CHAPTER 7

Phenotypical/functional characterization of \textit{in vitro} expanded mesenchymal stromal cells from Crohn’s disease patients


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Summary

Thanks to their capacity to modulate immune response and promote tissue repair, mesenchymal stromal cells (MSCs) represent a potential novel treatment for autoimmune/inflammatory diseases, including Crohn’s disease (CD). The aim of the study was the in vitro characterization of MSCs from active CD patients (CD-MSCs) for future clinical application. MSCs from bone marrow (BM) of 7 CD-patients (median age 32 years) were expanded ex vivo in the presence of 5% platelet lysate; cells were investigated for clonogenic efficiency, proliferative capacity, morphology, immunophenotype, differentiation potential, genetic stability and ability to suppress in vitro proliferation of both autologous and allogeneic lymphocytes to polyclonal mitogens. Results were compared with those of BM-MSCs of 4 healthy donors (HD).

MSCs were successfully expanded from all patients. Colony-forming unit-fibroblast (CFU-F) frequency and proliferative capacity were comparable in CD- and HD-MSCs. CD-MSCs showed the typical spindle-shaped morphology and differentiated into osteoblasts, adipocytes and chondrocytes. Surface immunological markers did not differ between CD- and HD-MSCs, with the only exception of sizeable levels of HLA-DR at early culture passages (12%-84% at P1) in the former. CD-MSCs ceased their growth at variable passages (from P8 to P25) and entered senescence, without any change in morphology/proliferation rate. Array-Comparative Genomic Hybridization demonstrated that CD-MSCs do not show imbalanced chromosomal rearrangements. CD- and HD-MSCs similarly inhibited in vitro proliferation of lymphocytes to mitogens.

CD-MSCs show biological characteristics similar to HD-MSCs and can be considered for approaches of anti-inflammatory and reparative cell therapy in patients with refractory disease.
Introduction

Within the bone marrow (BM) microenvironment, multipotent stromal cells, also referred to as mesenchymal stromal cells (MSCs), are known to be the precursor cells for stromal tissues that support hematopoiesis.\textsuperscript{1,2} For many years, MSCs have been considered mainly a component of marrow stroma, without any special function, and endowed only with structural support properties. It is now clear that MSCs give a substantial contribution to the creation of the hematopoietic stem cell (HSC) \textit{niche}, and play a crucial role in the development and differentiation of the lympho-hematopoietic system by secreting a number of growth factors and regulatory cytokines, and by promoting cell-to-cell interactions.\textsuperscript{3,5}

After the initial identification in post-natal BM,\textsuperscript{1} MSCs have been isolated from a variety of other human tissues, including muscle connective tissue, adipose tissue, foetal tissues, placenta and umbilical cord blood (UCB).\textsuperscript{6-10} The isolation and characterization of MSCs rely on their adherence to plastic, the ability to differentiate into the various mesenchymal differentiation lineages and on the expression or absence of a number of surface molecules on culture-expanded cells.\textsuperscript{11} MSCs possess unique immunological properties that are displayed on all cells involved in the immune response, including T- and B-lymphocytes, dendritic cells and Natural Killer (NK) cells, as demonstrated by several independent groups both \textit{in vitro} and \textit{in vivo}.\textsuperscript{12-16} Based on these biological and functional properties, MSCs have been already successfully employed in the clinical setting, either to enhance hematopoietic stem cell engraftment\textsuperscript{17,18} or to treat the most severe form of acute graft versus host disease (GvHD), refractory to conventional treatments.\textsuperscript{19,20}

Moreover, MSCs have been demonstrated to display chemotactic ability, to migrate to sites of inflammation and injury,\textsuperscript{21} as well as to secrete paracrine mediators able to reverse acute organ failure.\textsuperscript{22} Indeed, MSC infusions have been successfully used in repairing tissue injury secondary to allogeneic
Based on these findings, MSCs can be considered as a sort of novel treatment for inflammatory diseases, where cell loss is accompanied by local and systemic inflammatory response. Crohn’s disease (CD) is a chronic inflammatory enteropathy, whose prevalence in Western countries has dramatically increased in the last decade, and in which a dysregulation of the immune response towards intestinal bacteria in genetically susceptible individuals plays a pathogenetic role. Despite the large number of therapeutic options available, i.e. anti-inflammatory drugs, antibiotics, immunosuppressant drugs, biological agents and surgical strategies, there is a growing number of CD patients with refractory/recurrent disease. In view of this consideration and of the serious side effects of more aggressive therapies, alternative strategies are needed both to increase the proportion of patients achieving remission and to improve their quality of life. Very recently, the topical implantation of BM-derived MSCs has been demonstrated to be beneficial in the healing process of experimental colitis in rats, confirming the ability of MSCs to modulate immune-responses and to promote tissue repair through their trophic activity. Moreover, a phase I clinical trial for the treatment of perianal fistulas in 4 CD patients with autologous, adipose tissue derived-MSCs has been reported with promising results.

In this respect, we are currently investigating the potential role of autologous BM-derived MSCs as novel, anti-inflammatory cellular therapy to stimulate tissue repair in CD patients. However, no experimental data have been obtained, so far, on the biological and functional characterization of BM-derived MSCs from these patients. The aim of this study, therefore, was to evaluate the feasibility of isolating and expanding ex vivo MSCs from BM of CD patients with active disease, and to carry out a phenotypical and functional characterization of these cells in comparison with BM-MSCs isolated from
healthy subjects. In a perspective of future clinical use, in order to avoid any risk associated with the use of fetal calf serum (FCS), platelet lysate (PL, 5%) was employed as culture supplement to stimulate MSC growth.29

**Materials and methods**

**CD Patients and Healthy Donors**

Peripheral blood (PB) and BM cells were harvested from 7 patients with active CD (5 males, 2 females, median age 32 years, range 18-59) and 4 healthy HSCT donors (2 males, 2 females, median age 33 years, range 16-47), after obtaining written informed consent. The Institutional Review Board of Fondazione IRCCS Policlinico San Matteo Foundation approved the design of this study. Peripheral blood mononuclear cells (PBMCs) were isolated from CD patients and healthy donors (HDs) by means of density gradient centrifugation (Ficoll 1.077 g/ml; Lymphoprep, Nycomed Pharma, Oslo, Norway) and cryopreserved for future experiments. Patient characteristics at time of BM harvest are described in Table 1.
<table>
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<th>Disease duration (years)</th>
<th>Behaviour</th>
<th>Location</th>
<th>CDAI</th>
<th>Therapies</th>
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Table 1. Patients’ clinical features at time of bone marrow harvest
Pt: patient; F: female; M: male; BMI: body mass index; CDAI: Crohn’s disease activity index; MSZ: mesalazine; MTN: Metronidazole; PDN: prednisone; AB: antibiotics; AZT: azathioprine; surg.: surgery.
Disease behaviour and localization are classified according to the Vienna classification (*). Behaviour: B1 nonstricturing, nonpenetrating; B2 stricturing; B3 penetrating; location: L1 terminal ileum; L2 colon; L3 ileocolon; L4 upper gastrointestinal.
A CDAI value $\geq 150$ indicates active disease
Isolation and Culture of BM-derived MSCs
Mononuclear cells were isolated from BM aspirates (20 ml) of CD patients and HDs by density gradient centrifugation (Ficoll 1.077 g/ml; Lymphoprep) and plated in non-coated 75-175 cm² polystyrene culture flasks (Corning Costar, Celbio, Milan, Italy) at a density of 160,000/cm² in complete culture medium: LG-DMEM (Invitrogen, Paisley, PENN) supplemented with 5% PL, and gentamycin 50 µg/ml (Gibco-BRL, Life Technologies, Paisely, UK). PL was prepared as previously described and used as culture supplement for the generation and expansion of MSCs from all BM samples. Cultures were maintained at 37°C in a humidified atmosphere, containing 5% CO₂. After 48-hour adhesion, non-adherent cells were removed and culture medium was replaced twice a week. MSCs were harvested after reaching ≥ 80% confluence, using Trypsin (Sigma-Aldrich, Milano, Italy), and propagated at 4,000 cells/cm² until passage (P) 5. MSCs from 5 CD patients (CD-MSCs) were maintained continuously in culture until reaching senescence. Senescent MSCs were monitored for up to 8 weeks, in order to reveal any change in morphology and/or proliferation rate.

Characterization of ex vivo Expanded MSCs
The colony-forming unit-fibroblast assay (CFU-F) was performed as described previously. CFU-F formation was examined after incubation for 10 days; the clonogenic efficiency was calculated as the number of colonies per 10⁶ BM mononuclear cells seeded. CD-MSCs from all 7 patients and MSCs from the 4 HDs (HD-MSCs) were phenotypically characterized by flow-cytometry; fluorescein isothiocyanate (FITC) or phycoerythrin (PE)-conjugated monoclonal antibodies specific for CD45, CD14, CD34, CD13, CD80, CD31, HLA A-B-C, HLA-DR, CD90 (BD PharMingen, San Diego, CA), CD73, CD105 (Serotec, Kidlington, Oxford, UK) were used. Appropriate, isotype-matched, non-reactive fluorocrome-
conjugated antibodies were employed as controls. Analysis of cell populations was performed by means of direct immunofluorescence with a FACSCanto flow cytometer (BD PharMingen) and data were calculated using FlowJo software (Tree Star, Inc. Ashland, OR).

The osteogenic, adipogenic and chondrogenic differentiation capacity of MSCs was determined at P2 or P3 in 4 CD patients and 3 HDs, as previously described. To detect osteogenic differentiation, cells were stained for calcium deposition with Alzarin Red (Sigma-Aldrich). Adipogenic differentiation was evaluated through the morphological appearance of fat droplets stained with Oil Red O (Sigma-Aldrich). To detect chondrogenic differentiation, cells were stained for proteoglycans Toluidine Blue (Sigma-Aldrich).

In vitro PBMC Proliferation Assay with PHA and OKT3

The proliferation of PBMCs from 6 CD patients and 4 HDs, in response to both phytohemagglutinin (PHA-L; Boehringer, Mannheim, Germany) and anti-CD3 (OKT3; Ortho, Raritan, NJ), in the absence or in the presence of BM-derived MSCs was performed in triplicate in flat-bottom microwells (Corning Costar, Celbio).

In detail, irradiated (30 Gy) autologous or allogeneic MSCs were seeded at MSC:PBMC ratio 1:2, 1:10, 1:20 per well and allowed to attach overnight before adding 10^5 PBMCs per well. PBMCs, in RPMI 1640 medium (Gibco-BRL, Life Technologies) supplemented with 10% FCS (Euroclone, Celbio), were then added with or without PHA (4 μg/ml) and OKT3 (5 ng/ml). After a 3-day incubation at 37°C in a humidified 5% CO₂ atmosphere, ³H-thymidine (³HTdR 0.5 μCi/well; Amersham, Buckinghamshire, UK) incorporation was measured during the last 21 hours by standard procedure. The experiments were performed both in the autologous setting (i.e. CD-PBMCs/CD-MSCs;
HD-PBMCs/HD-MSCs) and in the allogeneic setting (i.e. CD-PBMCs/HD-MSCs; HD-PBMCs/CD-MSCs).

Molecular Karyotyping
Molecular karyotyping was performed by array-comparative genomic hybridization (array-CGH) with the Agilent kit (Human Genome CGH Microarray, Agilent Technologies, Santa Clara, CA). Since the minimum positive call was considered of three consecutive oligomeres with a log₂ ratio different from zero, the theoretical resolution of the 105kb 60mer oligonucleotide platform was of about 40kb. The genetic profile of 4 CD patients was tested before culture (defined time 0 or T₀) using PBMCs, and after in vitro culture on MSCs at P2 or P3 (T₁). CD-MSCs of 2 patients (pt. n. 3 and 4) were also evaluated at later passages (P10 and P13, respectively), after prolonged in vitro culture (T₂). DNA extracted from saliva of the 4 CD patients was used as control DNA to test T₀ cells. PBMC DNA of the analyzed patient was used as control DNA in every experiment on CD-MSCs. DNA was extracted using QIAamp DNA Blood Mini Kit (Qiagen, Milan, Italy) according to the manufacturer’s protocol. Array-CGH experiments were performed according to the manufacturer’s protocol version 5.0. and analyzed through the Agilent scanner and the Feature Extraction software (v9.1). Graphical overview was obtained using the CGH Analytics software (v3.4.27). Quality control parameters for every experiments were valuated using QC metric tool of CGH Analytics Agilent software.

Statistical Analysis
The non parametric Mann-Whitney test for independent samples was performed for the comparison of CFU-F numbers, cumulative cell
counts, inhibition of PBMC proliferation to mitogens. P values less than 0.05 were considered significant.

Results
Characterization of BM-derived MSCs
We have previously demonstrated that, as compared to MSCs cultured in 10% FCS, MSCs of HDs expanded in the presence of 5% PL display comparable morphology, phenotype and differentiation capacity, whereas are superior in terms of clonogenic efficiency and proliferative capacity. Based on these findings, we have chosen 5% PL as culture supplement for the ex vivo isolation and expansion of MSCs from BM of all CD patients and HDs evaluated in this study.

As compared to HD-MSCs, BM-derived MSCs from CD patients showed a similar spindle-shape morphology in culture (see Figure 1A). In addition, when BM samples were assayed for CFU-F frequency after 10-day culture the results were as follows: HD-MSCs (obtained from 4 subjects) showed a median value of 24.5 (range 18-31) CFU-Fs per 10⁶ mononuclear cells plated, whereas the median value for CD-MSCs (obtained from 7 patients) was 21.5 (range 16-29; P=NS) CFU-Fs.

Similar to HD-MSCs, CD-MSCs were able to differentiate into both osteoblasts, adipocytes and chondrocytes, as demonstrated by the histological detection of calcium depositions positive for Alizarin Red (Figure 1B), the morphological appearance of lipid droplets stained with Oil Red O (Figure 1C) and the histological detection of proteoglycans positive for Toluidine Blue, respectively (Figure 1D), respectively.

No differences were observed in terms of proliferative capacity, calculated as cumulative cell counts from P0 to P5, between HD- and CD-MSCs (P=NS; see Figure 1E).
Figure 1. Characterization of Crohn’s disease (CD)-MSCs, as compared to healthy donor (HD)-MSCs, expanded in the presence of platelet lysate (PL, 5%)-additioned medium.

A) Morphology of CD-MSCs from patient n. 2 at P3, as compared to HD-MSCs at P3.

B) Osteogenic differentiation capacity of CD-MSCs from patient n. 4 at P3, as compared to HD-MSCs at P3. The differentiation into osteoblasts is demonstrated by calcium deposition stained with Alzarin Red. Magnification x 20.

C) Adipogenic differentiation capacity of CD-MSCs from patient n. 4 at P3, as compared to HD-MSCs at P3. The differentiation into adipocytes is revealed by the formation of lipid droplets stained with Oil Red O. Magnification x 20.

D) Chondrogenic differentiation capacity of CD-MSCs from patient n. 4 at P3, as compared to HD-MSCs at P3. The differentiation into chondrocytes is demonstrated by deposition of extracellular matrix stained with Toluidine Blue (proteoglycans).

E) Calculated cumulative cell counts from P0 to P5 of CD-MSCs and HD-MSCs cultured in the presence of 5% PL; results are expressed as the mean calculated from data obtained from 7 CD patients and 4 healthy donors, respectively.
Moreover, the median time to reach 80% confluence for all passages from 1 to 5 was 6 days for both HD- and CD-MSCs.

MSCs from 5 CD patients (pt. n. 1, 2, 3, 4 and 6) were propagated for long term in vitro culture. CD-MSCs from pt. n. 1, 2, 3, and 6 ceased their growth at P11, 9, 11 and 8, and entered a senescence phase after 18-, 19-, 20- and 16-week culture, respectively. Patient n. 4 showed a late MSC growth arrest (P25), when the cells entered senescence after 28-week culture. Senescent CD-MSCs were monitored in culture daily for up to 8 weeks, without showing any change in morphology, immunophenotype and/or proliferation rate.

CD-MSCs from all 7 patients expressed high levels (>95% positive cells) of CD90, CD73, CD105, CD13 and HLA A-B-C surface antigens, whereas were negative for CD34, CD45, CD14, CD80, CD31 molecules (see Figure 2), this pattern being similar to that observed in HD-MSCs. However, while in HD-MSCs HLA-DR expression was always below 2% even at early passages (data not shown), CD-MSCs at P1-P3 expressed variably measurable levels of this marker (from 12 to 84% at P1; see Figure 2), which tended to decrease during in vitro culture and completely disappeared after P4.

Figure 2. Immunophenotypic characterization of CD-MSCs from patient n. 1 at P1 by flow cytometry. CD-MSCs stain positive for CD90, CD105, and HLA A-B-C surface antigens, whereas are negative for CD34 and CD45 molecules. Sixty % of CD-MSCs
from patient n. 1 at P1 are positive for HLA-DR. B) Expression of HLA-DR on CD-MSCs from patient n. 1 at P5. Only 2% of CD-MSCs are positive for HLA-DR.

The expression of HLA-DR was monitored throughout culture for all CD patients (see Table 2 for details).

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Table 2. Percentages of HLA-DR expression on *ex-vivo* expanded Crohn’s disease (CD)-MSCs by flow cytometry. CD-MSCs at early passages (P1-P3) express variable levels of HLA-DR which tend to decrease during *in vitro* culture and disappear after P4.

ND: not determined

Effect of MSCs on PBMC proliferation

The effect of CD- and HD-MSCs on the proliferation of PBMCs stimulated with PHA and OKT3 was evaluated in 6 CD patients and 4 HDs. Results are reported in Figure 3.

As far as the autologous setting (*i.e.* CD-PBMCs/CD-MSCs) is concerned, PHA- and OKT3-stimulated proliferation of CD-PBMCs was reduced by up to 61% ± 9.6% (MSC:PBMC ratio 1:10) and 59% ± 8.1% (MSC:PBMC ratio 1:20), respectively, by the addition of CD-MSCs. However, a direct correlation between the number of CD-MSCs added and the degree of inhibition of CD-PBMC proliferation was not found (see Figure 3A and 3B for the residual percentage of response). A comparable degree of inhibition
was observed when PHA- and OKT3-stimulated HD-PBMCs were tested in the presence of HD-MSCs: 44% ± 12.3% at MSC:PBMC ratio 1:10 for PHA (P=NS); 57% ± 11% at MSC:PBMC ratio 1:20 for OKT3 (P=NS, see also Figure 3C and 3D for the residual percentage of proliferation).

When the allogeneic setting was examined (i.e. CD-PBMCs/HD-MSCs), we found that HD-MSCs were comparable to CD-MSCs in the ability to reduce PHA- and OKT3-stimulated proliferation of CD-PBMCs (maximum inhibition for PHA at MSC:PBMC ratio 1:10 = 52.5% ± 21.2%, P=NS; maximum inhibition for OKT3 at MSC:PBMC ratio 1:20 = 45% ± 17%, P=NS). As already shown for the combination CD-PBMCs/CD-MSCs, also in this context a dose-dependent effect of MSC addition was not observed. Figure 3E and 3F report the residual percentage of response.

Figure 3. Residual proliferation of CD patient PBMCs (CD-PBMCs) and healthy donor PBMCs (HD-PBMCs), stimulated with PHA and OKT3, in the presence of CD
patient MSCs (CD-MSCs) and healthy donor MSCs (HD-MSCs). Each bar represents the percentage of proliferation of 100,000 PBMCs in the presence of decreasing numbers of MSCs (MSC:PBMC ratio 1:2; 1:10; 1:20). The cpm values at each cell concentration were normalized to the cpm of PBMCs without MSCs in each experiment. Each bar represents the mean ± SD of multiple experiments (4 to 6 MSC samples, each point being in triplicate). A) CD-PBMCs/CD-MSCs, PHA stimulation; B) CD-PBMCs/CD-MSCs, OKT3 stimulation; C) HD-PBMCs/HD-MSCs, PHA stimulation; D) HD-PBMCs/HD-MSCs, OKT3 stimulation; E) CD-PBMCs/HD-MSCs, PHA stimulation; F) CD-PBMCs/HD-MSCs, OKT3 stimulation.

The condition HD-PBMCs/CD-MSCs was also tested: CD-MSCs induced a reduction of HD-PBMC proliferation comparable to that observed when HD-MSCs were tested in the presence of HD-PBMCs (data not shown). Altogether, we did not find any significant difference between CD- and HD-MSCs in the ability to reduce the proliferation of both autologous and allogeneic PHA- and OKT3-stimulated PBMCs in vitro.

Molecular Karyotyping

To test the genetic profile of CD-MSCs, array-CGH experiments were performed on cells from 4 CD patients before culture (T₀), at early passages (T₁) and after prolonged in vitro culture (T₂; 2 patients). The comparison of results from two or three experiments for each patient (T₀, T₁ and T₂), allowed us to evidence the possible presence of chromosomal micro-unbalances which could be present only in CD-PBMCs (T₀) or both in CD-PBMCs and in ex vivo cultured CD-MSCs (T₁ and T₂). The use of DNA from saliva, as control DNA to test T₀ cells, permitted to exclude copy number variations (CNVs) constitutionally present in the patients’ genome and, therefore, to evidence only acquired chromosomal imbalances. Moreover, the DNA from saliva allowed to detect chromosomal deletions typical of T lymphocytes and, thus, present only in PBMCs, but not in MSCs. Results of array-CGH experiments demonstrated that T₀ cells were devoid of chromosomal imbalances, except for micro-deletions of different sizes in loci that undergo mitotic recombination.
specifically in T cells. These anomalies were not present in CD-MSCs expanded in vitro, which did not show imbalanced chromosomal rearrangements even after long term culture (see Figure 4). In fact, the array-CGH profiles of the repeated experiments from the same patient were perfectly overlapping and no deletions or duplications were present, considering the platform resolution of about 40kb.

Figure 4. Representative array-CGH profiles of chromosome 14 from patients n. 4: A) T0: PBMCs versus saliva; B) T1: MSCs at P3 versus PBMCs; C) The two overlapping experiments; red line applies to PBMCs, blue line to MSCs at P3. D) T2: MSCs at P13 versus PBMCs; E) The three overlapping experiments; red line applies to PBMCs, blue line to MSCs at P3 and green line to MSCs at P13. The first experiment (A) shows the presence of a small deletions in PBMCs in 14q11.2 which involves the TCA (T-cell receptor alpha chain C region) locus. This gene is rearranged specifically in T cells and its organization is similar to an Ig gene, with V, D, J, and C regions. The deletion is present in cellular mosaic, because PBMCs contain all peripheral blood mononuclear cells, a portion of whom is represented by T cells. The somatic deletion of PBMCs is also revealed in experiments on MSCs, where it appears as a duplication, because PBMCs are hybridized as control DNA. The array-CGH profile of CD-MSCs is linear and no unbalanced chromosomal rearrangements are present.
Discussion

Despite the large number of therapeutic options, disease control in CD remains hard to achieve in many patients.\textsuperscript{35} In such patients with refractory disease and in whom progression into an exacerbated form is associated with severe gut fibrosis and formation of strictures and/or fistulas, anti-inflammatory and immunosuppressive therapies, as well as biological agents and surgery, have limited success.\textsuperscript{35} In this context, adult stem cells and, in particular, MSCs are under investigation as a novel cell therapy-based approach that could ameliorate the management of CD patients.\textsuperscript{36,37}

In the present study, we have isolated and propagated in culture MSCs derived from the BM of 7 patients affected by active CD, with the aim of characterizing their in vitro biological and functional properties in view of their possible clinical use in patients refractory to conventional therapies. For this purpose, CD-MSCs have been cultured in the presence of a PL-additioned medium and compared to PL-cultured, BM-derived, MSCs obtained from HDs.

Our data demonstrate that both the isolation and the ex vivo expansion of BM-derived MSCs from CD patients are feasible and that this cell population exhibits similar morphology and differentiation potential into osteoblasts, adipocytes and chondrocytes, as compared to HD-MSCs (Figure 1A-D).

Moreover, CD-MSCs display similar CFU-F ability and proliferative capacity (Figure 1E), as compared to HD-MSCs, guaranteeing the possibility of expanding in vitro sufficient numbers of cells for clinical application.

As regards the immunophenotype, CD-MSCs displayed the panel of surface markers characteristic of MSC, with the only exception of HLA-DR (Figure 2). The consistent expression of this latter antigen in CD-MSCs at early passages (Table 2) might be related to the condition of active disease found in all patients when their BM was harvested, as demonstrated by the activity indexes (CDAI; see Table 1). In fact, the expression of HLA-DR is lost
shortly after *in vitro* culture, namely when cells are grown in a medium devoid of inflammatory signals/cytokines, which, by contrast, are potentially released by the cells involved in the immune/inflammatory response and present in the extracellular milieu of CD-BM. Support to this interpretation is provided by the observation that BM-derived MSCs from healthy volunteers, expanded in the presence of PL, did not show any expression of HLA-DR,\(^2\) this suggesting that the increased expression of HLA-DR is likely to be attributable to the disease status of the patients. It has been previously shown that *in vitro* exposure of MSCs to IFN-gamma, a cytokine known to be involved in inflammatory processes including those of CD,\(^3\) can induce the expression of HLA-DR on MSC cell surface.\(^4\) Moreover, Le Blanc et al.\(^5\) have already demonstrated that HLA-DR positive MSCs display comparable immunomodulatory properties as their counterpart not expressing HLA-DR, thus suggesting that this peculiarity of CD-MSCs should not affect their function and does not preclude their possible use in the clinical setting.

We also demonstrated that CD-MSCs are equally effective, as HD-MSCs, in inhibiting *in vitro* polyclonally-induced proliferation of both autologous (*i.e.* derived from the patients themselves) and allogeneic (*i.e.* derived from healthy subjects) PBMCs (Figure 3). In particular, it is noteworthy that CD-MSCs are able to display the same magnitude of inhibition on CD-PBMCs proliferation, as compared to the effect exerted by HD-MSCs on CD-PBMCs. This finding supports the use of patient-derived MSCs, instead of utilizing third party cells. The use of autologous MSCs offers significant advantages in light of the observations that MSCs can be lysed by both allogeneic T cells\(^6\) and NK cells.\(^7\) In the allogeneic setting, it is conceivable that an HLA-disparity possibly resulting in NK alloreactivity\(^8\) could be a condition where NK cells are particularly prone to kill mesenchymal progenitors. Finding an inhibitory effect of MSCs even in the presence of a low MSC:PBMC ratio suggests also
that a meaningful favorable clinical effect could be obtained even after the infusion of a low number of autologous mesenchymal progenitors.

BM-derived MSCs from CD patients could be cultured long term in vitro, without losing their peculiar morphological and phenotypical characteristics and maintaining a normal genetic asset, as demonstrated by array-CGH experiments (Figure 4). In this study, we used for the first time array-CGH with a higher resolution\(^3\) (about 40kb) to test MSCs, in order to have a deeper and more sophisticated evaluation of the genomic situation of ex vivo expanded cells, even if this approach is not able to unravel balanced chromosomal rearrangements (detectable only by conventional karyotype) and cell mosaicisms lower than 20%.\(^4\) When studying the genetic stability of ex vivo, extensively cultured BM-derived MSCs from HDs, we found that a normal array-CGH profile was associated with the absence of any abnormality in the conventional cytogenetic analysis.\(^3\) Our present results obtained through array-CGH indicate that CD-MSCs do not appear to be susceptible to malignant transformation even after long-term culture, thus rendering these cells suitable for cell-therapy approaches.

Both in animal models and in patients, it has been shown that BM-derived cells play a role in the healing process following intestinal injury and in the regeneration of various cellular components of the mucosa.\(^4-6\) MSCs, through the secretion of soluble factors (such as indoleamine 2,3-dioxygenase, prostaglandin E2, hepatocyte growth factor, etc.) as well as through a direct cell-to-cell contact, have been demonstrated to be able to inhibit T-lymphocyte proliferation to mitogens and allo-antigens, to inhibit cytotoxic T-lymphocytes generation, to influence the secretion of cytokines favouring the anti-inflammatory ones and to promote the differentiation of regulatory T cells.\(^40,47\) Thus, in view of their immunosuppressive properties, as well as of their role in tissue repair and trophism, BM-derived MSCs represent a promising tool in approaches of immunoregulatory and regenerative cell
therapy. Indeed, the potentiality of MSCs in the clinical setting has been already shown both in prevention and in treatment of GvHD occurring after allogeneic HSCT. In particular, a dramatic effect, in terms of complete resolution of the disease, has been observed in many patients suffering from acute GvHD of the gut refractory to conventional therapy. The therapeutic efficacy of BM-derived MSCs on this severe complication of HSCT, as well as the demonstration that MSCs colonize the site of histological injury in patients with gastrointestinal acute GVHD, have suggested their potential use in the treatment of other inflammatory and immune-mediated diseases, including CD. In this regard, recently, the results of a phase-I clinical trial, in which autologous, adipose tissue (AT)-derived MSCs have been used for the treatment of fistulizing CD in 4 patients, have been published. Eight weeks after MSC local infusion, 75% of the fistulas were considered healed and no adverse effect was observed. Based on these encouraging results, a phase-II trial on autologous AT-derived MSCs and a phase-III trial on third-party, BM-derived MSCs (Osiris Therapeutics, Inc. Columbia, MA) in CD patients refractory to conventional therapies, are underway. In this respect, it is worthy to note that ex vivo cultured human AT-derived MSCs have been reported to be prone to undergo spontaneous transformation characterized by significant chromosomal instability, while, as mentioned above, BM-derived MSCs do not. The biological and functional characterization of BM-derived CD-MSCs, which we have demonstrated to be comparable to HD-MSCs, confirm the plasticity of these cells, together with their immunomodulatory and differentiation properties, and provide the experimental background to consider their use as innovative therapeutic strategy in the management of CD patients with refractory disease. Moreover, the bio-safety profile of these cells is sustained not only by the genetic stability of their chromosome asset, but also by the fact that they were generated in the presence of PL as culture supplement. In fact, the utilization
of this reagent for the ex vivo expansion of MSCs allows to avoid the potential risks associated with FCS, such as the possible transmission of zoonoses and the rejection of the transplanted cells due to immune reactions against animal serum proteins,\textsuperscript{53} while providing rapid and efficient expansion.

In summary, this study provides, to our knowledge for the first time, a comprehensive characterization of BM-derived MSCs from CD patients; our results support the potential use of autologous BM-derived MSCs as novel, anti-inflammatory and reparative approach for treating patients with CD refractory to or relapsing after conventional therapies.

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References


