

## Adipose-Derived Mesenchymal Stem Cells Exert Antiinflammatory Effects on Chondrocytes and Synoviocytes From Osteoarthritis Patients Through Prostaglandin E<sub>2</sub>

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**Objective.** To examine the effect of different sources of Good Manufacturing Practice clinical grade adipose-derived mesenchymal stem cells (AD-MSCs) on inflammatory factors in osteoarthritic (OA) chondrocytes and synoviocytes.

**Methods.** AD-MSCs from infrapatellar Hoffa fat, subcutaneous (SC) hip fat, and SC abdominal fat were cocultured in Transwells with chondrocytes or synoviocytes. Inflammatory factors (interleukin-1 $\beta$  [IL-1 $\beta$ ], tumor necrosis factor  $\alpha$ , IL-6, CXCL1/growth-related oncogene  $\alpha$ , CXCL8/IL-8, CCL2/monocyte chemoattractant protein 1, CCL3/macrophage inflammatory protein 1 $\alpha$ , and CCL5/RANTES) were evaluated by quantitative reverse transcription–polymerase chain reaction or multiplex bead–based immunoassay. The role of different immunomodulators was analyzed.

**Results.** All the inflammatory factors analyzed were down-modulated at the messenger RNA or protein level independently by all 3 AD-MSC sources or by allogeneic AD-MSCs used in coculture with chondro-

cytes or synoviocytes. Inflammatory factor down-modulation was observed only when AD-MSCs were cocultured with chondrocytes or synoviocytes that produced high levels of inflammatory factors, but no effect was observed in cells that produced low levels of those factors, thus highlighting a dependence of the AD-MSC effect on existing inflammation. The immunomodulators IL-10, IL-1 receptor antagonist, fibroblast growth factor 2, indoleamine 2,3-dioxygenase 1, and galectin 1 were not involved in AD-MSC effects, whereas the cyclooxygenase 2 (COX-2)/prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) pathway exerted a role in the mechanism of antiinflammatory AD-MSC action.

**Conclusion.** The antiinflammatory effects of AD-MSCs are probably not dependent on AD-MSC adipose tissue sources and donors but rather on the inflammatory status of OA chondrocytes and synoviocytes. AD-MSCs seem to be able to sense and respond to the local environment. Even though a combination of different molecules may be involved in AD-MSC effects, the COX-2/PGE<sub>2</sub> pathway may play a role, suggesting that AD-MSCs may be useful for therapies in osteoarticular diseases.

Osteoarthritis (OA) is a chronic age-related disease of the “whole joint” characterized by the slowly progressive destruction of articular cartilage accompanied by changes to synovium and subchondral bone, degeneration of ligaments and menisci, and hypertrophy of the joint capsule (1). In vivo and in vitro studies indicate that proinflammatory cytokines (interleukin-1 $\beta$  [IL-1 $\beta$ ], tumor necrosis factor  $\alpha$  [TNF $\alpha$ ], and IL-6) and chemokines (CXCL1/growth-related oncogene  $\alpha$  [GRO $\alpha$ ], CXCL8/IL-8, CCL2/monocyte chemoattractant protein 1 [MCP-1], and CCL5/RANTES) produced by synoviocytes and chondrocytes, as well as cells from

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other joint tissues, can be measured in the synovial fluids of OA patients and contribute to the disruption of the balance between anabolism and catabolism (2–4). Existing drug therapies for OA provide, at best, symptomatic relief from pain and fail to prevent cartilage damage and subsequent destruction of other joint tissues (5).

Human mesenchymal stem cells (MSCs) have been identified in and can be isolated from a large number of adult tissues, including bone marrow, adipose tissue, and umbilical cord blood (6–8). MSCs have generated considerable medical interest since they have the potential to differentiate into numerous tissues (bone, cartilage, and fat) (9). Recent observations have shown that they also have tissue-regenerative properties, mainly via secretion of bioactive trophic factors that exert potent immunomodulatory, proangiogenic, anti-apoptotic, antifibrotic, and antiinflammatory effects (10–13). A clear therapeutic potential of MSCs in alloimmunity, autoimmunity, and inflammation has also been found in a high number of clinical trials (14–17).

In osteoarticular diseases, preclinical tests in animal models have also suggested that MSCs may lead to the development of innovative applications for the treatment of these diseases (18). In particular, it has been shown that intraarticular injections of bone marrow-derived MSCs (BM-MSCs) or their delivery on a degradable hyaluronan scaffold are effective in preventing the evolution of OA (19–21) in animal models. Recently, it has also been shown that intraarticular injections of adipose-derived MSCs (AD-MSCs) in OA mouse and rabbit models exert antiinflammatory and chondroprotective effects (22,23).

AD-MSCs are an interesting alternative source that share many properties with MSCs (7,24,25). They can be more easily collected by liposuction, and their proportion is several orders of magnitude higher than that of MSCs (26,27).

Because it is conceivable that soluble mediators produced by AD-MSCs might play a role in local inflammatory processes in the joint, we performed an extensive *in vitro* analysis of the effects of AD-MSCs in coculture with human OA chondrocytes and synovio-cytes. First, we analyzed different inflammatory factors (IL-1 $\beta$ , TNF $\alpha$ , IL-6, CXCL1/GRO $\alpha$ , CXCL8/IL-8, CCL2/MCP-1, CCL3/macrophage inflammatory protein 1 $\alpha$  [MIP-1 $\alpha$ ], and CCL5/RANTES) in cell monoculture and coculture. Then, we examined whether the effects observed were dependent on AD-MSC sources, patients, and the degree of inflammation of chondrocytes and synovio-cytes. Finally, we evaluated possible molecules involved in AD-MSC effects.

## MATERIALS AND METHODS

**Specimens.** Articular cartilage and synovia were harvested from the femoral condyles of 19 patients undergoing total knee replacement (12 women and 7 men, mean  $\pm$  SD age 72  $\pm$  10.6 years, mean  $\pm$  SD body mass index 28  $\pm$  2.01 kg/m<sup>2</sup>, mean  $\pm$  SD disease duration 5  $\pm$  2.83 years, Kellgren/Lawrence grade 3/4 [28]). Infrapatellar Hoffa fat and subcutaneous (SC) hip fat were obtained from OA patients, and SC abdominal fat was obtained from patients undergoing liposuction. The study was approved by the local ethics committee, and all patients provided their informed consent.

**Isolation and characterization of human AD-MSC cultures.** Clinical grade AD-MSCs were isolated from infrapatellar Hoffa fat, SC hip fat, and SC abdominal fat according to Good Manufacturing Practice as described (29). Briefly, fat was digested with collagenase at 37°C for 45 minutes and centrifuged at 600g for 10 minutes. The stromal vascular fraction was transferred into a CellStack chamber (Corning) at a density of 4,000 cells/cm<sup>2</sup> in  $\alpha$ -minimum essential medium supplemented with platelet lysate. On day 8, the cells were trypsinized and expanded at 2,000 cells/cm<sup>2</sup> until day 14. Cell counting and viability testing were performed using trypan blue exclusion dye on days 8 and 14. Phenotyping was carried out by flow cytometric analysis for the CD markers CD14, CD34, CD45, CD73, CD90 (BD PharMingen), and CD13 (eBioscience).

**Isolation and culture of chondrocytes and synovio-cytes.** Chondrocytes were isolated following a standardized procedure (30). Briefly, minced articular cartilage was digested at 37°C with 0.5% Pronase (Sigma) for 1 hour and 0.2% collagenase (Sigma) for 45 minutes in Dulbecco's modified Eagle's medium (DMEM; Sigma) with 25 mM HEPES (Sigma), 100 units/ml penicillin, 100  $\mu$ g/ml streptomycin (both from Life Technologies Italia), and 50  $\mu$ g/ml gentamicin (Life Technologies). The cells were seeded at a density of 25,000 cells/cm<sup>2</sup> and cultured at 37°C in 5% CO<sub>2</sub> in complete DMEM with 10% fetal calf serum (FCS).

Synovio-cytes were isolated from synovial tissue as previously reported (31). Briefly, the synovium was dissected from the underlying connective tissue, and minced fragments were cultured in 10 ml Optimem-1 (Life Technologies) supplemented with 15% FCS and 50  $\mu$ g/ml gentamicin. After 7 days of culture, tissue fragments were removed and the synovio-cytes were maintained in culture. First-passage cells were used for the experiments.

**Cell cocultures.** Cocultures were performed by seeding chondrocytes or synovio-cytes in the lower chamber of a 6-well plate and AD-MSCs in Transwells (0.4- $\mu$ m pore size; Corning) in DMEM with ascorbic acid (0.17 mmol/liter), proline (0.35 moles/liter), and sodium pyruvate (1 mole/liter) using a 1:8 cell ratio (further information is available at <http://www.ior.it/en/laboratori/lab-immunoreuma-rig-tis/laboratory-immunorheumatology-and-tissue-regeneration/supplementary-figures>). Control cells were monocultures of AD-MSCs, chondrocytes, and synovio-cytes, and as a control coculture chondrocytes were cocultured (lower chamber) with synovio-cytes (upper chamber). On days 2 and 7, both monocultures and cocultures were detached with trypsin, and cells were labeled with eosin vital dye and counted. The data were expressed as the number of viable cells. The cells were harvested on day 7 (further

**Table 1.** Oligonucleotide primers used for real-time polymerase chain reaction

Target gene*	Primers	Product size, bp	GenBank accession no.	Primer efficiency, %
RPS9	Forward: GATTACATCCTGGGCCTGAA Reverse: ATGAAGGACGGGATGTTTAC	161	NM_001013	94.5
IDO-1	Forward: GATGAAGAAGTGGGCTTTGC Reverse: CGCTGTGACTTGTGGTCTGT	179	NM_002164	102.7
COX-2	Forward: CAGCACTTACGCATCAGTTT Reverse: GCGCAGTTTACGCTGTCTA	129	NM_000963	96.9
CXCL8/IL-8	Forward: CCAAACCTTTCCACCC Reverse: ACTTCTCCACAACCCT	153	NM_000584	97.8
IL-6	Forward: TAGTGAGGAACAAGCCAGAG Reverse: GCGCAGAATGAGATGAGTTG	184	NM_000600	96.1
IL-1 $\beta$	Forward: CTGTCCTGCGTGTGAAAG Reverse: TGCTTGAGAGGTGCTGATG	177	NM_000576	98.1
TNF $\alpha$	Forward: AGCCCATGTTGTAGCAAACC Reverse: GGACCTGGGAGTAGATGAGGTA	149	NM_000594	96.2
IFN $\gamma$	Forward: TGTGGAGACCATCAAGGAAGA Reverse: ACAGTTCAGCCATCACTTGA	155	NM_000619	99.4

\* RPS9 = ribosomal protein S9; IDO-1 = indoleamine 2,3-dioxygenase 1; COX-2 = cyclooxygenase 2; IL-8 = interleukin-8; TNF $\alpha$  = tumor necrosis factor  $\alpha$ ; IFN $\gamma$  = interferon- $\gamma$ .

information is available at <http://www.ior.it/en/laboratori/lab-immunoreuma-rig-tis/laboratory-immunorheumatology-and-tissue-regeneration/supplementary-figures>) for quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis, and supernatant was stored at  $-80^{\circ}\text{C}$ .

Cocultures of chondrocytes in micromasses were also tested to confirm the results in monolayers. Briefly, each micromass was prepared by centrifuging 250,000 chondrocytes at passage 1 in a 15-ml tube and maintaining them for 4 days in an incubator at  $37^{\circ}\text{C}$  in DMEM with 10% fetal bovine serum and 50  $\mu\text{g}/\text{ml}$  ascorbic acid before starting the coculture experiments. Two chondrocyte micromasses (each with 250,000 cells) were inserted in the lower chamber of a 6-well plate, maintaining the same 1:8 cell ratio and time point, and cocultured with AD-MSCs as previously described.

**Effects of exogenous prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) on chondrocytes and synoviocytes.** Chondrocytes and synoviocytes were seeded in 6-well plates and untreated or treated with PGE<sub>2</sub> (Sigma) at 3 different concentrations (40  $\mu\text{g}/\text{ml}$ , 400  $\mu\text{g}/\text{ml}$ , and 4,000  $\mu\text{g}/\text{ml}$ ) for 24 hours, 48 hours, 72 hours, and 7 days. The cells were harvested for qRT-PCR analysis, and supernatant was stored at  $-80^{\circ}\text{C}$ .

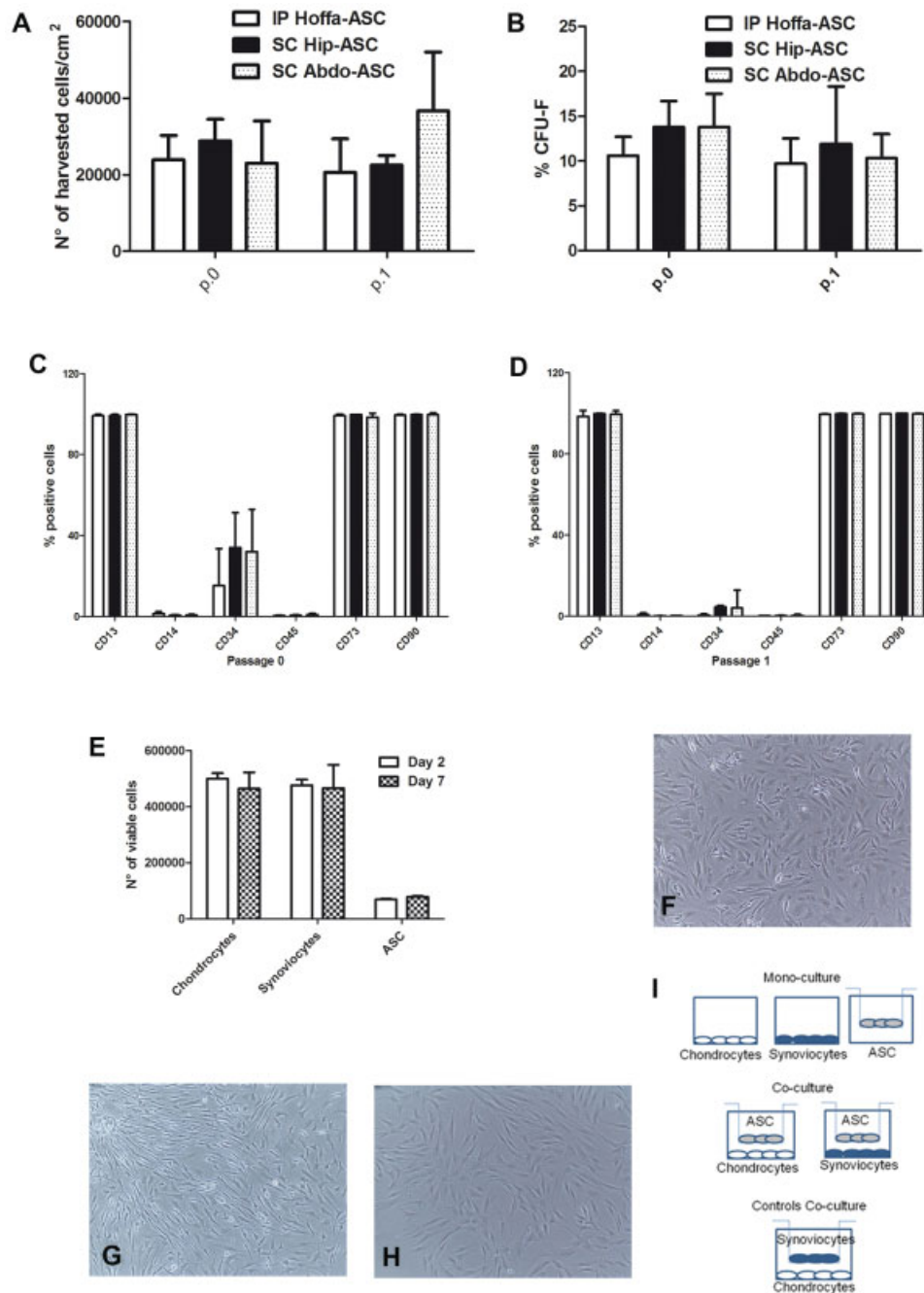
Blocking experiments were performed by treating cells for 6 hours with PGE<sub>2</sub> (400  $\mu\text{g}/\text{ml}$ ) or with IL-1 $\beta$  (10 units/ml) in the presence or absence of 10  $\mu\text{M}$  PGE<sub>2</sub> receptor EP<sub>4</sub> antagonist (EP<sub>4</sub>; Sigma). When indicated, cells were preincubated with EP<sub>4</sub> for 30 minutes before the addition of PGE<sub>2</sub> or IL-1 $\beta$ .

**Real-time qRT-PCR analysis.** Total RNA was extracted from human AD-MSC, chondrocyte, and synoviocyte monocultures and cocultures using RNA PURE reagent (EuroClone) according to the manufacturer's instructions and then treated with DNase I (DNA-free Kit; Ambion). Reverse transcription was performed using SuperScript VILO (Life Technologies) reverse transcriptase and random hexamers, following the manufacturer's protocol. Forward and reverse oligonucleotides for PCR amplification of IL-1 $\beta$ , TNF $\alpha$ , IL-6,

CXCL8/IL-8, cyclooxygenase 2 (COX-2), indoleamine 2,3-dioxygenase 1 (IDO-1), and interferon- $\gamma$  (IFN $\gamma$ ) are shown in Table 1. Real-time PCR was run in a LightCycler Instrument (Roche Molecular Biochemicals) using SYBR Premix Ex Taq (Takara) with the following protocol: initial activation of HotStar Taq DNA polymerase at  $95^{\circ}\text{C}$  for 10 minutes, then 45 cycles of  $95^{\circ}\text{C}$  for 5 seconds and  $60^{\circ}\text{C}$  for 20 seconds. Amplification efficiency (E) of each amplicon was determined using 10-fold serial dilutions of positive control complementary DNAs (cDNAs) and calculated from the slopes of the log input amounts (from 20 ng to 2 pg of cDNA) plotted versus the crossing point values, according to the formula  $E = 10^{-1/\text{slope}}$ . All primer efficiencies were confirmed to be high ( $>90\%$ ) and comparable (Table 1). For each target gene, messenger RNA levels were calculated, normalized to ribosomal protein S9 according to the formula  $2^{-\Delta\text{Ct}}$ , and expressed as a percentage of the reference gene.

**Cytokine quantification.** The concentrations of IL-1 $\beta$ , TNF $\alpha$ , IL-6, CXCL1/GRO $\alpha$ , CXCL8/IL-8, CCL2/MCP-1, CCL3/MIP-1 $\alpha$ , CCL5/RANTES, IL-1 receptor antagonist (IL-1Ra), IL-10, and fibroblast growth factor 2 (FGF-2) were simultaneously evaluated on days 2 and 7 using multiplex bead-based sandwich immunoassay kits (Bio-Rad) following the manufacturer's instructions. PGE<sub>2</sub> and galectin 1 concentrations were measured using enzyme-linked immunosorbent assays (R&D Systems) according to the manufacturer's instructions.

**Statistical analysis.** Statistical analysis was performed using mainly nonparametric tests since the data were not normally distributed and had a strongly asymmetric distribution (Friedman's nonparametric analysis of variance and Dunn's post hoc test for paired data, Kruskal-Wallis test and Dunn's post hoc test for unpaired data, and Mann-Whitney U test for unpaired two-group data). Parametric tests were also used for data with normal and symmetric distributions. Depending on the distribution, values were expressed as the

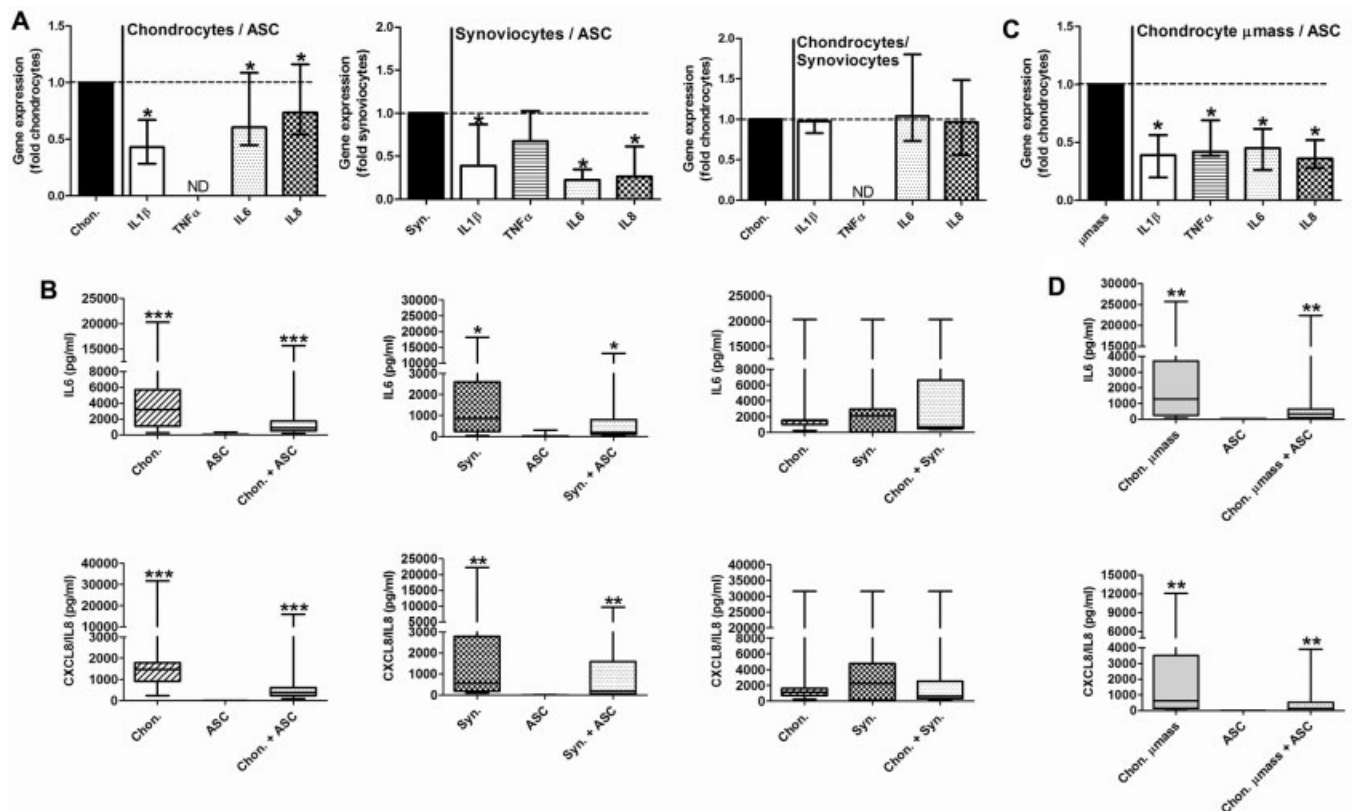


**Figure 1.** Characterization of Good Manufacturing Practice clinical grade adipose-derived mesenchymal stem cells (AD-MSCs; ASC) and evaluation of coculture conditions. **A–D**, AD-MSCs from 3 different sources (infrapatellar Hoffa fat [IP Hoffa-ASC], subcutaneous hip fat [SC Hip-ASC], and subcutaneous abdominal fat [SC Abdo-ASC]) were characterized at both passage 0 (p.0) and passage 1 (p.1) as number of harvested cells/cm<sup>2</sup> (**A**), percentage of colony-forming unit–fibroblast (CFU-F) colonies (**B**), and percentage of positive cells (**C** and **D**). Values are the mean  $\pm$  SD. **E**, Viability of isolated chondrocytes, synoviocytes, and AD-MSCs grown in Dulbecco’s modified Eagle’s medium with ascorbic acid, proline, and sodium pyruvate was analyzed on both day 2 and day 7. Values are the mean  $\pm$  SD. **F–H**, Morphology of chondrocytes (**F**), synoviocytes (**G**), and AD-MSCs (**H**) was evaluated on day 7. Original magnification  $\times$  100. **I**, A schematic of the coculture system is shown.

median and interquartile range, as the median and range (minimum–maximum), or as the mean  $\pm$  SD. CSS Statistica

Statistical Software (StatSoft) was used for analysis. *P* values less than 0.05 were considered significant.





**Figure 2.** Adipose-derived mesenchymal stem cells (AD-MSCs; ASC) down-modulate the main osteoarthritis inflammatory factors and chemokines of chondrocytes (Chon.) or synoviocytes (Syn.). **A**, Interleukin-1 $\beta$  (IL-1 $\beta$ ), tumor necrosis factor  $\alpha$  (TNF $\alpha$ ), IL-6, and CXCL8/IL-8 gene expression of chondrocytes or synoviocytes cocultured with AD-MSCs and in control coculture (chondrocytes plus synoviocytes). **B**, IL-6 and CXCL8/IL-8 release from monoculture of chondrocytes, synoviocytes, or AD-MSCs, from chondrocytes or synoviocytes cocultured with AD-MSCs, or from control coculture. **C**, IL-1 $\beta$ , TNF $\alpha$ , IL-6, and CXCL8/IL-8 gene expression of chondrocytes in micromasses ( $\mu$ mass) cocultured with AD-MSCs. **D**, IL-6 and CXCL8/IL-8 release from chondrocytes in micromasses either cultured alone or cocultured with AD-MSCs. Values in **A** and **C** are the median and interquartile range (IQR). \* =  $P < 0.05$  versus chondrocytes, synoviocytes, or chondrocytes in micromasses cultured alone. In **B** and **D**, data are shown as box plots. Each box represents the IQR. Lines inside the boxes represent the median. Whiskers represent the minimum and maximum values. \* =  $P < 0.05$ ; \*\* =  $P < 0.001$ ; \*\*\* =  $P < 0.0001$  for chondrocytes, synoviocytes, or chondrocytes in micromasses cocultured with AD-MSCs versus chondrocytes, synoviocytes, or chondrocytes in micromasses cultured alone. ND = not detectable.

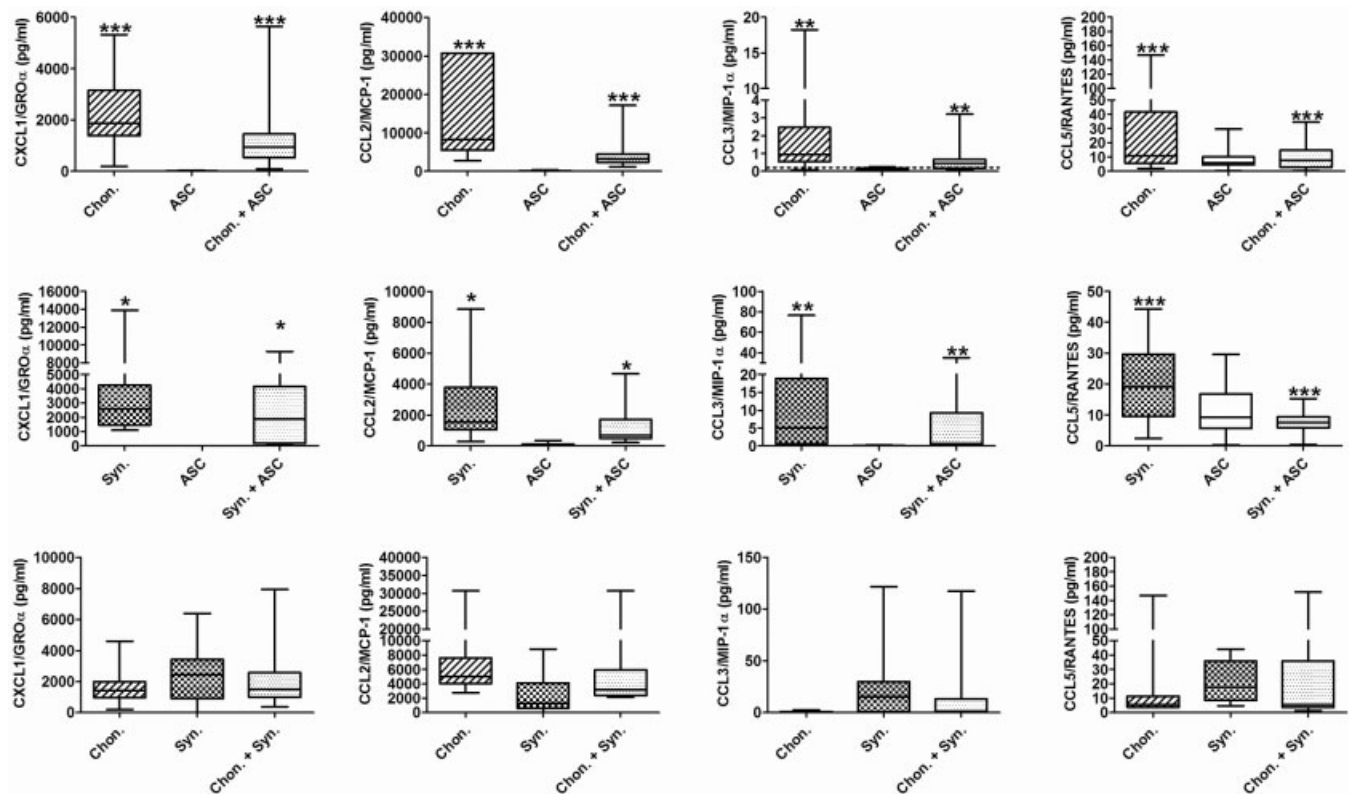
## RESULTS

**AD-MSC characterization and evaluation of coculture conditions.** AD-MSCs from infrapatellar Hoffa fat, SC hip fat, and SC abdominal fat were isolated according to Good Manufacturing Practice clinical grade procedures and characterized at passages 0 and 1. As shown in Figure 1, the number of harvested cells and the percentage of colony-forming unit–fibroblast colonies were similar in the 3 sources, with no significant changes from passage 0 to passage 1. Flow cytometric analysis showed a high expression of CD13, CD73, and CD90 at both passages 0 and 1 and a very low or absent expression of CD14 and CD45. CD34 was expressed at passage 0 but was significantly down-modulated at passage 1.

To evaluate coculture conditions, we performed

preliminary experiments to select a culture medium that did not induce proliferation of chondrocytes, synoviocytes, and AD-MSCs until day 7. DMEM with ascorbic acid, proline, and sodium pyruvate was chosen since, as shown in Figure 1E, it did not induce cell proliferation from day 2 to day 7. Moreover, the cells were all viable until day 7 and did not show any morphologic changes (Figures 1F–H). We used a Transwell system to avoid cell–cell contact (Figure 1I). On the basis of these results, the following experiments were performed on day 7.

**AD-MSCs exert antiinflammatory effects on both chondrocytes and synoviocytes.** First, the expression and release of the major inflammatory factors IL-1 $\beta$ , TNF $\alpha$ , IL-6, and CXCL8/IL-8 were analyzed on day 7. As shown in Figure 2A, AD-MSCs in coculture with these



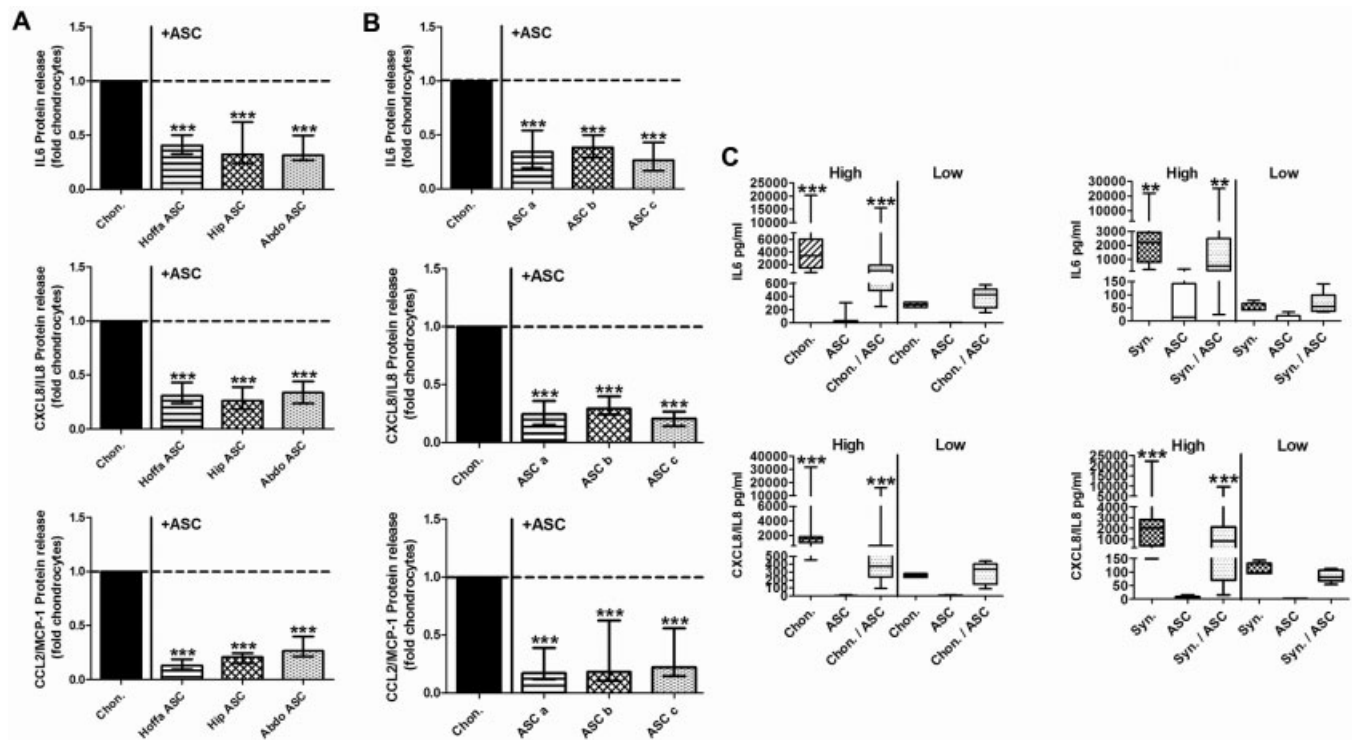
**Figure 3.** Effects of AD-MSCs on the chemokines CXCL1/growth-related oncogene  $\alpha$  (GRO $\alpha$ ), CCL2/monocyte chemoattractant protein 1 (MCP-1), CCL3/macrophage inflammatory protein 1 $\alpha$  (MIP-1 $\alpha$ ), and CCL5/RANTES. We tested the release of the chemokines from monoculture of chondrocytes, synoviocytes, or AD-MSCs, from chondrocytes or synoviocytes cocultured with AD-MSCs, or from control coculture. Data are shown as box plots. Each box represents the IQR. Lines inside the boxes represent the median. Whiskers represent the minimum and maximum values. \* =  $P < 0.05$ ; \*\* =  $P < 0.001$ ; \*\*\* =  $P < 0.0001$  for chondrocytes or synoviocytes cocultured with AD-MSCs versus chondrocytes or synoviocytes cultured alone. See Figure 2 for other definitions.

chondrocytes or synoviocytes significantly decreased the expression of IL-1 $\beta$ , IL-6, and CXCL8/IL-8 on both chondrocytes and synoviocytes. TNF $\alpha$  was not detected on chondrocytes, and its expression was not modulated on synoviocytes. Conversely, control cocultures of chondrocytes/synoviocytes showed opposite results. The analysis in the culture supernatants of these factors showed no production of IL-1 $\beta$  and TNF $\alpha$ . AD-MSCs in coculture with chondrocytes or synoviocytes significantly decreased the release of IL-6 and CXCL8/IL-8 on both chondrocytes and synoviocytes (Figure 2B). Coculture experiments with chondrocytes in micromasses were also performed to confirm our data in monolayers. As shown in Figure 2C, expression of IL-1 $\beta$ , TNF $\alpha$ , IL-6, and CXCL8/IL-8 was significantly down-modulated; the inhibition of IL-6 and CXCL8/IL-8 production was also confirmed (Figure 2D). In addition, IL-1 $\beta$  and TNF $\alpha$  were not produced under these conditions. The analysis of other inflammatory chemokines (CXCL1/GRO $\alpha$ , CCL2/MCP-1, CCL3/MIP-1 $\alpha$ , and CCL5/RANTES)

confirmed the same trend (Figure 3) under all culture conditions tested, whereas in the control coculture no modulation or up-regulation of these factors was found.

**AD-MSCs from different sources or patients down-modulate inflammatory factors on both chondrocytes and synoviocytes.** We then evaluated whether the 3 AD-MSC sources used for the experiments (infrapatellar Hoffa fat, SC hip fat, and SC abdominal fat) and whether different allogeneic AD-MSCs (from SC abdominal fat from 3 donor patients) exerted different antiinflammatory effects in coculture. All 3 sources of AD-MSCs (Figure 4A) and the different allogeneic AD-MSCs (Figure 4B) used in coculture with chondrocytes dampened the same percentages of IL-6, CXCL8/IL-8, and CCL2/MCP-1; the same trend was also observed with synoviocytes (data not shown).

**Dependence of the effect of AD-MSCs on existing inflammation.** As shown in Figure 2B, chondrocytes and synoviocytes in monoculture produced a wide range of IL-6 (median [minimum–maximum] 3,192 pg/ml [239–

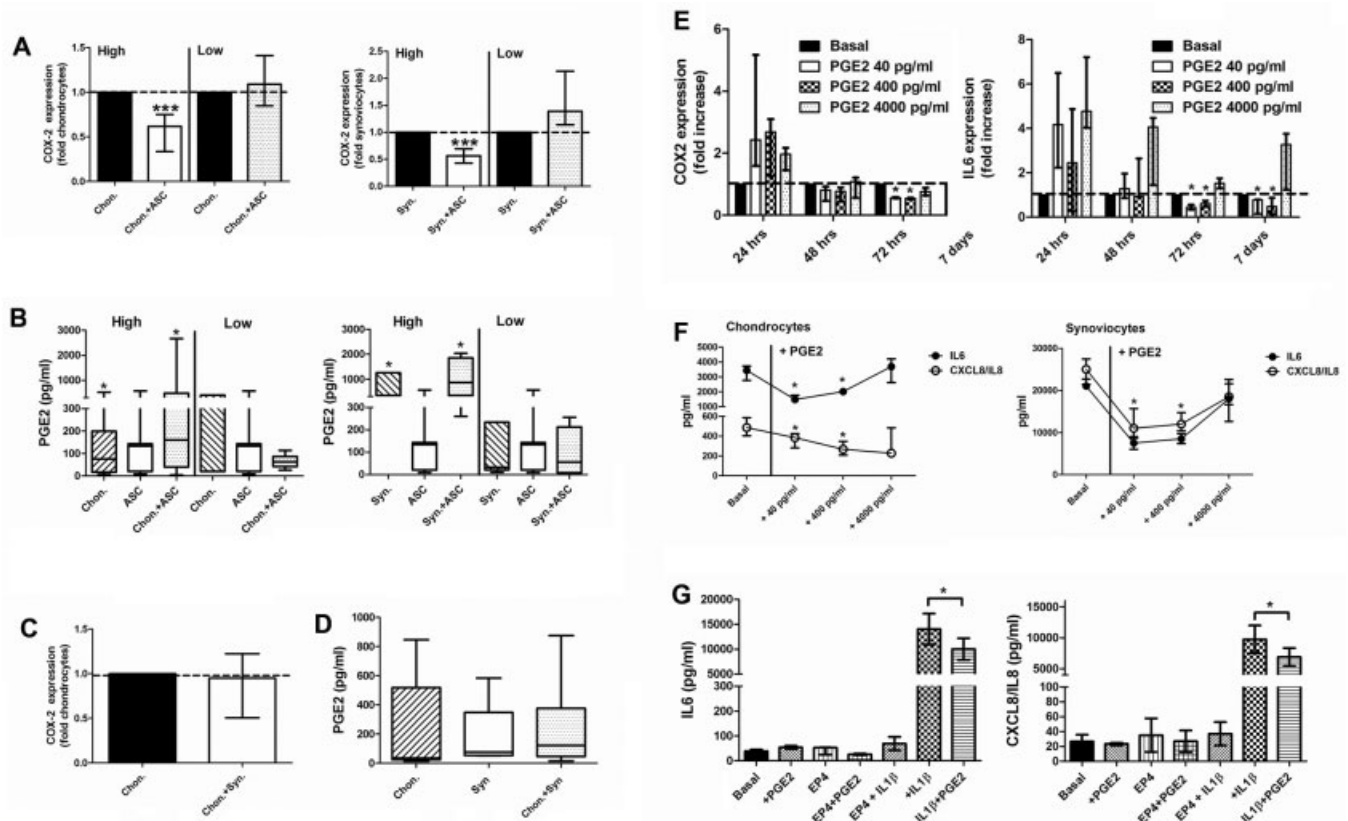


**Figure 4.** Effects of AD-MSCs are independent of sources and donors but depend on inflammation. **A**, Release of IL-6, CXCL8/IL-8, and CCL2/monocyte chemoattractant protein 1 (MCP-1) was independently down-modulated by AD-MSCs from the 3 sources used (infrapatellar Hoffa fat [Hoffa ASC], subcutaneous hip fat [Hip ASC], and subcutaneous abdominal fat [Abdo ASC]). **B**, Release of IL-6, CXCL8/IL-8, and CCL2/MCP-1 was independently down-modulated by allogeneic AD-MSCs (from subcutaneous abdominal fat from donor patients a, b, and c). **C**, AD-MSC modulation of IL-6 and CXCL8/IL-8 release was dependent on existing inflammation (High and Low) in chondrocytes and synoviocytes. Values in **A** and **B** are the median and IQR. \*\*\* =  $P < 0.0001$  versus chondrocytes cultured alone. In **C**, data are shown as box plots. Each box represents the IQR. Lines inside the boxes represent the median. Whiskers represent the minimum and maximum values. \*\* =  $P < 0.001$ ; \*\*\* =  $P < 0.0001$  for chondrocytes or synoviocytes cocultured with AD-MSCs versus chondrocytes or synoviocytes cultured alone. See Figure 2 for other definitions.

20,295 pg/ml] and 867 pg/ml [42–18,212 pg/ml], respectively) and CXCL8/IL-8 (median [minimum–maximum] 1,466 pg/ml [233–31,630 pg/ml] and 565 pg/ml [95–22,274 pg/ml], respectively), so a statistical analysis was performed to determine whether AD-MSCs were equally effective in inhibiting inflammatory factors independently of these factors' basal release. Based on this analysis, the monocultures of both chondrocytes and synoviocytes were divided into 2 groups based on whether they produced high or low levels of inflammatory factors. As shown in Figure 4C, we found that the effects of AD-MSCs depended on the existing basal inflammation of chondrocytes or synoviocytes. In particular, the production of IL-6 and CXCL8/IL-8 was down-modulated only when AD-MSCs were cocultured with chondrocytes or synoviocytes producing high levels of inflammatory factors; no effect was observed in chondrocytes or synoviocytes producing low levels of inflammatory factors. The same trend was also found when we analyzed the other inflammatory factors (data not shown).

**Involvement of COX-2 and PGE<sub>2</sub> in antiinflammatory effects of AD-MSCs.** To understand the mechanism responsible for the antiinflammatory effects of AD-MSCs, we first tested different immunomodulators (IL-10, IL-1Ra, FGF-2, IDO-1, and galectin 1). We did not find any modulation or production of these factors (data not shown); therefore, we analyzed COX-2 and PGE<sub>2</sub>, which were also reported to be involved in the antiinflammatory effect of MSCs (32,33). As shown in Figures 5A and C, COX-2 expression was significantly down-modulated only in chondrocytes and synoviocytes producing high levels of inflammatory factors when cocultured with AD-MSCs.

Conversely, compared to PGE<sub>2</sub> release in monoculture of chondrocytes or synoviocytes, PGE<sub>2</sub> release was higher only in chondrocytes and synoviocytes producing high levels of inflammatory factors when cocultured with AD-MSCs (Figures 5B and D). However, more PGE<sub>2</sub> was produced by AD-MSC monocultures than by chondrocyte coculture with AD-MSCs, whereas



**Figure 5.** Cyclooxygenase 2 (COX-2) and prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) are involved in antiinflammatory effects of AD-MSCs. **A** and **B**, COX-2 expression (**A**) and PGE<sub>2</sub> release (**B**) in chondrocyte and synovioocyte monocultures producing high or low levels of inflammatory factors and cocultured with AD-MSCs. **C** and **D**, COX-2 expression (**C**) and PGE<sub>2</sub> release (**D**) in control coculture. Values in **A** and **C** are the median and IQR. \*\*\* =  $P < 0.0001$  versus chondrocytes or synovioocytes cultured alone. In **B** and **D**, data are shown as box plots. Each box represents the IQR. Lines inside the boxes represent the median. Whiskers represent the minimum and maximum values. \* =  $P < 0.05$  for chondrocytes or synovioocytes cocultured with AD-MSCs versus chondrocytes or synovioocytes cultured alone. **E**, Time course analysis of COX-2 and IL-6 expression on chondrocytes treated for different periods of time with different concentrations of exogenous PGE<sub>2</sub>. Values are the median and IQR. \* =  $P < 0.05$  versus basal level. **F**, Release of IL-6 and CXCL8/IL-8 from chondrocyte and synovioocyte samples treated for 7 days with different concentrations of exogenous PGE<sub>2</sub>. **G**, Release of IL-6 and CXCL8/IL-8 from chondrocytes treated for 6 hours with PGE<sub>2</sub> (400 ng/ml) or IL-1 $\beta$  (10 units/ml) with or without PGE<sub>2</sub> receptor EP<sub>4</sub> antagonist (EP<sub>4</sub>). In **F** and **G**, values are the median and IQR. In **F**, \* =  $P < 0.05$  versus basal level. In **G**, PGE<sub>2</sub> significantly reduced IL-6 and CXCL8/IL-8 release in IL-1 $\beta$ -treated cells (\* =  $P < 0.05$ ). See Figure 2 for other definitions.

more PGE<sub>2</sub> was produced by monocultures of synovioocytes than by synovioocyte coculture with AD-MSCs.

**Time-dependent effect of exogenous PGE<sub>2</sub> on COX-2 and PGE<sub>2</sub> in chondrocytes and synovioocytes.** To test whether PGE<sub>2</sub> was responsible for the downmodulation of inflammatory factors on chondrocytes and synovioocytes, these cells were treated with exogenous PGE<sub>2</sub> at 3 different concentrations (40 pg/ml, 400 pg/ml, and 4,000 pg/ml). These concentrations were chosen on the basis of the results of PGE<sub>2</sub> release previously tested in AD-MSC monoculture supernatant (~400 pg/ml) (Figure 5B). As shown in Figure 5E, exogenously added PGE<sub>2</sub> at the lowest concentration

tested reduced both COX-2 and IL-6 expression only after a long period of stimulation (starting from 72 hours until day 7). Moreover, on day 7 also at the protein level (Figure 5F), IL-6 production was found to be inhibited only by the lowest concentrations of PGE<sub>2</sub> (40 pg/ml and 400 pg/ml) used on chondrocytes and synovioocytes, whereas CXCL8/IL-8 was also inhibited at the highest concentration (4,000 pg/ml) on chondrocytes.

Subsequently, to establish a specific involvement of PGE<sub>2</sub> in the inhibition of inflammatory factors, we treated cells with PGE<sub>2</sub> (400 pg/ml) or with IL-1 $\beta$  (to mimic an inflammatory condition) in the presence of a specific PGE<sub>2</sub> receptor antagonist (EP<sub>4</sub>). As shown in



Figure 5G, PGE<sub>2</sub> significantly reduced IL-6 and CXCL8/IL-8 release in IL-1 $\beta$ -treated cells. Moreover, when blocking cells with EP<sub>4</sub>, we did not find an increase in IL-6 or CXCL8/IL-8 release whether we stimulated cells with IL-1 $\beta$  or PGE<sub>2</sub>.

## DISCUSSION

MSCs have been identified and isolated from different tissues (bone marrow, adipose tissue, umbilical cord vein) (6–8) and have important immunomodulatory properties in vitro in coculture with mononuclear cells (T and B cells, natural killer cells, plasma cells, and dendritic cells) (13,34–36) as well as in vivo in various clinical trials (14–17). A recent report (18) suggested the possible use of MSCs in osteoarticular diseases. Based on these findings, we analyzed in vitro the effects of AD-MSCs on both chondrocytes and synoviocytes obtained from OA patients.

First, we found that Good Manufacturing Practice clinical grade AD-MSCs isolated from 3 different sources (infrapatellar Hoffa fat, SC hip fat, and SC abdominal fat) were able to down-modulate the release and expression of the main OA inflammatory factors (IL-1 $\beta$ , IL-6, and CXCL8/IL-8). Moreover, we also found a significant down-modulation of other chemokines (CXCL1/GRO $\alpha$ , CCL2/MCP-1, CCL3/MIP-1 $\alpha$ , and CCL5/RANTES) directly involved in the progression of the disease. It has been reported that CXCL8/IL-8 is one inducer of cartilage hypertrophy (37), whereas CXCL8/IL-8, CXCL1/GRO $\alpha$ , CCL2/MCP-1, and CCL5/RANTES are other selective inducers of matrix degradation (38,39). It is well known that low-density chondrocytes in monolayer lose their original phenotype and receptor profiles; therefore, to confirm these data we also evaluated chondrocytes grown in 3-dimensional micromasses, since they better mimic the cartilage-like structure. Consistent with monolayer data, we found that the major inflammatory OA factors were down-modulated with the same trend, indicating that the effects were independent from the culture conditions.

We then found that antiinflammatory effects of AD-MSCs were strictly dependent on the degree of inflammation of OA chondrocytes and synoviocytes, as also reported in an acute asthma lung model (40). It has been reported that in vitro human MSCs require activation, and activating stimuli appear to include proinflammatory cytokines (IL-1 $\beta$ , IFN $\gamma$ , TNF $\alpha$ ) or interaction with monocytes (11,34). Under the present culture conditions, AD-MSCs, chondrocytes, and synoviocytes in monoculture and coculture did not produce IL-1 $\beta$ ,

TNF $\alpha$ , and IFN $\gamma$ , so AD-MSCs might be activated by other factors specifically produced by inflamed chondrocytes and synoviocytes. Moreover, these AD-MSC effects were not dependent on the main immunomodulatory factors found to be involved in the immunosuppressive effect of BM-MSCs, such as IL-10, IL-1Ra, galectin 1, FGF-2, and IDO-1, since AD-MSCs did not produce or modulate these factors, thus suggesting a different pathway.

The inflammatory cascade is driven by a variety of enzymes, among which COX is prominent and catalyzes the formation of prostaglandins and thromboxanes from arachidonic acid. COX has at least 2 isoforms, a constitutive form (COX-1) and an inflammation-induced form (COX-2) (41). The present data show that in coculture, COX-2 expression by chondrocytes and synoviocytes was inhibited by AD-MSCs, thus suggesting a direct involvement of this pathway in the antiinflammatory effect exerted by these cells.

It has been reported that increased anabolic activity in OA cartilage may be associated with cytokine-induced synthesis of PGE<sub>2</sub>, whose feedback regulates COL2A1 transcription in a positive manner (42), which would in turn permit subsequent inhibition by cytokine-induced factors (43,44). It has also been shown that human OA cartilage explants express COX-2 and spontaneously produce PGE<sub>2</sub> (45,46) and their expression is modulated by proinflammatory cytokines, but PGE<sub>2</sub> is also produced by AD-MSCs (32). The present data showed that COX-2 inhibition was specifically associated with PGE<sub>2</sub> increase when AD-MSCs were cocultured with inflamed chondrocytes or synoviocytes. These results seem paradoxical, since it has been shown that COX-2 and PGE<sub>2</sub> increase or decrease is dependent on the degree of inflammation, but the increase of PGE<sub>2</sub> measured in the coculture was not due to a synergistic effect but was mainly dependent on the basal release of these cells in monoculture. In fact, the increase in PGE<sub>2</sub> secretion in the coculture was mainly associated with AD-MSCs, and it is well known that PGE<sub>2</sub> plays a key role in the immunosuppressive properties of AD-MSCs (32).

PGE<sub>2</sub> is reported to have both anabolic and catabolic effects on chondrocytes and synoviocytes (45–47); therefore, to confirm that PGE<sub>2</sub> was involved in the antiinflammatory effects exerted by AD-MSCs, 3 different concentrations of PGE<sub>2</sub> were tested, ranging from ~0.011 nM to ~11  $\mu$ M. We showed that COX-2 and IL-6 inhibition was evident only after a long period of stimulation and mainly using PGE<sub>2</sub> in the nM range (but not in the  $\mu$ M range), which, as reported (48), is the

physiologic concentration detected in the synovial fluid. We also confirmed a specific role of PGE<sub>2</sub> in modulating these effects under inflammatory conditions, with blocking and rescue experiments specifically involving its receptor EP<sub>4</sub>. It is interesting to note that PGE<sub>2</sub> in chondrocytes and synoviocytes plays a significant physiologic role in tissue homeostasis (49). Moreover, PGE<sub>2</sub> has been shown to inhibit in a dose-dependent manner the expression of matrix metalloproteinase 13, type X collagen, vascular endothelial growth factor, and alkaline phosphatase genes (50), consistent with data previously found in a collaborative work (Maumus M, et al: unpublished observations). These antiinflammatory effects might also be dependent on other factors released by AD-MSCs (51); a proteomic profiling of these cells in monoculture and coculture should help to reveal the role of other important molecules.

In conclusion, these data show that the antiinflammatory effects of AD-MSCs are probably not dependent on AD-MSC adipose tissue sources or donors but rather on the inflammatory status of OA chondrocytes and synoviocytes. AD-MSCs seem to be able to sense and respond to the local environment; the complexity of this antiinflammatory effect may be due to a combination of different molecules. However, the COX-2/PGE<sub>2</sub> pathway might be one of the modulators that plays a role in this mechanism of action.

#### AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Lisignoli had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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