

# Osteoarthritis and Cartilage



## Adipose stromal cells mediated switching of the pro-inflammatory profile of M1-like macrophages is facilitated by PGE2: *in vitro* evaluation

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### SUMMARY

**Objective:** To define if adipose mesenchymal stromal cell (ASC) treatment mediated switching of the pro-inflammatory profile of M1-like macrophages as a means to develop a tailored *in vitro* efficacy/potency test.

**Design:** We firstly performed immunohistochemical analysis of CD68, CD80 (M1-like) and CD206 (M2-like) macrophages in osteoarthritic (OA) synovial tissue. ASC were co-cultured in contact and in transwell with activated (GM-CSF + IFN $\gamma$ )-M1 macrophages. We analyzed IL1 $\beta$ , TNF $\alpha$ , IL6, MIP1 $\alpha$ /CCL3, S100A8, S100A9, IL10, CD163 and CD206 by qRT-PCR or immunoassays. Prostaglandin E2 (PGE2) blocking experiments were performed using PGE2 receptor antagonist.

**Results:** In moderate grade OA synovium we did not always find a higher percentage of CD80 with respect to CD206. M1-like-activated macrophage factors IL1 $\beta$ , TNF $\alpha$ , IL6, MIP1 $\alpha$ /CCL3, S100A8 and S100A9 were down-modulated both in contact and in transwell by ASC. However, in both systems ASC induced the typical M2-like macrophage markers IL10, CD163 and CD206. Activated-M1-like macrophages pre-treated with PGE2 receptor antagonist failed to decrease secretion of TNF $\alpha$ , IL6 and to increase that of IL10, CD163 and CD206 when co-cultured with ASC confirming a PGE2 specific role.

**Conclusions:** We demonstrated that ASC are responsible for the switching of activated-M1-like inflammatory macrophages to a M2-like phenotype, mainly through PGE2. This evidenced that activated-M1-like macrophages may represent a relevant cell model to test the efficacy/potency of ASC and suggests a specific role of ASC as important determinants in therapeutic dampening of synovial inflammation in OA. © 2017 The Authors. Published by Elsevier Ltd on behalf of Osteoarthritis Research Society International. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

### Introduction

Osteoarthritis (OA) is now generally accepted as an inflammatory rheumatic disease<sup>1</sup>. Synovitis has been shown to play a role in pathophysiology of OA<sup>2–4</sup>, specifically in promoting cartilage

destruction and pain<sup>3,5–7</sup>. Synovial tissue from OA patients evidenced clear signs of inflammation and infiltrating immune cells, which could be important determinants in OA severity and progression<sup>2,8</sup>. It has been described that macrophages, T cells and mast cells, are the most frequent immune cell types in synovial tissue<sup>8,9</sup>. The grading of synovial inflammation is mainly based on a rank that evaluates three characteristics of the synovial tissue: hyperplasia of the synovial lining layer, inflammatory infiltrate and stromal cell density<sup>10</sup>. Macrophages represent the cell population mainly located in the synovial lining layer that significantly increase in number with the increase of synovial inflammation grading from low to high. We have shown that macrophages are

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key effector cells in the synovial tissue<sup>11</sup> and it has been shown that these cells guide synovial inflammation<sup>12</sup>. Depletion of synovial macrophages in an OA mouse model reduces osteophyte formation and cartilage degeneration, typical features of OA progression<sup>13</sup>.

Two different subsets of macrophages have been described, the “classically activated” GM-Mφ or M1 macrophages (positive for CD80, CD86) that have a pro-inflammatory phenotype (TNF $\alpha$ , IL1 $\beta$  and IL6), and the “alternatively activated” M-Mφ or M2 macrophages (positive for CD163 and CD206) with an anti-inflammatory phenotype (IL10, IL1RA and TGF $\beta$ ) that are involved in tissue remodeling<sup>14–16</sup>. Classical macrophage activation requires priming with IFN $\gamma$ , the canonical cytokine generated by Th1 cells<sup>17</sup>. Alternatively activated macrophages are usually activated by Th2 cytokines IL4 and/or IL13<sup>17,18</sup>.

The presence of CD68 positive macrophages has been described in different studies in synovial tissue and their role in OA inflammation and progression is well defined<sup>19</sup>. Moreover, it is known that inflammatory M1-like cytokines are important determinants of destructive process that occur in cartilage tissue, mainly due to an increase of metalloproteinases (MMP3, MMP9, MMP13) that decrease aggrecan, collagen type II and favor osteophyte formation<sup>20</sup>. However, few studies have described the presence of both M1- and M2-like macrophages in OA synovial tissue and they have shown a large variation in the level of expression of their specific markers but without any specific focus on their functions<sup>21,22</sup>.

Mesenchymal stromal cells (MSCs) have an immunoregulatory function *in vitro* and *in vivo* and this feature suggests clinical application in the regulation of immunocompetent cell responses<sup>23,24</sup>. MSC enhanced repair or limited tissue destruction by paracrine secretion or cell to cell contact that modulated inflammation<sup>25–28</sup>. Recently, it has been shown that intra-articular injection of adipose derived stromal cells (ASCs) in OA mouse and rabbit models exert anti-inflammatory and chondroprotective effects<sup>29–31</sup> as well as in human<sup>32</sup>. MSC in culture secrete a large number of chemokines, cytokines and growth factors and some of these factors enhanced conversion of macrophages to an anti-inflammatory phenotype<sup>33–35</sup>. Prostaglandin E2 (PGE2) is a lipid mediator derived from the conversion of arachidonic acid to the prostaglandin through COX1 and COX2 enzyme action<sup>36</sup> and it is known to play a role in MSC-mediated immunoregulation. In an inflammatory environment these enzymes, with PGE2, are increased in MSC and it has been shown in several studies that PGE2 is an effector MSC molecule able to reprogramme M1-like to M2-like macrophages<sup>25,37–39</sup>.

We have focused our study on synovial tissue graded as moderate, firstly evaluating the pattern of expression of M1- and M2-like macrophages subsets with respect to the total macrophage population. Then, we have defined which macrophage subset was mainly modulated by anti-inflammatory action of ASC treatment, that we have previously described<sup>40,41</sup>. In particular, we tested in co-culture experiments if the effects were dependent on soluble factors and/or cell to cell contact. Finally, we have defined the involvement of PGE2 in macrophage subset polarization state and resolution of inflammation.

## Methods

### Patient characterization

Synovial tissues were obtained from 12 OA patients (seven women and five men; mean age: 64  $\pm$  10 years; body mass index: 28  $\pm$  4.5 kg/m<sup>2</sup>; disease duration: 7  $\pm$  4.8 years; Kellgren/Lawrence grade 3 or 4)<sup>42</sup>, undergoing total knee replacement surgery.

Subcutaneous abdominal fat was obtained from six healthy patients (four women and two men; mean age: 42.9  $\pm$  4.3 years) undergoing liposuction, as previously described<sup>43</sup>.

Human monocytes were isolated from the buffy coats of eight healthy donors, obtained from the Rizzoli Orthopaedic Institute, using a Ficoll-Hypaque density gradient.

The study was approved by the Rizzoli Orthopaedic Institute Ethical Committee and all patients provided their informed consent (Protocol number LIRT/ADIPOA2).

### Evaluation of macrophages in OA synovium

Synovial tissue specimens, with moderate grade OA<sup>10</sup>, were fixed in B5 solution (freshly prepared 9:1 mixture of mercuric-chloride/40% formaldehyde) at room temperature for 2 h and embedded in paraffin. Serial tissue sections (4  $\mu$ m thick) of each specimen were prepared and incubated overnight at 4°C with monoclonal anti-human-CD68 (10  $\mu$ g/ml; Dako Cytomation, Denmark), -CD80 (5  $\mu$ g/ml; Gentex Inc., Irvine CA, USA) and -CD206 (5  $\mu$ g/ml; Abcam, Cambridge, UK) diluted in TBS containing 0.1% bovine serum albumin (BSA) and developed as previously described<sup>11</sup>. Semi-quantitative analysis of immunohistochemistry stained slides was performed on 20 microscopic fields (20 $\times$  objective lens) for each section. The analysis was performed using Red/Green/Blue (RGB) with Software NIS-Elements and the Eclipse 90i microscope (Nikon Instruments Europe BV). Briefly, the total number of blue-stained nuclei and the total number of positive-stained red cells was acquired for each field. The data were expressed as percentage of positive cells for CD68, CD80 and CD206, respectively.

Synovial tissue specimens (from three cases) were digested using collagenase type I and recovered cells were analyzed by flow cytometry (FACSCanto 2, Becton Dickinson Italia, Milan) for the following markers: CD68, CD80, CD86, CD163 and CD206, as previously reported<sup>11</sup>.

### Human monocyte isolation and differentiation

CD14+ monocytes were isolated from human peripheral blood mononuclear cells (PBMC) using anti-CD14-coated magnetic beads (Miltenyi Biotec GmbH), followed by MACS LS column separation according to the manufacturer's recommendations. CD14+ monocytes obtained from peripheral blood were cultured for 5 days in RPMI containing 10% FBS, 1 mM L-alanyl-L-glutamine (Gluta-MAXTM, Gibco1, Life Technologies), 1 mM nonessential amino acids, 1 mM sodium pyruvate and 1 mM kanamycin sulfate, and supplemented with either 50 ng/ml granulocyte-macrophage colony-stimulating factor (GM-CSF, Novartis Pharma, Arnhem, The Netherlands) or 50 ng/ml macrophage colony-stimulating factor (M-CSF, R&D Systems, Inc., Minneapolis, MN) to obtain macrophages with proinflammatory (GM-CSF-M1, referred as M1-like) and tissue-remodeling anti-inflammatory (M-CSF-M2, referred as M2-like) activity<sup>15,44</sup>. Activated-M1-like macrophages were obtained by stimulating the M1-like macrophages with 10 ng/ml IFN $\gamma$  for 24 h. Both activated-M1- and M2-like macrophages were analyzed by flow cytometry (FACSCanto 2) using CD14, CD68, CD80, CD86, CD163 and CD206, as previously reported<sup>11</sup>.

CD14+ monocytes were also isolated from digested OA synovium using anti-CD14-coated magnetic beads (Miltenyi Biotec GmbH), as previously described<sup>45</sup>.

### Culture of ASCs and synovial fibroblasts (SFs)

ASCs were isolated from subcutaneous abdominal fat according to Good Manufacturing Practice (GMP)<sup>46</sup> and grown in  $\alpha$ MEM supplemented with platelet lysate (PLP) and characterized for the

CD markers CD14, CD34, CD45, CD73, CD90 and CD13 (eBioscience, San Diego, CA, USA) as previously described<sup>40,46</sup> (data not shown). SFs were isolated from OA synovium as previously described<sup>11</sup>.

#### Migration assay

ASC migration assays were performed using 8- $\mu$ m pore size HTS Transwell polycarbonate insert systems (Corning). Activated-M1-like, M2-like macrophages and SF cells were plated in the lower chamber at a density of  $5 \times 10^4$  cells/cm<sup>2</sup> and grown in culture for 24 h before starting the experiments.  $5 \times 10^4$  ASC were added to the upper chambers. PDGF-BB at a concentration of 50 ng/ml was added to the medium as a positive migration factor for ASC<sup>47</sup>. ASC were allowed to migrate through the membrane towards the stimulus during this incubation. After 18 h, the assay was blocked by removing the medium and the insert were washed with PBS. The cells that migrated through the membrane were stained with Calcein AM and finally fixed in 10% formaldehyde. The number of migrating ASC was quantified using fluorescent plate reader Synergy H1 – Hybrid reader (BioTek Instrument, Winooski, VT, USA). The fluorescence intensity of each experimental condition (in triplicate) was expressed as fold increase vs basal control, considered equal to 1.

#### ASC – macrophage co-cultures

Activated-M1-like macrophages ( $1 \times 10^5$  cells/well) were seeded in the lower chamber of a 24-well plate and co-cultured with clinical grade ASC ( $1 \times 10^5$  cells in contact or in Transwells<sup>®</sup>) for 48 h in incomplete DMEM using a defined cell ratio (1:1). Mono-cultures of ASC and activated-M1-like macrophages were used as controls. After 48 h co-culture the cells were harvested and analyzed for surface marker expression using flow cytometry (data not shown). RNA was extracted for gene expression analysis and supernatants were collected and stored at  $-80^\circ\text{C}$  for factors analysis.

#### Effects of exogenous PGE2 on activated-M1-like macrophages

Activated-M1-like macrophages were seeded in 12-well plates and treated or not with 400 pg/ml PGE2 (Sigma, Saint Louis, Missouri, USA) for 48 h, as previously reported<sup>40</sup>. Blocking experiments were performed by treating cells with PGE2 (400 pg/ml) in the presence or absence of 10  $\mu$ M PGE2 receptor EP4 antagonist (EP4; Sigma). When indicated, cells were pre-incubated with EP4 receptor antagonist (RA) for 30 min before the addition of PGE2. The cells were harvested for qRT-PCR analysis, and supernatant was stored at  $-80^\circ\text{C}$  for factors analysis.

#### Real-time qRT-PCR analysis

Total RNA was extracted from human ASC, M1-like, activated-M1-like macrophages in mono- and co-cultures, 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 SuperScript VILO (Life Technology) reverse transcriptase and random hexamers, following the manufacturer's protocol.

Forward and reverse oligonucleotides for PCR amplification of *IL1 $\beta$* , *IL6*, *IL10*, *IL1RA*, *TNF $\alpha$* , *CCL3/MIP1 $\alpha$* , *S100A8*, *S100A9*, *CD86*, *CD163*, *CD206*, *COX2* are described in Table I and real-time PCR was run and mRNA levels were calculated as previously described<sup>40</sup>.

#### Factors released in the supernatants

*IL1 $\beta$* , *CD163*, *PGE2* (R&D systems, Minneapolis, USA) and *S100A8*, *S100A9* (E Biosciences) concentrations were measured using sandwich ELISA. *IL6*, *IL10*, *CCL3/MIP1 $\alpha$*  and *TNF $\alpha$*  were analyzed using multiplex bead-based sandwich immunoassay kits (BioRad Laboratories Inc., Segrate, Italy) following the manufacturer's instructions.

#### Experimental design and statistical analysis

The ASC migration capacity was analyzed by Wilcoxon signed rank test (vs control = 1).

To define if M1-like macrophages were differently modulated in the absence or presence of ASC, we compared ASC group vs M1 groups (M1 mono-cultures combined with M1 in transwell and M1 in contact) using the non-parametric Mann–Whitney test. Then to verify if the presence of ASC influenced the effect of M1-like macrophages, we compared the M1 mono-cultures group with the ASC mono-cultures and with M1-like macrophages treated with ASC groups using the Kruskal–Wallis & Dunn's *post hoc* for unpaired data. Finally to define the effect of culture conditions (transwell and cell-contact), we compared the M1-like macrophages group with M1-like macrophages treated with ASC in transwell or contact using the Kruskal–Wallis & Dunn's *post hoc* for unpaired data.

To define if PGE2 was responsible for M1-like macrophages effects we compared M1-like macrophages in the presence of PGE2 with or without blocking the PGE2 receptor antagonist, using the non-parametric Mann–Whitney test with Bonferroni correction for four comparisons (M1-like macrophages vs M1-like macrophages treated with PGE2, M1-like macrophages vs M1-like macrophages treated with blocking PGE2 receptor antagonist, M1-like macrophages treated with blocking PGE2 receptor antagonist vs M1-like

**Table I**  
Oligonucleotide primers used for real-time PCR

Target gene	Primers (forward and reverse)	Product size (bp)	GenBank accession no.	Primer efficiency (%)
GAPDH	CGGAGTCAACGGATTGG CTGGAAGATGGTGATGG	218	NM_002046	99.2
IL1 $\beta$	CTGTCTCGCTGTGAAAG TGCTTGAGAGGTGCTGATG	177	NM_000576	98.1
IL6	TAGTGAGGAACAAGCCAGAG GCCAGAATGAGATGAGTTG	184	NM_000600	96.1
IL10	CITTAAGGGTTACCTGGGTTG CTTGATGCTGGGCTTTGG	100	NM_000572	99.7
IL1RA	ATGGAGGGAAGATGTGCCTGTC GTCTGCTTTCTGTTCTCGCTC	104	NM_173841.2 (V2)	94.4
TNF $\alpha$	AGCCATGTTGTAGCAAACC GGACCTGGAGTAGATGAGGTA	149	NM_000594	96.2
CCL3/MIP1 $\alpha$	ACCAGTCTCTGCATCACTT CTTGGTTAGGAAGATGACACC	146	NM_002983.2	99.5
S100A8	TAGAGACCGAGTGCTCTCA CGCCATCTTTATCACCAGA	126	NM_002964.4	93.4
S100A9	CCATCATCAACACCTTCCACCA CTGCTGTCTGCAITTTGTGTC	179	NM_002965.3	91.4
CD86	GGAACCAACAATGGAGAG AAACACGCTGGGCTTCATC	94	NM_006889	98.2
CD163	GCAATGGGGTGGACTTACCT TCACCATGCTTCACTCAACAC	126	NM_004244.5	99.1
CD206	TCGGGTTTATGGAGCAGGTG TGAACGGGAATGCACAGGT	121	NM_002438.3	96.6
COX2	CAGCACTTACCGCATCAGTTT GCGCAGTTTACGCTGTCTA	129	NM_000963	96.9



macrophages treated with both blocking PGE2 receptor antagonist and PGE2, M1-like macrophages treated with PGE2 vs M1-like macrophages treated with both blocking PGE2 receptor antagonist and PGE2). To define ASC effects on CD14+ synovial macrophages we used Friedman ANOVA & Dunn's *post hoc* for paired data. Values were calculated by GraphPad Prism 7 (GraphPad Software Inc., La Jolla, CA, USA) and expressed as the median and interquartile range or as mean with 95% confidence interval or as mean  $\pm$  SD depending on the distribution. Statistical analysis was performed using non-parametric tests since the data did not have a normal and strongly asymmetric distribution. All the statistical analyses were evaluated by Exact methods for small samples. CSS Statistica Statistical Software (Statsoft Inc., Tulsa, OK, USA) was used for the analysis and values of  $P < 0.05$  were considered statistically significant.  $N$  represents the number of cases used.

## Results

### Distribution of CD68, CD80, CD206 positive macrophages in OA synovial tissue

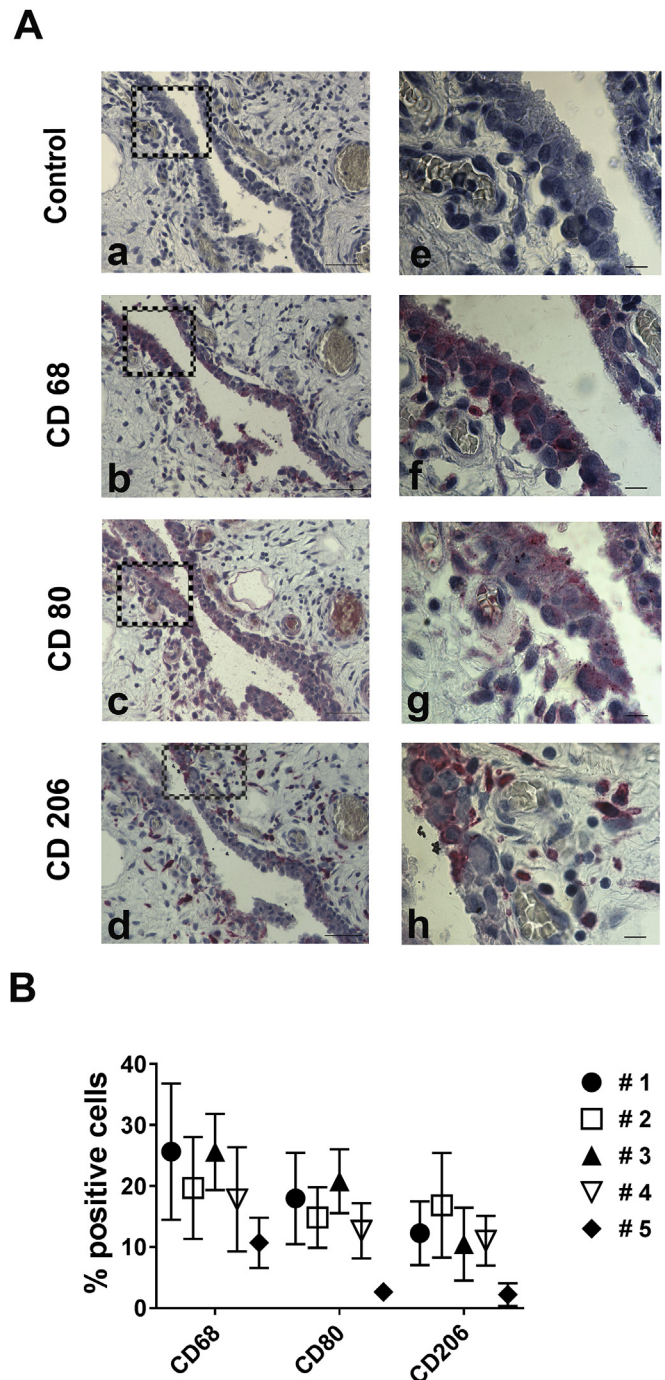
To identify the distribution of different macrophage subsets in OA synovial tissue with moderate grade synovitis, we evaluated the expression of CD68, CD80 and CD206 in five different cases (#1, #2, #3, #4, #5). Immunohistochemical analyses of CD68 positive cells confirmed their distribution in both lining layers, as previously reported<sup>11,19</sup>. CD80 as an M1-like and CD206 as an M2-like markers evidenced a characteristic distribution of these cells. M1-like macrophages were mainly located in the lining layer while M2-like macrophages were present in some areas only in the sublining, while in other areas were found both in the sublining and lining layers [Fig. 1(A)]. As shown in Fig. 1(B), in each case we found a high percentage of CD68 positive cells (with an average mean  $\pm$  SD of the five cases of  $20.12 \pm 8.06$ ). Interestingly, the percentage of CD80 (with an average mean  $\pm$  SD of the five cases of  $13.68 \pm 4.48$ ) and CD206 (with an average mean  $\pm$  SD of the five cases of  $10.57 \pm 5.13$ ) was higher for CD80 in two out of five cases compared to CD206, while in one case the percentage of CD206 was higher than that of CD80 and no differences were found in two cases. These data were confirmed by flow cytometry analysis on three digested synovial tissue samples. As shown in Supplementary Fig. 1 we found the comparable levels of CD68, CD80 and CD86 (typical M1-like markers) and CD163 and CD206 (typical M2-like markers) to that quantified using immunohistochemistry.

### Macrophages increase ASC migration

Migration assays were performed to determine if M1-like and M2-like macrophages influenced the migration of ASC. Firstly we characterized activated-M1-like and -M2-like macrophages by flow cytometry and found that both M1-like and M2-like were 100% positive for CD14 and CD68 (Supplementary Fig. 2). Moreover, we found that M1-like macrophages showed a high percentage of cells positive for CD80 and CD86 (approximately 95%) while M2-like macrophages were highly positive for CD163 and CD206 (approximately 82%). Both activated-M1-like and M2-like macrophages cells stimulated ASC migration (3 and 1.5 fold, respectively) compared to basal control, while SFs did not induce ASC migration as compared to a positive control (+PDGFBB) and to basal control (Fig. 2).

### Characterization of differentiated-M1-like vs activated-M1-like macrophages

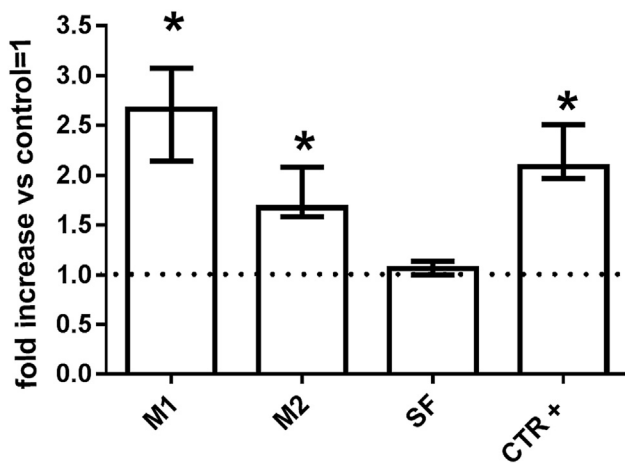
To characterize the differences between differentiated-M1- and activated-M1-like macrophages for establishing which cell model



**Fig. 1.** Characterization of synovial tissue in moderate grade OA synovial tissues. **A.** Immunohistochemical analysis of CD68, CD80 and CD206 in a representative sample. Bars a–d 50  $\mu$ m; e–h 10  $\mu$ m. Negative control (Control). **B.** Percentage of positive cells for CD68, CD80, CD206 in five representative cases with moderate grade synovitis. Data were expressed as mean  $\pm$  SD.

to use for further experiments, we evaluated gene expression of typical markers. As shown in Table II, IFN $\gamma$  used for activation of M1-like macrophages, induced an up-regulation of IL1 $\beta$ , MIP1 $\alpha$ , TNF $\alpha$ , COX2 and PGE2, and was associated with a down-regulation of CD163, CD206 and IL10. However, IL6, S100A8, S100A9, CD86 were not modified. In the following co-culture experiments we used activated-M1-like macrophages that better mimic a real inflammatory condition.

## ASC migration



**Fig. 2.** ASC migration to M1- and M2-like macrophages. ASC migration was tested after 18 h of co-culture with M1- and M2-like macrophages, SFs and positive control (culture medium with 50 ng/ml PDGF-BB) ( $N = 4$ ). Data are expressed as mean with 95% confidence interval of the fold increase vs control = 1. Statistical analysis was performed and  $^*P < 0.05$  was considered statistically significant using Wilcoxon signed rank test.

### ASC suppress M1-like macrophage factors and induce M2-like macrophage polarization

To assess the effect of ASC on the activated-M1-like macrophage phenotype we performed co-culture both in indirect (in transwell) and in direct (in contact) conditions. We selected activated-M1-like macrophages since preliminary co-culture experiments confirmed that differentiated-M1-like macrophages showed only a partial ability of ASC to down-modulate all inflammatory factors analyzed and were not able to induce M1-like macrophages switching (data not shown). As shown in Fig. 3(A), M1-like macrophages markers such as IL1 $\beta$ , IL6, TNF $\alpha$ , MIP1 $\alpha$ /CCL3, S100A8 and S100A9 expressed on activated-M1-like macrophages were significantly decreased by co-culturing them with ASC [Fig. 3(A)]. In particular, as evidenced

**Table II**  
Characterization of basal and IFN $\gamma$  activated-M1-like macrophages

	Basal (M1-like macrophages)	Activated-M1-like macrophages	P value
IL1 $\beta$ (% GAPDH)	0.39 $\pm$ 0.07	1.88 $\pm$ 0.20*	0.002
TNF $\alpha$ (% GAPDH)	0.13 $\pm$ 0.03	1.37 $\pm$ 0.27*	0.002
IL6 (% GAPDH)	0.16 $\pm$ 0.05	0.15 $\pm$ 0.02	0.807
MIP1 $\alpha$ /CCL3 (% GAPDH)	1.7 $\pm$ 0.6	5.09 $\pm$ 0.99*	0.006
S100A8 (% GAPDH)	1.35 $\pm$ 0.82	2.51 $\pm$ 0.96	0.126
S100A9 (% GAPDH)	5.26 $\pm$ 1.57	5.67 $\pm$ 1.86	0.512
IL10 (% GAPDH)	0.49 $\pm$ 0.27	0.10 $\pm$ 0.07†	0.002
CD86 (% GAPDH)	0.57 $\pm$ 0.46	0.65 $\pm$ 0.6	0.496
CD163 (% GAPDH)	11.24 $\pm$ 9.10	0.55 $\pm$ 0.28†	0.002
CD206 (% GAPDH)	87.68 $\pm$ 10	22.41 $\pm$ 8.36†	0.002
IL1RA (% GAPDH)	18.33 $\pm$ 1.61	23.21 $\pm$ 2.05*	0.003
COX2 (%GAPDH)	0.08 $\pm$ 0.015	0.24 $\pm$ 0.19*	0.003
PGE2 (pg/ml)	ND	29.23 $\pm$ 10.42*	0.003

Data are expressed as mean  $\pm$  SD. ND = not detected.

\* Up-regulated factors in activated-M1 ( $N = 12$ ) vs basal M1-like macrophages ( $N = 8$ ).

† Down-regulated factors in activated-M1 ( $N = 12$ ) vs basal M1-like macrophages ( $N = 8$ ).

by statistical analysis IL6, TNF $\alpha$ , MIP1 $\alpha$ /CCL3, S100A8 were decreased by co-culturing both in indirect or direct conditions. Although IL1 $\beta$  was decreased in both culture conditions, statistical significance was only noted in direct contact. By contrast, S100A9 was statistical significantly decreased only in direct contact. Interestingly, when we evaluated the same factors as released proteins [Fig. 4(A)] we found that all were decreased using both co-culture conditions.

IL10, CD163 and CD206 induction were significantly enhanced in activated-M1-like macrophages co-cultured with ASC both in transwell or direct contact [Fig. 3(B)]. In particular, statistical analysis evidenced that IL10 and CD163 were significantly up-regulated, whether co-cultured in indirect or direct conditions. By contrast, CD206 was statistical significantly increased only after indirect contact. As shown in Fig. 4(B) both IL10 and CD163 were also significantly up-regulated at protein level.

Moreover, to confirm the specific effect of ASC on M1-like macrophages, we also assessed if ASC modulated secretion of IL1 $\beta$ , TNF $\alpha$ , IL10 and CD163 by M2-like macrophages. As shown in Supplementary Fig. 3, ASC did not significantly affect these markers.

### PGE2 as ASC mediator of M2 polarization

To identify the ASC mediator of macrophage polarization, we firstly evaluated the release of PGE2 in activated-M1-like macrophages mono-cultures or co-cultured with ASC in direct or indirect conditions. We found that PGE2 was released in low amounts in M1-like macrophages mono-cultures (approximately 30 pg/ml) and in higher amounts in ASC alone (400 pg/ml). Interestingly when activated-M1-like macrophages were co-cultured with ASC we found a statistically significant increase in both co-culture conditions [Fig. 5(A)].

Then, to evaluate the effect of PGE2, we incubated activated-M1-like macrophages with 400 pg/ml PGE2 for 48 h; a concentration similar to that secreted by ASC<sup>40</sup>. As shown in Fig. 5(B–C) we found that exogenous PGE2 significantly decreased the expression and secretion of TNF $\alpha$  and IL6. By contrast, PGE2 increased the expression of IL10, CD163 and CD206 [Fig. 5(B–C)], both classical inducers of M2-like macrophage biomarkers.

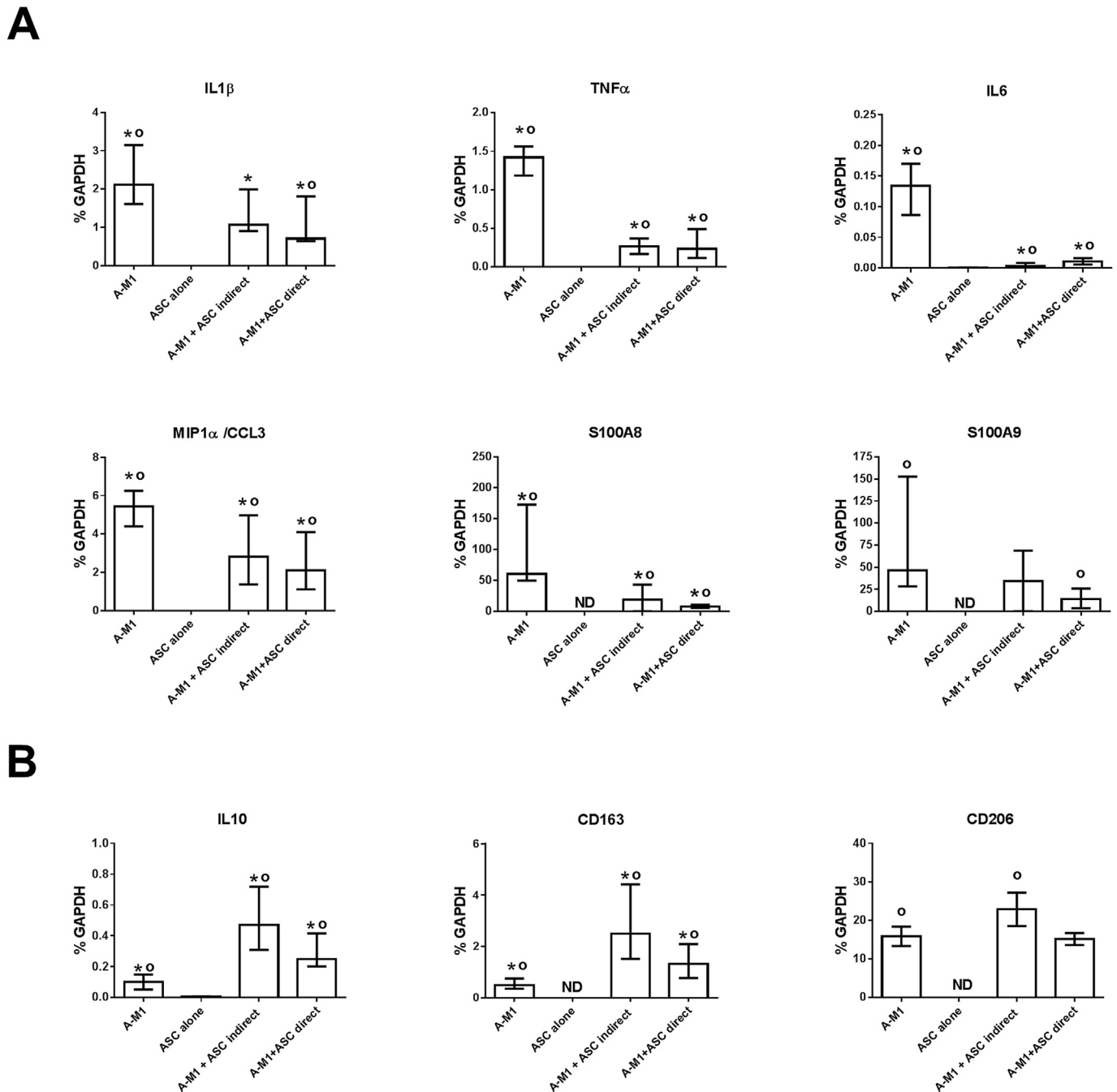
Blocking experiments, as shown in Fig. 5(B–C) showed that PGE2 failed to decrease secretion of TNF $\alpha$ , IL6 and to increase IL10, CD163 and CD206 when activated-M1-like macrophages were treated with PGE2 receptor antagonist (EP4). However, IL1 $\beta$ , MIP1 $\alpha$ /CCL3, S100A8 and S100A9 were not statistically significant modulated by PGE2 (data not shown).

### Involvement of PGE2/COX2 in CD14+ OA synovial macrophages

To confirm our data in an *ex vivo* model we tested PGE2 release and COX2 expression in isolated CD14+ macrophages from OA patient synovium. As shown in Fig. 6, we evidenced that ASC were responsible for the increase of PGE2 levels correlating with the decrease in COX2 expression.

## Discussion

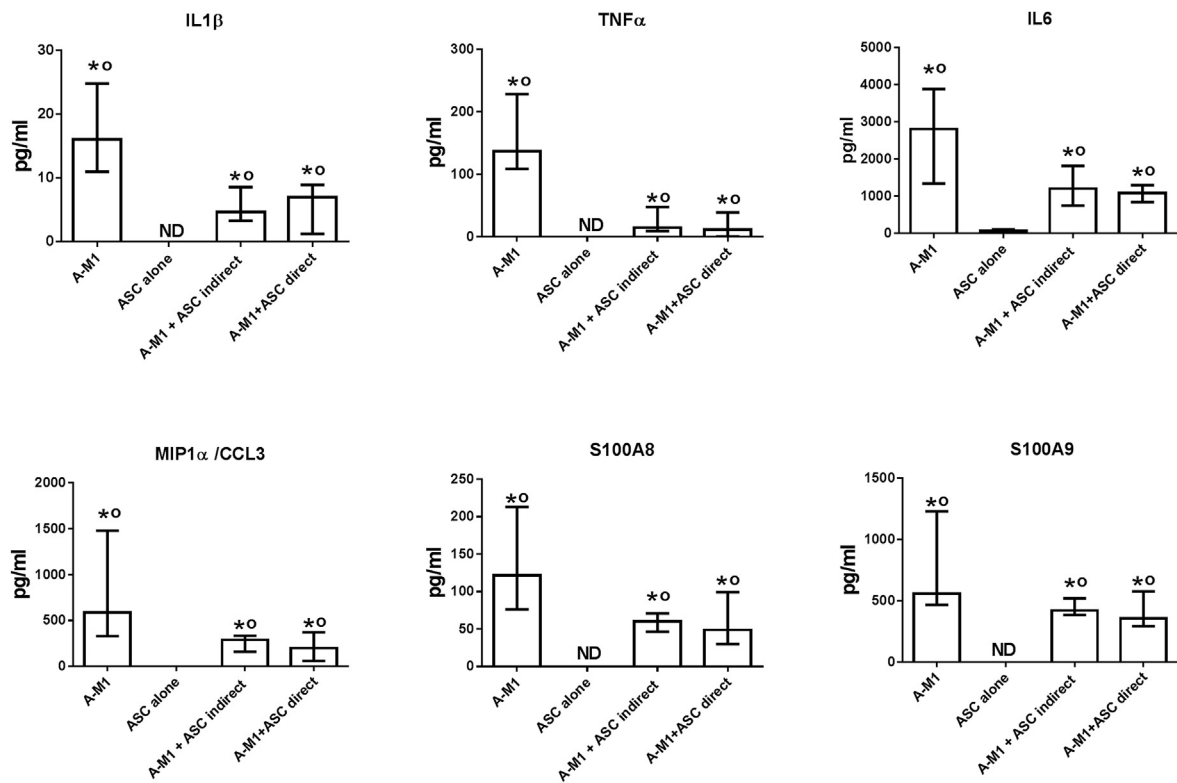
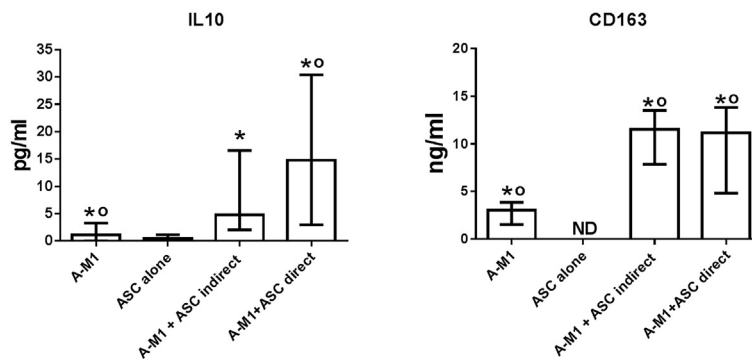
In OA severity and progression, it has been demonstrated that inflammation is mainly mediated by soluble factors and infiltrating immune cells. Macrophages are key effector cells in synovial tissue<sup>11</sup> significantly increasing in number and guiding synovial inflammation<sup>12</sup>. The presence of both M1 and M2 macrophages in human OA synovial tissue has been described in a few studies<sup>21,22</sup> where the focus has mainly been on the levels of their expression rather than on their function.



**Fig. 3.** Modulation of the expression of activated-M1-like factors by ASC co-cultured in transwell or in direct contact. **A.** The expression of IL1 $\beta$ , TNF $\alpha$ , IL6, MIP1 $\alpha$ /CCL3, S100A8, S100A9 was analyzed on activated-M1-like, ASC alone and on activated-M1-like co-cultured with ASC in transwell (A-M1 + ASC Indirect) or in contact (A-M1 + ASC Direct) ( $N = 10$ ). Data were expressed as median with 95% confidence interval. **B.** The expression of IL10, CD163, CD206 was analyzed on activated-M1-like, ASC alone and on activated-M1-like co-cultured with ASC in transwell (A-M1 + ASC Indirect) or in contact (A-M1 + ASC Direct) ( $N = 10$ ). Data were expressed as median with 95% confidence interval. Statistical analysis was performed with the Kruskal–Wallis & Dunn's *post hoc* for unpaired data, \* $P < 0.01$  indicates if the presence of ASC influenced the effect of M1-like macrophages by comparing the M1-like alone group with the ASC alone group and M1-like treated with the ASC group;  $^{\circ}P < 0.01$  indicates significant effects of culture conditions (transwell and cell-contact) by comparing M1-like group with M1-like treated with ASC in transwell group and M1-like treated with ASC in contact group. ND = not detected.

In this study we first analyzed the pattern of expression of *bona fide* M1-like (CD80 positive) and M2-like (CD206 positive) macrophage subsets with respect to the total macrophage population (CD68 positive) in OA synovial tissue with moderate grade synovitis, as reported by Krenn<sup>10</sup>. Immunohistochemical analysis demonstrated that the percentage of CD80 with respect to CD206-positive cells was not always higher in all cases evaluated, in line

with Fahy *et al.*<sup>21</sup> who also showed that the percentage of these cells varied from area to area. By contrast to CD80 positive cells, we showed that CD206 positive cells were located both in the lining and sublining layers suggesting that this distribution may be due to their different role in the tissue. In fact, as also reported by Mucke *et al.*<sup>48</sup>, we found that the number of CD68 positive cells decreased from lining to sublining layer, confirming their important role in

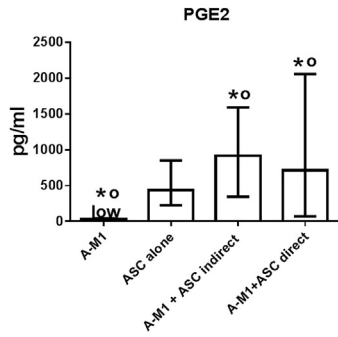
**A****B**

**Fig. 4.** Factors released by activated-M1-like macrophages modulated by ASC co-cultured in transwell or in direct contact. **A.** The release of IL1 $\beta$ , TNF $\alpha$ , IL6, MIP1 $\alpha$ /CCL3, S100A8, S100A9 was analyzed on activated-M1-like, ASC alone and on activated-M1-like co-cultured with ASC in transwell (A-M1 + ASC Indirect) or in contact (A-M1 + ASC Direct) ( $N = 10$ ). Data were expressed as median with 95% confidence interval. **B.** The release of IL10, CD163 was analyzed on activated-M1-like, ASC alone and on activated-M1-like co-cultured with ASC in transwell (A-M1 + ASC Indirect) or in contact (A-M1 + ASC Direct) ( $N = 10$ ). Data were expressed as median with 95% confidence interval. Statistical analysis was performed with the Kruskal–Wallis & Dunn's *post hoc* for unpaired data, \* $P < 0.005$  indicates if the presence of ASC influenced the effect of M1-like by comparing the M1-like alone group with the ASC alone group and M1-like treated with ASC group;  $^{\circ}P < 0.004$  indicates significant effects of culture conditions (transwell and cell-contact) by comparing M1-like group with M1-like treated with ASC in transwell group and M1-like treated with ASC in contact group. ND = not detected.

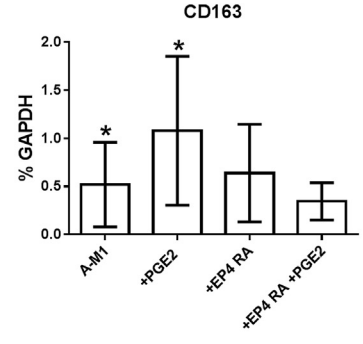
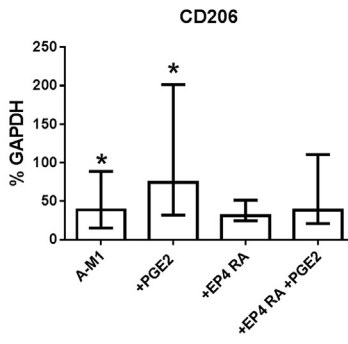
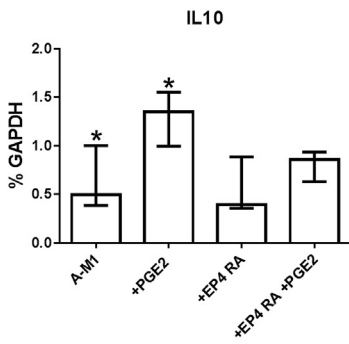
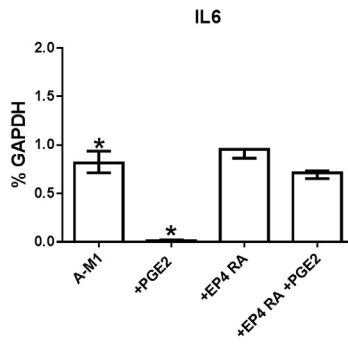
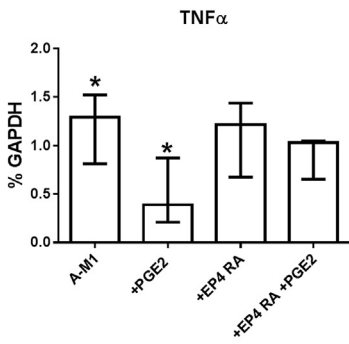
the inflammatory joint reaction<sup>49</sup>. It has been shown that macrophages polarization is strictly dependent on the factors used for their differentiation and activation<sup>15</sup>, therefore we firstly characterized differentiated-M1-like macrophages with activated-M1-like macrophages. We evidenced in activated-M1-like macrophages a high number of up-regulated factors (IL1 $\beta$ , TNF $\alpha$ , MIP1 $\alpha$ /CCL3, IL1RA, COX2 and PGE2), down-regulated factors typical of M2-like macrophages (CD163, CD206 and IL10) and non-

modulated factors like S100A8, S100A9 and IL6. This pattern was characteristic of IFN $\gamma$ -activated-M1-like macrophages, that showed an up-regulation of IL1RA and no modulation of IL6, by contrast to LPS-activated-M1-like macrophages<sup>25</sup>, evidencing their plasticity<sup>15</sup> and the importance of clearly describing the factors used to polarize monocytic cells. As such this cell model may represent a superior alternative to passage 1 OA synoviocytes<sup>11</sup> for testing the efficacy/potency of ASC. Activated-M1-like macrophage

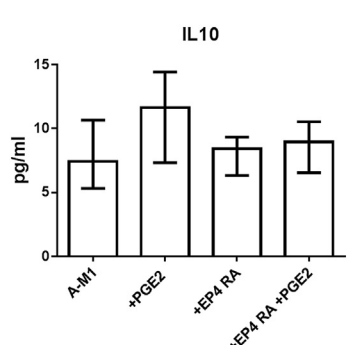
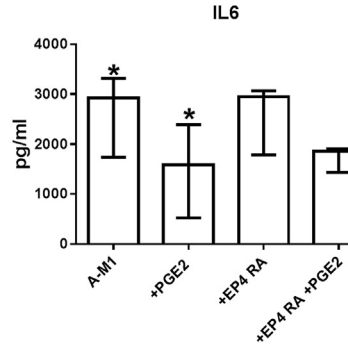
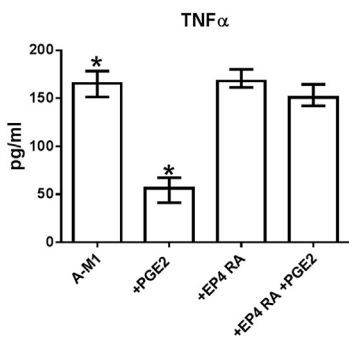
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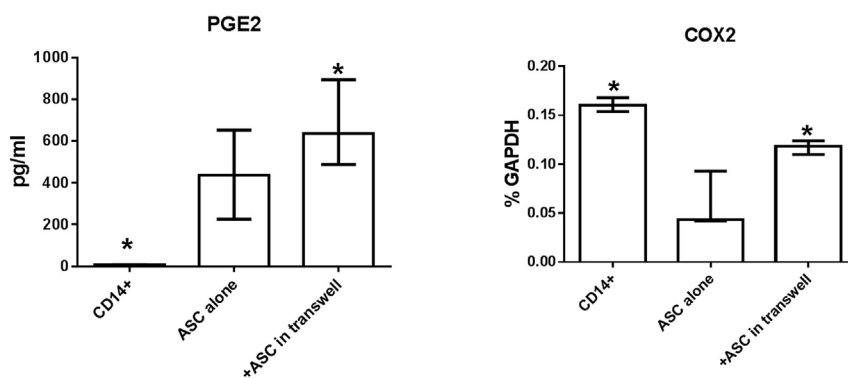
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**C**







**Fig. 6.** ASC modulation of PGE2 and COX2 on CD14<sup>+</sup> synovial macrophages. The release of PGE2 (pg/ml) and COX2 (% GAPDH) expression were evaluated on CD14<sup>+</sup> synovial macrophages obtained from OA patients, ASC alone and CD14<sup>+</sup> synovial macrophages co-cultured with ASC ( $N = 4$ ). Data were expressed as median with 95% confidence interval. \* $P < 0.05$  was considered statistically significant and indicates the differences between CD14<sup>+</sup> synovial macrophages alone and co-cultured with ASC groups using Friedman ANOVA & Dunn's *post hoc* for paired data.

subsets were more efficient in attracting ASC than M2-like macrophages suggesting that the inflammatory M1 milieu is preferred. Interestingly, OA SFs were unable to attract ASC confirming, as previously demonstrated<sup>11</sup>, a specific therapeutic action of ASC on macrophages. The ASC anti-inflammatory effects, found both at molecular and protein levels, were specifically against typical M1-like cytokines like IL1 $\beta$ , TNF $\alpha$  and IL6, as well as macrophage factors like MIP1 $\alpha$ /CCL3, S100A8 and S100A9. Our data, in line with a previous report<sup>37</sup>, showed that ASC at the same cell ratio (1:1) were also very efficient in reducing inflammation. The inhibition of inflammatory and macrophage markers (CD163, CD206) was similar whether mediated by soluble factors (culture in transwell) or by direct cell contact confirming that activated-M1-like macrophages might be used to evaluate the potency of ASC for therapeutic purposes. It has been previously shown that ASC injected in the joint of different OA animal models<sup>29,30</sup> reduce inflammatory and macrophage markers by specifically adhering to synovial macrophages. Our data suggest that ASC may modulate the synovium by release of factors as well as adhesion to resident cells and that the two different cell culture modalities could mimic different phases of ASC modulation after injection in the joint (firstly characterized by an interaction with soluble factors and then by adhesion to synovium). Interestingly, ASC reduced inflammation and were able to facilitate activated-M1-like macrophage reprogramming to an M2-like phenotype. Both co-culture conditions tested evidenced a statistically significant increased release of IL10 and CD163, as well as increased expression of CD163 and CD206, typical markers of M2-like macrophage phenotype as previously demonstrated using bone marrow MSC<sup>37</sup>. The up-regulation of CD206 in association with down-regulation of TNF $\alpha$  in LPS-activated M1-like macrophages is well described. We confirmed the modulation of the same factors by ASC co-cultured with IFN $\gamma$  activated-M1-like macrophages. Interestingly, IFN $\gamma$  activated-M1-like macrophages

expressed higher amounts of IL1RA, that was not modulated when co-cultured with ASC, as we previously found using OA passage 1 synoviocytes<sup>11</sup>, confirming that activated-M1-like macrophages are a cell model that may recapitulate the OA milieu.

Finally, we have defined the involvement of PGE2 in the IFN $\gamma$ -activated, M1-like macrophage subset polarization state and resolution of inflammation. PGE2 is responsible in part for the immunomodulatory role of MSC and the data presented suggests that this molecule may represent a regulatory checkpoint in an innate immune response. Our data demonstrate that PGE2, mainly produced by ASC, was directly responsible for inhibition of the inflammatory cytokines TNF $\alpha$  and IL6, as also reported by other studies<sup>37,50</sup> with bone marrow MSC, identifying this molecule as an important mediator of macrophage attenuation. By contrast, IL1 $\beta$ , MIP1 $\alpha$ /CCL3 and S100A9 inhibition was independent of PGE2 clearly suggesting the involvement of other factors. Interestingly, PGE2 as well as ASC were also able to induce production of the anti-inflammatory cytokine IL10 and expression of CD163 and CD206, typical of the M2-like macrophage phenotype.

PGE2 blocking experiments using the EP4 receptor antagonist confirmed the specificity of these effects in line with data reported by other authors<sup>37</sup>. Interestingly, the direct involvement of the PGE2/COX2 pathway was also confirmed on CD14<sup>+</sup> synovial macrophages, obtained from OA patients, corroborating our previous data<sup>40</sup>.

In conclusion, this study demonstrates a new role of ASC in switching activated-M1-like inflammatory macrophages to a M2-like phenotype, partially through the PGE2/COX2 pathway as occurred with isolated CD14<sup>+</sup> OA synovial macrophages. This data supports a specific role for ASC in therapeutic resolution of OA synovial inflammation and also clearly provided evidence that IFN $\gamma$  activated-M1-like macrophages are a good cell model to test the efficacy/potency of ASC.

**Fig. 5.** Effects of PGE2 released by ASC as a mediator of M2-like macrophage polarization. **A.** the release of PGE2 was analyzed on activated-M1-like macrophages, ASC alone and on activated-M1-like co-cultured with ASC in transwell (A-M1 + ASC Indirect) or in contact (A-M1 + ASC Direct) ( $N = 8$ ). Data were expressed as median with 95% confidence interval. Statistical analysis was performed with the Kruskal–Wallis & Dunn's *post hoc* for unpaired data, \* $P < 0.002$  indicates if the presence of ASC influenced the effect of M1-like by comparing the M1-like alone group with the ASC alone group and M1-like treated with ASC group; \* $P < 0.001$  indicates significant effects of culture conditions (transwell and cell-contact) by comparing M1-like group with M1-like treated with ASC in transwell group and M1-like treated with ASC in contact group. **B.** TNF $\alpha$ , IL6, IL10, CD163 and CD206 expression was analyzed on activated-M1-like, treated with PGE2 (400 ng/ml) with or without PGE2 receptor antagonist (EP4) ( $N = 6$ ). Data were expressed as median with 95% confidence interval. Statistical analysis was performed, \* $P < 0.01$  indicates the differences between groups using the non-parametric Mann–Whitney test with Bonferroni correction for four comparisons. **C.** TNF $\alpha$ , IL6 and IL10 release was analyzed on activated-M1-like, treated with PGE2 (400 ng/ml) with or without PGE2 receptor antagonist (EP4) ( $N = 6$ ). Data were expressed as median with 95% confidence interval. Statistical analysis was performed, \* $P < 0.01$  indicates the differences between groups using the non-parametric Mann–Whitney test with Bonferroni correction for four comparisons.

### Author contributions

CM, GL conceived and designed the study; CM, FP, EG, AP, LG performed the experiments; GF and SF-C collected and acquired data; CM, GL interpreted data and drafted the article; AB, MM, GL critically revised the manuscript. All the authors approved the final version of the manuscript.

### Conflict of interest

The authors have nothing to disclose.

### Role of funding source

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### Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.joca.2017.01.011>.

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