Biomaterials Influence Macrophage-Mesenchymal Stem Cell Interaction In Vitro.

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Abstract

Background

Macrophages and mesenchymal stem cells (MSCs) are important cells in wound healing. We hypothesized that the cross-talk between macrophages and adipose tissue-derived MSCs (ASCs) is biomaterial dependent, thereby influencing processes involved in wound healing.

Materials and Methods

The effect of macrophages cultured on polypropylene (PP) or polyethylene terephthalate coated with a collagen film (PET/Col) on ASCs in monolayer or on the same material was examined either through conditioned medium (CM) or in a direct coculture. ASC proliferation, collagen production, and gene expression were examined. As comparison, the effect of macrophages stimulated with lipopolysaccharide (LPS) and interferon gamma (IFNγ) [M(LPS/IFNγ)] or interleukin (IL) 4 [M(IL-4)] on ASCs was examined.

Results

Macrophage-CM increased collagen deposition, proliferation, and gene expression of MMP1, PLOD2, and PTGS2 in ASCs, irrespective of the material. Culturing ASCs and macrophages in coculture when only macrophages were on the materials induced the same effects on gene expression. When both ASCs and macrophages were cultured on biomaterials, PP induced COL1A1 and MMP1 more than PET/Col. M(LPS/IFNγ) CM increased PLOD2, MMP1, and PTGS2 and decreased TGFB in ASCs more than the M(IL-4) CM.

Conclusion

Biomaterials influence wound healing by influencing the interaction between macrophages and ASCs. We provided more insight into the behaviour of different cell types during wound healing. This behaviour appears to be biomaterial specific depending on which cell type interacts with the biomaterial. As such, the biomaterial will influence tissue regeneration.

Introduction

Biomaterials are often used in regenerative medicine. After implantation of a biomaterial, the body reacts with inflammation followed by a wound-healing reaction. The extent of this reaction depends on the type of biomaterial. Different cells are involved in this reaction and macrophages are believed to be key players in orchestration of this reaction (1).

Another cell type important in the foreign body reaction is the mesenchymal stem cell (MSC). These cells are recently discovered as candidates for the production of extracellular matrix in wound healing (1–3). Adipose tissue-derived MSCs (ASCs) also have this capacity and are likely to rapidly migrate to the wound site accelerating wound healing by enhancing angiogenesis, stimulating extracellular matrix remodelling and synthesis (4,5) as well as differentiating into different cell types to replace the damaged tissue (1–3). In addition, ASCs can be immunomodulatory and therefore are expected to have a great influence on the foreign body reaction (6). ASCs stimulate macrophages to produce interleukin (IL)-10 and express CD206 on their surface leading to an anti-inflammatory subtype (M2) (7,8).

Macrophages are likely candidates for attraction of stem cells. Macrophage products such as monocyte chemotactive protein-1 (MCP-1), macrophage inflammatory protein-1α, and IL-8 enhance the migration of stem cells (9,10). It is well known that biomaterials can influence the phenotype of macrophages (11–13) We previously found that macrophages differentiated toward a pro-inflammatory phenotype when cultured on polyethylene terephthalate coated with collagen film (PET/Col), whereas when cultured on polypropylene (PP), they differentiated toward an anti-

inflammatory phenotype. These two biomaterials had the most distinguishing reaction in our culture model *in vitro*; therefore, we choose these two for the following research (12).

In vivo, PP is a material used for many decades in reconstructive surgery. PP is known to induce fibrosis that leads to shrinkage of the mesh and encapsulation of nerves, leading to pain (14) PET/Col is a more recently developed material very often used for hernia repair and has a low complication rate with less adhesions and good tissue integration (15,16). The influence of biomaterials on the interaction between macrophages and ASCs and the contribution of these cells to the wound-healing process in response to biomaterials are largely unknown. We hypothesized that the cross-talk between macrophages and ASCs is biomaterial dependent and thereby influences processes involved in wound healing.

Since we found opposite reactions of macrophages to PP and PET/Col (12), we used these two biomaterials as model materials to evaluate the effect of macrophages in contact with the already mentioned biomaterials on the wound-healing responses of human ASCs as a model for *in vivo* wound healing in which macrophages and ASCs play a role. This was evaluated in experiments with conditioned medium (CM) of macrophages cultured on PP and PET/Col on ASCs and with a direct coculture of macrophages and ASCs in the presence of the same materials. We analysed proliferation and collagen production of ASCs.

Expression of genes important in wound-healing processes was also examined, namely collagen type 1 (COL1A1) as marker for the production of collagen, matrix-metalloprotease 1 (MMP1) as remodelling marker, procollagen-lysine, 2-oxoglutarate 5-dioxygenase (PLOD2, a gene encoding for an enzyme involved in collagen cross-linking),17 α-smooth muscle actin (ASMA)18 and transforming growth factor (TGFB1) as genes associated with fibrosis (12,19,20), and prostaglandin-endoperoxide synthase 2 (PTGS2) as an immunomodulatory marker based on the literature (21).

Materials and Methods

ASC isolation

Subcutaneous abdominal adipose tissue was harvested as left-over material from breast reconstruction of six different female patients, aged 46–69 years, with approval of the local medical ethics committee (MEC-2011-371). The tissue was incubated overnight with collagenase type I (Gibco, Carlsbad, CA), bovine serum albumin (BSA; Sigma-Aldrich, St. Louis, MO), and low glucose (LG) Dulbecco's modified Eagle's medium (DMEM) (Gibco) with 0.6% fungizone and 0.1% gentamycin (both Gibco) at 4°C followed by incubation at 37°C for 1 h on a shaker.

The solution was then centrifuged and washed in LG DMEM. After filtration through a 100 µm filter (BD Pharmingen, Franklin Lakes, NJ), cells were seeded at a density of 40,000 cells/cm2 and cultured in LG DMEM with 10% fetal calf serum (FCS; Lonza, Verviers, Belgium), ascorbic acid (10−4 M; Sigma-Aldrich), and 1 ng/mL fibroblast growth factor 2 (FGF2; AbD Serotec, Oxford, United Kingdom). This medium was changed every 3 to 4 days and cells were grown until an 80% confluence. Undifferentiated ASCs at passage 3 or 4 were used for experiments. In parallel, their multilineage differentiation capacity (i.e., osteogenic and adipogenic differentiation) was confirmed (data not shown).

Monocyte isolation

Monocytes were isolated with Ficoll density gradient (Ficoll-Paque™ PLUS; GE Healthcare) from buffy coats of male donors, age 21–63 years, obtained from the blood bank (Sanquin, The Netherlands). The buffy coat was diluted (1:5 ratio with phosphate-buffered saline [PBS]/BSA 0.1%) and 30 mL was layered on 15 mL of Ficoll and centrifugated for 15 min at 1000 g without brake. The interphase band, containing the peripheral blood mononuclear cells, was collected. The cells were washed in PBS/BSA 0.5% of 2 mM EDTA and labeled with anti-CD14+ magnetic beads (CD14 microbeads human, MACS separation columns LS and MidiMACS™ separator; all Miltenyi Biotec). The monocytes were then isolated according to the manufacturer's guidelines as done previously (12). This positive selection of monocytes will not activate the cells (22). After monocytes were isolated and attached to the biomaterial or culture well, they were referred to as macrophages.

Culture of cells on biomaterials

To evaluate the effect of biomaterials on macrophages or ASCs, monocytes were seeded on two different materials immediately after isolation from the buffy coat or ASCs after expansion in monolayer. The following materials with a mesh architecture were chosen because they initiate a different reaction *in vitro* (12,23): pure PP multifilament, and multifilament polyethylene terephthalate with an absorbable, continuous, and hydrophilic collagen film on one of its sides (PET/Col). Both materials were from Sofradim Production, A Medtronic Company.

The materials were cut into pieces of 1.5 cm by 1.5 cm with a sterile scalpel. Before cell seeding to provide protein attachment, materials were incubated in 100% nonheat-inactivated fetal bovine serum (Lonza, Verviers, Belgium) for 2 h. Monocytes or ASCs were adjusted to a concentration of 700,000/mL in a total volume of 25 mL in a 50 mL tube (Falcon, PP conical tube; Becton Dickinson, Franklin Lakes, NJ). Twelve samples were incubated per 25 mL for 2 h at 37°C.

Macrophage-conditioned medium on ASCs

Macrophages were cultured in monolayer with a seeding density of 500,000 cells/cm2 and stimulated to obtain a pro-inflammatory subtype by lipopolysaccharide (LPS) (100 ng/mL; Sigma-Aldrich, St. Louis, MO) and interferon gamma (IFNγ) (10 ng/mL; PeproTech, Rocky Hill, NJ),10 from now on referred to as M(LPS/IFNγ) (24) or to obtain an anti-inflammatory subtype by IL-4 (10 ng/mL; PeproTech) (10), from now on referred to as M(IL-4) (24) in X-vivo15 medium (Lonza, Verviers, Belgium) with 20% FCS (Lonza). Previously, it was seen that these different stimuli indeed lead to different phenotypes as based on gene expression and protein production (12,25).

Macrophages were also seeded on the biomaterials by rotational seeding for 2 h in a concentration of 700,000 cells/mL. After seeding, the biomaterials were transferred to a nonadherent 24-well plate (NUNC, nontreated multiplate, Rochester, NY) with X-vivo15 medium with 20% FCS. To generate CM, the medium was replaced after 2 days with LG DMEM (Gibco, Carlsbad,

CA) with 10% FCS, the medium more suitable for ASC culture. After 24 h, this CM was harvested, spun down, and supernatant was stored at −80°C until further use.

ASCs from three donors were seeded at a seeding density of 50,000 cells/cm2 in six-well plates in triplicate per condition in LG DMEM with 10% FCS and ascorbic acid (25 µg/mL). The medium containing 10% pooled macrophage-conditioned medium (MCM) was added 24 h after seeding. To account for the number of cells by which the MCM was produced, the average DNA contents of all macrophage phenotypes or macrophages cultured on biomaterials was defined as 10%. The percentage CM used in culture was adjusted for the DNA content per macrophage phenotype as described previously (25).

The control condition also received 10% medium that was treated in the same way as the CM, but without being in contact with cells. The end concentration of FCS in this condition was also 10%. The medium was refreshed at day 3 and day 6, and at day 7 the ASC monolayers were harvested in 500 µL PBS by scraping and stored at −20°C for later measurement of DNA and collagen.

Hydroxyproline assay

To determine the amount of collagen, samples of ASC monolayers without medium in PBS were digested with papain (250 µg/mL; Sigma) overnight at 56°C. Half of this papain-digested sample was hydrolyzed overnight with hydrochloric acid (final concentration HCl, 6 N). The next day, HCl was removed from the samples by use of a centrifugal evaporator. The dried samples were dissolved in 150 µL Milli-Q water and subsequently a hydroxyproline assay was performed. The samples were incubated for a period of 20 min at room temperature in a solution of assay buffer (0.24 M C6H8O7, 0.88 M NaAc ·3H2O, 0.85 M NaOH) with chloramine-T (0.07 g/reaction; Merck, Darmstadt, Germany).

This was followed by an incubation of 25 min at 60°C with a solution of PBS and 7,12-dimethylbenz(a)anthracene (DMBA) (Fluka; Sigma-Aldrich). Hydroxyproline (Merck) was used as a standard curve. The extinction was measured at 570 nm with a spectrophotometer.

DNA

The other half of the papain-digested sample consisting of ASC monolayers was used to determine the amount of DNA. The samples were treated with heparine (8.3 IU/mL; Leo pharmaceutical) and RNAse (0.05 mg/mL; Sigma). After 30 min of incubation at 37°C, ethidium bromide (25 µg/mL; Gibco) was added. Calf thymus DNA (Sigma) was used as a standard curve up to 25 µg/mL. The samples were analyzed by a spectrophotometer at excitation 340 nm and emission 590 nm.

The monolayer of macrophages and the macrophages on biomaterials were harvested in 0.1%Triton/PBS (Sigma-Aldrich) and analyzed with CyQUANT© cell proliferation assay kit (Invitrogen, Carlsbad, CA) to measure the amount of DNA according to the manufacturer's recommendation.

Transwell setup, reciprocal paracrine signalling

To investigate the influence of macrophages on ASCs in reciprocal paracrine signalling, a transwell system was used (Greiner bio-one; ThinCerts). ASCs were placed in the lower compartment in monolayer or as seeded on a biomaterial. Macrophages were placed in the upper compartment on a biomaterial. We choose to culture the macrophages always on a biomaterial since these cells *in vivo* are one of the first cells to react to the biomaterial. The experiment was performed with three different ASC donors and three different macrophage donors in triplicate for each donor. The cells were cultured for 3 days in 50:50 LG DMEM: X-vivo medium with a final concentration of 10% FCS. At day 3, both compartments were harvested in 175 µL RLT (Qiagen) lysis buffer with 1% β-mercaptoethanol (Sigma-Aldrich) for gene expression.

Gene expression (mRNA isolation, cDNA, qPCR)

mRNA was isolated from the RLT buffer containing cell lysate using Qiagen RNeasy microkit (Qiagen) according to manufacturer's protocol. The synthesis of cDNA was performed with the RevertAid First Strand cDNA Synthesis Kit (MBI Fermentas, Germany). Primers in case of Sybr green assays and primers with probe sequences in case of Taqman assays to analyze gene expression are shown in

Table 1 (all Eurogentec, Seraing, Belgium). For analysis of phenotype of macrophages, we used IL-6 as genes encoding pro-inflammatory proteins and IL-10, CCL18, and CD206 as genes encoding anti-inflammatory proteins since we have shown earlier that these genes discriminate between phenotypes (26). Either Taqman Universal PCR mastermix (Applied Biosystems) or SybrGreen (Eurogentec) was used in the quantitative polymerase chain reaction. Relative gene expression was calculated using the 2−ΔCT method.

Gene	Fw	Rev	probe
Reverence gene: Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)	GTCAACGGATTTGGTCGTATTGGG	TGCCATGGGTGGAATCATATTGG	FAM-CGCCCAATACGACCAAATCCGTTGAC-TAMRA
procollagen-Lysine, 2-Oxoglutarate 5-Dioxygenase (PLOD2)	CCCTCCGATCAGAGATGATT	AATGTTTCCGGAGTAGGGGAGTCTTTTT	FAM-CGTGCGCGTGATAAACTGGATCCTGATATGGCTTCTTCGCACG-Dabcyl
α-smooth muscle actin *(ASMA)*	CGTTGCCCCTGAAGAGCAT	CCGCCTGGATAGCCACATACA	
Collagen type 1 (*COL1A1*)	CAGCCGCTTCACCTACAGC	TTTTGTATTCAATCACTGTCTTGCC	
Prostaglandin-Endoperoxide Synthase 2 (*PTGS2*)	AATGGGGTGATGAGCAGTTGTTC	GGATGCCAGTGATAGAGGGTGTTA	
matrix metalloprotease (*MMP1)*	CTCAATTTCACTTCTGTTTTCTG	CATCTCTGTCGGCAAATTCGT	FAM-CGTGCCAAAGCCTTTCAACTCTGGAGCAATGTCACGGCACG-Dabcyl
transforming growth factor 1 (TGFB1)	GTGACAGCAGGGATAACATACTG	CATGAATGGTGGCCAGGTC	
Interleukin-6 (IL-6)	TCGAGCCCACCGGGAACGAA	GCAGGGAAGGCAGCAGGCAA	
Interleukin-10 (IL-10)	CCTGGAGGAGGTGATGCCCCA	GACAGCGCCGTAGCCTCAGC	
Chemokine Ligand 18 (CCL18)	GCACCATGGCCCTCTGCTCC	GGGCACTGGGGGCTGGTTTC	
Mannose receptor (CD206)	TGGCCGTATGCCGGTCACTGTTA	ACTTGTGAGGTCACCGCCTTCCT	

Table 1. Genes Used for Gene Expression. List of primers in case of Sybr green assays and primers with probe sequences in case of Taqman assays to analyse gene expression are shown in this table (all Eurogentec, Seraing, Belgium).

Data analysis

Data are presented as scatter dot plots with each dot representing an individual sample. All experiments were performed with three different ASC donors, in triplicate. The mean of these donors is indicated by a line in the graphs. All samples were normalized to the unstimulated monolayer of ASCs. We compared the groups in SPSS (IBM Corp. IBM SPSS for Windows, Version 21.0. Armonk, NY: IBM Corp.). The data were not normally distributed; therefore, the groups were compared by a Kruskal–Wallis test (independent samples median test) and a Mann–Whitney test. Bonferroni was used to correct for multiple testing, $p < 0.05$ was considered statistically significant.

Results

To first evaluate in a one-way direction how factors secreted by macrophages on biomaterials influence regeneration by ASCs, we measured the amount of collagen and DNA in the ASC monolayer after stimulation with MCM (Fig. 1A). MCM stimulated the collagen deposition and the amount of DNA of ASCs (Fig. 1B, C). No differences were found between ASCs exposed to medium from macrophages cultured on PP or on PET/Col, even though macrophages were differently influenced by the biomaterials in accordance with our earlier results where PET/Col stimulated macrophages to a predominant pro-inflammatory reaction and PP stimulated macrophages to a predominant anti-inflammatory reaction (data not shown, (12).

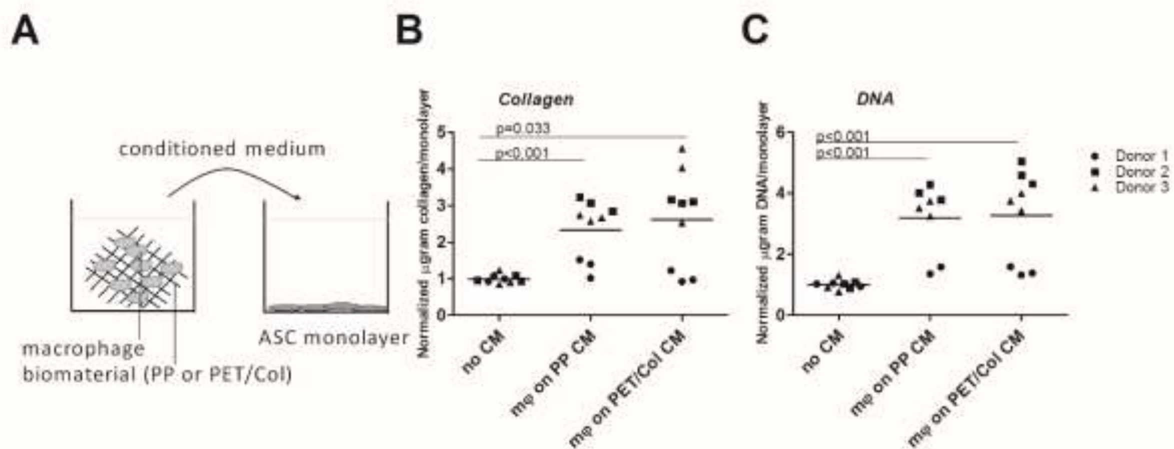


Fig. 1 Collagen production and proliferation of ASCs. (A) Schematic representation of the culture setup in which ASCs in monolayer were stimulated with MCM for 7 days. (B) Amount of collagen in

microgram per monolayer of ASCs stimulated with or without MCM. (C) Microgram of DNA per monolayer of ASCs stimulated with or without MCM. Values were normalized to their own control condition without pooled MCM within each separate experiment. Experiments were performed in triplicate for three ASC donors. ASC, adipose tissue-derived mesenchymal stem cells; MCM, macrophage-conditioned medium; PET/Col, Parietex™ composite; PP, polypropylene.

In addition to proliferation and collagen deposition, we analysed the expression of genes involved in collagen modification and immune modulation in the ASC monolayers. Macrophage-secreted factors stimulated remodelling of the extracellular matrix by increasing the gene expression of MMP1 in a monolayer of ASCs. PTGS2 and PLOD2, encoding for procollagen-lysine, 2-oxoglutarate 5-dioxygenase, an enzyme involved in collagen cross-linking, were also increased when ASCs in the monolayer were stimulated by medium conditioned by macrophages on biomaterials.

Again, no differences were seen between the conditioned media made from macrophages cultured on the two different materials. ASMA, transforming growth factor (TGFB1), and collagen type 1 (COL1A1) gene expression were unaffected by the MCM (Fig. 2).

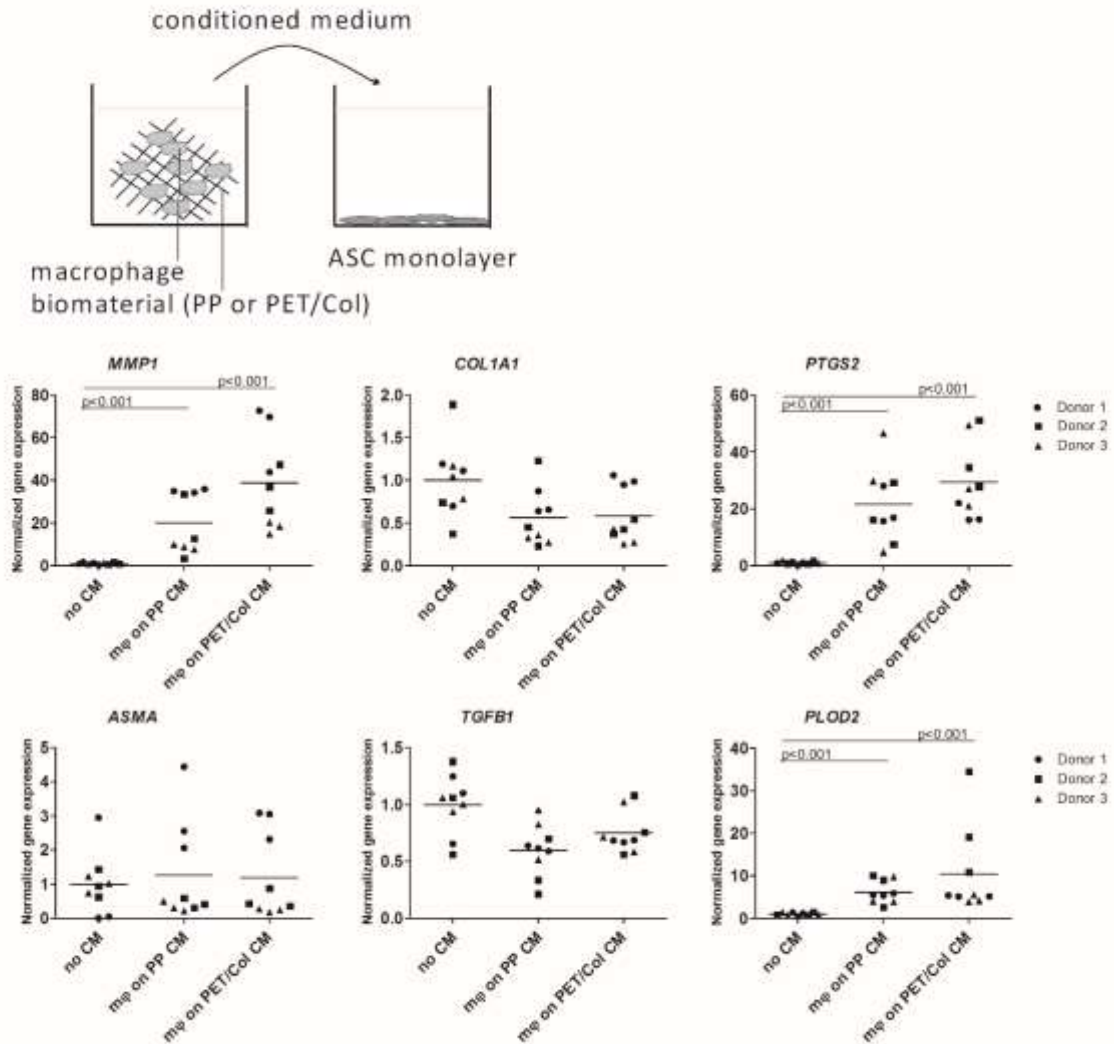


Fig. 2. Gene expression by ASCs stimulated with CM. Gene expression of ASCs cultured in monolayer with or without MCM of macrophages cultured on biomaterials (PP and PET/Col). The gene expression was normalized to the average of the control condition without MCM within each separate ASC experiment. Experiments were performed in triplicate for three ASC donors.

To investigate the direct interaction between ASCs and macrophages in the presence of a biomaterial, we cocultured macrophages on a biomaterial with ASCs in monolayer in a transwell system (Fig. 3). The effects were similar to the effects of medium conditioned by macrophages on biomaterials. In addition to a similar effect found for MMP1, PTGS2, and PLOD2, we found a decrease in gene expression for COL1A1 and TGFB1 when ASCs were cocultured with macrophages regardless of the biomaterial on which macrophages were cultured. The ASMA gene expression of ASCs in

monolayer was also decreased when cocultured with macrophages on biomaterials, although only statistically significantly lower when macrophages were on PET/Col (Fig. 3).

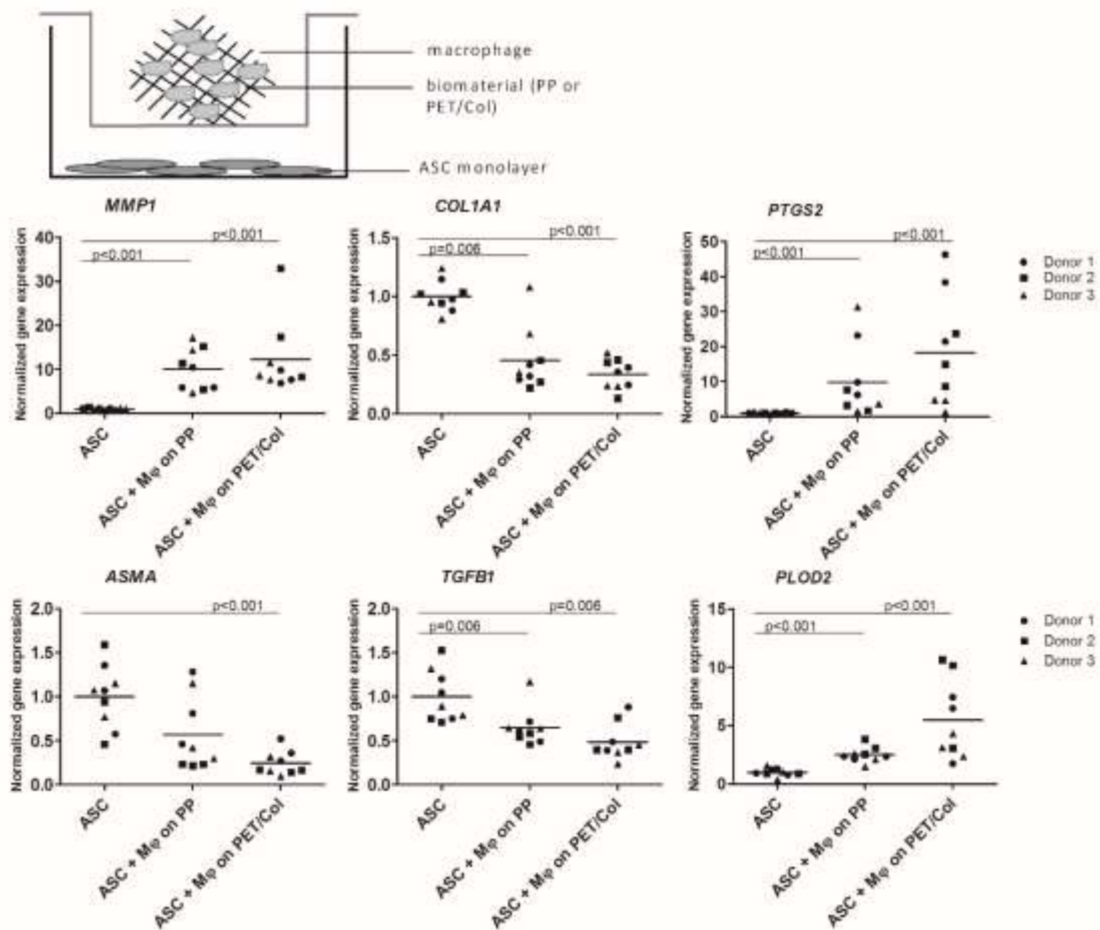


Fig. 3 Gene expression of ASCs in monolayer cocultured with macrophages. Gene expression of ASCs in monolayer cocultured with macrophages on biomaterials (PP and PET/Col) in a transwell system. The average gene expression of ASCs without macrophages was set to 1 for each ASC donor. Experiments were performed in triplicate for three ASC donors.

The experiments thus far describe the effects of factors secreted by macrophages on biomaterials on ASCs in monolayer. However, after being attracted by the macrophages, ASCs will also interact with the biomaterial. We therefore investigated the response when ASCs were seeded on PP and PET/Col with and without the presence of macrophages. Without macrophages, ASCs on PET/Col expressed less COL1A1, PLOD2, and ASMA and more PTGS2 than ASCs on PP (Fig. 4). The presence of macrophages on the same material lowered COL1A1 and increased MMP1 gene expression by ASCs.

Moreover, when ASCs and macrophages were cultured on PP, COL1A1 and MMP1 gene

expression was higher than when both cells were cultured on PET/Col. Differences between biomaterials were not detectable anymore for PTGS2, ASMA, and PLOD2 when ASCs were cocultured with macrophages, both cultured on the same material. TGFB in ASCs on biomaterials was unaffected by the type of biomaterial and the presence of macrophages on the same material (Fig. 4).

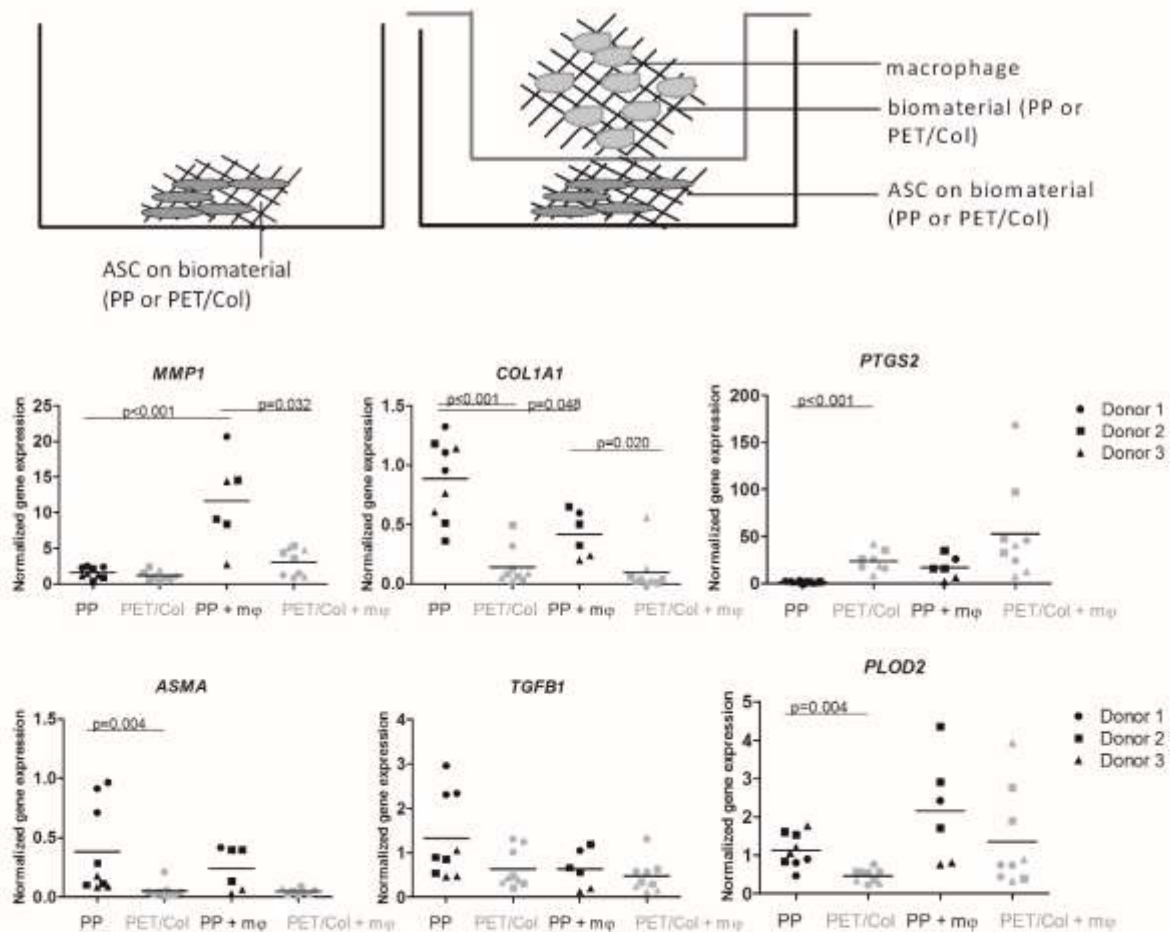
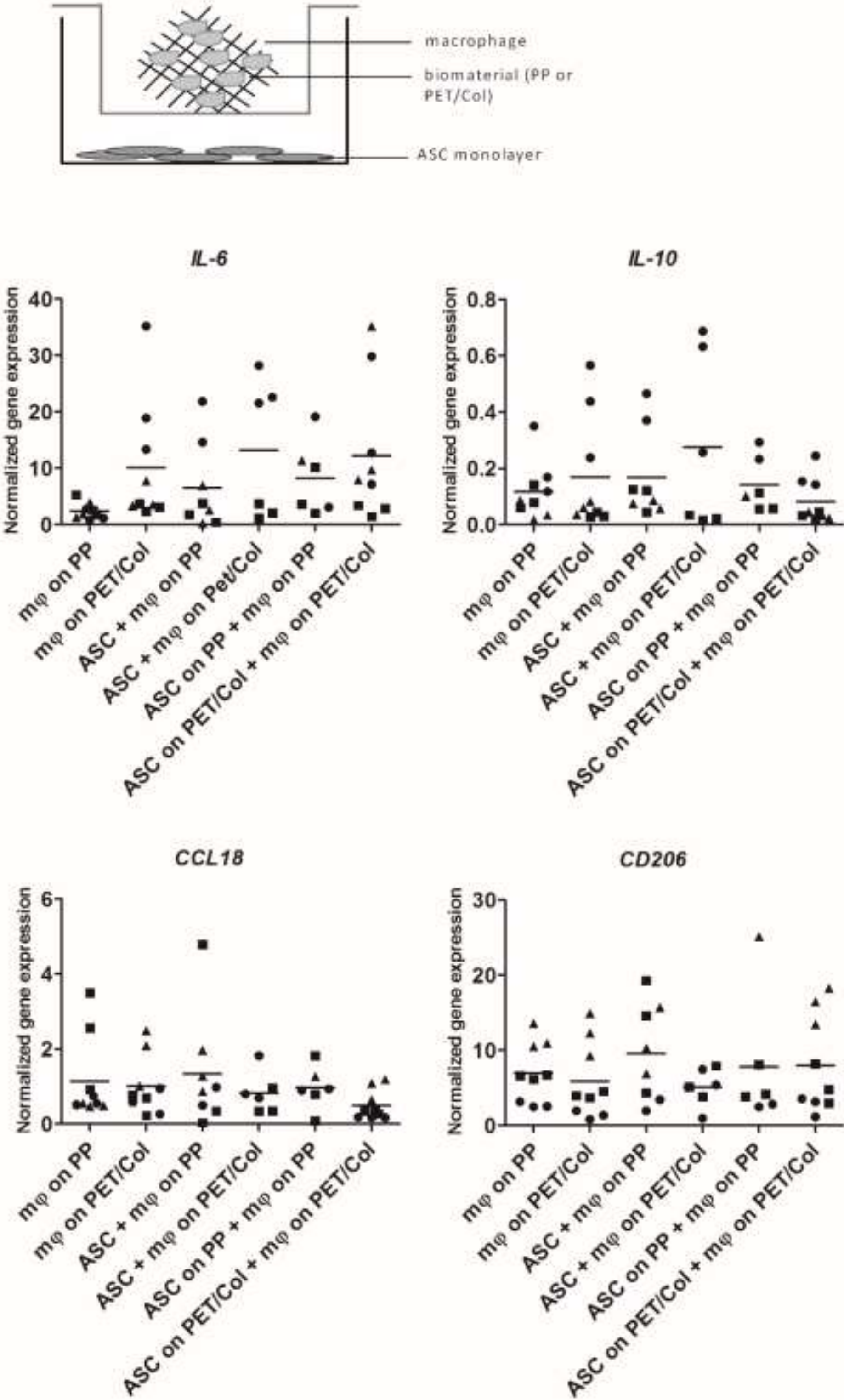


Fig. 4 The influence of biomaterials on ASCs. The effect of biomaterials on the gene expression of MMP1, COL1A1, PTGS2, ASMA, TGFB1, and PLOD2 by ASCs with or without the presence of macrophages on the same biomaterial. ASC PP, ASCs alone on PP; PET/Col, ASCs alone on PET/Col; PP+mφ, ASCs on PP and macrophages on PP in a transwell system; PET/Col+mφ, ASCs on PET/Col and macrophages on PET/Col in a transwell system (PP, PET/Col). Experiments were performed in triplicate for three ASC donors.

To investigate the effect of ASCs on macrophages, we analysed the macrophage gene expression of CCL18, IL-6, IL-10, and CD206 when the macrophages on the biomaterials were cocultured with ASCs on the same biomaterials or as monolayer. No statistically significant effects were seen in gene

expression of macrophages on biomaterials in response to the presence of ASCs in monolayer or on the same biomaterial (Supplementary Fig. S1).



Supplementory Figure: Gene expression of macrophages on biomaterials co-cultured with ASCs in monolayer or on the same biomaterials. Gene expression of macrophages on biomaterials (polypropylene: PP and ParietexTM Composite: PET/Col) in a transwell system. The average gene

expression of macrophages was set to 1 for each monocyte donor. Experiments were performed in triplicate for 3 monocyte-donors. ASC: adipose-derived mesenchymal stem cells.

To further understand the influence of macrophage phenotype on the interaction between macrophages and ASCs, we cultured ASCs in the presence of M(LPS/IFNγ) and M(IL-4) MCM. Both M(LPS/IFNγ) and M(IL-4)-CM increased PLOD2, MMP1, and PTGS2 in ASCs; however, M(LPS/IFNγ)-CM increased the gene expression more than M(IL-4)-CM. TGFB1 gene expression was lower in ASCs in monolayer stimulated with M(LPS/IFNγ)-CM than in ASCs in monolayer not exposed to MCM (Fig. 5).

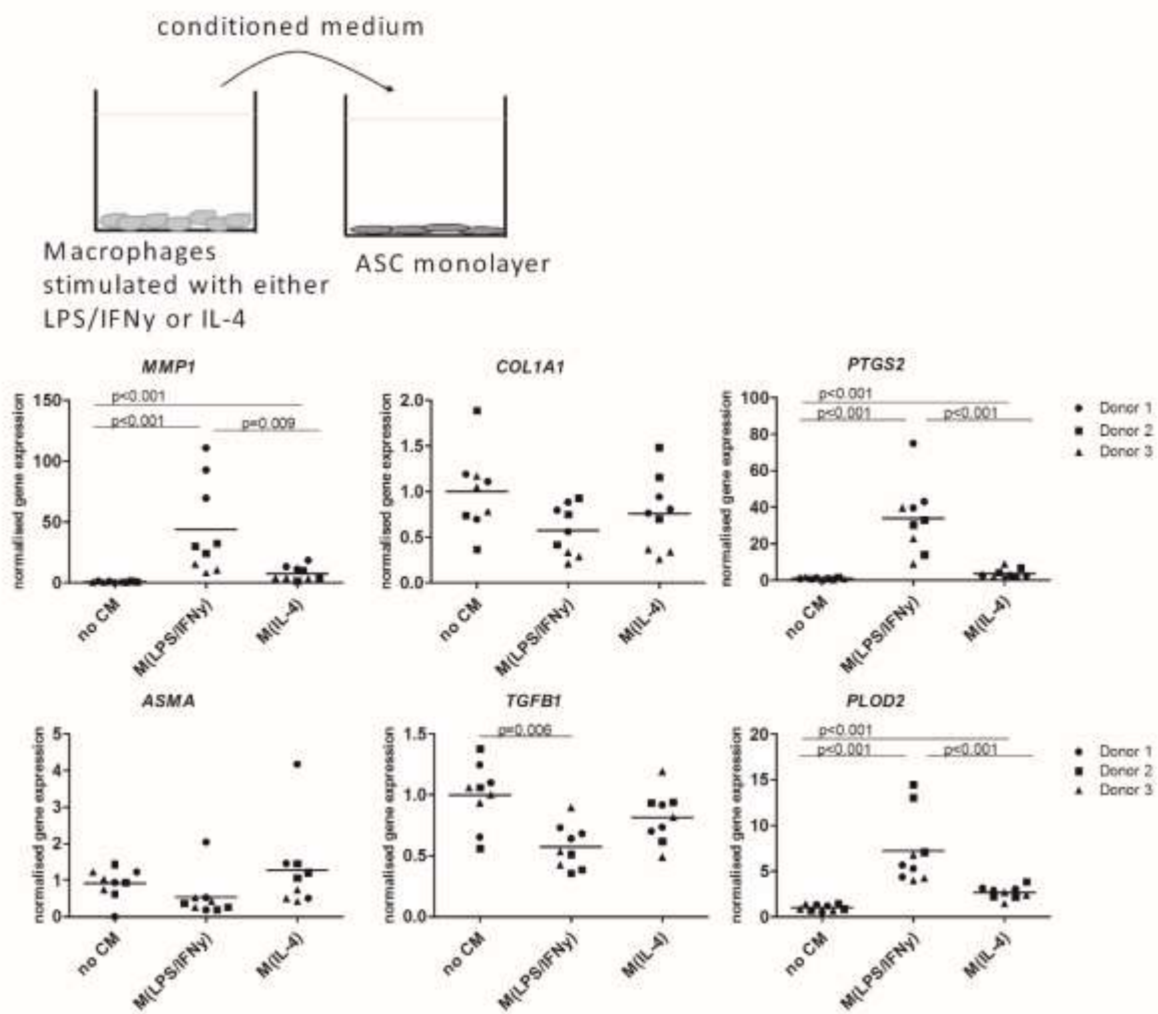


Fig. 5 Gene expression of ASCs with MCM. Gene expression of ASCs in monolayer stimulated with or without M(LPS/IFNγ) or M(IL-4)-CM. The average gene expression of ASCs without MCM was set to 1 for each ASC donor. Experiments were performed in triplicate for three ASC donors.

Discussion

Macrophages and MSCs can influence wound healing and tissue regeneration and the interplay between these cell types is important for the healing process (27). Biomaterials influence the behaviour of macrophages (12) and might also influence the cross-talk between macrophages and MSCs. Which processes are activated in each cell in this interaction is, however, not fully understood. Using a coculture model of macrophages and ASCs with biomaterials, our data indicate a biomaterial-dependent wound-healing reaction that is orchestrated by macrophages. We found indeed that macrophages on biomaterials induce a reaction in ASCs. Differences between the materials became obvious when the ASCs were in direct contact with the biomaterial.

MSCs are found to contribute to wound healing by migrating to the wound site and differentiate into different cell types, including extracellular matrix producers (5). Direct contact with biomaterials influenced the behaviour of ASCs, suggesting that the wound-healing process might not solely be directed by macrophages. In fact, MSCs are known to influence other cells such as T cells (28), macrophages (29), and fibroblasts (30) by producing many cytokines and growth factors and thereby coordinate the wound-healing process (30). This implicates a pivotal role for MSCs in wound healing and, therefore, MSCs isolated from adipose tissue (ASCs) were used in our culture models.

To investigate the role of each cell type in the cross-talk in reaction to biomaterials, several culture setups were used, starting with using CM from macrophages cultured on biomaterials for the culture of ASCs in monolayer. MCM increased collagen deposition by the ASCs, ASC proliferation, and the gene expression of MMP1, PLOD2, and PTGS2. This effect was independent of the biomaterial on which the macrophages were cultured.

Having the macrophages on the biomaterial and the ASCs in monolayer in a transwell coculture induced the same effects on MMP1, PLOD2, and PTGS2, with additional decrease of COL1A1, ASMA, and TGFB1. This means that biomaterials have a great influence on the reaction between ASCs and macrophages and thereby the wound healing, mainly influencing remodelling since the presence of biomaterials increased PLOD2 and MMP1. PTGS2 was increased in the ASCs, in

monolayer, and on biomaterials, when influenced by macrophages, indicating an immunomodulatory effect of ASCs, this immunomodulatory capacity is known from the literature (6).

Interestingly, biomaterials also influenced ASCs without the presence of macrophages. The genes COL1A1, PTGS2, ASMA, and PLOD2 were differentially expressed between the biomaterials. This could suggest that material differences *in vivo* regarding markers for fibrosis are less due to macrophage responses, but instead are due to MSCs that are recruited to the site. Material screening when focusing on fibrotic processes might be done using ASCs rather than macrophages.

Taken together, macrophages in general influence the behaviour of ASCs, especially processes related to wound healing, and when macrophages are cultured on different biomaterials in a coculture with ASCs, they also elicit biomaterial-specific reactions in the ASCs. Biomaterials themselves also elicit specific reactions in the ASCs, however, on other parameters related to collagen modification and immune regulation.

MCM and macrophages in coculture with ASCs stimulated the expression of PTGS2, the gene encoding the enzyme cyclooxygenase 2 (COX2). PTGS2 was also differentially expressed in the ASCs in response to the two different biomaterials. COX2 can stimulate cell proliferation and vasodilation (20,21), important factors in wound healing. Thus, this shows that biomaterials influence the reaction of ASCs and that macrophages can influence this reaction. The literature indicates that non-steroid anti-inflammatory drugs might have a negative influence on wound healing (21,31). These drugs inhibit the COX2 enzyme. Since these drugs are commonly used after surgery for analgesia, this might also have implications for biomaterial-specific wound healing.

Macrophages are key players in wound healing (32). Macrophage subtype can determine the wound-healing reaction, and the presence of biomaterials was demonstrated by us and others to have an effect on macrophage subtype (12,13,33). After the acute reaction, a predominant anti-inflammatory reaction is associated with a better wound healing (32,33).

Previously, we compared the effect of the used biomaterials on macrophages to the gene expression and protein production profile of M(LPS/IFNγ) and M(IL-4) (12). There, we found that IL-

1RA, regulated on activation, normal T-cell expressed and secreted, IL-6, CCL18, and macrophage-derived cytokine were differentially produced between M(LPS/IFNγ) and M(IL-4). When looking at the materials, again CCL18 was differentially produced between the materials, but also IL-1β, TNFα, and MCP-3. In this study, we questioned whether the effect of macrophages cultured on biomaterials on ASCs was comparable with that of pro-inflammatory macrophages or anti-inflammatory macrophages.

Our data indicate that the response of ASCs in monolayer to macrophages on both PET/Col and PP is similar to the response of ASCs to medium conditioned by M(LPS/IFNγ), since the macrophages on the materials induced MMP1, PTGS2, and PLOD2 and reduced COL1A1 and TGFB in ASCs. These effects were also seen after adding M(LPS/IFNγ)-CM to ASCs in monolayer. This suggests that the first reaction of ASCs in response to macrophages on biomaterials is predominantly pro-inflammatory, which is expected since pro-inflammatory macrophages are the first type of macrophages in the wound-healing cascade (32,33). Most likely, factors such as IL-6, IL-1β, or TNFα among many others have contributed to these effects since these factors were highly produced by M(LPS/IFNγ) (12,25) or by macrophages on PET/Col or PP (12).

Next, to an effect of macrophages on ASCs, ASCs are known to influence macrophage phenotype. Macrophages have been reported to produce more IL-10 and less IL-6, IL-12, and TNFα when they interact with stem cells, the so-called stem cell-educated macrophage (29). We found, however, no effect of the ASCs cultured in monolayer or on biomaterials on macrophages. This might be explained by the fact that the macrophages were already present on a biomaterial, which might be a stronger stimulus than the factors produced by ASCs.

Several culture setups were used to investigate the reaction between biomaterials, ASCs, and macrophages: experiments with CM to investigate the effect of one cell type on the other and cocultures to examine the interaction between ASCs and macrophages. Many different cytokines are produced by ASCs and macrophages, but it is unclear which cytokine is responsible for which reaction seen in our cultures.

More research is needed to investigate these reactions in more detail and to find out which soluble factor is responsible for which process, for instance with neutralizing antibodies. It is likely that cell–cell contact also contributes to the interaction between ASCs and macrophages. However, we did not include a culture setup in which we cultured macrophages and ASCs together on the material to allow cell–cell contact. Such a culture would not allow us to analyse the cells separately for gene expression or protein production and, therefore, we focused on different cells in different compartments.

The *in vitro* culture of macrophages and ASCs with biomaterials can be used as a model to investigate the wound healing in response to the implantation of a biomaterial. Thus making *in vitro* research an easy way to investigate this reaction that can lead to new hypotheses and ideas and maybe even predict what is happening *in vivo*, as we have seen earlier with our macrophage culture model (12). Future research might aim to show that our coculture system indeed can predict the *in vivo* situation.

Our culture systems contained FCS as prerequisite for the macrophage culture. FCS is a source of cytokines and growth factors, thereby having the possibility to interfere in our culture system. However, the presence of FCS does not prevent macrophages from polarizing to different phenotypes as we have seen before (18,26,34). In this study, even though FCS was present in the same amount for every culture and condition, we still see biomaterial-dependent reactions of macrophages and ASCs.

Although we found some donor variation (some donors had a higher overall gene expression than others), the results were very reproducible. Variation is not unexpected and comparable with the *in vivo* situation where each patient responds differently. Using different human macrophage and ASC donors represents the variety of patients. We did not use ASCs and macrophages from the same donor. Since we found clear differences between conditions, we assume that no immune reaction took place in our culture model. For this study, we have used monocytes and ASCs isolated from healthy donors. It is well known from the literature that in some patient groups comorbidities such as

diabetes or obesity impair wound healing. Macrophage subtype in obesity and diabetes is mainly pro-inflammatory (33) which will likely influence the wound healing (32,33). Therefore, more research is needed with nonhealthy donors.

Conclusions

Biomaterials influence tissue regeneration by influencing interaction between macrophages and ASCs but also by influencing the cell types separately as shown in this article. This article gives more insight into the behaviour of two different cell types during wound healing after implantation of a biomaterial. This behaviour appears to be biomaterial specific. As such, for the tissue-engineering field, the choice of a biomaterial can influence the wound-healing response.

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