

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

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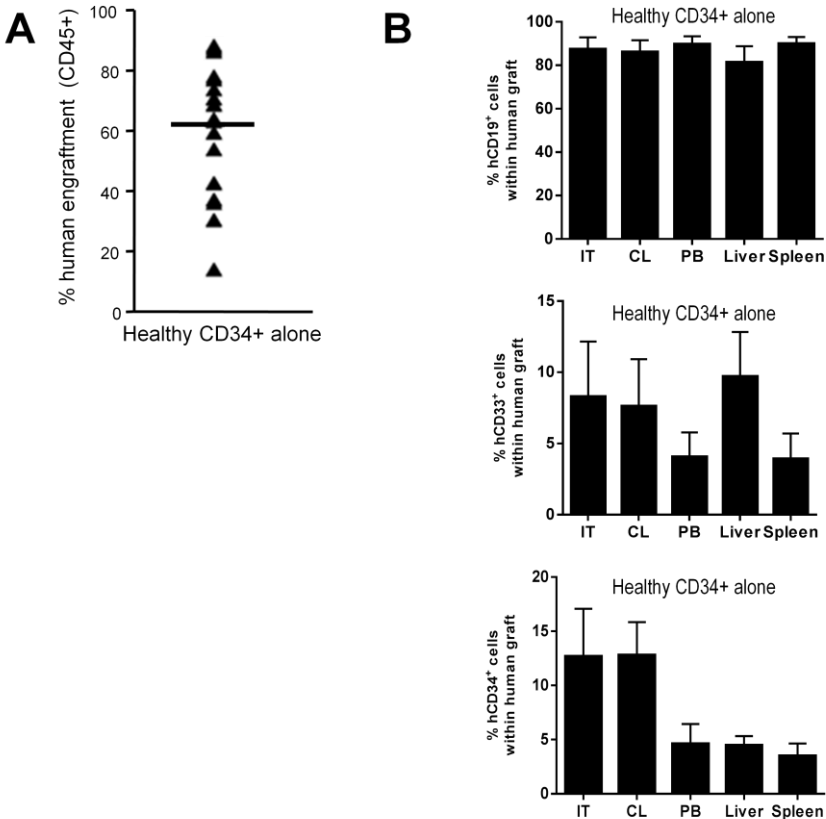


Figure 1S: *In vivo* engraftment (A) and multilineage repopulation (B) of CB-CD34+ HSPCs cultured in the absence of MSCs.

SUPPLEMENTAL METHODS

Patients

BM samples from nine newly diagnosed untreated idiopathic (non-inherited) AA patients were studied. Diagnosis of AA was based on the UK treatment guidelines (1). Seven normal bone marrow (NBM) samples were obtained from healthy volunteers and used as negative controls. All patients exhibited hematological parameters evidencing moderate-severe AA: i) white blood counts $<1.7 \times 10^3/\mu\text{L}$; ii) neutrophil count $<1 \times 10^3/\mu\text{L}$; iii) reticulocyte counts $<34 \times 10^3/\mu\text{L}$; iv) hemoglobin level $<8.5\text{g/dL}$; v) platelet count $<45 \times 10^3/\mu\text{L}$ (1). All patients showed severe hypocellular BM at presentation with absence of both paroxysmal nocturnal hemoglobinuria and abnormal karyotype clones. **Table 1** summarizes the main hematological parameters of each group. This study was approved by the Institutional Review Board of the Hospital Virgen de las Nieves, Granada, Spain.

Isolation and expansion BM-MSCs

Mononuclear cells (MNCs) from BM were isolated by centrifugation using Ficoll-Paque Plus (GE Healthcare) density gradient, as described (15, 21). MNCs were seeded at a density of 3×10^4 cells per cm^2 in Advanced DMEM supplemented with 10% FBS, 1% Glutamax and 1% penicillin/streptomycin, and incubated at 37°C in a 5% humidified CO_2 atmosphere. After 24 h, non-adherent cells were discarded and fresh medium was added. When cell culture achieved $>85\%$ of density, adherent cells were trypsinized, washed and replated at 5×10^3 cells/ cm^2 .

Characterization of MSC cultures

The immunophenotype of cultured BM-MSCs was analyzed by flow cytometry as previously described (21, 22). In brief, 2×10^5 cells were incubated with the fluorochrome-conjugated monoclonal antibodies CD90, CD73, CD105, CD44, CD45, CD34, HLA-DR, CD19, and CD14 (Miltenyi) or their respective isotype controls, washed in PBS and analyzed in a FACSCanto II cytometer (BD).

Functional BM-MSC differentiation studies were performed by plating BM-MSCs in specific differentiation inductive media for 2 weeks (23). For adipogenic differentiation, cells were cultured in Adipogenic MSCs Differentiation BulletKit (Lonza) and differentiated cultures were stained with oil red O (Sigma-Aldrich). For osteogenic differentiation, cells were cultured in Osteogenic MSCs Differentiation BulletKit (Lonza) and differentiated cultures were stained with alizarin red S (Sigma-Aldrich) (23). In addition, BM-MSC differentiation was analyzed and compared between AA patients and normal BM donors by q-RT-PCR for osteogenic (osteopontin, alkaline phosphatase, osterix) and adipogenic factors (total PPAR, C/EBP α). Primers and PCR conditions have been reported elsewhere (23).

Cord blood (CB) collection and CD34+ cell isolation

CB units from healthy neonates were obtained from local hospitals following approval from our local Ethics and Biohazard Board Committee. CB samples were pooled to reduce variability among individual units. MNCs were isolated using Ficoll-Hypaque and after lysing the red cells (Cytognos, Salamanca, Spain), CD34+ cells were purified by magnetic bead separation using the human CD34 MicroBead kit and the AutoMACS Pro separator (Miltenyi). The purity of the CD34+ fraction was assessed by flow cytometry and only CD34+ fractions showing purity >95% were used (24-26).

Co-culture of BM-MSCs and CB-CD34+ cells and *in vitro* analyses of CD34+ cells homeostasis

Isolated CD34+ cells (5×10^4) were co-cultured on irradiated (42 Gy) normal BM-MSCs or AA BM-MSCs (6×10^4) in a 6-well plates on StemSpan media (Stem Cell Technologies) supplemented with SCF (100 ng/mL), FLT3L (100 ng/mL) and IL3 (10ng/mL) (Peprotech). *In vitro* analyses were also performed with CD34+ cells alone (n=4), without MSC co-culture, as a base-line control for CD34-MSCs co-cultures. To determine the growth kinetics and CD34 phenotype, CD34+ cells cultured on

BM-MSCs were counted twice a week and assayed for CD34 expression (27, 28). The apoptotic status of CD34+ cells was assessed using the annexin-V apoptosis detection kit (BD Biosciences) (25, 27, 28). Briefly, cells were harvested and washed with PBS before staining with annexin V-PE and 7-amino actinomycin D. Apoptotic cells were detected by gating the annexin V-positive fraction. For cell cycle analysis, CD34+ cells were fixed in 70% ice-cold ethanol and stored at -20°C. Cells were washed with ice-cold PBS and suspended in propidium iodide (PI) buffer containing 5 mg of PI and 100 mg/mL of RNAase. Cell cycle distribution discriminating between quiescent cells (G0/G1) and cycling cells (S/G2/M phase) was analyzed on a FACSCanto cytometer using FACSDiva and ModFit softwares (BD) (25, 27, 28).

Human clonogenic progenitor assays were performed by plating 2000 CD34+ cells that had been cultured for 2 or 4 days with normal or AA BM-MSCs into methylcellulose H4434 (Stem Cell Technologies) containing the human growth factors SCF (50ng/mL), GM-CSF (10ng/mL), IL3 (10ng/mL) and erythropoietin (3U/mL). CD34+ cells not cultured with MSC (n=4) were also assayed as control for CD34-MSC co-cultures. Colonies were counted and scored on day 14 of the colony-forming unit (CFU) assay using standard morphological criteria.

Mice xenotransplantation and analysis of engraftment

NOD/LtSz-scid IL2R γ ^{-/-} mice (NSG) were housed under sterile conditions. The Animal Care Committee of the University of Granada approved all animal protocols. Mice (n=20) at 8 to 12 weeks of age were sublethally irradiated (2.5 Gy) before intrabone marrow transplantation (25, 29). A total of 1x10⁵ CD34+ cells that had been cultured for 4 days on either normal or AA BM-MSCs were transplanted in a volume of 20 μ L. CD34+ cells not cultured with MSC (n=10) were also transplanted as a base-line control for CD34-MSCs co-cultures. For pain relief, 0.1 mg/kg buprenorphine and 5 mg/kg carprofen were administered immediately after transplantation and 24 h after. Mice were killed

7 weeks after transplantation. Cells from the bone marrow (injected tibia [IT] and from the contralateral tibia and femur [CL]), spleen, liver and peripheral blood (PB) were stained with anti-hHLA-ABC-FITC and anti-hCD45-APC-Cy7 (BD) to analyze human chimerism by flow cytometry. All engrafted mice were assessed for multilineage analysis using anti-hCD33-PE for myeloid cells, anti-hCD19-APC for B cells, and anti-hCD34-PE-Cy7 for immature hematopoietic cells (BD)(25, 28).

Assessment of the immunosuppressive response in human T cells

Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood of 5 healthy volunteers by density sedimentation on Ficoll-Hypaque gradients. Cells recovered were washed in RPMI medium and immediately used for culture. To establish mixed lymphocyte cultures (MLC), responder PBMCs (1×10^5) from donor A were incubated with 10^5 allogeneic HLA-mismatched mitomycin C-treated stimulator PBMCs from donor B at 37°C and 5% CO₂ in round 96-well plates in 200 µl complete DMEM medium (DMEM containing 10% FBS, 2 mM glutamine and 1% penicillin/streptomycin) in the presence or absence of 2×10^4 Normal BM-MSCs or AA BM-MSCs. Cells were pulsed with 2.5 µCi/well [³H]-thymidine for the last 12 h of a culture of 96 h, harvested onto membranes and proliferation was determined by measuring [³H]-thymidine uptake in a liquid scintillation counter. After 48 h, cytokine determinations for IL-2, TNF-α and IFN-γ in the supernatants were determined by ELISA using capture/biotinylated detection antibodies from BD Pharmingen (13).

Determination of anti-inflammatory activity

Synovial membrane cells (SMCs) were isolated by digestion with collagenase of synovial tissue obtained from three patients with active rheumatoid arthritis (RA) at time of knee replacement surgery. SMCs cultures were conducted in 48-well plates in complete medium consisting of RPMI-1640 supplemented with heat-inactivated human pooled serum (8%), L-glutamine (20 mM), sodium

pyruvate (1%), 1% nonessential amino acids and 1% penicillin/streptomycin at 37°C and 5% CO₂. SMCs (2x10⁵) were stimulated with TNF- α (20 ng/ml, PreproTech) for 24 h in the presence or absence of 1x10⁵ either normal BM-MSCs (n=7) or AA BM-MSCs (n=7). Extracellular matrix-degrading activities of stimulated SMCs were determined in culture supernatants using the EnzChek gelatinase/collagenase assay kit (Molecular Probes), a fibril degradation assay that utilises self-quenched fluorescein-conjugated type I collagen, type IV collagen and gelatine, to determine the type I collagenase (MMP1/MMP8/MMP13) activity and the type IV collagenase and gelatinase (MMP2) activities, respectively (13). The content of MMP1 was determined in supernatants by using an ELISA kit (R&D Biosystems). Moreover, SMCs were stimulated with lipopolysaccharide (LPS; 1 μ g/ml, Sigma) in the presence or absence of 10⁵ either normal BM-MSCs (n=7) or AA BM-MSCs (n=7). After 48 h, culture supernatants were assayed for TNF- α by ELISA (BD Pharmingen).

Statistical Analysis

All data are expressed as mean \pm SD. Statistical comparisons between experimental groups were performed with either a paired Student's t test or Duncan's multiple range test after two-way analysis of variance. Statistical significance was defined as a *p value* <0.05.