Large-scale secretome analyses unveil a superior immunosuppressive phenotype from umbilical cord stromal cells compared to other adult mesenchymal stromal cells

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Abstract

Mesenchymal stromal cells (MSCs) with regenerative and immunomodulatory potential are being investigated as a potential therapeutic tool for cartilage lesions. MSCs express a wide variety of bioactive molecules including cytokines, trophic factors, and proteases, which act in a paracrine fashion to modulate the tissue microenvironment. Yet, little is known about the divergence of these signalling molecules between MSCs populations from adult or young tissues. This makes it challenging to decide the optimal source of MSCs for a specific clinical application. In this study, we investigated cell secretomes from cultured human stromal cells harvested from Hoffa's fat pad (HFPSCs), synovial membrane (SMSCs), umbilical cord (UCSCs) and cartilage (ACs) by quantitative LC-MS/MS proteomics. We also performed multiplex protein arrays and functional assays to compare the constitutive immunomodulatory capabilities of different MSCs. Proteins involved in extracellular matrix degradation and inflammation such as MMPs, IL-17, and complement factors were significantly downregulated in UCSCs compared to other cell types. Additionally, we found enhanced expression of TGF-β1 and PGE2 in UCSCs supernatants. UCSCs were superior in inhibiting peripheral blood mononuclear cells proliferation, migration and TNF-α and IFN-γ secretion compared to ACs, HFPSCs and SMSCs. Although all cell types could repress HLA-DR surface expression and cytokine release by activated macrophages, only UCSCs significantly blocked IL-6 and IL-12 production. Our data demonstrate that stromal cells from umbilical cords display superior anti-inflammatory and immunosuppressive properties than stromal cells from adult tissues. This Allogeneic cell source could potentially be considered as an adjuvant therapy for articular cartilage repair.

Introduction

Articular cartilage lesions associate with pain, discomfort, and inflammation in the synovial joint, which subsequently restrict the function of articular activities. Mechanical trauma or degenerative diseases are the major causes of articular cartilage injuries. Traumatic cartilage lesions, on the other hand, increase the risk of developing osteoarthritis (OA) by more than four times (Muthuri *et al.*, 2011). This fast-growing chronic disease is expected to be the fourth leading cause of disability by the year 2020 (Cross *et al.*, 2014). Commonly used surgical and nonsurgical OA treatment modalities include intra-articular injections of soluble materials such as corticosteroids or hyaluronate, autologous blood products, nonsteroidal anti-inflammatory drugs (NSAIDs), and arthroscopic lavage. These procedures improve OA symptoms to a certain degree but do not heal completely the progressive loss of joint functions (Lee and Wang, 2017; Wolfstadt *et al.*, 2015). Additionally, the treatment of localised cartilage injuries with cell-based therapies benefit patients from debilitative knee functions and also prevents the onset of developing secondary OA (Ogura *et al.*, 2017). Although autologous chondrocytes have been used as an intuitive source for cell-based therapy, in recent years, mesenchymal stromal cells (MSCs) are gaining attention as an alternative and potentially effective therapeutic tool for cartilage lesions.

MSCs have been successfully isolated and expanded *in vitro* from numerous tissues sources. Many scholars in the field support the notion that MSCs represent a defined population of multipotent progenitor cells residing in the perivascular niche of nearly all human tissues, although different views exist (Crisan *et al.*, 2008; Guimaraes-Camboa *et al.*, 2017). MSCs differentiation capacity and immunomodulatory properties have been demonstrated *in vitro* irrespective of tissue sources (Ghannam *et al.*, 2010). However, *in vitro* studies have shown that MSCs from different origins differ in their lineage-specific differentiation capacity and their functional potential (Garcia *et al.*, 2016; Islam *et al.*, 2016; Subramanian *et al.*, 2015). In

addition, a systematic review of intra-articular injection of bone marrow MSCs in humans concluded that articular stem cells therapies are safe (Peeters *et al.*, 2013). Some preclinical studies in animals have demonstrated the *in vivo* efficacy of MSCs from different sources in OA management including autogenic, allogeneic and xenogeneic cell sources. However, none of these studies has compared the optimal source of MSCs (Ozeki *et al.*, 2016; Saulnier *et al.*, 2015; Singh *et al.*, 2014; Yang *et al.*, 2015). Therefore, the choice of optimal source of MSCs for a given clinical implication has yet to be elucidated.

Mechanistically, it is not well established how MSCs exert their effects *in vivo*. It was previously believed that MSCs promote tissue regeneration by engraftment of cells in damaged areas and transdifferentiating into tissue forming cells to promote repair (Liechty *et al.*, 2000). Recently, the field has witnessed a paradigm shift in understanding the mechanism of action elicited by MSCs, which highlights paracrine signalling and the release of potent bioactive factors to modulate the microenvironment in benefit of tissue healing (Gnecchi *et al.*, 2016; Iso *et al.*, 2007; Prockop, 2009). In the field of cartilage repair and OA, the fate of implanted cells during biological repair procedures and their contribution to rebuilding the damaged tissue is mostly unknown. Previous studies in animals suggest that most of the repaired tissue is composed of cells of unknown origin migrating to the lesion (Dell'Accio *et al.*, 2003; Grande *et al.*, 1989). A recent human clinical trial concluded that Allogeneic bone marrow MSCs function as a source of stimulatory and trophic factors, which orchestrate tissue repair rather than differentiating into the host tissue (de Windt *et al.*, 2017). However, clinical procedures based on autologous MSCs transplantation, including bone marrow or adipose tissue MSCs, may provide beneficial effects, but are associated with invasive harvesting procedures, two-stage operations and long-time cell expansion ex *vivo*. Allogeneic MSCs harvested from umbilical cords, amniotic membrane and placenta might represent alternative sources for one-stage cell-based therapies. In addition to their pro-angiogenic properties, anti-inflammatory

phenotype, and multi-lineage differentiation potential, these Allogeneic MSCs are well tolerated and elicit low immunogenic responses as their adult counterparts (Balasubramanian *et al.*, 2012; Donders *et al.*, 2015). Unlike investigating neotissue forming ability, the role of secreted bioactive molecules in the context of paracrine signalling and immunomodulation have not been comprehensively explored between cells from adult joints and young cells. The aim of this study was to find a suitable cell source that could serve as a potent immunomodulator to mediate the tissue microenvironment.

In this study, we compared the secretome of culture-expanded cells harvested from four different tissues sources comprising cartilage (ACs), Hoffa's fat pad (HFPSCs), synovial membrane (SMSCs) and umbilical cords (UCSCs). For analyses, mechanisms and pathways relevant to cartilage and joint physiology including inflammation and immune regulation, extracellular matrix (ECM) remodelling, mitotic factors and chondro-inductive molecules have been considered. Proteins involved in ECM remodelling such as MMPs, complement factors, and serpins were significantly downregulated in UCSCs compared to other cell types, whereas cell signalling molecules such as TGF-β1, MCP-1, and PDGFD were upregulated in UCSCs. To evaluate the constitutive abilities of the different MSCs as immunomodulators, we compared the immunoregulatory properties of supernatants from the different cell types by functional immune assays. Our data revealed that UCSCs exhibit superior anti-inflammatory properties and low catabolic phenotypes compared to ACs, HFPSCs and SMSCs.

Materials and Methods

Human Materials and Ethical statements

Human samples were collected from the University Hospital of Northern Norway (UNN). The Regional Ethical Committee (REK Nord 2014/920 and 2010/586) at the University of Tromsø

approved the study. Adult stromal cells were isolated from knee joint tissues of 25 patients aged 45 to 75 undergoing total knee replacement (TKR) to complete this study. A macroscopic observation was made by the operating surgeon to assess the inflammatory states of the samples. Patients with inflammatory joint diseases and very advanced OA were excluded; however, both secondary posttraumatic and primary osteoarthritis patients were included in the study. Fat pad and synovial membrane tissues were harvested from TKR patients. UCSCs were isolated from seven umbilical cords immediately after birth. Buffy-coats for isolation of peripheral blood mononuclear cells (PBMCs) were collected from healthy donors from the local blood bank (REK Nord 2014/401). All patients provided written informed consent.

Isolation and culture of human stromal cells

Macroscopically good-looking cartilage without any traces of bone, collected from femoral heads during total knee replacements was used to isolate human chondrocytes. All cell types were isolated using a mixed enzymatic-explant method as previously described (Islam *et al.*, 2016; Islam *et al.*, 2017). Briefly, all tissue specimens were washed three times with sterile Dulbecco's phosphate buffered saline (PBS; Cat. no. D8537; Sigma-Aldrich) and minced into small pieces for enzymatic digestion in collagenase XI solution (Cat. no. C9407; ≥ 800 units/mg solid, Sigma-Aldrich) at a final concentration of 1.25 mg/mL on a shaker at 37 °C. Cartilage tissue specimens were digested for 3-4 h, and other adult tissue specimens were digested only for 1-1.5 h. UCSCs were isolated from cord matrix (also known as a mixed cord) using 1 h of digestion. Partially digested tissues were centrifuged for 10 min at 800 x*g* and resuspended in high glucose Dulbecco's Modified Eagle Medium (DMEM; Cat. no. D5796; Sigma-Aldrich) before plating on a T-75 culture flask (Cat. no. 156499; Thermo Scientific). The culture medium was supplemented with L-ascorbic acid (62 mg/L) (Cat. no.103033E; BDH Laboratory), penicillin and streptomycin (1 %) (P/S; Cat. no. P4333; Sigma-Aldrich) and 20 % foetal bovine serum to promote cell attachment (FBS; Cat. no. S0115; Biochrom). All cells were incubated

144 in a humidified atmosphere containing 5 % CO_2 at 37 °C. After the initial 24 h, primary cultures
145 were expanded in 10 % FBS supplemented medium, and the medium was changed every 3-4 d
146 until the cultures became confluent.

147 **Preparation of conditioned medium**

148 All cells were used for experimentation at passage 3-4. Serum-rich conditioned medium was
149 used in functional assays with peripheral blood mononuclear cells (PBMCs) and macrophages,
150 whereas serum-free conditioned medium was used for protein-arrays and secretomics. Upon
151 reaching 70-80 % confluence, culture flasks were thoroughly washed with warmed PBS, and
152 the medium was replaced with fresh medium containing high glucose DMEM and 1 % P/S
153 (with or without 10 % FBS). Serum-free medium was additionally supplemented with insulin-
154 transferrin-selenium supplement (1:1000) (ITS; Cat. no. 354351; Corning). Both serum-free
155 and serum-rich conditioned medium (CM) were collected after 48 h, centrifuged at 4500 x*g* for
156 10 min, filtered using 0.22 µm porous membrane and used immediately for experimentation or
157 stored at – 70 °C for further analysis. The number of cells was counted for each culture
158 condition and used to normalise the measured expression of cytokines and growth factors in
159 CM. In functional assays with PBMCs and macrophages, the fresh culture medium was diluted
160 with serum-rich CM (1:1) from different stromal cells.

161 **Quantitative and qualitative LC-MS/MS analysis**

162 Serum-free CM (6 mL) from all cultures were collected from T-75 culture flasks and
163 concentrated in PBS to a final volume of 500-800 µL using 5000 Da MWCO vivaspin column
164 (Cat. no. Z614440-25EA; Sigma-Aldrich) at 4500 x*g* for 20 min. Protein concentration was
165 measured using DC Protein Assay Kit (Cat. no. 5000116; Bio-Rad). Protein samples (100
166 µg/tube) were reduced in 5 mM dithiothreitol (Cat. no. D9779; Sigma-Aldrich) for 30 min at
167 70 °C. Samples were alkylated by incubation with 375 mM iodoacetamide (Cat. no. 90034;

Thermo Scientific) at room temperature for 30 min in the dark. Protein samples were collected as dry pellets after overnight precipitation in pre-chilled acetone (Cat. no. 270725; Sigma-Aldrich) at -20 °C. Dry pellets containing 100 µg protein were resuspended in 100 µL of 2 M Urea (Cat. no. U1250; Sigma-Aldrich) with 50 mM TEAB. Only 25 µg of protein per sample was taken for further analysis. Samples were pre-digested for 6 h with 1:100 (w/w) LysC endopeptidase (Cat. no. 125-05061; Wako Chemicals) with 1 mM final concentration of $CaCl_2$, followed by further dilution with 50 mM TEAB in 1 M Urea and digestion overnight in 1:20 (w/w) trypsin (Cat. no. V511A; Promega). A volume of 5 µL trifluoroacetic acid (10 %) (Cat. no. 28904; Thermo Scientific) was added to each tube and centrifuged at 13000rpm for 10 min. OMIX C18 tips were used for sample clean-up and concentration. Samples containing 0.2 % formic acid (FA; Cat. no. 28905; Thermo Scientific) were loaded to a Thermo Fisher Scientific EASY-nLC1000 system and EASY-Spray column (C18, 2 µm, 100 Å, 50 µm, 50 cm). Peptides were fractionated using a 2-100 % acetonitrile (Cat. no. 51101; Thermo Scientific) gradient in 0.1 % FA at a flow rate of 250 nL/min over 180 min. The separated peptides were analysed using a Thermo Scientific Q-Exactive mass spectrometer. Data were collected by a Top10 method in data-dependent mode. The raw data were processed using MaxQuant (v 1.5.6.0) for label-free protein quantification (LFQ). MS/MS data were searched against the UniProt human database from November 2016 to yield protein identification (false discovery rate (FDR) = 0.01). Parameters used for the search: fixed modification, carbamidomethylation of cysteines; variable modifications, oxidation of methionine and acetylation of protein N-terminal; ion mass tolerance, 4.5 ppm; fragment mass tolerance, 20 ppm; charge states, 2+, 3+ and 4+; Maximum missed cleavages, 2; enzyme specificity, trypsin; and minimum number of unique peptides, 2. Perseus 1.5.6.0 software was used for statistical analysis of identified proteins. All contaminants were filtered out before log10-transformation of data for further analysis. The log10-transformed intensities were normalised by subtracting the median. Data were grouped as ACs,

HFPSCs, SMSCs and UCSCs and analysed using a *t*-test, with a minimum of three valid values in each group. Volcano plots for each comparison were generated to identify differentially expressed proteins using FDR < 0.01.

Multiplex protein arrays

A panel of 36 specific proteins including cytokines, chemokines, matrix metalloproteinases (MMPs) and growth factors was measured in the serum-free CM of all four stromal cell types by immune-based protein arrays. A human cytokine magnetic 25-plex kit (Cat. no. LHC0009M; Thermo Scientific) was used to measure the concentration of 18 cytokines (1:4 dilution) involved in inflammation including GM-CSF, IFN-α, IFN-γ, IL-1β, IL-1RA, IL-2, IL-2R, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12 (p40/p70), IL-13, IL-15, IL-17 and TNF-α and 7 chemokines (1:4 dilution) including Eotaxin, IP-10, MCP-1, MIG, MIP-1α, MIP-1β and RANTES. Fluorokine MAP human MMP base kit (Cat. no. LMP000; R&D) was used to measure the concentration of MMP-1, MMP-3, MMP-7, MMP-9 and MMP-13 (1:5 dilution). Quantitative measurements (two replicates) were performed according to manufacturers' guidelines using Luminex Bio-Plex 200 system (Bio-Rad, USA). In addition, quantification of PGE2 (Cat. no. KGE004B) and five growth factors including TGF- β1 (Cat. no. DY240-05), BMP-2 (Cat. no. DY355-05), IGF-1 (Cat. no. DY291-05), PDGF-AB (Cat. no. DY222) and bFGF (Cat. no. DY233-05) was performed by ELISA. All ELISA kits were purchased from R&D Systems and performed according to manufacturer instructions. Measured protein concentrations were normalised with cell number at specific culture conditions and expressed as pg/mL/10^6 cells.

Isolation and culture of human PBMCs

PBMCs were isolated from buffy-coats (n = 5) of whole blood using lymphoprep (Cat. no. 1114545; Alere Technologies) following manufacturer instructions. Briefly, peripheral blood

was diluted in sterile PBS (1:1) and slowly layered over a lymphoprep gradient in a 50 mL tube. The tube was centrifuged without a break at 800 x*g* for 30 min at room temperature. PBMCs were collected from the gradient-interface with a Pasteur pipette, followed by washing three times for 10 min at 400 x*g* with PBSA containing PBS and 0.2 % bovine serum albumin (BSA; Cat. no. 130-091-376; Miltenyi Biotec). PBMCs were cultured in growth medium containing Rosewell Park Memorial Institute medium (RPMI-1640; Cat. no. R8758; Sigma-Aldrich), 1 % P/S and 10 % FBS in a humidified atmosphere (5 % CO_2) at 37 °C.

PBMCs activation assays

PBMCs proliferation was assessed using the carboxyfluorescein succinimidyl ester (CFSE) dilution assay (Cat. no. 10009853; Cayman). Cultured PBMCs were washed in pre-warmed sterile PBS and centrifuged at 400 x*g* for 5 min before incubating cells with CFSE for 15 min at 1:400 dilution. CFSE-stained PBMCs were cultured in RPMI, 1 % P/S and 10 % FBS in a 24-well plate at a density of 10^6 cells/well. PBMCs were stimulated with the mitogen phytohemagglutinin (10 µg/mL) (PHA; Cat. no. 1249738; Roche) for 5 d to induce proliferation. Half of the medium was replaced with fresh medium after the second day. The proliferation assay was performed on a BD FACSAria III flow cytometer, and the data were analysed by FlowJo software (Tree Star Inc., USA). CM from stromal cells was added to the culture of PHA activated PBMCs from 1 d at 1:1 ratio with fresh culture medium. CM from last 3 d during proliferation of PBMCs was collected to measure cytokine profiles. CM derived from PBMCS was centrifuged at 4500 x*g* for 5 min and filtered by 0.22 µm porous membrane before analysing TNF-α (Cat. no. DY210-05; R&D) and IFN-γ (Cat. no. DY285-05; R&D) contents using ELISA.

PBMCs migration assay

PBMCs migration assay was performed by a Boyden chamber assay. PBMCs were activated with PHA (10 μg/mL) for 48 h before performing the assay. Recombinant stromal cell-derived factor-1 (100 ng/mL) (SDF-1; Cat. no. 300-28A; PeproTech) was used as a chemoattractant. PBMCs (10^6 cells/100 μL) were added to the top chamber of Transwell culture inserts (6.5 mm diameter, 8 μm pores, Cat. no. CLS3464; Sigma-Aldrich). Bottom chambers contained either growth medium or serum-rich CM from different stromal cells. After 2 h, PBMCs that migrated to the lower chamber were harvested and washed in PBSA by centrifugation at 400 x*g* for 4 min followed by resuspending in 0.5 ml of PBSA. Lymphocytes population was gated using forward, and side scatter and counted on a BD FACSAria III flow cytometer.

Isolation and culture of monocyte-derived macrophages

Monocytes were isolated from PBMCs using CD14+ magnetic-activated cell sorting (MACS) (Cat. no. 130-050-201; Miltenyi Biotec) with minor modification from manufacturer guidelines. Briefly, PBMCs were washed in MACs buffer containing autoMACS rinsing solution (Cat. no. 130-091-222; Miltenyi Biotec) and BSA (20:1) for 10 min at 4 °C. PBMCs were incubated with CD14+ microbeads at a concentration of 10 μL/10^7 in 40 μL of MACs buffer for 15 min at 4 °C. PBMCs were rinsed in MACs buffer and resuspended in appropriate volume before passing through MS column. CD14+ monocytes were eluted from the column and washed with ice-cold MACs buffer. The purity of eluted monocytes was checked by flow cytometry using anti-CD14-FITC conjugated antibody (Cat. no. 130-098-063; Miltenyi Biotec) and its isotype control mouse IgG2a-FITC (Cat. no. 130-098-877). Fully transformed macrophages (M0-M) were achieved after six days incubation of CD14+ monocytes in a macrophage growing medium containing RPMI-1640, 1 % P/S, 10 % FBS and 100 ng/mL Macrophage Colony Stimulating Factor (M-CSF; Cat no. 300-25; Peprotech) in a humidified atmosphere (5 % CO_2) at 37 °C. The medium was replaced with fresh medium after 3 d.

Macrophage polarisation assays

For induction of M1 phenotype (M1-M), M0-M were plated in a 6-well plate at density of 2.5 x 10^6 cells/well and stimulated with lipopolysaccharides (100 ng/mL) (LPS; Cat. no. L6529-1MG; Sigma-Aldrich) and IFN-γ (20 ng/mL) (Cat. no. 300-02; PeproTech) for 48 h. To investigate the effects of cell secretomes on M1-M polarisation, macrophage culture medium was diluted with serum-rich CM (1:1) from different stromal cells. Macrophages were harvested after 48 h and washed in MACs buffer before analysing on BD FACSAria III flow cytometer. FlowJo software was used for analysing surface markers expression.

M1-M polarization was characterised by surface marker expression of CD40 (Cat. no. 130-099-385; Miltenyi Biotec), CD64 (Cat. no. 130-100-415; Miltenyi Biotec), CD80 (Cat. no. 130-110-371; Miltenyi Biotec), CD86 (Cat. no. 560957; BD Biosciences) and HLA-DR (Cat. no. 560943; BD Biosciences). All antibodies were PE-conjugated and analysed with respective isotype controls, including mouse IgG1 (Cat. no. 130-098-845; Miltenyi Biotec), REA control (Cat. no. 130-104-612; Miltenyi Biotec) and mouse IgG2a (Cat. no. 555574; BD Biosciences). For induction of M2 phenotype (M2-M), M0-M were stimulated with dexamethasone (4 µg/mL) (Cat. no. PZN-3103491; Galenpharma) for 48 h and characterised by surface marker expression of CD163-FITC (Cat. no. 130-099-969; Miltenyi Biotec) and its isotype control mouse IgG1-FITC (Cat. no. 130-098-847). To further investigate the effects of CM from all cell types on M1-M polarisation, macrophage-CM was collected after 48 h, centrifuged at 4500 x*g* for 5 min and filtered by 0.22 µm porous membrane. The concentration of TNF-α, IL-6 (Cat. no DY206-05) and IL-12 (Cat. no. DY1240-05) were measured by ELISA. All ELISA kits were purchased from R&D Systems.

Statistical analyses

All statistical analyses were performed using IBM SPSS statistics version 24 (Chicago, USA). Data were analysed using non-parametric Kruskal-Wallis test, and significance values were adjusted by Bonferroni correction for multiple comparisons. The level of significance was set

at $p < 0.05$. Results were presented as density graphs, where each donor plotted as a dot in the dataset.

Results

Comparative protein profiles in supernatants of different stromal cells by LC-MS/MS proteomics

All cell types were characterised by MSCs surface markers and retained similar characteristics as shown in our previous study (Islam *et al.*, 2016). The cell secretome established in serum-free conditioned media from each cell type (four unrelated donors per cell type) was analysed by LC-MS/MS proteomics. Only proteins identified in at least three donors of each cell type were considered for further analyses. Results showed more proteins identified in the supernatants of ACs (709) compared to HFPSCs (641), SMSCs (567) and UCSCs (653) (Fig. 1A). Comparative analysis of identified proteins revealed 472 proteins present in the supernatants of all cell types. Only a minor fraction of proteins was exclusively found in supernatants of specific cell types, including 50 differentially expressed by UCSCs, 44 by ACs, 22 by HFPSCs and two by SMSCs. Hierarchical clustering of identified proteins revealed two major clusters, where one cluster comprised the four donors of UCSCs, and the second cluster comprised all stromal cells from adult tissues (Fig. 1B). Furthermore, among the stromal cells from adult tissues, the four ACs donors were clearly separated from HFPSCs and SMSCs donors. Identified proteins were divided into six groups according to their functions using Gene Ontology Biological Process (GOBP) terms (Fig. 2A). Qualitative comparison of proteins in different pathways revealed no significant differences between cell sources. Proteins involved in the catabolic process and ECM remodelling were abundant in the supernatants of all cell types. In addition, all stromal cells released similar percentage of proteins involved in immunoregulation (~ 20 %) and secretion (~ 13 %) (Fig. 2A).

Quantitative analyses of protein expression were performed using the LFQ approach (Fig. 2B). Six volcano plots representing all possible comparisons show differentially expressed proteins by plotting Log10 of the fold change on the X-axis and –Log10 of the *p*-value on the Y-axis for each comparison (*e.g.* HFPSCs vs ACs). Results revealed largest differences in protein expression between UCSCs and adult stromal cells ($p < 0.01$). Proteins involved in cell signalling such as TGF-β1, PDGFD, and MCP-1 were significantly upregulated in UCSCs, while catabolic proteins such as MMPs, serpins, and complement factors were downregulated compared to stromal cells from the adult origin (Fig. 2B). Notably, minor differences particularly in ECM remodelling proteins such as MMPs, and serpins were observed while comparing stromal cells from cartilage and synovium (Fig. 2B). Protein profiles belonging to specific pathways (ECM remodelling, cell communication, and inflammation) were compared among the four cell types (Fig. 3). Several MMPs, serpins, some complement factors, and heat shock proteins were less expressed in UCSCs. On the other hand, some cell signalling molecules including MCP-1, ITG-β1, PDGFD, CSF-1, HLA-C and TGF-β1 were more abundant in the supernatants of UCSCs.

Determination of cytokines and growth factors in supernatants by multiplex protein arrays

A panel of 18 selected cytokines involved in inflammation and immunoregulation was measured in supernatants of all stromal cells. Only IL-4, IL-6, IL-8, IL-12 and IL-17 were detected in supernatants of all cell types, whereas GM-CSF, IFN-α, IFN-γ, IL-1β, IL-1RA, IL-2, IL-2R, IL-5, IL-7, IL-13, IL-15 and TNF-α could not be detected in any of the supernatants. From the panel of chemokines, MCP-1, MIP-1α and RANTES were detected in all supernatants, but Eotaxin, IP-10, MIG and MIP-1β could not be detected. Overall, the levels of IL-6, MPC-1 and PGE2 were increased in UCSCs supernatants compared to other cell types, whereas the levels of IL-17, MIP-1α and RANTESs were decreased (Fig. 4). The concentration

of IL-17 and MIP-1α was significantly lower in the supernatants of UCSCs compared to ACs, and the levels of PGE2 was significantly higher in UCSCs supernatants compared to HFPSCs (Fig. 4). Regarding expression of proteases, only MMP-13 was not detectable, whereas MMP-1, MMP-3, MMP-7 and MMP-9 were detected to some degree in all serum-free CM (Fig. 5). The secretion of MMP-1, MMP-3, MMP-7 and MMP-9 was in general lower in UCSCs cultures compared to all other cell types. Significant differences were found for MMP-3 and MMP-7 when comparing UCSCs and ACs. The anabolic growth factors TGF-β1, BMP-2 and bFGF were detected at low levels in supernatants of the four cell sources, whereas IGF-1 and PDGF-AB could not be detected. Importantly, TGF-β1 was significantly elevated by UCSCs compared to HFPSCs (Fig. 5).

UCSCs supernatants exert stronger immunosuppressive effects on mitogen-activated PBMCs

To investigate the immunomodulatory effects of cell supernatants on activated PBMCs, we performed *in vitro* proliferation and migration assays (Fig. 6A). Mitogen (phytohaemagglutinin, PHA)-activated PBMCs were incubated for 5 d in the presence or absence of serum-containing CM from the different cell types. Proliferation assays revealed that UCSCs supernatants blocked PBMCs proliferation ($p = 0.06$) when compared with PHA-treated controls (Fig. 6A and B). CM from ACs did not block PBMCs proliferation, while HFPSCs (71 ± 5 %) and SMSCs (68 ± 3 %) had a minor effect. In migration assays, both UCSCs and SMSCs blocked the migration of activated PBMCs compared to positive controls (38 ± 2.5 % and 38 ± 1.2 % vs 44.2 ± 0.5 %, respectively) (Fig. 6B). To further investigate the immunomodulatory effects of MSCs supernatants, we measured the expression of TNF-α and IFN-γ in PBMCs-CM. Values were normalised against residual expression levels present in supernatants of stromal cells. Supernatants from HFPSCs and SMSCs stimulated the production of TNF-α and IFN-γ above the levels achieved by PHA treatments. Importantly, supernatants from UCSCs suppressed the

production of both TNF-α and IFN-γ by activated PBMCs, reaching significant differences when compared to HFPSCs (Fig. 6C).

UCSCs supernatants exert superior anti-inflammatory effects on M1 activated macrophages

Macrophage polarisation assay was performed as previously reported (Ambarus *et al.*, 2012; Vogel *et al.*, 2014). A panel of costimulatory molecules and cytokines to characterise macrophage polarisation was chosen based on validation tests using different stimulants. We observed divergent expression of these markers in the presence of different stimulants. In addition, we found IL-10 production as an irrelevant marker for M2 polarised macrophages. In the validation study, we observed increased IL-10 production in the presence of LPS and IFN-γ compared to dexamethasone or TGF-β and IL-4 stimulation. These discrepancies have also been reported in other studies (Chanteux *et al.*, 2007; Vogel *et al.*, 2014). Moreover, we also observed CD163 as a suitable marker for dexamethasone-stimulated M2 polarised macrophages. The discrepancy concerning the expression of CD206 has also been demonstrated in a previous study (Jaguin *et al.*, 2013).

In this study, immunomodulatory effects on M1 polarised macrophages were investigated by characterisation of surface markers expression of CD40, HLA-DR, CD64, CD80, CD86 and inflammatory cytokines release (Fig. 7). Supernatants from all stromal cell types suppressed the surface expression of HLA-DR on activated macrophages, whereas only UCSCs supernatants were able to significantly suppress the expression of CD40. On the other hand, supernatants from ACs were able to increase the expression of the co-regulatory receptors CD80 and CD86 above the levels of M1 activation (Fig. 7B). In contrast to ACs and UCSCs, supernatants from HFPSCs and SMSCs increased the surface expression of CD64 above M1 activation levels. None of the supernatants was able to alter the expression of the M2 phenotype marker CD163. Regarding cytokines profiles, CM from all cell types was able to reduce the

production of TNF-α, IL-6 and IL-12 by M1-M. Of note, a significant reduction of IL-6 and IL-12 concentration was only achieved by UCSCs (Fig. 7C).

Discussion

The main objective of this study was to ascertain which source of stromal cells possesses the most favourable phenotype for the treatment of hyaline cartilage lesions or chronic inflammatory joint disorders. Given the importance of paracrine signalling of MSCs, we performed large-scale comparative analyses of cell secretomes and conducted functional studies with cell supernatants on immune cells to compare the constitutive immunomodulatory capabilities of different MSCs. Overall, our results demonstrate that stromal cells from umbilical cord matrix exhibit better anti-inflammatory and trophic effects when compared with ACs, HFPSCs and SMSCs.

In our study, all cell sources have been expanded in monolayer cultures in the serum-supplemented medium for some weeks, as done in standard cell transplantation procedures. To facilitate the analyses of secretory profiles by LC-MS/MS proteomics, the media were conditioned under serum-free conditions. Multiplex protein assays were performed with the same serum-free CM that was used for proteomics, which allowed us to make direct comparisons of results. However, functional assays with immune cells were done with serum-supplemented CM, as serum deprivation has been shown to affect proliferation and induce apoptosis in lymphocytes and macrophages, respectively (Sato *et al.*, 2009; Wei *et al.*, 2006). Short periods of serum deprivation have been shown to not affect the cell viability of mesenchymal cells (Boraldi *et al.*, 2008). However, some changes in the secretome could occur upon changes in serum supplementation. We have analysed in parallel the expression of TNF-α, IFN-γ, IL-6 and IL-12 in both serum-containing and serum-free CM from all four cell types and only the expression of IL-6 was considerably changed in the presence of serum (Fig. 8). Although we expect only minor phenotypic changes in cells associated with serum presence,

414 alterations in the expression of some bioactive molecules could occur and should be taken into
415 consideration.

416 Currently, MSCs are viewed as “drugstores” with the potential to modulate the phenotype,
417 migration and activation of resident tissue and inflammatory cells (Caplan and Correa, 2011).
418 These have lead researchers to study MSC-mediated paracrine effects and profiles of secreted
419 proteins from different mesenchymal stromal cell types. Previous studies comparing secretory
420 profiles from different MSCs sources highlight the existence of differentially expressed factors
421 with impact on angiogenesis, matrix remodelling, inflammation and immunosuppression
422 (Amable *et al.*, 2014; Dabrowski *et al.*, 2017; Hsiao *et al.*, 2012; Li *et al.*, 2015). Our qualitative
423 analyses using large-scale proteomic approach reveal similar protein profiles, where the
424 majority of identified proteins are present in all cell supernatants. However, after hierarchical
425 clustering of protein profiles from all donors, UCSCs secretomes single out from the other adult
426 cell sources (Fig. 1B). Quantitative analyses of the secretome data reveal that proteins involved
427 in cell signalling such as TGF-β1 and PDGFD were significantly upregulated in UCSCs
428 supernatants, while catabolic proteins such as MMPs, serpins, and complement factors were
429 downregulated compared to stromal cells from the adult origin. TGF-β1 is a master driver of
430 chondrogenesis and has been shown to ameliorate OA pathogenesis (Tang *et al.*, 2015; Zhang
431 *et al.*, 2015). In addition, TGF-β1 has been shown to possess anti-apoptotic effects (Rehman *et*
432 *al.*, 2004). Observations from other studies are disparate and include cell sources that we have
433 not used; however, the superior anabolic phenotype of UCSCs, including highest expression of
434 TGF-β among the compared cell types, has also been observed recently by others (Dabrowski
435 *et al.*, 2017). Data from our multiplex protein analyses also confirmed the findings of LC-
436 MS/MS. It revealed a significant reduction of MMPs and increased expression of TGF-β1 by
437 UCSCs, thus reasserting observations made in the large-scale proteomic approach. Altogether,

these observations highlight less catabolic phenotype of UCSCs compared to the three other adult MSCs in the context of cartilage repair.

The immunomodulatory profile of the different MSCs was also investigated by proteomics and multiplex arrays. Secretome analyses revealed comparable expression of complement components, heat shock proteins, galectins and immunoregulators such as CSF-1, MCP-1, MIF and TGF-β1 among the different cell sources. In addition, our data from multiplex protein arrays showed enhanced expression of the immunomodulators IL-6, MCP-1 and PGE2, and reduced expression of IL-17 and MIP-1α by UCSCs. IL-6 has an omnidirectional role in maintaining biological functions. It has been reported to have deleterious effects in the joint (Poree *et al.*, 2008; Sui *et al.*, 2009). However, selective depletion of IL-6 in animals is associated with accelerated joint degeneration upon ageing (de Hooge *et al.*, 2005). Other authors have demonstrated IL-6-dependent inhibition of local inflammation in experimental arthritis (Bouffi *et al.*, 2010).

MCP-1 (also called CCL2), MIP-1α (CCL3) and IL-17 are all potent inflammatory factors mediating recruitment and activation of myeloid cells. Their presence is associated with cartilage degeneration and progression of OA (Appleton *et al.*, 2015; Snelling *et al.*, 2017; Wang *et al.*, 2017; Xu *et al.*, 2015; Zhao *et al.*, 2015). Mechanistically, IL-17 has been reported to inhibit chondrogenesis and promote MMPs in chondrocytes (Benderdour *et al.*, 2002; Kondo *et al.*, 2013). PGE2 is known to regulate the phenotype and functions of pro-inflammatory macrophages and NK cells (Manferdini *et al.*, 2017), however, the overall role of this factor in OA progression and cartilage homeostasis is still controversial (Bouffi *et al.*, 2010; Miwa *et al.*, 2000; Otsuka *et al.*, 2009). Despite the difficulty to reach general conclusions due to the pleiotropic nature of many chemokines and cytokines, our observations based on the global expression of released factors indicate that UCSCs display a more immunosuppressive and anti-inflammatory phenotype than their adult counterparts.

To investigate further the paracrine potential of the different MSCs on immunomodulation we conducted functional assays on lymphocytes and macrophages. Results follow the same trend as the analyses made on protein profiles, highlighting the superior immunosuppressive phenotype of UCSCs compared to the other MSCs. The ability of MSCs to regulate inflammation and immunity has been the focus of intense research during recent years (Donders *et al.*, 2018; von Bahr *et al.*, 2012). Many *in vitro* studies have shown that mesenchymal cells from multiple sources, including differentiated connective tissue cells such as chondrocytes and fibroblasts, have the potential to regulate inflammation and T-cell functions to some extent (Bouffi *et al.*, 2011; Lohan *et al.*, 2016). Still, there is no consensus on which cell source is the most powerful in this respect.

Most published studies have compared bone marrow, adipose tissue and foetal tissues with different outcomes. Some comparative studies demonstrated bone marrow MSCs have slightly superior immunosuppressive capacity than other MSCs (Heo *et al.*, 2016; Karaoz *et al.*, 2017). In line with our observations, some groups have previously observed superior immunosuppressive abilities by MSCs from cords (Jin *et al.*, 2013; Najar *et al.*, 2012). It is important to mention that in our study MSCs were not primed with pro-inflammatory cytokines during medium conditioning. Such experimental condition allowed us to investigate the constitutive abilities of these cells without external stimuli. Priming of MSCs is extensively documented in the published literature and has been recommended as a mandatory step to unleash the full immunosuppressive potential of MSCs (Gomez-Aristizabal *et al.*, 2017; Najar *et al.*, 2012; van Buul *et al.*, 2012). In agreement with our study, constitutive immunoregulation by unstimulated MSCs has been previously observed (Saulnier *et al.*, 2015). In this context, *ex vivo* priming of MSCs has been related with few controversial outcomes *in vivo* describing increased immunogenicity of pre-stimulated MSCs (Papadopoulou *et al.*, 2012; Treacy *et al.*, 2014).

In clinical settings, MSCs from different sources have been investigated for the treatment of focal cartilage lesions and OA (Lee and Wang, 2017). Articular chondrocytes, bone marrow and adipose tissue stromal cells are the most commonly used sources for cartilage repair (Vonk *et al.*, 2015). These studies mostly assessed safety and efficacy of used MSCs for specific clinical implications. However, a comparative study in humans argued that autologous SMSCs exert superior healing outcomes (Akgun *et al.*, 2015). On the other hand, in preclinical models, MSCs from cords have been shown to exert immunosuppression and disease regression in experimental models of OA and autoimmune disorders (Donders *et al.*, 2015; Saulnier *et al.*, 2015; Yang *et al.*, 2015). In contrast to MSCs from autologous sources, there are few ongoing trials exploring the potential of allogeneic UCSCs for both OA management and focal cartilage repair in humans (NCT02580695, NCT02291926, NCT03166865 and NCT03358654), without published outcomes hitherto. Confirming the results of this comparative study in suitable animal models would provide more insight into the use of UCSCs in the clinics.

Conclusions

Traditionally, the regenerative potential of MSCs has been directly linked to their multipotent differentiation and tissue-forming capabilities. Nowadays, increasing attention is given to their role as cellular modulators. In the field of articular cartilage lesions and degenerative joint diseases, there is no consensus on the best cell source for treatment. Considering the relevance of paracrine signalling, in this study we have compared the secretomes among MSCs from different sources. Both the molecular analyses and the functional assays indicate that UCSCs display superior anti-inflammatory and trophic effects compared to other MSCs from adult tissues. The hypoimmunogenic nature of UCSCs, along with their high abundancy, simple isolation and favourable protein profiles makes this cell source an attractive tool for off-the-self allogeneic adjuvant therapy.

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Availability of data and materials

The datasets used in this study are available from the corresponding author upon request.

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Authors' Contributions

AI primarily conducted the laboratory work, planned the study and prepared the manuscript. IMZ participated in the conception of the study, data evaluation, edited and approved the final draft of the manuscript. IU and JAB performed LC-MS/MS. All authors contributed to the data interpretation of results, provided direction and comments on the manuscript.

Ethical statement

The Regional Ethical Committee of Northern Norway has approved the study (REK Nord 2014/920 and 2010/586).

Consent for publication

Not applicable

Competing interests

The authors declare no competing interests.

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Figure legends

Figure 1. Hierarchical clustering of identified proteins from secretomes of ACs, HFPSCs, SMSCs and UCSCs. A. Venn diagram depicts the percentage of identified proteins shared among four different stromal cell types. **B.** Dendrogram shows two major clusters of four different stromal cell types. All cell sources from adult mesenchyme origin clustered together, whereas the four donors of UCSCs from extra-embryonic origin clustered separately.

Figure 2. Protein expression analysis by LC-MS/MS from conditioned medium of ACs, HFPSCs, SMSCs and UCSCs. A. Distribution of identified proteins into six main categories according to their function annotated using GOBP terms. **B.** Volcano plot illustrates the results of the six sets of statistical comparisons made between HFPSCs vs ACs, SMSCs vs ACs, HFPSCs vs SMSCs, UCSCs vs ACs, UCSCs vs HFPSCs and UCSCs vs SMSCs. These plots show each protein with –Log10 (*p*-value) and Log10 of fold change of the comparison on the Y-axis and X-axis, respectively. Proteins with greater fold change and lower *p*-value are plotted further away from zero on each axis. Proteins that are significantly up and down-regulated ($p < 0.01$) are presented in green and red colour, respectively.

Figure 3. Comparative expression of selected proteins from conditioned medium of ACs, HFPSCs, SMSCs and UCSCs. The heat map shows proteins involved in **A.** ECM remodelling (proteases and inhibitors), **B.** cell signalling (growth factors) and **C.** inflammation/immune

responses. Down-regulated proteins are indicated in red, whereas up-regulated proteins are in blue.

Figure 4. Comparison of identified cytokines and chemokines from conditioned medium of ACs, HFPSCs, SMSCs and UCSCs. Dot density show concentration of cytokines involved in inflammation (IL-4, IL-6, IL-8, IL-12, IL-17 and PGE2) and chemokines (MCP-1, MIP-1α and RANTES) detected in supernatants of four different stromal cell types. Level of significance is p (*) < 0.05.

Figure 5. Comparison of identified MMPs and anabolic factors from conditioned medium of ACs, HFPSCs, SMSCs and UCSCs. Dot density shows the concentration of MMPs (MMP-1, MMP-3, MMP-7 and MMP-9) and growth factors (TGF-β1, bFGF and BMP-2) detected in supernatants of four different stromal cell types. Level of significance is p (*) < 0.05.

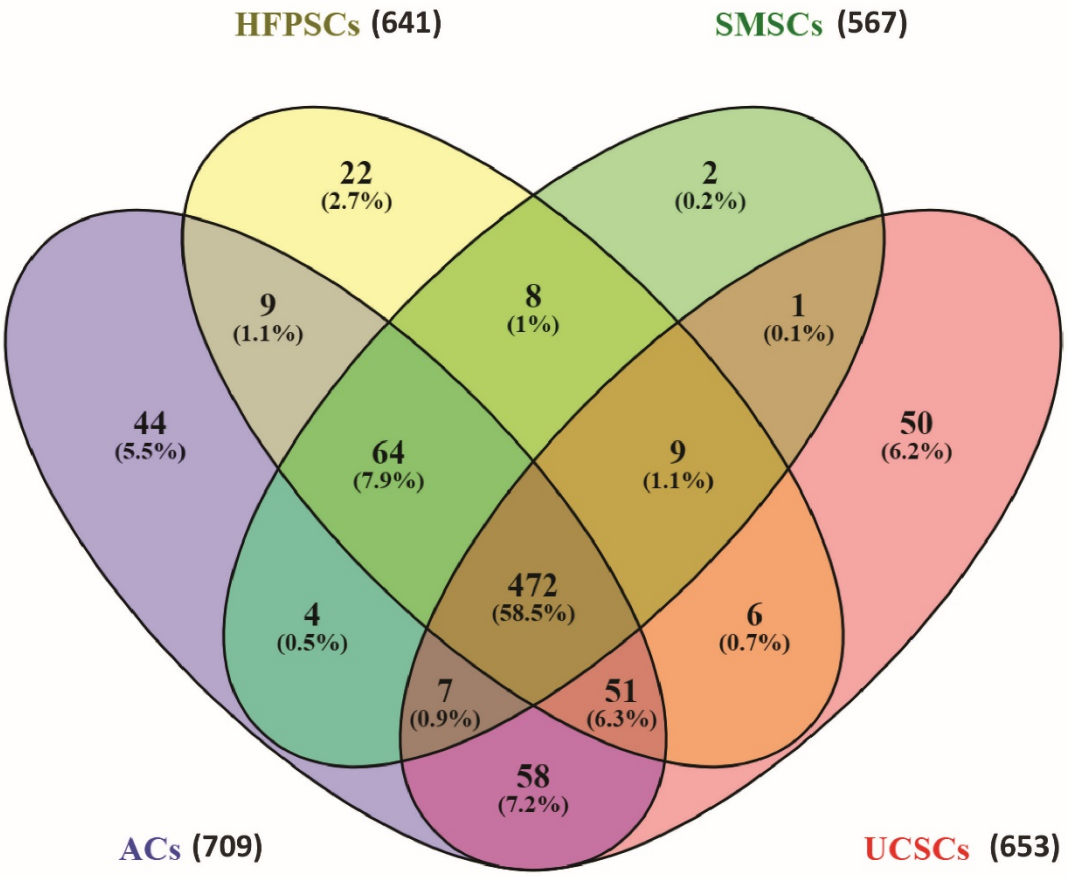
Figure 6. Differential modulation of PBMCs activation by MSCs conditioned medium. A. PBMCs proliferation assay: representative flow cytometry dot plots depict the percentage of CFSE labelled PBMCs stimulated with 10 ug/mL of PHA in presence and absence of CM from the four different stromal cell types. **B.** Quantitative analyses of PBMCs proliferation and migration in presence and absence of CM from four different stromal cell types. SDF-1 at 100 ng/mL was used for chemo-attraction in migration assays **C.** Total concentration of TNF-α, and IFN-γ detected in PBMCs-CM (10^6 cells/well) after incubation with PHA and CM from four different stromal cell types. Level of significance is p (*) < 0.05.

Figure 7. UCSCs secretomes can modulate macrophage-mediated inflammation. A. Characterisation of surface molecules during polarisation of M0-M into M1-M (CD40, CD64, CD80, CD86 and HLA-DR) and M2-M (CD163) by flow cytometry. Red, blue and orange peak represents isotype control, M0-M and activated macrophages, respectively. **B.** Dot density depicts M1-M activation and distinct blocking of M1-M activation by CM from ACs, HFPSCs,

SMSCs and UCSCs. M2-M polarisation was used as a negative control for surface expression of CD40 and HLA-DR. **C.** Levels of TNF-α, IL-6 and IL-12 detected in macrophage culture conditioned medium (2.5 x 10^6 cells/well) after incubation with CM from four different stromal cell types. Level of significances are $p < 0.05$ (*) and $p < 0.005$ (**).

Figure 8. Comparison between serum-free and serum-containing conditioned medium of ACs, HFPSCs, SMSCs and UCSCs. Presence of serum increases the production of IL-6 by all cell types.

A.



B.

808

809 Figure 1: Hierarchical clustering of identified proteins from secretomes of ACs, HFPSCs,

810 SMSCs and UCSCs.

811

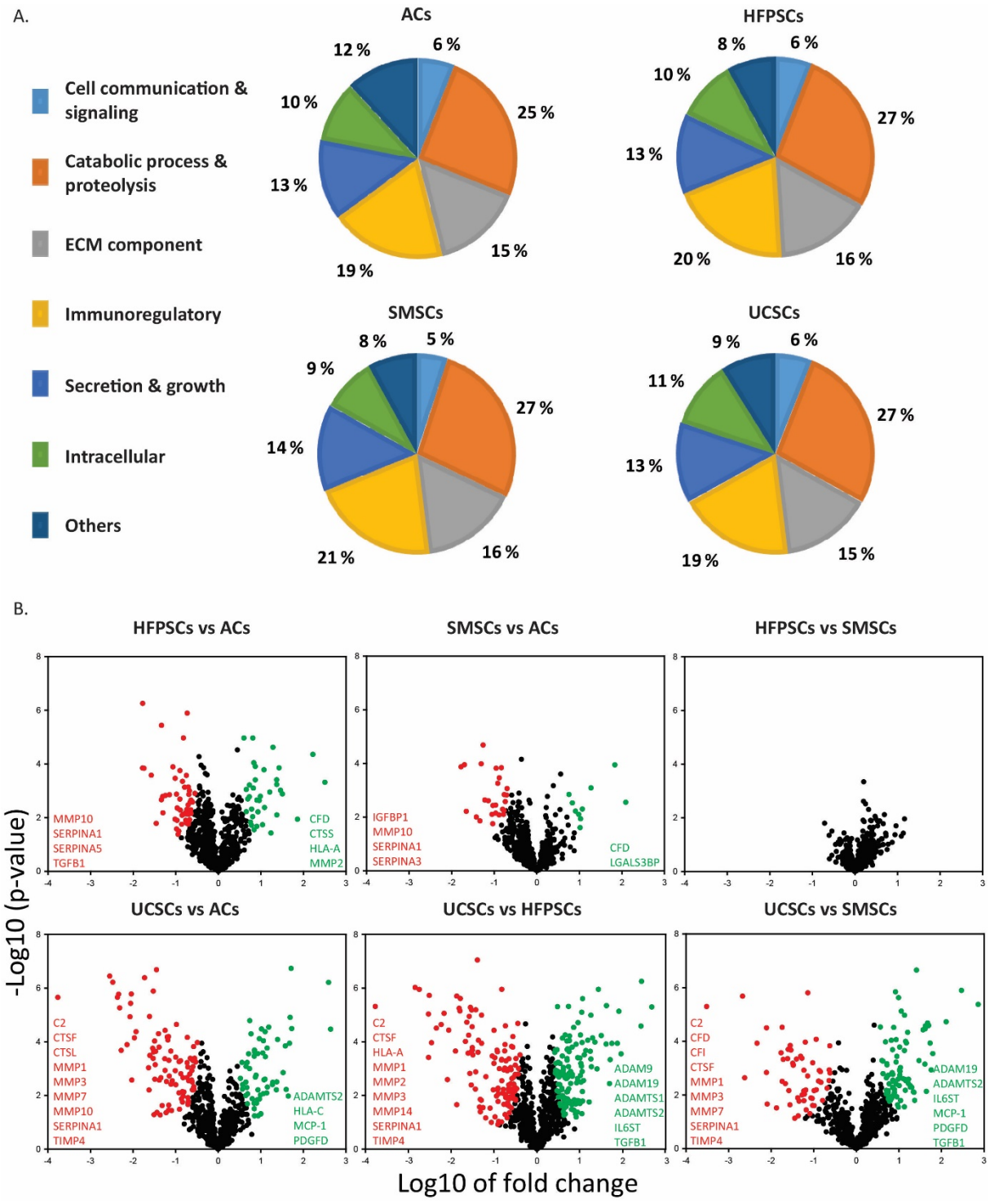
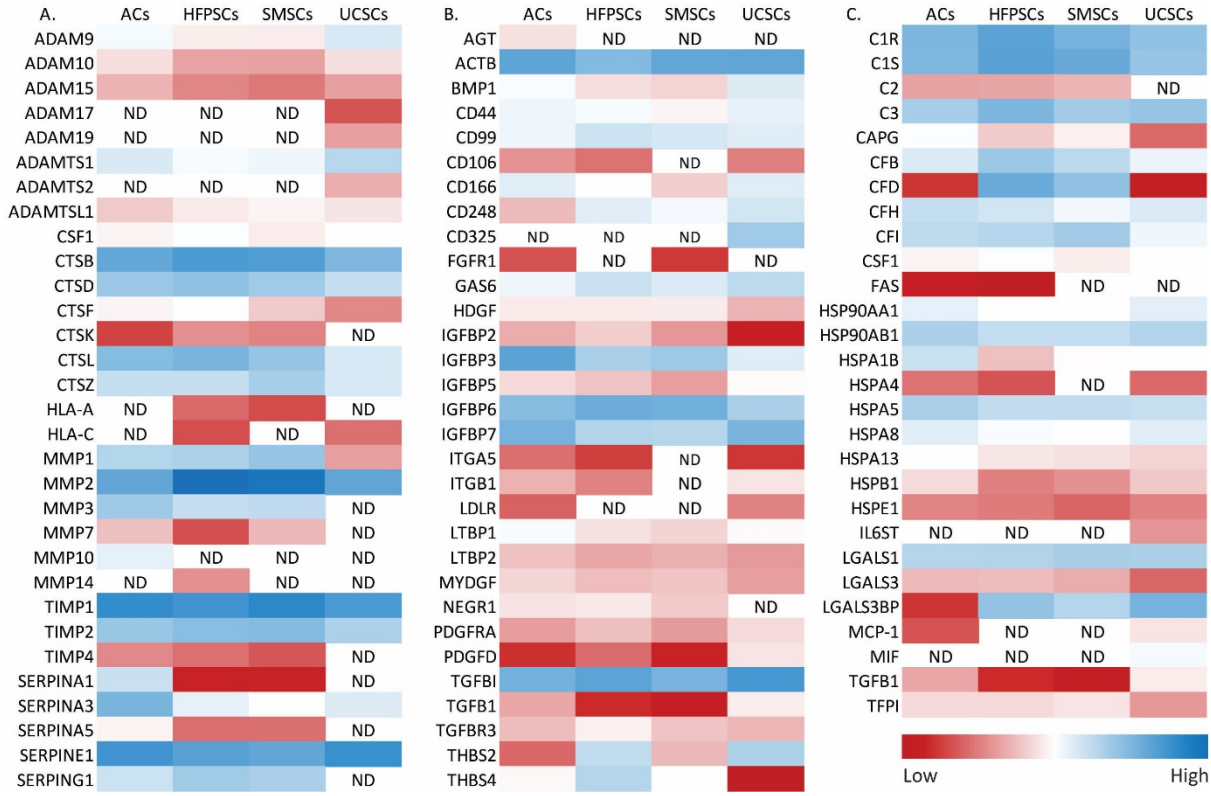


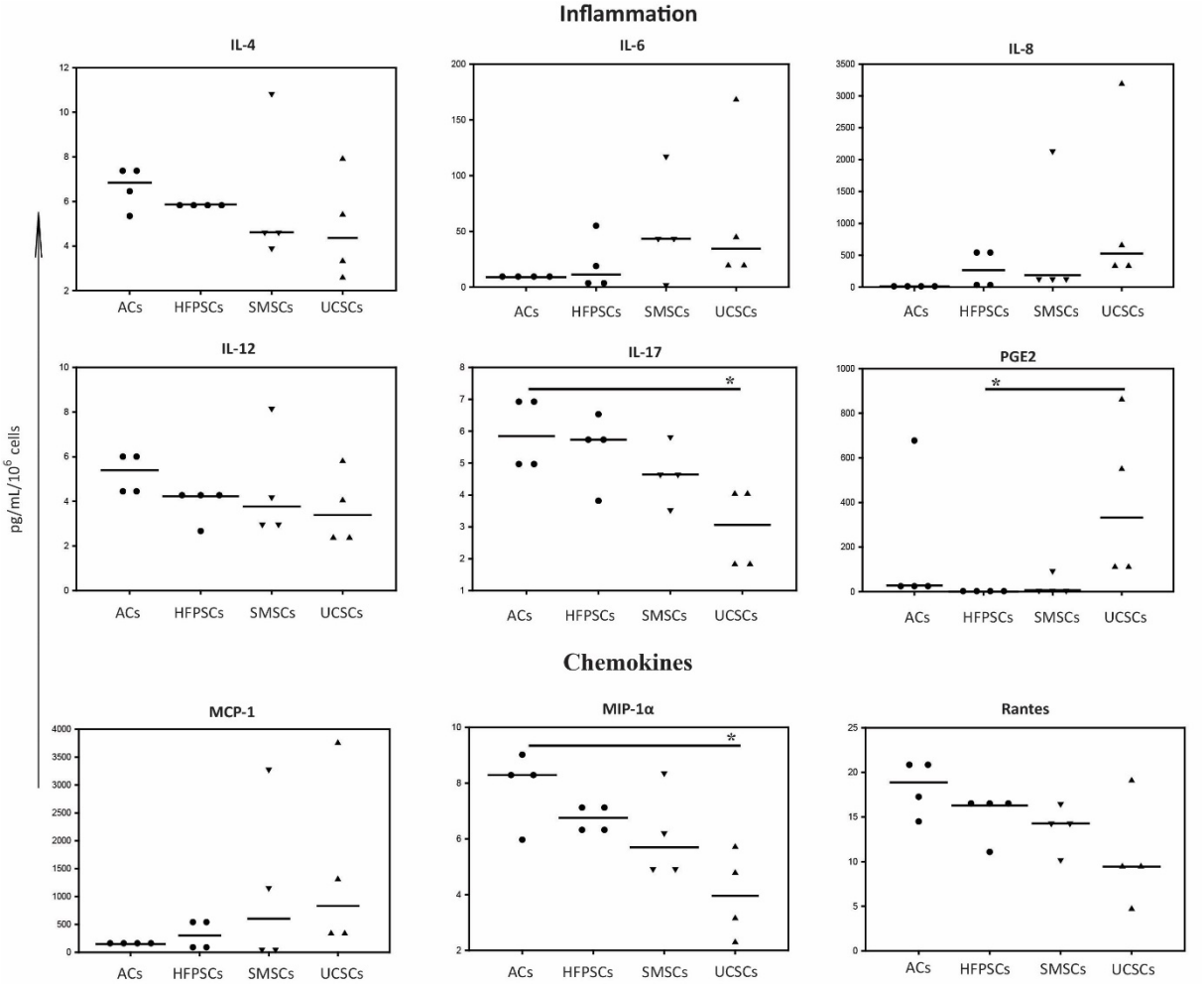
Figure 2. Protein expression analysis by LC-MS/MS from conditioned medium of ACs, HFPSCs, SMSCs and UCSCs.



817

818 Figure 3. Comparative expression of selected proteins from conditioned medium of ACs,

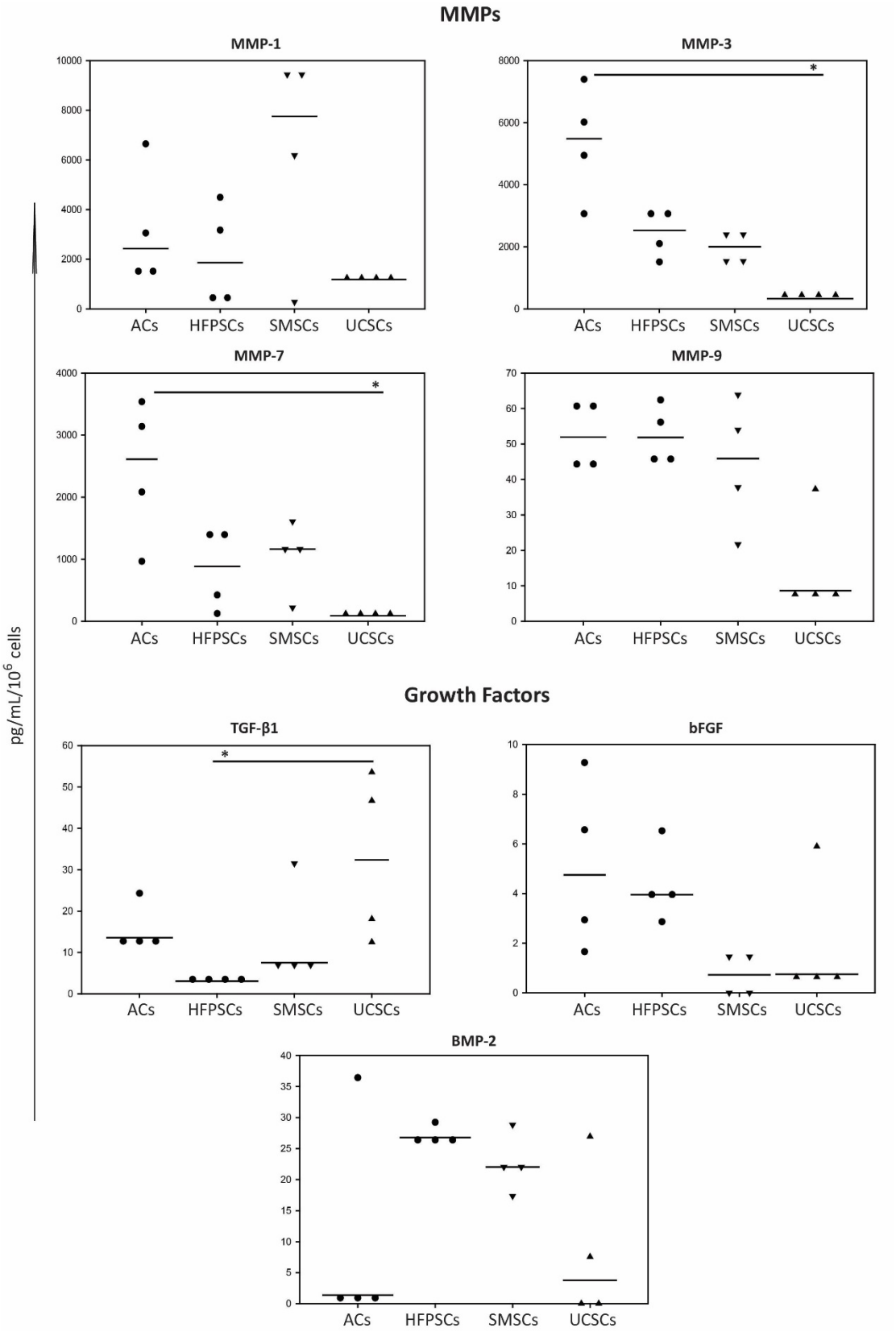
819 HFPSCs, SMSCs and UCSCs.



820

821 Figure 4. Comparison of identified cytokines and chemokines from conditioned medium of

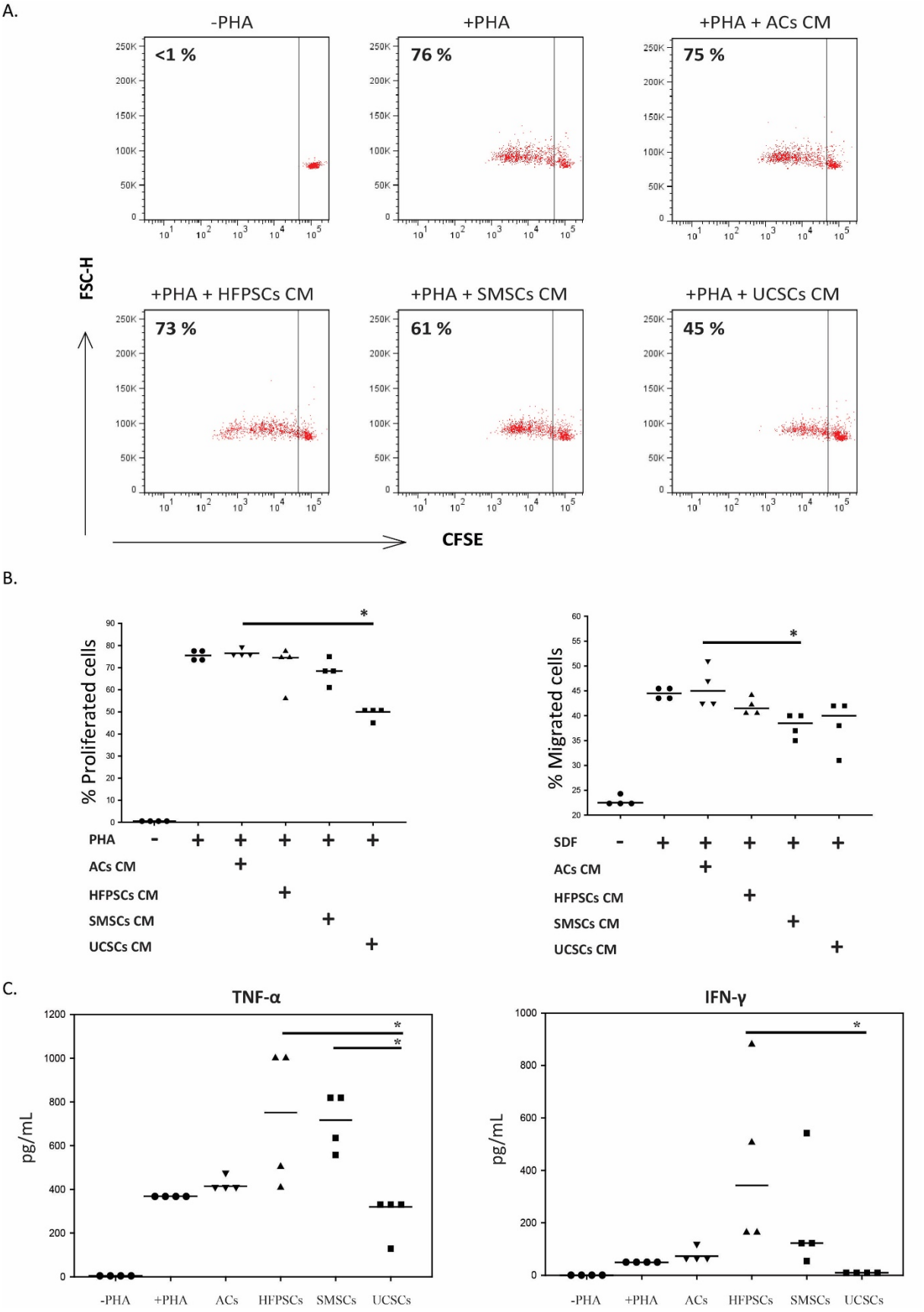
822 ACs, HFPSCs, SMSCs and UCSCs.



823

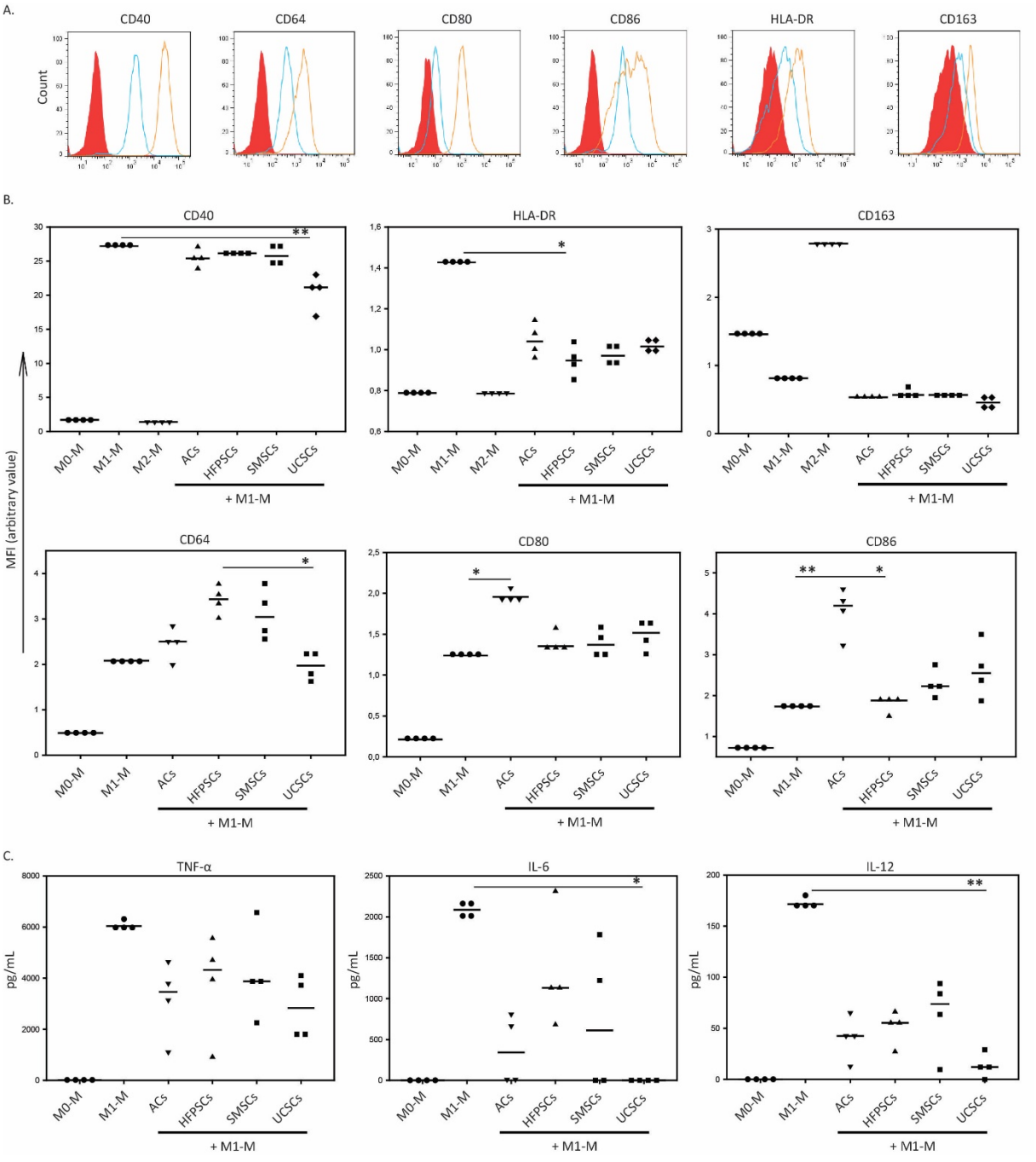
824 Figure 5. Comparison of identified MMPs and anabolic factors from conditioned medium of

825 ACs, HFPSCs, SMSCs and UCSCs.



826

827 Figure 6. Differential modulation of PBMCs activation by MSCs conditioned medium.



828

829 Figure 7. UCSCs secretomes can modulate macrophage-mediated inflammation.

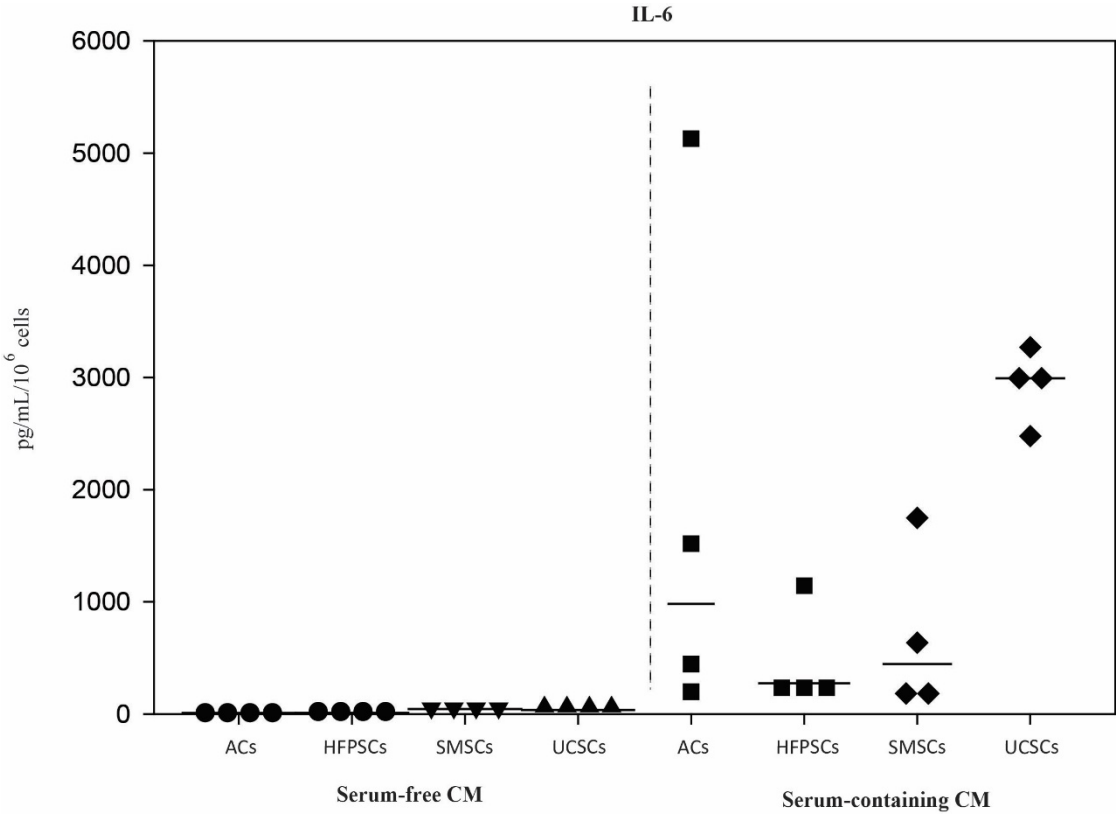


Figure 8. Comparison between serum-free and serum-containing conditioned medium of ACs, HFPSCs, SMSCs and UCSCs.