THE INFRAPATELLAR FAT PAD FROM DISEASED JOINTS INHIBITS CHONDROGENESIS OF MESENCHYMAL STEM CELLS

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Abstract

Cartilage repair by bone marrow derived mesenchymal stem cells (MSCs) can be influenced by inflammation in the knee. Next to synovium, the infrapatellar fat pad (IPFP) has been described as a source for inflammatory factors. Here, we investigated whether factors secreted by the IPFP affect chondrogenesis of MSCs and whether this is influenced by different joint pathologies or obesity. Furthermore, we examined the role of IPFP resident macrophages. First, we made conditioned medium from IPFP obtained from osteoarthritic joints, IPFP from traumatically injured joints during anterior cruciate ligament reconstruction, and subcutaneous adipose tissue. Additionally, we made conditioned medium of macrophages isolated from osteoarthritic IPFP and of polarised monocytes from peripheral blood. We evaluated the effect of different types of conditioned medium on MSC chondrogenesis. Conditioned medium from IPFP decreased collagen 2 and aggrecan gene expression as well as thionin and collagen type 2 staining. This anti-chondrogenic effect was the same for conditioned medium from IPFP of osteoarthritic and traumatically injured joints. Furthermore, IPFP from obese (Body Mass Index >30) donors did not inhibit chondrogenesis more than that of lean (Body Mass Index <25) donors. Finally, conditioned medium from macrophages isolated from IPFP decreased the expression of hyaline cartilage genes, as did peripheral blood monocytes stimulated with pro-inflammatory cytokines. The IPFP and the resident pro-inflammatory macrophages could therefore be targets for therapies to improve MSC-based cartilage repair.

Keywords: Cartilage repair, mesenchymal stem cells, infrapatellar fat pad, osteoarthritis, inflammation, obesity.

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Introduction

Articular cartilage has limited self-healing capabilities and surgical intervention using bone marrow stimulation techniques, such as the microfracture procedure, can be used to activate mesenchymal stem cells (MSCs) from the underlying bone marrow to repair the defect (Shapiro *et al.*, 1993; Steadman *et al.*, 2003). However, instead of a normal hyaline cartilage matrix, a fibrocartilaginous cartilage matrix fills up the defect (Frisbie *et al.*, 2003; Kaul *et al.*, 2012; Shapiro *et al.*, 1993). An explanation why hyaline cartilage production is inhibited could be that the cartilage defect is located in a post-traumatic inflammatory environment (Irie *et al.*, 2003). In this post-traumatic inflammatory environment, circulating inflammatory factors stimulate matrix degradation and inhibit chondrogenic differentiation (Heldens *et al.*, 2012).

Besides surgical intervention, research is currently focused on MSC-based tissue-engineered cartilage repair strategies (Hunziker *et al.*, 2015). In these strategies, repair tissues are engineered *in-vitro* using a combination of MSCs, scaffolds and growth factors and subsequently implanted into the cartilage defect. When implanted, the MSCs in these pre-engineered constructs will be exposed to the same post-traumatic inflammatory environment as the MSCs after the microfracture procedure. An inflammatory environment can inhibit hyaline cartilage matrix production (Wehling *et al.*, 2009).

Another factor that could influence MSC chondrogenic differentiation is obesity. The clinical results after the microfracture procedure are reported to be worse in patients with obesity (Mithoefer *et al.*, 2005). Obesity is known to cause systemic metabolic and inflammatory changes, with increased circulating inflammatory factors (Lumeng and Saltiel, 2011).

Inflammatory factors can be produced by different tissues of the knee joint, the most well described being the synovium and cartilage. The infrapatellar fat pad (IPFP) is an adipose tissue located extra-synovially and intra-capsularly. It is richly vascularised, innervated and known to also secrete many adipokines and cytokines (Clockaerts *et al.*, 2012; Clockaerts *et al.*, 2010). Adipose tissue in obese patients has been shown to have an inflammatory phenotype with abundant immune cell infiltration and secretion of inflammatory cytokines (Grant and Dixit, 2015). Furthermore, joint trauma and joint degradation can both increase inflammation in the IPFP and the secretion of inflammatory factors (Bastiaansen-Jenniskens *et al.*, 2012;

Clockaerts *et al.*, 2012; Gandhi *et al.*, 2011; Gierman *et al.*, 2013).

The IPFP contains immune cells such as macrophages (Bastiaansen-Jenniskens *et al.*, 2012; Klein-Wieringa *et al.*, 2011). Macrophages play an important role in inflammation and wound healing. Macrophages can be categorised into pro-inflammatory macrophages and anti-inflammatory or wound healing macrophages (Mosser and Edwards, 2008; Murray *et al.*, 2014). Inflammation or obesity often results in increased numbers of inflammatory macrophages. Previously, we have shown that only pro-inflammatory and not anti-inflammatory macrophages inhibit MSC chondrogenesis *in vitro* (Fahy *et al.*, 2014).

Since the IPFP from end-stage OA patients is a source of inflammatory and catabolic cytokines (Clockaerts *et al.*, 2012), it is to be expected that IPFP secreted factors influence other joint tissues. Against our expectation that the factors released from IPFP would stimulate tissue degradation, our previous studies demonstrated that factors released from IPFP from end-stage osteoarthritis (OA) patients inhibit catabolic mediators in cartilage (Bastiaansen-Jenniskens *et al.*, 2012) and stimulate fibrotic processes in synovial fibroblasts (Bastiaansen-Jenniskens *et al.*, 2013). This indicates that the factors released by the IPFP not only have a pro-inflammatory effect on joint environment and but that this effect could be different in each type of joint tissue.

Considering the IPFP's potential to produce factors that influence the joint environment, and the knowledge that the joint environment influences cartilage repair, we hypothesised that factors secreted by the IPFP affect MSC-based cartilage repair. In this study, we investigated whether and how factors secreted by the IPFP affect the chondrogenesis of MSCs *in vitro* and whether this could be influenced by joint pathology or obesity. Furthermore, we evaluated whether macrophages present in the IPFP could be responsible for the effect seen on MSC chondrogenesis.

Materials and Methods

Preparation of adipose tissue conditioned medium (CM)

IPFP tissue was obtained as leftover material from patients with OA who had undergone a total knee replacement (age 66.1 years (53.9-80.2); body mass index (BMI) 29.3 (21.5-43.7)), or from patients during anterior cruciate ligament (LR) reconstruction (age 29.3 (17.6-48.0); BMI 22.8 (19.3-29.0)), with time from trauma to reconstruction 5.4 months (2-12 months). Subcutaneous adipose tissue (SAT) from patients undergoing total hip replacement was used (age 63.0 (50.2-79.1); BMI 31.3 (24.0-35.8)). Consent was given in accordance with the guidelines of the Federation of Biomedical Scientific Societies (http://www.federa. org) after approval by the local ethical committee (MEC 2012-267). All culture media from now onwards were supplemented with 1.5 µg/mL fungizone and 50 µg/mL gentamicin (both Gibco, Carlsbad, CA, USA). To generate adipose tissue CM, adipose tissue was cut into small pieces of approximately 9 mm^2, washed two times with saline and cultured at a concentration of 100 mg/mL in Dulbecco's

Modified Eagle Medium with Glutamax (DMEM-HG; Gibco) supplemented with 1 % insulin-transferrin-selenium (ITS+; BD Biosciences, Erembodegem, Belgium) for 24 h at 37 °C. Afterwards, the medium was harvested, centrifuged at 250 ×g for 8 min and the supernatant was stored at −80 °C for culture experiments. Unconditioned medium was generated by incubation of DMEM-HG supplemented with ITS+ for 24 h at 37 °C, centrifuged at 250 ×g for 8 min and stored at −80 °C.

Isolation of macrophages from adipose tissue and preparation of IPFP macrophage CM

To isolate macrophages from the stromal vascular fraction of adipose tissue, six OA IPFP samples (age 66.6 (54.1-80.2), BMI 28.9 (21.9-33.9)) were used. Adipose tissue samples were cut into pieces of around 9 mm^2 and incubated overnight at 37 °C with 1 mg/mL collagenase B (Roche, Penzberg, Germany) in DMEM-HG with 10 % FCS. After centrifugation, the supernatant containing the floating adipocytes and fat was removed, and the cell pellet was re-suspended and filtered through a 100 µm filter followed by a 40 µm filter. The resulting cell suspension was layered on top of 15 mL Ficoll (Ficoll-Paque™ PLUS, GE Healthcare, Little Chalfont, UK) and separated by density gradient following centrifugation at 1,000 ×g for 15 min. The interphase was removed and washed with phosphate buffered saline (PBS; Gibco) containing 2 % foetal calf serum (FCS) followed by incubation with CD45-PE (BD Biosciences) for 1 h and incubation in 20 µL of anti-mouse-IgG magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany) *per* 10,000,000 cells for 30 min in the dark at 4 °C. CD45 positive cells were separated by magnetic activated cell sorting (MACS, MACS Separation Columns LS and MidiMACS™ Separator, Miltenyi Biotec) according to manufacturer's instructions. Flow cytometric analysis was performed to confirm a >90 % CD45+ population purity. The CD45 reduced fraction contained <10 % CD45+ cells. Isolated CD45+ cells were seeded at a cell density of 100,000 cells/cm^2 in 24 wells plates and cultured overnight at 37 °C in DMEM-HG supplemented with 10 % FCS. Cells were then carefully washed twice with phosphate-buffered saline (PBS) to remove non-attached cells and DMEM-HG with 1 % ITS+ was added. After 24 h, IPFP CD45+ CM was harvested, centrifuged at 250 ×g for 8 min and stored at −80 °C for further experiments.

Human monocyte isolation, differentiation and CM preparation

Peripheral blood monocytes were isolated from buffy coats with CD14+ magnetic beads (Miltenyi Biotec) and MACS according to manufacturer's instructions. Monocytes were then seeded at 500,000 cells/cm^2, stimulated to M(LPS+IFN-ɣ) with 100 ng/mL lipopolysaccharide (LPS; Sigma Aldrich, St. Louis, MO, USA) and 10 ng/mL interferon-ɣ (IFN-ɣ; PeproTech, Rocky Hill, NJ, USA) or M(IL-4) with 10 ng/mL interleukin-4 (IL-4; Preprotech) (Murray *et al.*, 2014). Subsequently, M(LPS+IFN-ɣ) and M(IL-4) CM were made as previously described (Fahy *et al.*, 2014; Grotenhuis *et al.*, 2014). Samples of these experiments were previously used in Fahy *et al.* (2014),

but additional gene expression analysis were performed for the current study.

Human dermal fibroblast expansion and CM preparation

Human dermal fibroblast from adult donors (HDFa) were acquired from Gibco (Cat. no. C-013-5C) and were seeded at 5,000 cells/cm^2 in DMEM-HG containing 10 % FCS. After reaching sub-confluency, HDFa were trypsinised and seeded again. For HDFa CM preparation, P4 cells were cultured until subconfluency, washed with PBS three times, and DMEM-HG with ITS+ was added. After 24 h, CM was harvested, centrifuged at 250 ×*g* for 8 min and stored at −80 °C for further experiments.

Isolation and culture of human MSCs

MSCs were isolated from heparinised bone marrow aspirates of patients with OA undergoing total hip replacement (9 donors; age 61 years (32-78)). Patients gave informed consent and the study was approved by the local ethical committee of the Erasmus MC, Rotterdam (MEC 2004-142) and Albert Schweitzer Hospital, Dordrecht (MEC 2011-07). Bone marrow aspirates were plated and washed after 24 h with PBS containing 2 % FCS (Lonza, Verviers, Belgium). MSCs were expanded in Minimum Essential Medium-Alpha (α-MEM; Gibco) supplemented with 10 % FCS, 1 ng/mL fibroblast growth factor 2 (FGF2; R&D Systems, Minneapolis, MN, USA) and 25 µg/mL ascorbic acid-2-phosphate (Sigma-Aldrich). After reaching sub-confluency, MSCs were trypsinised and seeded again at a density of 2,500 cells/cm^2. MSCs of passage 3 or 4 were used.

Chondrogenic differentiation of MSCs

MSCs were encapsulated in 1.2 % alginate (CP Kelco, Atlanta, GA, USA) in saline at a density of 4,000,000 cells/mL. Beads were formed by purging the MSC-alginate solution through a 23G needle and allowing droplets to fall into 102 mM CaCl$_2$ solution. Pellets were made by centrifugation of 200,000 cells in polypropylene tubes. Beads and pellets were cultured with DMEM-HG with ITS+ containing 25 % IPFP CM or unconditioned medium. As chondrogenic supplements, we used 0.1 µM dexamethasone (Sigma-Aldrich), 10 ng/mL transforming growth factor-β1 (TGFβ1; R&D Systems), 25 µg/mL ascorbic acid-2-phosphate (Sigma-Aldrich), 40 µg/ml L-proline (Sigma-Aldrich) and 1 mM sodium pyruvate (Gibco). MSC beads were cultured in 100 µL medium *per* bead and pellets were cultured in 500 µL medium *per* pellet. Medium was refreshed 3 times a week. Beads and pellets were harvested after 28 d.

For the experiments with macrophages, obese *versus* lean IPFP, osteoarthritic *versus* post-joint trauma IPFP, and SAT *versus* IPFP, beads were first cultured in DMEM-HG containing ITS+ and chondrogenic supplements for 14 d. From day 14-17, parts of the DMEM-HG with ITS+ was replaced by conditioned medium or an equal percentage of unconditioned medium. For the experiments with IPFP and SAT CM, we replaced 25 % of the DMEM-HG containing ITS+. For the macrophages experiments, we replaced 50 % of the DMEM-HG containing ITS+ with

CD45 CM or 20 % with M(LPS+IFNγ)/M(IL-4) CM. For the CD45 experiments, the lowest cell number used to generate CM (0.23 µg DNA) was set at 50 % and for the M(LPS+IFNγ)/M(IL-4) experiments, the average cell number used to generate CM (1.64 µg DNA) was set at 20 %. All subsequent donors were corrected accordingly to assure that medium conditioned by an equal number of cells was added. Chondrogenic supplements were always added after mixing the medium with CM or unconditioned medium. In this way, all conditions had the same final concentration of all supplements. Beads were harvested at day 17. All media were refreshed 24 h prior to harvest.

Gene expression analysis

MSC alginate beads were harvested in ice-cold 55 mM sodium citrate and dissolved after gentle agitation at 4 °C. The solution was then centrifuged at 400 ×*g* for 8 min at 4 °C and cell pellets were re-suspended in 650 µL RNA-Bee (TelTest, Friendswood, TX, USA) *per* bead. Chloroform (Sigma-Aldrich) was added to all samples at 200 µL/mL RNA-Bee. RNA was further purified using a RNeasy Micro Kit (Qiagen). RNA concentration and quality was measured using a NanoDrop ND1000 UV-Vis Spectrophotometer (Isogen Life Science, De Meem, The Netherlands). RNA was reverse-transcribed to cDNA using the RevertAid First Strand cDNA Synthesis Kit (Fermentas, Waltham, MA, USA) according to manufacturer's instructions. qRT-PCR was performed in 10 µL reactions on cDNA using the CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). Expression of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), hypoxanthine phosphoribosyltransferase 1 (*HPRT1*), collagen type II a1 (*COL2A1*), aggrecan (*ACAN*), collagen type I a1 (*COL1A1*) and versican (*VCAN*) were determined with qRT-PCR. The genes *GAPDH* and *HPRT* were used as housekeepers. Gene expression was analysed according to the ΔΔCt method, with the condition treated with unconditioned medium used as control and set at 1.0. Expression levels of other conditions are expressed relative to this control condition. The primer efficiency for *GAPDH* was 1.06, for *HPRT* 0.96, for *COL2A1* 1.02, for *ACAN* 0.95, for *COL1A1* 0.97 and *VCAN* 0.95. The mean Ct value for *GAPDH* was 22.9 and for *HPRT* 28.8. Difference in Ct values between the samples within each experiment was less than 1 Ct and there were no significant differences between the conditions (data not shown). Gene expression values after normalisation to housekeepers are used to calculate *COL2A1/COL1A* and *ACAN/VCAN* ratios.

Primer sequences were as follows: *GAPDH,* forward: GTCAACGGATTTGGTCGTATTGGG, reverse: TGCCATGGGTGGAATCATATTGG, and probe Fam: TGGCGCCCCAACCAGCC. *HPRT* forward: TATGGACAGGACTGAACGTCTTG, reverse: CACACAGAGGGCTACAATGTG, and probe Fam: CGCCCAATACGACCAAATCCGTTGAC. *COL2A1* forward: GGCAATAGCAGGTTCACGTACA, reverse: CGATAACAGTCTTGCCCCACTT, and probe Fam: CCGGTATGTTTCGTGCAGCCATCCT. *ACAN* forward: TCGAGGACAGCGAGGCC, reverse: TCGAGGGTGTAGCGTGTAGAGA, and probe Fam: ATGGAACACGATGCCTTTCACCACGA. *COL1A1*

forward: CAGCCGCTTCACCTACAGC, reverse: TTTTGTATTCAATCACTGTCTTGCC, and probe Fam: CCGGTGTGACTCGTGCAGCCATC. *VCAN* forward: TGGAATGATGTTCCCTGCAA, reverse: AAGGTCTTGGCATTTTCTACAACAG, and probe Fam: CTGGCCGCAAGCAACTGTTCCTTT.

Quantification of DNA and glycosaminoglycan (GAG) content

Alginate beads were dissolved in sodium citrate and digested with papain. (Sigma Aldrich). Pellets were digested with proteinase K (Sigma Aldrich). DNA was subsequently quantified by spectrophotometric detection of ethidium bromide (Gibco) binding at 340 and 590 nm. GAG content was determined with dimethylmethylene blue (Polysciences, Warrington, PA, USA) spectrophotometrically at 530 nm and 590 nm (Enobakhare *et al.*, 1996) and at pH 1.75.

Histological evaluation of MSC chondrogenesis in pellet culture

6 μm thick paraffin sections were made of the pellets and thionin staining was performed. For immunohistochemical staining, sections were incubated with monoclonal antibody against collagen type 2 (DSHB, Iowa City, IA, USA, #II-II 6B3) and collagen type 1 (Abcam, Cambridge, UK, #6308) or IgG1 isotype control (Dako Cytomation, Heverlee, Belgium, #X0931). Sections were then incubated with Link and Label (Biogenex, Fremount, CA, USA, HK-321-UK) and freshly prepared new-fuchsin was used as substrate.

Evaluation of macrophage subtypes in adipose tissue

10 μm cryosections were made of 13 samples of OA IPFP (age 65.3 (54.3-74.1), BMI 28 (21.5-35.8)), 7 samples of LR IPFP (age 29.9 (21.3-48), BMI 25.6 (19.3-38.5)) and 9 samples of SAT (age 63.1 (±50.2-79.1), BMI 32.3 (25.9-47.2)). After fixation in acetone, sections were incubated with monoclonal antibodies against CD68 (Dako, #EMB11), CD11c (Chemicon, Millipore, Billerica, MA, USA, #EP1347Y) and CD206 (Abcam, #64693). Mouse IgG (Dako) were used as isotype controls. Subsequently, sections were incubated with Link and Label (BioGenex) and freshly prepared new-fuchsin was used as substrate. A cell was considered positive when stained red. Samples were ranked based on their relative number of positive cells in three sections *per* sample by two blinded observers as previously described (Bastiaansen-Jenniskens *et al.*, 2012). Ranking was performed for each marker separately and for each sample, the average rank of the two observers (WW and RR) was used in the analysis.

Statistical analysis

IBM SPSS statistics 21 (IBM Corporation, Armonk, NY, USA) was used for statistical analysis. The Shapiro-Wilk test was used to test data for normality and the Levene's test for homogeneity of variance. No data were normally distributed. For gene analysis and GAG measurement, if not otherwise stated, the Kruskal-Wallis test was used followed by a *post-hoc* Bonferroni correction. For immunohistochemical ranking, the Mann-Whitney U test was used. $p < 0.05$ was considered statistically significant.

Results

IPFP negatively impacts chondrogenic differentiation of MSCs

COL2A1 and *ACAN* were only detectable after TGFβ1 was added (Fig. 1). IPFP from OA donors statistically significantly lowered the gene expression of MSCs for *COL2A1* ($p < 0.001$), *ACAN* ($p < 0.001$) and *VCAN*, without affecting *COL1A1* (Fig. 1A). This resulted in a lower *COL2A1/COL1A1* ($p < 0.001$) and *ACAN/VCAN* ratio ($p < 0.05$) (Fig. 1A). This effect was absent when CM was heated to 95 °C for 10 min before use, indicating that the effects are caused by proteins produced by IPFP (Fig. 1B). GAG content of the alginate beads was increased by IPFP (Fig. 2A). To investigate the effects of IPFP on matrix production further, pellet culture was used. IPFP decreased thionin and immunohistochemical staining of collagen type 2, whereas collagen type 1 staining was not influenced (Fig. 2C). Furthermore, IPFP decreased GAG content of pellets (Fig. 2D and E).

Effect of obese *versus* lean IPFP and osteoarthritic *versus* traumatic joint IPFP on MSC chondrogenesis

Obesity increases the release of inflammatory factors by adipose tissue. Therefore, we hypothesised that CM from obese IPFP donors (BMI 33.1 (30-35.8)) would reduce MSC chondrogenesis more than CM from lean IPFP (BMI 23.1 (21.5-24)) donors. However, chondrogenic markers were not more inhibited by CM from obese than by CM from lean donors (Fig. 3A).

Next, we compared the effect of CM from LR IPFP (BMI 22.9 (22.0-25.0)) with BMI matched OA IPFP (BMI 23.1 (21.5-24.0)). Chondrogenic gene expression was not statistically significantly different between MSCs exposed to CM from LR IPFP and CM from OA IPFP. Interestingly, LR IPFP CM statistically significantly increased *COL1A1* compared to control and OA IPFP CM ($p = 0.023$) (Fig. 3B).

Chondrogenic differentiation of MSCs is more affected by IPFP than by SAT

To investigate whether the effect of adipose tissue of the joint (IPFP) is different from subcutaneous adipose tissue (SAT), we added CM made from SAT to MSCs during chondrogenic differentiation. SAT statistically significantly lowered gene expression of *COL2A1* and *ACAN* ($p < 0.01$), but did not influence the *COL2A1/COL1A1* and *ACAN/VCAN* ratio (Fig. 4A). To investigate whether this is due to a difference in the concentration of factors released by IPFP and SAT, we increased the percentage of SAT CM added to culture up to 50 %. In contrast to the effect of IPFP, the effect of SAT on chondrogenesis was not dosage dependent (Fig. 4B).
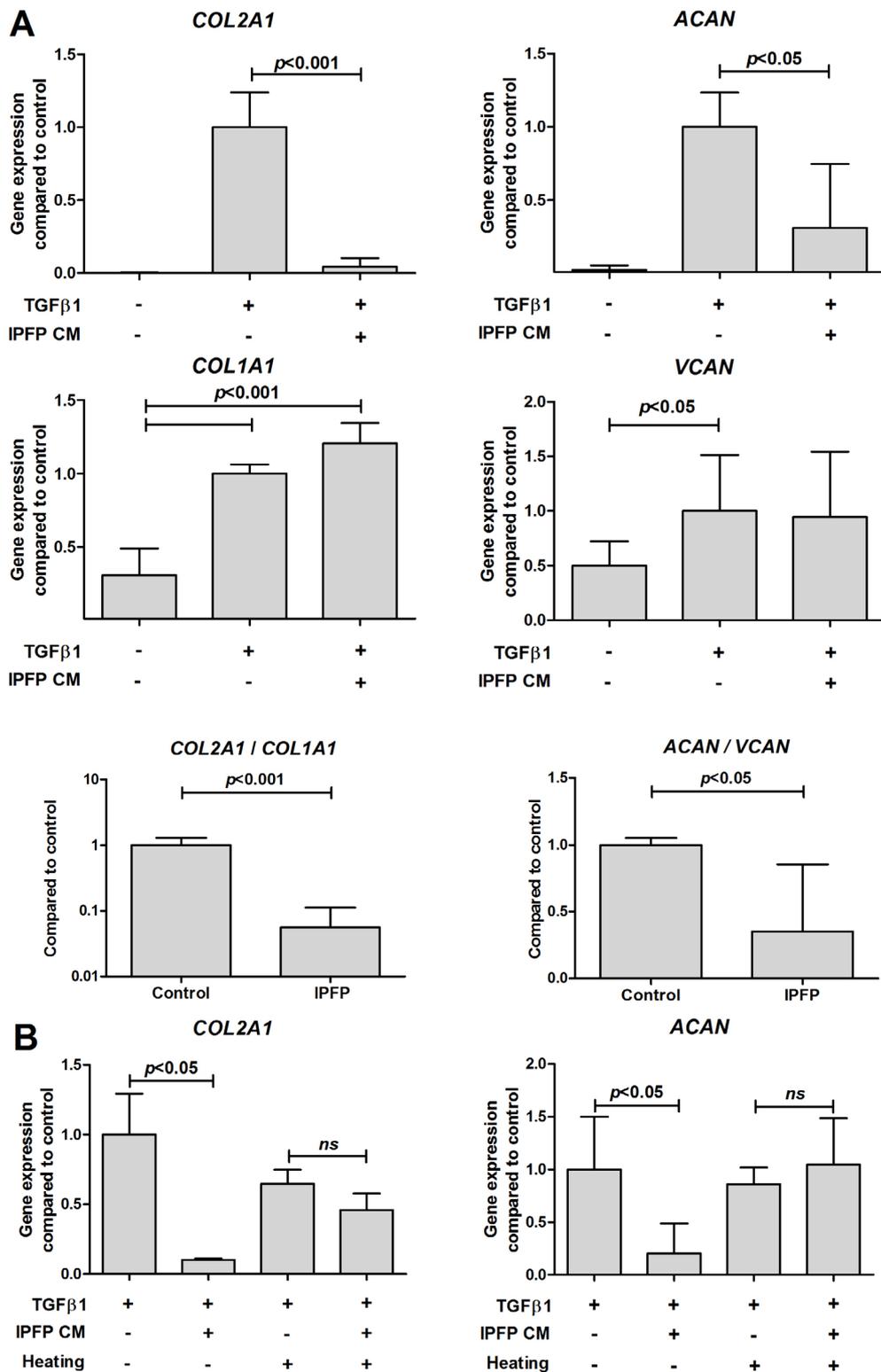
Fig. 1. IPFP inhibits chondrogenic differentiation of MSCs. (A) MSC alginate beads were treated with 25 % IPFP CM for 28 d of culture in chondrogenic medium with TGFβ1. The control condition was treated with 25 % unconditioned medium with chondrogenic medium with TGFβ1. Relative gene expression of COL2A1, ACAN, COL1A1 and VCAN normalised to the control condition and COL2A1/COL1A1 and ACAN/VCAN ratio. CM were pooled from 3-5 different IPFP donors and used to treat 3 MSC donors, with each condition performed in triplicate. **(B)** MSC alginate beads were treated with 25 % CM from IPFP from day 14-17 of chondrogenic stimulation. CM or unconditioned medium were heated for 10 min at 95 °C and allowed to cool down to room temperature before addition to MSC alginate beads. Relative gene expression of COL2A1 and ACAN in MSCs after treatment, normalised to the control condition. One pool of CM from 3 IPFP donors was used to treat 1 MSC donor, with each condition performed in triplicate. The mean Ct value in the control condition for COL2A1 was 23.2, for ACAN 27.3, for COL1A1 19.7 and for VCAN 24.3. Values represent the mean ± standard deviation (SD).
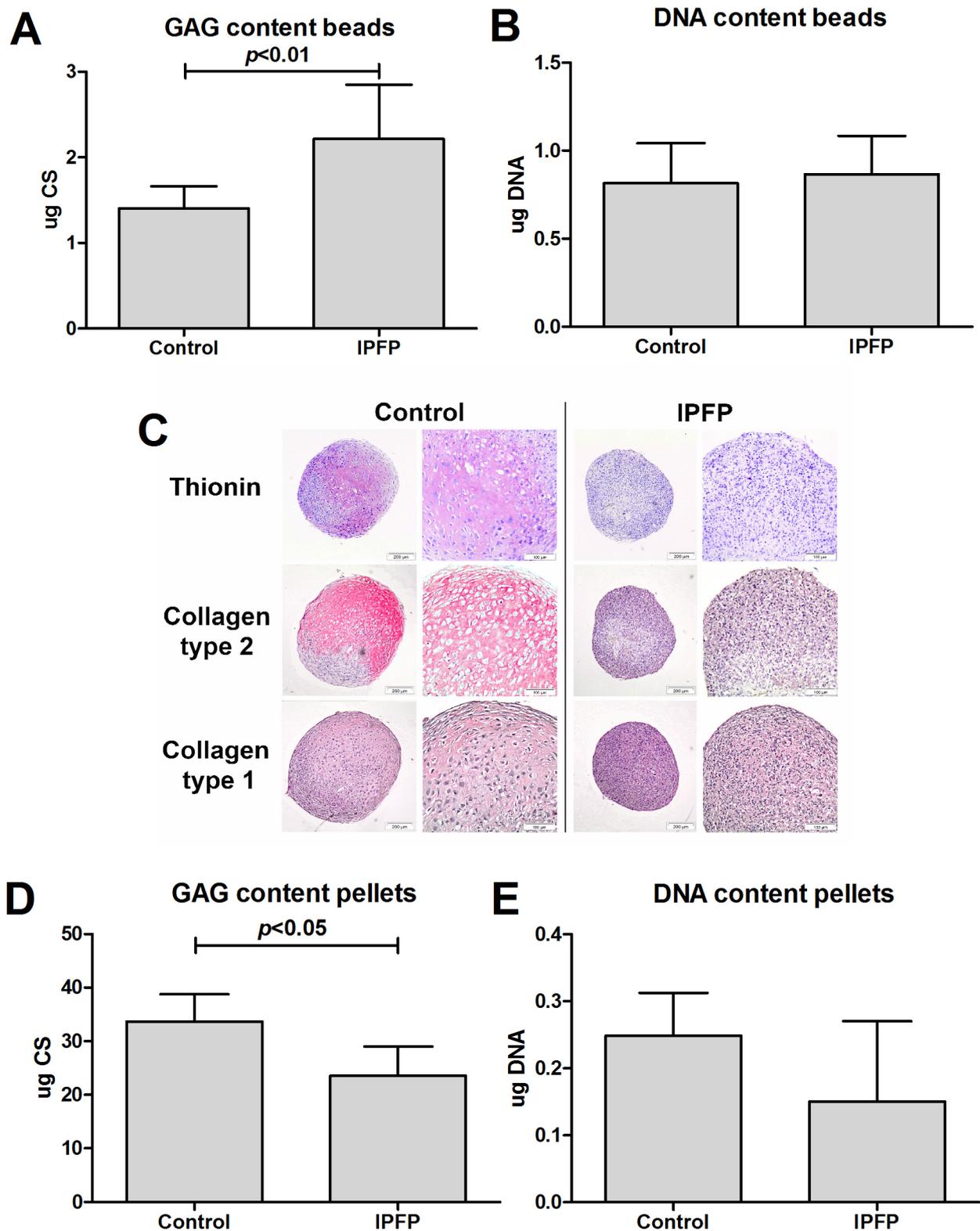
Fig. 2. IPFP inhibits chondrogenic differentiation of MSCs. All conditions were chondrogenically stimulated with TGFβ1 during the entire culture period. MSC alginate beads and pellets were treated with 25 % IPFP CM whereas the control condition was treated with 25 % unconditioned medium for 28 d. (**A**) Total GAG content and (**B**) total DNA content of alginate beads. (**C**) Representative images of pellets from 1 MSC donor stained for glycosaminoglycans with thionin or collagen type 2 and collagen type 1 using immunohistochemistry. (**D**) Total GAG content and (**E**) total DNA content of pellets. Different pools of CM from 3 different IPFP donors were used for each experiment. For total GAG and DNA content in beads (Panels A and B), 3 MSC donors were used with each condition performed in triplicate. For the pellet culture experiment (Panels C-E), 2 MSC donors were used with 5 pellets *per* condition. Values represent mean ± SD. The Mann-Whitney U test was used to determine statistical significance.
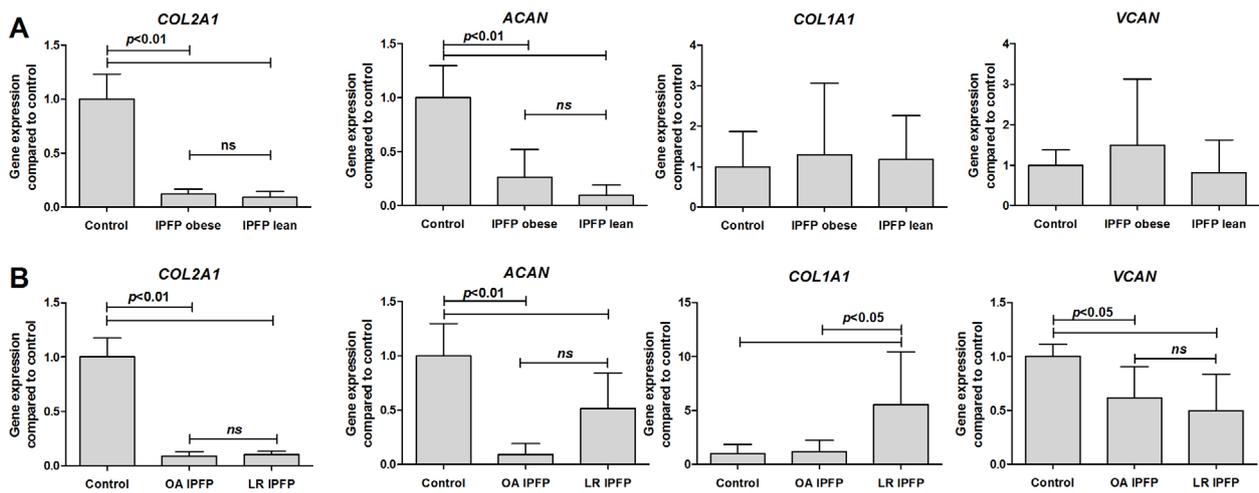
Fig. 3. Effect of obese *versus* lean IPFP and osteoarthritic *versus* traumatic joint IPFP on MSC chondrogenesis. All conditions were chondrogenically stimulated with TGFβ1 during the entire culture period. MSC alginate beads were treated with 25 % IPFP CM from day 14-17. The control condition was treated with 25 % unconditioned medium. Relative gene expression of COL2A1, ACAN, COL1A1 and VCAN in MSCs after treatment with CM from (**A**) obese (BMI>30) or lean (BMI <25) OA IPFP donors, and (**B**) OA or BMI-matched LR IPFP donors. Gene expression was normalised to the control condition *per* MSC donor. CM from 6 obese OA IPFP, 6 lean OA IPFP and 6 LR IPFP donors were used. Three different pools of CM were made with each pool a selection of three different IPFP donors. Three MSC donors were used, with each condition performed in triplicate. Values represent the mean ± SD.
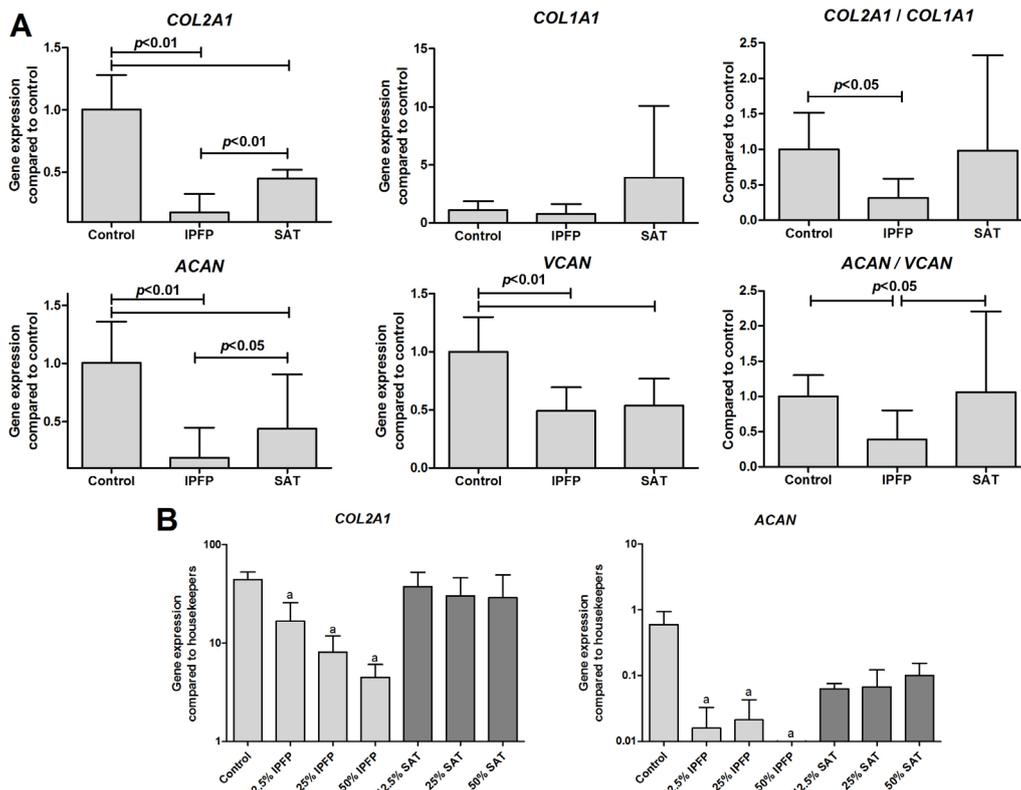
Fig. 4. MSC chondrogenesis is more influenced by IPFP than subcutaneous adipose tissue (SAT). MSC alginate beads were treated with CM from IPFP or SAT from day 14-17 of culture. The control condition was treated with 25 % unconditioned medium. All conditions were chondrogenically stimulated with TGFβ1 during the entire culture period. (**A**) 25 % of CM was used and CM were pooled from 3 pools of different 3 IPFP or 3 pools of different 3 SAT donors and used to treat 3 MSC donors. Each experiment was performed in triplicate. Relative gene expression of COL2A1, ACAN, COL1A1, VCAN, COL2A1/COL1A1 ratio and ACAN/VCAN ratio in MSCs after treatment. Gene expression was normalised to the control condition *per* MSC donor. (**B**) Different percentages of CM were used and CM were pooled from 3 IPFP or 3 SAT donors and used to treat 1 MSC donor, with each condition performed in triplicate. Relative gene expression of COL2A1 and ACAN compared to housekeeping genes in MSCs after treatment. All conditions received the same final concentration of all chondrogenic supplements. Values represent the mean ± SD. [a]*p* < 0.05 *versus* control using the Mann-Whitney-U test.
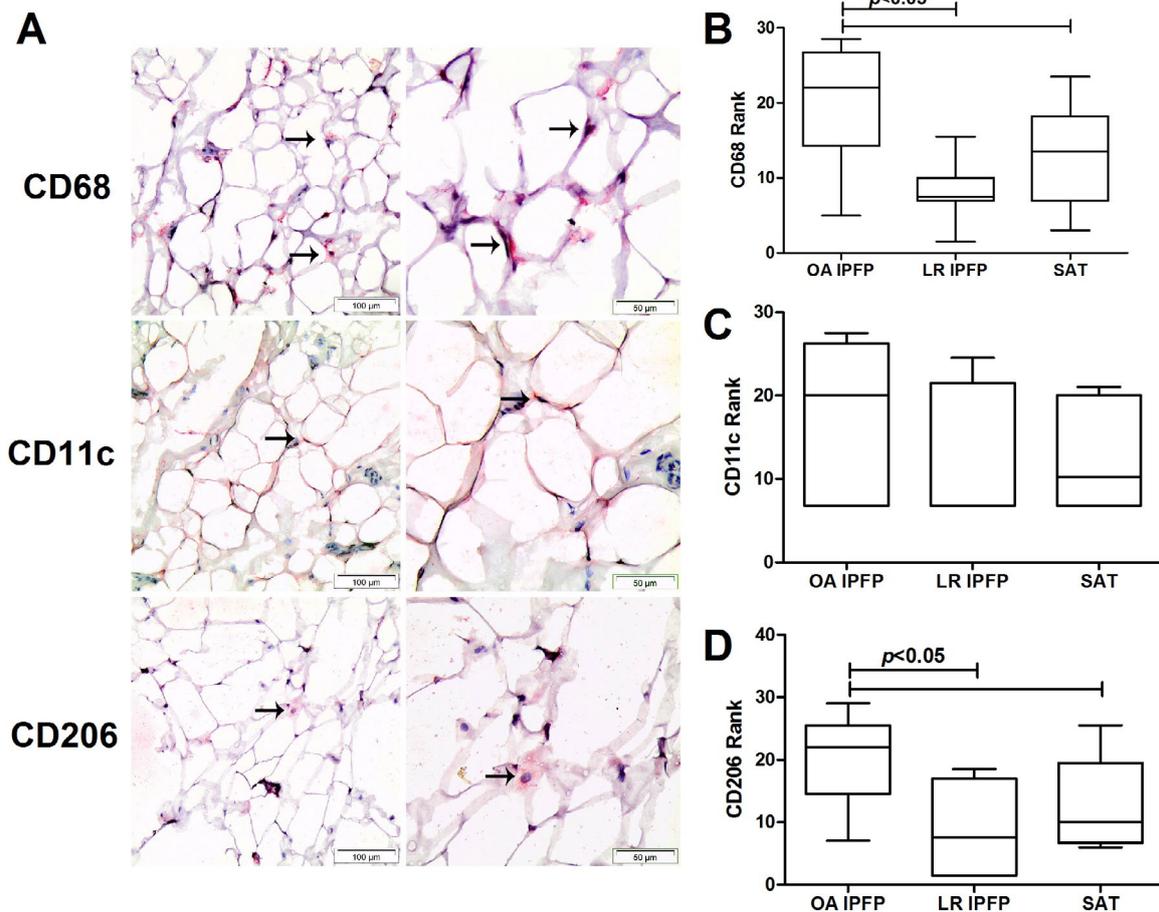
Fig. 5. Macrophages with different phenotypes are present in adipose tissue samples. 13 samples of OA IPFP, 7 samples of LR IPFP and 9 samples of SAT were used. (**A**) Representative images of immunohistochemcal staining of CD68, CD11c and CD206 positive cells in OA IPFP samples. Positive cells are stained red and indicated with arrows. Ranking of positive staining in adipose tissue samples for (**B**) CD68, (**C**) CD11c, and (**D**) CD206.

Difference between IPFP and SAT in number and phenotype of macrophages

We hypothesised that the difference in effects of IPFP and SAT on expression of chondrogenic and fibrous tissue genes could be due to the type of macrophages in the adipose tissue. We therefore first characterised the macrophages in the different adipose tissues. IPFP samples from OA donors ranked higher on CD68 ($p = 0.007$ and $p = 0.03$) and CD206 ($p = 0.02$ and $p = 0.049$) positive staining than LR IPFP and SAT samples (Fig. 5B and D). We could not detect differences between obese and lean donors.

Macrophages in IPFP contribute to the effects seen on MSC chondrogenesis

To investigate whether macrophages present in the IPFP were responsible for the effect seen on MSC chondrogenesis, we added CM from macrophages isolated from IPFP to MSCs during chondrogenesis. IPFP macrophages statistically significantly lowered the *COL2A1* and *ACAN* gene expression (Fig. 6A). Since we applied a very strict selection for CD45+ cells, the remainder fraction still contained a small number of CD45+ cells. This CD45 reduced fraction appeared to

be able to inhibit *COL2A1* and *ACAN* gene expression as well (Fig. 6B). To investigate whether this effect was specific for cells isolated from the IPFP, we added CM from human dermal fibroblasts (HDFa) to MSCs during chondrogenesis. CM from HDFa did not influence MSC chondrogenesis (Fig. 6B).

Finally we investigated whether the negative effects of IPFP-derived macrophages on MSC chondrogenesis could be explained by the phenotype of the macrophage. Due to the low number of macrophages we could obtain from IPFP, we used PBMCs that were stimulated to pro- or anti-inflammatory phenotype. MSCs exposed to CM of M(LPS+IFNγ) macrophages had lower hyaline cartilage genes expression compared to control and CM of M(IL-4) macrophages (Fig. 6C).

Discussion

IPFP secretes cytokines, growth factors and adipokines which could influence the joint environment (Clockaerts *et al.*, 2010). In this study, we have shown that the IPFP released factors that inhibit chondrogenic differentiation of MSCs and the production of hyaline cartilage matrix. This
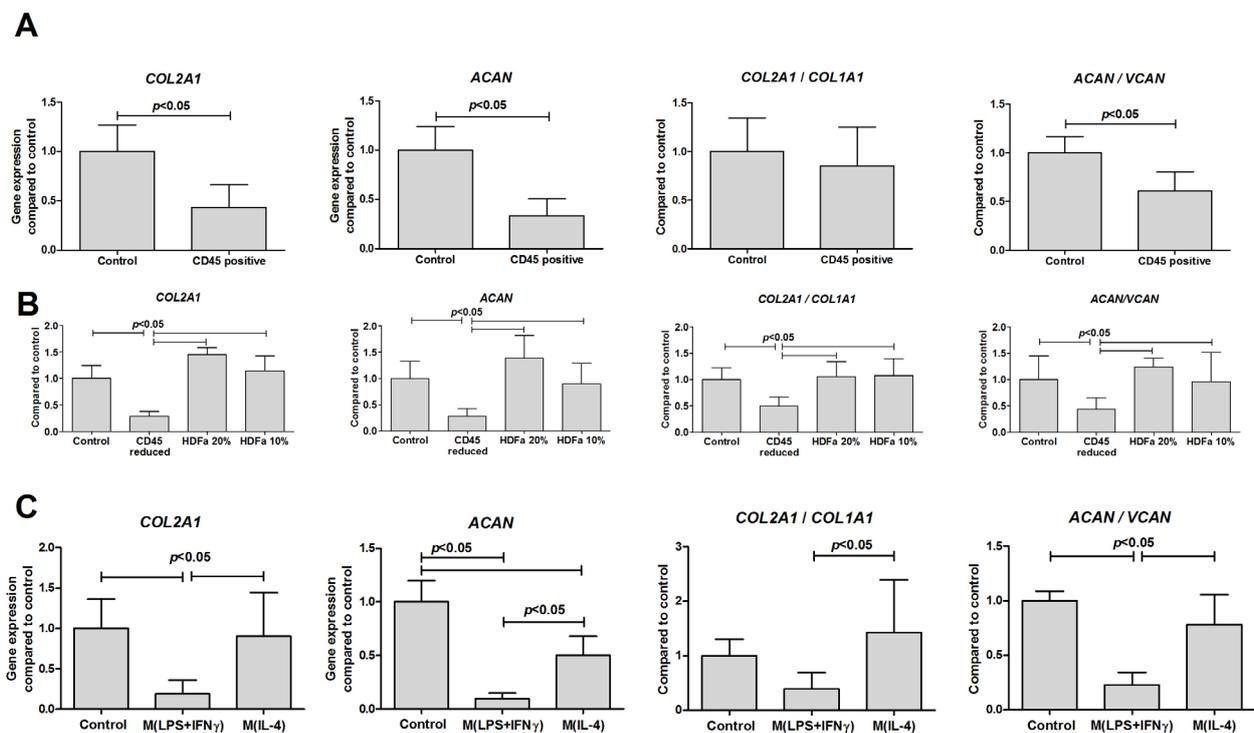
Fig. 6. Macrophages in IPFP contribute to the effects seen on MSC chondrogenesis. MSC alginate beads were treated with CM from day 14-17. The control condition was treated with 25 % unconditioned medium. All conditions were chondrogenically stimulated with TGFβ1 during the entire culture period. (**A**) Effect of 50 % CM from CD45+ cells from IPFP on MSC chondrogenesis; (**B**) Effect 50 % CM from CD45 reduced fraction from IPFP or CM from adult human dermal fibroblasts (HDFa) on MSC chondrogenesis; and (**C**) Effect of 20 % CM from M(LPS+IFNγ) or M(IL-4) macrophages on MSC chondrogenesis. Relative gene expression of COL2A1, ACAN, COL2A1/COL1A1 ratio and ACAN/VCAN ratio in MSCs after treatment. Gene expression was normalised to the control condition *per* MSC donor. CM were from 6 IPFP donors, 1 HDFa donor or 3 blood donors and used to treat 1 MSC donor. Conditions with CM derived from IPFP were performed in single well and all other conditions were performed in triplicate. Values represent the mean ± SD.

anti-chondrogenic effect was the same for patients with end stage OA and patients with joint trauma. Furthermore, the IPFP of obese patients did not inhibit chondrogenesis more than IPFP of lean patients. Finally, we have indications that macrophages residing in the IPFP might play an important role in this anti-chondrogenic effect.

Repair tissue formed after marrow stimulation techniques, such as the microfracture procedure, is mainly fibrocartilage, which is of less biomechanical quality than native hyaline cartilage (Furukawa *et al.*, 1980). In the present study, the decrease in *COL2A1/COL1A1* and *ACAN/VCAN* ratios suggests that IPFP not only inhibits chondrogenesis, but also shifts the production from hyaline to fibrocartilage. Fibrocartilage also contains proteoglycans, but instead of aggrecan this is versican. This could be an explanation why we found no reduction in glycosaminoglycan production in alginate beads after IPFP CM treatment, despite a decrease in aggrecan core-protein gene expression. However, this could also be caused by interference of the DMMB assay by alginate especially when low amount of GAG is involved (Enobakhare *et al.*, 1996; Zheng and Levenston, 2015). Analyses on matrix with DMMB and immunohistochemistry can be used more reliably on pellets (Yang *et al.*, 2004). The reduced thionin

staining and immunohistochemical staining for collagen type 2 in pellets treated with IPFP CM supports the gene expression results in alginate beads. Our findings indicate that fibrocartilage production by MSCs, taking place after microfracture procedure, could be partly due to factors secreted by the IPFP.

The secretion of factors by IPFP could be influenced by the disease state of the joint. The microfracture procedure and MSC-based treatments are recommended for the treatment of post-traumatic, rather than end-stage OA cartilage defects (Gomoll *et al.*, 2011). Higher levels of inflammatory cytokines were found in post-traumatic than end-stage OA knees (Beekhuizen *et al.*, 2013; Tsuchida *et al.*, 2012). Gandhi *et al.* (2011) reported that post-traumatic IPFP expresses more pro-inflammatory genes compared to end-stage OA IPFP. We found differences in macrophage number and in particular CD206 positive cells between IPFP from OA and LR knees, albeit factors secreted by both OA and LR IPFP inhibited chondrogenesis. However, we cannot exclude that these effects are due to different cytokines or adipokines.

Another factor that could influence both IPFP and the results of microfracture is obesity (Mithoefer *et al.*, 2005). In obese patients, macrophages in adipose tissue are changed

towards a chronic inflammatory phenotype with increased production of pro-inflammatory cytokines (Lumeng and Saltiel, 2011). An inflamed obese IPFP would possibly be an explanation for the worse results of microfracture in obese patients. In contrast to this hypothesis, we did not detect an increased anti-chondrogenic effect of IPFP from obese compared to lean donors. To investigate whether the OA process could be of more influence to the factors secreted by IPFP than being obese, experiments with IPFP donors with a low or high BMI but without OA would be required. It is however difficult to obtain sufficient IPFP from non-OA donors.

Independent from joint pathology or obesity, differences have been reported between IPFP and other adipose tissue. IPFP expresses more inflammatory genes, releases more inflammatory factors, and contains more immune cells than SAT (Klein-Wieringa *et al.*, 2011). In our study, the anti-chondrogenic effect of IPFP was stronger than that of SAT and even after increasing the dosage of SAT CM, the anti-chondrogenic effect was not increased to the level of IPFP. This suggests that the IPFP releases anti-chondrogenic factors not produced by subcutaneous adipose tissues.

IPFP secretes pro-inflammatory factors, such as TNF-α, IL1β and IL6, and adipokines, such as leptin, resistin and adiponectin (Clockaerts *et al.*, 2012). These factors could be produced by the fibroblasts, adipocytes and immune cells residing in the IPFP. A large proportion of the immune cells in the IPFP are macrophages (Bastiaansen-Jenniskens *et al.*, 2012; Klein-Wieringa *et al.*, 2011). Immunohistochemical analysis on IPFP and SAT indicated that IPFP contained more macrophages than SAT. Previously, we have shown that only pro-inflammatory macrophages inhibit chondrogenesis (Fahy *et al.*, 2014) and in the current study, only the pro-inflammatory macrophages shift MSCs to produce fibrocartilage matrix. As a proof of principle, we showed that isolated macrophages from the IPFP were anti-chondrogenic. Although we could not exclude that other cells in the IPFP could also secrete anti-chondrogenic factors, our study does suggest that macrophages in the IPFP play a vital role in inhibiting production of hyaline cartilage and the shift towards fibrocartilage matrix production.

Next to directly negatively affecting chondrogenesis of MSCs, IPFP can influence other joint structures and thereby indirectly influence chondrogenesis. IPFP factors have been shown to stimulate fibrotic processes (Bastiaansen-Jenniskens *et al.*, 2013) and production of pro-inflammatory factors in synovial fibroblasts (Eymard *et al.*, 2014). In this way, synovium can then inhibit chondrogenesis (Fahy *et al.*, 2014). In contrast to the effect on synovium, the IPFP inhibits catabolic mediators in cartilage explants stimulated with or without IL1β (Bastiaansen-Jenniskens *et al.*, 2012). This shows that different tissues in the joint can react differently to factors secreted by the IPFP and the interplay between all joint tissues will determine the eventual effect on cartilage repair. The use of co-cultures of several joint tissues would be required to elucidate this in future studies and to show the potential effect of modulation.

In summary, we have shown that the IPFP is a source of factors that inhibit chondrogenic differentiation of MSCs

and shift the production from hyaline to fibrocartilage matrix. This anti-chondrogenic effect of adipose tissue was specific for IPFP and not subcutaneous adipose tissue. Furthermore, this anti-chondrogenic effect was not more in obese donors than lean donors and was not different between donors with post-joint trauma or end-stage OA. Finally, the IPFP and specifically the macrophages in the tissue could therefore be targets for future therapies to improve the joint environment for MSC chondrogenesis and thereby improve the outcome of the microfracture procedure and future MSC-based treatments for the repair of cartilage defects.

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Discussion with Reviewers

Reviewer I: This study presents the intriguing interpretation that the infrapatellar fat pad but not subcutaneous adipose contains macrophages that impede chondrogenic differentiation of mesenchymal stromal/stem cells through a paracrine mechanism. Much of the interpretation hinges on qPCR data (presented in four of the six figures). Yet, quantitative aspects of the qPCR results are unclear, because values have been arbitrarily set to 1 for one of the samples. Before one can define ratios, it is important to establish that values of two parameters are sufficiently robust.

Authors: We understand that setting the condition which was treated with control medium to 1.0 can create concerns. However, since each MSC donor has a different chondrogenic potential, setting the "control" condition with unconditioned medium to 1.0 for each donor allows better visualisation of the effect of conditioned medium treatment. To be sure that our qPCR data are sufficiently robust, we have analysed the efficiency of the primers and confirmed stable expression of the two housekeeping genes used.

Reviewer II: Presuming that future studies confirm that activated, pro-inflammatory macrophages are the primary source of anti-chondrogenic factors secreted by the IPFP, how might this population be targeted to enhance MSC-based cartilage repair? For example, assuming that these cells could be specifically targeted *in vivo*, what might be the negative consequences of ablating them altogether? Alternatively, might it be possible to shift this subpopulation to an anti-inflammatory phenotype *in situ*?

Authors: Our study suggests that pro-inflammatory macrophages could be a target in the future to improve MSC-based cartilage repair. According to our work, anti-inflammatory macrophages are not anti-chondrogenic and removing all macrophages could increase inflammation by other inflammatory cells such as T-cells. However, it has been proven possible to shift macrophage phenotype from a pro-inflammatory to an anti-inflammatory phenotype using drugs (Van der Garde *et al.*, 2014; Zhang *et al.*, 2015). It would be interesting to investigate whether the use of these drugs can change the macrophage phenotype in the IPFP and therefore reduce the anti-chondrogenic effect of the IPFP. If delivered locally into the IPFP, this might provide a way to improve the joint environment before performing MSC-based cartilage repair procedures.

Reviewer II: Given the recent identification of a chronic inflammatory phenotype within the adipose tissues of obese patients, it was somewhat surprising IPFP from donors with high BMI did not demonstrate altered anti-chondrogenic effects compared to those with lower BMI. As you point

out, it would be interesting to make this comparison after eliminating the confounding factor of pre-existing joint disease. If patient enrolment were not a limiting factor, what clinical population and treatment effects would you include in future studies of IPFP anti-chondrogenic activity? For example, are there acute dietary changes for patients with high BMI that have been shown to lower the inflammatory phenotype within their adipose tissues?

Authors: Metabolic dysregulation rather than an increased BMI alone has been shown to be a risk factor for the development of OA (Zhuo *et al.*, 2012). It would be interesting to investigate whether IPFP from donors with metabolic syndrome would be more anti-chondrogenic than from donors without metabolic syndrome. In this way, we could maybe better identify the patients in most need of joint modulation prior to cartilage repair procedures. It has also been shown that an increased intake of omega-3 fatty acids could decrease chronic inflammation (Simopoulus, 2002). Furthermore, it has been shown in mice that dietary fatty acid content could regulate wound healing and OA severity following joint injury (Wu *et al.*, 2015). It would therefore be interesting to investigate whether the IPFP from donors, without OA, who had increased intake of omega-3 fatty acids are less anti-chondrogenic compared to those on a 'regular' diet. In this way, in the future, we can investigate whether dietary changes would be a possible strategy to modulate IPFP inflammation and improve cartilage repair procedures.

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Editor's Note: Scientific Editor in charge of the paper: Chris Evans.