Manuscript Details

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Title	Expression of inflammatory cytokines in mesenchymal stromal cells is sensitive to culture conditions and simple cell manipulations
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Abstract

Background; Mesenchymal stromal cells (MSCs) can be used in several clinical applications. While MSCs are frequently cultured in fetal bovine serum for in vitro experimentation, human serum supplements are required for cells to be used in patients. Here we show how different human serum supplements and in vitro manipulations used during the cell culture impact on MSC proliferation rate and expression of inflammatory molecules. Methods; MSCs were cultured in medium supplemented with human plasma or serum combined with human platelet lysate (PL) and/or basic fibroblast growth factor (FGF2). Real time RT-PCR and western blot were used to assess expression of inflammatory cytokines. Results; Serum with addition of FGF2 gave the fastest proliferation rate. However, serum with FGF2 also increased expression of genes encoding inflammatory cytokines. The most favorable expansion condition for chondrogenic differentiation and inhibition of cartilage matrix degrading enzymes was plasma supplemented with PL and FGF2. Detachment of cells using trypsin gave considerable upregulation of inflammatory cytokine mRNAs which lasted for up to 24 hours, with concomitant increase in protein levels. Even the gentle act of changing medium led to upregulation of cytokine mRNA, caused by addition of fresh serum. Discussion; Different culture conditions and simple cell manipulation influence proliferation rate and expression of inflammatory genes. Supplementing culture medium with allogeneic AB serum and FGF2 during monolayer expansion supported cell expansion better than other supplements, but also induced the highest levels of inflammatory cytokines and gave inferior results for chondrogenic differentiation. The importance of the composition of the culture medium and even gentle in vitro manipulation of the cells should be taken into account in the planning of procedures using in vitro expanded MSCs.

Keywords	MSCs, inflammation, chondrogenesis, serum, FGF2
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Dear Editor,

Thank you very much for giving us the opportunity to resubmit our article "Expression of inflammatory cytokines in mesenchymal stromal cells is sensitive to culture conditions and simple cell manipulations".

As suggested by you and the reviewer most of the experiments have now been reproduced in cells from several new donors. In total we have data from 9 different donors. Due to inclusion of data from several new donors and new interpretations the revised manuscript has been considerably rewritten. However, the main findings from the original manuscript are the same due to high reproducibility in the new experiments.

We hope you find the revised manuscript suitable for publication in Experimental Cell Research.

Sincerely,

Tommy A. Karlsen

Response to the reviewer

Thank you very much for your critical review. Your comments have been valuable for us. We have repeated many of the experiments in cells from several new donors. Although there were some donor variations we feel more confident in our results and conclusions due to the high reproducibility in the experiments. Due to inclusion of data from several new donors and your comments and suggestions the manuscript has been rewritten. Below is our response to your comments. We believe we have answered all your comments and performed the necessary experiments in a satisfactory way. We appreciate your help.

Major comments from the reviewer;

The text is well written, however in major need of structure and detail.

Response: The manuscript has been considerably rewritten and we believe it is easier to follow the text and the results provided.

Methods are not sufficiently described, which makes it difficult to evaluate the quality of data.

Response: in the "Materials and methods" section we have included a lot more details on how experiments were performed. See comments below for more details.

Results need confirmation by more repitions, - data based on one donor in 3 technical replicates, is simply not substantial. Donor variation is essential and as such results from one donor are neighter indicative nor conclusive.

Response: Although several of the experiments in the submitted manuscript were repeated in the next figures using cells from different donors, we agree that it is important to run these exact same experiments in biological replicates. Most of the experiments from the original manuscript have now been repeated using cells from several new donors. Results in Figure 1B, C and D, Figure 2B and Figure 3B have been repeated in three new donors while the results in Figure 2A have been repeated in two new donors. These results are presented as supplementary figures. In total we have included data from nine donors. The results are very similar to the originally presented results, although there are some donor variations, as expected. Thank you for this suggestion.

Discussion is actually good and relevant, but statements and conclusions remain speculative on the present basis.

Response: After inclusion of results from several new donors we believe our conclusions have been strengthened. Although the discussion has been rewritten the main conclusions from the original manuscript are the same.

Methods;

Information about donors, procurement and isolation method is lacking.

Response: We are not allowed by our regulatory authorities to provide age or sex of our donors. However in the " Isolation and culture of bone marrow mesenchymal stem cells" section, page 4-5, we have included details about the procurement and isolation of the cells.

Cell culture: no mentioning of passages used. Methods used for cell counting/proliferation not described.

Response: Except for the cumulative cell counts in Figure 1A all experiments were performed on cells from passage 2 or 3. The cells were counted using trypan blue stain and the Invitrogen™ Countess™ automated cell counter. This has been included under the section "Isolation and culture of bone marrow mesenchymal stem cells" on page 4-5.

Chondrogenic differentiation; seeding on discs not described, - methods used for analysis of differentiation not described - primers not mentioned.

Response: Details for making alginate discs and chondrogenic differentiation are included on page 5-6 under the section "Chondrogenic differentiation". Taqman assays used to determine gene expression are listed in Table 1, page 20.

PCR: purity and integrity of RNA not mentioned, stability of HKG during variations tested not addressed

Response: RNA purity and integrity assessment and HKG variation between samples are now included under section "Isolation of total RNA, cDNA synthesis and RT-qPCR" on page 6.

WB; how are lysates made, - methods description simply not present

Response: Details on WB and preparation of lysate is included on page 7 under the section "Western blotting".

Results;

Section should be "streemlined", - present data, not methods nor discussion of results

Response: The manuscript has been rewritten. We present our data and have removed text that could be interpreted as discussion from the results section.

Expression of inflammatory cytokines in mesenchymal stromal cells is sensitive to culture conditions and simple cell manipulations

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Short title: Culture-induced inflammatory cytokines in MSCs

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Abstract

Background; Mesenchymal stromal cells (MSCs) can be used in several clinical applications. While MSCs are frequently cultured in fetal bovine serum for in vitro experimentation, human serum supplements are required for cells to be used in patients. Here we show how different human serum supplements and in vitro manipulations used during the cell culture impact on MSC proliferation rate and expression of inflammatory molecules.

Methods; MSCs were cultured in medium supplemented with human plasma or serum combined with human platelet lysate (PL) and/or basic fibroblast growth factor (FGF2). Real time RT-PCR and western blot were used to assess expression of inflammatory cytokines.

Results; Serum with addition of FGF2 gave the fastest proliferation rate. However, serum with FGF2 also increased expression of genes encoding inflammatory cytokines. The most favorable expansion condition for chondrogenic differentiation and inhibition of cartilage matrix degrading enzymes was plasma supplemented with PL and FGF2. Detachment of cells using trypsin gave considerable upregulation of inflammatory cytokine mRNAs which lasted for up to 24 hours, with concomitant increase in protein levels. Even the gentle act of changing medium led to upregulation of cytokine mRNA, caused by addition of fresh serum.

Discussion; Different culture conditions and simple cell manipulation influence proliferation rate and expression of inflammatory genes. Supplementing culture medium with allogeneic AB serum and FGF2 during monolayer expansion supported cell expansion better than other supplements, but also induced the highest levels of inflammatory cytokines and gave inferior results for chondrogenic differentiation. The importance of the composition of the culture medium and even gentle in vitro manipulation of the cells should be taken into account in the planning of procedures using in vitro expanded MSCs.

Keywords: MSCs, inflammation, chondrogenesis, serum, FGF2

Introduction

Human mesenchymal stromal cells (hMSCs) have classically been expanded in vitro using fetal bovine serum. However, for fear of xenogeneic immune responses and zoonotic diseases, animal supplementation is undesirable in media intended for expansion of hMSCs to be used in human clinical applications [1, 2]. Autologous serum may support expansion of hMSCs without growth factor supplementation [3], but the amount of serum required for cultivation of 10^8 cells or more is not easily obtained from a single individual. Allogeneic human serum does not always support MSC proliferation in the absence of growth factors [3].

Human pooled plasma supplemented with human platelet lysate (PL) (platelet lysate in plasma, PLP) is perhaps the human protein source most commonly used for expansion of hMSCs [4]. It supports the establishment of many colonies following initial seeding and quick population doubling (PD) time during the subsequent log phase of proliferation, but the production of the PLP is cumbersome, and even with the addition of anticoagulants such as heparin there is a tendency for microcoagulation to occur in the medium. This is avoided when human pooled serum is used instead of plasma, where heparin is also not needed.

However, cell proliferation is not the only parameter required to make the best choice of a human serum source. hMSCs are known for their secretion of a wide range of cytokines and other soluble factors, and for many human therapeutic applications the profile of secreted cytokines will determine the success or failure of the use of the hMSCs. In particular, the secretion of pro-inflammatory cytokines may be problematic for several human therapeutic applications. Thus, in the present study we have compared the effect of supplementing standard MSC growth medium with plasma or serum combined with PL (PLP and PLS), fibroblast growth factor 2 (FGF2) (plasma/FGF2, serum/FGF2) or both (PLP/FGF2, PLS/FGF2) for their support of cell proliferation and their ability to induce expression of

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inflammatory cytokine genes and proteins. To investigate MSC functionality, chondrogenic differentiation was investigated after expansion in serum/FGF2, PLP and PLP/FGF2. The study was extended to also examine the impact of simple cell manipulation procedures on the expression of inflammatory cytokine genes.

Materials and methods

Chemicals and reagents.

Information about Taqman assays, antibodies, fatty acids and other laboratory reagents are listed in supplementary Table 1.

Isolation and culture of bone marrow mesenchymal stem cells.

Bone marrow mesenchymal stromal cells (BM-MSCs) were isolated as previously described [5, 6]. Approximately 50 mL of BM was aspirated from the iliac crest of 9 voluntary donors. The mononuclear cells were isolated using density gradient centrifugation (Lymphoprep; Fresenius Kabi, Bad Homburg, Germany). The mononuclear cells were seeded in 175 cm2 tissue culture flasks. After 48 h, the non-adherent cells were removed by medium exchange. The adhering cells were expanded in monolayer culture until colonies reached 70-80 % confluence and then passaged using trypsin-EDTA (Sigma Aldrich, St.Louis, MO). At each passage 875.000 cells were seeded out in new 175 cm2 flasks. The cells were counted using trypan blue stain and the Invitrogen™ Countess™ automated cell counter (Thermo Fisher Scientific).

The culture medium consisted of DMEM F12 + glutamax (Gibco/Thermo Fisher Scientific, Waltham, MA) containing either 1) 10% AB serum (Sigma Aldrich and Oslo Blood Bank,

Oslo, Norway), 2) 10% AB plasma (Octaplas AB, Oslo Blood Bank), 3) 10% plasma

supplemented with 10 ng/ml FGF2 (R&D Systems, Minneapolis, MN) (plasma/FGF2, 4) 10%

AB serum supplemented with 10 ng/ml of FGF2 (serum/FGF2), 5) 10% human AB serum

supplemented with PL (corresponding to 10^9 platelets/ml of serum) (PLS) or in 6) 10% AB

plasma supplemented with PL (10^9 platelets/ml plasma) (PLP). 2 IU/ml of heparin

(Wockhardt, Wrexham, UK) was added to the cultures containing plasma to avoid

coagulation [4]. All conditions contained 100 units/mL penicillin, 100 mg/mL streptomycin,

and 2.5 mg/mL amphotericin B (Sigma). The culture medium was changed every 3–4 days.

After the first passage amphotericin B was removed. Except for calculated cumulative cell

counts in Figure 1A all experiments were performed on cells in passage 2 or 3.

All donors provided written, informed consent. The study was approved by the

Regional Committee for Medical Research Ethics, Southern Norway, Section A. All methods

and experiments were performed in accordance with the relevant guidelines and

regulations. Cells from nine donors were used in the study.

Chondrogenic differentiaiton

BM-MSCs were trypzinised and washed with DMEM-F12. PRONOVA-LVG

Alginate and calcium-alginate particles from the self-gelling system (NovaMatrix, Sandvika,

Norway) were prepared to a final alginate concentration of 1% using a 4.6% mannitol solution.

$1,2 \times 10^6$ cells were resuspended in 60 μL of the PRONOVA-LVG solution. 60 μL of the

calcium-alginate particle solution was added and the cell/alginate suspension was thoroughly

mixed before 100 μl of the mix, corresponding to 10^7 cells/mL alginate, was transferred to 16-

well chamber slides (Lab-Tek, Nunc/Sigma Aldrich) for gelling. The gelling process was

supported by washing with a 50 mM $CaCl_2$ solution. After stabilization of the alginate

structure the discs were washed three times with DMEM-F12. Chondrogenic differentiation

was induced by high-glucose DMEM (4.5 g/L) supplemented with 1 mM sodium pyruvate (Gibco), 0.1 mM ascorbic acid-2-phosphate, 1% ITS (insulin 25 mg/mL, transferrin 25 mg/mL, and sodium selenite 25 ng/mL), 1.25 mg/mL human serum albumin (Octapharma, Lachen, Switzerland), 500 ng/mL bone morphogenic protein-2 (InductOs; Wyeth Pharmaceuticals, Taplow Berks, UK), and 10 ng/mL recombinant human transforming growth factor beta 1 (R&D Systems). Medium was changed every second or third day. Chondrogenic differentiation was evaluated using RT-qPCR to quantify levels of *SOX9*, *COL2A1*, *ACAN*, *ADAMTS5* and *MMP13* after 10 days of differentiation.

Isolation of total RNA, cDNA synthesis and RT-qPCR.

Total RNA was isolated using the miRNeasy mini kit according to the protocol from the manufacturer (Qiagen, Hilden, Germany). cDNA synthesis and RT-qPCR were performed following protocols from the manufacturer using the High Capacity cDNA Reverse Transcription Kit and the Taqman MicroRNA Reverse Transcription Kit (Thermo Fisher Scientific). For cDNA synthesis 200 ng of total RNA was reverse transcribed into cDNA in a total volume of 20 µl. All samples were run in technical triplicates. Each replicate contained 1.0 µl cDNA in a total volume of 25 µl. The thermocycling parameters were 95ºC for 10 minutes followed by 40 cycles of 95ºC for 15 seconds and 60ºC for 1 minute. *GAPDH* was used as endogenous control. RT-qPCR results are shown as relative fold changes using mean values from technical triplicates with a 95% confidence interval. All donors are shown separately. *GAPDH* did not vary more than 1-1.5 Ct-values between the different samples and were considered to be stably expressed. RNA concentration and purity were assessed by spectrophotometry (Nanodrop, Wilmington, DE). The 260/280 ratios were ~ 2.0 and 260/230 ratios were between 1.8 and 2.2. RNA integrity was assessed by gel electrophoresis.

Western blotting

One million cells were lysed in 100 µl of 1x Laemmli Sample Buffer (Sigma Aldrich) and incubated at 98ºC for 10 minutes to denature proteins. Cell lysates corresponding to 250.000 cells were loaded onto a 4-20 % gradient polyacrylamide gel (Biorad, Hercules, CA). Proteins were separated by gel electrophoresis, transferred to PVDF membranes using the TransBlot Turbo system (Biorad) and incubated with appropriate antibodies before visualizing the bands using the myECL imager (Thermo Fisher Scientific). All antibodies were diluted in 1X TBS, 5 % nonfat dry milk, 0.1 % Tween 20 and used at concentrations listed in Table 1.

Stimulation with fatty acids and lipofectamine 2000.

500.000 cells were stimulated with fatty acids (50 µM) overnight in culture medium containing SF. The fatty acids were purchased from Nu-chek prep, Inc (Elysian, MN) and dissolved in PBS containing 0.5 % BSA. Lipofectamine 2000 (Invitrogen, Carlsbad, CA) was used as a positive control to induce inflammation. For each reaction 8 µl Lipofectamine 2000 were added to 500.000 cells in a total volume of 2.5 ml culture medium

Results

Cells cultivated in serum with FGF2 (serum/FGF2) proliferated much faster than cells cultivated with other culture supplements (Figure 1A). hMSCs cultured in serum or plasma without FGF2 or PL quickly ceased to proliferate and are not included in Figure 1A. hMSCs cultured in plasma with FGF attached well to plastic upon initial seeding, but subsequently proliferated very poorly and ceased to proliferate after 2-3 passages. This condition is also not included in Figure 1A. Serum/FGF2 conditions induced higher levels of interleukin 1 beta

(*IL1B*) and *IL8* mRNA than serum with PL (PLS) or plasma with PL (PLP) (Figure 1B and Supplementary Figure 1).

Heparin has been shown to have anti-inflammatory properties [7]. Thus, we wanted to determine the possible effect of the heparin component (2 IU/ml) added to PLP during culture to avoid coagulation in this model system. Surprisingly, addition of heparin increased rather than decreased *IL1B* mRNA. *IL6* and *IL8* mRNA levels differed too much between donors to draw robust conclusions (Figure 1C and Supplementary Figure 2).

Next, the importance of serum vs plasma and the addition of FGF2 were investigated by changing the culture condition for hMSCs initially expanded in serum/FGF2 (Figure 1D and Supplementary Figure 3). In the absence of FGF2 there was little difference between serum and plasma for their ability to induce markers of inflammation. The addition of FGF2 to PLP and PLS increased *IL1B* and *IL8* mRNAs, while the results for IL6 were modest and varied between donors.

One way to compare the culture media for their ability to support hMSC functionality would be to induce chondrogenic differentiation in hMSCs cultured under different conditions. Here, across three donors, hMSCs cultured in PLP/FGF2 and then subjected to chondrogenic differentiation for 10 days gave the highest levels of essential chondrogenic molecules *COL2A1* and *ACAN* mRNA and, importantly, by far the lowest levels of cartilage degrading enzymes *ADAMTS5* and *MMP13* mRNA (Figure 2A and Supplementary Figure 4).

We next went on to determine if simple cell manipulations such as trypsinization would also impact on the synthesis of inflammatory cytokines in the PLP and serum/FGF2 conditions. Detachment of cells using trypsin induced rapid and considerable upregulation *IL1B* mRNA under both culture conditions (Figure 2B). Under serum/FGF2 conditions IL6, IL8 and TNFα mRNA levels were also upregulated following trypsinization (Figure 2B and supplementary

Figure 5). The increase was usually sustained for more than 5 hours, sometimes for more than 24 hours. The rapid and sustained upregulation of inflammatory cytokines was observed also at the protein level (Figure 2C).

The gentle act of changing medium should a priori not traumatize the cells. However, medium change induced upregulation of all cytokine mRNAs, particularly for the fresh serum and serum/FGF2 conditions (Figure 3A and Supplementary Figure 6). Interestingly, when the used cell culture supernatant (SUP) or used supernatant with fresh FGF2 (SUP/FGF2) were added back to the cultures, no upregulation of inflammatory cytokines was seen (Figure 3B and Supplementary Figure 6), showing that the addition of fresh serum was responsible for the inflammatory response after medium change.

Another moderate manipulation of the cells, the use of the synthetic fatty acid composite Lipofectamine to facilitate gene transfection, has already been shown to induce inflammatory cytokines [8]. This was confirmed in the present project, and shown to be caused by the synthetic fatty acids present in the transfection reagent, since naturally occurring fatty acids did not induce inflammation (Figure 3C).

Discussion

Different cell culture media are known to affect gene expression, proliferation and differentiation of MSCs [3]. In this study hMSCs did not proliferate in culture medium containing human pooled AB plasma or human pooled AB serum without addition of other supplements. This is consistent with our previous observations, but differs from results published by others [9, 10]. This difference may be caused by differences in the donor population, as the two studies with successful cell expansion used self-made AB serum from a small number of donors (10 and 6 donors, respectively), while the current study used a

commercial pool of a large number of donors. There may also be differences in the production procedures, which may impact greatly on the content of cytokines and growth factors in the resulting plasma or serum [11, 12]. When plasma was supplemented with FGF2 the cells proliferated slowly, but stopped dividing after only 2-3 passages, while addition of FGF2 to serum (serum/FGF2) resulted in fast and sustainable proliferation for 50-60 days. In contrast, addition of platelet lysate (PL) enabled expansion when added to both serum (PLS) and plasma (PLP), but the cells did not proliferate as fast as the serum/FGF2 condition.

However, while the MSCs proliferated fastest with serum/FGF2 supplementation, these cells also expressed higher levels of *IL1B* and *IL8* mRNA compared with MSCs cultured in PLP or PLS. This was not found to be due to the presence of heparin in the PLP supplement, because heparin was found to increase, not decrease the levels of *IL1B* mRNA. Instead, FGF2 led to upregulation of *IL1B* and *IL8*, most robustly on a PLP background. This is consistent with previous findings were FGF2 induced expression of *IL1B* in human BM-MSCs[13].

IL1B has previously been shown to inhibit expression of key chondrogenic markers when added to cells in vitro [14]. Thus, addition of FGF2 during expansion should, in theory, inhibit the chondrogenic differentiation capability of the cells. However, expansion in the PLP/FGF2 condition resulted in the highest expression of *ACAN* and *COL2A1* and reduced expression of the two most important cartilage matrix degrading enzymes, *MMP13* and *ADAMTS5,* after chondrogenic differentiation. Thus, of all the different culture media tested in this study, PLP/FGF2 seemed to be the most favorable expansion medium for chondrogenic differentiation. The chondrogenic differentiation medium was serum free, and cells pre-expanded in the different conditions were all differentiated using the same serum free differentiation medium. Thus, the inflammatory stimulus of FGF2 was removed when differentiation was started. This may explain why the PLP/FGF2 was the most favorable expansion medium despite the high expression of *IL1B* during the expansion phase. Since the

cells did not proliferate in serum alone, it was impossible to compare the chondrogenic potential of cells expanded in serum with and without FGF2. Although we did not investigate how FGF2 increased the chondrogenic potential of MSCs in this study, regulation of the IGF-I, TGFβ, MAPK and WNT signaling pathways have been shown by others to be involved [15, 16]. It has also been shown that FGF2 increased SOX9 protein levels and chondrogenic differentiation of MSCs [17].

Passaging using trypsin and changing medium are unavoidable when culturing MSCs. As shown here both trypsination and change of medium resulted in small bursts of inflammation that lasted for up to 24 hours. We have not investigated the mechanisms behind these inflammatory responses, but detachment from plastic adherence may resemble trauma to the cells, a situation which in vivo has been shown to lead to the release of inflammatory cytokines [18, 19]. Inflammation as a result of medium change was shown to be caused by addition of fresh serum and not by fresh FGF2, nor by the mechanical stimulus of the medium moving over the adherent cells. Since serum and plasma contain many growth factors and cytokines, including IL1B, we speculate that this is the main cause of inflammation after medium change.

In conclusion, cytokine release may impact on the effect exerted by hMSC in many experiments in vitro and in different clinical applications. The results presented here will help scientists avoid the confusion created by cytokines released from hMSCs as a result of the medium used or simple manipulations to which the cells may need to be exposed.

Acknowledgement.

Competing financial interest

The authors have no potential financial conflicts of interest

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Figure legends

Figure 1. Proliferation, and expression of cytokine mRNAunder different culture conditions. (A) Bone marrow-derived MSCs (BM-MSCs) were expanded in medium supplemented with human pooled AB serum containing FGF2 (serum/FGF2) or serum with the addition of platelet lysate (PL) (PLS) or plasma with the addition of PL (PLP). Cells were counted at each passage and shown as calculated cumulative cell counts. (B) RT-qPCR was used to measure mRNA levels of IL1B, IL6 and IL8 in BM-MSCs expanded in PLP, PLS and serum/FGF2. (C) Expression of IL1B, IL6 and IL8 mRNA in BM-MSC cultured in PLP containing heparin or serum/FGF2 with or without of heparin. (D) BM-MSCs were cultured in PLP until passage 2. The cell culture was then split in six parallel cultures and expanded for three days in serum/FGF2, PLS/FGF2, PLP/FGF2, serum, PLS or PLP before RT-qPCR analysis. Results are given as fold change mRNA levels relative to the serum/FGF2 condition. BM-MSCs used in (A) and (B) are from donor 1, (C) from donor 2 and (D) from donor 3.

Figure 2. Chondrogenic differentiation and inflammatory response after trypsinization. (A) BM-MSCs expanded in serum/FGF2, PLP and PLP/FGF2 were differentiated for 10 days before measuring mRNA levels of SOX9, ACAN, COL2A1, ADAMTS5 and MMP13. (B). BM-MSCs were cultured in PLP or serum/FGF2 until passage 2. Expression of IL1B, IL6, IL8 and TNF was analyzed 0, 1, 5 and 24 hours after passage 2 trypsinization. Results are given as fold change relative to values obtained for cells cultured in PLP (serum/FGF2 for TNF). (C) BM-MSCs were pre-expanded in serum/FGF2F until passage 2 and then cultured for three days in PLP or serum/FGF2. IL1B, IL6 and TNF protein were investigated 0, 0.5, 1, 3 and 5 hours after trypsinization using western blotting. ACTB (beta-actin was used as loading control). IL1B and IL6 were developed from the same membrane and TNF on a

separate membrane. The bands of each protein were cropped in order to make one image with all proteins. Cells used in (A) are from donor 4, (B) from donor 1 and (C) from donor 3.

Figure 3. Inflammatory response after change of culture medium, exposure to lipofectamine and fatty acids. (A) BM-MSCs were cultured in PLP or serum/FGF2 until passage 2. Expression of IL1B, IL6 and IL8 mRNA was analyzed 0, 1, 5 and 24 hours after medium change. Results are given as fold change relative to results obtained from cells grown in PLP. (B) Importance of FGF2 versus serum as inflammatory stimulators. BM-MSCs were expanded in serum/FGF2 until passage 2. Three days later medium was either not changed (UNSTIM), fresh medium without FGF2 (serum) or with FGF2 (serum/FGF2), the removed supernatant without (SUP) or with addition of fresh FGF2 (SUP/FGF2) was added to the cells. One hour later IL1B, IL6 and IL8 mRNA was measured using RT-qPCR. Results are given as fold change relative to UNSTIM. In all experiments, both the fresh medium and the add-back supernatant had been heated to 37ºC, to avoid the potential effect of temperature. (C) BM-MSCs were cultured in serum/FGF2 until passage 2. Cells were then exposed to Lipofectamine, bovine serum albumin (BSA, as control) or one of nine different fatty acids. The next day RT-qPCR was performed to measure IL1B, IL6 and IL8 mRNA levels. EPA – eicosapentaenoic acid, DPA - docosapentaenoic acid , DHA – docosahexaenoic acid. ND – not detected. BM-MSCs used in (A) are from donor 1, (B) and (C) from donor 2.

Figures

Figure 1

Figure 2

3
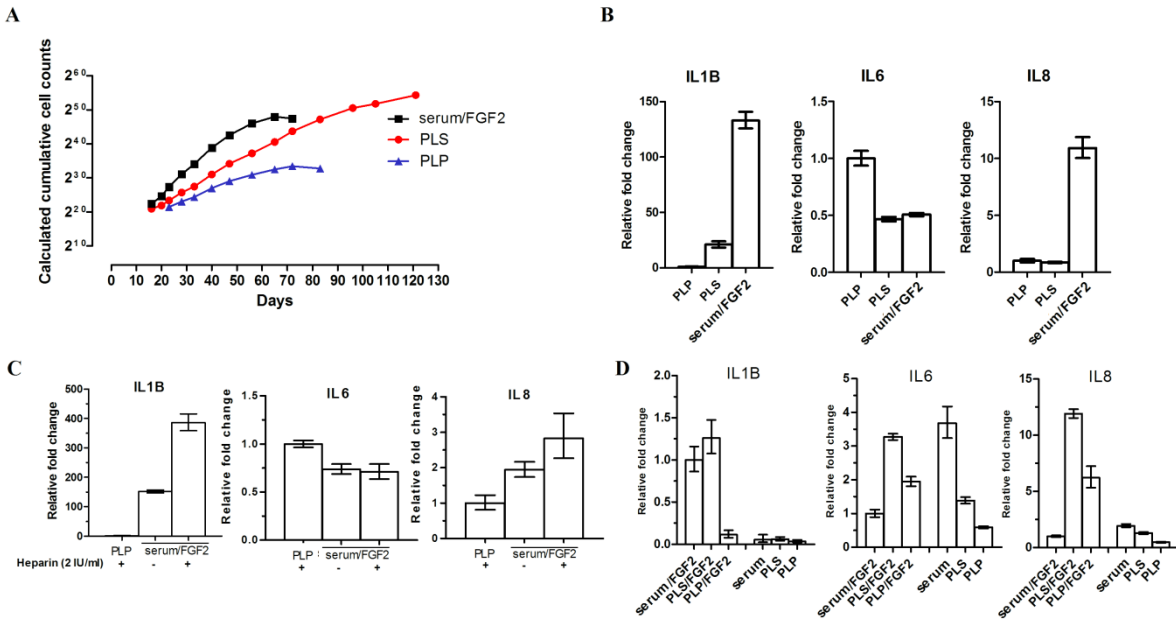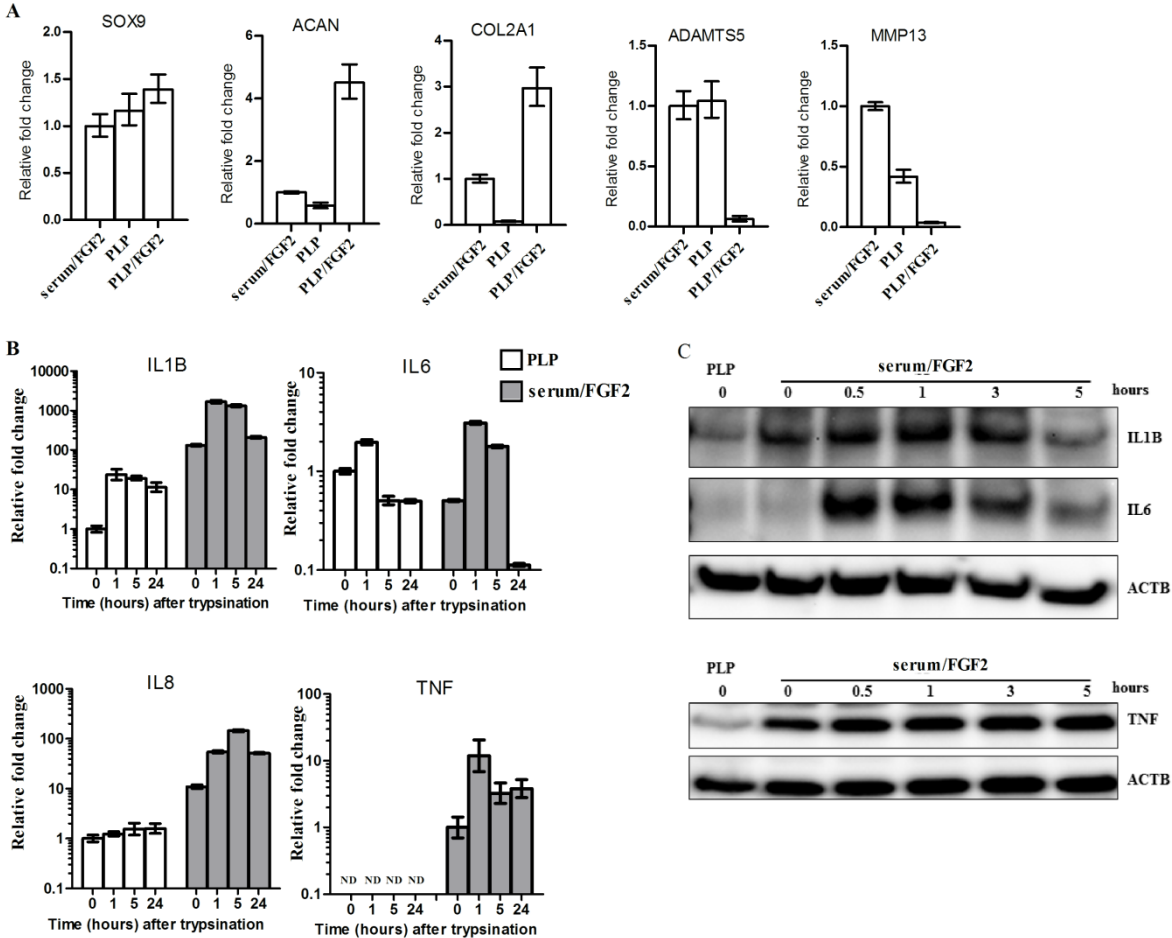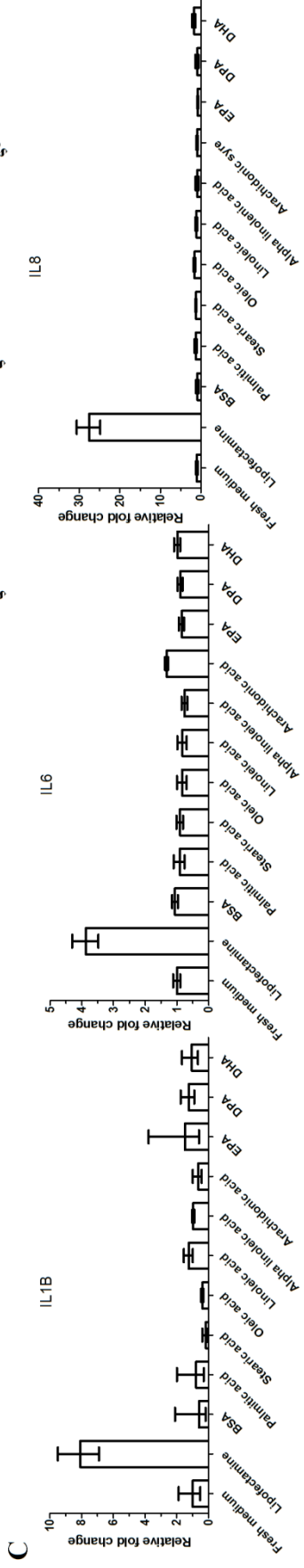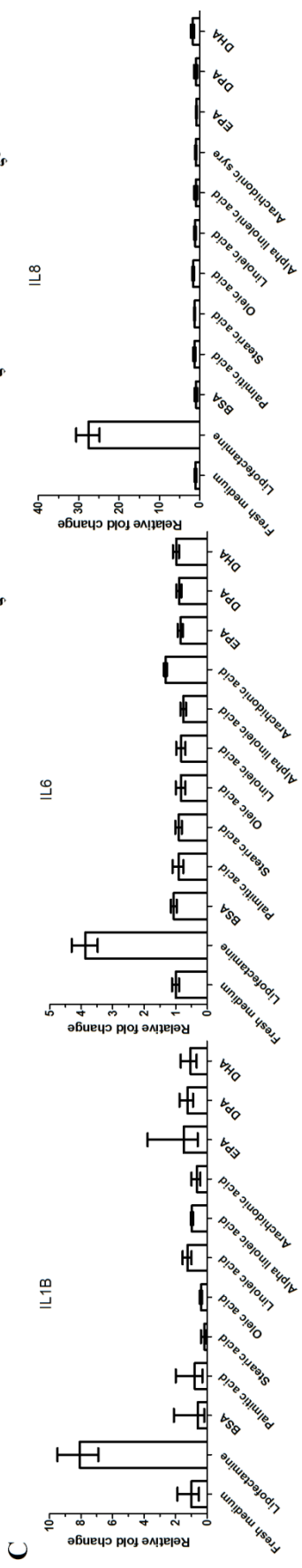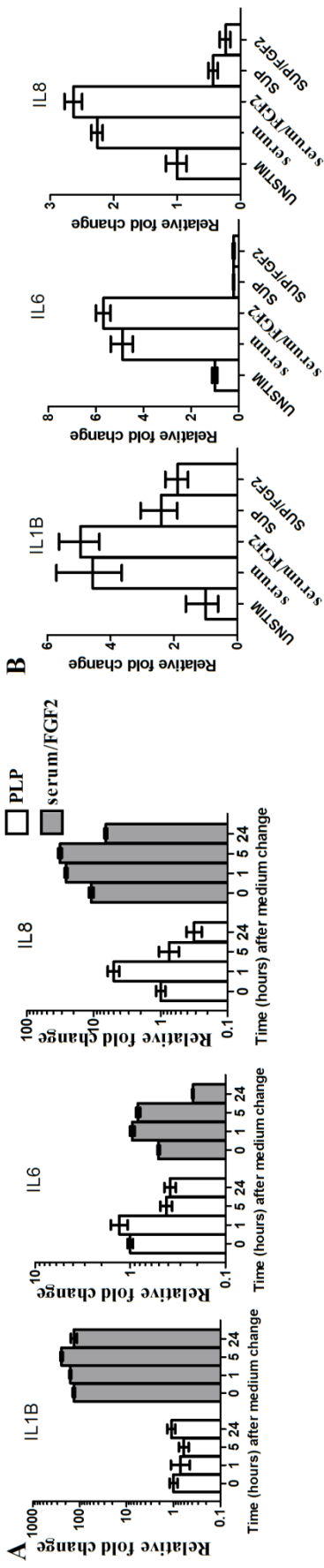
Table 1

<u>**Taqman Assays**</u>

Official symbol	Official full name	Company	Cat no.
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	Thermo Fisher Scientific	Hs99999905_m1
IL1B	Interleukin 1 beta	Thermo Fisher Scientific	Hs01555410_m1
IL6	Interleukin 6	Thermo Fisher Scientific	Hs00985639_m1
IL8	Interleukin 8	Thermo Fisher Scientific	Hs00174103_m1
TNF	Tumor necrosis factor alpha	Thermo Fisher Scientific	Hs01113624_g1
SOX9	SRY-box 9	Thermo Fisher Scientific	Hs00165814_m1
COL2A1	Type II collagen	Thermo Fisher Scientific	Hs00264051_m1
ACAN	Aggrecan	Thermo Fisher Scientific	Hs00202971_m1
ADAMTS5	ADAM metallopeptidase with thrombospondin type 1 motif 5	Thermo Fisher Scientific	Hs00199841_m1
MMP13	Matrix metallopeptidase 13	Thermo Fisher Scientific	Hs00233992_m1

<u>**Fatty acids and BSA**</u>

Name		Company	Cat no.
Palmitic acid		Nu-Chek Prep	S-1109
Stearic acid		Nu-Chek Prep	S-1111
Oleic acid		Nu-Chek Prep	S-1120
Linoleic acid		Nu-Chek Prep	S-1127
Alpha linoleic acid		Nu-Chek Prep	S-1129

Arachidonic acid		Nu-Chek Prep	S-1133
EPA		Nu-Chek Prep	S-1144
DPA		Nu-Chek Prep	S-1145
DHA		Nu-Chek Prep	U-84-A
BSA (low fatty acid)		Sigma	A8806

Antibodies (western blot)

Primary antibodies	Dilution/concentration	Company	Cat.no
Mouse anti-ACTB	1:2000	Abcam	ab8226
Rabbit anti-IL6	1:1000	Abcam	ab32530
Rabbit anti-TNF	1:1000	Abcam	ab183896
Mouse anti-IL1B	1:500	Millipore	MABF18
Secondary antibodies	**Dilution/concentration**	**Company**	**Cat no.**
Goat anti-Rabbit (HRP)	1:10000	Vector labs	PI-1000
Horse anti-Mouse (HRP)	1:2000	Vector labs	PI-2000

Cell culture reagents

Trypsin-EDTA solution		Sigma	T4049
Penicillin and streptomycin		Sigma	P4333
Amphotericin B		Sigma	A2942
Tryple		Thermo Fisher Scientific	A12177-01
DMEM F12 + glutamax		Thermo Fisher Scientific	31331-028

Supplementary Figures

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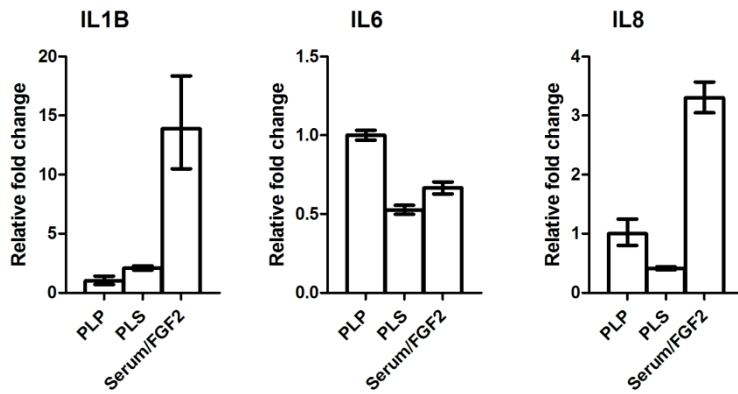
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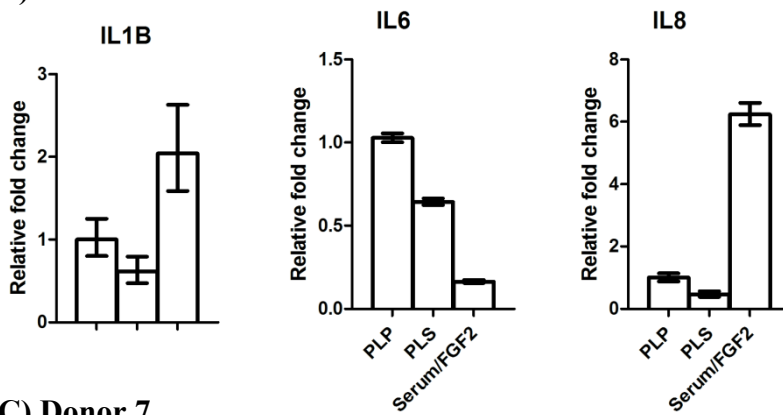
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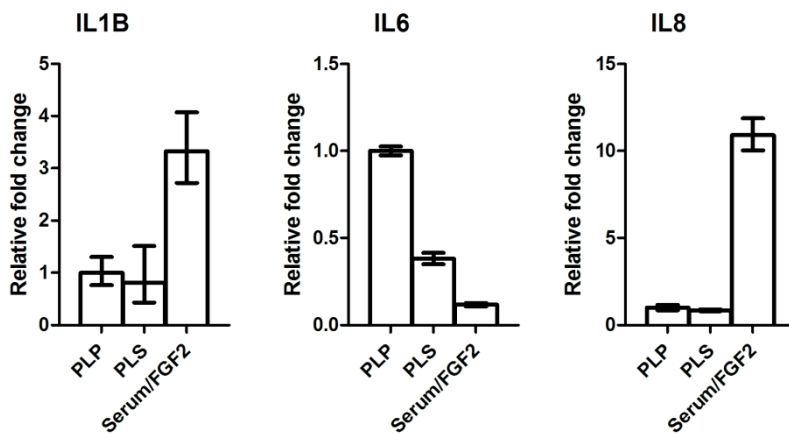
Supplementary Figure 1. Expression of cytokine mRNA under different culture conditions. RT-qPCR was used to measure mRNA levels of IL1B, IL6 and IL8 in BM-MSCs that were pre-expanded in PLP for one passage and continued in PLP or changed to PLS and serum/FGF2 for two additional passages. Results in (A), (B) and (C) are from three different donors.
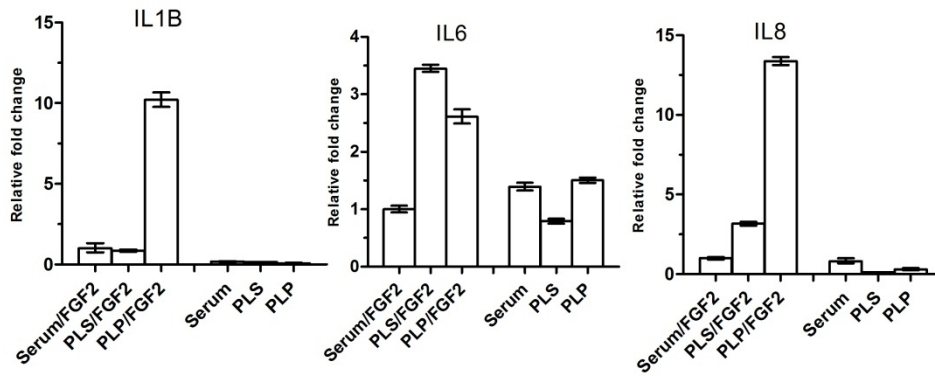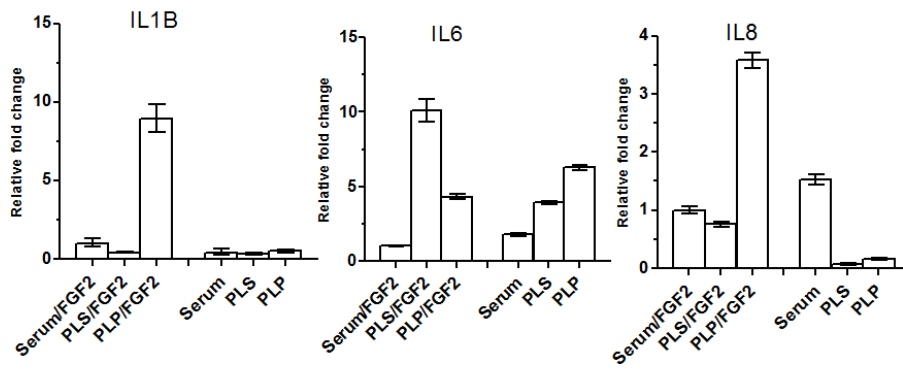
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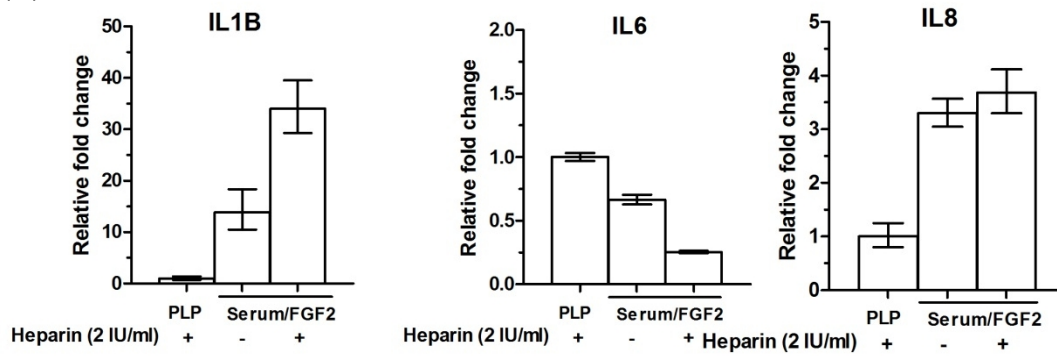
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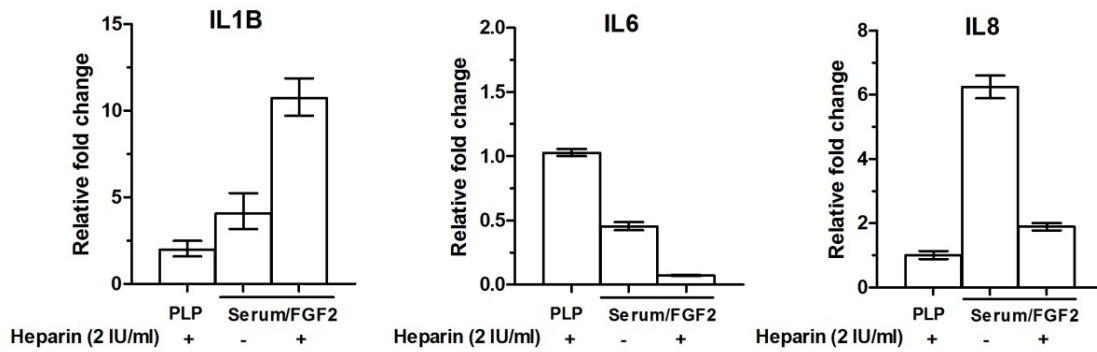
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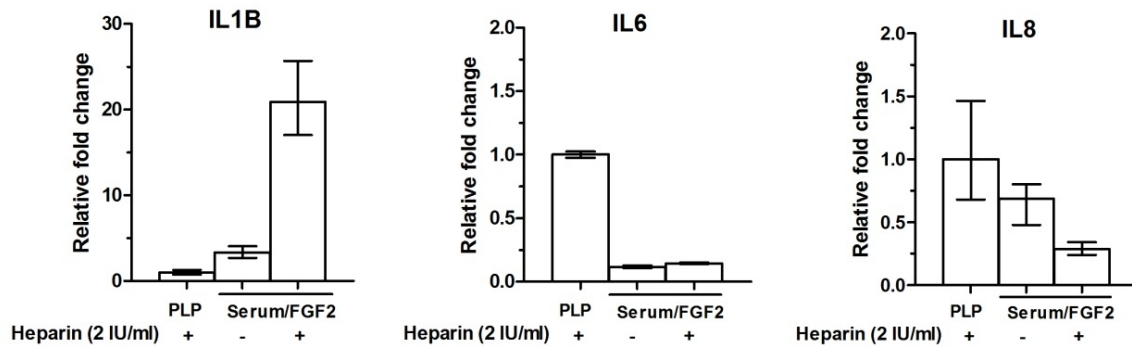
Supplementary Figure 2. Effect of heparin. Relative expression of IL1B, IL6 and IL8 mRNA in BM-MSCs cultured in PLP containing heparin or serum/FGF2 with or without heparin. Results in (A), (B) and (C) are from three different donors.

(A) Donor 5

(B) Donor 6

(C) Donor 7
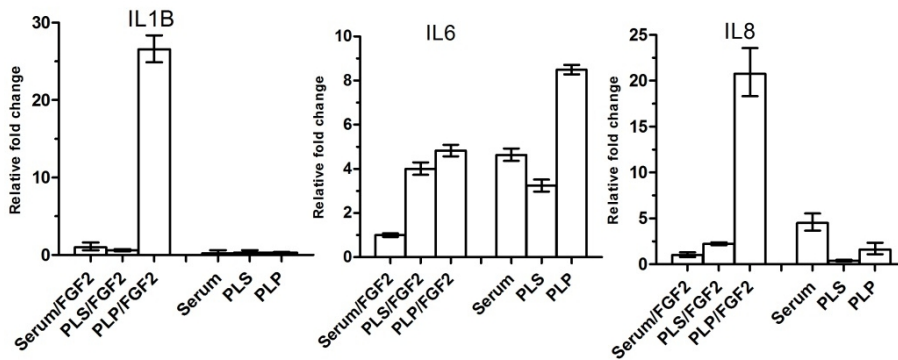
Supplementary Figure 3. Effect of FGF2. BM-MSCs were cultured in PLP until passage 2. The cell culture was then split in six parallel cultures and expanded for three days in serum/FGF2, PLS/FGF2, PLP/FGF2, serum, PLS or PLP before RT-qPCR analysis. Results are given as fold change mRNA levels relative to the serum/FGF2 condition. (A), (B) and (C) represent results from three different donors.
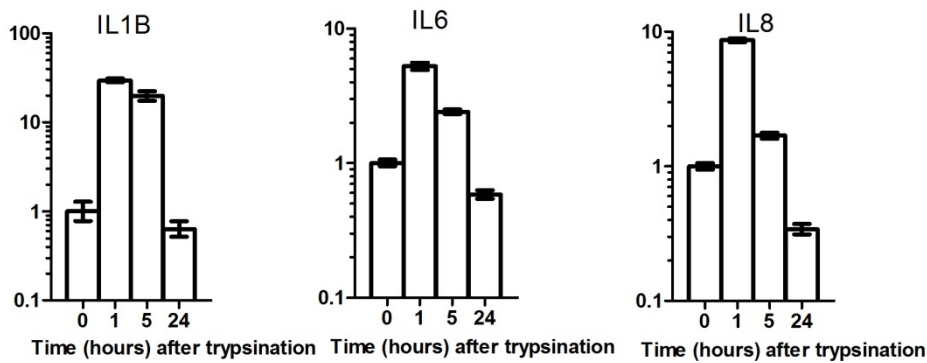
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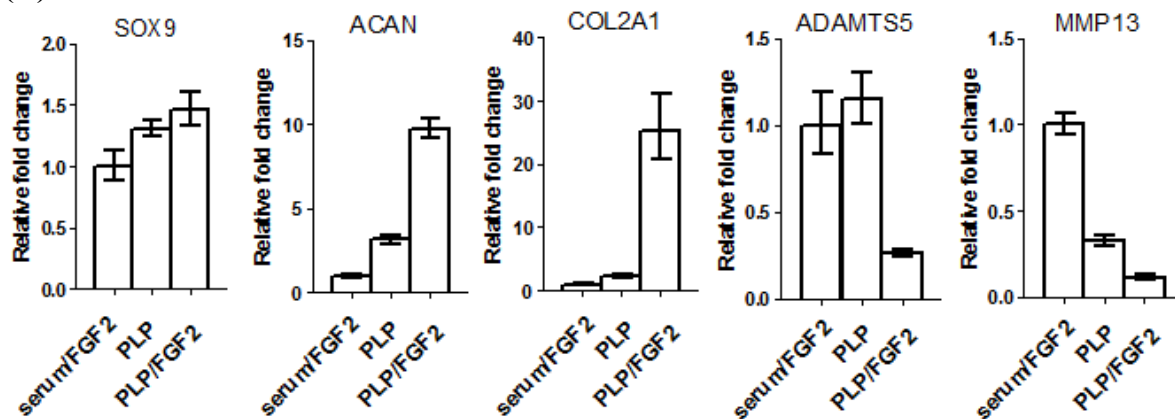
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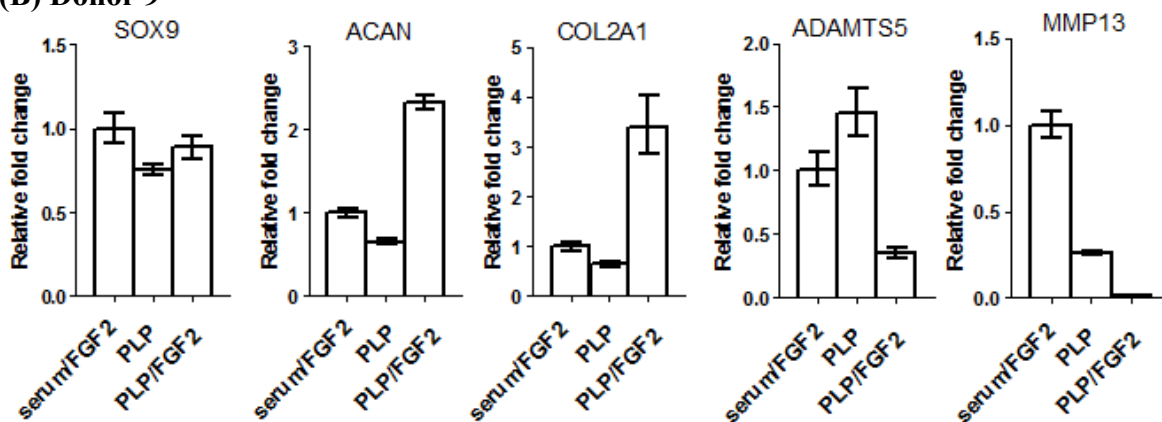
Supplementary Figure 4. Chondrogenic differentiation. BM-MSCs expanded in serum/FGF2, PLP and PLP/FGF2 were differentiated for 10 days before measuring mRNA levels of SOX9, ACAN, COL2A1, ADAMTS5 and MMP13 using RT-qPCR. Results in (A) and (B) are from two different donors.
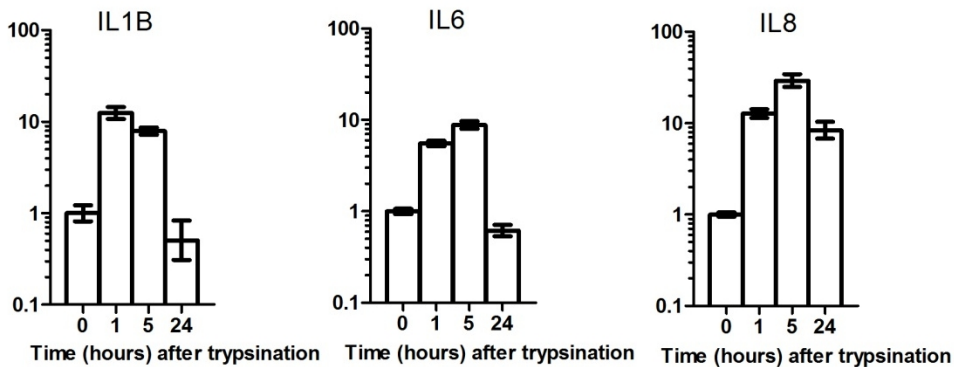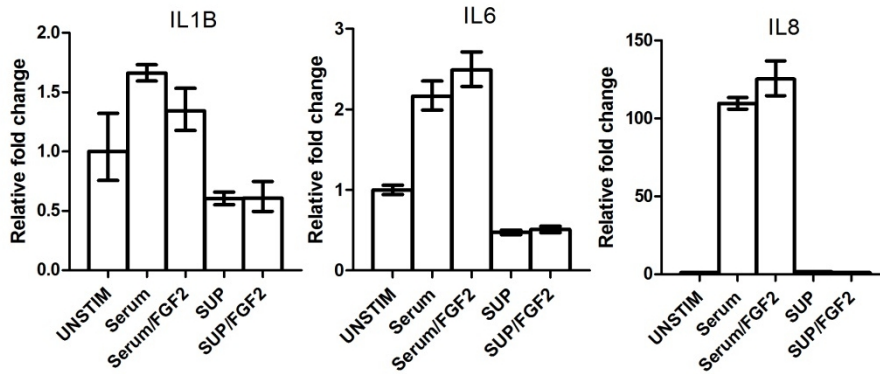
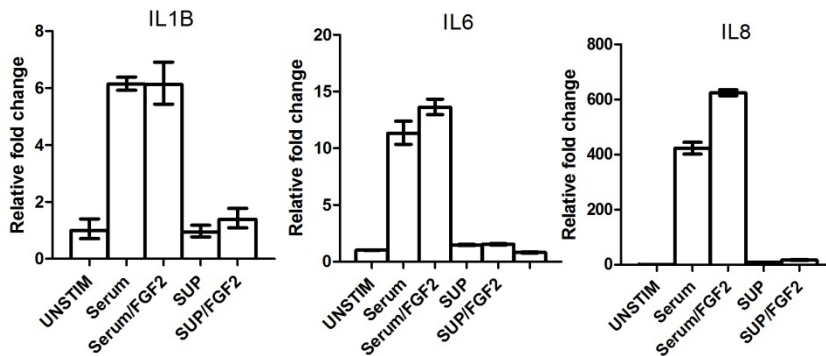**(A) Donor 5**
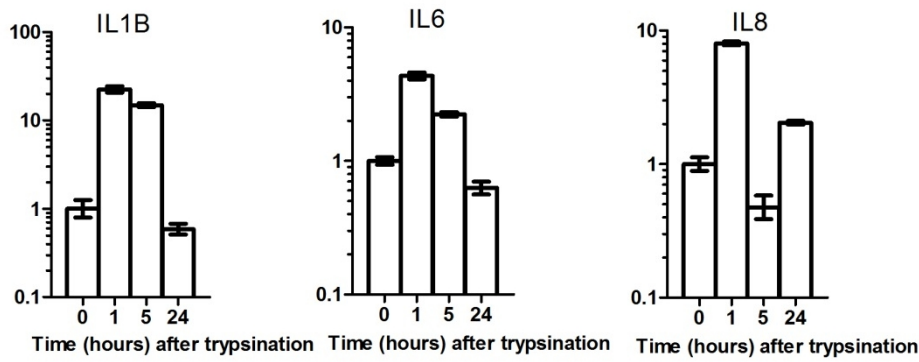
(B) Donor 6

(C) Donor 7

Supplementary Figure 5. Trypsination. BM-MSCs were cultured serum/FGF2 until

passage 2. Expression of IL1B, IL6 and IL8 was analyzed 0, 1, 5 and 24 hours after passage 2

trypsinization. Results are given as fold change relative to time 0. Results in (A), (B) and (C)
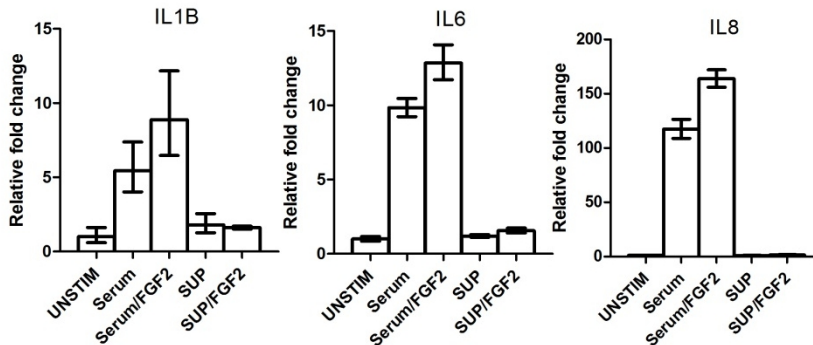
are from three different donors.

(A) Donor 5

(B) Donor 6

(C) Donor 7

Supplementary Figure 6. Importance of FGF2 versus serum as inflammatory stimulators. BM-MSCs were expanded in serum/FGF2 until passage 2. Three days later medium was either not changed (UNSTIM), fresh medium without FGF2 (serum) or with FGF2 (serum/FGF2), the removed supernatant without (SUP) or with addition of fresh FGF2 (SUP/FGF2) was added to the cells. One hour later IL1B, IL6 and IL8 mRNA was measured using RT-qPCR. Results are given as fold change relative to UNSTIM. In all experiments, both the fresh medium and the add-back supernatant had been heated to 37°C, to avoid the potential effect of temperature. Results in (A), (B) and (C) are from three different donors.