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Immunoregulatory effects on T lymphocytes by human mesenchymal stromal cells isolated from bone marrow, amniotic fluid, and placenta

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Mesenchymal stromal cells (MSCs) are a promising tool in cell therapies because of their multipotent, bystander, and immunomodulatory properties. Although bone marrow represents the main source of MSCs, there remains a need to identify a stem cell source that is safe and easily accessible and yields large numbers of cells without provoking debates over ethics. In this study, MSCs isolated from amniotic fluid and placenta were compared with bone marrow MSCs. Their immunomodulatory properties were studied in total activated T cells (peripheral blood mononuclear cells) stimulated with phytohemagglutinin (PHA-PBMCs). In particular, an in vitro co-culture system was established to study: (i) the effect on T-lymphocyte proliferation; (ii) the presence of T regulatory lymphocytes (Treg); (iii) the immunophenotype of various T subsets (Th1 and Th2 naïve, memory, effector lymphocytes); (iv) cytokine release and master gene expression to verify Th1, Th2, and Th17 polarization; and (v) IDO production. Under all co-culture conditions with PHA-PBMCs and MSCs (independently of tissue origin), data revealed: (i) T proliferation inhibition; (ii) increase in naïve T and decrease in memory T cells; (iii) increase in T regulatory lymphocytes; (iv) strong Th2 polarization associated with increased interleukin-10 and interleukin-4 levels, Th1 inhibition (significant decreases in interleukin-2, tumor necrosis factor-α, interferon-γ, and interleukin-12) and Th17 induction (production of high concentrations of interleukins-6 and -17); (v) indoleamine-2,3-dioxygenase mRNA induction in MSCs co-cultured with PHA-PBMCs. AF-MSCs had a more potent immunomodulatory effect on T cells than BM-MSCs, only slightly higher than that of placenta MSCs. This study indicates that MSCs isolated from fetal tissues may be considered a good alternative to BM-MSCs for clinical applications.

Mesenchymal stromal cells (MSCs) are adult stem cells (SCs) that maintain the capacity to self-renew and have high plasticity. Clonogenic MSCs are a heterogeneous mixture of progenitors, in which a subset population is capable of differentiating not only into mesenchymal tissue cells (tenocytes, skeletal myocytes, stromal cells, adipocytes, osteoblasts, chondrocytes), but also into neurons, astrocytes, and hepatocytes. However, their ability to transdifferentiate into ectodermal and endodermal cells is still controversial. In addition to stemness, MSCs have other intriguing features, which include support of hematopoiesis, tissue remodeling/repair properties, and angiogenesis. Although none of these properties is the main reason for the popularity of MSCs in current medical practice, their unique immunomodulatory property makes them an attractive source for several clinical uses [1].

Among several applications, the possibility of using MSCs in autoimmune, chronic inflammatory and degenerative diseases has led to notable therapeutic uses such as treatment of graft-versus-host disease and autoimmune conditions [2]. These cellular therapy applications might represent new therapeutic approaches with benefits and expectations for patients.

Mesenchymal stromal cells have intermediate levels of HLA major histocompatibility complex (MHC) class I

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molecules, but do not have HLA class II antigens, FAS ligand, and the co-stimulatory molecules [3,4]. Several articles indicate that T-cell proliferation induced with specific antigens or polyclonal mitogens and allogeneic cells is inhibited by MSCs [5]. This capacity is confirmed in vivo also because MSCs are able to control graft-versus-host disease after bone marrow (BM) transplantation [6,7].

The arrest of T cells occurs in the G0/G1 phase of the cell cycle, and T-cell proliferation inhibition is mediated by both autologous and allogeneic MSCs, so this mechanism is not MHC restricted. Moreover, this phenomenon seems to be caused by both cell-to-cell contact [8,9] and humoral factors such as indoleamine-2,3-dioxygenase (IDO), galectin-1, prostaglandin E2, and B7-H [10]. These factors are responsible for inducing decreases in tumor necrosis factor (TNF)-α and interferon (IFN)-γ and an increase in interleukin (IL)-10 secretion from T cells in contact with MSCs [11,12]. MSCs also induce T helper (Th) 2-type lymphocytes and T regulatory (Treg) cell differentiation through HLA-G5 and other molecules [13]. Moreover, MSCs block maturation and activation of antigen presenting cells (APCs) [14], modulate production of cytokines and growth factors by dendritic and T cells [15], and increase the number of Treg cells in a mixed lymphocyte reaction [16].

Although BM is the main source of MSCs, the use of BM-MSCs is not always acceptable because of the invasive harvesting procedure. Moreover, the number of BM-MSCs declines with increasing age [17,18]. MSCs have been isolated from a variety of tissues, including adipose tissue, umbilical cord, Wharton's jelly, placenta, and amnion. These all represent promising sources of MSCs, as they are abundant and easily obtained by noninvasive procedures.

Our previous studies have proven that multipotent MSCs can be isolated from amniotic fluid (AF) [19] and placenta (PL) (data not published) with MSC characteristics as defined by the International Cellular Therapy Society [20]. Interestingly, MSCs derived from these sources exhibit greater proliferative and differentiative potentials than BM-MSCs, most likely because of the early embryologic origin of AF- and PL-MSCs as compared with BM-MSCs. These data support the importance of investigating the properties of AF- and PL-MSCs as an essential prerequisite to allow their clinical use in cell therapy protocols for regenerative medicine.

For this purpose, in our study, we compared the effects of MSCs isolated from AF and PL with those of MSCs isolated from BM on T lymphocytes (Ly) using an in vitro direct co-culture system.

Methods

Isolation and culture of human MSCs

All studies adhered to the Declaration of Helsinki. Human BM, AF, and PL samples were collected after submission of written consent, in accordance with the Ospedale Infantile Regina Margherita-Sant'Anna-Ordine Mauriziano hospitals' ethics committees, which approved collection of the samples.

Bone marrow cells were harvested from the iliac crest of adult or pediatric Caucasian donors who underwent BM collection for a related patient after submitting informed consent. When available, an unfiltered BM collection bag was also used (Baxter Healthcare, Deerfield, IL) and was normally discarded before the BM infusion. The bag was washed three times with 1× phosphate-buffered saline (PBS) (Lonza, Versviers, Belgium), and the cells were collected and washed at 200*g* for 10 min. An aliquot of whole BM was counted and plated directly in T25 or T75 flasks (Becton Dickinson, Franklin Lakes, NJ, USA) at 1×10^4 cells/cm^2.

Amniotic fluid was harvested from women undergoing amniocentesis for routine prenatal diagnosis at 14–16 weeks of pregnancy. AF samples were centrifuged, and the resulting pellets were plated in 25-cm^2 T flasks as previously described [19].

Placenta was collected immediately after elective cesarean section (to avoid contamination with vaginal pathogens) after receiving informed consent. On receipt of the PL, the decidua tissue and amniotic membrane were dissected, and a piece of central PL was harvested from the basal plate. Each piece of PL was rinsed three times with PBS and digested mechanically and enzymatically using the gentle MAC Dissociator (Milthenyi Biotec). After two digestion steps using trypsin (Sigma-Aldrich, Saint Louis, MO) and collagenase (StemCell Technologies, Vancouver, BC, CA) with DNase (Sigma-Aldrich), the cells were plated directly in T75 flasks.

The culture medium used for all sources was α-MEM (Biochrome, Berlin, Germany) supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich), 2 mmol/L L-glutamine (Sigma-Aldrich), penicillin/streptomycin 1X (Euroclone, Pero, MI, Italy). The culture was maintained at 37°C in a 5% CO_2 atmosphere. After 5–7 days, nonadherent cells were removed, and the adherent cells were re-fed every 3–4 days. To expand the isolated cells, the adherent semiconfluent monolayer was detached with trypsin/EDTA (Sigma-Aldrich) for 5 min at 37°C and expanded for several passages until they no longer reached confluence.

Only those cells that met all MSC criteria of the International Cellular Society [20] and that were not senescent were frozen as MSCs in FBS with 10% dimethyl sulfoxide (DMSO, Euroclone) and then thawed immediately before use.

MSC analysis and characterization

Bone marrow, placenta, and amniotic fluid MSCs used for this study were analyzed with respect to viability, immunophenotype, and differential and proliferative potential to verify that the freezing had not altered the characteristics of the MSCs.

To analyze the immunophenotype, flow cytometry analysis was performed on MSCs using the following antibodies: anti-CD90 fluorescein isothiocyanate (FITC), CD73 phycoerythrin (PE), CD34 FITC, CD14 FITC, CD45 FITC (Becton Dickinson, San Jose, CA) and CD105 allophycocyanin (APC) and CD146 APC (Miltenyi Biotec, Bologna, Italy). Details of the cytofluorometric analysis are described below.

To analyze multipotent capacity, MSCs isolated from the different sources were cultured in osteogenic (StemCell Technologies), adipogenic (StemCell Technologies), and chondrogenic (Lonza, Cologne, Germany) media for 21 days, according to the

manufacturer's instructions. Briefly, 5,000 and 10,000 cells, for control samples and differentiation experiments, were seeded in a six-well plate for osteogenic and adipogenic culture conditions, respectively. Osteogenic differentiation was demonstrated by the accumulation of crystalline hydroxapatite on Von Kossa staining, and adipogenic differentiation, by the presence of intracellular lipid vesicles assessed with oil red O. MSC chondrogenic differentiation was achieved as previously described [21] and was evaluated by alcian blue staining, which identifies the presence of hyaluronic acid and sialomucin.

Preparation of human peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMCs) were separated from buffy coats by centrifugation on a Ficoll Histopaque density gradient. The buffy coats were isolated in the Blood Component Production and Validation Center, City of Science and Health of Turin, S. Anna Hospital, from healthy donors, after informed consent, using an automated blood component separator (Compomat G5, Fresenius Kabi, Bad Homburg, Germany). Donors were negative for infectious markers (hepatitis B and C, HIV-1,2, and *Treponema pallidum*) in accordance with Italian laws and European guidelines.

To isolate naïve T cells, the PBMCs were magnetically labeled with CD45RA microbeads (Miltenyi Biotech) according to the manufacturer's instructions. The magnetically labeled CD45RA+ cells were retained on the column and were eluted as the positively selected cell fraction. An aliquot of the isolated cell population was labeled with the CD45RA FITC antibody to check the purity of the separate cell population.

MSC/T-cell co-culture

On the basis of the experiments to be performed, BM-, AF-, and PL-MSCs were plated in 6-, 24-, or 96-well plates or T flasks (25 cm^2) containing total PBMCs from an unrelated donor (the MSC/T-cell ratio was 1:10). To trigger T lymphocytes, PBMCs were stimulated with phytohemagglutinin (PHA, 2.5 μg/mL). The culture groups were: (i) MSCs alone; (ii) unstimulated PBMCs; (iii) PHA-stimulated PBMCs; (iv) co-culture of MSC-T cells with unstimulated PBMCs; (v) co-culture of MSCs with stimulated PBMCs or Th1- or Th2-induced $CD45RA^+$.

To trigger T lymphocytes, PBMCs were stimulated with recombinant human (rh) IL-2 (300 U/mL), or PHA (2.5 μg/mL). To trigger PBMCs, we used anti-CD3 (5 μg/mL), anti-CD28 (1 μg/mL), rhIL-2 (4 ng/mL), rhIL-12 (1 μg/mL), and anti-IL-4 (1 μg/mL). To trigger Th1 or Th2 cells, we used anti-CD3 (5 μg/mL), anti-CD28 (1 μg/mL), rhIL-2 (4 ng/mL), rhIL-4 (1 μg/mL), anti-IFN-γ (1 μg/mL), and PHA (2.5 μg/mL).

After 5 days, co-cultures of the nonadherent cells were harvested and counted for total RNA extraction and molecular, cytofluorometric, and proliferative analyses. The supernatants were collected for analysis of Th1 (IL-2, IL-12, IFN-γ, TNF-α) and Th2 (IL-10, IL-17, IL-4, IL-6) cytokine release performed using enzyme-linked immunosorbent assay (Mabtech, ELISA Assay). MSCs were detached with a scraper and collected for total RNA extraction and molecular analysis.

Proliferation assay

Mesenchymal stromal cells were plated in triplicates, after irradiation at 3000 rad, into 96-well plates at 2×10^4 cells/mL in 100 μL complete α-MEM medium and allowed to adhere to the plate for 24 hours. PBMCs, resuspended at 2×10^5 cells/mL, were added to wells (in 100-μL volumes) containing or lacking MSCs in the presence of the mitogen PHA (2.5 μg/mL). The MSC/PBMC ratio was 1:10. Experiments were performed using three preparations of MSCs from each source with PBMCs from an unrelated donor and one MSC preparation from each source with PBMCs from three additional unrelated donors. Co-cultures without PHA were used as controls. The culture was continued, and [^{3}H]thymidine (1 μCi, 0.037 MBq) was added 4 hours before the end of the 72-hour culture. Cells were harvested and counted using a 1450 Microbeta TriLux apparatus (Perkin Elmer, Boston, MA). T-Cell proliferation was assessed as the incorporated radioactivity in counts per minute (cpm).

Cytofluorometric analysis

Peripheral blood mononuclear cells were characterized using the following monoclonal antibody (mAb) panels: anti-human CD45RA-FITC/CD45RO-PE/CD3-peridinin-chlorophyll protein cyanine 5.5 (PerCP-Cy5.5)/CD8-APC, CD45RA-FITC/CD45RO-PE/CD3-PercP-Cy5.5/CD4-APC, CD62L-FITC/CD27-PE/CD3-PerCP-Cy5.5/CD4-APC/CD8APCCy7, CD45RA-FITC/CD27-PE/CD3-PerCP-Cy5.5/CD4-APC/CD8-APCCy7. All antibodies were from Becton Dickinson.

Briefly, 2×10^5 to 2.5×10^5 cells for each mAb panel were stained with the appropriate amount of antibody for 20 min according to the antibody titration method described by Rustichelli and colleagues [22]. The labeled cells were thoroughly washed with 1× PBS (200*g* for 10 min) and analyzed on a FACSCanto II (Becton Dickinson) with the DIVA software program.

The percentage of positive cells was calculated using the unstained cells as a negative control and used to calculate the absolute number on the basis of the cell number counted after 5 days of co-culture (stimulated PHA-PBMCs + BM-, AF-, and PL-MSCs). Mean fluorescence intensity (MFI) was analyzed on the positive cells.

Treg analysis

For Treg analysis, the co-cultures were performed in 24-well plates using 100,000 PBMCs in each set. The proportion of Treg cells was determined by flow cytometry analysis, in which the cells were labeled with CD4-APC, CD25-PE, and Fox-P3-FITC antibodies using a specific kit (Anti-Human Foxp3 Staining Set FITC, eBioscience). Preliminary experiments were performed on IL-2-stimulated PBMCs, and only the percentage of total $CD4^+/CD25^+/Foxp\text{-}3^+$cells was analyzed without counting the cells in the culture. For T-cell proliferation, the absolute number of Treg cells was evaluated in the co-cultures after counting the absolute number of cells present in the wells after 5 days.

Expression of STAT5B and Foxp3 was also analyzed at a molecular level by real-time polymerase chain reaction (PCR) as described below.

Evaluation of cytokine release with ELISA

The supernatants were collected for Th1 (IL-2, IL-12, IFN-γ, TNF-α) and Th2 (IL-10, IL-17, IL-4, IL-6) cytokine release analysis performed using an ELISA kit coated at home (Mabtech, ELISA Assay). Briefly, 96-well high protein-binding ELISA plates (Nunc) were coated with mAb13A5 diluted, according to the manufacturer's instructions, to 0.5 μg/mL in PBS, pH 7.4, and incubated overnight at 4°C. After being washed (PBS with

0.05% Tween 20), the plate was blocked for 1 hour at room temperature (RT) with a blocking solution (PBS with 0.05% Tween 20 containing 0.1% bovine serum albumin). The standard for each cytokine was prepared by reconstituting the contents of each vial with incubation buffer (PBS with 0.05% Tween 20 containing 0.1% bovine serum albumin) at the indicated dilution range.

Standards and samples were incubated at RT for 2 hours. Five washes were then performed. The plate was then incubated with mAb 39C3–biotin at 1 μg/mL in an incubation buffer for 1 hour at RT, washed five times, and incubated for 1 hour at RT with streptavidin–horseradish peroxidase. At the end of the procedure, an appropriate substrate solution was used, conferring at the reaction a coloring with an intensity (evaluated with a spectrophotometer at a wavelength of 450 nm) directly proportional to the cytokine concentration. Data were expressed as the cytokine concentration in picograms per milliliter.

HPLC analysis of IDO activity

The chromatographic determination of tryptophan (Trp)-to-kynurenine (Kyn) conversion was performed by HPLC assays in the Clinical Biochemistry Laboratory of the University Hospital City of Science and Health of Turin. The supernatants of MSCs alone and of MSC co-cultures were collected after 5 days and frozen at −20°C. Samples were thawed, and 1-mL volumes were deproteinized with 100 μL 30% trichloroacetic acid (Sigma-Aldrich, Italy). A 250-μL volume of supernatant was added to 50 μL of an aqueous solution of 49.4 μmol/L theophylline as an internal standard (IS, Sigma-Aldrich, Italy). Standard stock aqueous solutions (2.47 mmol/L for Kyn and 4.41 mmol/L for Trp, both from Sigma-Aldrich, Italy) were prepared and kept frozen at −80°C. Working standard solutions were made by appropriately diluting standard mixtures.

Separation was achieved on the HP1100 LC system (Agilent Technologies Italia) using a Phenomenex Kynetex C18 100A column (150 × 4.6 mm, 5μ) by isocratic elution in 7 min. The mobile phase consisted of 50 mmol/L acetic-acetate (Sigma-Aldrich, Italy) buffer, pH 4.6, and HPLC-grade methanol (VWR International PBI, Italy) (85:15 v/v) at a flow rate of 0.9 mL/min at 40°C.

Eluates were monitored with a diode array detector (DAD) set at $\lambda = 360$ nm for Kyn and $\lambda = 275$ nm for Trp and the IS. Absorbances at $\lambda = 220$ nm and $\lambda = 302$ nm were also acquired: absorbance ratios were used for the identification and purity assessment of each peak. The sample injection volume was 50 μL.

The transcripts for IDO-1 and IDO-2 on MSCs were also analyzed by real-time PCR (assay IDs: Hs00984148_m1 and Hcg1646605_m1, respectively [Applied Biosystems, Foster City, CA]).

Molecular analysis of co-cultures

Total RNA was extracted from PBMCs and MSCs after 5 days of co-culture using the TRI-Reagent kit (Sigma) according to the manufacturer's protocol. One microgram of total RNA was reverse-transcribed with 5 μL of 10× PCR buffer II, 11 μL of 25 mmol/L $MgCl_2$, 2 μL of 50 U/L reverse transcriptase MuLV (murine Moloney leukemia virus), 1 μL of 20 U/L RNase inhibitor, 5 L of 50 μmol/L random hexamers (Applied Biosystems), 1 μL/L of a mixture of 100 nmol/L dNTPs (Amersham), and double-distilled water in a final volume of 50 μL. The reaction mixture was prepared in a GeneAmp PCR system 9700 Thermal Cycle (Applied Biosystems) under the following conditions: 10 min at 20°C, 45 min at 42°C, and 5 min at 99°C for the enzyme inactivation; the cDNAs were stored at −80°C.

The real-time PCR array was performed to quantify cytokines, chemokines, molecules for antigen uptake, signal transduction, regulators of T-cell activation, and regulators of Th1 and Th2 development and differentiation. The forward and reverse primers (Table 1) and hydrolysis probe were specific to each assay. Five μL of cDNA was added at 15 μL of reaction mixture containing 900 nmol/L forward and reverse primers, 200 nmol/L hydrolysis probe MGB or TAMRA, and 1× Master Mix in a final volume of 20 μL.

We used the Hs00984148_m1 and Hcg1646605_m1 assays and TaqMan Universal PCR Master Mix (Applied Biosystems) to detect the transcripts for IDO-1 and IDO-2, respectively, on MSCs.

Amplifications were performed on the ABI 7500 real-time PCR system (Life Technologies) in a 96-well plate at 95°C for 10 min, followed by 45 cycles at 95°C for 15 s and 60°C for 1 min. Relative quantification of mRNA expression of selected genes was achieved by Taqman amplification. To normalize the PCR results, hypoxanthine phosphoribosyltransferase 1 (HPRT1) was used as a reference gene (assay HPRT1:Hs02800695_m1).

Table 1. Sequences of primers used for real-time polymerase chain reaction

Name	Sequence
IFN	
-Forward	CTAATTATTCGGTAACTGACTTGA
-Reverse	ACAGTTCAGCCATCACTTGGA
Probe	6FAM-TCCAACGCAAAGCAATACATGAAC-TAMRA
Tbet	
-Forward	ACACGCATATCTTTACTTTCCAAGAA
-Reverse	TCAGCTGAGTAATCTCGGCATTC
Probe	6FAM-CCCAGTTCATTGCCGTGACTGCC-TAMRA
GATA3	
-Forward	TTCCCCAAGAACAGCTCGTT
-Reverse	GGCTCAGGGAGGACATGTGT
Probe	6FAM-AACCCGGCCGCCCT-MGB
FOXP3	
-Forward	TCACCTACGCCACGCTCAT
-Reverse	ATTGAGTGTCCGCTGCTTCTC
Probe	6FAMCTGGGCCATCCTGGA-MGB
STAT3	
-Forward	TCCTGGTGTCTCCACTGGTCTA
-Reverse	TTCCGAATGCCTCCTCCTT
Probe	6FAM-CTCTATCCTGACATTCC-MGB
STAT6	
-Forward	TCCATCCCCCCGTATCAA
-Reverse	GGCTCCTGGAAGGCTGACA
Probe	6FAM-CCTCTCCCCAGAAGAATCAGTCAACGTG-TAMRA
STAT5b	
-Forward	TGATTACAGTGGCGAGATCTTGA
-Reverse	GCCTGTGGCTTGGTGGTACT
Probe	6FAM-CAACTGCTGCGTCATG-MGB
RORγ	
-Forward	CGGGCCTACAATGCTGACA
-Reverse	GCCACCGTATTTGCCTTCAA
Probe	6FAM-CCGCACGGTCTTT-MGB

Each sample was run in triplicate. Furthermore, to confirm that there was no DNA genomic contamination, a control PCR was performed with RNA before reverse transcription using the same primers and probe described above. Relative quantification of target gene expression was performed with the $\Delta\Delta Ct$ method.

Statistical analysis

All data obtained in this work were analyzed with Graph PAD Prism statistical software. The statistical tests used to compare the differences between the analyzed groups were chosen on the basis of the number of samples and the distribution of the data. The Kolmogorov–Smirnov test with Dalla–Wilkinson–Lille was used for the first analysis to verify the normality of the data distribution. The differences between paired samples were evaluated with analysis of variance or Friedman's test depending on whether data distribution was normal or not, respectively. Dunn's multiple comparison test in one-way analysis of variance was used to compare the mean ranks of preselected pairs of columns with the mean ranks of a control column (PHA-PBMCs). Bonferroni's multiple comparison test was used to compare each cell mean with the control cell mean on that row.

All statistical tests were considered significant at $p < 0.05$, highly significant at $p < 0.001$, and very highly significant at $p < 0.0001$.

Results

MSC cultures

Frozen BM-, PL-, and AF-MSC samples, stored in liquid nitrogen, were used for this study. We used BM-MSC aliquots from the second to fourth passages and AF-MSCs and PL-MSCs from the second to seventh passages so that the cells were in the exponential phase of their cellular growth. Postthawing cell viability was always >70% in all MSC samples. Two to three days postthawing, nonadherent cells and debris were discarded, and single cells in BM-MSCs or colonies in AF-MSCs and PL-MSCs were left to expand until reaching 70%–80% of confluence.

We observed that the morphology after thawing was similar to that observed in the passage before freezing and maintained the immunophenotype characteristics. BM-, AF-, and PL-MSCs used in this study were negative for hematopoietic antigens and for HLA-DR, and expressed more than 90% of CD90, CD73, CD105, and CD146 (Fig. 1A, C, E). No statistical differences were observed among the three different media in terms of percentage or mean fluorescence in the positive cells.

The MSCs obtained from the three different sources exhibited multipotent capacity as all samples used differentiated into osteoblasts, adipocytes, and chondrocytes (Fig. 1 B, D, F).

MSC/T cell interaction: Proliferation assay

The mean radioactivity in cultures of PBMCs alone was < 1,000 cpm.

Comparison of the mean radioactivity levels of the groups with that of the control group (PHA-PBMCs) revealed that the decrease in proliferation was very highly significant in the presence of BM-MSCs ($p < 0.001$), highly significant in the presence of AF-MSCs ($p < 0.01$), and significant in the presence of PL-MSCs ($p < 0.05$), under the co-culture conditions (Fig. 2). No significant differences were observed between BM-, AF-, and PL-MSCs.

These experiments indicated that MSCs from different sources had a significant inhibitory effect on PHA-PMBCs and an inhibitory, but not statistically significant, effect in induced Th1 effector cells, under all co-culture conditions. The proliferation data obtained on Th2 effector cells were not homogenous and were controversial. For this reason, we focused our experimental study on total PHA-PBMCs.

T-cell subset determination

Multiparameter flow cytometric analysis allowed identification of the following T subsets, based on the antibody combination used:

- Naïve CD8+ T cells: CD45RA+/CD3+/CD8+
- Naïve Th cells: CD45RA+/CD3+/CD4+
- Memory CD8+ T cells: CD45RO+/CD3+/CD8+
- Memory Th cells: CD45RO+/CD3+/CD4+

The percentage obtained by cytofluorometric analysis was used to calculate the absolute number based on the cell number counted after 5 days of co-culture (stimulated PBMCs + BM-, AF-, and PL-MSCs). The gating strategy using for the cytofluorometric analysis is illustrated in Supplementary Figure E1 (online only, available at www.exphem.org)

We observed high variability in naïve and memory T cells among the donors, so the standard deviations of the means were high, but the modulation of the T-cell subsets simultaneously analyzed in the different co-cultures were the same in all experiments. Indeed, in all experiments, the number of memory T cells was always higher than the number of naïve T cells in PHA-PBMCs, and after co-culture with MSCs, independent of the source, this ratio reversed: the naïve T cells increased (Fig. 3A, C), whereas the memory T subpopulation decreased (Fig. 3B, D).

Moreover, we observed that (i) the increase in naïve T cells (CD3+/CD4+/CD45RA+ and CD3+/CD8+/CD45RA), observed in all co-cultures, was statistically significant when PHA-PBMCs were co-cultured with AF- and PL-MSCs (Fig. 3A, C); and (ii) the decrease in memory T cells (CD3+/CD4+/CD45RO+ and CD3+/CD8+/CD45RO+) was statistically significant in co-cultures with PHA-PBMCs and BM- and PL-MSCs (Fig. 3B, D).

We also analyzed CD4+ T-effector memory cell subsets (CD4+/CD45RA–/CD27–/CD62L–) and CD8+ T-effector memory subsets (CD8+/CD45RA–/CD27–/CD62L–) and observed that total memory T cells decreased under all

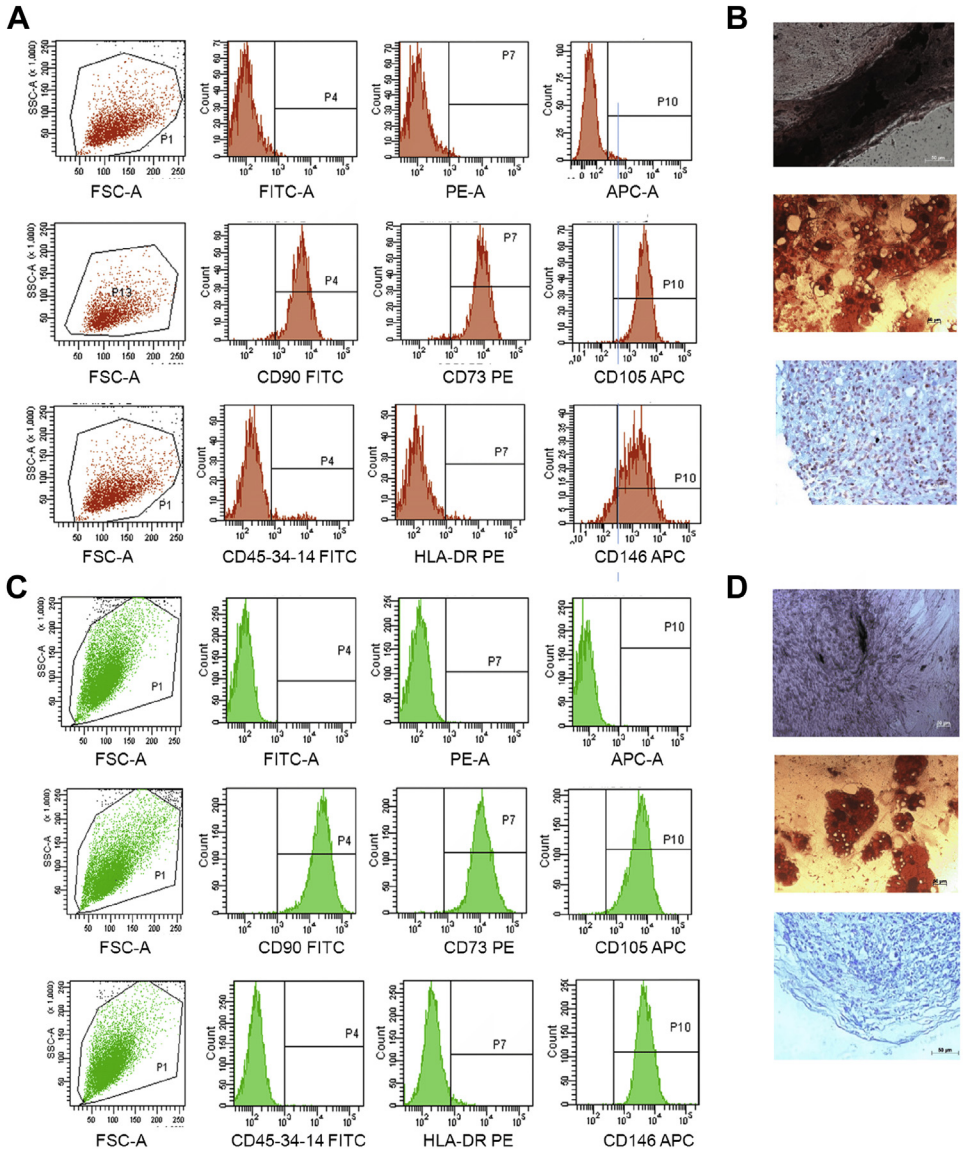

Figure 1. Immunophenotype and differentiative potential of BM-MSCs (**A**, **B**), AF-MSCs (**C**, **D**), and PL-MSCs (**E**, **F**). (**A**–**C**) Flow cytometry histograms of the histotype control in the top row and the characteristic immunophenotype of MSCs in the other rows. No statistical differences between the three sources were observed. (**B**–**F**) Top: Osteogenic differentiation reveals differentiated cells containing mineralized matrices, which were strongly positive for Von Kossa; middle: adipogenic differentiation reveals morphologic changes in the formation of neutral lipid vacuoles containing numerous oil red O-positive lipid droplets; bottom: chondrogenic differentiation reveals the presence of acid mucopolysaccharides and sulfated and carboxylated sialomucins positive for alcian blue.

co-culture conditions, but, interestingly, the Th subset (CD4+) remained unchanged and the CD8+ subset decreased, especially in AF-MSC co-culture (data not shown).

Treg evaluation

Regulatory T cells were evaluated with respect to absolute number either in PBMCs alone under basal conditions and after stimulation with PHA or in co-culture with MSCs isolated from the three sources. An increase in absolute numbers of Treg cells was observed in all co-culture experiments. Also, comparison of the cell means of all experiments with PHA-stimulated PBMCs revealed a significant increases in absolute numbers of Treg cells in co-cultures with AF-MSCs ($p < 0.05$) and PL-MSCs ($p < 0.05$) (Fig. 3E). No significant differences were observed in unstimulated co-cultures.

Cytokine release

Phytohemagglutinin-stimulated PBMCs were used as the control and the range of analyzed cytokines was similar to those reported in literature [23–25]. MSCs constitutively

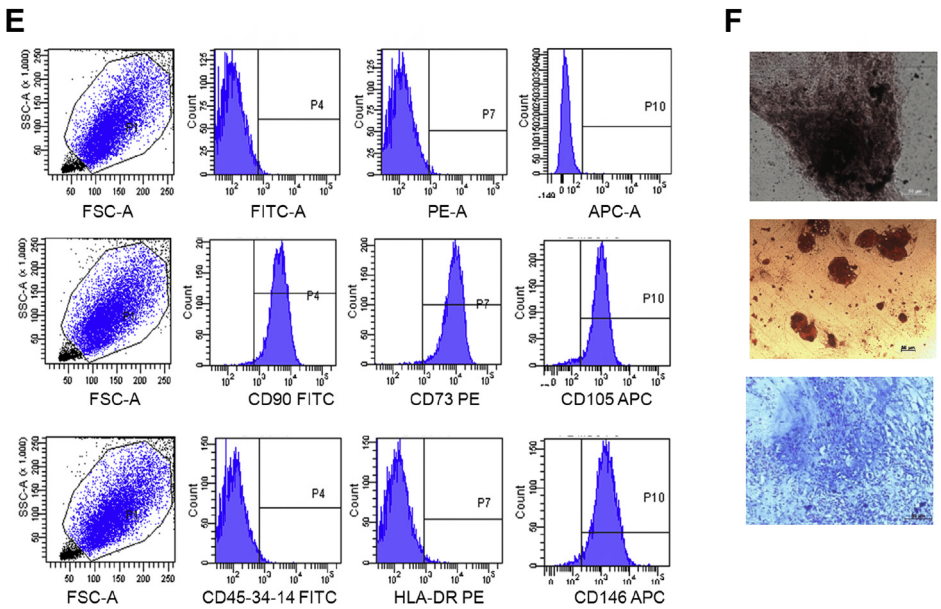

Figure 1. (*continued*)

produced: (i) negligible levels of Th1 cytokines, except IL-12, which was higher than IL-12 levels in PHA-PBMCs; (ii) very low levels of Th2 cytokines; and (iii) significant levels of IL-6 and IL-17.

As also reported elsewhere [19–21], PHA-PBMCs had high levels of IL-2, IL-12, TNF-α, and IFN-γ, which decreased under all co-culture conditions with MSCs. In particular, IL-2 concentrations significantly decreased under all co-culture conditions (Fig. 4A). Bonferroni's multiple comparison test revealed high statistical significance ($p < 0.01$) in two experiments for PHA-PBMCs vs PHA-PBMCs + BM-MSCs or AF-MSCs or PL-MSCs. Interestingly, even though BM-, AF-, and PL-MSCs constitutively produced higher levels of IL-12 than PHA-PBMCs, a decrease in IL-12 was observed in all co-cultures (Fig. 4B). Dunn's multiple comparison test revealed a significant difference in the presence of PL-MSCs ($p < 0.05$). The high level of TNF-α produced by PHA-PBMCs significantly decreased in all MSC co-cultures (Fig. 4C). Dunnett's multiple comparison test revealed significant decreases in TNF-α levels in BM-, AF-, and PL-MSCs ($p < 0.05$ in all samples). IFN-γ also significantly decreased in the co-cultures with MSCs. Dunn's multiple comparison tests revealed a significant difference in co-cultures with AF-MSCs ($p < 0.05$) and PL-MSCs ($p < 0.05$) (Fig. 4D). PHA-PBMCs produced moderate amounts of IL-4 and IL-10, which increased in the presence of MSCs in all co-cultures.

Comparison of the mean rank of each column with the mean rank of PBMCs revealed a significant increase in IL-4 in the co-cultures with AF- and PL-MSCs ($p < 0.05$ and $p < 0.05$, respectively) (Fig. 4E). Similar results were obtained in analysis of IL-10 release (Fig. 4F): the increase in IL-10 was significantly higher in the co-cultures with AF-MSCs ($p < 0.05$) and PL-MSCs ($p < 0.05$) (Dunn's multiple comparison test).

Phytohemagglutinin-stimulated PBMCs produced moderate levels of IL-17 and IL-6, which increased in all co-culture experiments with MSCs (Fig. 4). Interestingly, these two cytokines were produced constitutively by BM-, AF-, and PL-MSCs, and Dunn's multiple comparison test revealed a significant increase ($p < 0.05$) in IL-17 in AF-MSC co-culture (Fig. 4H).

Master gene expression: Th1/Th2/Th17/Treg molecular pathway

It is well known that signature cytokines and master transcription factors play important roles in the differentiation of Th1/Th2/Th17/Treg cells. To elucidate the mechanisms underlying the regulatory effect of MSCs on the Th1/Th2/Th17/Treg paradigm, the mRNA of master transcription factors for Th1/Th2/Th17/Treg cell differentiation (IFN-α, T-bet, GATA-3, STAT-6, RORγ, STAT-3, STAT5b, and Foxp3) was analyzed by real-time PCR in the co-culture of MSCs with unstimulated PBMCs and PHA-PBMCs. Molecular analysis performed after 5 days of co-culture revealed significant polarization versus Th2 and Th17 induction in all experiments in unstimulated PBMCs. The relative quantification calculated on unstimulated PBMCs showed a higher mRNA level of each analyzed transcript in the co-cultures with PL-MSCs vs PBMCs with BM- and AF-MSCs. Expression of GATA-3, STAT-6, RORγt and STAT-3 mRNA increased in unstimulated PBMCs with BM-, PL- and AF-MSCs, whereas expression of IFN-γ and T-bet mRNA decreased. Treg polarization was mixed with STAT5b mRNA expression, which increased, and FOXP3 mRNA expression, which decreased in unstimulated PBMCs with BM-, PL-, and AF-MSCs

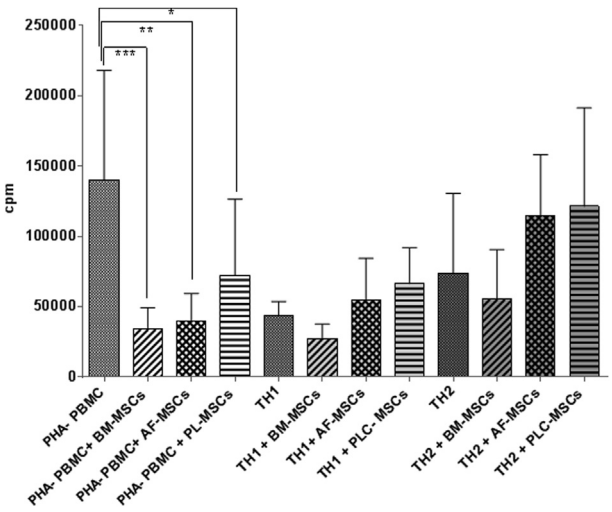

Figure 2. Proliferative assay of stimulated PBMCs and induced Th1 and Th2 cells alone and in co-culture with BM-, AF-, and PL-MSCs. The results reported are averages of five experiments of identical design. T-cell proliferation cultures were performed in triplicate, and the results averaged. Bars represent SD. *$p < 0.05$, significant, **$p < 0.01$, highly significant, and ***$p < 0.001$, very highly significant versus control cultures (PHA-PBMCs) without MSCs.

(Fig. 5A). When we performed the test on PMBCs in presence of PHA, the results were different: Th2 polarization with increased expression of GATA-3 and STAT-6 mRNA was observed only in PHA-PBMCs with BM- and PL-MSCs. PHA-PBMCs with AF-MSCs exhibited only an increase in STAT-6 mRNA expression. Th17 polarization with increased expression of RORγt and STAT-3 mRNA was observed only in PHA-PBMCs with BM-MSCs, whereas PHA-PBMCs with PL-MSCs exhibited only an increase in RORγt mRNA expression. The results for Th1 and Th17 were mixed: In cultures of PHA-PBMCs with BM-MSCs, there was Th1 and Th17 polarization with increased expression of IFN-γ, T-bet, STAT5b and Foxp3 m-RNA; in cultures of PHA-PBMCs with PL- and AF-MSCs only increased expression levels of IFN-γ mRNA were observed; in cultures of PHA-PBMCs with PL-, only increased expression of STAT5b mRNA was observed; and in cultures of PHA-PBMCs with AF-MSCs, there was only increased expression of FOXP3 mRNA.

IDO expression

We analyzed IDO-1 and IDO-2 mRNA expression on MSCs after 5 days of co-culture. We observed that in human MSC cultures, co-cultured PBMCs were responsible for modulation of IDO at both the gene and protein levels. Interestingly, unstimulated BM-MSCs do not express detectable levels of IDO-1 and IDO-2 transcripts (uncalculated *Ct*). However, after treatment with PBMCs, expression of IDO-1 transcripts was observed. In contrast, unstimulated AF- and PL-MSCs already had detectable levels of IDO-1 transcripts, but in this case we observed a stimulatory effect of co-culture quantifiable as 2.3 and 1.3 log for AF and PL, respectively. This increment increases in co-culture with PHA-BPMCs to 2.57 and 1.8 log for AF and PL, respectively. The fold increment in IDO-1 transcripts for MSCs derived from BM was not calculable because the initial level in single cultures was not detectable. Moreover, unstimulated human AF- and PL-MSCs did not express detectable levels of IDO-2 transcripts. However, after 5 days of co-culture with PBMCs, expression of IDO-2 transcripts was observed only in MSCs derived from PL. In contrast, we observed a stimulatory effect of co-culture with PHA-PBMCs. This increment was 0.77 log for PL and undetectable for BM and AF.

To verify that IDO-1 and IDO-2 mRNA expression correlated with its activity, we also determined IDO activity by quantifying conversion of Trp to Kyn under the same experimental conditions. Untreated BM-, AF-, and PL-MSCs produced negligible amounts of Kyn, but co-culture with PBMCs and PHA-PBMCs induced an increase in Kyn and total abolition of the tryptophan in the culture. In particular, we observed mean Kyn values of 11.71 ± 13.4, 5.12 ± 6.83, and 10.63 ± 4.64 mmol/L in co-cultures of unstimulated PBMCs and 10.30 ± 9.0, 12.08 ± 9.6, and 7.0 ± 3.9 mmol/L in co-cultures of PHA-PBMCs with BM-, AF-, and PL-MSCs, respectively. No statistical differences were observed among the three MSC sources, although AF-MSCs produced lower levels of Kyn.

Discussion

Mesenchymal stromal cells represent a promising tool for cell therapies in regenerative medicine because of their multipotent, bystander, and immunomodulatory properties. An increasing number of phase I, phase II, and phase I/II studies using MSCs are in progress for a range of therapeutic applications. Most MSCs used in these clinical trials are isolated from BM and are considered safe and efficacious for their multipotent and immunomodulatory properties (www.clinicaltrials.gov). However, the clinical application of BM-derived cells is limited by the relatively invasive procedure required for sample collection, the difficulty involved in obtaining a sufficient number of MSCs to perform studies, and the marked reduction in cell number, proliferation, and differentiation capacity with age [18]. Therefore, there remains a need to identify a SC source that is safe and easily accessible, yields large numbers of cells, and for which cell procurement does not provoke debates over ethics. We isolated multipotent SCs from AF [26] that showed greater proliferative and differentiative potential than BM-MSCs. We also isolated multipotent SCs, with characteristics similar to those of AF-MSCs, from term placentas (data not published).

For this study, BM-, AF-, and PL-MSC aliquots were thawed and (i) their immunophenotypic, proliferative, and

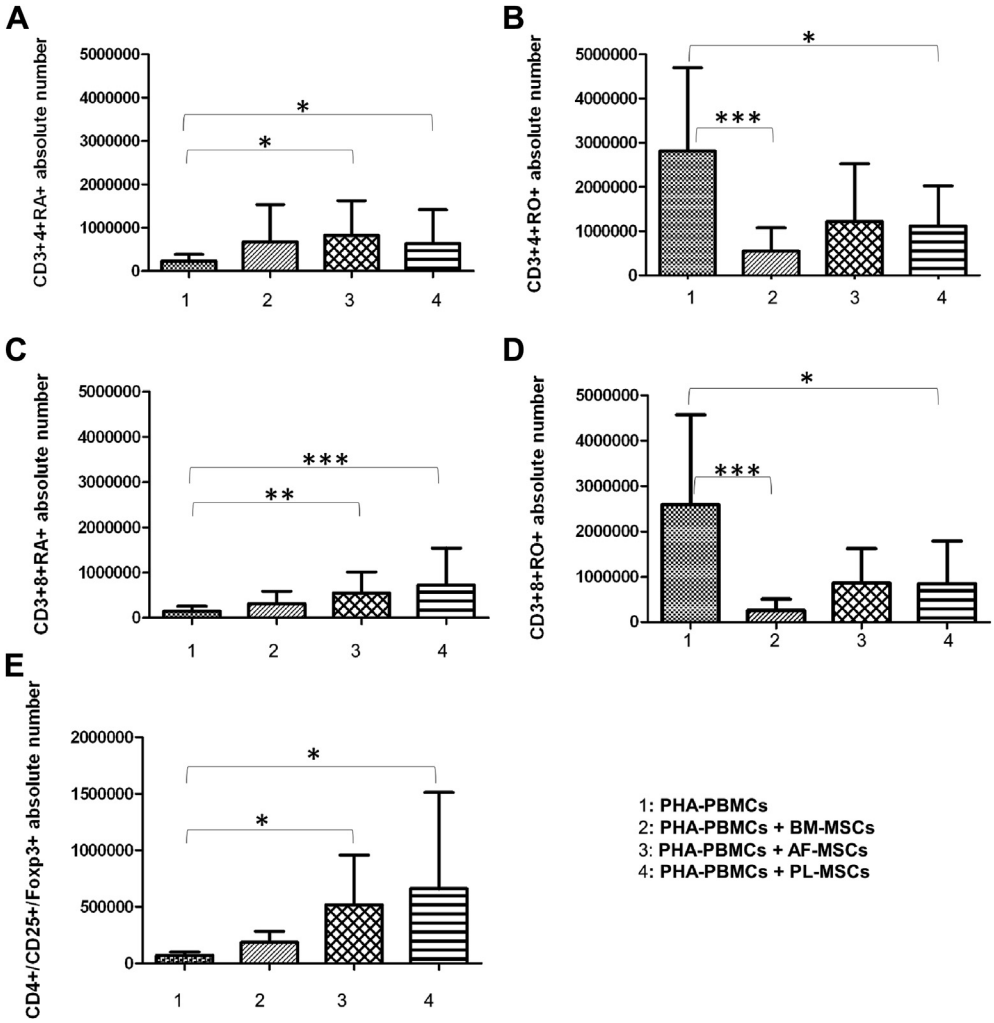

Figure 3. Naïve and memory T and Treg subsets in PBMCs alone and in co-culture with BM-, AF-, and PL-MSCs. In PHA-PBMCs, memory T cells (**B,D**) were higher than naïve Th cells (**A,C**), and after co-culture, regardless of the source, this number was seen to reverse in favor of naïve T cells, especially CD8+ naïve Th subsets (**A,C**). The CD4+ and CD8+ naïve Th subset increased in all co-culture conditions (**A,C**), whereas the CD4+ and CD8+ memory T subpopulation decreased (**B,D**). In PHA-PBMCs with AF- and PL-MSCs, the Treg absolute number of Treg cells was significantly higher (**E**). Bars represent SD. Asterisks *, **, *** indicate statistically significant differences: respectively $p < 0.05$, $p < 0.01$ and $p < 0.001$.

differentiative characteristics were not modified by cryopreservation; and (ii) their immunomodulatory effects on T cells of healthy donors were compared. We found that MSCs isolated from the three different sources are multipotent SCs with the immunophenotypic characteristics and differentiative potential established by guidelines by the Cellular Therapy Society [20], even though MSCs derived from fetal tissues have a greater proliferative capacity associated with the expression of embryonic markers [27].

The ability to modulate the alloreactive immune response has been documented for MSCs derived from human BM, and to our knowledge, there are no studies comparing BM-MSCs with AF- and PL-MSCs, although single articles, with controversial results, describe the immunomodulatory properties of AF-MSCs [28,29] and PL-MSCs [30–32].

Because T cells are the primary cells in adaptive immune response, we evaluated and compared the inhibitory effects of MSCs on PHA-PBMC. A T-cell proliferation assay revealed inhibitory effects on PHA-stimulated PMBCs under all co-culture conditions. We also tested the effects of MSCs on naïve T cells induced to differentiate in Th1 and Th2 effector cells, but in these experiments, different modulations of proliferative activity, were observed. However, these results suggest that T-cell inhibition might be closely related to an interaction of these cells with other cells from innate immunity (such as dendritic and NK cells) not present in co-cultures with Th1- and Th2- induced cells alone.

There was greater inhibition of T-cell proliferation in co-cultures with BM-MSCs, but the absolute numbers of Treg cells, under the same experimental conditions, were

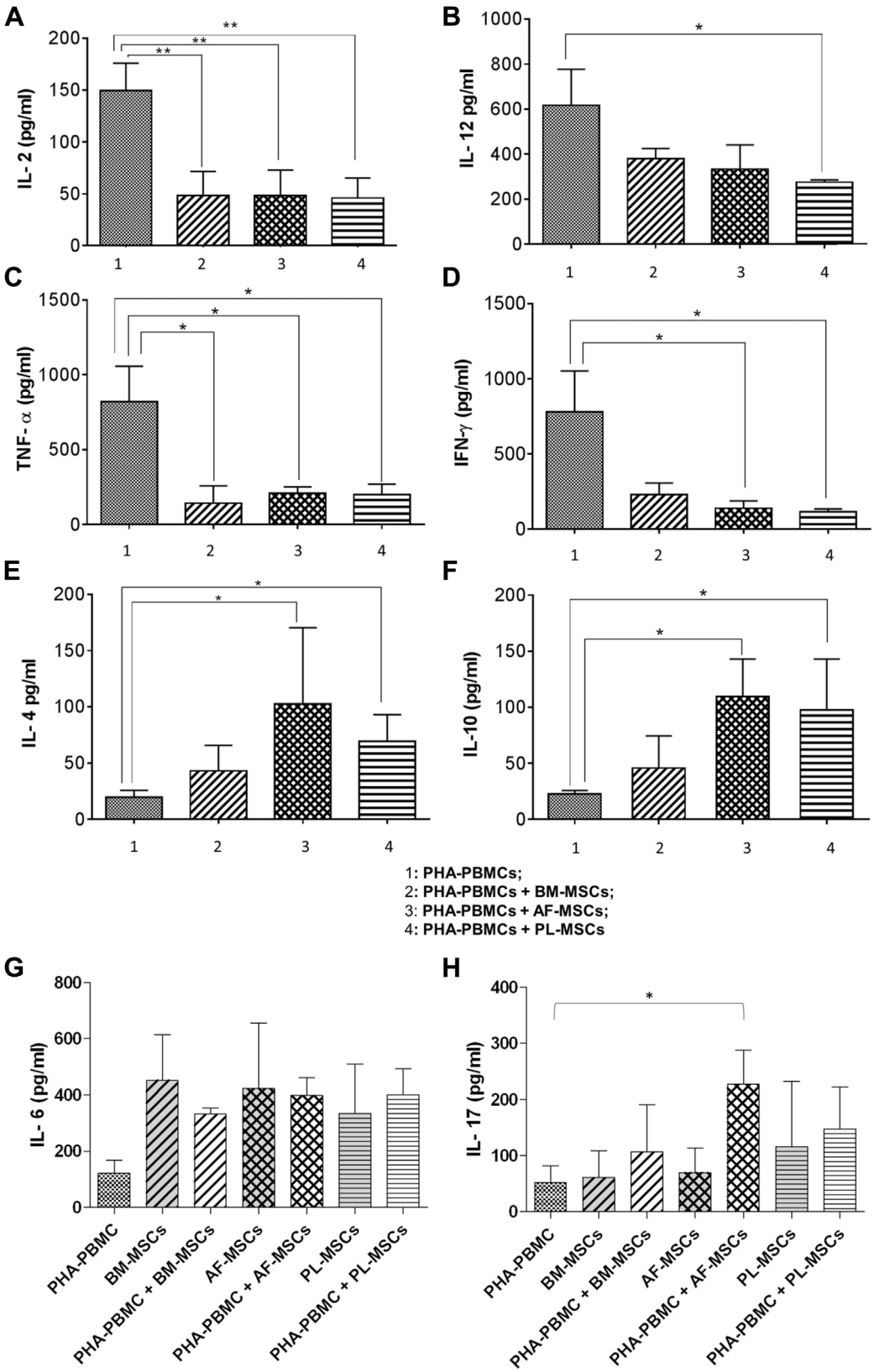

Figure 4. Cytokine release. Release of IL-2 (**A**), IL-12 (**B**), TNF-α (**C**), IFN-γ (**D**), IL-4 (**E**), IL-10 (**F**), IL-6 (**G**), and IL-17 (**H**) detected on PHA-PBMCs and PHA-PBMCs co-cultivated with BM-, AF-, and PL-MSCs. Each column represents the mean and SD of five experiments. * and ** indicate respectively significant ($p < 0.05$) and highly significant ($p < 0.01$) differences versus control cultures (PHA-PBMCs) without MSCs.

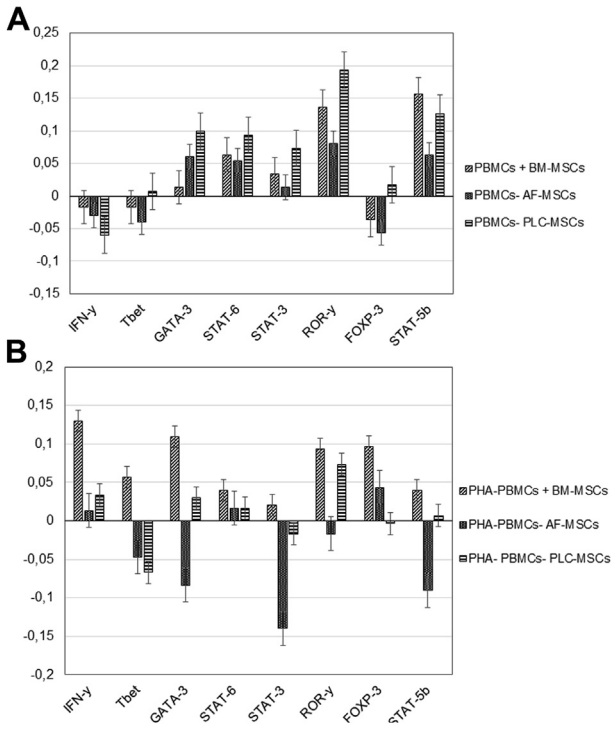

Figure 5. Master gene expression: Th1, Th2, Th17, Treg molecular pathway. mRNA expression of IFN-γ Tbet, and GATA-3 in (**A**) unstimulated PBMCs and (**B**) PHA-PBMCs with BM-, AF and PL-MSCs. The arbitrary unit was calculated as *Ct*(housekeeping gene MSCs)/*Ct*(target gene MSCs) – *Ct*(housekeeping gene PBMCs)/*Ct*(target gene PBMCs).

significantly higher in co-cultures with AF- and PL-MSCs than in those with BM-MSCs. Moreover, analysis of the various T subsets revealed a statistically significant increase in naïve T cells in the co-cultures of PHA-PBMCs with AF- and PL-MSCs and a decrease in memory T cells in the co-cultures with BM- and PL-MSCs. Also, the presence of MSCs induced a reversal of the ratio (compared with PHA-PBMCs) of these subsets in the co-cultures in favor of both $CD4^+$ and $CD8^+$ naïve T-cell subpopulations.

The beneficial effects of MSCs in cell therapy can be explained by the paracrine action of secreted cytokines. For this reason, major cytokines associated with pro-inflammatory and anti-inflammatory functions were analyzed and compared in our experiments. We observed a distinctly high concentration of IL-12 in MSCs, especially in BM- and PL-MSCs. Independent of their origin, MSCs also produced a moderate concentration of TNF-α and negligible amounts of IL-2 and IFN-γ. PHA-PBMCs had high levels of Th1 cytokines, as just reported [23–25]. In all co-culture experiments, we observed decreases in all Th1 cytokines. The MSC/T-cell interaction might block Th1 polarization because this phenomenon was also observed for IL-12 produced in high concentrations by MSCs. It is interesting to note that IL-4 and IL-10, the major anti-inflammatory cytokines, significantly increased in co-cultures with AF- and PL-MSCs. The same effect was observed for Th17 cytokines. As the MSCs, independent of their sources, produced high levels of both IL-6 and IL-17, it is difficult to interpret the significant increases in these cytokines in the co-culture of PHA-PBMCs with AF-MSCs. IL-6 is a well-known immune modulator that also inhibits apoptosis in antigen-stimulated [33] and resting T cells by sustaining the expression of the anti-apoptotic molecule Bcl-2 [34]. The inhibition of IL-6 produced by MSCs results in an additional decrease in the proliferation of activated T lymphocytes in vitro in co-cultures with MSCs [35] and increases apoptosis in neutrophils [36]. IL-17 is a cytokine that has attracted attention because of its involvement in chronic inflammation, having a critical role in the pathogenesis of such autoimmune diseases as rheumatoid arthritis, psoriasis, inflammatory bowel disease, diabetes, and multiple sclerosis [37]. Moreover, Guo et al. reported that fetal BM-MSCs promote the expansion of human Th17 cells, but still inhibit the production of Th1 cells in an in vitro assay using $CD4^+$ T cells stimulated with PHA and recombinant IL-2 [38]. Moreover, a recent work identified a new subset of IL-17+ MSCs capable of inhibiting *Candida albicans* growth and attenuating cell-based immunosuppression [39]. The authors described IL-17^+ MSCs distinct from the bulk MSC population, which were unable to upregulate Treg or downregulate Th17 cells, suggesting that IL-17 production in MSCs directly impairs MSC-based immunomodulatory functions.

The molecular data indicated that co-culture of unstimulated PBMCs with BM-, AF-, and PL-MSCs promoted expression of the signature cytokines and master transcription factors directed to Th2 and Th17 cell differentiation, but inhibited cytokines and master transcription factors directed to Th1 differentiation. The results of Treg polarization were mixed: co-culture of unstimulated PBMCs with PL-MSCs seems directed to the differentiation of Treg with upregulation of Foxp3 and STAT5b mRNA transcription levels; culture of unstimulated PBMCs with BM- and AF-MSCs caused upregulation of STAT5b but down-regulation of FOXP3. The data indicated that the results for co-culture of PHA-PBMCs with BM-, PL-, and AF-MSCs were mixed with respect to the role of the MSC effect of signature cytokines and master transcription factors directed to the differentiation of Th1/Th2/Th17 and Treg cells, probably because of a confounding effect of PHA stimulus. Moreover, we have to consider, as demonstrated by Fan et al, that the highest expression of mRNA encoding IL-2, IL-6, IL-10, TNF-α, and IFN-γ occurred almost at the same time (an average of 8 hours) after PHA-stimulation [40]. On the basis of this observation, the mRNA levels detected after 5 days of co-culture with MSCs might be inversely related to protein production. These results confirmed the detection of high, intermediate, and almost no mRNA in co-cultures of PHA-PBMCs with BM-, PL-, and AF-MSCs, respectively, which reflected data obtained in protein detection.

All of these data, in line with other authors [41], indicate that MSCs inhibit or limit inflammatory responses and promote the mitigating and anti-inflammatory pathway with an increase in Treg cells. The inhibition occurs only on Th1 cells, leading to a paradoxical increase in pro-inflammatory Th17 cells. As already suggested by other authors, a mechanism that might explain the late stimulating effect of MSCs on pro-inflammatory Th17 cells is the upregulation of IL-6 levels in the cultures, as IL-6 is a main mediator of Th17 cell differentiation [35,38,42]. Furthermore, in agreement with the results in this study, IL-6 might also inhibit differentiation of the Th1 subset [35]. The increase of Treg cells might also be induced by the high concentration of IL-10 released. However, from these studies we cannot draw conclusions on the separate roles of the different cytokines in mediating inhibition either directly or through induction of Treg cells (e.g., by IL-10).

In addition, IL-4 and IL-10 are indicative of a Th2-deviated immune response and might be produced by a cellular compartment other than the T cells in PBMCs. It is therefore important to investigate the role of MSCs in contact with dendritic cells. Our preliminary results indicate that AF-MSCs block dendritic cell maturation to a greater extent than BM- and PL-MSCs.

Luo et al. recently reported that PBMC proliferation was suppressed by AF-MSCs in a dose-dependent manner and the inhibitory effect was caused by increased induction of IL-10 and IDO after co-culture [43]. Moreover, IDO was also considered a key mediator of the immunosuppressive effect of PL-MSCs. For this study, IDO-1 mRNA and IDO-2 mRNA were undetectable in basal MSCs (independent of the source), but were present in significantly higher concentrations in co-cultures with PBMCs alone and in PHA-PBMCs. PL-MSCs produced larger amounts of IDO-1 and IDO-2 mRNA.

Conclusions

Analysis of the properties and effects of MSCs on T cells revealed that AF-MSCs have a more potent immunomodulatory effect on T cells than BM-MSCs and only a slightly greater effect than PL-MSCs. This study indicates that MSCs isolated from fetal tissues may be considered a good alternative to BM-MSCs for clinical applications, as recently reported by Fierabracci et al. [44] and in Ullah et al. [45]. The clinical trials website (www.clinicaltrials.gov) reports about 30 trials based on the use of "cells derived from placenta," but most of these studies have an unknown status. We believe that safety should be the main focus in cell therapy and stem cell research today and that this also concerns MSCs isolated from fetal tissues. For this reason, further studies are needed to provide a complete understanding of the mechanisms underlying the immunomodulatory effects of AF- and PL-MSCs, which will ultimately allow the development of new and more effective strategies for regenerative medicine and transplantation to treat a wide range of conditions.

Another fact that should not be overlooked is that AF-MSCs are isolated from AF harvested from women undergoing amniocentesis, which, although considered routine, is still seen as an invasive procedure used for prenatal diagnosis at 14–16 weeks of pregnancy. Moreover, we isolated AF-MSC clones only from the most abundant samples that contained at least 6 mL of AF [19]. In all other samples, we observed heterogeneous clones also presenting epithelial characteristics, making it difficult to establish a standard MSC isolation and expansion protocol for AF. On the other hand, although the method of isolating MSCs from placenta could be critical [46], placenta is a more abundant discharged fetal tissue compared with AF and might well be considered an excellent source of MSCs without any ethics problems and, thus, a good alternative to BM-MSCs for clinical applications.

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Conflict of interest disclosure

All the authors have reported no conflicts of interest and no competing financial interests exist.

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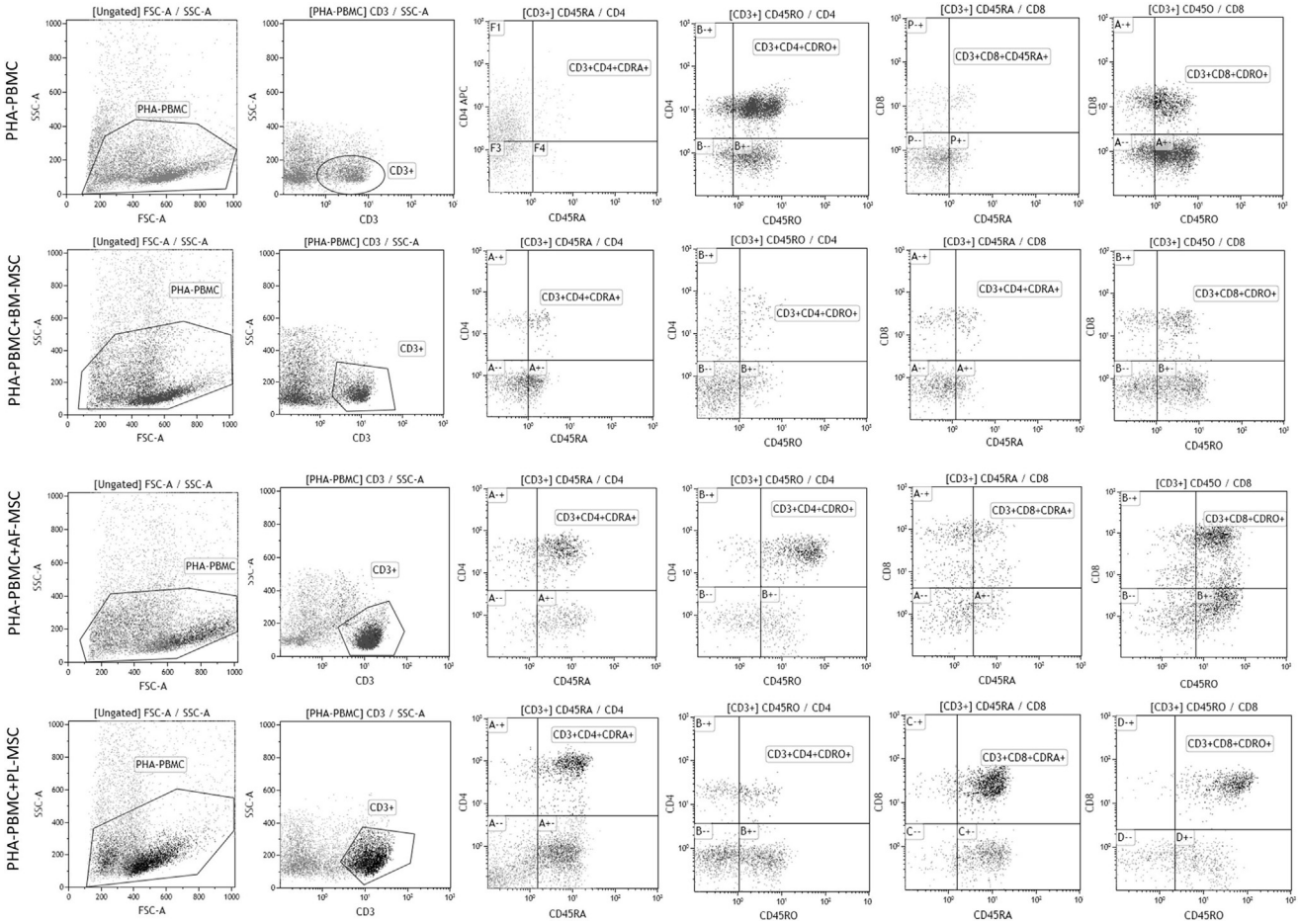

Supplementary Figure E1. Representative dotplots during cytofluorometric analysis illustrate the gating strategy used to evaluate T-cell subsets in PHA-PBMCs alone (upper row) and in co-culture with BM-, AF-, and PL-MSCs. As illustrated on the upper side of each dot plot, we obtained the total percentages of CD3+/CD4+/CD45RA+, CD3+/CD4+/CD45RO+, CD3+/CD8+/CD45RA+, and CD3+/CD8+/CD45RO+. We calculated this percentage from the total number of cells counted after 5 days of co-culture.