Bone marrow mesenchymal stem/stromal cells from risk-stratified acute myeloid leukemia patients are anti-inflammatory in *in vivo* preclinical models of hematopoietic reconstitution and severe colitis

Rafael Diaz de la Guardia,[1,] Belen Lopez-Millan,[1,*] Heleia Roca-Ho,[1] Clara Bueno,[1] Francisco Gutiérrez-Agüera,[1] Jose Luis Fuster,[2] Eduardo Anguita,[3] Samanta Romina Zanetti,[1] Susana Vives,[4] Josep Nomdedeu,[5] Robert Sackstein,[6] Jessie Lavoie,[7] Elena Gónzalez-Rey,[8] Mario Delgado,[8] Michael Rosu-Myles[7,#] and Pablo Menendez[1,9,10,$,#]*

[1]Josep Carreras Leukemia Research Institute, Department of Biomedicine, School of Medicine, University of Barcelona, Spain; [2]Sección de Oncohematología Pediátrica, Hospital Virgen de Arrixaca, Murcia, Spain; [3]Servicio de Hematología, Hospital Clínico San Carlos, IdISSC, Medicina UCM, Madrid, Spain; [4]Hematology Department, ICO-Hospital Germans Trias i Pujol and Josep Carreras Leukemia Research Institute, Barcelona, Spain; [5]Servicio de Hematología, Hospital de la Santa Creu i Sant Pau and Josep Carreras Leukemia Research Institute, Barcelona, Spain; [6]Department of Medicine and Program of Excellence in Glycosciences, Brigham and Women's Hospital, Harvard Medical School, Boston, MA, USA; [7]Biologics and Genetic Therapies Directorate, Health Products and Food Branch, Health Canada, Ottawa, ON, Canada; [8]Instituto de Parasitología y Biomedicina López-Neyra, CSIC, Granada, Spain; [9]Instituciò Catalana de Reserca i EstudisAvançats (ICREA), Barcelona, Spain and [10]Centro de Investigación Biomédica en Red de Cancer (CIBER-ONC), Barcelona, Spain

*RDdG and BL contributed equally to this work.

#MRM and PM should be equally considered senior authors.

Correspondence: pmenendez@carrerasresearch.org
doi:10.3324/haematol.2018.196568

Supplemental Methods

Patients

Fresh BM aspirates were obtained at disease presentation from 15 patients (median age 53±25 years) with cytogenetically different AML (**Table S1**). The diagnosis of AML was based on French-American-British (1) and World Health Organization classifications (2-4). AML was classified as low-risk AML (LR-AML, displaying favorable cytogenetics/molecular features), intermediate-risk AML (IR-AML, with normal karyotype and lacking mutations in NPM, FLT3, or cEBPa), and high-risk AML (HR-AML, displaying unfavorable cytogenetics/molecular features) disease. Additionally, BM from 5 age-matched healthy donors (HD) were used as controls. The study was IRB-approved by the Clinic Hospital of Barcelona and samples were accessed upon signed informed consent.

BMSCs and cord blood (CB)-derived CD34+ cells

Mononuclear cells (MNCs) from BM were isolated by Ficoll-Paque^{Plus} density gradient and seeded at $2x10^5$ cells/cm^2 for 48h. Then, non-adherent cells were washed off and fresh medium added. When cultures were ~85% confluent, adherent cells were trypsinized and serially replated at 1.6×10^3 cells/cm^2. BMSCs were fully characterized as previously described (5-8). CB procurement, purification of CD34+ cells and Luminex Multiplex assays were performed as previously detailed (9-12).

Co-culture of BMSCs and CD34+ cells, *in vitro* assays and xenotransplantation in NSG mice

CD34+ cells were co-cultured on irradiated (40Gy) BMSCs (iBMSCs) from HD or risk-stratified AMLs on serum-free media supplemented with SCF, FLT3L and IL3. CD34+ cells not exposed to iBMSCs were always used a base-line control. Growth kinetics, CD34 phenotype, apoptosis, cell cycle analysis were performed as detailed (11, 13, 14). CD34+ cells were counted using Countess-II-FL Automated Cell Counter (Thermo Fisher) (15). For serial clonogenic colony forming cell (CFC) assays, $1x10^4$ FACS-purified CD45+CD33+ AML blasts were plated in methylcellulose. Colonies were

counted/scored 10-12 days later using standard morphological criteria (11, 13, 14). For secondary re-plating, CFU colonies from each experimental condition were harvested and a single-cell suspension was achieved and re-plated as above. Eight-ten weeks-old NOD/LtSz-scidIL2Rγ−/− mice (NSG) were sublethally irradiated before intra-BM transplantation (IBMT) with $5x10^4$ CD34+ cells alone or co-cultured for 4 days with $3x10^5$HD- or AML-BMSCs. Mice were killed 6-7 weeks later and human chimerism analyzed by flow cytometry in the injected (IT) and the contralateral (CL) tibiae, spleen and peripheral blood (PB) (9-11). Serial transplantations were performed as described elsewhere (9-11). Animal protocols were approved by our local University Animal Care Committee. **Figure 1A** summaries our experimental design *in vitro* and *in vivo*.

Induction and treatment of experimental severe colitis

A well-established experimental mouse model for bowel inflammatory disease/acute colitis was employed (16, 17). Colitis was induced by intrarectal administration of 3mg of 2,4,6-trinitrobenzenesulfonic acid (TNBS,Sigma) in 50% ethanol in 7-week-old Bagg Albino/c male mice. Control mice received 50% ethanol alone. Animals (n=10/group) were treated intraperitoneally (i.p.) 12h after TNBS instillation with PBS (controls) or with either 10^6 HD-BMSCs or 10^6 AML-BMSCs. Animals were monitored daily for the appearance of diarrhea, body weight loss and survival. Colitis clinical scores, macroscopic damage of colons and histological analysis are described in detailed in Lopez-Millan *et al* (18). The animal care committee of the IPBLN-CSIC approved all procedures.

Colitis clinical scores, macroscopic damage of colons and histological analysis

Scores for colitis were assessed by two blinded observers at day 3 based in stool consistency and rectal bleeding as follows: 0, normal stool appearance, 1, slight decrease in stool consistency, 2, moderate decrease in stool consistency, 3, moderate decrease in stool consistency and presence of blood in stools, 4, severe watery diarrhea and moderate/severe blooding in stools Sera and colons

were collected immediately after death or at day 9 by cardiac puncture and dissection, respectively. Sera were assayed for cytokine levels by specific sandwich ELISAs using capture/biotinylated detection antibodies from BD Pharmingen according to the manufacture's recommendations. Colons were evaluated for macroscopic damage (graded on a scale 0-10) based on criteria reflecting inflammation (ie, hyperemia, bowel thickening, and extent of ulceration) by two independent researcher in a blinded fashion: ulceration (0=normal appearance, 1=focal hyperemia, no ulcers, 2=ulceration without hyperemia or bowel wall thickening, 3=ulceration with inflammation at 1 site, 4=two or more sites of ulceration and inflammation, 5=major sites of damage extending >1 cm along length of colon, 6-10=when an area of damage extended >2 cm along length of colon, score is increased by 1 for each additional cm of involvement). For histopathologic analysis, colon was fixed in 10% buffered formalin phosphate, paraffin embedded and sectioned. Sections were stained with Masson´s thrichromic, and inflammation was graded from 0 to 4 by an independent pathologist as follows: 0, no signs of inflammation; 1, low leukocyte infiltration; 2, moderate leukocyte infiltration; 3, high leukocyte infiltration, moderate fibrosis, high vascular density, thickening of the colon wall, moderate goblet cell loss, focal loss of crypts; and 4, transmural infiltrations, massive loss of goblet cells, extensive fibrosis, diffuse loss of crypts.

Statistical analysis

The impact of BMSCs on parameters reflecting *in vitro* homeostasis was analyzed with unpaired t-test analysis. Differences in colitis scores and survival between BMSCs and TNBS groups were compared using the t-test for two independent samples, using the GraphPad Prism software. P-values <0.05 were considered statistically significant.

REFERENCES

1. Bennett JM, Catovsky D, Daniel MT, et al. Proposed revised criteria for the classification of acute myeloid leukemia. A report of the French-American-British Cooperative Group. Ann Intern Med. 1985;103(4):620-5.
2. Dohner H, Estey EH, Amadori S, et al. 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):453-74.
3. Grimwade D. Screening for core binding factor gene rearrangements in acute myeloid leukemia. Leukemia. 2002;16(5):964-9.
4. Swerdlow SH, International Agency for Research on Cancer., World Health Organization. WHO classification of tumours of haematopoietic and lymphoid tissues. 4th ed. Lyon, France: International Agency for Research on Cancer, 2008.
5. Diaz de la Guardia R, Lopez-Millan B, Lavoie JR, et al. Detailed Characterization of Mesenchymal Stem/Stromal Cells from a Large Cohort of AML Patients Demonstrates a Definitive Link to Treatment Outcomes. Stem Cell Reports. 2017;8(6):1573-86.
6. de la Guardia RD, Correa JG, Lopez-Millan B, et al. Detection of inflammatory monocytes but not mesenchymal stem/stromal cells in peripheral blood of patients with myelofibrosis. Br J Haematol. 2018;181(1):133-7.
7. Sanchez L, Gutierrez-Aranda I, Ligero G, et al. Enrichment of human ESC-derived multipotent mesenchymal stem cells with immunosuppressive and anti-inflammatory properties capable to protect against experimental inflammatory bowel disease. Stem Cells. 2011;29(2):251-62.
8. Rodriguez R, Rosu-Myles M, Arauzo-Bravo M, et al. Human bone marrow stromal cells lose immunosuppressive and anti-inflammatory properties upon oncogenic transformation. Stem Cell Reports. 2014;3(4):606-19.
9. Prieto C, Stam RW, Agraz-Doblas A, et al. Activated KRAS Cooperates with MLL-AF4 to Promote Extramedullary Engraftment and Migration of Cord Blood CD34+ HSPC But Is Insufficient to Initiate Leukemia. Cancer Res. 2016;76(8):2478-89.
10. Bueno C, Roldan M, Anguita E, et al. Bone marrow mesenchymal stem cells from patients with aplastic anemia maintain functional and immune properties and do not contribute to the pathogenesis of the disease. Haematologica. 2014;99(7):1168-75.
11. Romero-Moya D, Bueno C, Montes R, et al. Cord blood-derived CD34+ hematopoietic cells with low mitochondrial mass are enriched in hematopoietic repopulating stem cell function. Haematologica. 2013;98(7):1022-9.
12. Belen Lopez-Millan, Rafael Diaz de la Guardia, Heleia Roca-Ho, et al. IMiDs mobilize Acute Myeloid Leukemia blasts to peripheral blood through downregulation of CXCR4 but fail to potentiate AraC/Idarubicin activity in preclinical models of non del5q/5q- AML. OncoImmunology. 2018; https://doi.org/10.1080/2162402X.2018.1477460.
13. Bueno C, Montes R, de la Cueva T, Gutierrez-Aranda I, Menendez P. Intra-bone marrow transplantation of human CD34(+) cells into NOD/LtSz-scid IL-2rgamma(null) mice permits multilineage engraftment without previous irradiation. Cytotherapy. 2010;12(1):45-9.
14. Montes R, Ayllon V, Gutierrez-Aranda I, et al. Enforced expression of MLL-AF4 fusion in cord blood CD34+ cells enhances the hematopoietic repopulating cell function and clonogenic potential but is not sufficient to initiate leukemia. Blood. 2011;117(18):4746-58.
15. Shariatmadar S, Sharma S, Cabana R, Powell S, Ruiz P, Krishan A. Electronic volume of CD34 positive cells from peripheral blood apheresis samples. Cytometry B Clin Cytom. 2008;74(3):182-8.
16. Gonzalez-Rey E, Anderson P, Gonzalez MA, Rico L, Buscher D, Delgado M. Human adult stem cells derived from adipose tissue protect against experimental colitis and sepsis. Gut. 2009;58(7):929-39.

17. Anderson P, Souza-Moreira L, Morell M, et al. Adipose-derived mesenchymal stromal cells induce immunomodulatory macrophages which protect from experimental colitis and sepsis. Gut. 2013;62(8):1131-41.
18. Lopez-Millan B, Diaz de la Guardia R, Roca-Ho H, et al. Therapeutic effect of the immunomodulatory drug lenalidomide, but not pomalidomide, in experimental models of rheumatoid arthritis and inflammatory bowel disease. Exp Mol Med. 2017;49(2):e290.

Table S1. Biological and cytogenetic-molecular characteristics of blasts and BM-MSCs from diagnostic AML patients

Patient ID	Diagnostic	Cytogenetics	Molecular	Age (y)	Gender	Blasts (%)	Risk
AML04	AML	46, XX	$NPM1^{MUT}$, $IDH1^{MUT}$	20	F	92	Low
AML05	AML	46, XY	$NPM1^{MUT}$	18	M	32	Low
AML07	AML-M4	46, XX	$NPM1^{MUT}$	37	F	80	Low
AML14	AML	46, XX	$NPM1^{MUT}$	78	F	67	Low
AML16	AML-M3	46, XY, inv(16)	Cbfb-MYH11	27	M	40	Low
AML17	AML	46, XY	-	10	M	92	Int
AML19	AML	46, XY	-	77	M	45	Int
AML24	AML-M4	46, XX	-	44	F	65	Int
AML25	AML	46, XY	-	61	M	1	Int
AML27	AML	46, XY	-	56	M	40	Int
AML30	AML-M2	46, XX, t(8;21)	AML1-ETO, FLT3-ITD	4	F	40	High
AML33	AML-M5	46, XY	$NPM1^{MUT}$, $FLT3^{del}$	77	M	80	High
AML34	AML-M5	46, XX	$NPM1^{MUT}$, FLT3-ITD	65	F	90	High
AML43	AML	46, XY, -7q	-	53	M	56	High
AML46	AML	46, XX	FLT3-ITD	8	F	95	High
HD02	Normal	46, XY	-	34	M	0	H. D
HD06	Normal	46, XX	-	34	F	0	H. D
HD07	Normal	46, XY	-	28	M	0	H. D
HD08	Normal	46, XX	-	49	F	0	H. D
HD09	Normal	46, XY	-	56	M	0	H. D

Abbreviations: HD, Healthy donor; CK, Complex karyotype; Int, Intermediate risk; M, male; F, female;-: no mutations found for FLT3, NPM1, cEBPa, WT and IDH1.