Brief Report

blood

MYELOID NEOPLASIA

inv(16) and NPM1^{mut} AMLs engraft human cytokine knock-in mice

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Key Points

- Humanized cytokine KI mice support engraftment of human favorable-risk AML.
- Engraftment and gene-enrichment analysis suggest M-CSF dependency of inv(16) AML.

Favorable-risk human acute myeloid leukemia (AML) engrafts poorly in currently used immunodeficient mice, possibly because of insufficient environmental support of these leukemic entities. To address this limitation, we here transplanted primary human AML with isolated nucleophosmin (NPM1) mutation and AML with inv(16) in mice in which human versions of genes encoding cytokines important for myelopoiesis (macrophage colony-stimulating factor [M-CSF], interleukin-3, granulocyte-macrophage colony-stimulating factor, and thrombopoietin) were knocked into their respective mouse loci. NPM1^{mut} AML engrafted with higher efficacy in cytokine knock-in (KI) mice and showed a trend toward higher bone marrow engraftment levels in comparison with NSG mice. inv(16) AML engrafted with high efficacy and was serially transplantable in cytokine KI mice but, in contrast, exhibited virtually no engraftment in NSG mice. Selected use of cytokine KI mice revealed that human M-CSF was required for inv(16) AML engraftment. Subsequent transcriptome profiling in an independent AML patient study cohort demonstrated high expression of M-CSF receptor and enrichment of M-CSF inducible genes in inv(16) AML cases. This study thus provides a first xenotransplantation mouse model for and informs on the disease biology of inv(16) AML. (*Blood*. 2016;128(17):2130-2134)

Introduction

Biologically less aggressive, so-called favorable-risk acute myeloid leukemia (AML) constitutes up to 40% of newly diagnosed cases of AML and is relatively more common in patients younger than 60 years.[1] This risk group is currently defined by 4 distinct cytogenetic and molecular constellations according to the European LeukemiaNet 2010 guidelines,[2,3] that is, AML with t(8;21), inv(16)/t(16;16), isolated nucleophosmin (NPM1) mutation (cytogenetically normal without recurrent FLT3 mutations), or mutated CEBPA. Upon state-of-the-art therapy, these patients benefit from significantly longer survival when compared with intermediate- or adverse-risk cases. However, only 66% of younger patients and 33% of older patients with favorable-risk AML are alive 3 years after diagnosis.[1] Studying the biology of these entities in in vivo model systems has been inherently difficult. In fact, faithful xenoengraftment of human AML to immunodeficient mice has been limited to higher-risk cases,[4] and there is no published mouse model supporting robust engraftment of lower-risk disease.[5] We recently developed humanized mice expressing human cytokines central to myeloid development and hematopoietic stem cell maintenance (macrophage colony-stimulating factor [M-CSF], interleukin-3 [IL-3], granulocyte-macrophage colony-stimulating factor [GM-CSF], and thrombopoietin) under their endogenous promoter, as well as human SIRPα as a transgene. These mice have displayed superior human healthy CD34$^+$ cell engraftment and myelopoiesis.[6-11] We hypothesized that this human myelopoiesis-supportive environment may also permit xenoengraftment of human favorable-risk AML.

Study design

Humanized cytokine knock-in (KI) mice were generated as reported previously.[6-11] Bone marrow aspirate was collected from patients with newly diagnosed AML after obtaining informed consent. The study was approved by the Cantonal Ethics Committee Zurich. Human blast cells were purified by density gradient centrifugation followed by immunomagnetic bead depletion of lymphocytes. Newborn mice were sublethally irradiated and intrahepatically injected with 1×10^6 cells per mouse. Mice were analyzed 16 to 24 weeks after transplantation (Figure 1A). All animal experiments were approved by the Cantonal Veterinary Office. For further details, see supplemental Methods (available on the *Blood* Web site).

Results and discussion

Our studies focused on 2 entities that together make up 60% of favorable-risk AML cases encountered in the clinical setting, namely

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There is an Inside *Blood* Commentary on this article in this issue.

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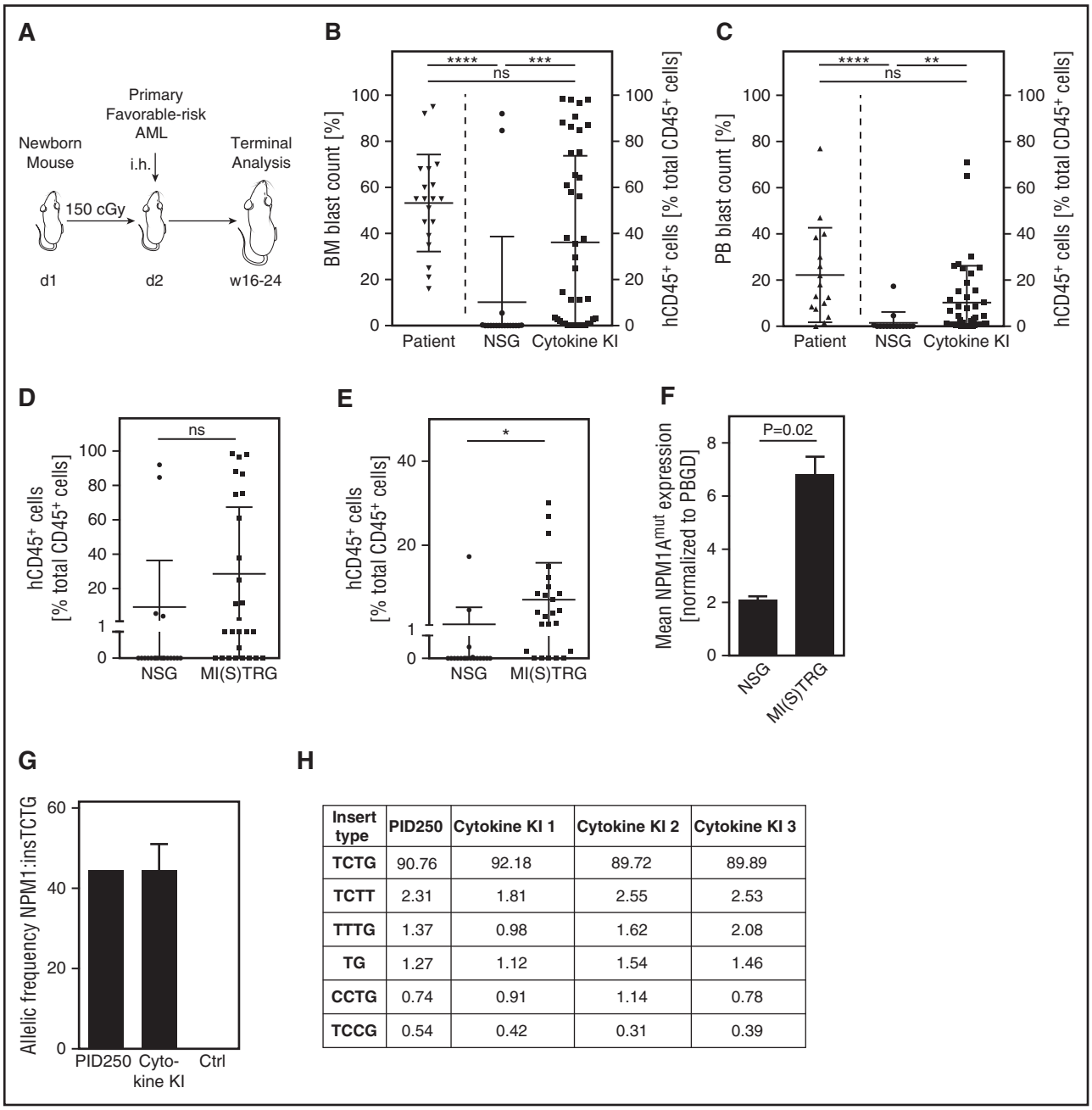

Insert type	PID250	Cytokine KI 1	Cytokine KI 2	Cytokine KI 3
TCTG	90.76	92.18	89.72	89.89
TCTT	2.31	1.81	2.55	2.53
TTTG	1.37	0.98	1.62	2.08
TG	1.27	1.12	1.54	1.46
CCTG	0.74	0.91	1.14	0.78
TCCG	0.54	0.42	0.31	0.39

Figure 1. Humanized cytokine KI mice support robust engraftment of favorable-risk AML and closely model disease biology. (A) Schematic representation of experimental setup. Scatter plots depict blast count in patients with inv(16) or NPM1^{mut} AML (left columns) and human engraftment in transplanted NSG or cytokine KI mice, in the bone marrow (B) and peripheral blood (C) (mean ± standard deviation [SD], N = 15-45 per group, 1-way ANOVA $P < .0001$ for both bone marrow and peripheral blood). Data stem from >20 independent experiments. Scatter plots compare human engraftment in NSG and MI(S)TRG mice transplanted with NPM1^{mut} AML in the bone marrow (D) and peripheral blood (E) (mean ± SD, N = 19-36 per group). (F) Mean expression of NPM1A^{mut} allele normalized to PBGD in engrafted NSG and MISTRG mice (mean ± SD, N = 4 per group). (G) Allelic frequency of NPM1 mutation (TCTG insertion at position chr5:170837542) in patient (PID250), transplanted cytokine KI mice (n = 3) and untransplanted cytokine KI mouse (ctrl) when mapping to HG19:chr5:170837542 (human reference genome). (H) Frequency of subclones of NPM1 insertion at HG19:chr5:170837542 in patient (PID250) and 3 cytokine KI mice.

AML with isolated mutations in the nucleophosmin 1 gene and AML with inv(16)(p13.1q22). Flow cytometric analysis (supplemental Figure 1) of bone marrow and blood of cytokine KI vs NSG mice, the current standard xenotransplantation model,[12] 16 to 24 weeks after transplantation revealed profound differences: average engraftment, defined as the proportion of cells expressing human CD45 among all cells expressing human and murine CD45, was 36% in the bone marrow of cytokine KI mice compared with only 10% in NSG mice, across all patient samples transplanted in this study (Figure 1B; supplemental Table 1; supplemental Figures 2A and 3). A similar pattern was observed in the peripheral blood, where human engraftment reached 10% in KI mice compared with >2% in NSG mice (Figure 1C; supplemental Figure 2B). In line with engraftment levels, engraftment efficacy, that is, overall engraftment vs no engraftment (defined as ≥1% hCD45$^+$/all CD45$^+$ cells[1]), was higher in cytokine KI mice (supplemental Table 2). Importantly, average engraftment in cytokine KI mice approached bone marrow and peripheral blast count observed in human AML patients at diagnosis, whereas NSG engraftment levels were

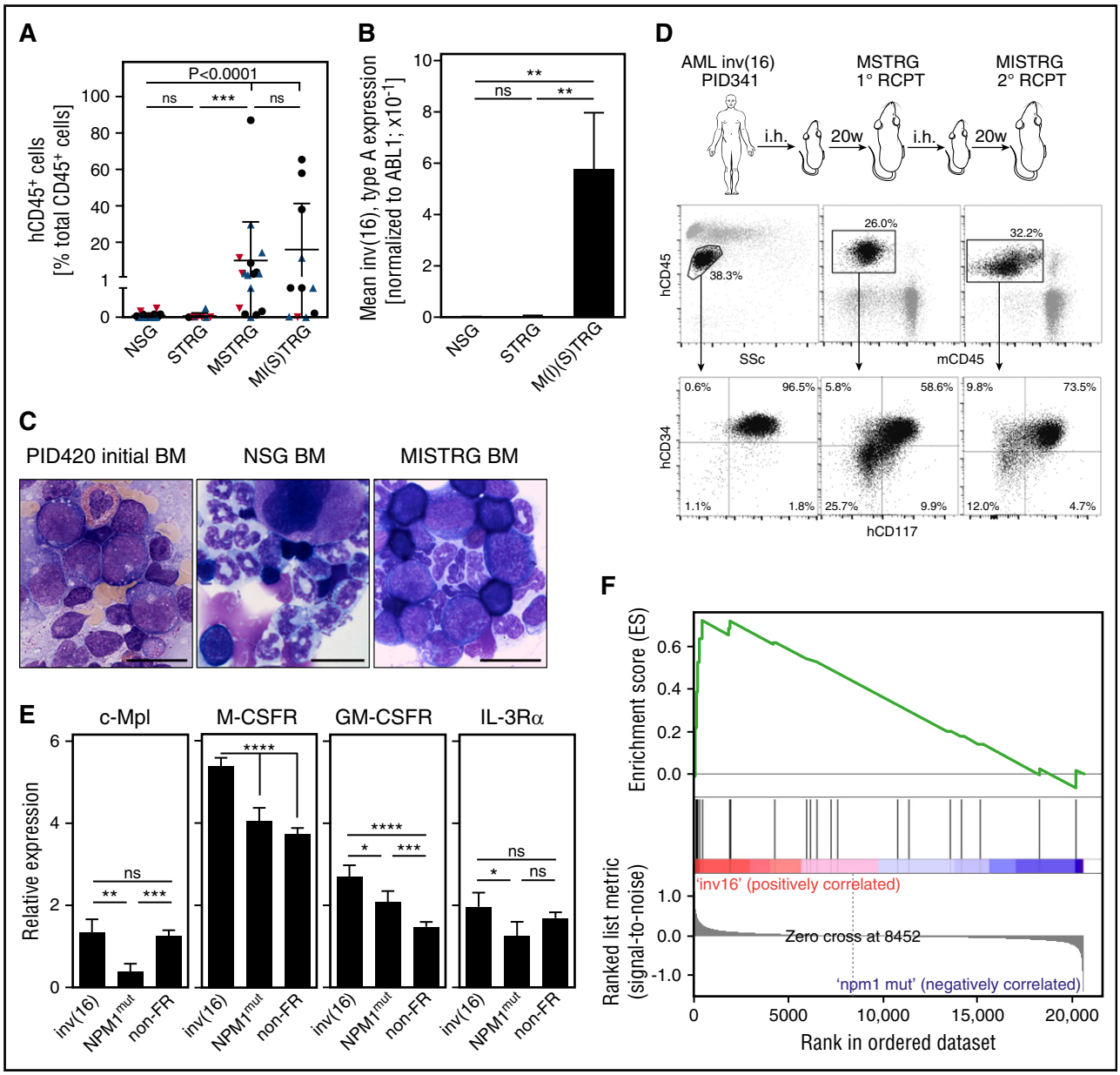

Figure 2. M-CSF is required for engraftment of AML with inv(16). (A) Scatter plot compares human engraftment in the bone marrow of mice of the indicated strains transplanted with inv(16) AML. Split samples are indicated by blue (PID330) and red (PID420) symbols (mean ± SD; N = 8-16 per group; Mann-Whitney *U* test $P < .0001$ for aggregate NSG vs cytokine KI, $P = .01$ for PID330, $P = .03$ for PID420; 1-way ANOVA $P = .0002$). (B) Mean expression of inv(16), type A normalized to ABL1 in transplanted mice of the indicated strains (mean ± SD, N = 4-7 per group, 1-way ANOVA $P < .0001$). (C) Brightfield microscopy images depict bone marrow aspirate smear from PID420 at initial diagnosis (left) and cytospins from NSG (middle) and MISTRG (right) mice transplanted with PID420 AML. Bars represent 20 μm. Data are representative of N = 6. (D) Serial transplantation: cartoon shows experimental setup; dot plots show immunophenotype of engrafted human cells. Data are representative of 2 independent experiments. (E) Bar graphs depict expression of indicated cytokine receptors in indicated AML subgroups (non-FR, nonfavorable risk) by microarray analysis (mean ± SD; N = 32-298 per group; 1-way ANOVA $P < .0001$ for c-Mpl, M-CSF receptor [M-CSFR], and GM-CSF receptor [GM-CSFR]; $P = .02$ for IL-3Rα). (F) Gene set enrichment analysis of M-CSF inducible genes in inv(16) compared with NPM1A^{mut} AML (N = 33 for inv(16), N = 46 for NPM1A^{mut}; enrichment score 0.72, significant at nominal *P* value <5%).

significantly lower (Figure 1B-C). AML engraftment in cytokine KI mice was found to be independent of expression of the SIRPα transgene in mice expressing all 4 cytokines (supplemental Figure 4).

Next, we set out to define the phenotype of favorable-risk AML engrafted in MI(S)TRG mice, the most advanced type of human cytokine KI mice,[10] in comparison with NSG mice, utilizing animals matched by donor and time of analysis. Because virtually no engraftment of inv(16) AML could be observed in NSG mice, this comparative analysis was limited to NMP1^{mut} AML. This subtype showed a trend toward higher bone marrow engraftment in MI(S)TRG vs NSG mice, and the difference in the peripheral blood reached statistical significance (Figure 1D-E). Engraftment of NMP1^{mut} AML in MI(S)TRG mice faithfully reproduced the original leukemia-associated phenotype by flow cytometry, and the same leukemia in NSG mice displayed loss of CD117 (c-kit) expression (supplemental Figure 5A-B), suggesting an environmentally driven alteration of the initial AML blast population or selective support of nondominant cell populations in the xenograft. Because of low engraftment efficacy in NSG mice, future studies will need to determine whether this finding is a general feature of NPM1^{mut} AML cases or if individual AML cases within this risk group might behave differently. As an independent measure of engraftment of the diseased clone, we also assessed

expression of the mutant NPM1 allele. $NPM1^{mut}$ expression was more than threefold higher in MI(S)TRG mice compared with NSG mice, suggesting more faithful AML engraftment in MI(S)TRG mice (Figure 1F). Performing targeted next-generation sequencing in patient and engrafted cytokine KI mouse bone marrow (supplemental Figure 6A-B), we found a comparable allelic frequency ranging from 38% to 51% for NPM1 mutation (TCTG insertion at position chr5: 170837542) in the patient's leukemia (PID250) and 3 engrafted mice, and no reads mapped to a nontransplanted cytokine KI mouse serving as control (Figure 1G). Of note, even the composition of subclones of the NPM1 mutation and additional identified mutations was constant between the analyzed patient and respective cytokine KI recipient mice (Figure 1H; supplemental Figure 6C-D).

In contrast to $NPM1^{mut}$ AML engraftment, a striking difference in quantitative inv(16) AML engraftment was observed between cytokine KI and NSG mice, both by aggregate and split-sample analysis (Figure 2A). This could be verified by molecular analysis (Figure 2B) and microscopy, which demonstrated marrow infiltration by large granulated blasts with cytoplasmatic vacuolization in both patient and transplanted MISTRG recipients, whereas NSG recipients displayed murine hematopoiesis only (Figure 2C); we did not observe bone marrow eosinophilia, which might be because of the lack of additional human cytokines, particularly human IL-5.[11,13] Serial transplantation further validated engraftment of an inv(16) leukemia-initiating cell population in cytokine KI mice (Figure 2D; supplemental Figure 7). To dissect the contribution of respective single human KI cytokines to inv(16) AML propagation, we analyzed engraftment in $Rag2^{-/-}gc^{-/-}$ mice carrying, in addition to a human SIRPα transgene, subsequent additions of KI genes for human thrombopoietin (STRG), M-CSF and thrombopoietin (MSTRG), and M-CSF, IL-3, GM-CSF, and thrombopoietin (MI(S)TRG). STRG mice, as NSG mice, did not support engraftment. In contrast, addition of M-CSF in MSTRG mice led to robust engraftment of inv(16) AML, whereas addition of IL-3 and GM-CSF in MI(S)TRG mice resulted in only marginal, if any, improvement of engraftment over MSTRG mice (Figure 2A). Of note, KI of human M-CSF has previously been shown to be sufficiently cross-reactive to rescue the murine M-CSF knockout phenotype.[9] We thus reasoned that the engraftment advantage observed in MSTRG mice was likely because of human M-CSF effects on blasts and not because of reduction in mouse macrophages. Together, this establishes a central role for M-CSF in maintenance of inv(16) AML.

To further decipher the molecular basis of M-CSF dependency observed in inv(16) AML, we profiled cytokine receptor expression for thrombopoietin, M-CSF, IL-3, and GM-CSF on inv(16) AML blasts in comparison with $NPM1^{mut}$ AML and an aggregate of nonfavorable-risk AML cases, using microarray data from an international cohort study.[14] inv(16) AML showed significantly higher expression of each of the 4 receptors compared with $NPM1^{mut}$ AML and significantly higher expression of M-CSFR and GM-CSFR compared with nonfavorable-risk AML, with the overall highest difference in expression observed for M-CSFR (Figure 2E). Moreover, gene set enrichment analysis[15] demonstrated enrichment of M-CSF-inducible genes[16] in inv(16) AML compared with $NPM1^{mut}$ AML (Figure 2F) and nonfavorable-risk AML cases (supplemental Figure 8), confirming increased signaling through the M-CSF pathway. Of note, core-binding factor is a known regulator of M-CSFR transcription,[17,18] yet the functional consequences for inv(16) (CBFβ-MYH11) driven leukemogenesis are incompletely understood.[19]

In sum, we here describe a faithful and highly efficacious xenotransplantation model of favorable-risk, specifically $NPM1^{mut}$ and, importantly, inv(16) AML. We further demonstrate a strong dependency of inv(16) AML on M-CSF, a finding that might provide a basis for therapy optimization studies informed by an improved understanding of favorable-risk AML biology.

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Authorship

Contribution: J.M.E. and P.J.R. designed research, performed experiments, analyzed data, and wrote the manuscript; L.V.K., R.M., U.W., Y.S., and J.S.G. performed experiments and analyzed data; N.W.-V.v.W., C.F., A.R., V.L., E.D., A.P.T., and D.S. performed experiments; and R.A.F. and M.G.M. directed the study and wrote the manuscript.

Conflict-of-interest disclosure: R.A.F. and M.G.M. filed patent applications on genetically modified mice and the use thereof. MISTRG mice were donated for public access to the Jackson Laboratory. The remaining authors declare no competing financial interests.

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References

1. Mrózek K, Marcucci G, Nicolet D, et al. Prognostic significance of the European LeukemiaNet standardized system for reporting cytogenetic and molecular alterations in adults with acute myeloid leukemia. *J Clin Oncol.* 2012;30(36): 4515-4523.
2. Döhner H, Estey EH, Amadori S, et al; European LeukemiaNet. Diagnosis and management of acute myeloid leukemia in adults: recommendations from an international expert panel, on behalf of the European LeukemiaNet. *Blood.* 2010;115(3):453-474.
3. Döhner H, Weisdorf DJ, Bloomfield CD. Acute myeloid leukemia. *N Engl J Med.* 2015;373(12): 1136-1152.
4. Kennedy JA, Mitchell A, Chen WC, et al. Leukemic engraftment in NOD.SCID mice is correlated with clinical parameters and predicts outcome in human AML [abstract]. *Blood.* 2013; 122(21). Abstract 50.
5. Theocharides APA, Rongvaux A, Fritsch K, Flavell RA, Manz MG. Humanized hematolymphoid system mice. *Haematologica.* 2016; 101(1):5-19.
6. Willinger T, Rongvaux A, Takizawa H, et al. Human IL-3/GM-CSF knock-in mice support human alveolar macrophage development and human immune responses in the lung. *Proc Natl Acad Sci USA.* 2011;108(6): 2390-2395.
7. Rongvaux A, Willinger T, Takizawa H, et al. Human thrombopoietin knockin mice efficiently support human hematopoiesis in vivo. *Proc Natl Acad Sci USA.* 2011;108(6):2378-2383.
8. Strowig T, Rongvaux A, Rathinam C, et al. Transgenic expression of human signal regulatory

protein alpha in Rag2-/-gamma(c)-/- mice improves engraftment of human hematopoietic cells in humanized mice. *Proc Natl Acad Sci USA.* 2011;108(32):13218-13223.

9. Rathinam C, Poueymirou WT, Rojas J, et al. Efficient differentiation and function of human macrophages in humanized CSF-1 mice. *Blood.* 2011;118(11):3119-3128.
10. Rongvaux A, Willinger T, Martinek J, et al. Development and function of human innate immune cells in a humanized mouse model. *Nat Biotechnol.* 2014;32(4):364-372.
11. Rongvaux A, Takizawa H, Strowig T, et al. Human hemato-lymphoid system mice: current use and future potential for medicine. *Annu Rev Immunol.* 2013;31:635-674.
12. McDermott SP, Eppert K, Lechman ER, Doedens M, Dick JE. Comparison of human cord blood engraftment between immunocompromised mouse strains. *Blood.* 2010;116(2):193-200.
13. Haferlach T, Winkemann M, Löffler H, et al. The abnormal eosinophils are part of the leukemic cell population in acute myelomonocytic leukemia with abnormal eosinophils (AML M4Eo) and carry the pericentric inversion 16: a combination of May-Grünwald-Giemsa staining and fluorescence in situ hybridization. *Blood.* 1996;87(6):2459-2463.
14. Wouters BJ, Löwenberg B, Erpelinck-Verschueren CA, van Putten WL, Valk PJ, Delwel R. Double CEBPA mutations, but not single CEBPA mutations, define a subgroup of acute myeloid leukemia with a distinctive gene expression profile that is uniquely associated with a favorable outcome. *Blood.* 2009;113(13):3088-3091.
15. Subramanian A, Tamayo P, Mootha VK, et al. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. *Proc Natl Acad Sci USA.* 2005;102(43):15545-15550.
16. Caescu CI, Guo X, Tesfa L, et al. Colony stimulating factor-1 receptor signaling networks inhibit mouse macrophage inflammatory responses by induction of microRNA-21. *Blood.* 2015;125(8):e1-e13.
17. Zhang DE, Hetherington CJ, Meyers S, et al. CCAAT enhancer-binding protein (C/EBP) and AML1 (CBF alpha2) synergistically activate the macrophage colony-stimulating factor receptor promoter. *Mol Cell Biol.* 1996;16(3):1231-1240.
18. Ward AC, Loeb DM, Soede-Bobok AA, Touw IP, Friedman AD. Regulation of granulopoiesis by transcription factors and cytokine signals. *Leukemia.* 2000;14(6):973-990.
19. Shigesada K, van de Sluis B, Liu PP. Mechanism of leukemogenesis by the inv(16) chimeric gene CBFB/PEBP2B-MHY11. *Oncogene.* 2004;23(24):4297-4307.