


Effect of microfragmented adipose tissue on osteoarthritic synovial macrophage factors

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Funding information

Italian Ministry of Health, Grant/Award Number: RF-2011-02352638; Istituto Ortopedico Rizzoli di Bologna, Grant/Award Number: Funds "5 per mille and Ricerca corrente"

Abstract

Cell-based therapies using adipose-derived mesenchymal stromal cells (ADMSCs) have shown promising results for the treatment of osteoarthritis (OA). In fact, ADMSCs are now indicated as one of the most powerful cell sources through their immunomodulatory and anti-inflammatory activities. Recently, an innovative one-step closed device was developed to obtain microfragmented adipose tissue (MF) to avoid the need for good manufacturing practices for ADMSCs expansion while maintaining their regenerative potential. The aim of this study was to assess the mechanisms of action of MF and ADMSCs from MF (MF-ADMSCs) on an inflammatory cell model of OA synoviocytes. We found that MF produced low levels of inflammatory factors such as interleukin 6 (IL-6), CC-chemokine ligand 5/receptor-activated normal T-cell expressed and secreted (CCL5/RANTES), CC-chemokine ligand 2/monocyte chemoattractant protein-1 (CCL2/MCP-1), and CC-chemokine ligand 3/macrophage inflammatory protein-1 α (CCL3/MIP-1 α), and a higher level only of CXC-chemokine ligand 8/interleukin 8 compared with MF-ADMSCs. Matrix metalloproteinase 9 (MMP-9) degradative factor but released a lower level of its inhibitor tissue inhibitor of the metalloproteinase (TIMP-1). MF in coculture with synoviocytes significantly induced both the metabolic activity and the release of IL-6. In contrast, MF, not MF-ADMSCs, partially decreased CCL5/RANTES. Moreover, MF reduced the release of both macrophage-specific chemokines (CCL2/MCP-1 and CCL3/MIP-1 α) and degradative marker MMP-9. Interestingly, MF increased TIMP-1 (the MMP-9 inhibitor) and down-modulated toll-like receptor (TLR4) receptor and key molecules of NF κ B pathways.

These data evidenced different effects of MF versus MF-ADMSCs on inflamed synoviocytes. MF reduced typical macrophages markers and its potentiality by switching off macrophages activity was strictly dependent on TLR4 and NF κ B signaling.

KEYWORDS

adipose mesenchymal stromal cells, inflammation, macrophage factors, microfragmented adipose tissue (MF), osteoarthritis (OA)

1 | INTRODUCTION

In recent years the use of cell-based therapies has been proposed to address the limited repair and regenerative capacity of some tissues (Andriolo et al., 2018; Filardo et al., 2013). Mesenchymal stromal cells (MSCs) represent a promising approach for the treatment of various rheumatic diseases, including osteoarthritis (OA; de Girolamo et al., 2016; Perdisa et al., 2015). OA is a whole joint disease characterized by progressive degeneration of articular cartilage, inflammation of the synovium, and subchondral bone changes (Loeser, Goldring, Scanzello, & Goldring, 2012; Scanzello & Goldring, 2012).

Synovial inflammation is a process characterized by thickening of the synovial membrane (hypertrophy and hyperplasia) and infiltration of cells (lymphocytes and macrophages), which lead to clinical symptoms such as joint swelling and pain (Bondeson et al., 2010). The synovial tissue is mainly characterized by two cell populations; macrophages and fibroblasts. Synovial fibroblasts (SF) express vimentin, CD90, CD106, intracellular adhesion molecule-1 and are able to consistently produce interleukin 6 (IL-6), CXC-chemokine ligand 8/interleukin 8 (CXCL8/IL-8), transforming growth factor- β (TGF- β), and CC-chemokine ligand 5/receptor-activated normal T-cell expressed and secreted (CCL5/RANTES; Bartok & Firestein, 2010). Synovial macrophages (SM) population instead expresses CD11b, CD14, CD16, and CD68 and produces catabolic mediators, such as matrix metalloproteinases (MMPs), aggrecanases, and proinflammatory mediators, such as IL-6, interleukin 1 β (IL-1 β), tumor necrosis factor α (TNF- α), CC-chemokine ligand 2/monocyte chemoattractant protein-1 (CCL2/MCP-1) and CC-chemokine ligand 3/macrophage inflammatory protein-1 α (CCL3/MIP-1 α ; Manferdini et al., 2016).

MMPs have been suggested as important cofactors or disease mediators in the progression of OA (Murphy & Nagase, 2008). MMPs are secreted as inactive proenzymes and are activated by the cleavage of propeptide domain by proteolytic enzymes (Muthukuru & Cutler, 2015); when they reach high levels, they have destructive functions, as occurs in inflammatory processes (Scian et al., 2011). The activity of MMPs is counterbalanced by the activity of inhibitors, including those belonging to the tissue inhibitor of the metalloproteinase (TIMP) family (Scian et al., 2011). All these mediators trigger the inflammation processes that alter the balance between regeneration and destruction of the cartilage matrix and amplifies the synovial inflammation, creating a vicious circle (Bijlsma, Berenbaum, & Lafeber, 2011).

Hyaluronic acid (HA), known to be a major component of healthy synovial tissue, has a joint lubrication function as well as an anti-inflammatory effect by suppressing MMPs/aggrecanase a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS) pathways (Masuko, Murata, Yudoh, Kato, & Nakamura, 2009). Interleukin 10 (IL-10) is another important factor that shows potent anti-inflammatory properties by inhibiting the synthesis of proinflammatory cytokines such as TNF- α and IL-1 (Iyer & Cheng, 2012; Katsikis, Chu, Brennan, Maini, & Feldmann, 1994).

Current treatments for OA are only able to manage symptoms and inflammation, but they do not counteract the pathological

processes. To this end, the use of adipose-derived mesenchymal stromal cells (ADMSCs) in regenerative medicine is a rapidly growing area of research, due to their abundance, easy availability, and potential to differentiate (Ferng et al., 2016; Schaffler & Buchler, 2007). Besides their capacity for multilineage differentiation (bone, cartilage, tendons, skeletal muscle, and fat), these cells represent a valid cell therapy, because *in vitro* and *in vivo* studies have shown immunomodulatory, anti-inflammatory, antiapoptotic, proangiogenic, proliferative, or chemoattractive effects, through direct cell-cell interaction or by secretion of bioactive factors (Desando et al., 2013; Djouad, Bouffi, Ghannam, Noël, & Jorgensen, 2009; Hoogduijn et al., 2010; Manferdini et al., 2013; ter Huurne et al., 2012). Moreover, recent studies have demonstrated that MSC secreted soluble factors can influence macrophage function and polarization (Eggenhofer & Hoogduijn, 2012; Luz-Crawford et al., 2016; Manferdini et al., 2017).

The production of clinical-grade MSCs requires their isolation in accordance with current Good Manufacturing Practice (cGMP) guidelines (Regulation [EC] No. 1394/2007 of the European Parliament and of the Council) and the identification and control of all the phases of cell manipulation and release (Neri et al., 2013; Roseti et al., 2008). To overcome this problem, new procedures and strategies have been developed (Hamdi et al., 2011; Sekine et al., 2011). The Lipogems[®] device allows to obtain microfragmented adipose tissue (MF) in a short time, without expansion or enzymatic treatment, thus reducing the phase of cell manipulation that are fundamental for clinical application (Fantasia & Santos Cortes, 2016; Russo, Condello, Madonna, Guerriero, & Zorzi, 2017; Striano, Bilbool, Azatullah, Hilado, & Horan, 2015; Tremolada, Ricordi, Caplan, & Ventura, 2016). Furthermore, it appears that the injection of autologous MF in patients affected by diffuse degenerative chondral lesions with nonresponsive knee pain is a promising viable and safe treatment (Russo et al., 2017; Striano et al., 2015).

Different authors have extensively studied and characterized *in vitro* the MF by immunohistochemical analysis, and they found a higher number of cells positive for CD34, CD146, S-100 protein, and α -smooth actin compared with fat tissue lipoaspirate (Bianchi et al., 2013). In particular, Ceserani et al. (2016) observed that MF was composed of an abundant number of microvascular endothelial cells (positive for CD31, CD34, and CD146) surrounded by several stromal cells expressing the typical mesenchymal markers (CD44, CD105, and TGF- β 1), most of them positive for NG2. Moreover, Carelli et al. (2015) confirmed positive staining of MF for S-100, vimentin, β -tubulin III, and fatty acid-binding protein 4.

It has been shown that MF contains viable and functional human ADMSCs able to secrete bioactive molecules that act to trigger and sustain angiogenic, antifibrotic, antiapoptotic, antimicrobial, and immunomodulatory responses in the target tissue (Bianchi et al., 2013; Ceserani et al., 2016; García-Contreras, Jimenez, & Mendez, 2014; Giori et al., 2015).

The aim of this study was to investigate the potential of MF or ADMSCs derived from microfragmented adipose tissue

(MF-ADMSCs) to better assess the anticatabolic and proanabolic effects of these sources in an inflammatory environment.

2 | MATERIALS AND METHODS

2.1 | Processing of microfragmented adipose tissue

Fat from subcutaneous adipose tissue was obtained from seven subjects; three males and four females, aged between 42 and 66 years, undergoing elective plastic surgical procedures at the Image Institute (Milan, Italy). A written informed consent was obtained from all patients before sample collection.

The harvested fat was immediately processed through the Lipogems[®] processing kit (Lipogems International SRL, Milan, Italy) to obtain MF. Briefly, the adipose tissue was introduced and shook inside the Lipogems[®] device containing beads that allow the fragmentation of the fat tissue, removal of red blood cells, and oil

residues (Marfia et al., 2016). The entire process was carried out in one surgical step, and the resulting MF was collected in a 10-cm³ syringe.

2.2 | Cell isolation and culture

Inflamed synovial tissue (moderate synovitis) was obtained from patients with OA (Kellgren-Lawrence Grades II-III [Kellgren & Lawrence, 1957]) undergoing joint surgery. The study was approved by the Ethics Committee of the Rizzoli Orthopedic Institute.

Synoviocytes were isolated from synovial tissue ($n = 5$) as previously described (Manferdini et al., 2016). Briefly, the synovium was dissected from the underlying connective tissue, and minced fragments were cultured in 12 ml Optimum-1 (Life Technologies Grand Island, NY) supplemented with 15% fetal calf serum (FCS) and 50 $\mu\text{g/ml}$ gentamicin (Life Technologies) and incubated in a humidified atmosphere at 37°C with 5% CO₂. After 7 days of culture, tissue

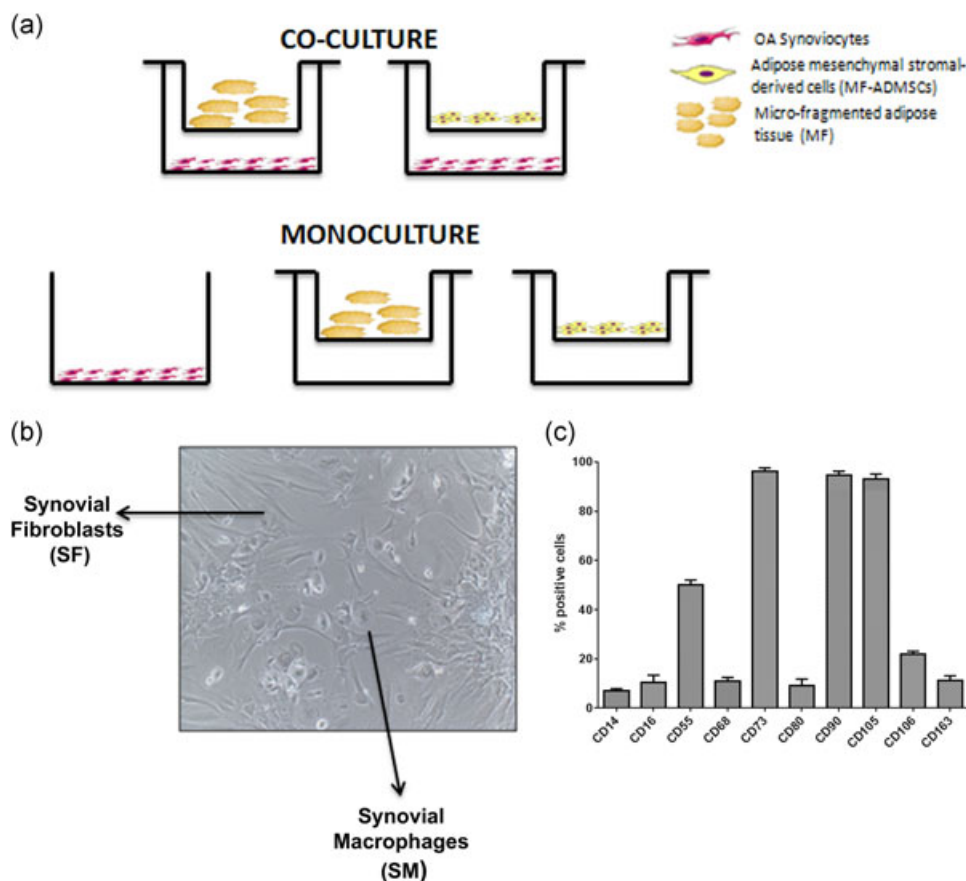


FIGURE 1 Experimental design and characterization of OA synoviocytes. (a) A schematic of the coculture system to investigate the paracrine interaction between Passage 1 synoviocytes and MF or MF-ADMSCs using a transmembrane system with 0.4- μm pores (transwell) is shown. In coculture, synoviocytes were seeded in the bottom of the wells, whereas MF or MF-ADMSCs in the transwell, instead of the monoculture system of synoviocytes, MF and MF-ADMSCs are used as a control. (b) Passage 1 synoviocytes are characterized by a mix of cells with a spindle- (SF) and a star-shaped (SM) morphology. (c) Flow cytometry analysis of Passage 1 synoviocytes showing the percentage of positive fibroblastic (CD14, CD16, CD55, CD68, CD73, CD80, CD90, CD105, CD106, and CD163) and macrophages markers. Data are expressed as the mean \pm SD. ADMSC: adipose-derived mesenchymal stromal cells; MF: microfragmented adipose tissue; OA: osteoarthritis; SD: standard deviation [Color figure can be viewed at wileyonlinelibrary.com]

fragments were removed, and synoviocytes were maintained in the culture. First-passage synoviocytes used for the experiments were characterized by flow cytometry using specific antibodies: CD14 and CD16 (5 µg/ml, Dako Cytomation, Glostrup, Denmark), CD55 (2.5 µg/ml, Millipore, Temecula, CA), CD68 (10 µg/ml, Dako), CD73, CD90 and CD105 (5 µg/ml, BD Pharmingen, San Jose, CA), CD80 (2 µg/ml, GeneTex Inc., Irvine, CA), CD106 (10 µg/ml, Millipore), and CD163 (10 µg/ml, Abcam, Cambridge, UK) to discriminate SF from SM.

Adipose mesenchymal stromal-derived cells were obtained from MF by enzymatic digestion. Briefly, the MF was washed in phosphate-buffered saline (PBS) and digested with 0.05% collagenase A type I (Sigma-Aldrich, St. Louis, MO) under gentle agitation at 37°C for 1 hr and centrifuged at 650g for 10 min to separate the stromal vascular fraction from adipocytes. The supernatant was discarded, and the cell pellet was resuspended and filtered with a 100-µm cell strainer.

The cells were seeded into culture flasks and maintained in α -MEM (Sigma-Aldrich) supplemented with 15% FCS and penicillin-streptomycin (100 U/ml–100 µg/ml Life Technologies) and incubated in a humidified atmosphere at 37°C with 5% CO₂. At confluence, cells were detached by treatment with trypsin-EDTA. All experiments were performed on cells at Passage 2.

2.3 | Cocultures experiments

Cocultures were performed by seeding 1 × 10⁵ OA synoviocytes at Passage 1 in the lower chamber of a six-well plate and 0.1 gr/ml MF or 1 × 10⁵ MF-ADMSCs (number of cells obtained from 0.1 gr/ml MF) in transwell (0.4 µm pore size, Corning, Toledo, OH). Cultures were maintained for 48 hr in Dulbecco modified Eagle medium (Life Technologies) with ascorbic acid (0.17 mM/l), proline (0.35 M/l), and sodium pyruvate (1 M/l; Figure 1a). Synoviocytes, MF, and MF-ADMSCs in monocultures under the same conditions were used as control groups. Supernatants were stored at –20°C.

2.4 | Quantification of secreted factors

Supernatants from cocultures and controls were used to quantify secreted factor concentration of IL-6, CXCL8/IL-8, CCL2/MCP-1, CCL3/MIP-1 α , CCL5/RANTES, IL-1 β , TNF- α , IL-10, matrix metalloproteinase 9 (MMP-9) using multiplex bead-based sandwich immunoassay kits (Bio-Rad, Marnes-la-Coquette, France) following the manufacturer's instructions. TIMP-1 and HA were evaluated using ELISA assays (R&D Systems, Minneapolis, MN) following the manufacturer's instructions.

2.5 | Metabolic activity

After 48 hr of coculture, synoviocytes were washed with PBS and stained using the colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (MTT assay). Briefly, MTT solution was added to the cells and incubated at 37°C for 3 hr. Afterward, each well was washed with PBS, 1 ml of HCl 0.01N was added, and aliquots were transferred into a 96-well plate. The absorbance of each well was

TABLE 1 Oligonucleotide primers used for real-time PCR

Target gene	Primers (forward and reverse)	Product size (bp)
RPS9	AAGTCCATCCACCACGCTC GGAATCCAGGCGGACAATGA	99
TLR4	GGCTTACTTTCACTTCCAAC ATACTTTAGGCTGGTTGTCC	146
RelA	CGAGCTTGTAGGAAAGGACTG TGACTGATAGCCTGCTCCAG	132
NF κ B1	CAGGAGACGTGAAGATGCTG AGTTGAGAATGAAGGTGGATGA	109
NF κ BIA	TCCTGAAGCTACCAACTACA CATTGACATCAGCACCCAAG	108

Note. PCR: polymerase chain reaction.

detected at 570 nm using automated spectrophotometric plate reader TECAN Infinite[®] 200 PRO (Tecan Italia S.r.l., Cernusco Sul Naviglio, Italy).

2.6 | Real-time quantitative reverse transcription PCR (qRT-PCR) analysis

Total RNA was extracted from OA synoviocytes in mono- and cocultures, using RNA PURE reagent (Euroclone Spa, Pero, Italy) according to the manufacturer's instructions, and then treated with DNase I (DNA-free Kit, Life Technologies). Reverse transcription was performed using Super-Script VILO (Life Technology) reverse transcriptase and random hexamers, following the manufacturer's protocol. Forward and reverse oligonucleotides for polymerase chain reaction (PCR) amplification of toll-like receptor (TLR4), nuclear factor NF- κ -B p65 subunit (RelA), nuclear factor NF- κ -B p105 subunit (NF κ B1) and I κ B- α (NF κ BIA) are described in Table 1; real-time PCR was run, and messenger RNA levels were calculated, as previously described (Manferdini et al., 2013).

2.7 | Statistical analysis

Statistical analysis was performed with GraphPad Prism 7 software (GraphPad Software Inc., La Jolla, CA). Statistical differences were evaluated using nonparametric tests since the data were not normally distributed (the Kruskal–Wallis test and Dunn's post hoc test for unpaired data or the Mann–Whitney *U* test for unpaired two-group data). Data were expressed as median and range (minimum–maximum) or as median and interquartile range. The data were considered significant at $p \leq 0.05$.

3 | RESULTS

3.1 | Characterization of Passage 1 synoviocytes

Cellular morphology of isolated Passage 1 synoviocytes showed the presence of the main two cell populations: SF and SM. As shown in Figure 1b, the SF has spindle-shaped morphology with cytoplasmic

extensions, whereas the SM has star-shaped morphology. The cytofluorimetric analysis confirmed the expression of fibroblastoid and macrophage markers (CD14, CD16, CD55, CD68, CD73, CD80, CD90, CD105, CD106, and CD163) on Passage 1 isolated synoviocytes (Figure 1c), whereas negative to CD3, CD34, and CD31 (data not shown).

As shown in Table 2, Passage 1 synoviocytes after 48 hr of culture show different amounts of released factors. In particular, high levels of IL-6, CXCL8/IL-8, CCL2/MCP-1, HA, TIMP-1 and lower of CCL5/RANTES, CCL3/MIP-1 α , MMP-9 and IL-10 were detected. IL-1 β and TNF- α were not produced.

3.2 | Factors released by MF and MF-ADMSC

First, the basal level of secreted factors was evaluated both in 0.1 gr of MF and in 10^5 MF-ADMSC (the number of cells isolated from 0.1 gr of MF). We found that the levels of inflammatory factors, IL-6 and CCL2/MCP-1, were significantly lower ($p = 0.0007$ and $p = 0.0007$, respectively) in MF than in MF-ADMSC. Only CXCL8/IL-8 was significantly higher in MF ($p = 0.0007$). The production of CCL5/RANTES and CCL3/MIP-1 α did not show differences (Figure 2a). As shown in Figure 2b, the anti-inflammatory factors, IL-10 and HA, were significantly lower in MF than

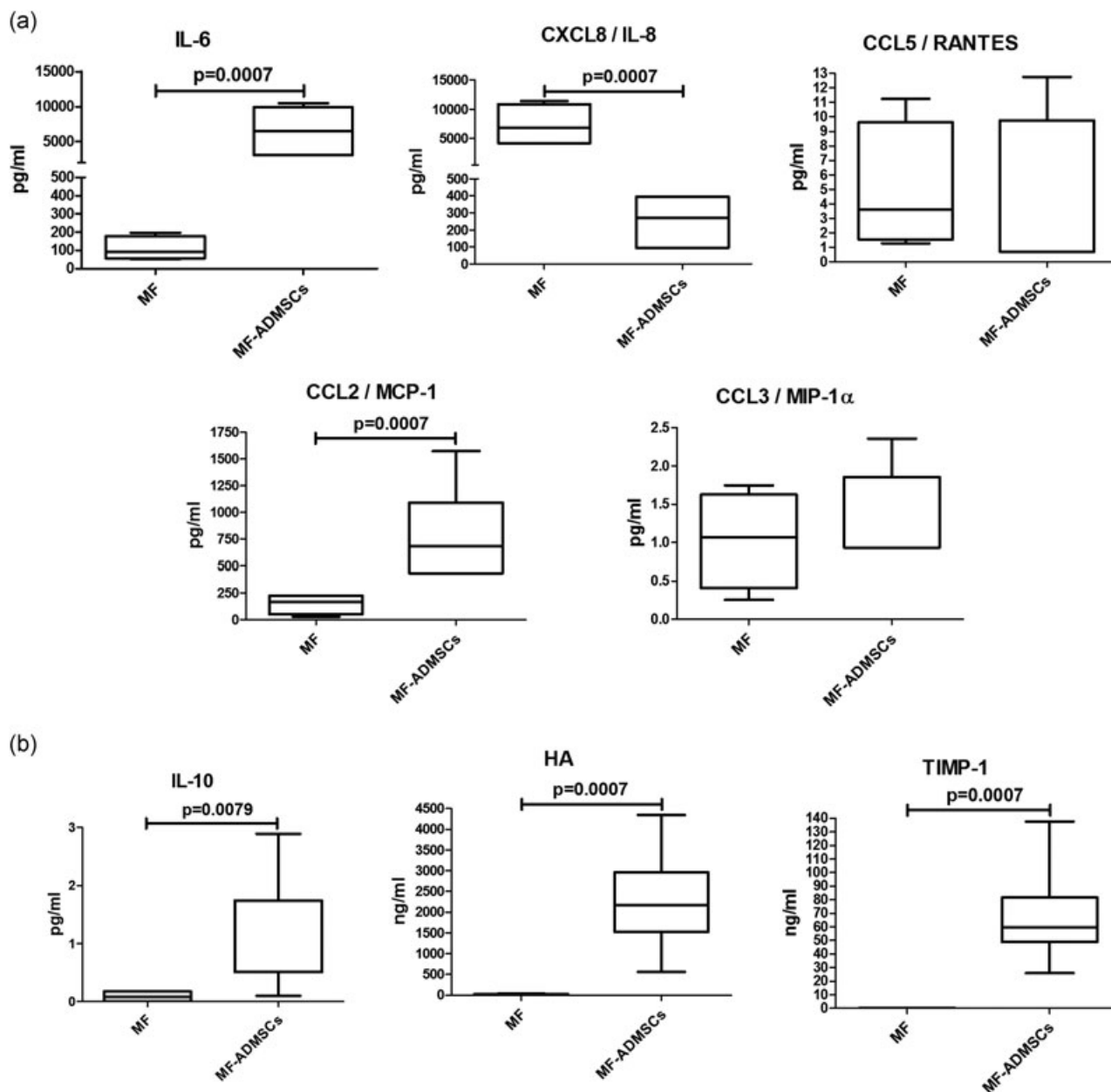


FIGURE 2 Characterization of MF and MF-ADMSCs. (a) Inflammatory factors (IL-6, CXCL8/IL-8, CCL5/RANTES, CCL2/MCP-1, and CCL3/MIP-1 α) released after 48 hr by MF (0.1 gr) and MF-ADMSCs (10^5 cells). (b) Anti-inflammatory factors (IL-10 and HA) and metalloproteinase inhibitor (TIMP-1) released after 48 hr by MF (0.1gr) and MF-ADMSCs (10^5 cells). Data are shown as boxes and whiskers. Lines inside the boxes represent the median, and whiskers represent the minimum and maximum values. Data are analyzed with a Mann-Whitney test for nonparametric data and $p \leq 0.05$ is considered statistically significant. ADMSC: adipose-derived mesenchymal stromal cells; MF: microfragmented adipose tissue; HA: hyaluronic acid; OA: osteoarthritis

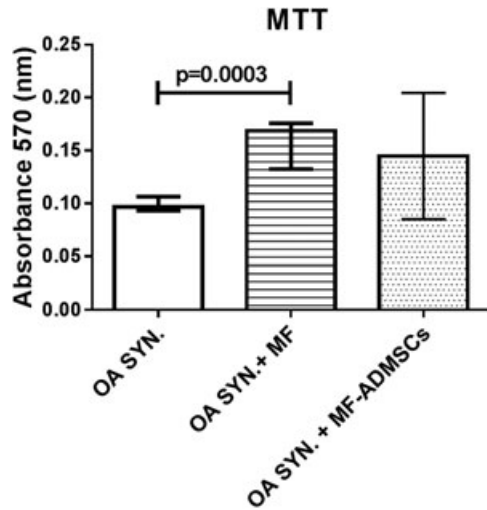


FIGURE 3 Metabolic activity of cocultured OA Passage 1 synoviocytes with MF or MF-ADMSCs. Cells were evaluated by the MTT assay at 48 hr. Data are expressed as median with interquartile range, and $p \leq 0.05$ is considered statistically significant. ADMSC: adipose-derived mesenchymal stromal cells; MF: microfragmented adipose tissue; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; OA: osteoarthritis

in MF-ADMSC ($p=0.0079$ and $p=0.0007$, respectively). Also, the metalloproteinase inhibitor TIMP-1 followed the same trend ($p=0.0007$), whereas MMP-9 was not detected.

3.3 | Metabolic activity of synoviocytes in coculture experiments

Synoviocytes alone or in coculture with MF or MF-ADMSCs maintained their morphology for up to 48 hr in all the experimental groups (data not shown). The MTT assay, which is directly related to mitochondrial activity, showed a significant increase with MF ($p=0.0003$) but not with MF-ADMSCs. (Figure 3).

3.4 | Effects of MF or MF-ADMSCs on synoviocytes secreted factors in coculture

Different secreted factors were tested after 48 hr of cocultures. As shown in Figure 4, in the coculture systems, the release of IL-6 inflammatory factor was significantly increased only by MF ($p=0.00143$), whereas CXCL8/IL-8 and CCL5/RANTES were not affected by both MF and MF-ADMSCs. IL-1 β and TNF- α were not detected under these conditions.

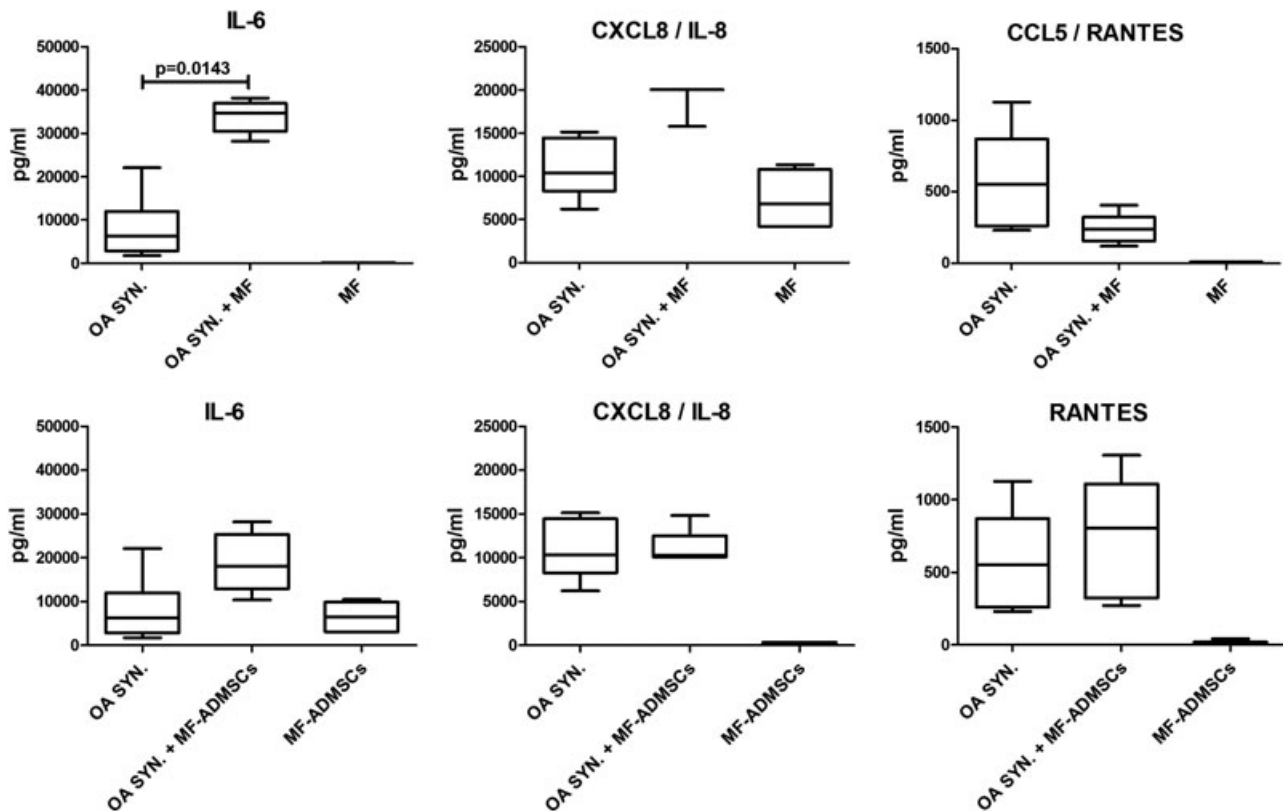


FIGURE 4 Effects of MF or MF-ADMSCs on inflammatory factors released by OA Passage 1 synoviocytes. Data are shown as boxes and whiskers. Lines inside the boxes represent the median, and whiskers represent the minimum and maximum values. Data are analyzed with the Kruskal-Wallis test and Dunn's post hoc test for unpaired data, and $p \leq 0.05$ is considered statistically significant. ADMSC: adipose-derived mesenchymal stromal cells; MF: microfragmented adipose tissue; OA: osteoarthritis

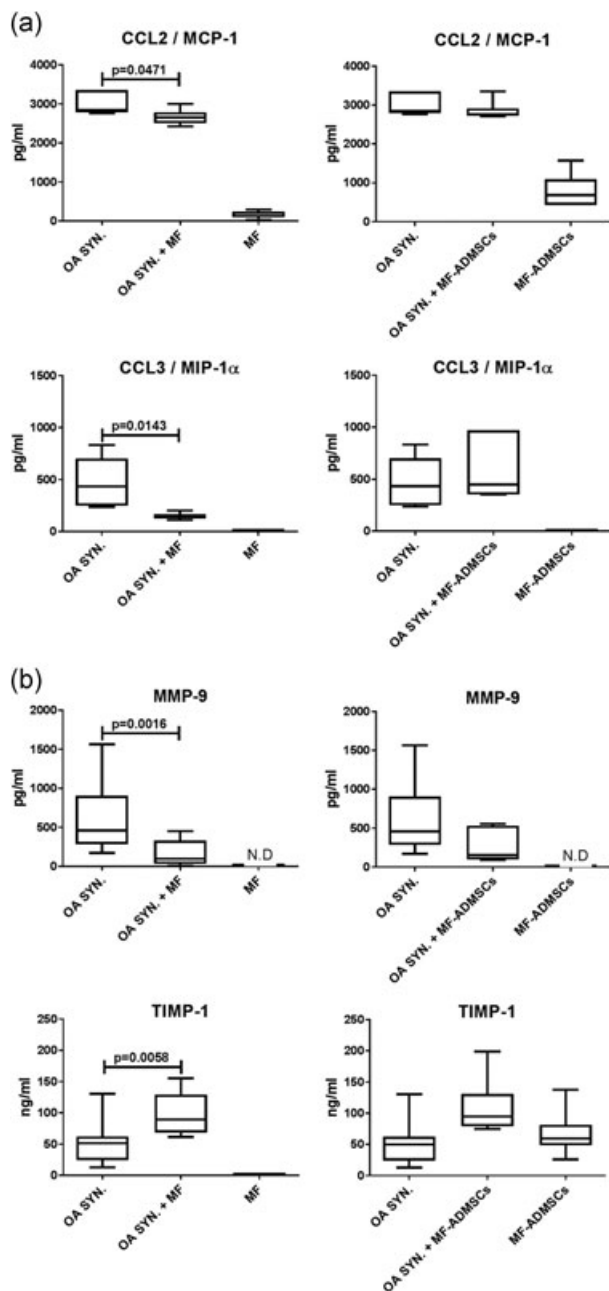


FIGURE 5 Effects of MF or MF-ADMSCs on macrophage factors released by OA Passage 1 synoviocytes. (a) CCL2/MCP-1 and CCL3/MIP-1α released from OA Passage 1 synoviocytes cocultured with MF or MF-ADMSCs. (b) MMP-9 and its specific inhibitor TIMP-1 released from OA Passage 1 synoviocytes cocultured with MF or MF-ADMSCs. Data are shown as boxes and whiskers. Lines inside the boxes represent the median, and whiskers represent the minimum and maximum values. Data are analyzed with the Kruskal–Wallis test and Dunn’s post hoc test for unpaired data, and $p \leq 0.05$ is considered statistically significant. ADMSC: adipose-derived mesenchymal stromal cells; MF: microfragmented adipose tissue; OA: osteoarthritis

3.5 | Effect of MF or MF-ADMSCs on synovial macrophage factors in cocultures

To investigate the effect of both cocultures on SM, the production of factors mainly associated with macrophage functions was analyzed,

including CCL2/MCP-1, CCL3/MIP-1α, and MMP-9. We found that after 48 hr of coculture, the levels of CCL2/MCP-1, CCL3/MIP-1α, and MMP-9 decreased significantly only when synoviocytes were cocultured with MF ($p = 0.0471$, $p = 0.00143$, and $p = 0.0016$, respectively; Figure 5a,b). Interestingly, the release of TIMP-1, a specific inhibitor of MMP-9 (Figure 5b), was significantly increased only by MF ($p = 0.0058$).

3.6 | TLR4 receptor and NFκB pathways involved in anti-inflammatory effects

To assess the effects of MF and MF-ADMSCs, we evaluated the gene expression of proteins involved in regulating OA synoviocytes inflammatory signaling: TLR4 receptor and some key molecules involved in the NFκB pathways. As shown in Figure 6, we found that MF significantly reduced the gene expression of TLR4 receptor. Moreover, on analyzing the NFκB pathway, we evidenced that RelA was not modulated by either MF or MF-ADMSC, whereas NFκB1 and the NFκB signaling inhibitor NFκBIA were significantly decreased by both MF and MF-ADMSC ($p = 0.0313$, $p = 0.0313$, $p = 0.0499$, and $p = 0.0032$, respectively). Interestingly, the ratio between NFκB1 and NFκBIA showed a significant increase ($p = 0.0499$) only when synoviocytes were treated with MF, mainly because of the reduction of NFκBIA. These proteins being NFκB target genes, the level of their gene expression is strictly connected with the level of inflammation (Oeckinghaus & Ghosh, 2009). Collectively, these results suggest that the anti-inflammatory effects observed were strictly dependent on both TLR4 and NFκB signaling pathways.

3.7 | Effect of MF or MF-ADMSCs on synoviocytes anti-inflammatory factors in cocultures

Anti-inflammatory factors HA and IL-10, measured in the supernatants for both cell coculture systems, showed that only HA was significantly increased by MF ($p = 0.0143$) and not affected by MF-ADMSCs. In contrast, IL-10 was not modulated by either MF or MF-ADMSCs (Figures 6,7).

4 | DISCUSSION

The emerging field of regenerative medicine has identified adipose tissue as an abundant source of stromal cells for tissue engineering applications. Studies have shown that ADMSCs demonstrates immunomodulatory, proangiogenic, antiapoptotic, anti-inflammatory, antifibrotic, proliferative, and chemoattractant positive effects both in vitro and in vivo on chondrocytes and synoviocytes (Desando et al., 2013; Djouad et al., 2009; Hoogduijn et al., 2010; Manferdini et al., 2013; Maumus et al., 2013; ter Huurne et al., 2012). The clinical use of ADMSCs requires their expansion in vitro and production in GMP condition, which could be avoided by alternative methods without enzymatic digestion. Recently, a new device has been proposed based on the use of mechanical forces

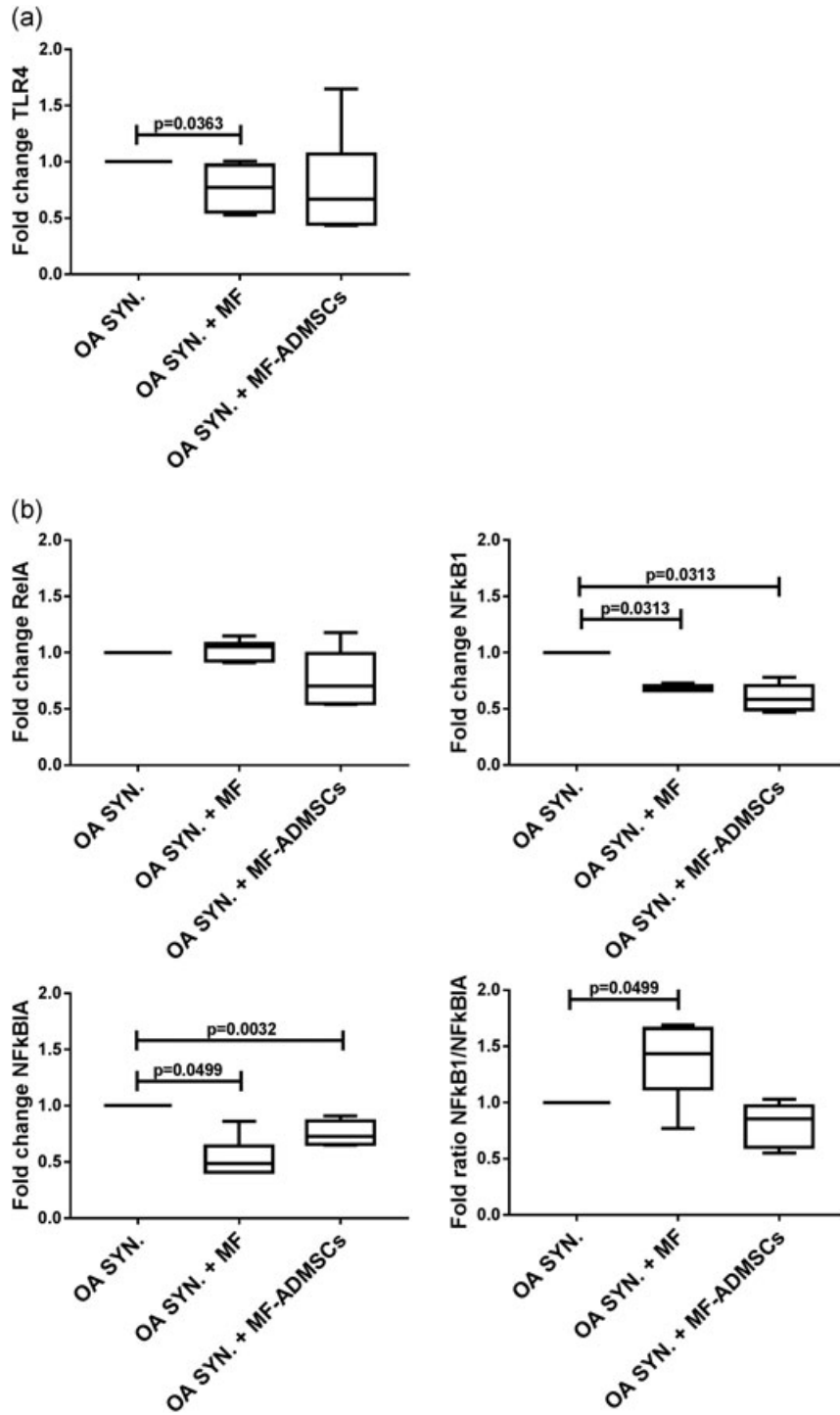


FIGURE 6 Effects of MF or MF-ADMSCs on TLR4 receptor and NFκB pathways on OA Passage 1 synoviocytes. (a) TLR4 gene expression of OA synoviocytes cocultured with MF or MF-ADMSCs. (b) RelA, NFκB1, and NFκB inhibitor; NFκBIA genes expression of OA synoviocytes cocultured with MF or MF-ADMSCs. Data are expressed as fold change of mRNA (messenger RNA) expression respect to OA synoviocytes alone (considered equal to one). Data are represented as boxes and whiskers. Lines inside the boxes represent the median, and whiskers represent the minimum and maximum values. ADMSC: adipose-derived mesenchymal stromal cells; MF: microfragmented adipose tissue; OA: osteoarthritis

for the production of a microfragmented purified adipose tissue graft (MF) that is ready to use and does not require cell expansion. It has been shown that MF obtained with this methodology well preserves the stromal vascular fraction and the stem cell niche (Tremolada, Colombo, & Ventura, 2016).

To define and compare the potential of MF with that of ADMSCs isolated from a definite amount of MF, their effects were compared on OA synoviocytes. OA synoviocytes at Passage 1, previously characterized as *in vitro* model (because it well recapitulates the characteristics of inflamed tissue; Manferdini et al., 2013; Manferdini

et al., 2016), were used to define the paracrine effects of MF and MF-ADMSCs by means of a transwell system. The release of inflammatory factors from MF and the same number of MF-ADMSCs obtained from 0.1 gr/ml of MF was analyzed, showing that MF produced lower levels of inflammatory IL-6, CCL5/RANTES, CCL2/MCP-1, CCL3/MIP-1 α , and only a higher level of CXCL8/IL-8, compared with MF-ADMSCs isolated from the same sample. The phenotypical characterization of MF and MF-ADMSCs evidencing the positive expression of classical mesenchymal markers (CD44, CD73, CD90, CD105 and CD166) is already well defined (Bianchi et al., 2013;

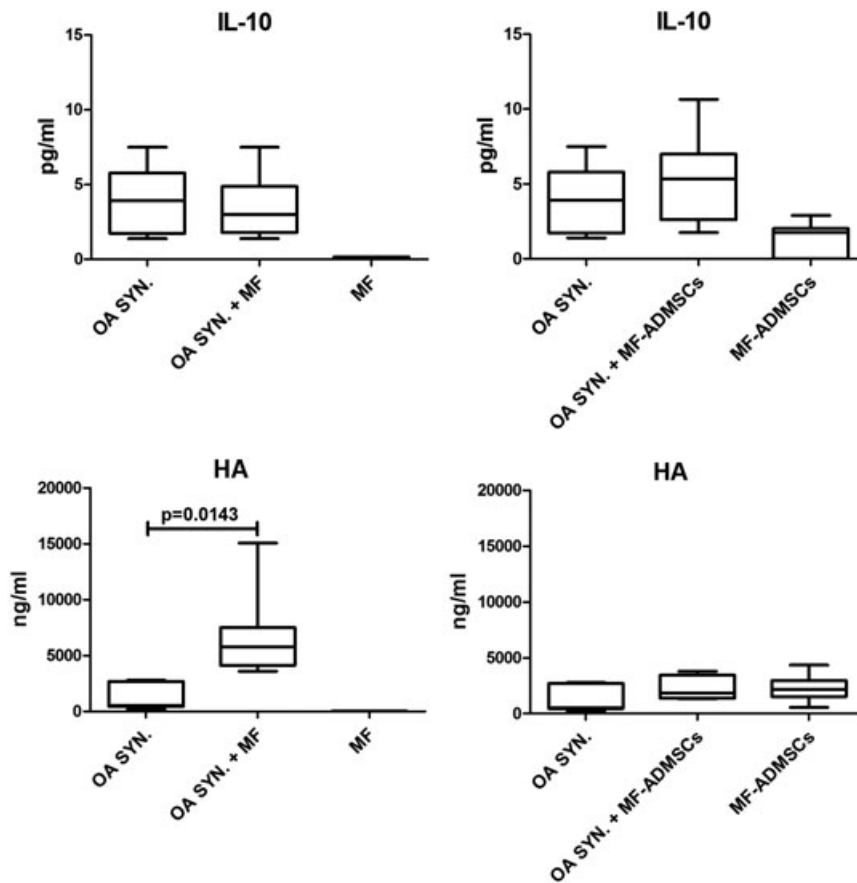


FIGURE 7 Effect of anti-inflammatory factors on OA synoviocytes cocultured with MF or MF-ADMSCs. Data are shown as boxes and whiskers. Lines inside the boxes represent the median, and whiskers represent the minimum and maximum values. Data are analyzed with the Kruskal-Wallis test, and Dunn's post hoc test for unpaired data and $p \leq 0.05$ is considered statistically significant. ADMSC: adipose-derived mesenchymal stromal cells; MF: microfragmented adipose tissue; OA: osteoarthritis

Carelli et al., 2015), whereas only less data are available on inflammatory factors. These findings, in line with Marfia et al. (2016), confirmed that MF produced lower levels of basal inflammatory factors compared with unprocessed lipoaspirate, confirming that MF could be a good alternative to lipoaspirate or MF-ADMSCs. It is important to note that MF-ADMSCs were derived from shaken MF and not produced in GMP conditions (as used in our previous studies; Manferdini et al., 2013; Manferdini et al., 2015; Maumus et al., 2013) that could have determined a higher release of inflammatory factors. Interestingly, the fresh MF used in this study did not release classical inflammatory factors IL-1 β and TNF- α , whereas they were detected in frozen MF (Marfia et al., 2016). These results confirm the importance of fresh MF for clinical use (Fantasia & Santos Cortes, 2016; Russo et al., 2017; Striano et al., 2015).

In contrast to what has been previously reported (Marfia et al., 2016), in our study, MF released lower amounts of anti-inflammatory IL-10 and HA, compared with MF-ADMSCs, which is probably due to different experimental conditions. Moreover, fresh MF did not produce MMP-9 degradative factors, but it produced lower levels of their inhibitor TIMP-1, whereas another study reported a higher amount of MMP-9 in conditioned medium obtained from MF after 5 days of culture (Ceserani et al., 2016). It is well documented that soluble factors from MSCs provide local signals to stimulate anti-inflammatory, antiapoptotic, and antifibrotic effects through paracrine mechanisms, as also reported for lipoaspirate (Doornaert et al., 2012; Randelli et al., 2016).

This study evidenced that coculturing Passage 1 synoviocytes for 48 hr with MF or MF-ADMSCs did not affect their morphology. In contrast, MF significantly increased their metabolic activity. These results are in line with other reports (Bosetti et al., 2016; Randelli et al., 2016) using a different cell model, that also did not evidence morphological changes but instead increased cell proliferation.

Previous studies have demonstrated that SM are mainly responsible for synovial inflammation condition (Bondeson et al., 2010; Han, Lee, Seong, & Lee, 2014), which is fundamental for priming MSCs to exert anti-inflammatory effect (Manferdini et al., 2013; Manferdini et al., 2015). However, no evidence has been

TABLE 2 Level of factors released by OA synoviocytes at Passage 1

Factors	Synoviocytes (pg/ml/10 ⁵ cells)
IL-6	6,292 (1,765–22,101)
CXCL8/IL-8	10,355(6,214–15,136)
CCL5/RANTES	552 (231–1,127)
CCL2/MCP-1	2,849 (2,765–3,354)
CCL3/MIP-1 α	433 (236–833)
IL-10	3.92 (1.4–7.49)
HA	532,000 (195,000–2,769,000)
MMP-9	459 (171–1564)
TIMP-1	49,700 (12,900–130,300)

Note. Data are expressed as median with range (min–max). OA: osteoarthritis.

previously described with MF containing a niche with MSCs (Randelli et al., 2016; Tremolada, Colombo et al., 2016). In this coculture system, the release of IL-6, and not CXCL8/IL-8, was significantly increased only by MF, whereas CCL5/RANTES showed a decreasing trend by MF and was not affected by MF-ADMSCs. Interestingly, this study demonstrated that MF reduced the release of SM-specific chemokines, such as CCL2/MCP-1 and CCL3/MIP-1 α . In line with these data, Ceserani et al. (2016), who applied a monocytic cell line U937, also confirmed the down-modulation of CCL2/MCP-1 and CCL5/RANTES using conditioned medium from MF.

In our study, we demonstrated that the anti-inflammatory effects of MF were determined by a decrease of TLR4 receptor and down-modulation of the NF κ B signaling pathway, as suggested by a decreasing expression of the NF κ B1 (p105 subunit) and of its inhibitor (NF κ BIA). Because these proteins are also NF κ B target genes (Oeckinghaus & Ghosh, 2009), our results indicate that MF attenuates NF κ B activation.

Interestingly, MF decreased the production of the degradative MMP-9 factor compared with MF-ADMSCs, reaffirming a specific effect on SM cell population. These data are also supported by the evidence that MF was able to induce at the same time TIMP-1-specific MMP-9 inhibitor. It is well known that TIMPs contribute to counteract MMPs activity by regulating their release during inflammatory conditions. In fact, macrophage-derived MMP-9 and its inhibitor TIMP-1 play an important role in re-establishing the tissue homeostasis (Bondeson et al., 2010; Russell et al., 2002). Finally, with regard to anti-inflammatory factors, HA, but not IL-10, was significantly increased only by MF, reaffirming a positive effect of MF and its potential to induce an anti-inflammatory effect in light of its use in the clinical setting to address OA.

In conclusion, to our knowledge, this is the first study of its kind that systematically compares the same number of ADMSC in both MF and MF isolated cells, demonstrating that fresh MF has the potential of counteracting macrophages factors inducing specific inhibitors. The use of MF eliminates the need for GMP as well as reduces expansion time and relative costs, which represents an important parameter for clinical use. MF well preserves the stromal vascular fraction and the stem cell niche, maintaining the biologically intact structure of cell-cell junctions and preserving the basal ECM proteins, as demonstrated with ADMSC cell sheet (Hamdi et al., 2011) could represent an alternative therapy to counteract inflammation. In this current study, MF-ADMSCs were only partially effective, as previously demonstrated using GMP-ASC, probably due to a less standardized isolation procedure and culture condition that could have interfered with their potential. All in all, MF seems to have good properties that could be applied to various inflammatory conditions or pathologies, such as OA, which requires to switch off macrophage activity for adequate therapeutic treatment.

ACKNOWLEDGMENTS

F. Paoletta and G. Lisignoli conceived and designed the study; F. Paoletta, C. Manferdini, E. Gabusi, and L. Gambari performed the

experiments; F. Paoletta and C. Manferdini collected and acquired data; F. Paoletta and G. Lisignoli interpreted data and drafted the article; G. Lisignoli, E. Kon, G. Filardo, and E. Mariani critically revised the manuscript. All the authors approved the final version of the manuscript. All authors are acknowledged for their contribution to the study, and we thank Patrizia Rappini for her assistance in the preparation of the manuscript. This study was supported by Funds "5 per mille" and "Ricerca corrente Rizzoli Orthopaedic Institute" under contract grant number Italian Ministry of Health RF-2011-02352638.

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

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How to cite this article: Paoletta F, Manferdini C, Gabusi E, et al. Effect of microfragmented adipose tissue on osteoarthritic synovial macrophage factors. *J Cell Physiol*. 2018;1-12. <https://doi.org/10.1002/jcp.27307>