Testing the potency of anti-TNF-α and anti-IL-1ß drugs using spheroid cultures of human osteoarthritic chondrocytes and donor-matched chondrogenically differentiated mesenchymal stem cells

Supplemental information

Supplement 1a: *In vitro* characterization of isolated MSCs and OACs

After isolation from surgical waste material *in vitro* lineage differentiation potential of MSCs into adipocytes, chondroblasts and osteoblasts was assessed. In case of adipogenic and osteogenic differentiation, the cells were grown in 6-well plates (Costar) containing MSC expansion medium, which was changed 3 times per week. Upon reaching confluence, the expansion medium was exchanged with either adipogenic - high glucose DMEM (Gibco) supplemented with 10% v/v FBS (Gibco), 50 µg/mL gentamicin (Gibco), 100 nM dexamethasone (Sigma-Aldrich), 5 µg/mL human insulin (Sigma-Aldrich), 200 nM indomethacin (Sigma-Aldrich) and 450 nM 3-isobutyl-1-methylxanthine (Sigma-Aldrich), chondrogenic (described in the article section 2.1.) or osteogenic medium - low glucose DMEM (Gibco), supplemented with 10% (v/v) FBS (Gibco), 50 µg/mL gentamicin (Gibco), 1% (v/v) 1M HEPES buffer (Mediatech), 5 mM Sodium ß-glycerophosphate (Sigma-Aldrich), 100 nM dexamethasone, 50 µg/mL L-ascorbic acid (Sigma-Aldrich) and 10 ng/mLTGF-ß3 (R&D). Cells were then cultured for 3 weeks.

Subsequently, the differentiated cells were rinsed with PBS (Mediatech), fixed for 30 min by adding 3.7% formaldehyde solution (Sigma-Aldrich) and washed with demineralized water. Adipogenic differentiation was confirmed with Oil Red O (Fisher Scientific) staining, while osteogenic transformation was visualized after von Kossa staining. The Oil Red O dye staining was performed according to the manufacturer's instructions, while the von Kossa treatment was carried out using silver nitrate, sodium thiosulphate and Nuclear fast red solution (all from Sigma-Aldrich). Briefly, the cells were incubated with 2mL/well of 1% silver nitrate solution and exposed to UV light for 30 min. Then, 2 mL/well of 5% sodium thiosulphate solution was added and the cells were incubated for 5 min at room temperature. Subsequently they were washed with demineralized water, counterstained with Nuclear fast red solution for 5 min, washed with demineralized water again and visualized under the inverted light microscope.

Chondrogenic differentiation of MSCs was carried out in spheroid cultures containing 2 x 10^5 cells. Spheroids formed spontaneously after centrifuging MSCs in a deep U-bottomed 96-well microplate (Costar) for 5 min at 250 x g, using the Eppendorf 5810R centrifuge (Eppendorf, Hamburg, Germany) and the following 2-day incubation under standard conditions. Chondrogenic differentiation medium was changed 3-times per week during 3 weeks of cultivation. Subsequently the spheroids were washed with PBS and fixed overnight in 3.7% formaldehyde solution at 4 °C. After that they were dehydrated in gradient solutions of 30, 50 and 70% (v/v) ethanol (Sigma-Aldrich) with each step lasting 1h. For histology analyses dehydrated spheroids were paraffinized and cut in 5 µm thin sections. These were first deparaffinized in Citrisolv solution (Fisher Scientific) for 3 min, then gradually hydrated in 100, 95, 70 and 50% (v/v) ethanol solutions (for 1 min in each one) and finally washed in demineralized water. Afterwards they were immersed for 3 min in 3% acetic acid (Sigma-Aldrich), then for 15 min in 1% (m/v) Alcian blue dye solution (pH = 1; Sigma Aldrich) and finally thoroughly rinsed with 1% (v/v) of 12 N HCl solution (Fisher Scientific) in 70% (v/v) ethanol. The last step was dehydration which was carried out for 1 min in 95 and 1 min in 100% (v/v) ethanol. Slices were cleared in Citrisolv for an additional minute and mounted on coverslips by using mounting medium (Fisher Scientific).

After fixing and dissecting microspheroid tissues, chondrogenic differentiation of MSCs and re-differentiation of OACs in histological slices were determined by Alcian blue staining of GAGs (as described above) and by detecting collagen II fibers with specific primary anti-collagen type II antibody (Abcam, Eugene, OR, USA), followed by HRP-labeled secondary antibody and other components of the Vectastain® Universal Elite® ABC Kit (Vector laboratories, Burlingame, CA, USA), according to manufacturer's instructions. The images were generated and by using a light microscope.

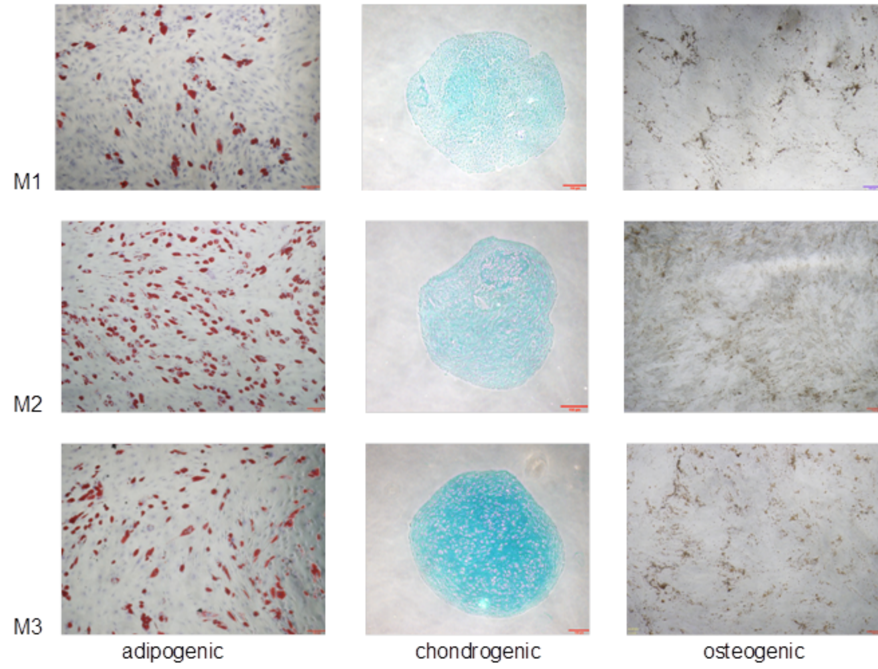
Supplement 1b: MSC immunophenotyping

Fluorescence activated cell sorting (FACS) technique was used for detection of CD73, CD90 and CD105 surface molecules on isolated human MSCs. Prior to analysis, 2 x 10^5 MSCs from monolayer cell culture passages P1 to P4 were obtained after trypsinization, washed with PBS and resuspended in 300 µL of PBS containing 2% (m/v) bovine serum albumin (BSA; Sigma-Aldrich) and 1 mM ethylenediaminetetraacetic acid (EDTA; Sigma-Aldrich). The cells were incubated for 30 min at 4 °C and centrifuged at 1,800 x g for 5 min (Eppendorf 5415R centrifuge, Eppendorf, Germany). Subsequently, aliquots of MSCs were resuspended in 300 µL of FACS staining buffer, containing 0,5% (m/v) BSA and 1 mM EDTA in PBS. To each aliquot of cells 3 µL of either CD73, CD90 or CD105 mouse anti-human APC-conjugated antibodies (100 µg/mL) or APC Mouse IgG1 isotype control solution (BD Biosciences, San Diego, CA, USA) were added. After 40-60 min incubation at 4 °C, the cells were centrifuged and the cell pellet was washed twice with 1 mL of FACS staining buffer. Then, the MSCs were resuspended in 250 µL of Cytofix solution (BD Sciences), incubated 15 min at 4 °C, and washed by centrifugation. In the final step the cell pellets were resuspended in 300 µL of FACS buffer and stored at 4°C protected from light, until use. Flow cytometry analysis was always performed within one week on the FACS Calibur S/N E5240 flow cytometer (Beckton-Dickinson, USA), and the results obtained were analyzed by using Flow.Jo version 7.6.1 software (Flow.Jo, Ashland, OR, USA).

Supplement 2: OHP quantification

OHP was determined as follows: to 250 µL of each papain digested sample in 1.5 mL plastic tube, 250 µL of 12 N HCl solution (Fisher Scientific) were added and then the opened tubes were incubated in a fume hood at 100 °C. To dry the residues, 350 µL of assay buffer [10x diluted solution of stock buffer: 50 g of citric acid monohydrate (Sigma-Aldrich), 12 mL of glacial acetic acid (99.8%; Sigma-Aldrich), 120 g of sodium acetate trihydrate (Fisher-Scientific), 34 g of sodium hydroxide (Sigma-Aldrich), dissolved in demineralized water up to 1,000 mL] were added and then the samples were vortexed and centrifuged for 5 min at 8,000 rpm (Eppendorf 5415 R centrifuge; Eppendorf, Germany). Subsequently Chloramine T and Dimethylaminobenzaldehyde reagents were prepared as follows: Chloramine T - 52 mg of chloramine T (Sigma-Aldrich) were mixed with 5.2 mL of demineralized water, 6.5 mL of n-propanol (Sigma-Aldrich) and 13.3 mL of stock buffer (10x); Dimethylaminobenzaldehyde - 3.75 g of p-dimethylaminobenzaldehyde (Fluka, India) were mixed with 15 mL of n-propanol and 5.5 mL of perchloric acid (Fisher). Sample aliquots of 300 µL were transferred in plastic tubes, diluted with 150 µL of Chloramine T reagent and incubated for 20 min at room temperature. Subsequently 150 µL of Dimethylaminobenzaldehyde reagent were added into each tube and the samples were incubated for 15 min at 60 °C. Then they were cooled to room temperature, transferred in duplicates (250 µL each) into wells of a transparent 96-well plate and analyzed by measuring absorbances at 540 nm on the TECAN Infinite M200 spectrophotometer (TECAN, Switzerland).

(1a)



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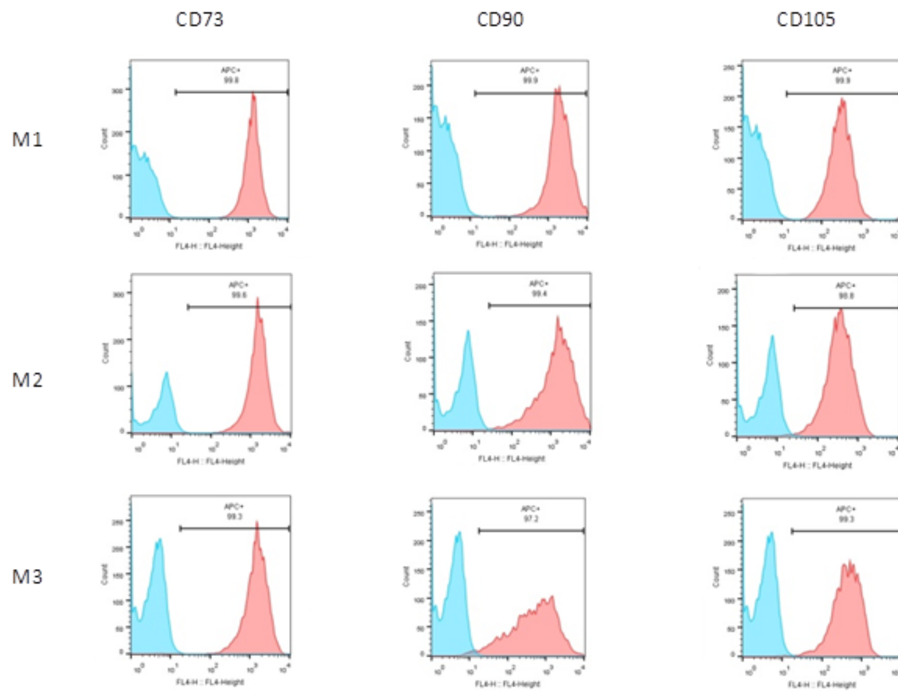

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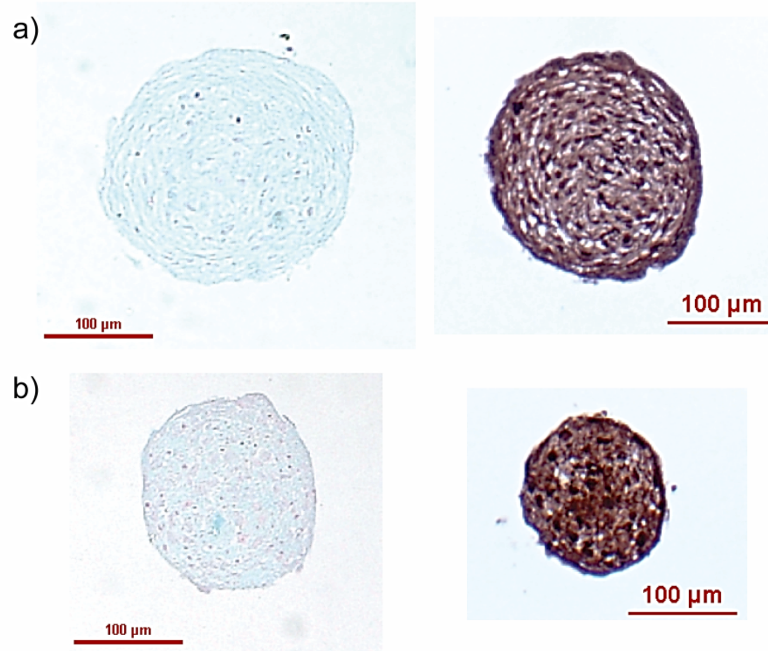
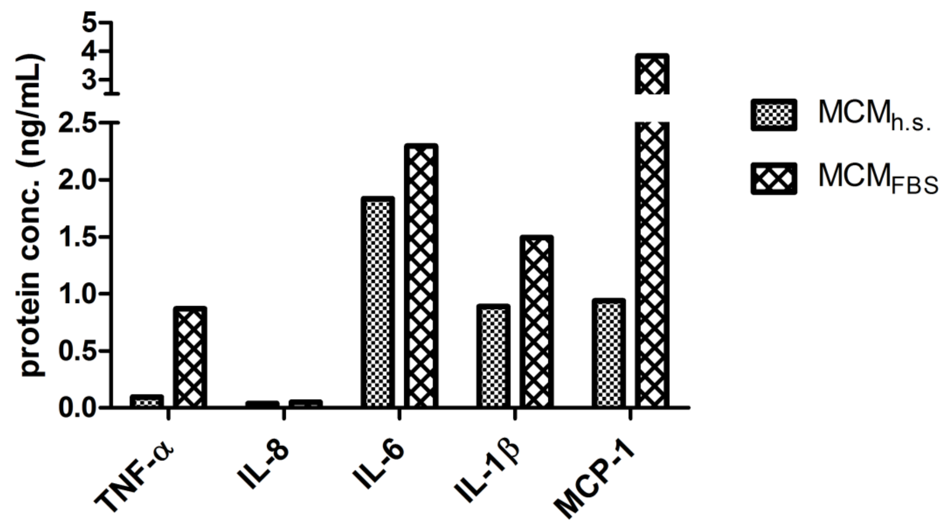
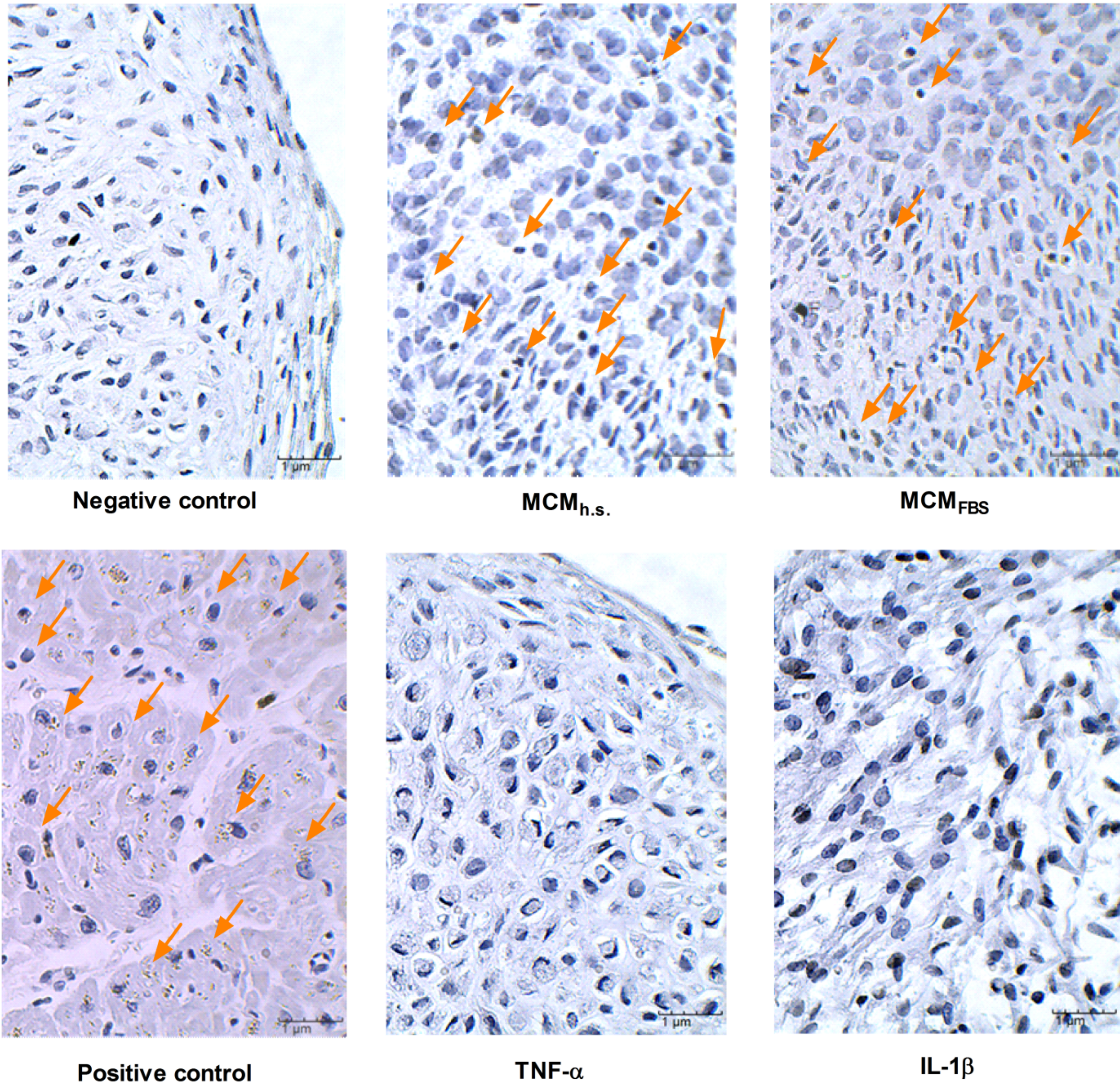


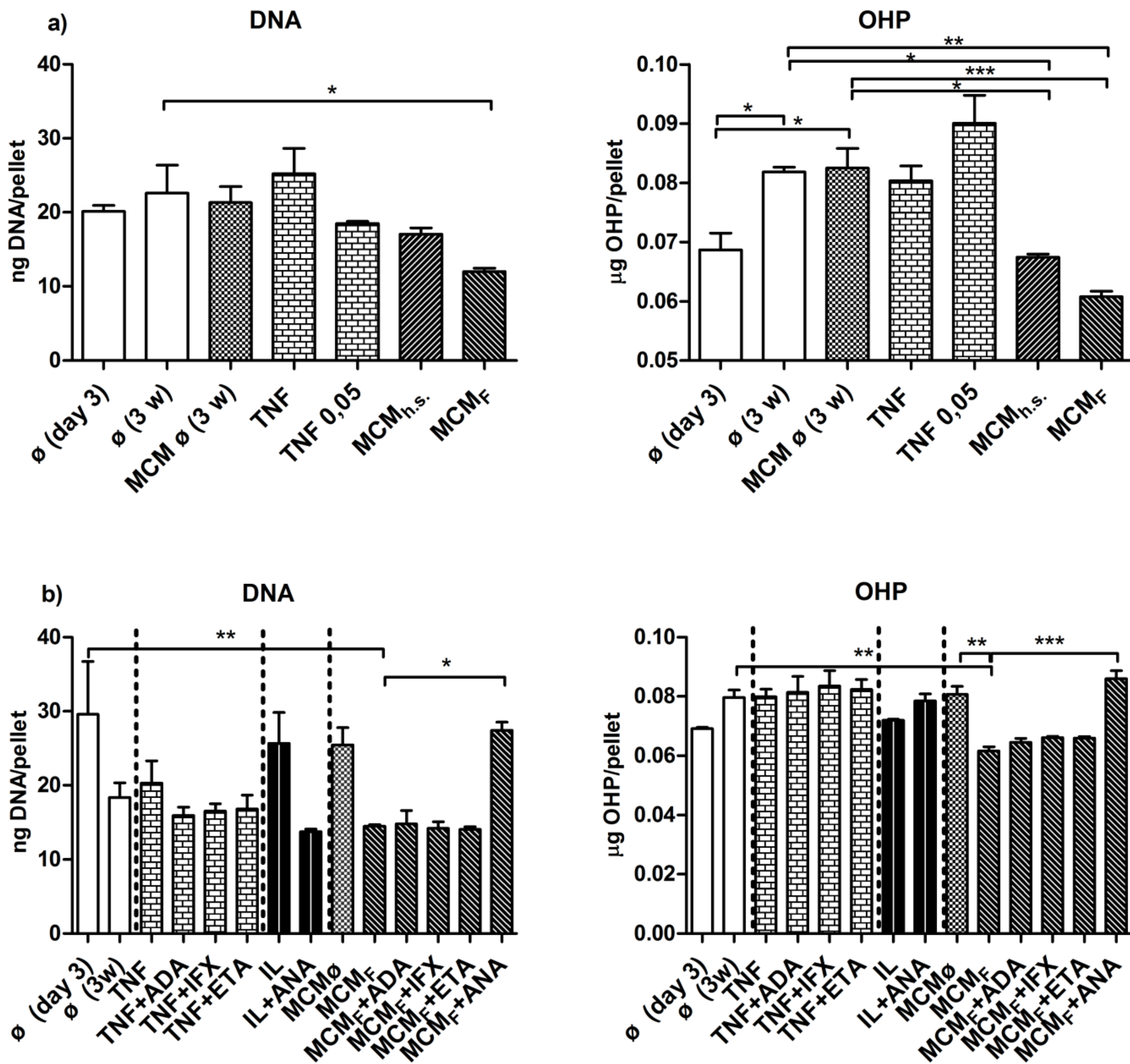
Supplement Figure 1: Characterization of MSCs and OACs. (1a) Confirmation of MSC (M) tri-lineage differentiation potential following Oil Red O, Alcian blue and von Kossa staining for adipogenic, chondrogenic and osteogenic differentiation, respectively; each scale bar represents 150 µm. (**1b)** Surface stem cell markers of MSCs (M) defined by FACS. Legend: **isotype control**; **specific CD markers**. **(1c)** Proof of MSC chondrogenic differentiation and OAC re-differentiation in microspheroid cultures**.** Alcian blue (left) and collagen II (right) staining of histology sections obtained from microspheroids containing 10,000: **a)** OACs (donor 1 – p ch1, passage P2), which were grown in hanging drops for 7 days, and **b)** MSCs (donor 1 – M1, passage P2), which were chondrogenically differentiated in hanging-drops for 21 days.



Supplement Figure 2: Concentrations of TNF-α, IL-1ß, IL-6, IL-8 and MCP-1 pro-inflammatory proteins in undiluted MCM$_{h.s.}$ and MCM$_{FBS}$. Values were obtained by measurements of single (pooled) samples (two technical replicates).



Supplement Figure 3: Cell apoptosis in chondral macrospheroids. Analyses of apoptosis in histological sections of macrospheroid chondral tissues made of 100,000 OACs (p ch2), following their 3 weeks-long cultivation. Negative control – OACs cultured in chondrogenic medium; MCM$_{h.s.}$ – cells cultured in the presence of MCM working solution prepared with the use of human serum; MCM$_{FBS}$ – cells cultured in the presence of MCM working solution prepared with the use of FBS; positive control – infarcted human cardiac tissue; TNF-α – cells cultured in the presence of 1 ng/mL of human recombinant TNF-α; IL-1ß – cells cultured in the presence of 1 ng/mL of human recombinant IL-1ß. Apoptotic cells and surrounding caspase III (brownish debris) are indicated by orange arrows. Each scale bar represents 5 µm in total.



Supplement Figure 4: DNA and OHP contents in chondral tissue macrospheroids. Differences in DNA and OHP contents in macrospheroids made of 100,000 OACs (p ch 2), following their 3 weeks-long cultivation in the presence of either TNF-a, IL-1ß, or $MCM_{h.s.}$ (**a**) or MCM_{FBS} (**b**) working solutions alone, combined with the tested biologicals. Results (n=3) were compared by one-way ANOVA; $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***). Legend: ø- chondrogenic medium (control 1); MCM ø - ½ chondrogenic medium + ½ THP-1 medium (control 2); $MCM_{h.s.}$ or MCM_F - MCM prepared with human serum or FBS; TNF - TNF-α (1 ng/mL, if not specified otherwise); IL - IL1ß (1 µg/mL); ADA= adalimumab (1 µg/mL); IFX = infliximab (1 µg/mL); ETA = etanercept (1 µg/mL); ANA = anakinra (1 µg/mL).