukemia patient-derived xenograft models

To test the effect of female *versus* male recipient sex on leukemogenicity, we transplanted equal numbers of leukemic cells from the same patients into pre-conditioned age-matched male and female mice. Of note, donor AML cells from both female and male patients were used. Interestingly, female recipients transplanted with female donor cells showed highest leukemic infiltration (BM: 80.8 ± 28.0%), followed by female recipients receiving male AML donor cells (BM: 54.5 ± 24.7%). The latter were also better repopulated compared to male recipients transplanted with male AML cells (BM: 29.7 ± 31.3%), while male recipients transplanted with female AML donor cells showed the lowest repopulation rates compared to all other groups (BM: 16.0 ± 18.9%) at all analyzed timepoints and in multiple tissues (Figure 1A). Limiting dilution assays excluded a possible cell dose-mediated artefact (Figure 1B). Similar results were observed in secondary transplantation assays, in which leukemic cells derived from female mice successfully expanded in female recipients, but almost entirely failed to engraft in male secondary recipients (Figure 1C). Interestingly, next-generation sequencing analysis of leukemic cells retrieved from mice transplanted from the same AML sample showed similar clonal compositions between female and male recipients (Figure 1D), indicating that the female environment promoted engraftment across

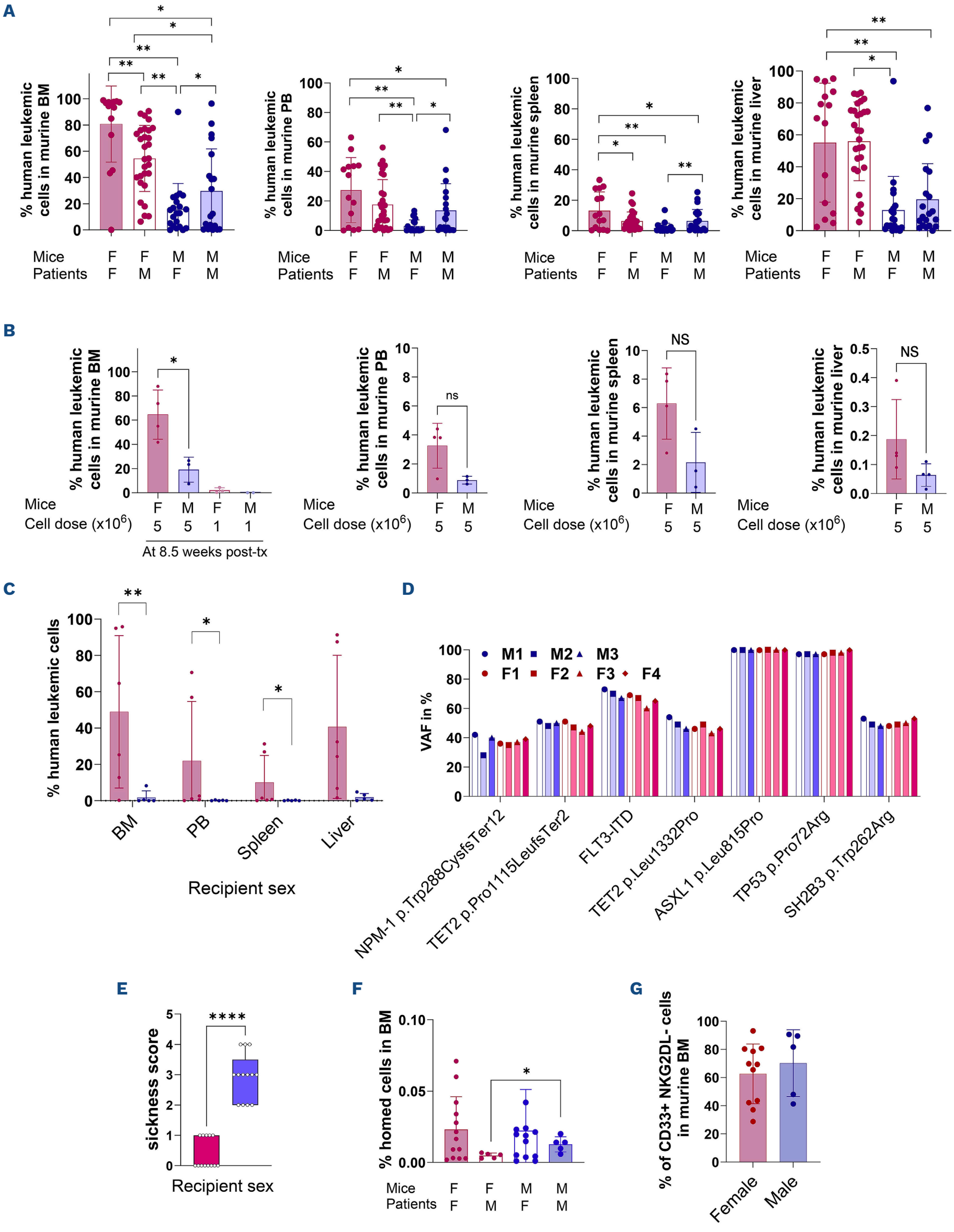

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Figure 1. Female NSG mice promote leukemic outgrowth in patient-derived xenograft models. (A) Engraftment assessment using flow cytometry in bone marrow (BM; left panel) and peripheral blood (PB) and organs (right panels) at endpoint analysis. Cells isolated from the indicated tissues from both male and female NSG mice were screened at the same timepoint for $CD33^+$ acute myeloid leukemia (AML) cells using flow cytometry (9 primary AML samples, 14 female mice with female donor cells, 28 female mice with male donor cells, 21 male mice with female donor cells and 19 male mice with male donor cells). (B) Limiting dilution assay. Mice were transplanted with $5x10^6$, $1x10^6$ and $0.1x10^6$ cells per mouse and screened at the same timepoint for $CD33^+$ AML cells using flow cytometry (1 AML sample with 3-4 mice per group). (C) Secondary transplantation assay; AML cells expanded in female NSG recipients were re-transplanted into female and male secondary recipients. Engraftment of $CD33^+$ AML cells was assessed by flow cytometry in the BM, PB and spleen from male and female NSG mice at endpoint analysis (2 primary AML samples, 6 female and 6 male NSG recipients). (D) Next-generation sequencing analysis of bulk cells retrieved from female and male recipients (1 AML sample). Mice (3 males and 4 females) were analyzed separately. (E) Sickness score of female and male recipients transplanted with the same AML samples (2 AML with 10 mice per group and sample). (F) Homing assay analyzing $CFSE^+$ cells in the murine BM as assessed by flow cytometry 16 hours after transplantation (5 primary AML samples, 19 female and 19 male NSG animals). (G) Flow cytometric quantification of NKG2DL-negative cells in murine BM (3 primary AML samples, 11 female and 5 male NSG animals). D'Agostino & Pearson test was used to test for normality. For statistical significance, Student *t* test: (A) for BM, PB and spleen, (B) and (F); Mann-Whitney *t* test: (A) for liver, (E) and (G). **P*<0.05, ***P*<0.01, *****P*<0.0001. NS: not statistically significant; F: female; M: male; post-Tx: after transplantation.

all investigated genetic backgrounds. Intriguingly, despite harboring lower numbers of leukemic cells, male mice showed more symptoms of disease than female recipient mice, as quantified by our "sickness score" (see Methods and Figure 1E).

Next, we asked why female mice had higher numbers of human leukemic cells than experimentally matched male recipients. A potential explanation would be enhanced cell homing to the BM environment after transplantation. To test this, we injected $1x10^6$ human leukemic cells from the same donor (male and female) into age-matched male and female mice by tail vein injection and assessed the percentages of human leukemic cells among murine BM cells 16 hours later (*Online Supplementary Table S2*). In contrast to the clear differences observed in long-term repopulation assays, male and female recipients showed similar homing rates (Figure 1F), suggesting that the observed long-term effects are independent of this early phase of leukemic engraftment.

Another possibility would be that recipient sex biases leukemic (stem) cell expansion. In previous studies, we showed that AML cells contain LSC-enriched subpopulations, identified by the absence of NKG2D ligand (NKG2DL) expression.[12] However, female and male mice engrafted with AML cells of the same donors showed comparable percentages from NKG2DL-negative AML cells, indicating that recipient sex did not specifically affect LSC (Figure 1G).

We next sought to investigate mechanistic influences of sex hormones mediating AML repopulation in PDX, and compared AML engraftment in ovariectomized *versus* control female recipients, and respectively in orchiectomized (castrated) *versus* control male mice. Figure 2A summarizes the results obtained by transplanting three different AML samples (2 male and 1 female donor) side-by-side in these

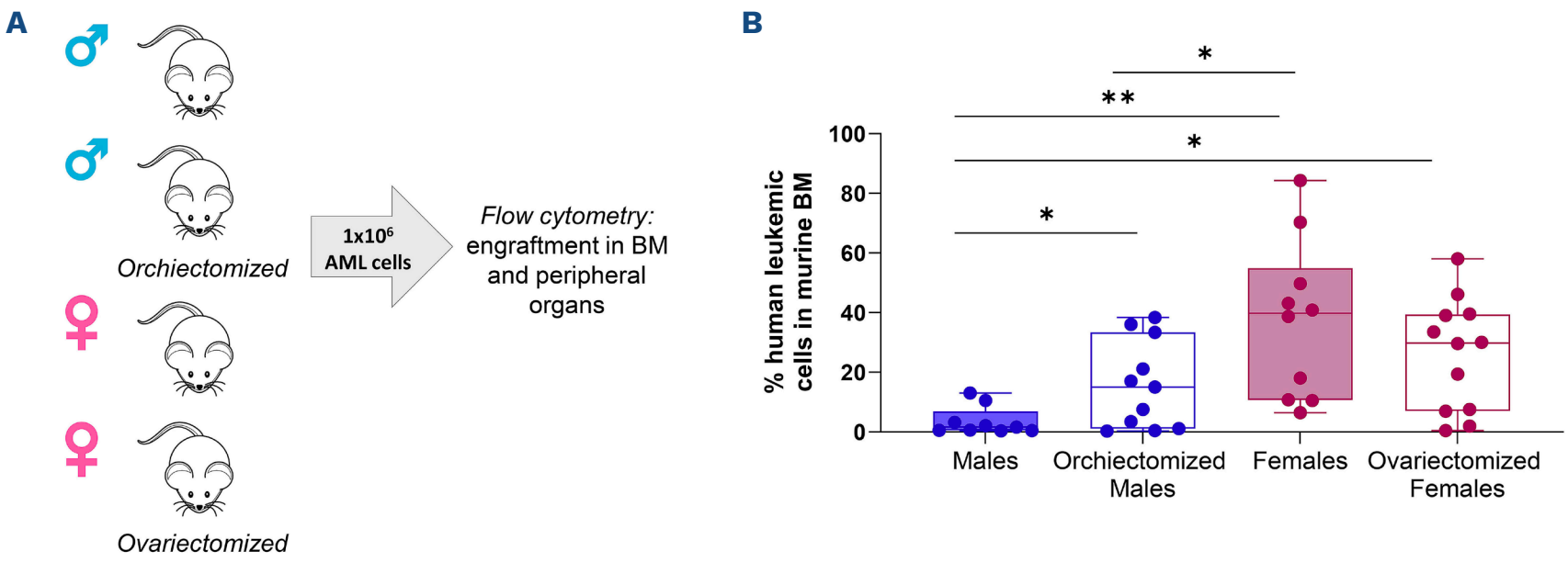

Figure 2. Different sex hormones influence leukemic bone marrow engraftment in patient-derived xenograft models. (A) Schematic overview of the study. (B) Engraftment assessment using flow cytometry in bone marrow (BM) at endpoint analysis. Cells isolated from all groups, ovariectomized female and orchiectomized (castrated) male next to control female and control male NSG mice, were collected at the same timepoints and screened for $CD33^+$ AML cells using flow cytometry (3 primary AML samples, 11 orchiectomized (castrated) male, 12 ovariectomized female, 10 regular male and 12 regular female NSG recipients). D'Agostino & Pearson test was used to test for normality, a Student *t* test was performed for statistical significance. **P*<0.05, ***P*<0.01.

recipients. Strikingly, castrated male recipients engrafted better than control male recipients while ovariectomy did not alter engraftment efficiency in female mice (Figure 2B, *Online Supplementary Figure S1*).

Molecular acute myeloid leukemia cell characteristics have an impact on leukemogenesis in patient-derived xenograft models

Favorable-risk AML cases were reported to engraft poorly in PDX models. Based on our results, we hypothesized that transplantation into female recipients may improve engraftment across all AML, including favorable-risk subtypes, thus allowing a more comprehensive *in vivo* analysis of heterogeneous AML subtypes.

We transplanted 61 AML cases stratified into favorable (N=23), intermediate (N=30) or adverse (N=8) European LeukemiaNet (ELN) risk groups[13] (*Online Supplementary Table S1*) into 175 NSG female mice and followed them for up to 1 year after the transplantation.[11] We screened mice for leukemic repopulation by routinely performing PB analyses and BM punctures. Detectable repopulation increased over time and was documented in 36 out of the total 61 (59.02%) transplanted AML cases (14/23 favorable-, 18/30 intermediate-, and 4/8 adverse-risk AML) (Table 1). Of note, animals negative in early routine BM punctures later turned positive, showing robust leukemic repopulation. This demonstrates that leukemia is initiated by rare cell populations that can remain undetectable for extended periods of time in this model – reminiscent of outcomes in patients, in whom relapses are often detected after several months of apparent post-therapy remission.

Interestingly, mice injected with favorable-risk AML cells, despite receiving no treatment, showed a particularly late onset of leukemia (48 weeks, *versus* 39 or 18 weeks for intermediate- or adverse-risk AML cells, respectively; P=0.0031 and P=0.0001, respectively) when all mice were considered and 38 weeks *versus* 28 weeks for intermediate-risk or 18 weeks for adverse-risk AML cells when only engrafted mice and samples were considered (Figure 3A).

Recently, Mian *et al*. showed that donor sex influences the engraftment of leukemic cells.[14] Subdivision of the data set according to donor sex revealed decreased survival with transplanted female *versus* male donor cells in favorable-risk AML cells (*Online Supplementary Figure S2A*), while no differences were observed with intermediate- or adverse-risk subtypes. Favorable-risk AML cells also showed reduced homing rates (*Online Supplementary Table S2*) compared to intermediate-risk (P=0.0131) or adverse-risk (P=0.0970) samples (Figure 3B) with more pronounced effects when female donor cells were transplanted (*Online Supplementary Figure S2B*).

FLT3 mutational status predicted time to leukemia and murine survival, highlighting the oncogenic nature of this mutation either alone or in the setting of *NPM1* mutations (Figure 3C). Interestingly, double-mutated *FLT3*-ITD/*NPM1*-mutant AML showed poor survival (Figure 3C) and enhanced homing by trend (Figure 3D), when compared to *FLT3* wild-type, or *FLT3*-ITD mutated/*NPM1* wild-type cases, suggesting that the female environment has a particularly negative impact in the context of this AML subtype (Figure 3D, E). No further homing differences could be documented (Figure 3F). Of note, while *NPM1* and *FLT3* mutational status was assessed in all cases, next-generation sequencing for further leukemia-specific mutations was not routinely performed. The impact of other genetic aberrations on the observed results cannot, therefore, be further explored.

Next, we asked whether mouse leukemia kinetics can predict clinical outcome in patients. AML cells collected at first diagnosis from patients in whom treatment subsequently induced complete remission showed delayed leukemogenesis in xenograft models when compared to AML cells derived from patients who had residual leukemia after treatment (Figure 4A). Lastly, we found that the French-American-British AML category was predictive of

Table 1. Engraftment kinetics in NSG animals.

Time points	N of engrafted samples per risk group (%)	Total engrafted samples, %	N of engrafted animals per risk group (%)	Total engrafted NSG mice, %
16 weeks	4/23 favorable (17.39) 3/30 intermediate (10.00) 1/8 adverse (12.50)	13.11	8/175 favorable (2.86) 6/175 intermediate (3.43) 2/175 adverse (1.14)	9.14
26 weeks	5/23 favorable (21.74) 8/30 intermediate (26.67) 2/8 adverse (25.00)	24.59	9/175 favorable (5.14) 14/175 intermediate (8.00) 15/175 adverse (8.57)	21.71
39 weeks	11/23 favorable (47.83) 12/30 intermediate (40.00) 3/8 adverse (37.50)	42.62	16/175 favorable (9.14) 26/175 intermediate (14.86) 16/175 adverse (9.14)	33.14
52 weeks	14/23 favorable (60.87) 18/30 intermediate (60.00) 4/8 adverse (50.00)	59.02	17/175 favorable (9.71) 37/175 intermediate (21.14) 18/175 adverse (10.29)	41.14

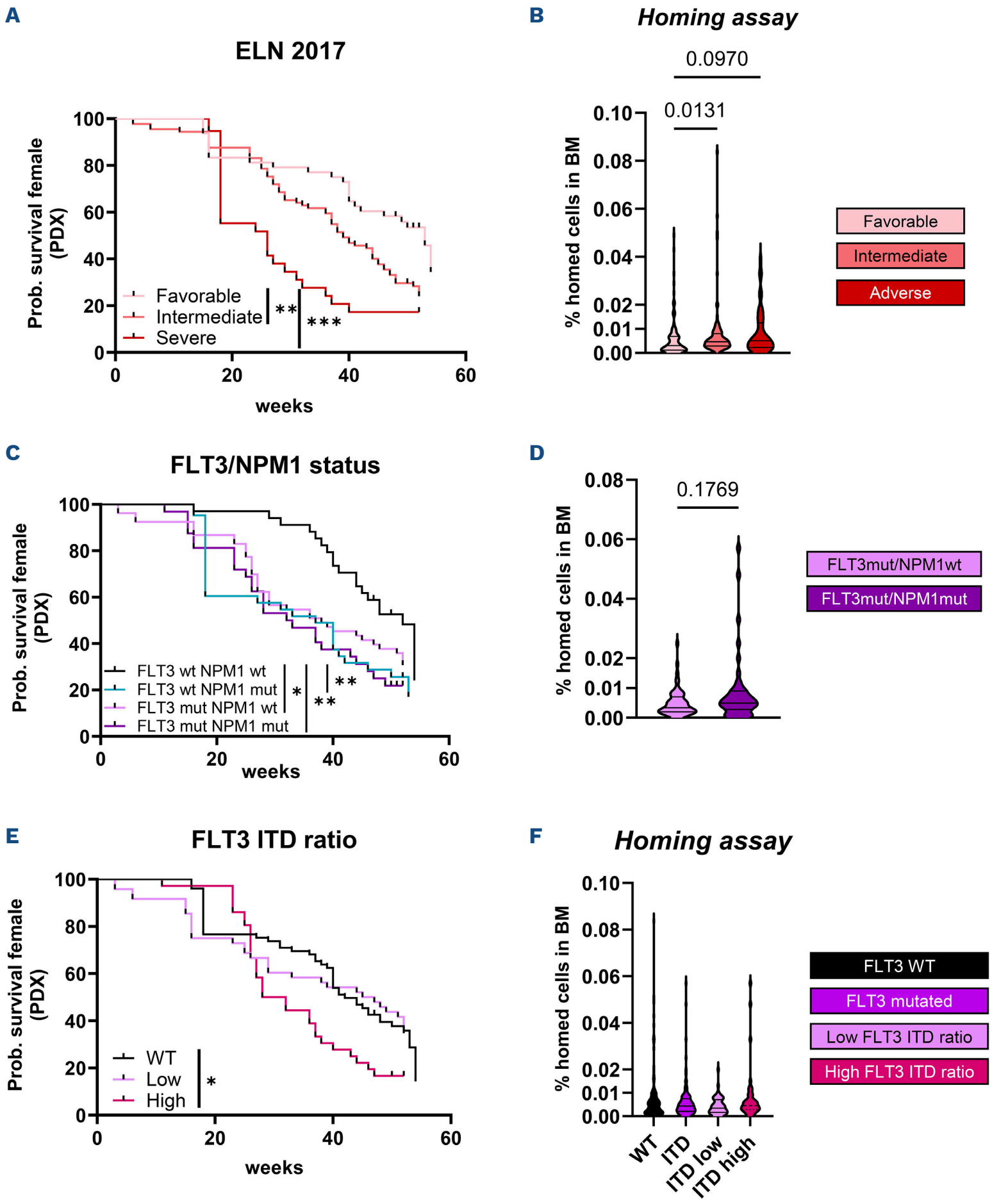

Figure 3. Influence of leukemic characteristics in patients on leukemic cell repopulation in NSG mice. (A) Murine survival based on the European LeukemiaNet 2017 risk classification (23 favorable-risk samples, 48 NSG mice; 30 intermediate-risk samples, 89 NSG mice; 8 adverse-risk samples, 38 NSG mice). (B) Homing assay analyzing CFSE⁺ cells in the murine bone marrow (BM) as assessed by flow cytometry 16 hours after transplantation (34 favorable-risk samples, 114 NSG mice; 34 intermediate-risk samples, 104 NSG mice; 15 adverse-risk samples, 46 NSG mice). (C) Murine survival based on *FLT3/NPM1* mutational status (16 wild-type samples, 34 NSG mice; 11 *NPM1* single-mutant samples, 43 NSG mice; 17 *FLT3* single-mutant, 53 NSG mice; 11 double-mutant samples, 32 NSG mice). (D) Homing assay analyzing CFSE⁺ cells in the murine BM as assessed by flow cytometry 16 hours after transplantation (18 single *FLT3* internal tandem duplication (ITD) samples, 58 NSG mice; 14 double-mutant samples, 46 NSG mice). (E) Murine survival based on *FLT3* mutational status and ITD/wild-type (WT) ratio (27 *FLT3*-WT samples, 77 NSG mice; 17 *FLT3*-ITD low samples, 48 NSG mice; 10 *FLT3*-ITD high samples, 36 NSG mice). (F) Homing assay analyzing CFSE⁺ cells in the murine BM as assessed by flow cytometry 16 hours after transplantation (38 WT samples, 119 NSG mice; 32 *FLT3*-mutated samples, 104 NSG mice; 17 *FLT3*-ITD low samples, 54 NSG mice; 12 *FLT3*-ITD high samples, 41 NSG mice). Statistical analysis: log-rank test for survival analysis, Mann-Whitney U test for homing. *P<0.05, **P<0.01, ***P<0.001. ELN: European LeukemiaNet; PDX: patient-derived xenograft.

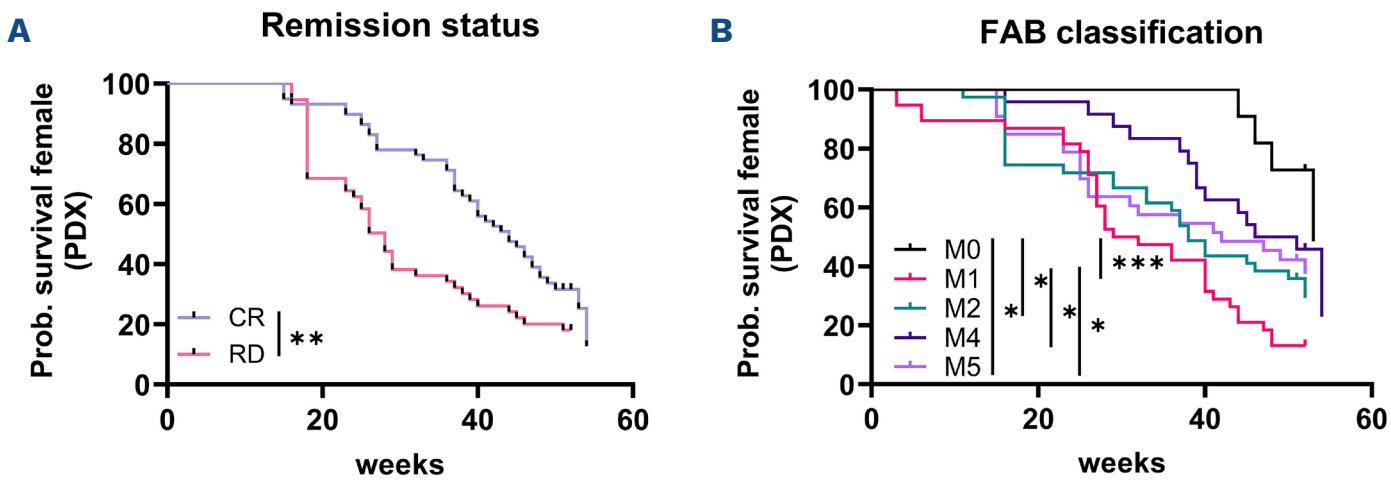

Figure 4. Influence of therapy response and French-American-British classification in patients on leukemic cell repopulation in NSG mice. Murine survival based on (A) remission status (24 complete remission samples, 59 NSG mice; 12 resistant disease samples, 57 NSG mice) and (B) French-American-British classification (4 M0 samples, 11 NSG mice; 12 M1 samples, 38 NSG mice; 14 M2 samples, 39 NSG mice; 11 M4 samples, 24 NSG mice; 13 M5 samples, 33 NSG mice). Statistical analysis: log-rank test for survival. 3B) and, vice versa, PDX: patient-derived xenograft; CR: complete remission; RD: residual disease; FAB: French-American-British. *P<0.05, **P<0.01, ***P<0.001.

leukemia kinetics (Figure 4B). Together, these data indicate that our PDX model in female recipient mice reliably mimics leukemogenesis and reflects differences in AML classifications established in of cohorts of clinical patients (*FLT3*-ITD mutations, European LeukemiaNet risk group, French-American-British class). It further intriguingly suggests sex disparities generally affect AML but might have more relevant effects in certain genetic backgrounds.

In contrast, patient´s age (younger *versus* older than 70 years) did not significantly influence results in PDX models (*Online Supplementary Figure S2C*), although as expected, patients over 70 years old more frequently displayed AML with adverse molecular subtypes, when compared to patients under 70 years old (*Online Supplementary Figure S2D*).

High expression of leukemia stem cell markers in adverse molecular risk acute myeloid leukemia

The data showing lower homing capacity with favorable-risk, compared to intermediate-risk or adverse-risk AML (Figure 3B), and *vice-versa*, enhanced homing with *FLT3*-ITD/*NPM1* double-mutated samples (Figure 3D), suggest that lower LSC content may be a determinant of better outcome in AML. We thus sought to investigate our primary and engrafted AML cells for phenotypic heterogeneity to specifically assess LSC content.

AML is known for its heterogeneity regarding cell surface marker expression.[3,15] In addition, AML cells were reported to change phenotypically during *in vitro* and *in vivo* experiments,[16-18] which complicates the interpretation of experimental results. Thus, we first investigated whether propagation of patient-derived AML cells in xenograft models can maintain their phenotypic properties.

Using flow cytometry, we assessed the expression of different stem/progenitor cell surface markers on AML cells collected from female recipient xenograft animals and compared it to the expression on originally transplanted AML cells. We observed that CD38, CD123, CD47, cKIT, and GPR56 expression did not change significantly in response to the *in vivo* transplantation experiment, while there was a decrease in CD34, and a concomitant increase in NKG-2DL expression (*Online Supplementary Figure S3A*). These data highlight that PDX experiments using female mice as transplant recipients in general maintain the original phenotype of the patient's AML cells, but in some cases may increase differentiation.

Due to female recipient mice having an environment that has little influence on AML cell phenotype, we next assessed phenotypic differences in molecular subtypes of AML that have been previously understudied. We hypothesized that LSC subpopulations might be increased in adverse molecular risk AML, accounting for their greater aggressiveness in patients. Indeed, adverse-risk AML samples had higher expression of the LSC marker CD34 compared to favorable- or intermediate-risk AML cells (Figure 5A, left panel), which was then maintained in the mouse xenografts (Figure 5A, right panel). *Vice-versa*, there was a trend for NKG2DL to be more highly expressed on favorable-risk *versus* intermediate-risk or adverse-risk AML, and this again was reflected in the phenotype of cells collected from PDX mice (Figure 5B). Furthermore, cKIT expression was higher on adverse-risk *versus* favorable-risk AML (Figure 6A), while GPR56, CD123, or CD47 showed similar distribution among molecular AML subtypes (Figure 6B-E). Interestingly, $NKG2DL^{-}cKIT^{+}$ subpopulations were significantly expanded in mice transplanted with intermediate- and adverse-risk AML compared to favorable-risk AML samples (Figure 6E).

Leukemia stem cell frequency among acute myeloid leukemia subtypes predicts leukemogenesis

We next explored whether the expression of the LSC markers CD34 and cKIT, and absence of NKG2DL, correlated with leukemogenesis in our female recipient PDX model. Indeed, in line with the observation that adverse-risk AML

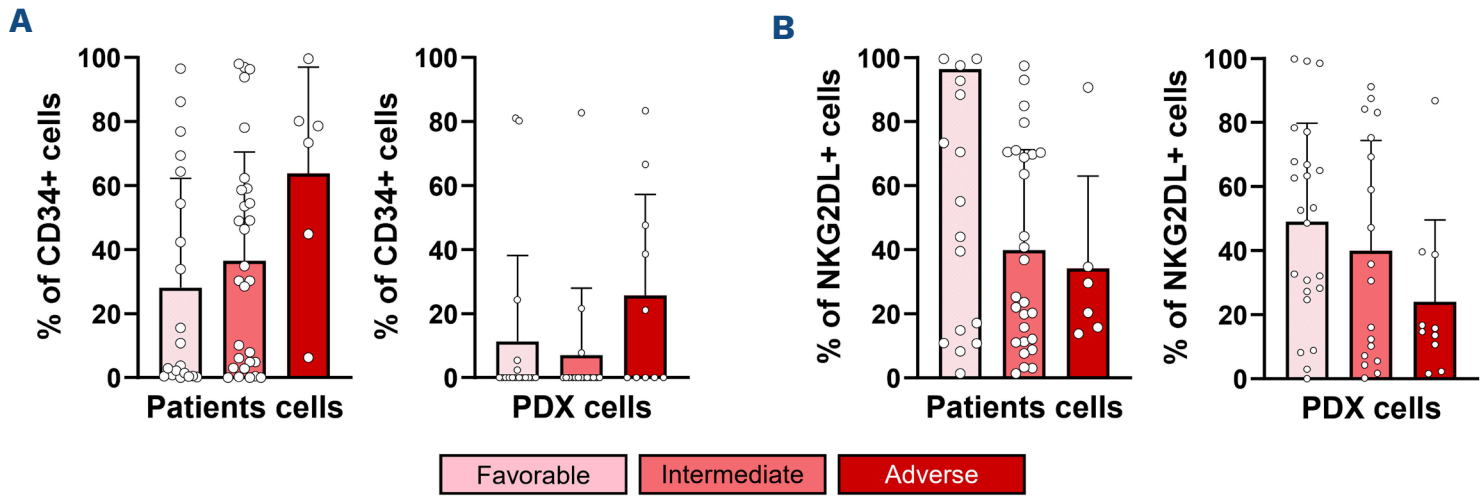

Figure 5. Correlation of CD34 and NKG2DL expression with acute myeloid leukemia risk group. Samples were distributed according to their molecular characteristics into favorable-, intermediate- and adverse-risk group samples. (A) Distribution of CD34$^+$ cells (left) directly from patients' biopsy and (right) patient-derived xenograft (PDX) cells. (B) Distribution of NKG2DL$^+$ cells (left) directly from patients' biopsy and (right) PDX cells. Acute myeloid leukemia (AML) cells were identified using CD33 positivity. Fifty primary samples (left panel A and B) and 24 primary samples in 51 NSG mice (right panel A and B) were analyzed. Statistical analysis: Kruskal-Wallis analysis of variance test.

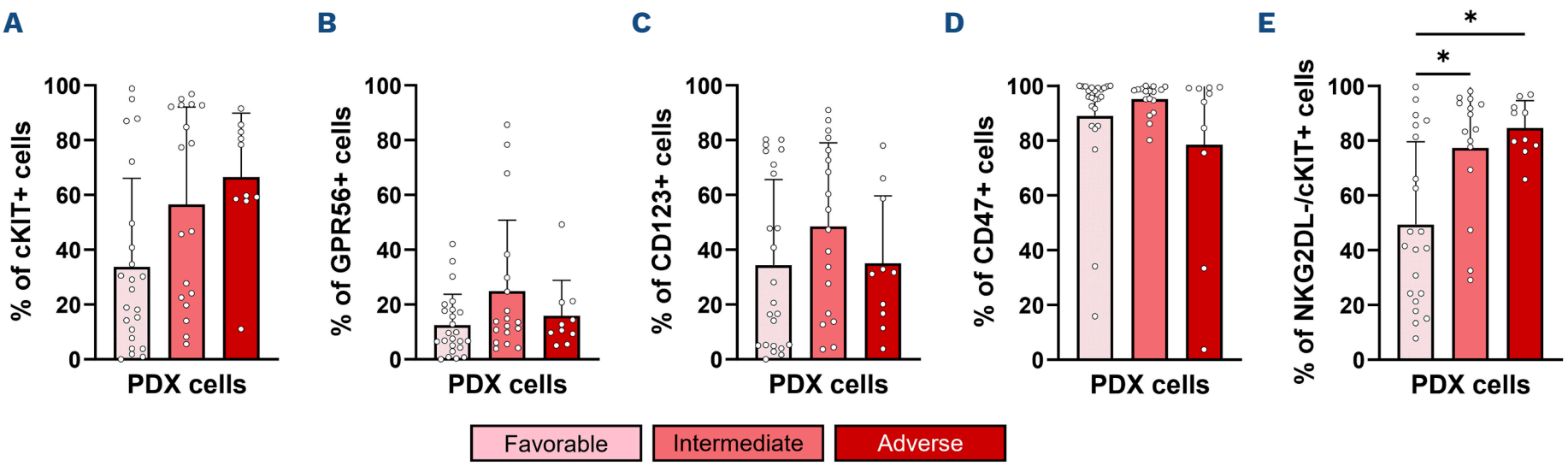

Figure 6. Association of leukemia stem cell frequency and acute myeloid leukemia risk group. (A-E) Distribution of marker expression between favorable-, intermediate- and adverse-risk group samples. (A) Distribution of cKIT$^+$ cells from patient-derived xenograft (PDX) cells. (B) Distribution of GPR56$^+$ cells from PDX cells. (C) Distribution of CD123$^+$ cells from PDX cells. (D) Distribution of CD47$^+$ cells from PDX cells. (E) Distribution of NKG2DL$^-$/cKIT$^+$ cells from PDX cells. Twenty-four primary samples in 51 NSG mice were analyzed in all datasets shown. Statistical analysis: Kruskal-Wallis analysis of variance test. *P<0.05.

exhibits decreased expression of NKG2DL, mice transplanted with AML cells characterized by low (bottom 25%) NKG-2DL expression showed increased leukemogenesis (Figure 7A). Surprisingly, while there was a significant increase of CD34$^+$ cells in adverse-risk *versus* favorable-risk AML, we did not find any segregation between mice transplanted with AML having different levels of CD34 expression (Figure 7B). Lastly, we found that there was a highly significant relationship between leukemogenesis and cKIT expression, with cKIT-expressing samples leading to higher leukemia burden (Figure 7C), which also correlated with increased BM homing capacity (Figure 7D). Overall, these data indicate that high LSC content promotes leukemia aggressiveness in PDX models, and supports the notion that the environment generated by transplanting AML samples into female mice allows an accurate representation of AML cell function in patients.

Discussion

Sex disparities are increasingly recognized as important in cancer development and therapy response.[19-23] The incidence of AML and myeloid neoplasia is higher in males than in females. In this report, we show that female recipient sex promotes leukemia induction in PDX models, enabling reconstitution across heterogeneous AML subtypes of all risk groups. Supporting the notion that the female environment promotes AML aggressiveness and/or impairs therapy response, *FLT3*-ITD mutated AML showed worse clinical outcome in female than in male patients.[19]

The limited cross-species reactivity hampers growth of human leukemic cells in the mouse microenvironment, and particularly influences selected AML subtypes. "Improved" NSG strains with transgenic expression of human interleukin-3, human granulocuyte-monocyte colony-stimulating

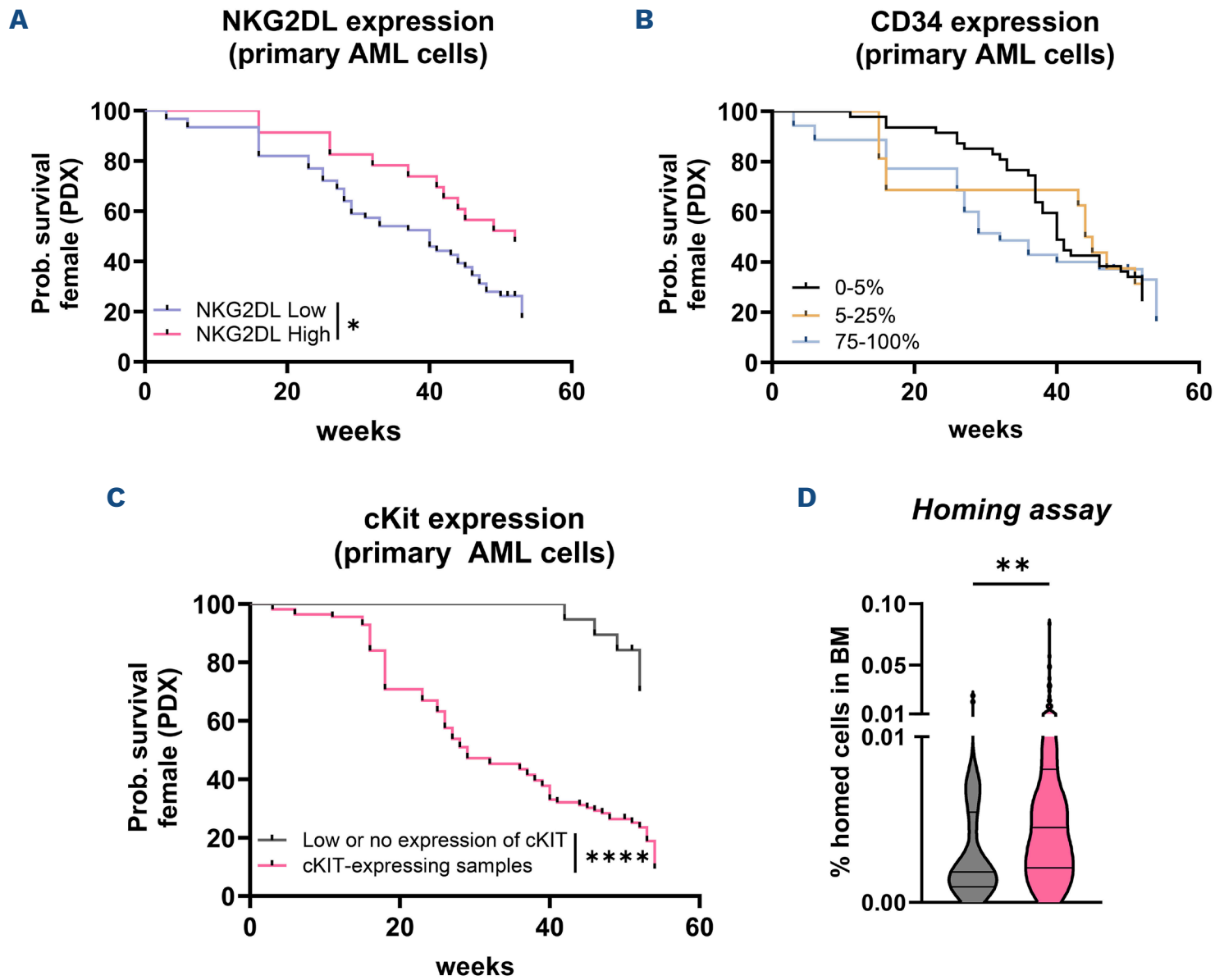

Figure 7. Donor-dependent parameters influence leukemia kinetics in NSG mice. Murine survival based on: (A) NKG2DL-expression status (25 bottom 25%, 64 NSG mice; 10 top 25%, 23 NSG mice); (B) CD34-expression status (19 CD34-non-expressing samples, 47 NSG mice; 6 CD34-expressing samples 5-25%, 16 NSG mice; 11 CD34-expressing samples 75-100%, 35 NSG mice); (C) cKIT-expression status (7 cKIT-non-expressing samples, 19 NSG mice; 35 cKIT-expressing samples, 93 NSG mice). (D) Homing assay analyzing CFSE+ cells in the murine bone marrow as assessed by flow cytometry 16 hours after transplantation (12 cKIT-non-expressing samples, 37 NSG mice; 46 cKIT-expressing samples, 144 NSG mice). Statistical analysis: log-rank test for (A-C) and Mann-Whitney U-test for (D). *P<0.05, **P<0.01, ****P<0.0001. PDX: patient-derived xenograft; AML: acute myeloid leukemia; BM: bone marrow.

factor and human stem cell factor, also called NGS-S, were shown to improve engraftment of inv(16) favorable-risk AML.[10,24-26] However, our data indicate that using female recipients and extending observation times can already enable robust repopulation with all ELN risk groups, including favorable-risk AML. As expected, adverse-risk AML show faster mouse repopulation and shorter survival.[9,11,24] Of note, the revised ELN classification groups *FLT3*-mutated AML into intermediate disease, regardless of allelic ratio and *NPM1* mutational status.[13]

Phenotypic markers were largely conserved during the *in vivo* leukemic cell propagation in female PDX models. Higher LSC frequency was observed in adverse-risk *versus* favorable- and intermediate-risk AML and correlated with enhanced long-term repopulation as well homing capacity. Consistently, LSC from adverse-risk AML showed enhanced repopulation capacity in serial re-transplantation assays.[24] Overall, these data suggest that adverse-risk AML contain LSC less accessible to differentiation induction which may contribute to their ability to repopulate NSG mice as well as leukemia aggressiveness in patients. However, the higher leukemic expansion in female PDX was not restricted to LSC.[27]

Our data indicate an underappreciated role of the female environment in PDX leukemogenesis and raise intriguing questions on the effects of sex disparities in patients with leukemia. A potential explanation for the sex-dependent differences could be the differences in estrogen levels. However, modulators of estrogen and its receptor were, in contrast, described to induce pro-apoptotic effects in *in vitro* assays and in *in vivo* syngeneic leukemia mouse models.[28,29] In AML, as also in some other forms of hematologic and solid cancers, lower incidence and better outcomes have consistently been reported for females.[22,23,30] This is in general considered to result from a more adverse genetic landscape in male AML cases,[20] and to be potentially associated with exposure to pro-tumorigenic environmental factors such as smoking or chemicals. Interestingly, the excess of myeloid malignancies and especially myelodys-

plastic neoplasms in males *versus* females is reversed between the onset of puberty and approximately the age of 50 years, and afterwards again shows an increase.[30] Our results with castrated and ovariectomized animals suggest that, in fact, androgen deprivation, rather than estrogen addition enhances leukemogenesis in female mice. This notion is supported by a phase III trial including 330 elderly AML patients which showed improved disease-free and overall survival after the addition of androgens to post-remission maintenance therapy.[31] However, since female mice show better engraftment than castrated males, estrogens or other factors in the female environment might still play a role. For example, the ratio of estrogens to androgens may be a critical force driving the sex disparities seen in AML outcomes as well as leukemic blast expansion *in vitro* and *in vivo*. As recently shown by Mian *et al.*,[14] donor sex was also found to influence engraftment, although in our samples to a lesser extent. Future analyses will show whether leukemic cells with the same genetic background may more readily expand in female patients than in male patients, e.g., showing shorter time to relapse in the former. Finally, inflammatory signaling is involved in myeloid leukemogenesis and might contribute to the increased incidence of myelodysplastic neoplasms in younger women. It is recognized that female individuals have an overall higher propensity for chronic low-grade inflammation,[32] but also for stronger immune responses. Hence, the female immune system might facilitate a higher rate of (pre-)leukemic clone expansion. At the same time, more efficient immune eradication of highly malignant myeloid disease clones might occur, resulting in an overall lower rate of adverse-risk genetics in female AML cases. Since immune responses are blunted in PDX models, the female microenvironment factors triggering expansion might become more evident. Intriguingly, while female PDX mice showed a greater leukemic burden, at the same time they had lower sickness scores compared to male mice engrafted with cells from the same patients. Higher androgen levels, which also inhibit leukemogenesis, may be responsible for the higher sickness score, as suggested by our comparison of leukemogenesis in castrated *versus* control male mice. Future studies are required to further analyze sex-dependent differences mediating disparities not only in leukemic cell growth but also in sickness score in females *versus* males, and how these findings may apply to patients with leukemia.

Together, these results reinforce the concept that PDX models faithfully model AML but at the same time reveal recipient sex as a new essential variable that requires careful evaluation to avoid important experimental biases. Future studies will show whether they can also be used to investigate *in vivo* other still challenging-to-engraft hematologic or solid cancers. Our findings highlight the impact of sex differences on AML biology and treatment outcome as an intriguing area for future research.

Disclosures

No conflicts of interest to disclose.

Contributions

MA, AMPS, PH and LMK designed and performed experiments, analyzed data and generated tables. MA, AMPS and LMK generated figures and prepared the manuscript. JK, SG, EG, LK and MB supported in vitro and in vivo experiments, respectively, and JK assisted with data analyses. TSM, SR, JCS and MK helped with proofreading, data discussion and data interpretation. CL supervised the study, designed the experiments, interpreted data and supported writing the manuscript.

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Data-sharing statement

The data that support the findings of this study are available from the corresponding author, CL, upon reasonable request.

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