TIM3, a human acute myeloid leukemia stem cell marker, does not enrich for leukemia-initiating stem cells in B-cell acute lymphoblastic leukemia

Here, we prompted to determine in molecularly distinct B-cell acute lymphoblastic leukemia (B-cell ALL) patients whether TIM3 represents a leukemia-initiating stem cell (LIC) marker enabling the prospective isolation of LIC-enriched B-cell ALL cells and found that in contrast to what has been shown in acute myeloid leukemia (AML), TIM3 does not enrich for LIC in B-ALL.

Relapse remains a major challenge in the clinical management of both AML and B-cell ALL and is driven by rare therapy-resistant LIC that reside in specific bone marrow (BM) niches.[1] The clinical implications of LIC are beyond any doubt, as evidenced by the large number of preclinical and clinical studies elucidating the phenotype and molecular determinants of LIC.[2,3] In human AML, where the hierarchical leukemic stem cell model is well established, multiple surface proteins that have been proposed to enrich for AML-LIC.[4-8] Among these, stands out T-cell immunoglobulin mucin-3 (TIM3), a human AML stem cell marker which has been shown to enable the prospective isolation of LIC-enriched AML cells.[9] In fact, TIM3 has been suggested to be a promising target to selectively eliminate AML-LIC and several TIM3 inhibitors are being clinically tested in patients with advanced AML.[10,11] The stem cell model picture is less clear in B-cell ALL. Conflicting studies could not resolve the phenotype of the B-cell ALL-LIC yet. Consequently, whether B-cell ALL follows a hierarchical leukemogenic model, driven by a rare population of LIC, or a stochastic leukemogenic model where most of the blasts (even at different maturational stages) can reconstitute in serial xenotransplantation assays and re-establish the complete leukemic phenotype remains unresolved.

Here, we initially profiled by fluorescence-activated cell sorting (FACS) the expression of TIM3 in 85 BM samples from both pediatric and adult B-cell patients (*Online Supplementary Table S1*) and found that TIM3 protein is heterogeneously expressed in B-cell ALL blasts from both diagnostic (Dx, n=47) and relapsed (n=38) patients (Figure 1A-C). The proportion of TIM3-expressing $CD34^{+}CD19^{+}$ B-cell blasts at Dx was ~2-fold higher than that observed in normal B-cell/B-cell progenitor counterparts from healthy

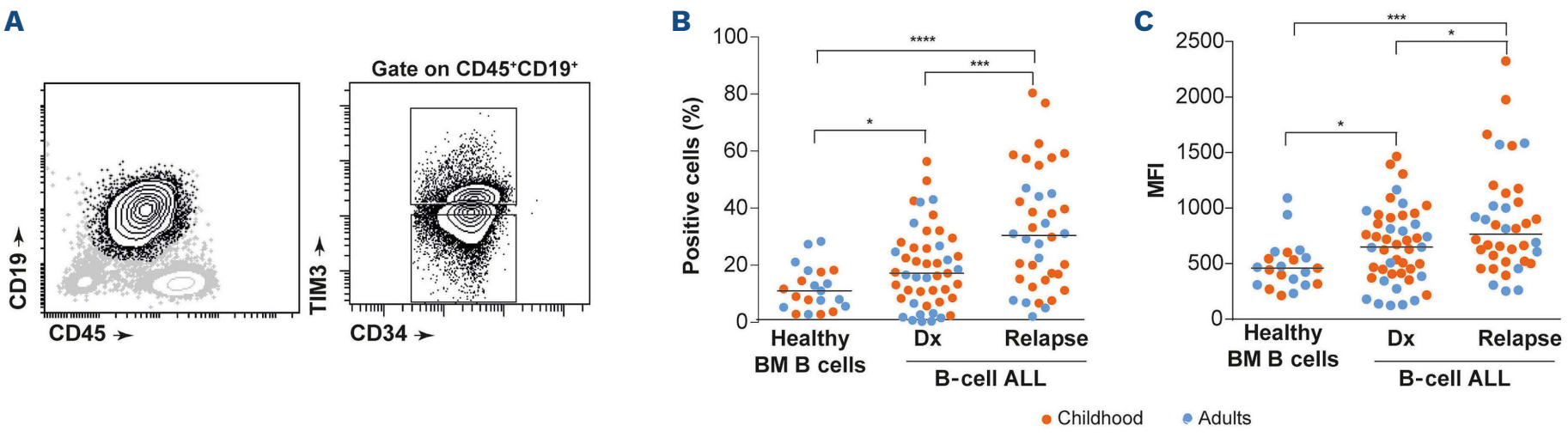
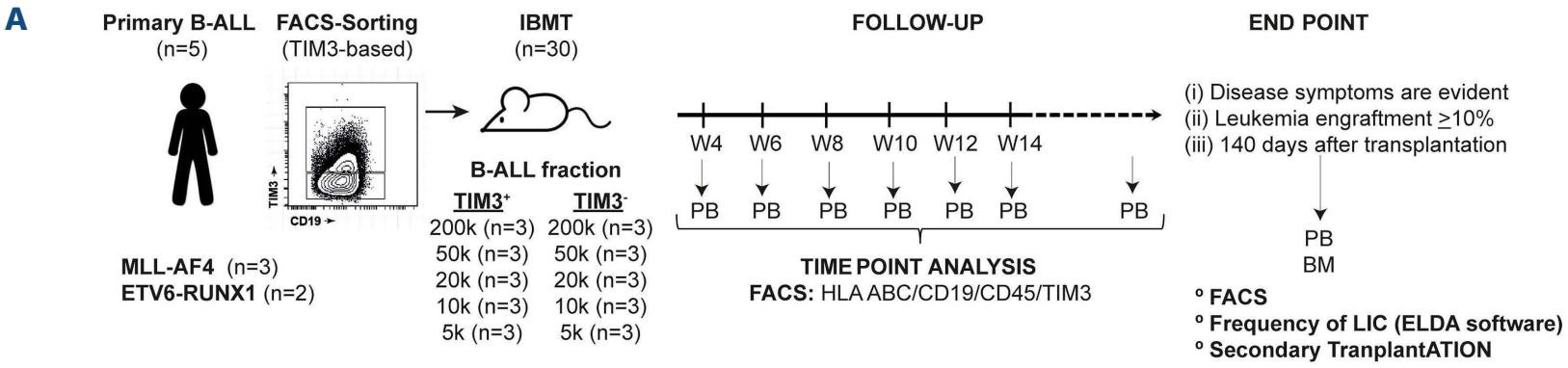
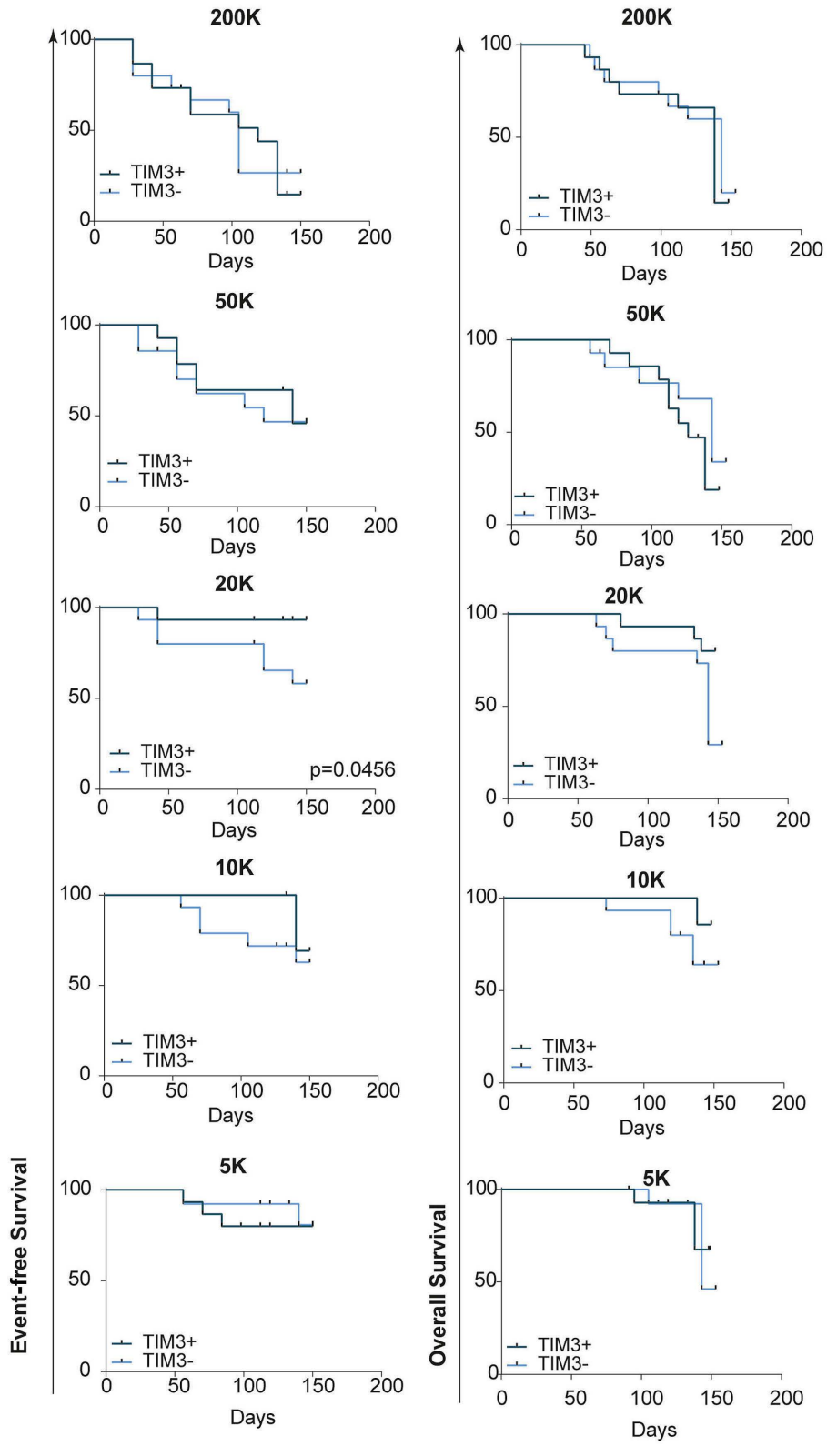
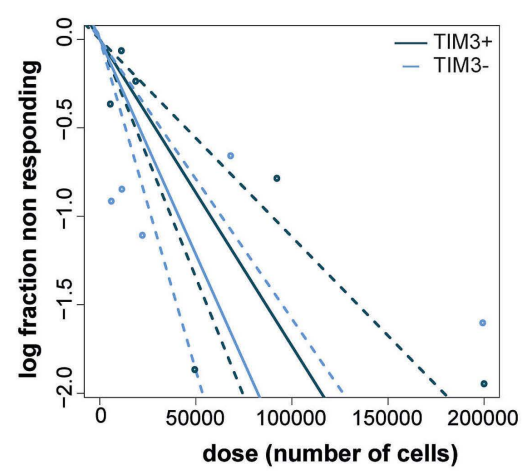


Figure 1. Expression of TIM3 in blasts from B-cell acute lymphoblastic leukemia patients at disease presentation and relapse. (A) Representative fluorescence-activated cell sorting analysis showing the expression of TIM3 in B-cell acute lymphoblastic leukemia (ALL) blasts ($CD45^{low/+}$ $CD34^{+}CD19^{+}$). (B, C) Percentage (B) and mean fluorescence intensity (MFI) (C) of $TIM3^{+}$ B-cell ALL cells in bone marrow (BM) at diagnosis (Dx) and relapse. $CD19^{+}$ B-cell progenitors and mature B cells were analyzed in healthy BM. Healthy BM, n=21; Dx B-cell ALL, n=47; relapsed B-cell ALL, n=38. (D) Bulk RNA sequencing-based *TIM3* expression obtained at Dx from n=10 pediatric $MLL4\text{-}AF4^{+}$ proB-cell ALL patients and in the corresponding matched B-cell ALL (no lineage switch) relapse (n=5) or myeloid lineage-switched relapse (n=5).







Confidence intervals for LIC frequency

	Lower	**Estimate**	Upper
B-ALL TIM3⁺	1/89574	**1/57703**	1/37172
B-ALL TIM3⁻	1/63322	**1/41148**	1/26739

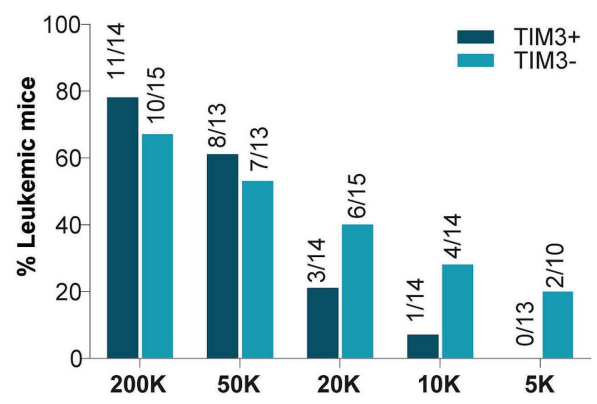
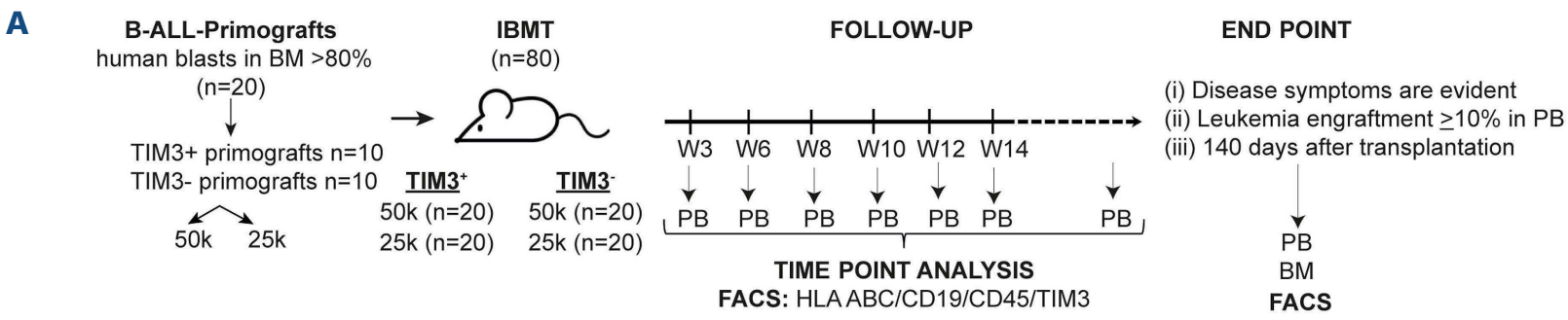
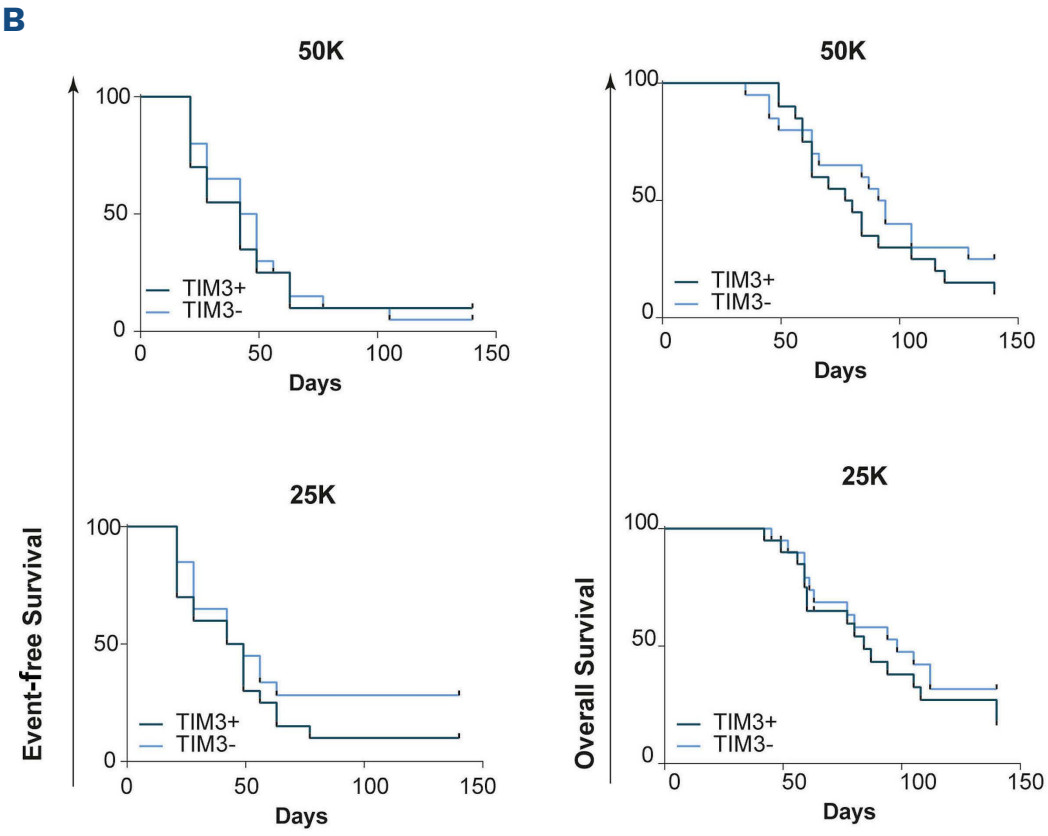


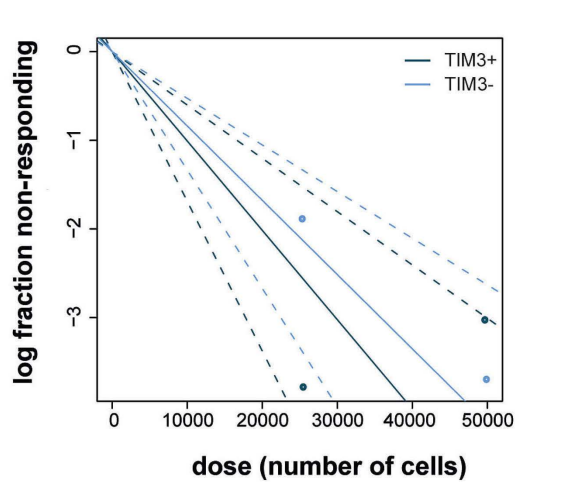
Figure 2. Leukemia reconstitution *in vivo* of TIM3+ and TIM3- B-cell acute lymphoblastic leukemia blasts in cell dose limiting dilution primograft xenotransplantation assays. (A) Detailed experimental design for assessing *in vivo* the leukemia-initiating stem cells (LIC) frequency in cell dose-limiting dilution primograft xenotransplantation assays using 5 primary B-cell acute lymphoblastic leukemia (B-ALL) samples (2 ETV6-RUNX1⁺ and 3 MLL-AF4⁺). Decreasing doses (200,000 down to 5,000) of TIM3⁺ and TIM3⁻ B-cell ALL blasts were intra-bone marrow (intra-BM) transplanted into NSG mice. Mice health and leukemia development was monitored over 20 weeks. Mice were sacrificed when i) signs of disease were evident, ii) B-cell ALL graft was >10% in peripheral blood in the absence of signs of disease or iii) at day 140 (end point) in the absence of symptoms or leukemia engraftment. (B) Kaplan-Meier event-free and overall survival curves for each cell fraction (TIM3⁺ *vs.* TIM3⁻) and cell dose (200,000 down to 5,000). N=150 mice studied in total: 30 mice/leukemia; 75 mice/cell fraction, 10 mice/cell dose. (C) Estimated frequency (and 95% confidence interval) of LIC in primografts transplanted with TIM3⁺ and TIM3⁻ blasts. (D) Penetrance of leukemic mice at end point (number engrafted mice/total number transplanted mice). IBMT: intra-bone marrow transplant.

BM (20%, range, 0.3-56.4 *vs*. 12%, range, 2.8-28.4; *P*=0.0028), and the proportion of TIM3$^+$CD34$^+$CD19$^+$ cells further increased significantly in B-cell blasts at relapse (32%, range, 2-80; *P*=0.0008) (Figure 1A, B). The levels of TIM3 expression in CD34$^+$CD19$^+$ B-cell blasts, measured by mean intensity fluorescence, showed a very similar trend to the proportion of TIM3$^+$CD34$^+$CD19$^+$ cells, further confirming an upregulation of TIM3 expression in B-cell ALL patients during disease progression (Dx>relapse) (Figure 1C). We next analyzed the *TIM3* RNA expression in ten KMT2A$^-$AFF1$^+$ proB-cell ALL patients at Dx and in matched relapses (Figure 1D).[12] Half of these MLL$^-$AF4$^+$ proB-cell ALL patients relapsed as CD19$^+$ B-cell ALL while the other half relapsed as a CD19$^-$ myeloid lineage-switched. Very interestingly, the expression levels of *TIM3* were dramatically higher in all KMT2A$^-$AFF1$^+$ myeloid lineage-switched relapses than in KMT2A$^-$AFF1$^+$ CD19$^+$ B-cell relapses or the Dx samples, further linking TIM3 with AML-LIC/AML progression.

We next interrogated the ability of highly purified (FACS







Confidence intervals for LIC frequency

	Lower	Estimate	Upper
Mice TIM3$^+$	1/16626	**1/9925**	1/5925
Mice TIM3$^-$	1/19008	**1/11932**	1/7490

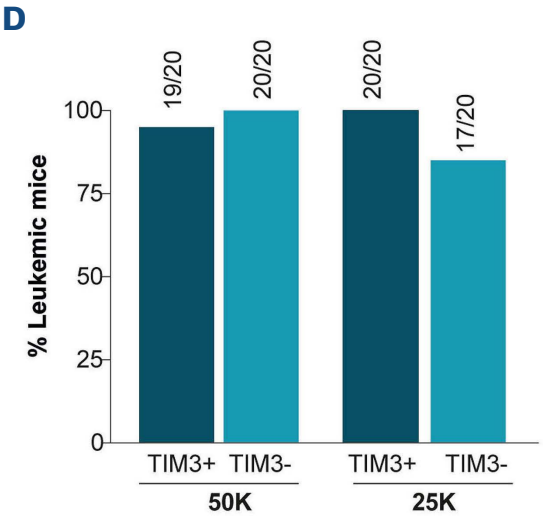


Figure 3. TIM3 expression does not enrich for B-cell acute lymphoblastic leukemia-initiating stem cells capacity in secondary recipients. (A) Detailed experimental design for assessing *in vivo* the leukemia-initiating stem cells (LIC) frequency in serial xenotransplantation assays. Bone marrow (BM) cells were retrieved from 20 primografts engrafted (>80%) with TIM3$^+$ (n=10) or TIM3$^-$ (n=10) B-cell acute lymphoblastic leukemia (B-ALL) blasts and transplanted at 2 different doses (50,000 and 25,000) in secondary irradiated recipients (1 primograft into 4 secondary; n=80 secondary mice, 16 mice/leukemia, 40 mice/cell fraction). Mice health and leukemia development was monitored over 20 weeks. Mice were sacrificed when i) signs of disease were evident, ii) B-cell ALL graft was >10% in peripheral blood (PB) in the absence of signs of disease or iii) at day 140 (end point) in the absence of symptoms or leukemia engraftment. (B) Kaplan-Meier event-free and overall survival curves for each cell fraction (TIM3$^+$ *vs*. TIM3$^-$) and cell dose (50,000 and 25,000). N=80 mice studied in total: 16 mice/leukemia; 40 mice/cell fraction, 40 mice/cell dose. (C) Estimated frequency (and 95% confidence interval) of LIC in secondary mice. (D) Penetrance of leukemic mice at end point (number engrafted mice/total number transplanted mice). IBMT: intra-bone marrow transplant.

purity >98%, *data not shown*) TIM3$^+$ and TIM3$^-$ blasts to initiate B-cell ALL *in vivo* upon intra-BM transplantation into NSG mice (n=150) in limiting dilution assays (LDA) (Figure 2A). All primary leukemias (2 ETV6$^-$RUNX1$^+$ and 3 KMT2A$^-$AFF1$^+$) engrafted onto primografts reproducing the Dx phenotype (*data not shown*). As expected, both the event-free survival (EFS) and the overall survival (OS) decreased with increasing doses of transplanted blasts (Figure 2B); however, no significant differences were found in either EFS or OS between TIM3$^+$ and TIM3$^-$ populations across cell doses (Figure 2B). Importantly, the estimated LIC frequency, calculated in LDA using the ELDA software[13] was similar between TIM3$^+$ and TIM3$^-$ B-cell blast populations (Figure 2C). Similarly, despite a trend towards a slightly higher frequency of engrafted mice (leukemia penetrance) in primografts transplanted with lower doses of TIM3$^-$ blasts, no significant differences were observed overall in the frequency of engrafted mice between TIM3$^+$ and TIM3$^-$ B-cell blast populations (Figure 2D).

For serial transplantation experiments, 50,000 and 25,000 B-cell ALL cells from primografts were intra-BM transplanted into secondary mice, rendering a significantly lower EFS and OS (higher aggressiveness) (Figure 3A, B) than that observed in primary recipients; however, no differences in either EFS or OS were observed between secondary recipients transplanted with TIM3$^+$ or TIM3$^-$ B-cell blast populations (Figure 3B). Similarly, the estimated LIC frequency (Figure 3C) and leukemic penetrance (frequency of engrafted mice) were very similar between secondary recipients transplanted with TIM3$^+$ or TIM3$^-$ B-cell blast populations (Figure 3D). Taken together, our data demonstrate that despite an increased expression of TIM3 in B-cell ALL blasts during disease progression, TIM3 does not enrich for LIC in B-cell ALL.

We report here that, in contrast to what has been shown in AML, TIM3 does not represent a stem cell marker capable of prospectively isolating LIC in either high-risk KMT2A$^-$AFF1$^+$ and low/standard-risk ETV6$^-$RUNX1$^+$ B-cell ALL. All individual high-risk and standard-risk leukemic primary BM samples engrafted in NSG mice even at very low doses, validating this immunodeficient mouse model to functionally assess for candidate human leukemia stem cell populations. Furthermore, our intra-BM transplantation assay provides a highly sensitive and specific assay for interrogating LIC in acute leukemia because it overcomes potential survival and BM homing intrinsic deficiencies of transplanted cells.[14] These findings are in line with previous reports and reinforce that distinct immunophenotypically defined B-cell ALL blast populations, even at different maturation stages, have stem cell properties,[14,15] reinforcing that some hematopoietic malignancies (as for AML and other myeloid neoplasms) are maintained by a rare population of LIC (hierarchical model) whereas in B-cell ALL most of the blasts possess “stemness” features (stochastic model) being capable of initiating and recapitulating the disease *in vivo*. Further work is needed to understand the role of, and alteration in, the expression of the immune checkpoint receptor TIM3 in blasts patients with acute leukemia, especially for rationalizing and interpreting current clinical trials testing TIM3 inhibitors in relapsing/refractory AML and myelodysplastic syndrome patients.[11]

Authors

Clara Bueno,[1,2,3] Alba Martínez,[1,2] Paola Alejandra Romecin,[1,2] Samanta Romina Zanetti,[1] Ricky Tirtakusuma,[4] Eulalia Genesca,[1] Mireia Camós,[5,6] Manuel Ramírez-Orellana,[2,7] Eduardo Anguita,[8] Paola Ballerini,[9] Franco Locatelli,[10] José Luis Fuster[11] and Pablo Menéndez[1,2,3,12,13]

[1]Josep Carreras Leukemia Research Institute, Barcelona, Spain; [2]Red Española de Terapias Avanzadas (TERAV)-Instituto de Salud Carlos III (ISCIII) (RICORS, RD21/0017/0029), Madrid, Spain; [3]CIBER-ONC, ISCIII, Barcelona, Spain; [4]Newcastle University, Newcastle upon Tyne, UK; [5]Hematology Laboratory, Hospital Sant Joan de Déu, Barcelona, Spain; [6]Leukemia and other Pediatric Hemopathies, Developmental Tumor Biology Group, Instiutut de Recerca Hospital Sant Joan de Déu, Barcelona, Spain; [7]Oncohematología, Hospital Niño Jesús, Madrid, Spain; [8]Servicio de Hematología, Hospital Clínico San Carlos, IdISSC, Medicina UCM, Madrid, Spain; [9]Pediatric Hematology, Armand Trosseau Hospital, Paris, France; [10]Department of Hematology and Oncology, Ospedale Bambino Gesú, Rome, Italy; [11]Sección de Oncohematología Pediátrica, Hospital Clínico Universitario Virgen de la Arrixaca and Instituto Murciano de Investigación Biosanitaria (IMIB), El Palmar, Murcia, Spain; [12]Institució Catalana de Recerca i Estudis Avançats (ICREA), Barcelona, Spain and [13]Department of Biomedicine, School of Medicine, University of Barcelona, Barcelona, Spain

Correspondence:
P. MENENDEZ - pmenendez@carrerasresearch.org
C. BUENO - cbueno@carrerasresearch.org

https://doi.org/10.3324/haematol.2022.282394

Received: November 22, 2022.
Accepted: January 5, 2023.
Early view: January 19, 2023.

Disclosures

PM is founder of the spin-off OneChain Immunotherapeutics which has no connection with the present research. All other authors have no conflicts of interest to disclose.

Contributions

CB conceived the study, designed, and performed experiments, analyzed/interpreted data, prepared figures, wrote the manuscript and financially supported the study. AM, PAR and RT performed experiments. SRZ conceived the study, performed experiments and analyzed/interpreted data. EG, MC, MRO, EA, PB, FL and JLF contributed patient's samples and clinical data. PM conceived the study, designed experiments, interpreted data, wrote the manuscript, and financially supported the study. All authors have read and agreed to publish the manuscript.

Acknowledgments

We thank Francisco Gutierrez-Agüera for technical help. We thank CERCA/Generalitat de Catalunya and Fundació Josep Carreras-Obra Social la Caixa for core support.

Funding

Competitive financial support for this work was obtained from Spanish Ministry of Economy and Competitiveness (PID2019-108160RB-I00) to PM and (PLE2021-007518) to CB, the Carlos III Health Institute (ISCIII/FEDER PI20/00822) to CB, ISCIII-RICORS within the Next Generation EU program (plan de recuperación, transformación y resiliencia) to PM and Asociación Española Contra el Cancer (AECC) (PRYGN211192BUEN) to CB. SRZ was supported by a Marie Sklodowska Curie Fellosip (GA795833).

Data-sharing statement

Original data is available upon reasonable request to the corresponding author.

References

1. Ishikawa F, Yoshida S, Saito Y, et al. Chemotherapy-resistant human AML stem cells home to and engraft within the bone-marrow endosteal region. Nat Biotechnol. 2007; 25(11):1315-1321.
2. Hoang VT, Zepeda-Moreno A, Ho AD. Identification of leukemia stem cells in acute myeloid leukemia and their clinical relevance. Biotechnol J. 2012; 7(6):779-788.
3. Mohamed MMI, Aref S, Agdar MA, Mabed M, El-Sokkary AMA. Leukemic stem cell (CD34(+)/CD38(-)/TIM3(+)) frequency in patients with acute myeloid leukemia: clinical implications. Clin Lymphoma Myeloma Leuk. 2021;21(8):508-513.
4. Bonnet D, Dick JE. Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. Nat Med. 1997;3(7):730-737.
5. Ding Y, Gao H, Zhang Q. The biomarkers of leukemia stem cells in acute myeloid leukemia. Stem Cell Investig. 2017;4:19.
6. Jaiswal S, Jamieson CH, Pang WW, et al. CD47 is upregulated on circulating hematopoietic stem cells and leukemia cells to avoid phagocytosis. Cell. 2009;138(2):271-285.
7. Jin L, Lee EM, Ramshaw HS, et al. Monoclonal antibody-mediated targeting of CD123, IL-3 receptor alpha chain, eliminates human acute myeloid leukemic stem cells. Cell Stem Cell. 2009;5(1):31-42.
8. van Rhenen A, van Dongen GA, Kelder A, et al. The novel AML stem cell associated antigen CLL-1 aids in discrimination between normal and leukemic stem cells. Blood. 2007;110(7):2659-2666.
9. Jan M, Chao MP, Cha AC, et al. Prospective separation of normal and leukemic stem cells based on differential expression of TIM3, a human acute myeloid leukemia stem cell marker. Proc Natl Acad Sci U S A. 2011;108(12):5009-5014.
10. Bosch M, Sanchez-Alvarez M, Fajardo A, et al. Mammalian lipid droplets are innate immune hubs integrating cell metabolism and host defense. Science. 2020;370(6514):eaay8085.
11. Rezaei M, Tan J, Zeng C, Li Y, Ganjalikhani-Hakemi M. TIM-3 in leukemia; immune response and beyond. Front Oncol. 2021;11:753677.
12. Tirtakusuma R, Szoltysek K, Milne P, et al. Epigenetic regulator genes direct lineage switching in MLL/AF4 leukaemia. Blood. 2022;140(17):1875-1890.
13. Hu Y, Smyth GK. ELDA: extreme limiting dilution analysis for comparing depleted and enriched populations in stem cell and other assays. J Immunol Methods. 2009;347(1-2):70-78.
14. Prieto C, Lopez-Millan B, Roca-Ho H, et al. NG2 antigen is involved in leukemia invasiveness and central nervous system infiltration in MLL-rearranged infant B-ALL. Leukemia. 2018;32(3):633-644.
15. le Viseur C, Hotfilder M, Bomken S, et al. In childhood acute lymphoblastic leukemia, blasts at different stages of immunophenotypic maturation have stem cell properties. Cancer Cell. 2008;14(1):47-58.