Osteoarthritis and Cartilage 20 (2012) 1186–1196

Osteoarthritis and Cartilage

OARSI OSTEOARTHRITIS RESEARCH SOCIETY INTERNATIONAL

Mesenchymal stem cells secrete factors that inhibit inflammatory processes in short-term osteoarthritic synovium and cartilage explant culture

G.M. van Buul †‡, E. Villafuertes †§, P.K. Bos †, J.H. Waarsing †, N. Kops †, R. Narcisi †, H. Weinans †||, J.A.N. Verhaar †, M.R. Bernsen ‡¶, G.J.V.M. van Osch †#*

† *Department of Orthopaedics, Erasmus MC, Rotterdam, The Netherlands*
‡ *Department of Radiology, Erasmus MC, Rotterdam, The Netherlands*
§ *Rheumatology Service, Hospital Clinico San Carlos, Madrid, Spain*
|| *Department of Biomechanical Engineering, Delft University of Technology, Delft, The Netherlands*
¶ *Department of Nuclear Medicine, Erasmus MC, Rotterdam, The Netherlands*
# *Department of Otorhinolaryngology, Erasmus MC, Rotterdam, The Netherlands*

ARTICLE INFO

Article history:
Received 26 March 2012
Accepted 20 June 2012

Keywords:
Mesenchymal stem cell
MSC
Osteoarthritis
OA
Immune modulation
Paracrine

SUMMARY

Objective: Mesenchymal stem cells (MSCs) are promising candidates for osteoarthritis (OA) therapies, although their mechanism of action remains unclear. MSCs have recently been discovered to secrete anti-inflammatory cytokines and growth factors. We studied the paracrine effects of MSCs on OA cartilage and synovial explants *in vitro*.

Design: MSC-conditioned medium was prepared by stimulating primary human MSCs with tumour necrosis factor alpha (TNFα) and (50 ng/ml each). Human synovium and cartilage explants were cultured in MSC-conditioned medium or in control medium, containing the same amount of added TNFα and IFNγ but not incubated with MSCs. Explants were analyzed for gene expression and the production of nitric oxide (NO). The presence of the inhibitor of nuclear factor kappa B alpha (IκBa) was assessed by Western blot analysis.

Results: Synovial explants exposed to MSC-conditioned medium showed decreased gene expression of interleukin-1 beta (*IL-1β*), matrix metalloproteinase (*MMP*)*1* and *MMP13*, while suppressor of cytokine signaling (*SOCS*)*1* was upregulated. In cartilage, expression of IL-1 receptor antagonist (*IL-1RA*) was upregulated, whereas a disintegrin and metalloproteinase with thrombospondin motifs (*ADAMTS*)*5* and collagen type II alpha 1 (*COL2A1*) were downregulated. MSC-conditioned medium reduced NO production in cartilage explants and the presence of IκBa was increased in synoviocytes and chondrocytes treated with MSC-conditioned medium.

Conclusions: In an inflammatory environment, MSCs secrete factors which cause multiple anti-inflammatory effects and influence matrix turnover in synovium and cartilage explants. Thereby, the presented data encourage further study of MSCs as a treatment for joint diseases.

Introduction

Osteoarthritis (OA) is characterized by a catabolic and inflammatory joint environment. To this date, no drugs are available to structurally modify OA processes or prevent progression of the disease[1]. The use of mesenchymal stem cells (MSCs) as a treatment option in cartilage regenerative therapies is under extensive investigation[2]. MSCs have chondrogenic potential and are experimentally being implanted in focal cartilage defects, showing promising results[3]. In OA, more generalized cartilage lesions and joint inflammation are present, thereby limiting the usefulness of focal treatments. In order to treat the joint as a whole, MSCs have been injected intra-articularly in pre-clinical and some initial clinical studies as a treatment for OA[4–6]. Animal studies have shown beneficial effects of MSCs on cartilage morphology and histology in various OA models[4,5,7]. Interestingly, studies using cell tracking in cartilage repair show only limited cartilage formation by

* Address correspondence and reprint requests to: G.J.V.M. van Osch, Department of Otorhinolaryngology, Room Ee 1655, Erasmus MC, Dr. Molewaterplein 50, 3015 GE Rotterdam, The Netherlands. Tel: 31-107143661; Fax: 31-107044690.

E-mail addresses: g.vanbuul@erasmusmc.nl (G.M. van Buul), evillafuertes@gmail.com (E. Villafuertes), p.k.bos@erasmusmc.nl (P.K. Bos), e.waarsing@erasmusmc.nl (J.H. Waarsing), n.kops@erasmusmc.nl (N. Kops), r.narcisi@erasmusmc.nl (R. Narcisi), h.weinans@erasmusmc.nl (H. Weinans), j.verhaar@erasmusmc.nl (J.A.N. Verhaar), m.bernsen@erasmusmc.nl (M.R. Bernsen), g.vanosch@erasmusmc.nl (G.J.V.M. van Osch).

1063-4584/$ – see front matter © 2012 OsteoArthritis Society International. Published by Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.joca.2012.06.003

chondrogenic differentiation of the injected MSCs[4,5]. Instead, the applied cells are mostly retrieved from other articular structures, like the synovium. Apparently, the intra-articularly injected MSCs only occasionally differentiate into chondrocytes to actively produce extracellular matrix. This implies a different OA modifying mechanism, like influencing the micro-environment by paracrine actions, stimulating locally present progenitor cells to repair OA damage or by attracting circulating endogenous progenitor cells to enable repair[8]. We studied the influence of MSCs on their local micro-environment by the secretion of bioactive factors. Some of these factors, including interleukin-6 (*IL-6*), IL-10, indoleamine 2,3-dioxygenase (*IDO*), hepatocyte growth factor (*HGF*) and transforming growth factor beta (*TGFβ*), have immunomodulatory properties[9,10],whereas others are involved in extracellular matrix turnover such as matrix metalloproteinases (MMPs) and their inhibitors, tissue inhibitors of metalloproteinases (TIMPs)[11]. In addition, trophic effects of MSCs to stimulate chondrocyte proliferation and matrix deposition have been shown[12]. Aim of our study was to explore the protective effects of MSCs on OA related processes in a controlled and standardized environment, by investigating the paracrine effects of MSCs on OA synovium and cartilage explants *in vitro*. These paracrine effects were studied by means of MSC-conditioned medium; medium containing factors secreted by MSCs. Since MSCs increase their immunomodulatory properties in response to an inflammatory stimulus, and inflammation plays a substantial role in OA pathology, we challenged our cells with the inflammatory cytokines tumour necrosis factor alpha (TNFα) and interferon gamma (IFNγ)[13,14]. We measured the presence of factors in MSC-conditioned medium involved in inflammation, tissue regeneration and extracellular matrix turnover. Furthermore, we evaluated the effects of MSC-conditioned medium on osteoarthritic cartilage and synovium by analyses of the expression of genes related to inflammation and matrix turnover, the production of nitric oxide (NO) and prostaglandin E_2 (PGE2) and activity of p38 mitogen-activated protein (MAP) kinase and nuclear factor kappa B (NFκB) pathways. These two pathways are major orchestrators in transducing inflammatory and catabolic signals in joint degeneration[15,16].

Materials and methods

Cells and tissue preparation

Human MSCs (hMSCs) were isolated and cultured from heparinized femoral-shaft marrow aspirate of nine patients undergoing total hip arthroplasty (after written informed consent; protocol # MEC-2004-142) using previously described procedures[17]. This procedure was previously confirmed to yield MSC on the basis of morphological criteria, expression of CD105 marker and absence of CD34 marker and an adipogenic, osteogenic and chondrogenic differentiation potential[17]. Cells were seeded at a density of 2300 cells/cm^2 and cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 1 g/l glucose, 10% fetal calf serum (FCS), 50 μg/ml gentamicin, 1.5 μg/ml fungizone, 1 ng/ml fibroblast growth factor-2 and 0.1 mM L-ascorbic acid 2-phosphate (MSC culture medium). Human synovial explants (approximately 10 mm^3) and cartilage explants (approximately 70 mm^3) were obtained as surgical waste material from seven patients undergoing total knee replacement surgery. All patients implicitly consented to the use of these tissues for scientific research (protocol # MEC-2004-322). Explants were pre-cultured for 48 h in DMEM containing 1 g/l glucose, 2% FCS, 50 μg/ml gentamicin and 1.5 μg/ml fungizone. Synoviocytes and chondrocytes were isolated from synovium and cartilage respectively as described previously[18] by treating explants from either tissue with 0.2% protease (Sigma–Aldrich, Zwijndrecht, Netherlands) and subsequent overnight digestion in DMEM containing 4.5 g/l glucose, 10% FCS, 50 μg/ml gentamicin and 1.5 μg/ml fungizone, supplemented with 0.15% collagenase B (Roche Diagnostics, Mannheim, Germany).

Conditioned medium preparation and explant culture

Subconfluent hMSC monolayer cultures (passage two) were used to obtain conditioned medium. MSC-conditioned medium from four donors was prepared by incubating hMSCs for 24 h in MSC culture medium. To stimulate the secretion of immunomodulatory factors by MSCs, TNFα and IFNγ (50 ng/ml each, PeproTech, London, UK) were added to the MSC culture medium for five other MSC donors. After 24 h the medium was collected and centrifuged for 8 min at 700*g* to remove cellular debris. MSCs and the supernatant of the medium (MSC-conditioned medium) were harvested separately and stored at −80°C until further use. MSC processing for gene expression analysis is described later in this section. Control (unconditioned) medium was made of plain MSC culture medium for the first four MSC donors, and MSC culture medium supplemented with TNFα/IFNγ (50 ng/ml each) for the five cytokine-stimulated MSC donors. Both control media, with or without TNFα/IFNγ, were incubated without MSCs at 37 °C for 24 h and stored at −80 °C until further use. TNFα/IFNγ. Except for the absence of MSCs during incubation, the control medium was treated identical to MSC-conditioned medium. In experiments using MSC-conditioned media from non-stimulated donors, control medium without TNFα/IFNγ was used. Stimulated MSC-conditioned media were compared to control medium with TNFα/IFNγ.

Synovium and cartilage explants were cultured in MSC-conditioned medium or in control medium for 48 h. Explants from each synovium or cartilage donor were cultured in triplicate samples per condition in 24-well plates in a total volume of 1.0 ml, consisting of 500 μl MSC-conditioned medium and 500 μl freshly added DMEM containing 50 μg/ml gentamicin and 1.5 μg/ml fungizone. Depending on the amount of explant material that could be obtained from a given donor, explants were cultured in conditioned medium from one to four separate MSC donors. After culturing for 48 h, explants for gene expression analyses and media were harvested and stored at −80°C until further use. MSC-conditioned medium without TNFα/IFNγ from four MSC donors was used on two synovium and cartilage donors. Conditioned medium from MSCs stimulated with TNFα/IFNγ from five MSC donors was used on five synovium and cartilage donors.

We additionally studied the effect of MSCs in a co-culture system of synovium and cartilage explants[19]. In short, synovium and cartilage explants from the same donor were cultured together, thereby preventing direct contact between cartilage and synovium by using Millicell filter inserts with a pore size of 0.4 μm (Millipore, Amsterdam, the Netherlands). These experiments were performed on synovium and cartilage from one donor in triplicates using pooled MSC-conditioned media from five different MSC donors.

Gene expression analysis

The frozen explants were processed using a Mikro-Dismembrator S (B. Braun Biotech International GmbH, Melsungen, Germany). RNA from explants was extracted using RNA-Bee™ (TEL-TEST, Friendswood, USA) according to manufacturer's guidelines and subsequently precipitated with chloroform 20% (v/v). RNA from MSCs was extracted using RNeasy lysis buffer (Qiagen, Venlo, the Netherlands) and beta-mercaptoethanol 1% (v/v). All RNA were further purified using RNeasy Micro Kit (Qiagen, Hilden, Germany) with on-column DNA digestion. Nucleic acid content was determined spectrophotometrically (NanoDrop ND1000; Isogen Life Science, IJsselstein, The Netherlands). Complementary DNA and

polymerase chain reactions (PCRs) were performed as described before[20]. Reverse transcriptase (RT)-PCR primer nucleotide sequences are listed in Table I. Data were normalized to a best keeper index (BKI) of three reference genes glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), ubiquitin C (*UBC*), hypoxanthine-guanine phosphoribosyltransferase (*HPRT1*)[21]. Relative expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method[22].

NO and PGE2 measurements

NO and PGE2 secretion by synovium and cartilage was analyzed in the cryopreserved media from the synovium and cartilage explants culture experiments. NO secretion was determined by quantifying its derived product, nitrite, in medium using a spectrophotometric method based upon the Griess reaction[23]. Briefly, 100 μl of culture medium or sodium nitrite ($NaNO_2$) standard dilutions were mixed with 100 μl of Griess reagent (0.5% sulphanilamide, 0.05% naphtyl ethylenediamine dihydrochloride, 2.5% H_3PO_4). The absorption was measured at 540 nm. PGE2 secretion in the media was determined using the PGE2 assay (R&D systems, Minneapolis, MN, USA) according to manufacturer's guidelines. NO measurements were performed on triplicate samples from five synovium and cartilage donors separately. For the PGE2 assay the triplicate samples per condition were pooled before measurement to obtain values from four synovium and four cartilage donors.

Western blotting for p38 and NFκB signaling pathway analyses

The amount of native and phosphorylated p38 MAP kinase and the amount of inhibitor of nuclear factor kappa B alpha (IκBa) were determined by Western blot analysis of total protein extracts from fibroblast-like synoviocytes and chondrocytes exposed to MSC-conditioned medium. Synoviocytes and chondrocytes from two donors each were used. Subconfluent monolayers of synoviocytes and chondrocytes (passage 2–4) were cultured in pooled MSC-conditioned medium (from five MSC donors) or control medium for either 10 min (for IκBa determination) or for 3 h (for p38 MAP kinase signaling). The chosen incubation times were based on an experiment using different durations of IFNγ/TNFα treatment to predefine the optimal time-point to evaluate possible paracrine MSC effects (data not shown). Based on previous reports, IFNγ/TNFα dependent IκBa degradation was determined in a period ranging from 5 min to 1 h and p38 MAP kinase phosphorylation in a period of 3–24 h[24,25].

Total protein fractions were isolated using Mammalian Protein Extraction Reagent (M-PER) (#78501, Thermo Scientific) with 1% protease inhibitor complete (Roche, Manheim, Germany). Protein concentrations were determined by the bicinchoninic acid (BCA) protein assay (Pierce Chemical, Rockford, IL, USA). For each sample, 8 μg of total protein fraction was separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels and transferred on polyvinylidene fluoride (PVDF) membranes by Western blotting. Membranes were blocked for 2 h in 0.1% tris-buffered saline-tween (TBS-T) containing 5% dry milk powder, washed three times in 0.1% TBS-T and incubated with primary antibodies against α-Tubulin, p38 MAP kinase, phosphorylated p38 (all diluted 1:1000; respectively 2148, 9212 and 9211, Cell Signaling Technology, Leiden, the Netherlands) or IκBa (1:500; sc-371, Santa Cruz Biotechnology, Heidelberg, Germany) overnight at 4°C following manufacturer's protocol. An anti-rabbit horseradish peroxidase (HPR)-linked secondary antibody (1:1000; 7074, Cell Signaling) was added and incubated for 1 h at room temperature. The blots were visualized by means of SuperSignal Chemiluminescent system (34077, Thermo Scientific) using manufacturer's instruction. Image analysis and quantification were performed using the National Institute of Health Image J freeware (release 1.44X; http://rsb.info.nih.gov/ij/).

Table I
Primer nucleotide sequences of the tested genes

Gene	Primer
IL-6	Fw: TCGAGCCCACCGGGAACGAA Rv: GCAGGGAAGGCAGCAGGCAA
HGF	Fw: GGCTGGGGCTACACTGGATTG Rv: CCACCATAATCCCCCTCACAT
TGFβ-1	Fw: GTGACAGCAGGGATAACACACTG Rv: CATGAATGGTGGCCAGGTC
IDO	Assay-on-demand (Hs00158027.m1, Applied Biosystems, Capelle a/d IJssel, the Netherlands)
IL-1β	Fw: CCCTAAACAGATGAAGTGCTCCTT Rv: GTAGTCGGATGCCGCCAT
IL-1RA	Fw: AACAGAAAGCAGGACAAGCG Rv: CCTTCGTCAGGCATATTGGT
TNFα	Fw: GCCGCATCGCCGTCTCCTAC Rv: AGCGCTGAGTCGGTCACCCT
SOCS1	Fw: CCCTGGTTGTTGTAGCAGCTT Rv: TTGTGCAAAGATACTGGGTATATGT
SOCS3	Fw: TCGGACCAGCGCCACTT Rv: CACTGGATGCGCAGGTTCT
MMP1	Fw: CTCAATTTCACTTCTGTTTTCTG Rv: CATCTCTGTCGGCAAATTCGT
MMP13	Fw: CTCAATTTCACTTCTGTTTTCTG Rv: CATCTCTGTCGGCAAATTCGT
TIMP1	Fw: TGCCGCATCGCCGAGAT Rv: ATGGTGGGTTCTCTGGTG
TIMP2	Fw: ATGGTGGGTTCTCTGGTG Rv: CGGTACCACGCACAGGA
ADAMTS4	Fw: CAAGGTCCCATGTGCAACGT Rv: CATCTGCCACCACCAGTGTCT
ADAMTS5	Fw: CAAGGTCCCATGTGCAACGT Rv: CATCTGCCACCACCAGTGTCT
COL2A1	Fw: GGCAATAGCAGGTTCACGTACA Rv: CGATAACAGTCTTGCCCCACTT
ACAN	Fw: TCGAGGACAGCGAGGCC Rv: TCGAGGGTGTAGCGTGTAGAGA

Analyses of MSCs and conditioned media

Stimulated and non-stimulated MSCs from four donors were analyzed for gene expression of *IL-6*, *TIMP2*, *HGF*, *TGFβ-1* and *IDO* as described in the gene expression analysis section. TIMP2, HGF, IL-6 and TGFβ-1 protein levels were measured in stimulated and non-stimulated MSC-conditioned media from three donors by means of enzyme-linked immunosorbent assays (ELISAs) (R&D systems, Abingdon, UK) according to the protocol supplied by the manufacturer. All factors were corrected for the amounts present in standard MSC culture medium. To determine the amount of IDO enzymatic activity in MSC-conditioned media, the level of its metabolite, kynurenine, was measured spectrophotometrically as described before[26]. In brief, 100 μl of 30% trichloroacetic acid (Sigma–Aldrich, St. Louis, USA) was added to 200 μl of culture supernatant, which was incubated at 50°C for 30 min, and then centrifuged at 10,000*g* for 5 min. 75 μl of supernatant was then added to an equal volume of Ehrlich's reagent (100 mg *p*-dimethylbenzaldehyde and 5 ml glacial acetic acid; Sigma–Aldrich St. Louis, USA) and optical density was measured at 490 nm.

Data analyses

All data are presented as mean ± standard deviation. Statistical analyses were performed using a mixed model analysis of variance (ANOVA) [statistical package social sciences (SPSS) 17.0.2; SPSS Inc., Chicago, USA], which takes the within donor correlation into account. Treatment using MSC-conditioned media vs control

medium was considered a fixed factor and these effects were statistically considered as independent observations. The synovium or cartilage donors were considered a random factor and measurements of the separate samples per donor were regarded correlated observations. Donor was included into the model to adjust for absolute differences in expression levels between explant donors. A log-transformation was applied to all gene expression data before statistical analyses to approach normal data distribution. A P-value < 0.05 was considered statistically significant.

Results

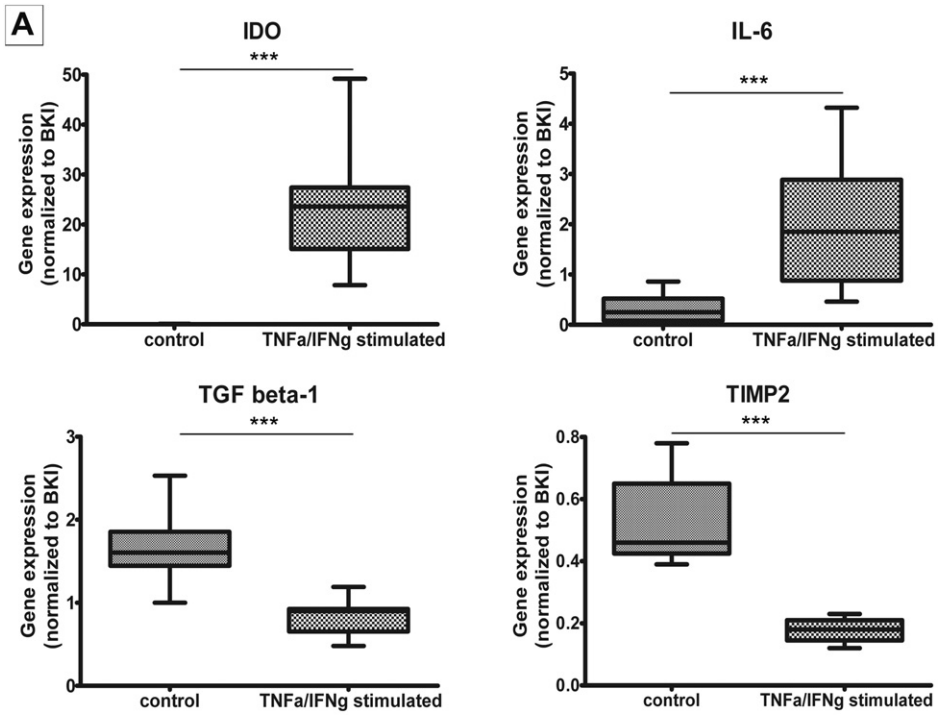
MSCs display immunomodulatory properties in response to inflammatory cytokine exposure

To determine which factors might be involved in the anti-inflammatory effects caused by the MSC-conditioned medium, we analyzed TNFα/IFNγ stimulated and non-stimulated MSCs for gene expression and protein secretion of various immunomodulatory factors and growth factors. Stimulation of MSC's with the inflammatory cytokines upregulated gene expression of *IDO* and *IL-6*, while *TIMP2* and *TGFβ-1* were markedly downregulated [Fig. 1(A); $P < 0.001$ for all genes]. *HGF*, *IL-1β* and *TIMP1* gene expressions were not significantly altered by the priming procedure (data not shown).

The amount of secreted IL-6, HGF, TIMP2, TGFβ-1 and IDO enzymatic activity in conditioned medium from TNFα/IFNγ stimulated and unstimulated MSCs was measured [Fig. 1(B)]. IL-6 was significantly elevated in TNFα/IFNγ stimulated MSC-conditioned medium (approximately 15-fold, $P < 0.001$), as was IDO activity (approximately 60-fold, $P < 0.001$) compared with non-stimulated conditioned medium. HGF, TIMP2 and TGFβ-1 were found in equal amounts in both stimulated and non-stimulated conditioned medium.

Factors secreted by stimulated MSCs modify genes related to inflammation and matrix turnover in synovium and cartilage

We evaluated whether factors secreted by MSCs affected inflammatory and catabolic processes in osteoarthritic synovium and cartilage. First we performed experiments in which we cultured synovium and cartilage explants in conditioned medium



B

Factor	24 hour secretions by MSCs (ng/ml)	
	Non TNFa/IFNg stimulated	TNFa/IFNg stimulated
Kynurenine (IDO activity)	0.2 ± 0.4 (x10³)	12.6 ± 1.0 (x10³)***
IL-6	2.6 ± 3.0	40.2 ± 15.0***
TIMP2	25.2 ± 6.8	23.5 ± 7.7
TGF beta-1	0.9 ± 0.9	0.9 ± 0.9
HGF	4.6 ± 3.4	4.3 ± 3.5

Fig. 1. Influence of TNFα/IFNγ stimulation on MSC gene expression (A) and MSC secretions (B). In order to stimulate the immunomodulatory capacity, MSCs were treated with TNFα and IFNγ for 24 h. Media from TNFα/IFNγ stimulated and unstimulated MSCs were analyzed for various factors and corrected for control medium containing 10% FCS. Gene expression data are presented as boxplots for four experiments performed in triplicate. Cell secretion data are presented as means ± standard deviations for three experiments performed in triplicate. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.005$.

from MSC donors which were not stimulated with TNFα/IFNγ (unstimulated MSC-conditioned medium). None of the genes analyzed (mentioned later in this section) in either synovium or cartilage were significantly affected or affected more than twofold by this unstimulated MSC-conditioned medium (Figs. 2, 3). Therefore, we did not include this condition in our further experiments. All further presented experiments were performed using conditioned medium from TNFα/IFNγ stimulated MSCs (designated as MSC-conditioned medium).

In synovial explants, MSC-conditioned medium downregulated *IL-1β* ($P = 0.014$), *MMP1* ($P = 0.034$) and *MMP13* ($P = 0.016$) gene expressions, while suppressor of cytokine signaling (*SOCS*)*1* expression was upregulated ($P = 0.002$) compared to control medium (Fig. 4). MSC-conditioned medium did not evidently affect IL-1 receptor antagonist (*IL-1RA*) or *SOCS3* gene expression of synovial explants. In cartilage explants, MSC-conditioned medium upregulated *IL-1RA* gene expression, while a disintegrin and metalloproteinase with thrombospondin motifs (*ADAMTS*)*5* and collagen type II alpha 1 (*COL2A1*) expressions were downregulated (Fig. 5; $P < 0.001$ for all genes). *ADAMTS4* and *SOCS3* were non-significantly downregulated by 2.5-fold and 1.6-fold respectively. *SOCS1*, *MMP1*, *MMP13* and aggrecan (*ACAN*) expressions of cartilage explants were not clearly influenced by factors secreted by MSCs.

The interaction between cartilage and synovium is an important aspect in the pathogenesis of OA. To evaluate the validity of our results in a more complex environment resembling more closely the *in vivo* situation, we exposed co-cultures of cartilage and synovium explants to MSC-conditioned medium (pooled from five MSC donors). Control experiments using pooled MSC-conditioned medium on separate cartilage and synovium explants revealed effects on gene expression level matching the effects observed using MSC-conditioned medium from individual donors (data not shown). MSC-conditioned medium affected gene expression levels in our co-culture model similar to our single explant culture model (Supp. Fig. 1(A, B)). Synovium showed downregulation of *IL-1β* (2.7-fold) and upregulation of *SOCS1* (4.6-fold). *MMP1* and *MMP13* appeared uninfluenced by MSC-conditioned medium. In cartilage, gene expression of *IL-1RA* (3.3-fold) and *SOCS1* (1.8-fold) was upregulated, whereas *ADAMTS5* (2.0-fold) and *COL2A1* (1.9-fold) were downregulated by MSC-conditioned medium compared to

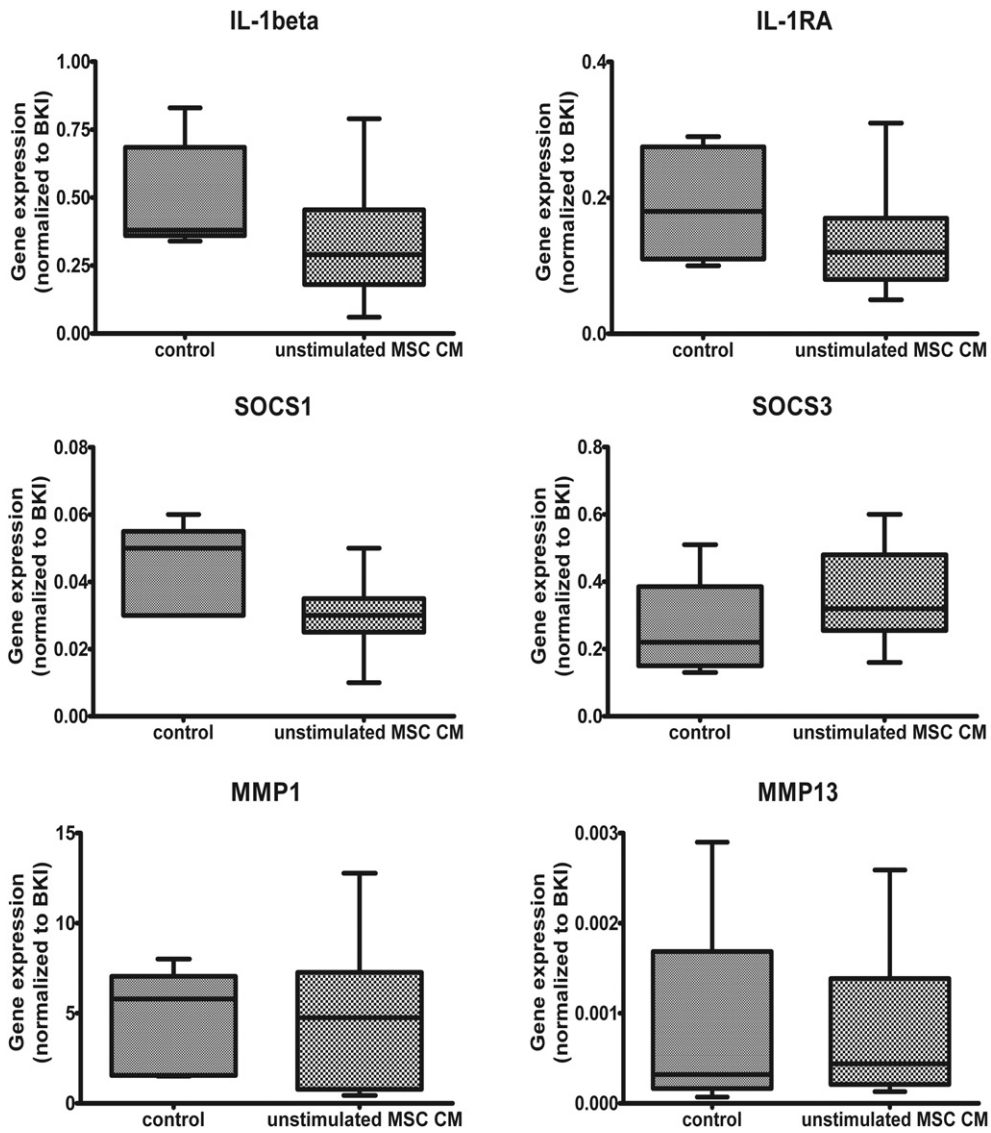


Fig. 2. Effects of factors secreted by non-stimulated MSCs on expression of genes related to inflammation and matrix degradation in human osteoarthritic synovial explants. Data are presented as boxplots for three experiments, in which MSC-conditioned medium from three MSC donors was applied to triplicate explants from three OA donors; explants were treated for 48 h.

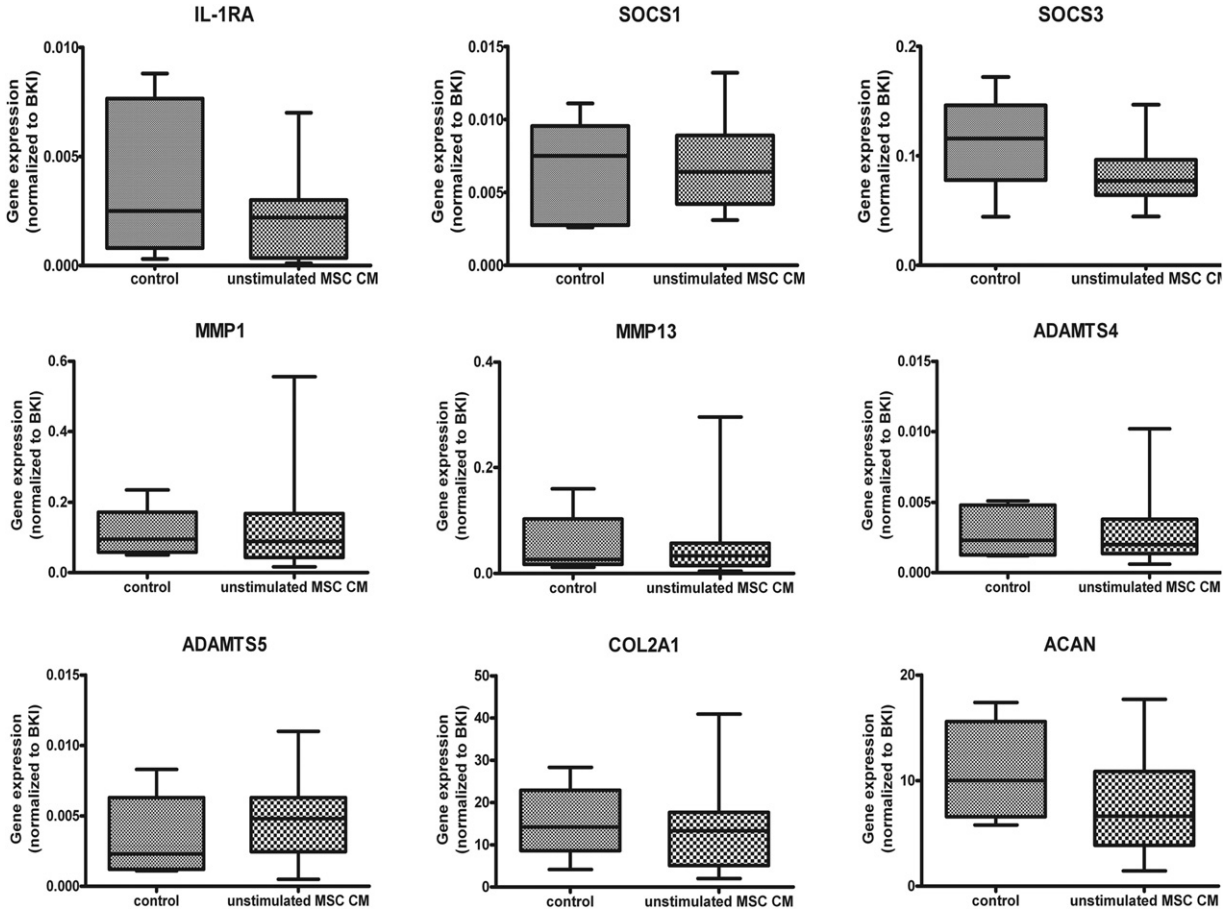


Fig. 3. Effects of factors secreted by non-stimulated MSCs on expression of genes related to inflammation and matrix degradation in human osteoarthritic cartilage explants. Data are presented as boxplots for three experiments, in which MSC-conditioned medium from three MSC donors was applied to triplicate explants from three OA donors; explants were treated for 48 h.

control medium. These results confirm the previously described results obtained in separate cultures using synovial or cartilage tissue, indicating that MSCs can have an effect in a more complex system that better mimics *in vivo* conditions.

Effects of MSCs on NO and PGE2 secretion

Cartilage explants secreted more NO than synovial explants, whereas the latter produced the most PGE2 [Fig. 6(A, B)]. In cartilage explants, MSC-conditioned medium exerted a small but significant ($P = 0.005$) decrease in the NO secretion from 112 ± 20 μM to 93 ± 18 μM, together with a 50% decrease in PGE2 secretion (not statistically significant) from 0.98 ± 0.78 ng/ml to 0.49 ± 0.18 ng/ml. No effects were seen in synovial explants.

Effects of MSCs on intracellular signaling pathways

To evaluate possible signaling pathways that are influenced by MSCs, we cultured isolated human synoviocytes and chondrocytes in MSC-conditioned medium and analyzed phosphorylated p38 MAP kinase and IκBa amounts by Western blot. MSC-conditioned medium did not influence p38 MAP kinase phosphorylation in either cell-type [Fig. 7(A)]. Non-phosphorylated p38 MAP kinase was constitutively expressed for all conditions (data not shown), as was α-Tubulin. Treatment with MSC-conditioned medium increased the presence of IκBa [Fig. 7(B)] in both cell-types, thereby indicating that MSCs secrete factors which inhibit NFκB activation.

Discussion

OA is a disabling disease where many catabolic and inflammatory processes play a role[27]. MSCs have chondrogenic potential, but can also play a role in immunomodulation and tissue regeneration by secretion of soluble factors[28]. In this study we showed for the first time such an effect of MSCs on OA tissues. Exposure of synovial and cartilage explants to MSC-secreted factors resulted in gene expression profiles and production of factors consistent with anti-inflammatory and anti-catabolic activity in these tissues. This included beneficial effects on the expression of genes related to inflammation (*IL-1β*, *IL-1RA*, *SOCS1*) and matrix degradation (*MMP1*, *MMP13* and *ADAMTS5*) in synovium or cartilage. These results were confirmed in a co-culture model of synovium and cartilage, a system resembling more closely the *in vivo* situation. Next to the effects on gene expression, MSC-conditioned medium reduced production of the inflammatory mediator NO in cartilage explants and increased presence of IκBa in synoviocytes and chondrocytes. The phosphorylation and degradation of IκBa, which normally binds NFκB, is an essential and first step in activation of the NFκB pathway. NFκB has been reported to induce gene expression of, amongst others, *IL-1β*, *TNFα*, *MMP1*, *MMP3* and *MMP13*, indicating it's role as a main pathway involved in inflammation and matrix degradation[16].

We needed to stimulate MSCs to achieve secretion of sufficient amounts of immunomodulatory factors to influence OA cartilage and synovial explants. We challenged our cells with TNFα and IFNγ

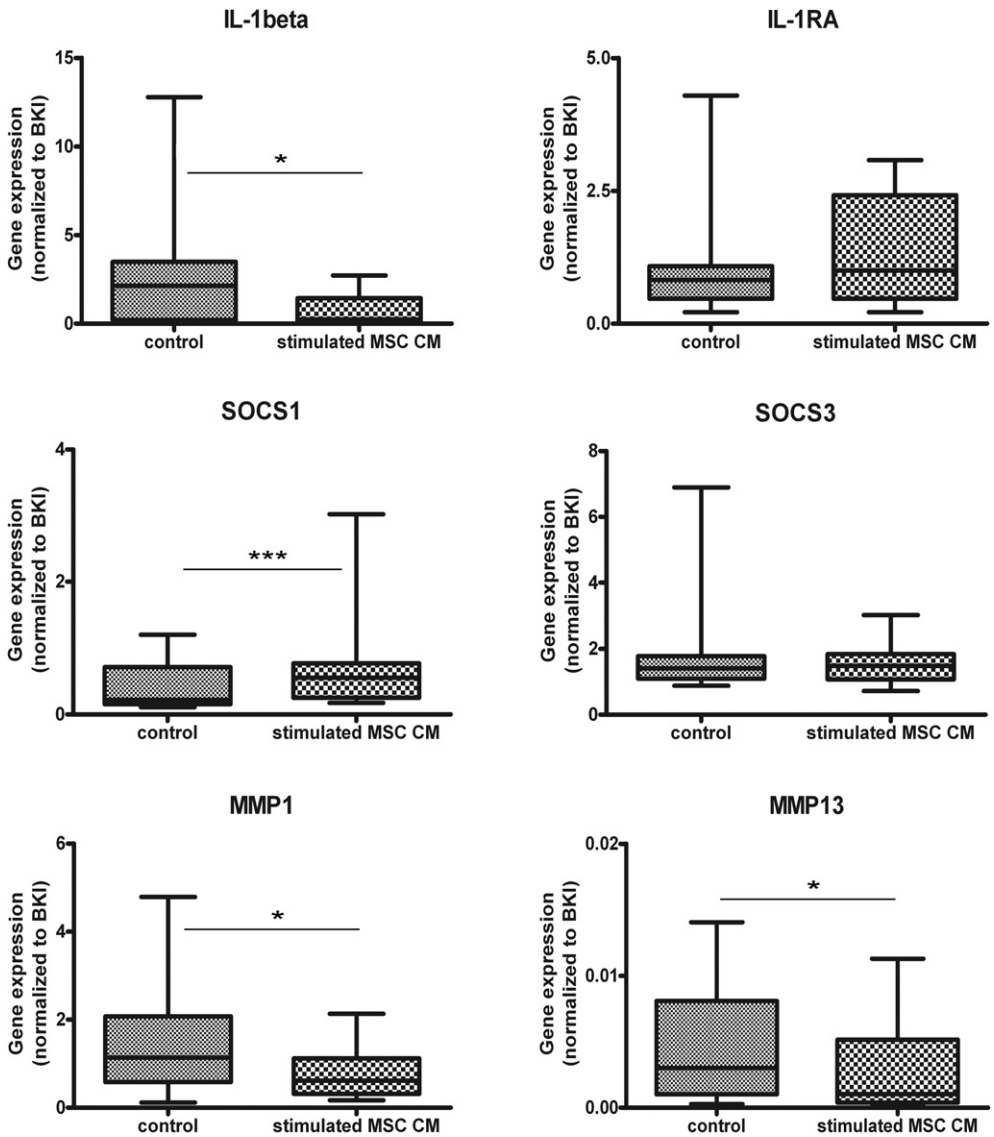


Fig. 4. Effects of factors secreted by TNFα/IFNγ stimulated MSCs on expression of genes related to inflammation and matrix degradation in human osteoarthritic synovial explants. Data are presented as boxplots for five experiments, in which MSC-conditioned medium from five MSC donors was applied to triplicate explants from five OA donors; explants were treated for 48 h; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.005$.

as described before[13,14]. TNFα is an extensively studied cytokine in OA research[27,29] and the presence of IFNγ producing T-cells in OA synovium has been indicated[30]. To study whether this model reflected pathological OA processes, we tested the effects of TNFα/IFNγ on explants from three of the five patients (Supp. Figs. 2, 3). Several inflammatory and catabolic effects were observed in both tissues at gene expression level, together with a significantly increased NO and PGE2 production as well as p38 phosphorylation and IκBa degradation. Taken together, this indicates that TNFα/IFNγ induced various processes which are relevant in OA[16,27].

The stimulated MSCs in our experiments produced IL-6, HGF, TIMP2 and TGFβ-1 and displayed a high enzymatic IDO activity. The selection of this panel of factors was based on the fact that they are known to be secreted by MSCs at high levels and their known involvement in general inflammation processes or joint metabolism[29,31]. IDO is an important factor for the inhibition of T-cell proliferation and has been reported to decrease joint inflammation[32]. The role of IL-6 in OA, however, is controversial (reviewed in Ref. 27). Although it is in general considered an inflammatory mediator, IL-6 deficient mice were shown to have a lower proteoglycan synthesis with a higher incidence of subchondral bone sclerosis[33], and increased cartilage damage which was reduced by IL-6 injection[34]. These results indicate possible advantageous roles of IL-6 in immune modulation. The other factors we determined in MSC-conditioned medium, HGF, TIMP2 and TGFβ-1, all play a role in tissue regeneration and cartilage matrix turnover[35–37].

MSC-conditioned medium upregulated *SOCS1* gene expression in synovium and caused a trend toward *SOCS3* downregulation in cartilage. *SOCS1* is a negative regulator of macrophage and dendritic cell activation, while *SOCS3* is a positive regulator of these immune cells (reviewed in Ref. 38). Furthermore, the presence of *SOCS1* has been reported to limit joint destruction in inflammatory arthritis, whereas *SOCS3* upregulation in chondrocytes has been shown to contribute to cartilage damage[39,40].

OA synovium and cartilage are known to have a very heterogeneous gene expression pattern between patients or between different areas within the same patient[41,42]. Due to this high variation it is challenging to obtain consistent results. Nevertheless, we

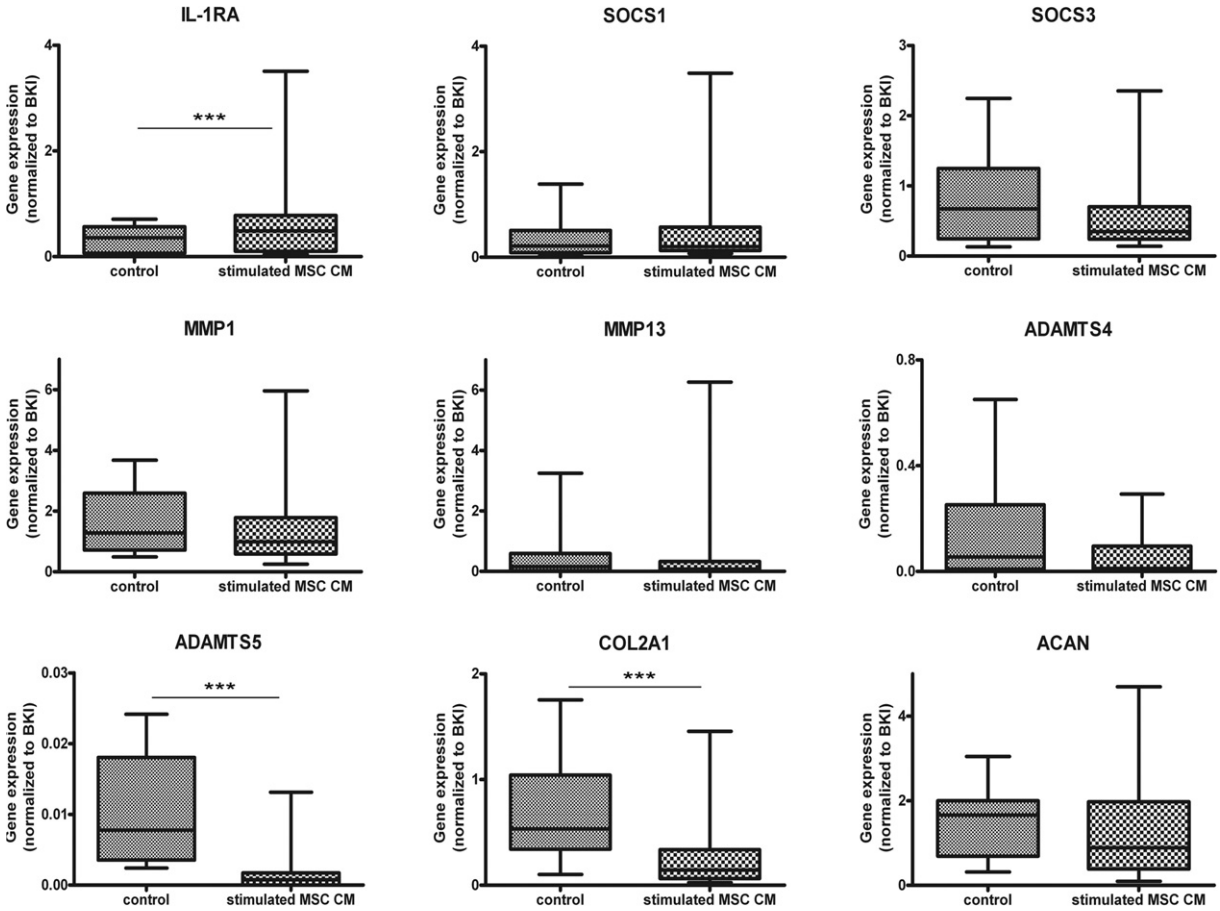


Fig. 5. Effects of factors secreted by TNFα/IFNγ stimulated MSCs on expression of genes related to inflammation and matrix turnover in human osteoarthritic cartilage explants. Data are presented as boxplots for five experiments, in which MSC-conditioned medium from five MSC donors was applied to triplicate explants from five OA donors; explants were treated for 48 h; $^{***}P < 0.005$.

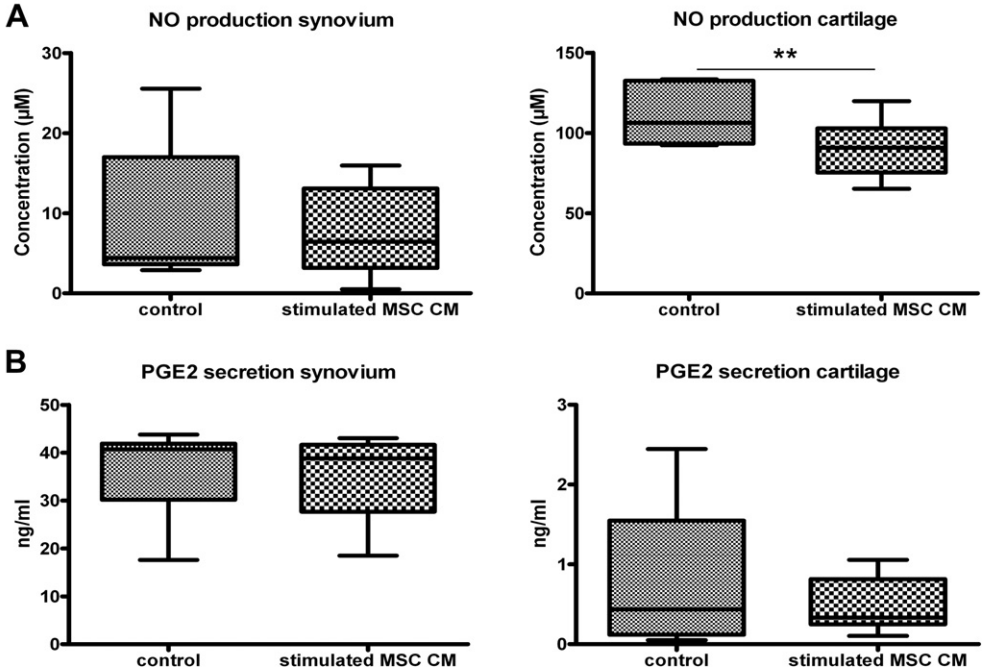


Fig. 6. Influence of factors secreted by TNFα/IFNγ stimulated MSCs on NO (A) and PGE2 secretion (B) by synovium (left panels) and cartilage (right panels) explants. Data are presented as boxplots for five experiments (NO) or four experiments (PGE2), in which MSC-conditioned medium from five MSC donors was applied to triplicate explants from five, respectively four, OA donors. Regarding NO measurements, all triplicate samples were measured individually; regarding PGE2 measurements, triplicates were pooled and measured in duplicate. Explants were treated for 48 h; $^{**}P < 0.01$.

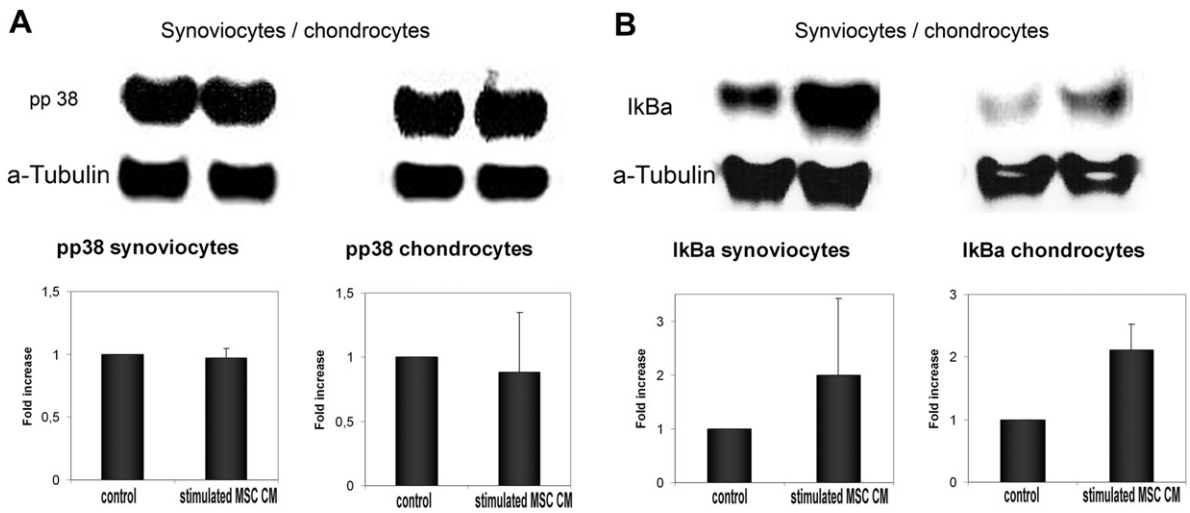


Fig. 7. Influence of factors secreted by TNFα/IFNγ stimulated MSCs on p38 MAP kinase phosphorylation (A) and IκBa presence (B) in both synoviocytes and chondrocytes was measured by Western blot. Presence of IκBa was increased in both cell-types by MSC-conditioned medium, thereby indicating an inhibitory effect on NFκB activation. Data are presented as means ± standard deviations for single values obtained in two experiments; cells were treated for 3 h for p38 MAP kinase evaluation or 10 min for IκBa analysis.

found significant effects of factors secreted by MSCs on inflammatory and matrix degrading processes. Although no clear effects of stimulated MSC-conditioned medium were observed on various other genes and factors we analyzed, many trends were seen pointing toward an overall decreased inflammatory and catabolic environment. Synovial explants exhibited a lower average gene expression of *SOCS3* and a higher expression of *IL-1RA* after treatment with factors secreted by MSCs. In cartilage, *SOCS1* was higher expressed and *ADAMTS4*, *MMP1* and *MMP13* genes were lower expressed on average in MSC-conditioned medium treated samples. Next to this, in cartilage a trend toward a diminished PGE2 production was found. Even though these effects were small and not significant, partially due to a low sample size, they supported our confidence in the potential of MSCs as environmental modulators and their beneficial role in modifying OA tissues.

Wu *et al.* found a beneficial trophic effect of MSCs on glycosaminoglycan (GAG) production by bovine chondrocyte pellets in a non-inflammatory environment[12]. We did not observe an effect on *ACAN* gene expression and found a down-regulation of *COL2A1* by MSC-conditioned medium. It could be hypothesized that in an inflammatory environment, MSCs are mainly triggered to counteract inflammation instead of stimulating matrix formation. This is further supported by the observed increase of the immunomodulatory factors IDO and IL-6 in conditioned media from TNFα/IFNγ stimulated MSCs, although the amount of growth factors between stimulated and non-stimulated MSCs remained similar. Possible anabolic effects of secreted growth factors may become more obvious in conditions without inflammation.

We have not assessed whether our observed paracrine effects were specific for MSCs. It was recently shown that skin fibroblasts suppress inflammation in an arthritis model[43], while others found no immunosuppressive properties of skin fibroblasts in a sepsis model[44]. We did not study fibroblasts since we consider this cell-type unsuitable for intra-articular application. MSCs have the advantage of being immune privileged, and they have the capacity of chondrogenic differentiation, a mechanism of action which is likely to be at least part of their regenerative capacity for cartilage repair[45]. Next to this, MSCs are locally present in multiple joint tissues[46] and are able to react to the joint environment, as shown by the increased presence of MSCs in synovium and synovial fluid after joint injury[47,48]. This suggests that the increased intra-articular presence of MSCs is part of a natural healing process. The administration of MSCs in an osteoarthritic joint could be a therapy for OA mimicking and enhancing this healing process and thereby provide a natural and autologous treatment for OA.

To our knowledge, this is the first study indicating that factors secreted by MSCs cause multiple anti-inflammatory and anti-catabolic effects in osteoarthritic cartilage and synovium. We performed culture experiments for 48 h, a common time-point to evaluate processes at a gene expression level in our group[49]. Generally, this time-point is too soon to evaluate effects at a protein level on matrix components like proteoglycans or ACAN. Therefore we evaluated proteins which are known to respond very fast and induce other processes such as NO, PGE2, IκBa or p38 MAP kinase. Further studies are warranted to investigate the effects on structural properties of the cartilage using longer-term cultures and *in vivo* experiments.

MSC-conditioned medium undoubtedly contained many more factors than the ones we have measured, including for instance TNFα stimulated gene-6[50]. The whole panel of bioactive factors probably works in concert to achieve the anti-osteoarthritic effects observed in our study. Since intra-articularly injected MSCs have been shown to survive in an intra-articular environment up to at least 4–6 weeks[5,7], they could provide the ultimate long term delivery of a cocktail of OA modifying factors.

Author contributions

GMvB: design study, data acquisition, data analysis, data interpretation, drafting the article, final approval submitted manuscript. EV: design study, data acquisition, data analysis, data interpretation, drafting the article, final approval submitted manuscript. PKB: design study, drafting the article, final approval submitted manuscript. JHW: data analysis, data interpretation, drafting the article, final approval submitted manuscript. NK: data acquisition, data analysis, drafting the article, final approval submitted manuscript. RN: design study, data acquisition, data analysis, data interpretation, drafting the article, final approval submitted manuscript. HW: data analysis and interpretation, drafting the article, final approval submitted manuscript. JANV: data analysis and interpretation, drafting the article, final approval submitted manuscript. MRB:

design study, data analysis, data interpretation, drafting the article, final approval submitted manuscript. GJVMvO: design study, data analysis, data interpretation, drafting the article, final approval submitted manuscript.

Role of the funding source
We acknowledge the Osteoarthritis Research Society International for providing a fellowship granted to E.V.; and the support of the Smart Mix Program of the Netherlands Ministry of Economic Affairs and the Netherlands Ministry of Education, Culture and Science (SSM06004). None of the funding sources influenced the study design, data collection, data analysis, data interpretation, writing the manuscript or in the decision for submission.

Conflicts of interest
None of the authors have a conflict of interest to declare.

Acknowledgments

We acknowledge Fons van de Loo for providing RT-PCR primers for *SOCS1* and *SOCS3*; Qiuwei Pan for providing the RT-PCR primers for *HGF*; Lianne van de Laar for providing the IκBa antibody and Benjamin Fernandez-Gutierrez for general support.

Supplementary data

Supplementary data related to this article can be found online at http://dx.doi.org/10.1016/j.joca.2012.06.003.

References

1. Harvey WF, Hunter DJ. The role of analgesics and intra-articular injections in disease management. Rheum Dis Clin North Am 2008;34:777–88.
2. Coleman CM, Curtin C, Barry FP, O'Flatharta C, Murphy JM. Mesenchymal stem cells and osteoarthritis: remedy or accomplice? Hum Gene Ther 2010;21:1239–50.
3. Marquass B, Schulz R, Hepp P, Zscharnack M, Aigner T, Schmidt S, *et al*. Matrix-associated implantation of predifferentiated mesenchymal stem cells versus articular chondrocytes: in vivo results of cartilage repair after 1 year. Am J Sports Med 2011.
4. Matsumoto T, Cooper GM, Gharaibeh B, Meszaros LB, Li G, Usas A, *et al*. Cartilage repair in a rat model of osteoarthritis through intraarticular transplantation of muscle-derived stem cells expressing bone morphogenetic protein 4 and soluble Flt-1. Arthritis Rheum 2009;60:1390–405.
5. Murphy JM, Fink DJ, Hunziker EB, Barry FP. Stem cell therapy in a caprine model of osteoarthritis. Arthritis Rheum 2003;48:3464–74.
6. Centeno CJ, Schultz JR, Cheever M, Robinson B, Freeman M, Marasco W. Safety and complications reporting on the re-implantation of culture-expanded mesenchymal stem cells using autologous platelet lysate technique. Curr Stem Cell Res Ther 2010;5:81–93.
7. Agung M, Ochi M, Yanada S, Adachi N, Izuta Y, Yamasaki T, *et al*. Mobilization of bone marrow-derived mesenchymal stem cells into the injured tissues after intraarticular injection and their contribution to tissue regeneration. Knee Surg Sports Traumatol Arthrosc 2006;14:1307–14.
8. Caplan AI, Correa D. The MSC: an injury drugstore. Cell Stem Cell 2011;9:11–5.
9. Chen L, Tredget EE, Wu PY, Wu Y. Paracrine factors of mesenchymal stem cells recruit macrophages and endothelial lineage cells and enhance wound healing. PLoS One 2008;3:e1886.
10. Meisel R, Zibert A, Laryea M, Gobel U, Daubener W, Dilloo D. Human bone marrow stromal cells inhibit allogeneic T-cell responses by indoleamine 2,3-dioxygenase-mediated tryptophan degradation. Blood 2004;103:4619–21.
11. Lozito TP, Tuan RS. Mesenchymal stem cells inhibit both endogenous and exogenous MMPs via secreted TIMPs. J Cell Physiol 2011;226:385–96.
12. Wu L, Leijten JC, Georgi N, Post JN, van Blitterswijk CA, Karperien M. Trophic effects of mesenchymal stem cells increase chondrocyte proliferation and matrix formation. Tissue Eng Part A 2011;17:1425–36.
13. Crop MJ, Baan CC, Korevaar SS, Ijzermans JN, Pescatori M, Stubbs AP, *et al*. Inflammatory conditions affect gene expression and function of human adipose tissue-derived mesenchymal stem cells. Clin Exp Immunol 2010;162:474–86.
14. Ryan JM, Barry F, Murphy JM, Mahon BP. Interferon-gamma does not break, but promotes the immunosuppressive capacity of adult human mesenchymal stem cells. Clin Exp Immunol 2007;149:353–63.
15. Fan Z, Yang H, Bau B, Soder S, Aigner T. Role of mitogen-activated protein kinases and NFkappaB on IL-1beta-induced effects on collagen type II, MMP-1 and 13 mRNA expression in normal articular human chondrocytes. Rheumatol Int 2006;26:900–3.
16. Roman-Blas JA, Jimenez SA. NF-kappaB as a potential therapeutic target in osteoarthritis and rheumatoid arthritis. Osteoarthritis Cartilage 2006;14:839–48.
17. de Mos M, Koevoet WJ, Jahr H, Verstegen MM, Heijboer MP, Kops N, *et al*. Intrinsic differentiation potential of adolescent human tendon tissue: an in-vitro cell differentiation study. BMC Musculoskelet Disord 2007;8:16.
18. Das RH, van Osch GJ, Kreukniet M, Oostra J, Weinans H, Jahr H. Effects of individual control of pH and hypoxia in chondrocyte culture. J Orthop Res 2010;28:537–45.
19. Beekhuizen M, Bastiaansen-Jenniskens YM, Koevoet W, Saris DB, Dhert WJ, Creemers LB, *et al*. Osteoarthritic synovial tissue inhibition of proteoglycan production in human osteoarthritic knee cartilage: establishment and characterization of a long-term cartilage-synovium coculture. Arthritis Rheum 2011;63:1918–27.
20. Hellingman CA, Koevoet W, Kops N, Farrell E, Jahr H, Liu W, *et al*. Fibroblast growth factor receptors in in vitro and in vivo chondrogenesis: relating tissue engineering using adult mesenchymal stem cells to embryonic development. Tissue Eng Part A 2010;16:545–56.
21. van der Windt AE, Haak E, Das RH, Kops N, Welting TJ, Caron MM, *et al*. Physiological tonicity improves human chondrogenic marker expression through nuclear factor of activated T-cells 5 in vitro. Arthritis Res Ther 2010;12:R100.
22. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 2001;25:402–8.
23. Green LC, Wagner DA, Glogowski J, Skipper PL, Wishnok JS, Tannenbaum SR. Analysis of nitrate, nitrite, and [15N]nitrate in biological fluids. Anal Biochem 1982;126:131–8.
24. Ishinaga H, Jono H, Lim JH, Komatsu K, Xu X, Lee J, *et al*. Synergistic induction of nuclear factor-kappaB by transforming growth factor-beta and tumour necrosis factor-alpha is mediated by protein kinase A-dependent RelA acetylation. Biochem J 2009;417:583–91.
25. Fitzgerald JB, Jin M, Chai DH, Siparsky P, Fanning P, Grodzinsky AJ. Shear- and compression-induced chondrocyte transcription requires MAPK activation in cartilage explants. J Biol Chem 2008;283:6735–43.
26. Kang JW, Kang KS, Koo HC, Park JR, Choi EW, Park YH. Soluble factors-mediated immunomodulatory effects of canine

adipose tissue-derived mesenchymal stem cells. Stem Cells Dev 2008;17:681–93.
27. Kapoor M, Martel-Pelletier J, Lajeunesse D, Pelletier JP, Fahmi H. Role of proinflammatory cytokines in the pathophysiology of osteoarthritis. Nat Rev Rheumatol 2011;7:33–42.
28. Singer NG, Caplan AI. Mesenchymal stem cells: mechanisms of inflammation. Annu Rev Pathol 2011;6:457–78.
29. Goldring MB, Otero M, Plumb DA, Dragomir C, Favero M, El Hachem K, *et al*. Roles of inflammatory and anabolic cytokines in cartilage metabolism: signals and multiple effectors converge upon MMP-13 regulation in osteoarthritis. Eur Cell Mater 2011;21:202–20.
30. Ishii H, Tanaka H, Katoh K, Nakamura H, Nagashima M, Yoshino S. Characterization of infiltrating T cells and Th1/Th2-type cytokines in the synovium of patients with osteoarthritis. Osteoarthritis Cartilage 2002;10:277–81.
31. Sze SK, de Kleijn DP, Lai RC, Khia Way Tan E, Zhao H, Yeo KS, *et al*. Elucidating the secretion proteome of human embryonic stem cell-derived mesenchymal stem cells. Mol Cell Proteomics 2007;6:1680–9.
32. Szanto S, Koreny T, Mikecz K, Glant TT, Szekanecz Z, Varga J. Inhibition of indoleamine 2,3-dioxygenase-mediated tryptophan catabolism accelerates collagen-induced arthritis in mice. Arthritis Res Ther 2007;9:R50.
33. de Hooge AS, van de Loo FA, Bennink MB, Arntz OJ, de Hooge P, van den Berg WB. Male IL-6 gene knock out mice developed more advanced osteoarthritis upon aging. Osteoarthritis Cartilage 2005;13:66–73.
34. van de Loo FA, Kuiper S, van Enckevort FH, Arntz OJ, van den Berg WB. Interleukin-6 reduces cartilage destruction during experimental arthritis. A study in interleukin-6-deficient mice. Am J Pathol 1997;151:177–91.
35. Takebayashi T, Iwamoto M, Jikko A, Matsumura T, Enomoto-Iwamoto M, Myoukai F, *et al*. Hepatocyte growth factor/scatter factor modulates cell motility, proliferation, and proteoglycan synthesis of chondrocytes. J Cell Biol 1995;129:1411–9.
36. Visse R, Nagase H. Matrix metalloproteinases and tissue inhibitors of metalloproteinases: structure, function, and biochemistry. Circ Res 2003;92:827–39.
37. Blaney Davidson EN, van der Kraan PM, van den Berg WB. TGF-beta and osteoarthritis. Osteoarthritis Cartilage 2007;15:597–604.
38. Kubo M, Hanada T, Yoshimura A. Suppressors of cytokine signaling and immunity. Nat Immunol 2003;4:1169–76.
39. Egan PJ, Lawlor KE, Alexander WS, Wicks IP. Suppressor of cytokine signaling-1 regulates acute inflammatory arthritis and T cell activation. J Clin Invest 2003;111:915–24.
40. Smeets RL, Veenbergen S, Arntz OJ, Bennink MB, Joosten LA, van den Berg WB, *et al*. A novel role for suppressor of cytokine signaling 3 in cartilage destruction via induction of chondrocyte desensitization toward insulin-like growth factor. Arthritis Rheum 2006;54:1518–28.
41. Okabe T, Ohmori Y, Tanigami A, Hishigaki H, Suzuki Y, Sugano S, *et al*. Detection of gene expression in synovium of patients with osteoarthritis using a random sequencing method. Acta Orthop 2007;78:687–92.
42. Fukui N, Miyamoto Y, Nakajima M, Ikeda Y, Hikita A, Furukawa H, *et al*. Zonal gene expression of chondrocytes in osteoarthritic cartilage. Arthritis Rheum 2008;58:3843–53.
43. Bouffi C, Bony C, Jorgensen C, Noel D. Skin fibroblasts are potent suppressors of inflammation in experimental arthritis. Ann Rheum Dis 2011;70:1671–6.
44. Nemeth K, Leelahavanichkul A, Yuen PS, Mayer B, Parmelee A, Doi K, *et al*. Bone marrow stromal cells attenuate sepsis via prostaglandin E(2)-dependent reprogramming of host macrophages to increase their interleukin-10 production. Nat Med 2009;15:42–9.
45. Niemeyer P, Krause U, Kasten P, Kreuz PC, Henle P, Sudkam NP, *et al*. Mesenchymal stem cell-based HLA-independent cell therapy for tissue engineering of bone and cartilage. Curr Stem Cell Res Ther 2006;1:21–7.
46. van Buul GM, van Osch GJVM. Musculoskeletal stem cells. In: Steinhoff G, Ed. Regenerative Medicine: From Protocol to Patient. 1 edn. London: Springer; 2011:397–420.
47. Kurth TB, Dell'accio F, Crouch V, Augello A, Sharpe PT, De Bari C. Functional mesenchymal stem cell niches in the adult knee joint synovium in vivo. Arthritis Rheum 2011.
48. Morito T, Muneta T, Hara K, Ju YJ, Mochizuki T, Makino H, *et al*. Synovial fluid-derived mesenchymal stem cells increase after intra-articular ligament injury in humans. Rheumatology (Oxford) 2008;47:1137–43.
49. Bastiaansen-Jenniskens YM, Clockaerts S, Feijt C, Zuurmond AM, Stojanovic-Susulic V, Bridts C, *et al*. Infrapatellar fat pad of patients with end-stage osteoarthritis inhibits catabolic mediators in cartilage. Ann Rheum Dis 2012;71:288–94.
50. Prockop DJ, Oh JY. Mesenchymal stem/stromal cells (MSCs): role as guardians of inflammation. Mol Ther 2012;20:14–20.