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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ABSTRACT

Aplastic anemia is a life-threatening bone marrow failure disorder characterized by peripheral pancytopenia and marrow hypoplasia. The majority of cases of aplastic anemia remain idiopathic, although hematopoietic stem cell deficiency and impaired immune responses are hallmarks underlying the bone marrow failure in this condition. Mesenchymal stem/stromal cells constitute an essential component of the bone marrow hematopoietic microenvironment because of their immunomodulatory properties and their ability to support hematopoiesis, and they have been involved in the pathogenesis of several hematologic malignancies. We investigated whether bone marrow mesenchymal stem cells contribute, directly or indirectly, to the pathogenesis of aplastic anemia. We found that mesenchymal stem cell cultures can be established from the bone marrow of aplastic anemia patients and display the same phenotype and differentiation potential as their counterparts from normal bone marrow. Mesenchymal stem cells from aplastic anemia patients support the *in vitro* homeostasis and the *in vivo* repopulating function of $CD34^+$ cells, and maintain their immunosuppressive and anti-inflammatory properties. These data demonstrate that bone marrow mesenchymal stem cells from patients with aplastic anemia do not have impaired functional and immunological properties, suggesting that they do not contribute to the pathogenesis of the disease.

Introduction

Aplastic anemia (AA) is a rare and life-threatening heterogeneous bone marrow (BM) failure disorder characterized by peripheral pancytopenia and marrow hypoplasia.[1,2] The majority of AA cases are idiopathic with an unknown primary etiology.[1-3] In some patients, a drug or infection is implicated in the etiology of AA although it is unclear why only some individuals are susceptible.[4-7] In ~15% of patients the disease is inherited or congenital, for example Fanconi anemia.[1,3] The main suggested underlying mechanism in AA is a primary hematopoietic stem cell (HSC) deficiency or a secondary HSC defect due to an abnormal balance between HSC death and differentiation.[3,8] Importantly, pathological autoimmune responses also seem to be involved in AA BM failure, given the good responses to immunosuppressive treatments.[1,9]

Mesenchymal stem/stromal cells (MSC) are rare BM multipotent cells that constitute a source of progenitors for mesodermal tissues.[10] MSC have emerged as excellent candidates for clinical applications thanks to their immunomodulatory properties and their ability to support hematopoiesis.[11-13] Importantly, MSC are an essential component of the BM hematopoietic microenvironment. The BM hematopoietic microenvironment regulates the homeostasis of hematopoiesis through the production and secretion of cytokines and extracellular matrix molecules.[14] Furthermore, the BM hematopoietic microenvironment plays a role in the pathogenesis of a variety of hematologic malignances including acute lymphoblastic[15] and myeloblastic leukemias,[16] multiple myeloma,[17] lymphomas,[18] chronic myeloid leukemia[19] and myelodysplastic syndromes.[16,20]

Because HSC failure and impaired immune responses underlie the pathogenesis of AA, it is plausible that BM-MSC may also contribute, directly or indirectly, to the pathogenesis of AA. However, there is almost no information on whether the functional and immunological properties of BM-MSC are impaired in AA patients or on the potential contribution of

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these cells to the pathogenesis of the disease. Here we report that BM-MSC from AA patients display the same phenotype and differentiation potential as their counterparts from normal BM, support *in vitro* homeostasis and *in vivo* repopulating function of CD34$^+$ hematopoietic stem and progenitor cells, and fully maintain immunosuppressive and anti-inflammatory properties. These data indicate that BM-MSC from AA patients do not have impaired functional and immunological properties, suggesting that they do not contribute to the pathogenesis of AA.

Methods

Patients

BM samples from nine newly diagnosed AA patients were studied. The diagnosis of AA was based on the UK treatment guidelines.[1] Seven normal BM samples were obtained from healthy volunteers and used as controls. Table 1 summarizes the main hematologic parameters of each group. Extended clinical/biological information is provided in the *Online Supplementary Methods.*

Isolation and expansion of bone marrow mesenchymal stem/stromal cells

Mononuclear cells from BM were isolated by Ficoll-Paque and seeded at 3x10^4 cells/cm^2. After 24 h, non-adherent cells were discarded and fresh medium added. When cultures achieved >85% density, adherent cells were trypsinized and replated at 5x10^3 cells/cm^2.[15,21]

Characterization of mesenchymal stem/stromal cell cultures

The flow cytometry immunophenotype and osteogenic and adipogenic differentiation of BM-MSC was analyzed as previously described.[21-24]

Cord blood collection and CD34$^+$ cell isolation

Cord blood (CB) units from healthy neonates were obtained from local hospitals following approval from our local Ethics Board Committee. Mononuclear cells were isolated using Ficoll-Hypaque and CD34$^+$ cells purified using the CD34 MicroBead kit and the AutoMACSPro. The purity was consistently >95%.[25-27]

Co-culture of bone marrow mesenchymal stem/stromal cells and cord blood CD34$^+$ cells and in vitro analyses of CD34$^+$ cell homeostasis

CD34$^+$ cells were co-cultured on irradiated BM-MSC from normal subjects or patients with AA on serum-free media supplemented with stem cell factor, FLT3 ligand and interleukin-3. *In vitro* analyses were performed with CD34$^+$ cells without MSC co-culture, as a baseline control for CD34-MSC co-cultures. Growth kinetics, CD34 phenotype, apoptosis, cell cycle analysis and clonogenic progenitor assays were performed, as detailed.[26,28,29]

Mice xenotransplantation and analysis of engraftment

NOD/LtSz-scidIL2Rγ$^{-/-}$ mice (NSG) were housed under sterile conditions. The Animal Care Committee of our University approved animal protocols. Mice at 8-12 weeks of age were sublethally irradiated before intra-BM transplantation.[26,30] CD34$^+$ cells (1x10^5) that had been cultured on normal or AA BM-MSC were transplanted. CD34$^+$ cells not cultured with MSC were transplanted as a control for CD34-MSC co-cultures. Mice were killed 7 weeks after transplantation and human chimerism was analyzed by flow cytometry in the injected and contralateral tibiae, spleen, liver and peripheral blood.[26,29]

Assessment of the immunosuppressive response in human T cells

Peripheral blood mononuclear cells were isolated from healthy volunteers. To establish mixed lymphocyte cultures, responder peripheral blood mononuclear cells (1x10^5) from donor A were incubated with 1x10^5 allogeneic HLA-mismatched mitomycin C-treated stimulator peripheral blood mononuclear cells from donor B in the presence or absence of 2x10^4 normal BM-MSC or AA BM-MSC. Cells were pulsed with 2.5 μCi/well [^{3}H]-thymidine for the last 12 h and harvested onto membranes; proliferation was determined by measuring [^{3}H]-thymidine uptake. After 48 h, interleukin-2, tumor necrosis factor-α and interferon-γ were determined by enzyme-linked immunoassay (ELISA).[13]

Determination of anti-inflammatory activity

Synovial membrane cells were obtained from patients with rheumatoid arthritis. These cells (2x10^5) were stimulated with tumor necrosis factor-α (20 ng/mL) for 24 h in the presence or absence of 1x10^5 normal BM-MSC (n=7) or AA BM-MSC (n=7).[13] Extracellular matrix-degrading activities were determined as described elsewhere.[13] The MMP1 content was determined in supernatants by ELISA. Synovial membrane cells were stimulated with lipopolysaccharide 1 μg/mL in the presence or absence of 1x10^5 normal BM-MSC (n=7) or AA BM-MSC (n=7). After 48 h, culture supernatants were assayed for tumor necrosis factor-α by ELISA.

Results

Bone marrow mesenchymal stem/stromal cells from patients with aplastic anemia have a normal phenotype and differentiation potential

MSC cultures were successfully established and expanded from the BM of nine patients with AA and seven age-matched healthy donors for further investigations. Table 1 presents the main biological and hematologic features of both groups. Established AA BM-MSC cultures were consistently devoid of contaminating hematopoietic cells, being negative for CD45, CD34, HLA-DR, CD19 and CD14 but expressed common MSC markers including CD90, CD73, CD105 and CD44 (Figure 1). They had typical fibroblastoid morphology (Figure 2). To further characterize MSC from AA patients, adipogenic and osteoblastic differentiation assays were performed at early MSC passages (p3-p5) (Figure 2A).[15,31] The efficiency of osteoblastic and adipogenic differentiation was similar to

Table 1. Main biological and hematologic parameters of AA patients and healthy donors.

	Aplastic anemia patients	Healthy BM donors
Age (years)	52±20 (9-72)	54±10 (42-65)
WBC (x10^9/L)	1.39±0.4 (0.5-1.7)	9.1±0.7 (7.7-10.5)
Neutrophils (x10^9/L)	0.69±0.2 (0.28-1)	5.5±0.7 (4.1-6.3)
Reticulocytes (x10^9/L)	13.3±4 (3-17)	71±31 (41-89)
Hemoglobin (g/dL)	7.9±0.5 (7-8.5)	12.3±4.2 (10.1-13.5)
Platelet count (x10^9/L)	29±13 (10-47)	213±47 (174-249)
% CD34$^+$ cells in BM	0.12±0.1 (0.08-0.19)	0.72±0.4 (0.19-1.37)

Range is shown in brackets. WBC: white blood cell counts; BM: bone marrow.

that of normal BM-MSC (Figure 2A). Osteoblastic and adipogenic differentiation potential was further analyzed by quantitative reverse transcriptase polymerase chain reaction. Upon osteogenic differentiation, the expression of the master osteogenic markers, osteocalcin, alkaline phosphatase and osterix, was almost identical in normal BM-MSC and AA BM-MSC. Similarly, upon adipogenic differentiation, the expression of the late adipogenic transcription factors, PPAR and CEBPα, was similar between normal BM-MSC and AA BM-MSC (Figure 2B). Thus, BM-MSC derived from AA patients are phenotypically and functionally similar to those from healthy donors.

Bone marrow mesenchymal stem/stromal cells from patients with aplastic anemia support the in vitro *homeostasis and the* in vivo *repopulating function of cord blood CD34⁺ cells*

Whether AA BM-MSC can maintain the homeostasis of purified CB-CD34⁺ cells was analyzed *in vitro* by co-culturing CB-CD34⁺ cells with early passage (p4-p8) BM-MSC. CB-CD34⁺ cells expanded equally in BM-MSC cultures from AA patients or healthy donors (Figure 3A). In the absence of MSC support, CD34⁺ cells grew slightly slower (Figure 3A). There were no differences in apoptosis (~2-6%; Figure 3B) or cycling status (~50%; Figure 3C) of CB-CD34⁺ cells when co-cultured on BM-MSC from either normal subjects or from patients with AA, supporting a similar expansion of CB-CD34⁺ cells in any of the BM-MSC co-cultures (Figure 3A). In contrast, the slower growth of CD34⁺ cells maintained in the absence of MSC was accompanied by a higher apoptotic rate (~18% by day 12; Figure 3B). We then analyzed the *in vitro* differentiation kinetics of CB-CD34⁺ cells by tracing the loss of the CD34 antigen, and found that CD34⁺ cells progressively disappeared within ~23 days on either normal or AA BM-MSC co-cultures (Figure 3D). Interestingly, in the absence of MSC support the differentiation of CD34⁺ cells was more pronounced. We next tested whether co-culture of CB-CD34⁺ cells with AA BM-MSC affects their hematopoietic progenitor cell and/or HSC function. We utilized *in vitro* clonogenic colony-forming unit (CFU) assays as a read-out for hematopoietic progenitor cell function. Equal numbers of CB-CD34⁺ cells that had been cultured for 2 or 4 days with normal or AA BM-MSC were plated in CFU assays and hematopoietic colonies were counted after 14 days (Figure 3E,F). Scoring the CFU revealed that co-culture with AA BM-MSC did not influence either the clonogenic ability or the colony phenotype of CB-CD34⁺ cells. The CFU capacity of CD34⁺ cells that had not been previously cultured on MSC was slightly diminished (P>0.05).

Xenotransplantation assays into NSG mice were undertaken as an *in vivo* read-out for SCID-repopulating HSC function. CB-CD34⁺ cells (1x10⁵) that had been cultured for 4 days with normal or AA BM-MSC were transplanted intratibia and mice were sacrificed 7 weeks later for chimerism analysis in multiple hematopoietic organs. Human multilineage reconstitution was determined by flow cytometry using anti-CD45, anti-HLA-ABC, anti-CD19, anti-CD33 and anti-CD34 (Figure 4A). CD34⁺ cells co-cultured on either normal or AA BM-MSC displayed similar levels of engraftment (54% *versus* 61%; P>0.05. Figure 4B). The migratory ability of CD34⁺ cells was assessed by analyzing the level of chimerism in the injected tibiae, contralateral tibiae, spleen, liver and peripheral

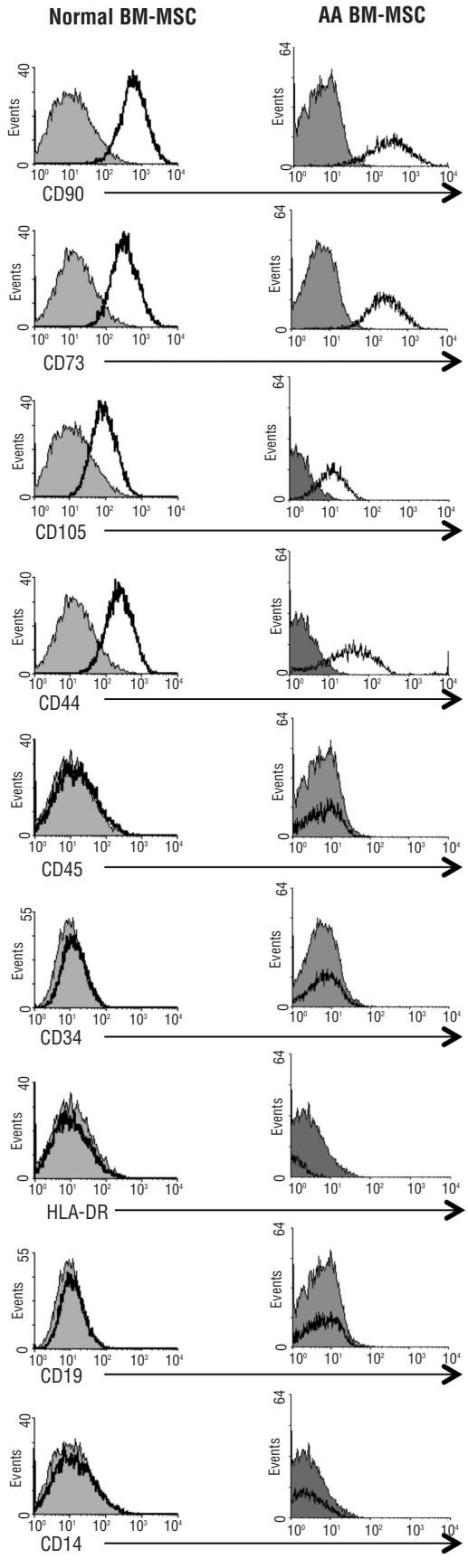


Figure 1. Immunophenotypic profile of BM-MSC from AA patients analyzed by flow cytometry. Filled lines represent the control isotypes. Empty lines show antibody-specific staining.

blood (Figure 4B). Co-culture with either normal or AA BM-MSC did not influence the migratory capacity of CB-CD34+ hematopoietic stem and progenitor cells as demonstrated by the similar capacity to colonize other hematopoietic tissues in all the animals (Figure 4B). We next characterized the engraftment composition, and found a very similar multilineage composition in all tissues reconstituted with CB-CD34+ cells that had been co-cultured with either normal or AA BM-MSC (Figure 4C). The engraftment and multilineage reconstitution were very similar between CD34+ cells cultured alone or with MSC (*Online Supplementary Figure S1*). Taken together, these findings indicate that BM-MSC from AA patients support the *in vitro* homeostasis and the *in vivo* repopulating function of CB-CD34+ cells.

Bone marrow mesenchymal stem/stromal cells from patients with aplastic anemia maintain immunosuppressive and anti-inflammatory properties

Human BM-MSC display robust immunomodulatory and anti-inflammatory properties. Because an impaired immune response is suggested to be at the origin of the BM failure in AA we investigated whether the capacity of BM-MSC from AA patients to inactivate T-cell responses and to inhibit inflammatory responses is impaired. The addition of BM-MSC to mixed lymphocyte cultures of peripheral blood mononuclear cells from different donors significantly reduced the proliferative response (Figure 5A) and the production of Th1 cytokines (interferon-γ, interleukin-2 and tumor necrosis factor-α) by responder T cells (Figure 5B). The immunomodulatory activity of AA BM-MSC was comparable to that observed for normal BM-MSC (Figure 5A,B). Moreover, BM-MSC isolated from AA patients were very efficient at inhibiting the inflammatory response of resident cells of the synovial membrane in patients with active rheumatoid arthritis. BM-MSC isolated from AA patients or healthy subjects similarly inhibited the production of pro-inflammatory cytokines (tumor necrosis factor-α) and matrix-degrading enzymes (MMP1/MMP8/MMP13 type I collagenase and MMP2 gelatinase and type IV collagenase activities) by activated synovial membrane cells (Figure 5C). These data indicate that BM-MSC from AA patients fully retain their immunomodulatory capacities.

Discussion

AA is a rare, heterogeneous disorder in which the majority of cases are idiopathic, because the primary etiology is unknown.[1-3] In a subset of patients, a drug or infection has been implicated in the etiology, although it is unclear why only some individuals are susceptible.[4-7] AA is generally considered as an immune-mediated BM failure syndrome

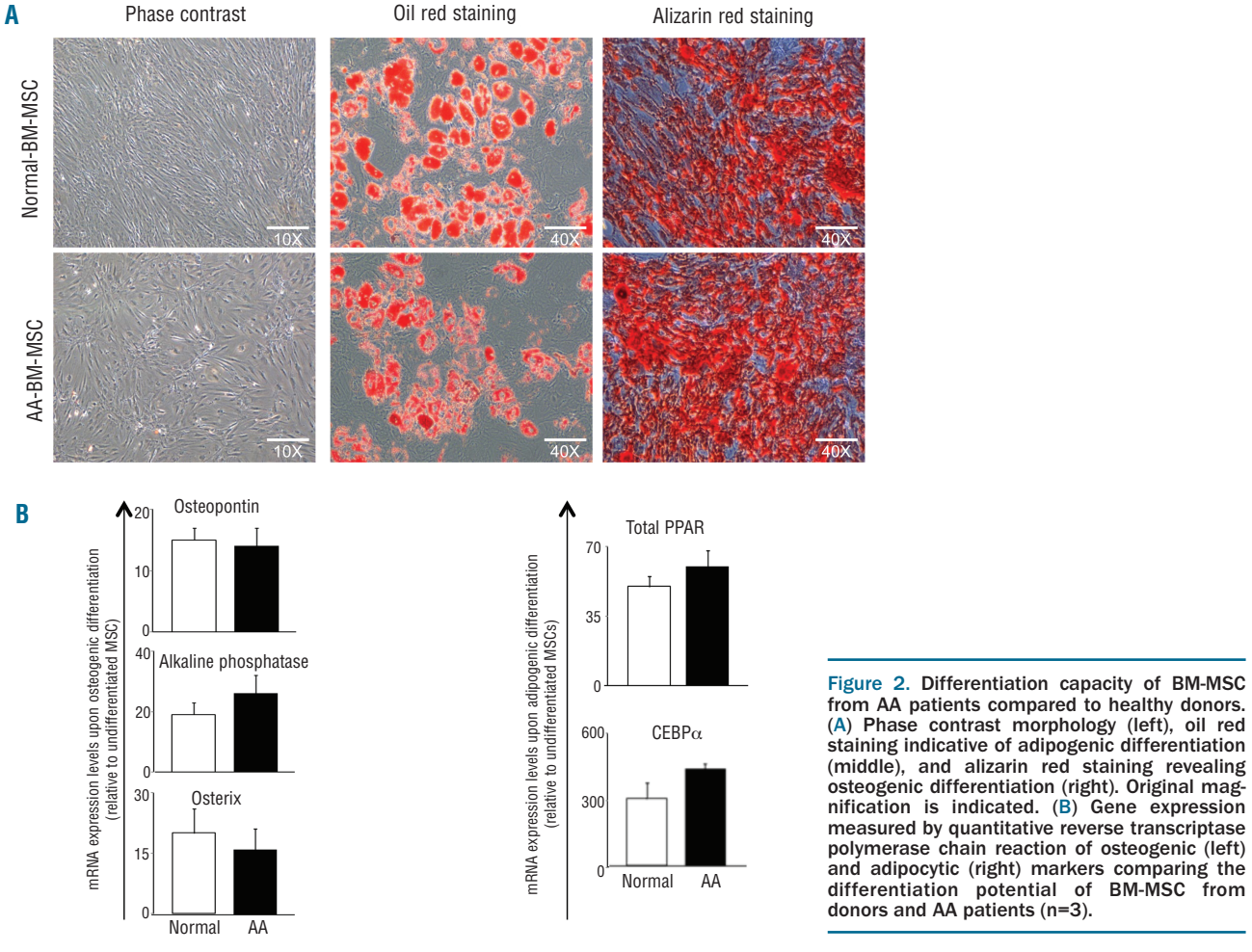


Figure 2. Differentiation capacity of BM-MSC from AA patients compared to healthy donors. (A) Phase contrast morphology (left), oil red staining indicative of adipogenic differentiation (middle), and alizarin red staining revealing osteogenic differentiation (right). Original magnification is indicated. (B) Gene expression measured by quantitative reverse transcriptase polymerase chain reaction of osteogenic (left) and adipocytic (right) markers comparing the differentiation potential of BM-MSC from donors and AA patients (n=3).

with defective HSC.[3,32] Previous studies demonstrated the defective HSC as well as aberrant T-cell immunity in AA.[3,33-35] Immunosuppressive therapy and allogeneic BM transplantation are the initial treatments of choice for newly diagnosed patients with severe AA.[1,36] On the one hand, the good responses to immunosuppressive treatments such as antithymocyte globulin and cyclosporine A support the belief that pathological T-cell-mediated autoimmune responses are a cause of the BM failure in AA.[1,34,36] On the other hand, several studies have shown that co-transplantation of allogeneic BM- or CB-derived MSC and HSC enhances hematopoietic engraftment and also improves stromal function in patients with AA,[37-40] suggesting a potential underlying role of the BM microenvironment in the pathogenesis of AA. In fact, AA patients have a hypocellular BM which is "physiologically" replaced by fatty BM, likely of mesenchymal origin, further supporting a potential contribution of the BM microenvironment to the pathogenesis of AA.[41] MSC have robust immunomodulatory and anti-inflammatory properties[11-13] and are an essential component of the BM hematopoietic microenvironment, which regulates the homeostasis of hematopoiesis through the production and secretion of cytokines and extracellular matrix molecules.[14] Importantly, the BM hematopoietic microenvironment has been shown to play a role in the pathogenesis of a variety of hematologic malignances including acute lymphoblastic[15] and myeloblastic leukemia,[16] multiple myelo-

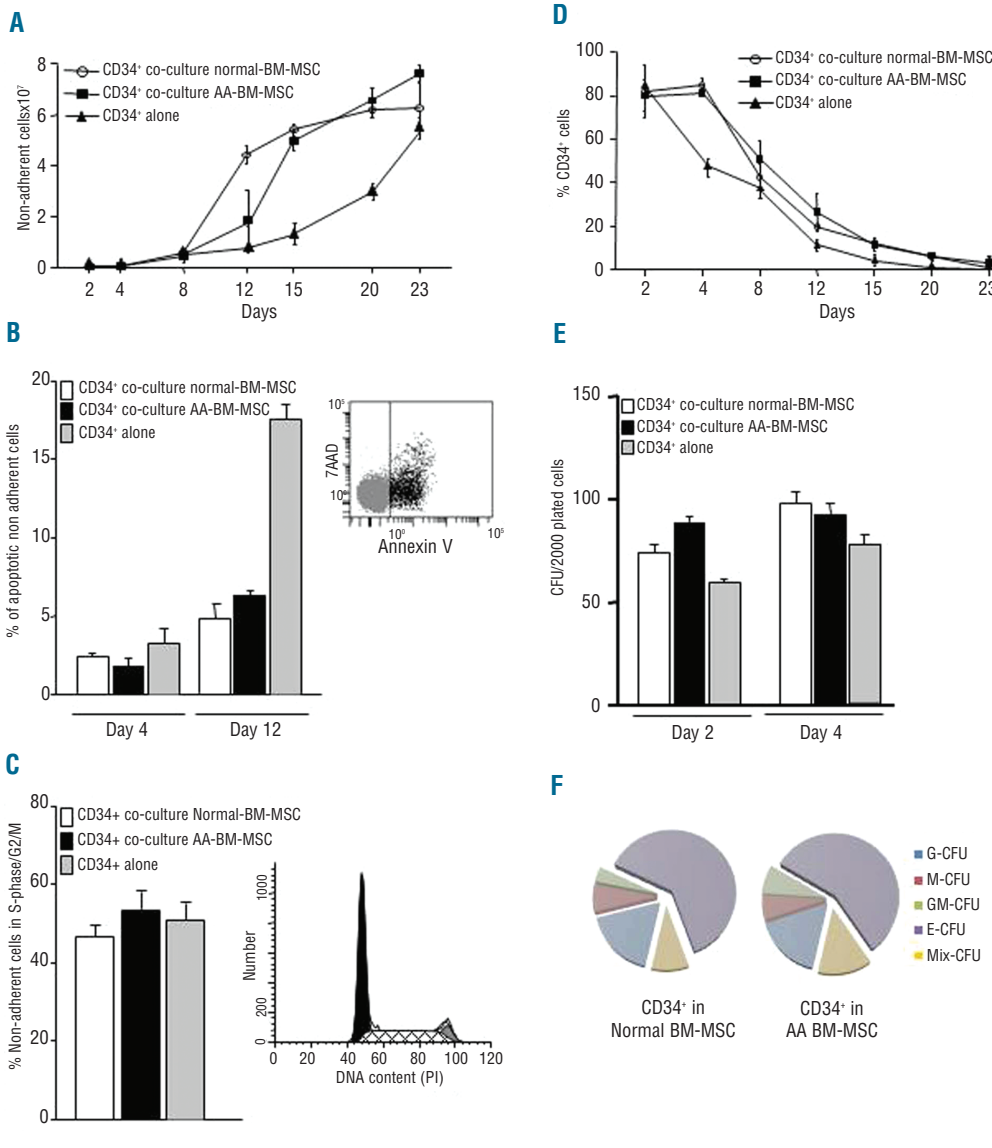
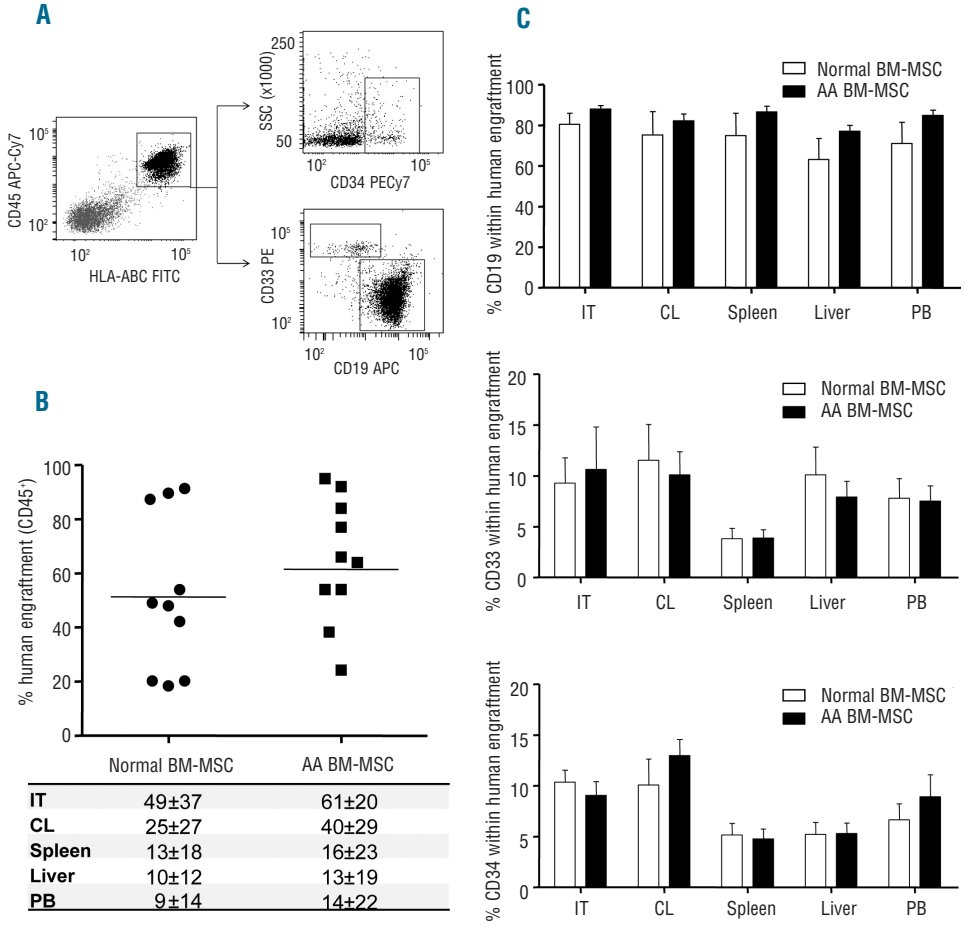


Figure 3. BM-MSC from AA patients support *in vitro* homeostasis of CB-CD34⁺ hematopoietic stem and progenitor cells (HSPC). (A) *In vitro* expansion of CD34⁺ HSPC co-cultured on BM-MSC from healthy donors (n=5) and AA patients (n=7). (B) Proportion of apoptotic CD34⁺ cells (annexin V⁺) measured at days 4 and 12 of CD34/BM-MSC co-cultures (n=2). (C) Proportion of cycling CD34⁺ cells measured at day 12 of CD34/BM-MSC co-cultures (n=2). (D) Loss of CD34 antigen over time in co-cultures of CD34⁺ HSPC with BM-MSC from healthy donors and AA patients (n=6). (E) Clonogenic potential (colony-forming units) of CD34⁺ HSPC previously co-cultured for 2 or 4 days with BM-MSC from healthy donors or AA patients (n=6). (F) Scoring of CFU obtained in (E). All the assays were also performed with CD34⁺ cells not previously cultured on MSC (n=4), as a baseline control for CD34-MSC co-cultures. Because CD34⁺ cells differentiate into CD34⁻ cells after a few days in culture, we refer to them as "non-adherent cells" rather than "CD34⁺ cells".

ma,[17] lymphomas,[18] chronic myeloid leukemia[19] and myelodysplastic syndromes.[16,20] We, therefore, hypothesized that BM-MSC may contribute, directly or indirectly, to the pathogenesis of AA.

There are limited studies with conflicting results on the properties of BM-MSC in AA patients.[8,41-48] These studies mainly claim that AA BM-MSC have aberrant morphology, impaired adipogenic and osteogenic potential, changes in gene expression, and a reduced ability to support hematopoiesis *in vitro*. However, to the best of our knowledge, no study so far has prospectively addressed in depth the ability of AA BM-MSC to maintain hematopoietic homeostasis and progenitor function *in vitro*, their *in vivo* repopulating function in xenotransplant models, or the immunosuppressive and anti-inflammatory properties on these cells. We comprehensively analyzed whether the functional and immunological properties of BM-MSC are impaired in AA patients and the potential contribution of these cells to the pathogenesis of the disease. We report that BM-MSC from AA patients have the same phenotype and differentiation potential as their counterparts from normal BM, support *in vitro* homeostasis and *in vivo* repopulating function of CD34$^+$ hematopoietic stem and progenitor cells, and fully maintain immunosuppressive and anti-inflammatory properties. Our data indicate that BM-MSC from AA patients do not have impaired functional and immunological properties and retain the ability to support hematopoiesis, suggesting that they do not contribute to the pathogenesis of the disease. Interestingly, it has been reported that BM-MSC from AA patients overexpress membrane-bound interleukin-15 which may indirectly participate in the T-cell-mediated autoimmune attack of HSC in AA patients by recruiting T cells to the BM and stimulating them *in situ*.[46] Our data are in partial disagreement with those of other studies suggesting that AA BM-MSC are aberrant.[8,41,42,44,46,48] From a methodological point of view, we assessed the features of AA BM-MSC beyond morphology, gene expression, differentiation potential and proliferation by analyzing the cells' ability to support hematopoiesis *in vitro* and *in vivo* and their immune properties. Biologically, all our patients, but one, were elderly patients while other studies focused on chil-



	Normal BM-MSC	AA BM-MSC
IT	49±37	61±20
CL	25±27	40±29
Spleen	13±18	16±23
Liver	10±12	13±19
PB	9±14	14±22

Figure 4. BM-MSC from AA patients do not impair *in vivo* multilineage repopulating function of CB-CD34$^+$ hematopoietic stem and progenitor cells (HSPC). (A) Representative flow cytometry analysis of the graft. The human graft is identified as the CD45$^+$HLA-ABC$^+$ fraction. The CD45$^+$ human graft comprises B-lymphoid cells (CD19$^+$), myeloid cells (CD33$^+$) and immature cells (CD34$^+$). (B) Long-term (7 weeks) hematopoietic reconstitution of NSG mice (n=20) after intra-BM injection of CD34$^+$ HSPC co-cultured for 4 days in BM-MSC from normal donors *versus* AA patients. Each dot represents an individual mouse and the horizontal line indicates the mean of each experimental cohort. The table shows the levels of human chimerism in the distinct hematopoietic tissues analyzed. (C) Multilineage and multiorgan human chimerism in the injected tibia (IT), contralateral tibia and femur (CL), spleen, liver, and peripheral blood (PB) demonstrating migration of human cells from the IT. No differences in graft composition were found between CD34$^+$ HSPC co-cultured with BM-MSC from normal donors *versus* AA patients.

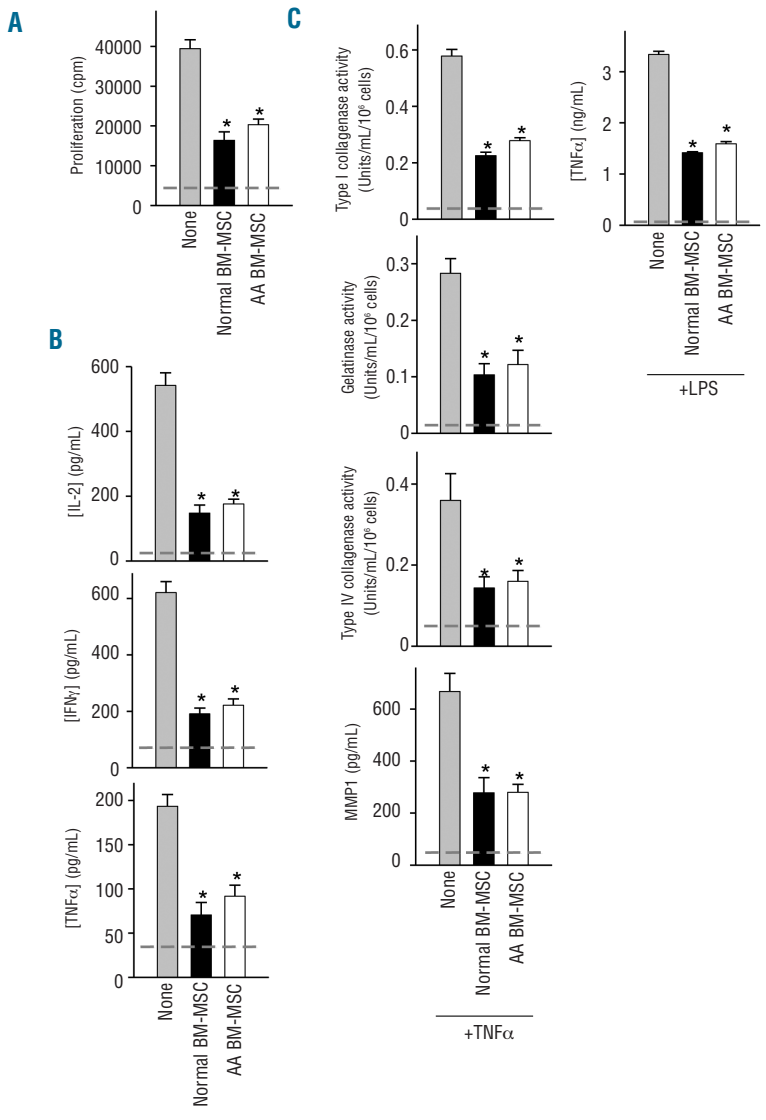


Figure 5. BM-MSC from AA patients maintain immunosuppressive and anti-inflammatory properties. (A) Mixed lymphocyte cultures (MLC) were established by co-culturing peripheral blood mononuclear cells (PBMC) from donor A ($1x10^5$ cells) and responder PBMC from donor B ($1x10^5$ cells). A total of $2x10^4$ BM-MSC from healthy donors (n=7) or AA patients (n=7) were added to the MLC, and the proliferative response was determined after 4 days of culture by thymidine incorporation. Dashed gray lines correspond to basal cell proliferation by unstimulated PMBC from donor A ($2x10^5$ cells). *, *P* value <0.001 *versus* MLC without MSC. (B) BM-MSC from AA patients decrease the production of cytokines by activated lymphocytes. BM-MSC ($2x10^4$) were added to allogeneic MLC ($1x10^5$ PBMC from donor A and $1x10^5$ PBMC from donor B). Interleukin-2 (IL2), interferon γ (IFNγ) and tumor necrosis factor-α (TNFα) contents in the supernatants were determined by ELISA after 48 h of culture. Dashed gray lines correspond to basal cytokine production by unstimulated PMBC from donor A ($2x10^5$ cells). *, *P* value <0.001 *versus* MLC without MSC. (C) BM-MSC from AA patients inhibit the inflammatory response in synovial membrane cells (SMC) from patients with rheumatoid arthritis. SMC ($2x10^5$) isolated from three patients with rheumatoid arthritis were stimulated with lipopolysaccharide or with TNFα in the absence (none) or presence of BM-MSC ($1x10^5$) from healthy donors (n=7) or AA patients (n=7). Culture supernatants were assayed for type I collagenase, type IV collagenase and gelatinase activities (after 24 h) or for TNFα and MMP1 contents (after 48 h). *, *P* value <0.001 *versus* SMC alone (none). Dashed gray lines correspond to basal TNFα production and matrix-degrading enzyme activities by unstimulated SMC ($2x10^5$). Data are the mean ± SD of seven experiments performed in triplicate.

dren/young adults with AA.[41,42] Importantly, there are also several degrees of severity of AA. Our patients were diagnosed as having moderate-severe AA while other studies analyzed patients with very severe AA. In brief, further studies involving larger numbers of AA patients are necessary to unravel whether age at diagnosis and disease severity are key factors determining the homeostasis and function of the BM microenvironment in patients with "*de novo*" AA.

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Authorship and Disclosures

Information on authorship, contributions, and financial & other disclosures was provided by the authors and is available with the online version of this article at www.haematologica.org.

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