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Lack of anti-inflammatory and anti-catabolic effects on basal inflamed osteoarthritic chondrocytes or synoviocytes by adipose stem cell-conditioned medium

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SUMMARY

Objective: To define whether good manufacturing practice (GMP)-clinical grade adipose stem cell (ASC)-derived conditioned medium (CM) is as effective as GMP-ASC in modulating inflammatory and catabolic factors released by both osteoarthritis (OA) chondrocytes or synoviocytes.

Methods: OA chondrocytes and synoviocytes were treated with ASC-CM or co-cultured with ASC. Inflammatory factors (IL6, CXCL1/GROα,CXCL8/IL8, CCL2/MCP-1, CCL3/MIP-1α and CCL5/RANTES) and proteinases, such as metalloproteinase (MMP13), a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS4, ADAMTS5) and their tissue metalloproteinase inhibitors (TIMP1, TIMP3) were evaluated by qRT-PCR or immunoassays. The involvement of prostaglandin E2 (PGE2) was also analyzed.

Results: Most ASC-CM ratios tested did not decrease IL6, CCL2/MCP-1, CCL3/MIP-1-α, CCL5/RANTES on basal inflamed chondrocytes or synoviocytes in contrast to what we found using ASC in co-culture. CXCL8/IL8 and CXCL1/GROα were not decreased by ASC-CM on synoviocytes but were only partially reduced on chondrocytes. Moreover, ASC-CM was less efficient both on basal inflamed OA chondrocytes and synoviocytes in reducing proteinases, such as MMP13, ADAMTS4, ADAMTS5 and increasing TIMP1 and TIMP3 compared to ASC in co-culture. The different ratios of ASC-CM contain lower amounts of PGE2 which were not sufficient to reduce inflammatory factors.

Conclusions: These data show that ASC-CM has a limited ability to decrease inflammatory and proteinases factors produced by OA chondrocytes or synoviocytes. ASC-CM is not sufficient to recapitulate the beneficial effect demonstrated using ASC in co-culture with inflamed OA chondrocytes and synoviocytes and shows that their use in clinical trials is fundamental to counteract OA progression.

Introduction

Mesenchymal stem cell (MSC)-based therapy is a new emerging clinical strategy that holds great promise for treating immune, hematological disorders, cardiovascular diseases, and cancer and for regenerative medicine[1–4].

MSC from adult donors obtained from different sources (bone marrow, adipose tissue etc.)[5,6] are considered to be among the most promising candidate cell types in regenerative medicine applied to rheumatic diseases such as osteoarthritis (OA)[7–9]. In particular, adipose stem cell (ASC) as well as MSC shows the minimal criteria

provided by the International Society for cellular therapy for defining MSC, by displaying an immunophenotype positive for CD73, CD90, CD105, and negative for CD3, CD14, CD45, CD31[10]. Moreover, in contrast to MSC, ASC express CD34, which is lost during the early phase of culture[11]. They exert their function using different pathways, not yet completely understood, that contribute to augmenting tissue regeneration. Their effects are mainly due, at least in part, to the "trophic" function of ASCs, characterized by the production of a large amount of secreted factors, such as chemokines, cytokines and growth factors, which are able to influence the behavior of the other cells[12,13]. In particular, it has been shown that ASC-secreted factors exert immunomodulatory, anti-inflammatory, anti-apoptotic, pro-angiogenic, proliferative, or chemoattractive effects[13–16]. Early studies using MSCs injected into animal models of OA well documented that they were able to orchestrate the differentiation process together with differentiated or undifferentiated resident cells for functional tissue restoration[17–19]. Moreover, in osteoarticular diseases, it has been shown that the evolution of OA in animal models might be prevented by intra-articular injections of bone marrow MSC or their delivery on a degradable hyaluronan scaffold[20–22]. Recent studies have confirmed that ASC injected intra-articularly in OA mice or rabbit models also showed anti-inflammatory and chondroprotective effects[23,24].

A body of evidence suggests that pro-inflammatory cytokines (IL1β, TNFα, IL6), chemokines (CXCL1/GROα, CXCL8/IL8, CCL2/MCP-1, CCL5/RANTES), metalloproteinases (MMP1, 3, 13) disintegrins and metalloproteinase with thrombospondin motifs (ADAMTS4, ADAMTS5) produced by different cell types of the joint tissues, are released in the synovial fluids of OA patients and contribute to the disruption of the balance between anabolism and catabolism[25–27], thereby causing progressive destruction of articular cartilage, changes to the synovium, subchondral bone, degeneration of ligaments, and menisci, and hypertrophy of the joint capsule[28].

New alternative therapies to counteract OA progression and restore joint tissue features are needed. The clinical use of these cells requires ASC production under good manufacturing practice (GMP) as well as the evaluation of their biosafety and purity[29,30]. We previously showed that GMP-clinical grade ASC in vitro were able to exert anti-inflammatory effects on chondrocytes and synoviocytes[31] and protect chondrocytes from degeneration[32], a typical feature associated with OA.

Cell-free-based therapy might overcome the limitations and risks associated with the cell-therapy approach. However, comparative studies are necessary to establish the potentiality of using conditioned medium (CM) from ASCs instead of ASC in OA cell-based treatment.

Knowing that ASC preparation is influenced by medium factors used[32], to gain new insight into this issue, we evaluated the effects of clinical grade GMP-ASC-CM on both OA chondrocytes and synoviocytes and compared these results with our previously published data. We focused our analysis on the main catabolic and inflammatory factors involved in the progression of OA. Our results clearly show that ASC-CM was less effective than ASC in reducing inflammatory and catabolic factors secretion by both on OA inflamed chondrocytes and synoviocytes.

Method

Specimens

Articular cartilage and synovia were harvested from 25 OA patients (14 women and 11 men; mean age: 69 ± 10.5 years; body mass index (BMI): 28 ± 4.04; disease duration: 5 ± 3.08 years, Kellgren–Lawrence: 3/4)[33] undergoing total knee replacement. Subcutaneous abdominal fat was obtained from 10 healthy patients undergoing liposuction. The study was approved by the local ethical committee and all patients provided their informed consent (Protocol number 15274).

Isolation of human ASC and ASC-CM preparation

Clinical grade ASCs were isolated from subcutaneous abdominal fat according to GMP[30] grown in αMEM supplemented with platelet lysate (PLP) and characterized for the CD markers CD14, CD34, CD45, CD73, CD90 (BD Pharmingen, San Jose, CA, USA) and CD13 (eBioscience, San Diego, CA, USA) as we previously described[30,31] (data not shown).

ASC-CM was prepared by collecting the supernatant, on day 2 and day 7, of ASC seeded at a concentration of 100,000/well in Transwells® (0.4 μm pore size, Corning, Toledo, OH, USA) in DMEM with ascorbic acid (0.17 mmol/L), proline (0.35 mol/L) and sodium pyruvate (1 mol/L) (complete DMEM), previously defined[31], and stored at 4°C before use.

Chondrocyte and synoviocyte cultures

Chondrocytes and synoviocytes were isolated following a standardized procedure previously described[34,35] and used for the experiments at the first passage. Chondrocytes and synoviocytes were seeded in the lower chamber of a 6-well plate and both treated for 7 days (medium was changed on day 2) with ASC-CM or co-cultured with ASC in Transwells® in complete DMEM using a defined cell ratio (1:8) as we previously reported[31]. ASC-CM (obtained by pooling the supernatant of day 2 and 7 to have all factors produced by ASC during the co-culture experiments) was mixed with DMEM at three different ratios (DMEM:CM 75:25, 50:50, 25:75) and added to chondrocytes or synoviocytes seeded in a 6-well plate. Control cells were mono-cultures of ASC, chondrocytes and synoviocytes. The cells were harvested on day 7 for RT-qPCR analysis and supernatant stored at −80°C. Co-cultures of chondrocytes in micromasses were also tested to confirm the results in monolayers. Briefly, chondrocytes in micromass culture were inserted in the lower chamber of a 6-well plate, maintaining the same 1:8 cell ratio and time point, and co-cultured with ASC as previously described[31].

Real time RT-qPCR analysis

Total RNA was extracted from human ASC, chondrocyte and synoviocyte mono- and co-cultures, using RNA PURE reagent (EuroClone S.p.A., Pero, Italy) according to the manufacturer's instructions, and then treated with DNase I (DNA-free Kit, Ambion, Austin, TX, USA). 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 IL6, CXCL8/IL8, ADAMTS4, ADAMTS5, TIMP1, TIMP3 and MMP13 are described in Table I. Real-time PCR was run in a LightCycler Instrument (Roche Molecular Biochemicals, Mannheim, Germany) using the SYBR Premix Ex Taq (TaKaRa Biomedicals, Tokyo, Japan) with the following protocol: initial activation of HotStarTaq DNA polymerase at 95°C for 10 min, 45 cycles of 95°C for 5 s and 60°C for 20 s. Amplification efficiency (E) of each amplicon was determined using 10-fold serial dilutions of positive control cDNAs and calculated from the slopes of the log input amounts (from 20 ng to 2 pg of cDNA) plotted vs 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 I). For each target gene, mRNA levels were calculated, normalized to RPS9 according to the formula

Table I
Oligonucleotide primers used for real-time PCR

Target gene	Primers (forward and reverse)	Product size (bp)	GenBank accession n°	Primer efficiency (%)
RPS9	GATTACATCCTGGGCCTGAA ATGAAGGACGGGATGTTCAC	161	NM_001013	94.5
IL6	TAGTGAGGAACAAGCCAGAG GCGCAGAATGAGATGAGTTG	184	NM_000600	96.1
CXCL8/IL8	CCAAACCTTTCCACCC ACTTCTCCACAACCCT	153	NM_000584	97.8
ADAMTS4	CTGCCTACAACCACCG GCAACCAGAACCGTCC	293	NM_005099.4	99.1
ADAMTS5	GCACTTCAGCCACCATCAC AGGCGAGCACAGACATCC	187	NM_007038.3	92.4
TIMP1	CGGTTCGTCTACACCC CACAAGCAATGAGTGCC	266	NM_003254.2	94.8
TIMP3	GGTCGCGTCTATGATGG CAGGCGTAGTGTTTGG	236	NM_000362.4	100.1
MMP13	TCACGATGGCATTGCT GCCGGTGTAGGTGTAGA	277	NM_002427	94.5

$2^{-\Delta Ct}$ and expressed as a percentage of the reference gene since expressed in the same amount in all conditions tested.

Immunoassays

The concentrations of IL6, CXCL1/GROα, CXCL8/IL8, CCL2/MCP-1, CCL3/MIP1-α and CCL5/RANTES were simultaneously evaluated on day 7 using multiplex bead-based sandwich immunoassay kits (BioRad Laboratories, Segrate, Italy) following the manufacturer's instructions.

Prostaglandin E2 (PGE2), MMP13, TIMP1 and TIMP-3 were measured using ELISA assays (R&D Systems, Minneapolis, MN, USA), according to the manufacturer's instructions. ADAMTS4 and ADAMTS5 were detected with the ELISA kit from Uscn life Science Inc. (Wuhan Hubei, China).

Statistical analysis

Statistical analysis was performed using mainly non-parametric tests since the data did not have a normal and strongly asymmetric distribution (Friedman ANOVA & Dunn's *post hoc* for paired data, Kruskal–Wallis & Dunn's *post hoc* for unpaired data and Mann–Whitney *U* test for unpaired two-group data). Values were expressed as the median and interquartile range. CSS Statistica Statistical Software (StatSoft Inc., Tulsa, OK, USA) was used for analysis and values of $P < 0.05$ were considered significant.

Results

Effects of different concentrations of ASC-CM on chondrocytes and synoviocytes

To establish the effects of ASC-CM, three different ratios of ASC-CM (DMEM:CM 75:25, 50:50, 25:75) were tested on both chondrocytes and synoviocytes and the expression of the main inflammatory and catabolic factors (proteinases) involved in OA progression were evaluated. In Fig. 1(A) the expression levels of IL6 and CXCL8/IL8 are shown. IL6 was not modulated on chondrocytes or synoviocytes and there were no notable differences between the ASC-CM ratios tested. A similar trend was also observed for the chemokines, CCL2/MCP-1, CCL3/MIP1-α and CCL5/RANTES (data not shown). Conversely, CXCL8/IL8 was significantly down-modulated in chondrocytes only by the 50:50ASC-CM ratio, as occurred for CXCL1/GROα (data not shown), whereas it was not modulated by any of the different ASC-CM ratios on synoviocytes.

Interestingly, the analysis of ASC-CM ratio on proteinases and inhibitor [Fig. 1(B)] markers show the same significant differences in chondrocytes and synoviocytes. In particular ADAMTS5 was significantly inhibited on both chondrocytes and synoviocytes only by the 50:50 ASC-CM ratio, whereas TIMP1 was not affected on either cell type. Based on these results, we chose the 50:50 ASC-CM:DMEM ratio to perform the subsequent tests.

ASC-CM exerts partial anti-inflammatory effects on chondrocytes but not on synoviocytes

In our previous report[30] we established that ASCs were able to reduce the release of inflammatory factors only when ASC were co-cultured with chondrocytes or synoviocytes producing high level of inflammatory factors (IL6 and CXCL8/IL8 not lower than 400 pg/ml and 300 pg/ml, respectively for chondrocytes and not lower than 200 pg/ml and 100 pg/ml, for synoviocytes, respectively). We therefore tested the effects of ASC-CM only on chondrocytes and synoviocytes matching these characteristics. As shown in Fig. 2(A) and (C), and in contrast to ASC in co-culture, the effects of ASC-CM were limited to a decrease of CXCL8 and CXCL1/GROα, whereas it showed no effect on the other factors analyzed: furthermore, the effect was limited to chondrocytes. Co-culture experiments with chondrocytes in micromasses were also performed to confirm our data in monolayers. As shown in Fig. 2(B), the inhibition of CXCL8, but not IL6, by ASC-CM was also confirmed in this 3D culture system.

ASC-CM effect on metalloproteinases and inhibitors

To establish whether ADAMTSs, TIMPs and MMP13 were also modulated by ASC-CM treatment or ASC co-culture, we tested their expression on both chondrocytes and synoviocytes at mRNA and protein level. As shown in Fig. 3(A) in chondrocytes, ACS-CM significantly induced the expression of MMP13 and reduced ADAMTS5, whereas ASC in co-culture significantly reduced the expression of TIMP3 and MMP13. At the protein level [Fig. 3(B) and (C)], ASC-CM had no effects on the proteinase markers analyzed except for TIMP1 that was down-modulated, whereas in co-culture we found a significant decrease of ADAMTS4, ADAMTS5 and MMP13 and an increase of TIMP1.

As shown in Fig. 4(A), ASC-CM increased the expression of ADAMTS4 and decreased ADAMTS5 expression but it did not affect MMP13, TIMP1 and TIMP3 in treated synoviocytes. Conversely, ASC in co-culture with synoviocytes significantly increased TIMP-1
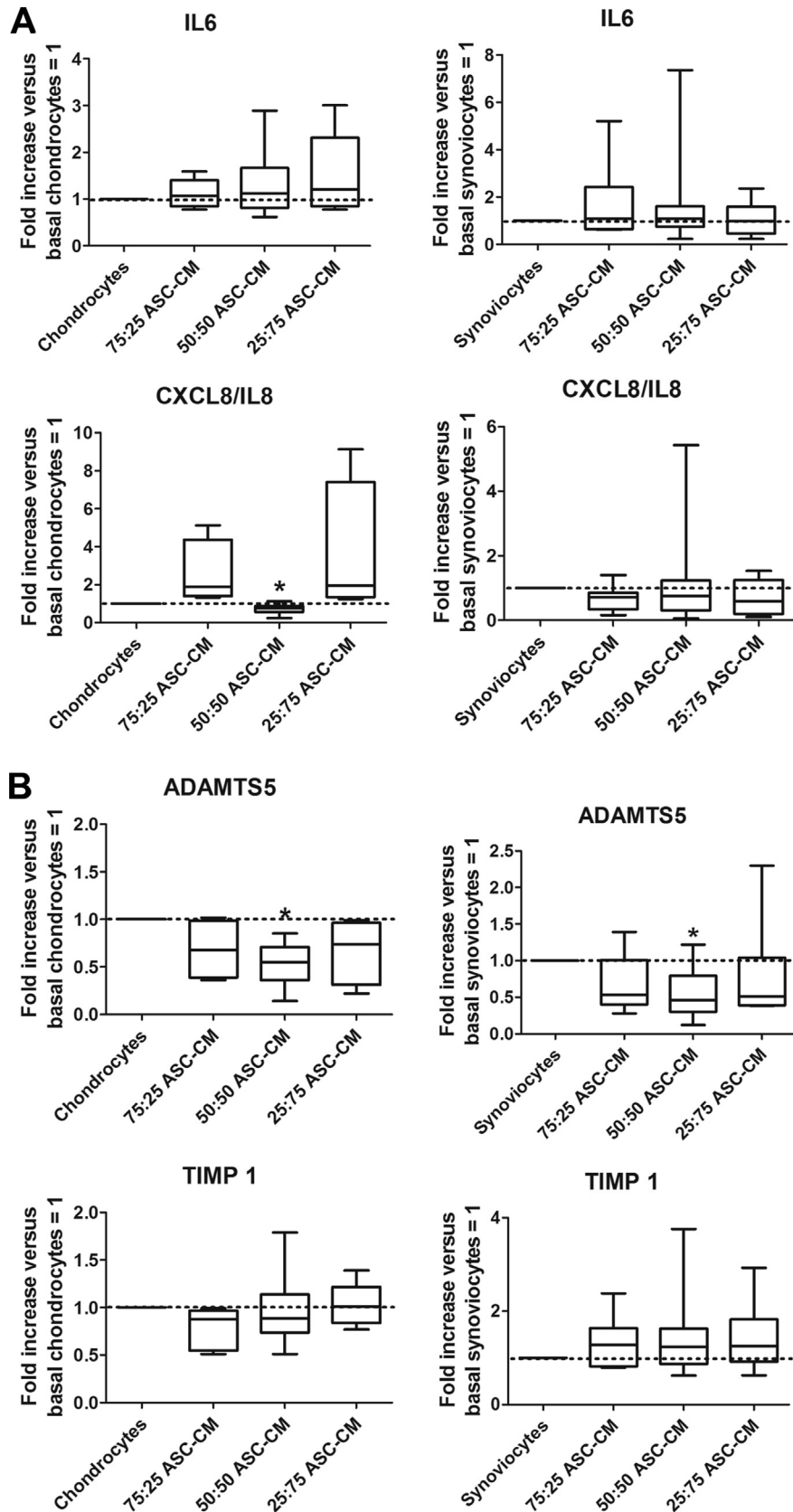
Fig. 1. Effects of different ratios of ASC–CM (DMEM:CM 75:25, 50:50, 25:75) both on chondrocytes ($n = 5$) and synoviocytes ($n = 5$). **A.** Gene expression of IL6 and CXCL8/IL8 tested on chondrocytes or synoviocytes treated with three different ratio of ASC–CM. **B.** ADAMTS5 and TIMP1 gene expression of chondrocytes or synoviocytes treated with different ratios of ASC–CM. Data show fold increase mRNA expression vs basal chondrocytes or synoviocytes (median with interquartile range). Significant differences vs basal *$P < 0.05$.

expression, decreased ADAMTS5 and did not affect ADAMTS4, TIMP3 or MMP13. At protein level [Fig. 4(B) and (C)], ASC-CM induced only the release of TIMP-1 and did not affect the other factors. Conversely, ASC in co-culture significantly decreased the release of ADAMTS4 and 5, increased TIMP-1 and did not affect TIMP3 and MMP13.

PGE2 involvement

We previously showed in co-culture experiments[26] that PGE2 produced by ASC was directly involved in the anti-inflammatory effects through its receptor EP4. Since we found limited anti-inflammatory effects of ASC-CM we analyzed PGE2 levels in
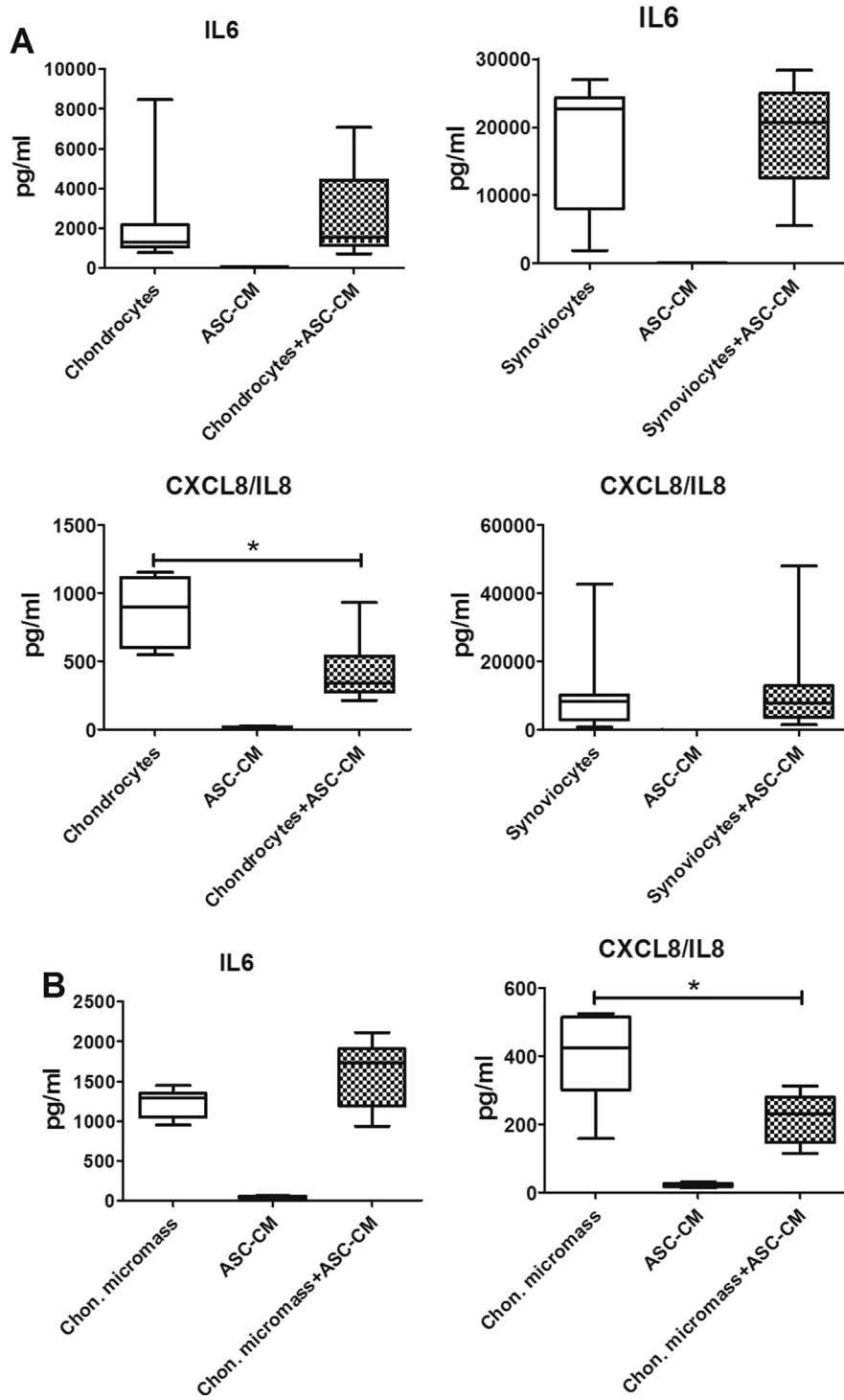

Fig. 2. ASC-CM effects on IL6 and CXCL8/IL8 chemokines released by chondrocytes ($n = 8$) and synoviocytes ($n = 12$). **A.** IL6 and CXCL8/IL8 (pg/ml) detected in chondrocytes or synoviocytes monoculture alone or treated with ASC-CM. Significant differences *$P < 0.001$. **B.** IL6 and CXCL8/IL8 (pg/ml) detected in chondrocytes micromass alone or treated with ASC-CM. Data are expressed as median with interquartile range. Significant differences * $P = 0.0175$. **C.** ASC-CM effects on CCL2/MCP-1,CCL3/MIP-1α,CCL5/RANTES and CXCL1/GROα chemokines released by chondrocytes ($n = 8$) and synoviocytes ($n = 12$). CCL2/MCP-1,CCL3/MIP-1α,CCL5/RANTES and CXCL1/GROα (pg/ml) detected in chondrocytes or synoviocytes alone or treated with ASC-CM. Data are expressed as median with interquartile range. Significant differences *$P < 0.001$.
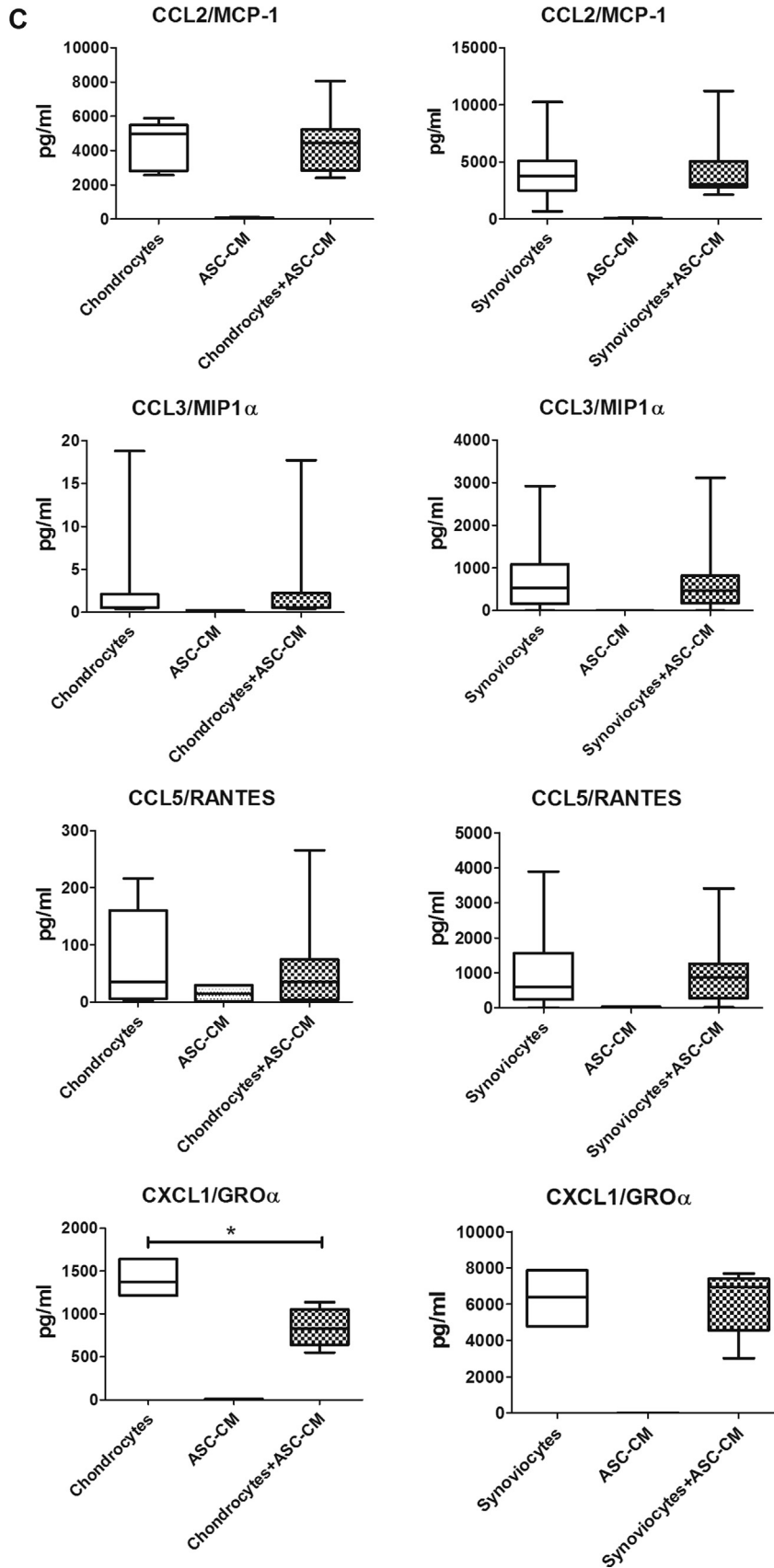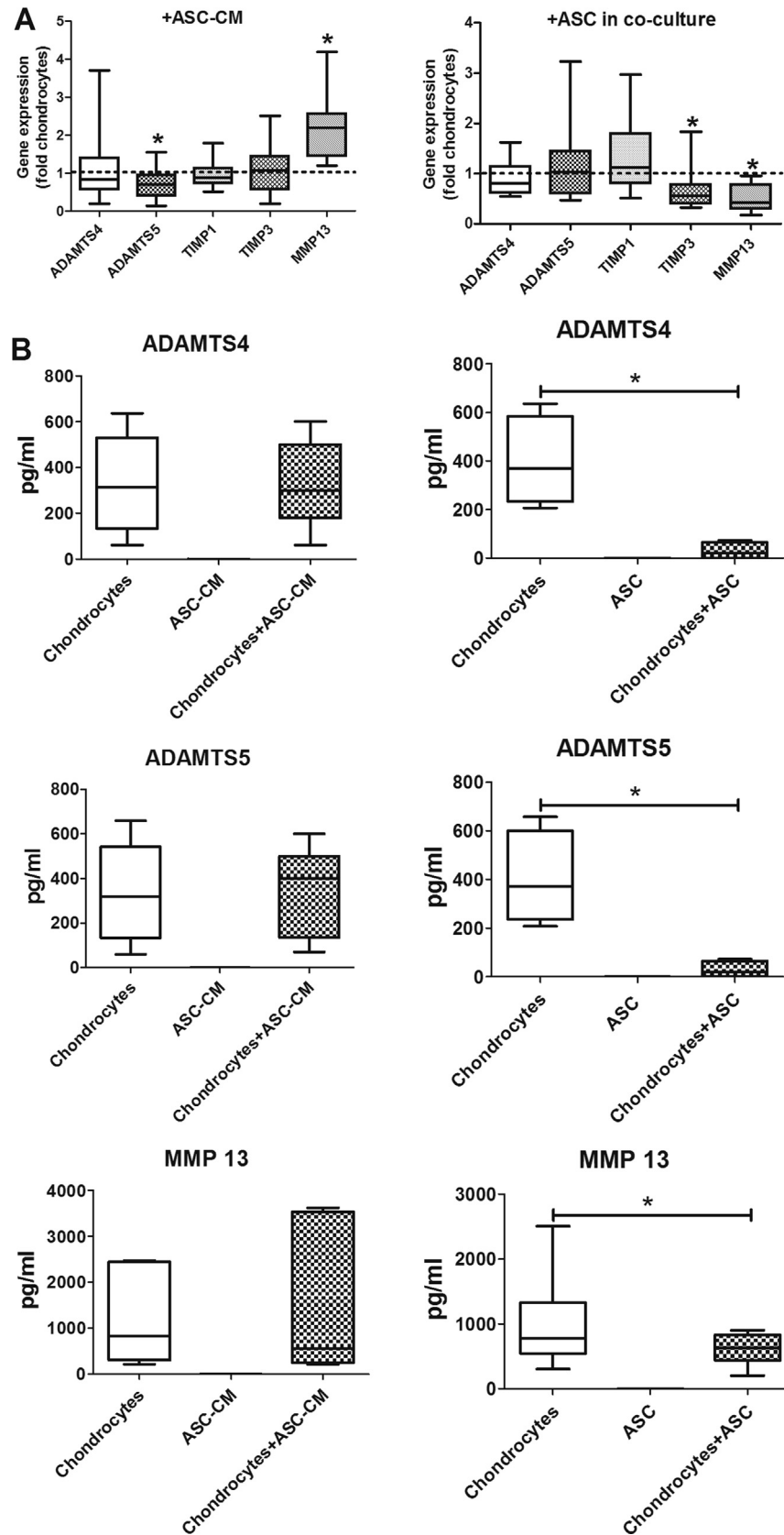
Fig. 2. (*continued*).

Fig. 3. ASC-CM effects on ADAMTS4, ADAMTS5, TIMP1, TIMP3 and MMP13 gene expression and protein release of chondrocytes ($n = 8$). **A**. Gene expression of ADAMTS4, ADAMTS5, TIMP1, TIMP3 and MMP13 was tested on chondrocytes in basal conditions and after treatment with ASC-CM (left side) or co-cultured with ASC (right side). Data are expressed as median with interquartile range of fold basal release. **B**. Protein release of ADAMTS4, ADAMTS5, and MMP13 (pg/ml) was tested on chondrocytes in basal conditions and after treatment with ASC-CM (left side) or co-cultured with ASC (right side). Significant differences: ADAMTS4 $P = 0.0286$, ADAMTS5 $P = 0.0286$, MMP13 $P = 0.048$. **C**. Protein release of TIMP1 (ng/ml) and TIMP3 (pg/ml) was tested on chondrocytes in basal conditions and after treatment with ASC-CM (left side) or co-cultured with ASC (right side). Data are expressed as median with interquartile range. Significant differences: TIMP1 (both left and right side) *$P = 0.048$.
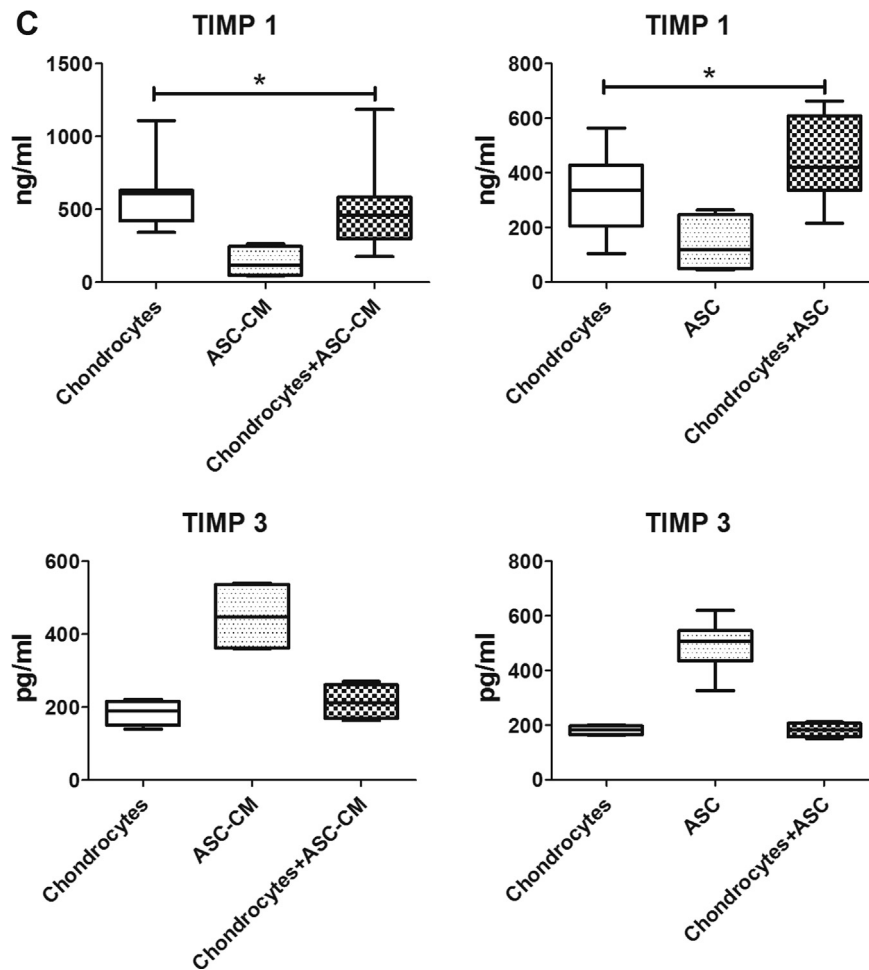
Fig. 3. (*continued*).

different ASC-CM ratios (DMEM:CM 75:25, 50:50, 25:75) before treating chondrocytes and synoviocytes for 7 days. As shown in Fig. 5(A), PGE2 levels were approximately 100, 200 and 300 pg/ml, respectively, a concentration close to that previously detected in ASC (400 pg/ml) [Fig. 5(A)]. However, when we evaluated PGE2 in chondrocytes and synoviocytes treated for 7 days with three different ASC-CM ratios we found that only by using the 50:50 ASC-CM ratio the amount of PGE2 detected was significantly higher selectively in chondrocytes; when tested in synoviocytes, the same ratio showed a trend towards an increase which however did not reach statistical significance [Fig. 5(B)]. The amount of PGE2 detected was lower both on 75:25 and 25:75 ASC-CM-treated chondrocytes and synoviocytes.

Discussion

MSC-based therapy for OA treatment is an emerging approach stemming from the observation that trophic factors released by these cells can exert anti-inflammatory, anti-fibrotic, anti-apoptotic effects[13,14]. Studies that compare the efficacy of cell-therapy approach with cell-free-based therapy are not available. Therefore, since we have already shown that GMP-clinical grade ASC *in vitro* exert anti-inflammatory effects on chondrocytes and synoviocytes[31] and protect chondrocytes from degeneration[32], in this study we compared GMP-ASC-CM and GMP-ASC to define their efficacy in reducing the release of pathogenic soluble factors, mainly involved in OA progression.

Firstly, we found that the factors analyzed were affected in the same way by the 25:75 and 75:25 ASC-CM ratios, but the 50:50 ratio that was used to perform the experiment showed a peculiar pattern of modulation. We showed that ASC-CM was not effective at reducing IL6, CXCL1/GROα, CXCL8/IL8, CCL2/MCP-1, CCL3/MIP-1α or CCL5/RANTES produced by OA synoviocytes or chondrocytes except for CXCL8/IL8 and CXCL1/GROα in chondrocytes both in monolayers or micromasses. In line with Tsuchida A[36] *et al.* we found that chondrocytes in micromasses release a lower of amount of inflammatory factors than in monolayers, although we confirmed that these molecules were down-modulated by ASC-CM. This finding indicates that the anti-inflammatory effects of ASC, which we previously demonstrated in co-culture experiments[31], were strictly dependent on the cross-talk between cells and was not recapitulated by the soluble factors secreted by unstimulated ASC. In fact, other reports have found that CM from TNFα/IFNγ-stimulated MSC was able to inhibit inflammatory processes[37], thus suggesting that unstimulated ASC could not reduce basal inflammation of chondrocytes or synoviocytes since they are not primed to release immunomodulatory factors. Even though CM from stimulated ASC is rich in immunosuppressive factors[37], activation with TNFα/IFNγ is mainly used *in vitro* to compare unstimulated and activated ASC-CM, but does not ensure that activated ASC *in vitro* produce the same factors when co-cultured with inflamed chondrocytes and synoviocytes, as occurs *in vivo*. This aspect is fundamental for planning what to use for clinical trials, since the *in vivo* local activation of ASC, exerted by OA chondrocytes or
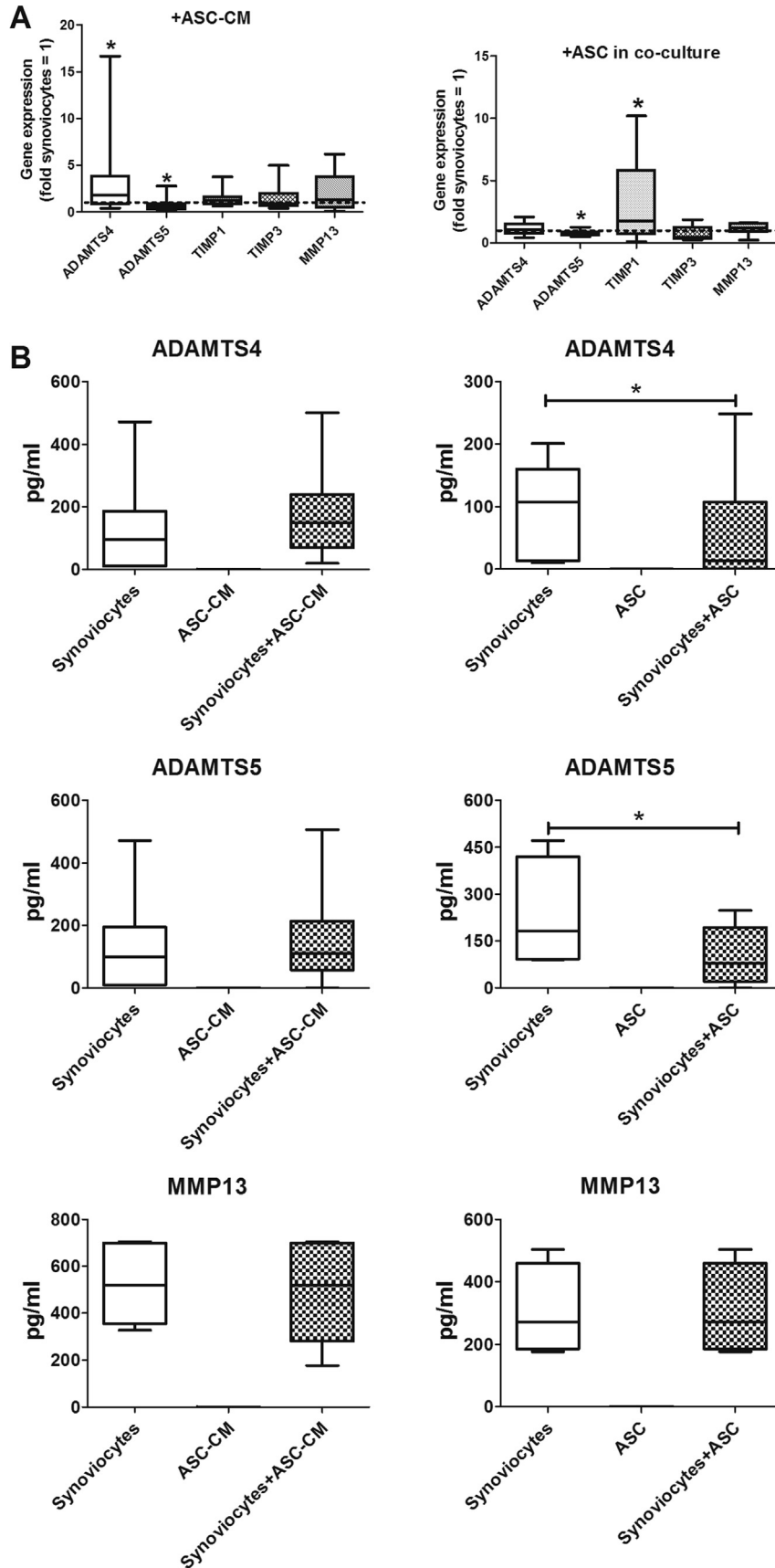
Fig. 4. ASC-CM effects on ADAMTS4, ADAMTS5, TIMP1, TIMP3 and MMP13 gene expression and protein release of synoviocytes ($n = 8$). **A**. Gene expression of ADAMTS4, ADAMTS5, TIMP1, TIMP3 and MMP13 was tested on synoviocytes in basal conditions and after treatment with ASC-CM (left side) or co-cultured with ASC (right side). Data are expressed as median with interquartile range of fold basal release. **B**. Protein release of ADAMTS4, ADAMTS5, and MMP13 (pg/ml) was tested on synoviocytes in basal conditions and after treatment with ASC-CM (left side) or co-cultured with ASC (right side). Significant differences: ADAMTS4 $P = 0.042$, ADAMTS5 $P = 0.045$. **C**. Protein release of TIMP1 (ng/ml) and TIMP3 (pg/ml) was tested on synoviocytes in basal conditions and after treatment with ASC-CM (left side) or co-cultured with ASC (right side). Data are expressed as median with interquartile range. Significant differences: TIMP1 (left side) *$P = 0.0225$, TIMP1 (right side). *$P = 0.0177$.
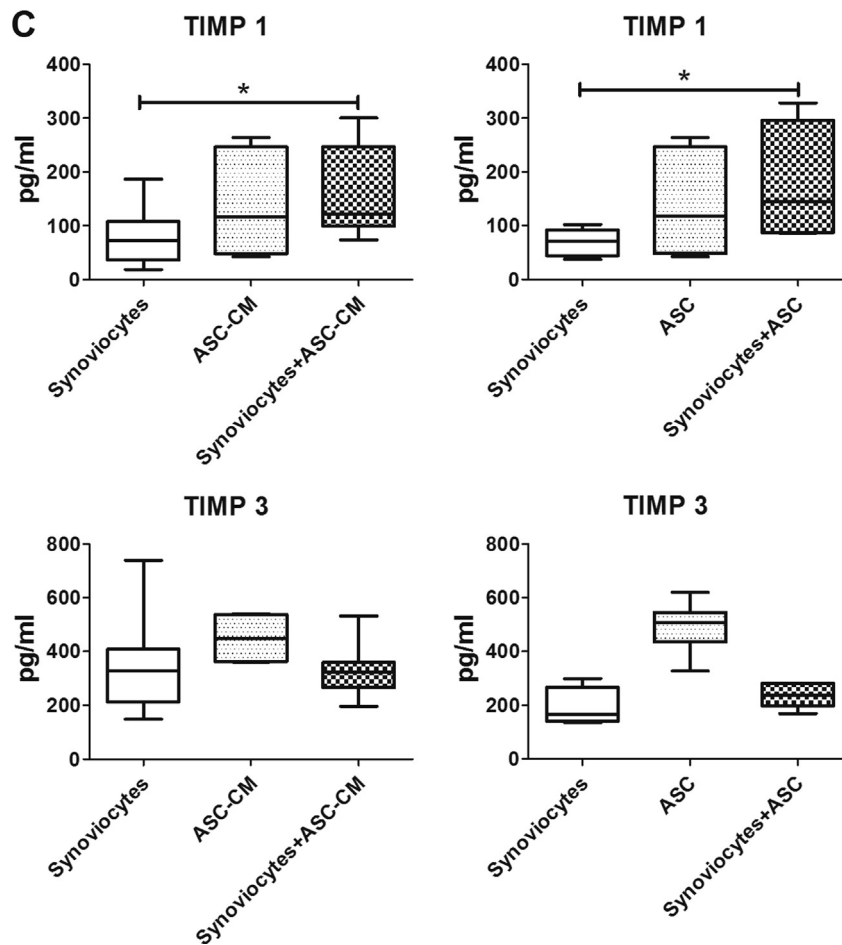
Fig. 4. (continued).

synoviocytes, cannot be recapitulated using ASC-CM, which is only a mixture of soluble factors.

Subsequently, we evaluated, both on chondrocytes and synoviocytes, the main proteases (ADAMTS4, ADAMTS5, MMP13), as well as their inhibitors (TIMP1 and TIMP3), involved in matrix degradation. In chondrocytes treated with ASC-CM the expression of ADAMTS5 was decreased and MMP13 was increased whereas in co-culture ADAMTS5 was not affected and MMP13 was decreased. However, the release of these two factors was significantly down-modulated only by ASC in co-culture, thus confirming their inhibitory role also on these factors. Our findings are coherent with those of a recent paper[38] that evaluated the effects of ASC-CM on basal and IL1β-activated OA chondrocytes and found an increase of MMP13 in basal but not in activated chondrocytes. Moreover, we found that in chondrocytes ADAMTS4 and TIMP3 release was not modulated but TIMP1 was down-modulated by ASC-CM, whereas ASC in co-culture significantly decreased the release of ADAMTS4 and increased TIMP1. These data showed that only ASC in co-culture were able to reduce the production of these degradative matrix proteases. In particular, ADAMTS4, ADAMTS5, MMP13 in the cartilage are modulated by inflammatory factors and constitute the main enzymes involved in cleavage of not only aggrecan and collagen type 2 but also sulfate proteoglycans (brevican and versican), cartilage oligomeric matrix protein (COMP), fibromodulin and decorin, thus indicating the wide spectrum of action of these enzymes[39,40]. Even if ASC in co-culture with chondrocytes decreased the release of the degradative matrix enzymes and

increase TIMP1 inhibitor, this effect was not sufficient to increase collagen type 2 and aggrecan expression, as we previously reported[32]. Conversely, in synoviocytes, ADAMTS4 mRNA expression was up-regulated by ASC-CM but not by ASC in co-culture. However, the release of the proteins was significantly down-modulated only by ASC in co-culture. Different inflammatory factors, such as IL1β, TNFα, IL6 and TGFβ are able to induce ADAMTS4[41,42] at mRNA level. Our previous data[32] showed that ASCs alone do not release these inflammatory factors involved in ADAMTS4 up-regulation. Therefore, we hypothesize that inflammatory factors produced by synoviocytes (i.e., IL6) treated with ASC-CM, were directly responsible for ADAMTS4 induction, and TIMP1 induced by ASC-CM seems insufficient to counteract this induction. Conversely, ADAMTS5 expression in synoviocytes decreased regardless of culture conditions (synoviocytes treated with ASC-CM or in co-culture), thus confirming that both ADAMTS4 and ADAMTS5 in this condition are inducible[43]. Conversely, it has been shown that different inflammatory factors (TNFα, IL1β, IL6)[40] increase ADAMTS4 on synoviocytes associated with a decrease of ADAMTS5[42], which suggests that ADAMTS4 is inducible by these factors, whereas ADAMTS5 is not modulated[44]. Overall these data confirm the lower effect of ASC-CM on both chondrocytes and synoviocytes proteinases and inhibitors again showing the efficacy of ASC in reducing a wide spectrum of inflammatory and degradative factors responsible for OA progression.

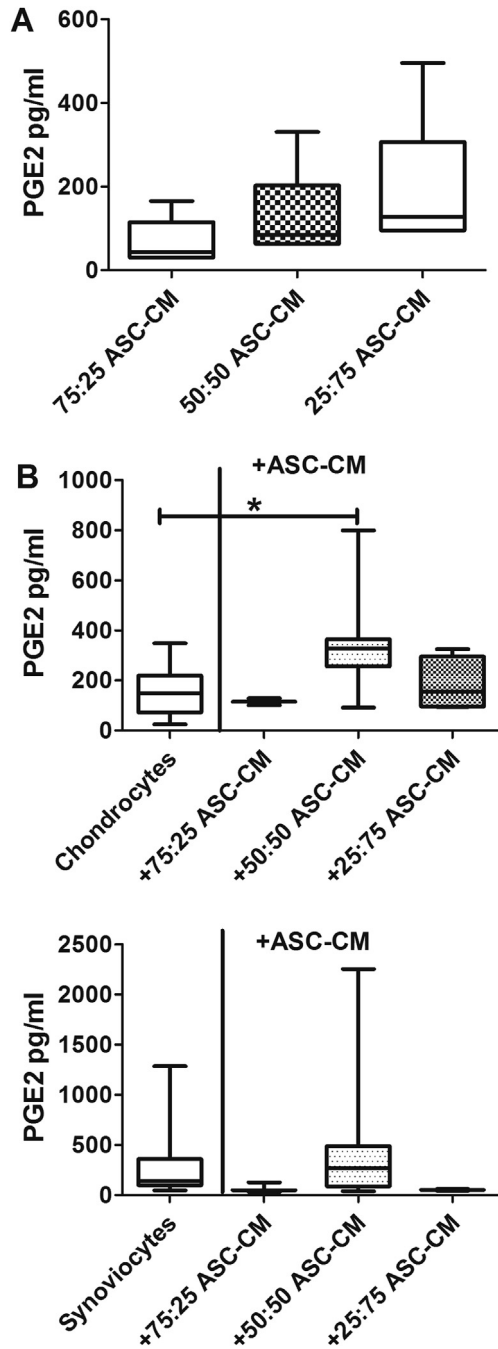We demonstrated that PGE2 was directly involved in anti-inflammatory effects exert by ASC in co-culture with inflamed

Fig. 5. PGE2 released by ASC-CM alone or after chondrocytes ($n = 8$) or synoviocytes ($n = 8$) treatment. **A**. PGE2 (pg/ml) was tested on different ratios of ASC-CM (DMEM:CM 75:25, 50:50, 25:75) **B**. PGE2 (pg/ml) was tested on chondrocytes or synoviocytes alone or treated with different ratios of ASC-CM. Data are expressed as median with interquartile range. Significant differences *$P = 0.0036$.
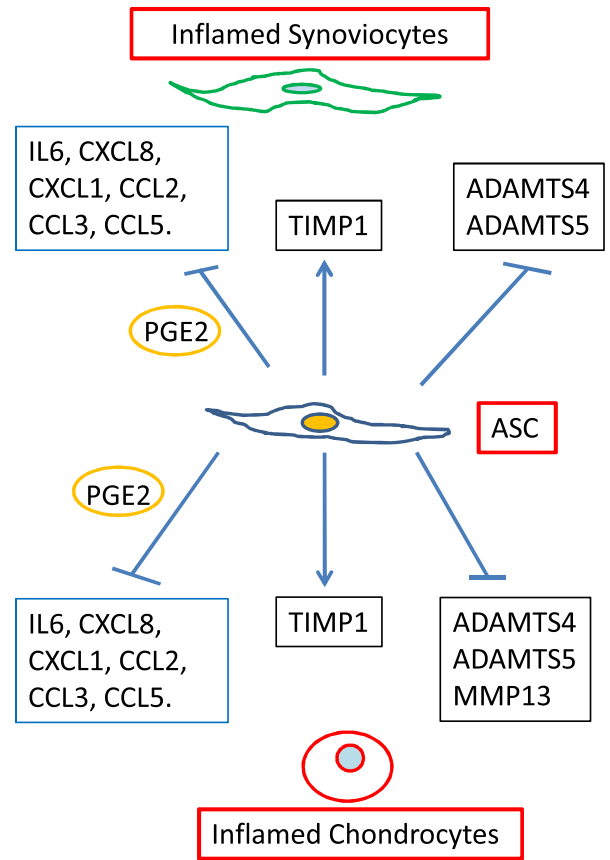

Fig. 6. ASC co-culture effects on inflamed chondrocytes and synoviocytes. A schematic model that summarizes all the ASC effects demonstrated by co-culturing ASC with inflamed chondrocytes or synoviocytes. ASC down-modulated cytokine/chemokines (IL6, IL8/CXCL8, CCL2/MCP-1, CCL3/MIP-1α, CCL5/RANTES and CXCL1/GROα), a dis-integrin and metalloproteinase with thrombospondin motifs (ADAMTS4, ADAMTS5), but increased tissue metalloproteinase (TIMP1) both on chondrocytes and synoviocytes. In chondrocytes ASC down-modulated also metalloproteinase (MMP13). The down-modulation of cytokine/chemokines was mediated by PGE2.

effects observed and their released could be dependent by inflammatory cells activation.

These data contribute to explaining, as summarized in Fig. 6 that ASCs primed by inflammatory chondrocytes or synoviocytes-secreted factors become more potent to exert their therapeutic effects counteracting the expression of crucial factors that favor OA evolution. These data help to clarify the importance of using ASC for the clinical treatment of OA disease.

Authors contribution

Cristina Manferdini: Conceived and participated in the study design and data acquisition.

Marie Maumus: Carried out immunoassay.

Elena Gabusi: Carried out Molecular biology.

Francesca Paolella: Carried out cell culture.

Francesco Grassi: Revised the article critically for important intellectual content.

Christian Jorgensen: Revised the article critically for important intellectual content.

Sandrine Fleury-Cappellesso: Carried out GMP-clinical grade ASC.

Danièle Noël: Participated in its design and helped to draft the manuscript.

chondrocytes and synoviocytes through its receptor EP4. Conversely using ASC-CM we only observed a partial anti-inflammatory effect, which can be due in part to the lower amount of PGE2 detected in ASC-CM compared to ASC, but also to the lower efficiency of unstimulated ASC-CM to recapitulate all the effects mediated by a co-culture with ASC. Moreover, it is known that secretome of mesenchymal stromal cells is enriched not only in secreted factors but also in plasma membrane-derived particles (MPs) which act as a shuttle for selected MSC-derived bioactive molecules, mRNAs and microRNAs[45–48] that could contribute to the

Gina Lisignoli: Conceived the study, performed statistical analysis and wrote the manuscript.

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