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A culture model to analyze the acute biomaterial-dependent reaction of human primary macrophages

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ABSTRACT

Macrophages are important in foreign body reactions. We devised a culture model with human primary macrophages to evaluate the acute response of macrophages to biomaterials. First we selected proteins representative for pro-inflammatory (M1) or anti-inflammatory/repair (M2) response of monocytes isolated from blood of healthy human donors by exposing them to LPS+IFNγ or IL-4. A relative M1/M2 index was calculated using IL-1β, IL-6, tumor necrosis factor (TNF)α, monocyte chemotactic protein (MCP)-3 and macrophage inflammatory protein (MIP)-1α as M1 markers, and IL-1 receptor antagonist (IL-1RA), CCL18, regulated and normal T-cell expressed and secreted (RANTES), and macrophage-derived chemokine (MDC) as M2 markers. Then monocytes were cultured for 3 days on 4 materials selected for known different foreign body reactions: Permacol™, Parietex™ Composite, multifilament polyethylene terephthalate and multifilament polypropylene. Macrophages on polypropylene produced high levels of anti-inflammatory proteins with a low M1/M2 index. Macrophages on Parietex™ Composite produced high levels of inflammatory and anti-inflammatory proteins, with a high M1/M2 index. Macrophages on polyethylene terephthalate also resulted in a high M1/M2 index. Macrophages on Permacol™ produced a low amount of all proteins, with a low M1/M2 index. This model with human primary macrophages and the panel of read-out parameters can be used to evaluate the acute reaction of macrophages to biomaterials *in vitro* to get more insight in the foreign body reaction.

1. Introduction

Biomaterials are widely used in regenerative medicine. Worldwide there are over 100 different commercially available biomaterial-based medical devices, with different composition and formulation for clinical use. All biomaterials elicit a reaction of the body, the foreign body reaction, more or less marked according to the nature of biomaterials. This reaction differs between patients [1–3]; in hernia surgery, for example, 14–52% of the patients have complaints, usually of pain, seroma (wound fluid production), or excessive production of scar tissue [4,5].

Macrophages play a pivotal role in the foreign body reaction [6,7]. Their subtype can change in response to environmental factors, giving rise to different populations of macrophages with distinct functions. Classically activated macrophages, or M1 macrophages, are the most thoroughly characterized and well-described activated macrophages. They propagate pro-inflammatory responses by producing cytokines such as interleukin (IL)-1β, tumor necrosis factor (TNF)α and interleukin (IL)-6 [8–10]. Another subtype of macrophages is represented by the alternatively activated macrophages, also referred to as M2 macrophages. These cells can arise when exposed to, for example, IL-4 or immune complexes. M2 macrophages produce among others IL-10 and chemokines CCL18 and CCL22 (macrophage derived chemokine, MDC) and are able to produce growth factors, promoting angiogenesis and extracellular matrix production [8–10].

After implantation, biomaterials are immediately coated with serum proteins and extracellular matrix proteins that activate the immune system [11,12]. These proteins drive the very first

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steps of the foreign body reaction, in particular, in stimulating neutrophils and activating monocytes to become macrophages. Neutrophils and macrophages then release different cytokines and chemokines to attract more cells to the wound site [13].

The foreign body reaction to biomaterials is mostly examined with animal experiments. Although studies have also been performed *in vitro*, most of these involve cell-lines or non-human cells. We devised an *in vitro* model based on human primary macrophages and designed an M1/M2 index to evaluate the involvement of macrophages in the foreign body reaction to a biomaterial. Four different biomaterials were chosen based on a different host response *in vivo*; polyethylene therephthalate with a fast resorbing coating made from purified pepsinized porcine collagen (Parietex™ Composite), porcine acellular dermis matrix, mainly composed of 'natural' collagen (Permacol™), polypropylene (mPP) and a polyethylene therephthalate (mPET) biomaterial. All the selected materials are representative of a large palette of surgical materials. For example, mPP and mPET based biomaterials are widely used as prostheses for vascular, abdominal, orthopedic surgeries and as permanent suture materials [14]. Acellular dermis matrices are reported to be used in surgical reconstructions of soft tissues such as head and neck soft tissues, breast and abdominal wall [15].

The synthetic biomaterials, especially polypropylene, are described to give a predominantly fibrotic response and naturally derived biomaterials firstly elicit an M1 response [16]. For our *in vitro* model we used primary human macrophages and selected a panel of genes and proteins based on literature and own experiments that can be used to discriminate between pro-inflammatory and anti-inflammatory macrophages.

2. Materials and methods

2.1. Monocyte isolation

Ficoll density gradient (Ficoll-Paque™ PLUS, GE Healthcare) was used to isolate monocytes from 7 buffy coats of healthy donors (men and women aged 21–63), obtained from the blood bank (Sanquin Bloodbank, Rotterdam, the Netherlands) Thirty milliliter of diluted buffy coat (1:5 ratio with PBS/BSA 0.1%) was layered on 15 mL of Ficoll. After 15 min centrifugation at 1000*g* without brake, the interphase band containing peripheral blood mononuclear cells was removed and washed in PBS/BSA 0.5% 2 mM EDTA and labeled with 100 μL of anti-CD14+ magnetic beads (CD14 microbeads human, MACS Separation columns LS and MidiMACS™ Separator; all Miltenyi Biotec), and isolated according to the manufacturer's guidelines. This positive selection of monocytes will not activate the cells [17]. To measure purity of the isolation, 1×10^6 monocytes were incubated for 15 min at room temperature with the following antibodies: FITC-conjugated CD14 and PerCP-conjugated CD45 (all BD Pharmingen, Franklin Lakes, NJ, USA). After incubation, cells were washed in PBS/BSA 0.1%. FACS analysis was performed with cellquest Pro (BD) on a FACSCalibur (BD).

2.2. Macrophage stimulation towards an M1 and M2 subtype

To validate the read out parameters, monocytes were cultured in monolayer non-stimulated and stimulated to M1 or M2 using cytokines as described previously [11,18–21]. The monocytes were plated in 6-well plates (polystyrene, Costar, Corning Inc. NY, USA) in a concentration of 100,000 monocytes per cm^2 and cultured in X-vivo 15 medium (Lonza, Verviers, Belgium) with 0.6% fungizone (Amphotericine, Gibco, Carlsbad) and 0.1% gentamycine (Gibco). Different media were tested for culture of human monocytes; on the basis of cell attachment and cell survival, X-vivo 15 was considered the optimal medium (data not shown). Directly after plating, monocytes were either not stimulated or stimulated with 100 ng/mL LPS (Lipopolysaccharide, Sigma–Aldrich, St. Louis, MO, USA) and 10 ng/mL IFNγ (recombinant human interferon-γ, PeproTech, Rocky Hill, NJ, USA) to obtain an M1 subtype or 10 ng/mL IL-4 (recombinant human interleukin 4 PeproTech) to obtain an M2 subtype [11,18–21]. Attached monocytes will be referred to as macrophages. Macrophages were cultured in a humidified incubator at 37 °C, 5% CO2 (Binder, Tuttlingen, Germany) for a total of 3 days.

The monolayer cultures were harvested after 1 day of culture for gene expression and after 3 days for protein production.

2.3. Culturing macrophages on biomaterials

To evaluate the effect of biomaterials on macrophages, the monocytes were seeded on four different materials immediately after isolation from the buffy coat. The following materials were chosen because they initiate a different reaction *in vivo*: multifilament polypropylene (mPP), multifilament polyethylene terephthalate (mPET), Permacol™ (collagen derived from porcine skin, crosslinked) and Parietex™ Composite (multifilament polyethylene terephthalate with an absorbable, continuous and hydrophilic collagen film on one of its sides) (all Covidien–Sofradim Production, Trevoux, France). The materials were cut into pieces of 1.5 by 1.5 cm with a sterile scalpel. Before cell seeding, to provide protein attachment, materials were incubated in 100% non-heat inactivated fetal bovine serum (Lonza, Verviers, Belgium) for 2 h. Freshly isolated monocytes were adjusted to a concentration of 700,000/mL in a total volume of 25 mL in a 50 mL tube (Falcon, polypropylene conical tube, Becton Dickinson, Franklin Lakes, NJ, USA). Twelve samples were incubated per 25 mL for 2 h at 37 °C. Afterwards, samples were placed in a 24-well non-adherent plate (NUNC, non-treated multiplate, Rochester, NY, USA) and cultured for a total of 3 days in serum free X-vivo 15 medium. The samples were harvested after 1 day of culture for gene expression and after 3 days for protein production and DNA analysis.

2.4. RNA isolation and qPCR

Monolayer samples were harvested in 1 mL RLT-buffer (Qiagen, Hilden, Germany). Seeded biomaterials were harvested in 350 μL RLT-buffer. Samples were kept at −80 °C until further RNA isolation was performed using the RNeasy Microkit (Qiagen) according to the manufacturer's instructions with on column DNA digestion. RNA concentration was measured using a spectrophotometer (NanoDrop ND1000 UV–VIS, Isogen Life Science B.V., the Netherlands). cDNA was prepared using RevertAid First Strand cDNA Synthesis Kit (MBI Fermentas, Germany) according to the manufacturer's instructions. qPCR was performed on an ABPrism 7000 system (Applied Biosystems, Foster City, CA, USA) using either Taqman Universal PCR mastermix (Applied Biosystems) or SybrGreen (Eurogentec, Seraing, Belgium). After testing several housekeeping genes, we found Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*, Fw:GTCAACGGATTTGGTCGTATTGGG; Rev:TGCCATGGGTGGAATCATATTGG; probe:Fam-TGGCGCCCCAACCAGCC-Tamra) to be the most suitable for our experiments. For analysis, the following distinctive genes were used chosen based on literature and pilot experiments [8,10,22,23]: *tnfα* and *il-6* as M1 genes, *cd206* and *ccl18* as M2 genes. *CCL18* FW GCACCATGGCCCTCTGCTCC, Rev GGGCACTGGGGGCTGGTTTC; *IL-6* FW TCGAGCCCACCGGGAACGAA, Rev GCAGGGAAGGCAGCAGGCAA; *CD206* FW TGGCCGTATGCCGGTCACTGTTA, Rev ACTTGTGAGGTCACCGCCTTCCT; *TNFα* Fw GCCGCATCGCCGTCTCCTAC, Rev AGCGCTGAGTCGGTCACCCT (all Eurogentec). Relative gene expression was calculated using the $2^{-\Delta CT}$ method [24].

2.5. Protein analysis

Proteins of interest were based on literature [8,10,22,23] and on a pilot experiment where production of 42 cytokines was measured in 25 μL cell-culture supernatants after 1 and 3 days of culture using Milliplex (Millipore, MPXHCYTO-60K, Billerica, MA, USA) according to manufacturer's recommendation to search for discriminating proteins (EGF, Eotaxin, FGF-2, FLt-3L, Fractalkine, G-CSF, GM-CSF, GRO, IFNα2, IFNγ, IL-1α, IL-1β, IL-1RA, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12(p40), IL-12(p70), IL-13, IL-15, IL-17, IP-10, MCP-1, MCP-3, MDC, MIP-1α, MIP-1β, PDGF-AA, PGGF-AB/BB, RANTES, sCD40L, sIL-2Ra, TGFα, TNFα, TNFβ, VEGF). CCL18 was measured in addition using a CCL18 DuoSet ELISA (R&D systems, Minneapolis, MN, USA) in 100 μL cell-culture supernatants according to the manufacturer's recommendation. From this pilot experiment the most discriminative 9 cytokines and the time point day 3 were chosen (data not shown) and measured in the subsequent experiments using an eight-plex Milliplex (Millipore) and a CCL18 DuoSet ELISA (R&D), namely: interleukin (IL)-1β, IL-6, tumor necrosis factor (TNF)α, monocyte chemotactic protein (MCP)-3, and macrophage inflammatory protein (MIP)-1α, IL-1 receptor antagonist (IL-1RA), regulated upon activation normal T-cell expressed and secreted (RANTES or CCL5), macrophage derived chemokine (MDC), and CCL18.

The data were corrected for number of cells by measuring DNA. Macrophages were lysed in 0.1%Triton/PBS (Sigma–Aldrich, St. Louis, MO, USA) and frozen at −80 °C before analyzing with CyQUANT© cell proliferation assay kit (Invitrogen, Carlsbad, CA, USA). DNA was measured according to the manufacturer's recommendation.

2.6. Statistics

The monolayer experiments were performed with four donors for 1 day of culture, three overlapping donors for the 3 days of culture, with for each donor three monolayers per condition. The biomaterial experiments were performed with three different donors, each donor in triplicate. All data are presented as scatter dot plots with each dot representing one single measurement with the mean of the different donors. To compare the effect of the four materials on macrophage subtype, a relative M1/M2 index was calculated. The percentage of the mean production per cytokine was calculated, followed by dividing the mean percentage of M1 cytokines (MIP-1α, TNFα, MCP-3, IL-1β, IL-6) by the mean percentage of M2 cytokines (MDC, RANTES, IL-1RA and CCL18) per sample.

Groups were compared in SPSS (20.0, IBM Corporation, Armonk, New York, USA) using a Kruskal–Wallis test (independent samples median test) and a Mann–Whitney test because the data were not normally distributed. Differences were considered statistically significant when $p < 0.05$.

3. Results

3.1. Determination of read-out parameters for M1/M2 subtype

Purity of freshly isolated CD14+ monocytes was always > 95%, as measured by FACS analysis (data not shown). To determine read out parameters for the M1 and M2 subtypes we first confirmed the subtypes using qPCR. After one day of culture in monolayer, expression of *tnfα* and *il-6*, genes specific for M1 macrophages, was significantly higher in the LPS+IFNγ-stimulated cells than in the IL-4 stimulated cells. In addition, LPS+IFNγ resulted in a decrease relative to the unstimulated cells of *cd206*, a gene characteristic for M2 macrophages. The genes specific for M2 macrophages, *ccl18* and *cd206*, were significantly higher expressed in the IL-4 stimulated cells than in the LPS+IFNγ-stimulated cells (Fig. 1).

After this confirmation of the pro-inflammatory (M1) and anti-inflammatory/repair (M2) subtype of macrophages we analyzed the production of the proteins MIP-1α, TNFα, MCP-3, IL-1β, IL-6, MDC, RANTES, IL-1RA and CCL18. The medium of LPS+IFNγ-stimulated cells contained significantly more IL-6 than the medium of IL-4 stimulated cells (Fig. 2A). The culture medium of IL-4 stimulated cells contained significantly more CCL18, IL-1RA, RANTES and MDC than medium of LPS+IFNγ stimulated cells (Fig. 2B).

3.2. Response of macrophages to biomaterials

Interaction of macrophages with different biomaterials resulted in differences in gene expression. For the inflammatory cytokines no significant differences were found, however gene expression

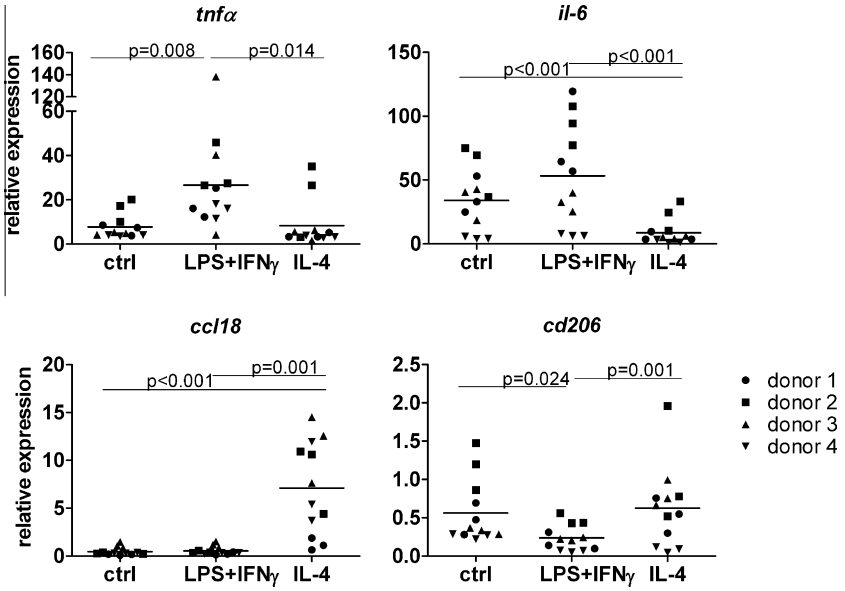

Fig. 1. Gene expression by stimulated macrophages. Macrophages were stimulated with LPS+IFNγ or IL-4 cultured for 1 day, $n = 4$ donors with samples in triplicate for each donor. *tnfα* and *il-6* were used as M1 markers; *ccl18* and *cd*206 as M2 markers. Gene expression was normalized for GAPDH.

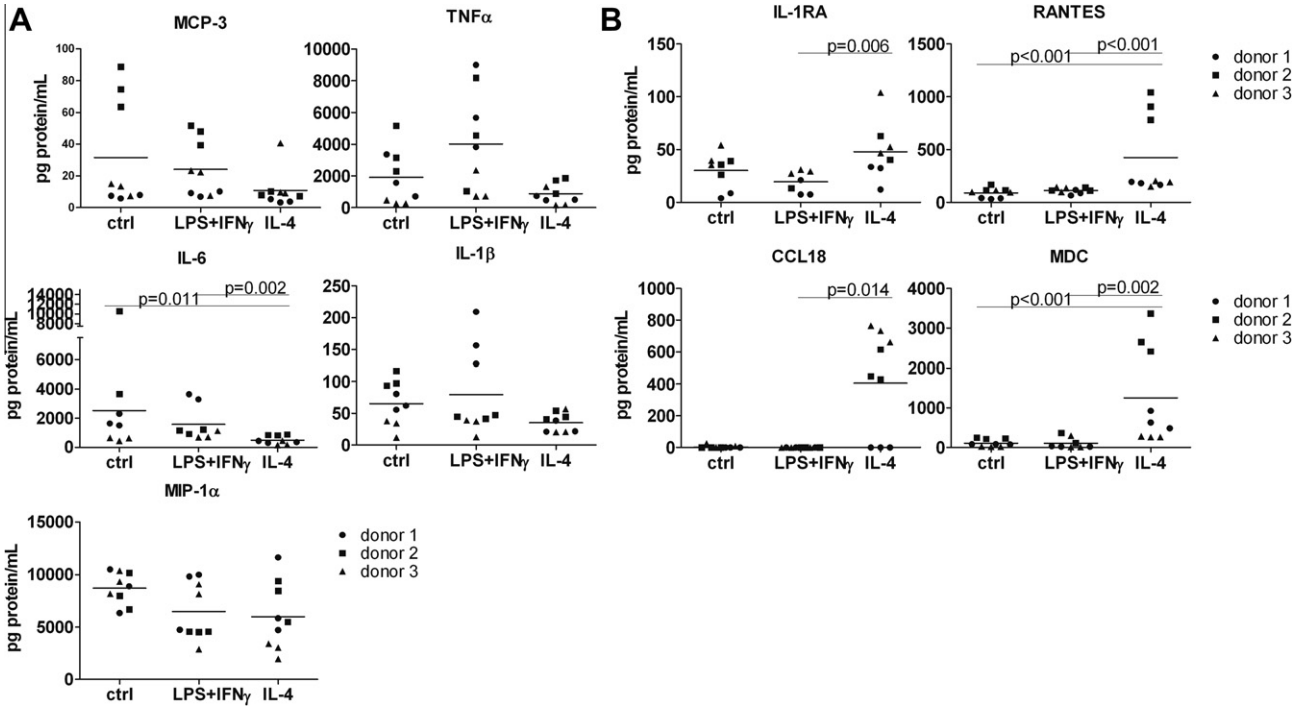

Fig. 2. Protein production of stimulated macrophages. Protein production of (A) pro-inflammatory markers (B) anti-inflammatory markers by macrophages in response to LPS+IFNγ or IL-4 stimulation after 3 days, $n = 3$ donors with samples in triplicate for each donor.

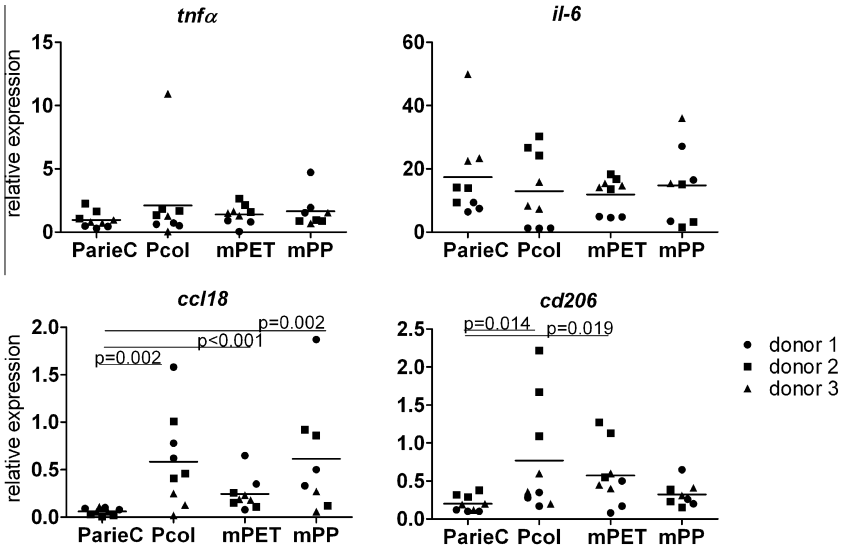

Fig. 3. Gene expression of macrophages seeded on biomaterials. Gene expression of macrophages seeded on biomaterials cultured for 1 day. $n = 3$ different donors, samples in triplicate. Gene expression was normalized for GAPDH. ParieC = Parietex™ Composite, Pcol = Permacol™, mPET = multifilament polyethylene terephthalate, mPP = multifilament polypropylene.

of *ccl18* was significantly lower for macrophages seeded on Parietex™ Composite than the other three biomaterials. Gene expression of *cd206* was significantly higher in macrophages on Permacol™ and mPET than in macrophages on Parietex™ Composite (Fig. 3).

All proteins, besides CCL18, were produced in higher amounts by macrophages on Parietex™ Composite than macrophages on the other materials. CCL18 secretion was significantly higher by macrophages on mPP than by macrophages on the other biomaterials. The lowest amounts of all proteins were secreted by macrophages on Permacol™ (Fig. 4A and B).

To facilitate comparison of the different biomaterials a relative M1/M2 index was calculated for each biomaterial. Macrophages on Parietex™ Composite and mPET have a high M1/M2 index, meaning a more pro-inflammatory subtype in comparison to mPP and Permacol based on our protein panel. Macrophages on Permacol™ and mPP had a low M1/M2 index, meaning a more anti-inflammatory subtype than the other two materials (Fig. 4C).

4. Discussion

A well-characterized *in vitro* model can be of great value to study the mechanisms of foreign body reactions. In this study, we presented an *in vitro* model of healthy human primary macrophages with an M1/M2 index as one of the read-out parameters. With this model we could show that the acute inflammation reac-

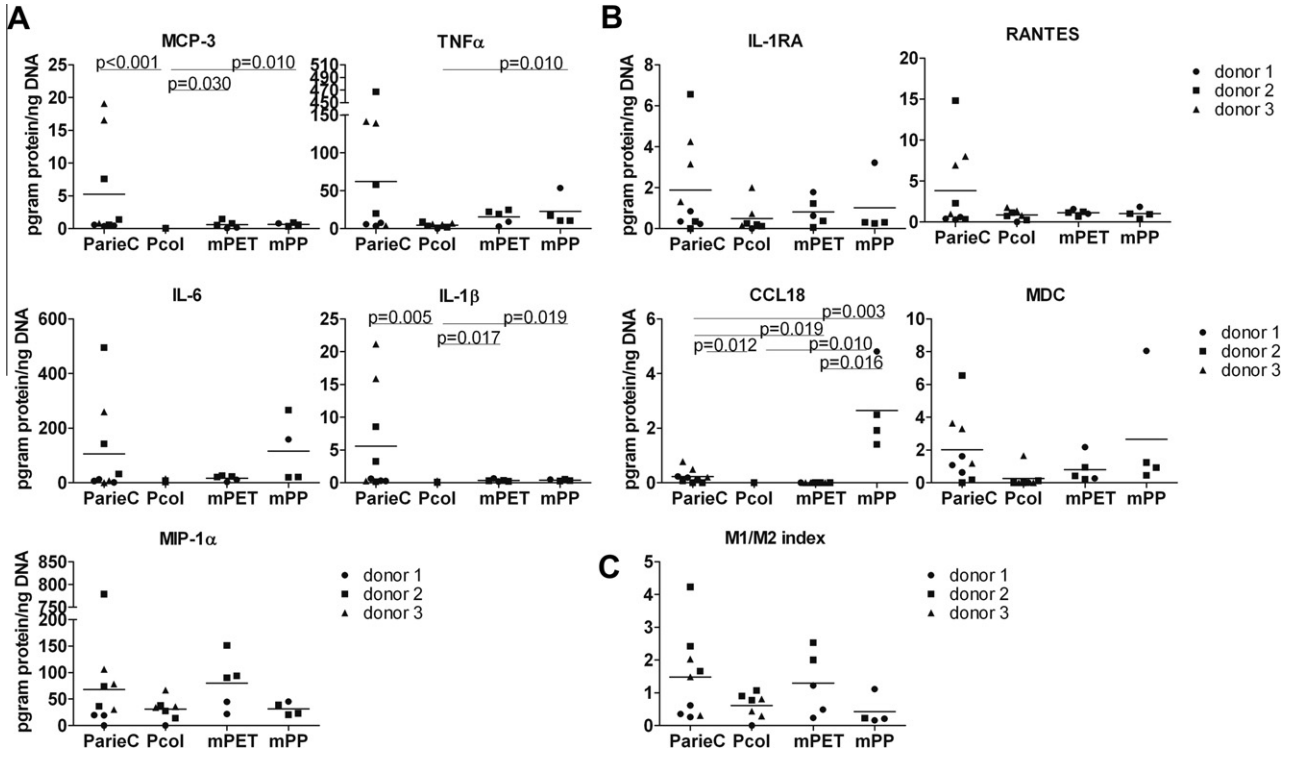

Fig. 4. Protein production by macrophages seeded on biomaterials. Protein production by macrophages seeded on biomaterials cultured for 3 days corrected for DNA. (A) Pro-inflammatory markers (B) anti-inflammatory markers (C) M1/M2 index, $n = 3$ different donors, samples in triplicate. The percentage of the mean production per cytokine was calculated, followed by dividing the mean percentage of M1 cytokines (MIP-1α, TNFα, MCP-3, IL-1β, IL-6) by the mean percentage of M2 cytokines (MDC, RANTES, IL-1RA and CCL18) per sample. ParieC = Parietex™ Composite, Pcol = Permacol™, mPET = multifilament polyethylene terephthalate, mPP = multifilament polypropylene.

tion of macrophages is different in response to different biomaterials.

We determined a panel of proteins and genes to define several distinguishing markers for pro-inflammatory and anti-inflammatory macrophages using LPS+IFNγ and IL-4 stimulated macrophages. We measured protein production for nine proteins, all except for MCP-3, were described in literature related to M1 or M2 macrophages; MIP-1α (or CCL3), TNFα, IL-1β and IL-6 are described as pro-inflammatory M1 markers, MDC (CCL22), RANTES (CCL5), IL-1RA and CCL18 are described as anti-inflammatory M2 markers [10,12,18,19,25]. Although IL-6 and RANTES are known to be able to act either pro-inflammatory or anti-inflammatory, depending on the environment [8,20,21,26], in our monolayer experiments with stimulated macrophages *il-6* gene expression was higher in LPS+IFNγ stimulated macrophages than in IL-4 stimulated macrophages. Therefore IL-6 was selected as pro-inflammatory marker. RANTES protein levels on the other hand were higher in IL-4 stimulated cells than in LPS+IFNγ stimulated cells and therefore selected in our model as an anti-inflammatory marker. When we measured the above nine proteins in all experiments, we detected donor differences, some proteins appeared discriminative in some, but not in other donors. This represents the human *in vivo* situation, where the macrophage reaction to a foreign material is also dependent on the recipient.

Taking together using gene expression of *tnfα*, *il-6*, *ccl18* and *cd206*, and the protein panel with MIP-1α, TNFα, MCP-3, IL-1β, IL-6, MDC, RANTES, IL-1RA and CCL18, we believe that we have a good read-out panel to determine the acute reaction of macrophages to biomaterials, based on the monolayer experiments. Not only can these parameters be used as positive marker for a pro-inflammatory or an anti-inflammatory/repair reaction, also as negative markers as for instance *cd206* that was downregulated by addition of LPS+IFNγ or *il-6* that was downregulated by addition of IL-4.

The differences found when investigating the response of macrophages on different biomaterials indicated that biomaterials can directly influence the differentiation of macrophages. Macrophages cultured on mPP produced high levels of CCL18 protein, and their overall M1/M2 index was low in comparison to the other biomaterials tested, suggesting that mPP induces an anti-inflammatory/repair subtype of macrophages. High levels of CCL18 are associated with fibrotic reactions [23,27]. From the *in vivo* situation, multifilament polypropylene is indeed known to generate a significant fibrotic reaction [28–30]. Macrophages on Permacol™ produce a low amount of both pro-inflammatory and anti-inflammatory/repair proteins, suggesting a mild reaction to the biomaterial. The M1/M2 index is low, meaning that the mild reaction is mostly anti-inflammatory. Indeed Permacol™ is known to induce a very low foreign body reaction *in vivo* [31,32]. Macrophages on mPET have a high M1/M2 index, meaning a predominantly pro-inflammatory subtype of macrophages on this biomaterial. Macrophages on Parietex™ Composite produced high levels of both types of proteins, with a relatively high M1/M2 index. This marked acute reaction may be generated by soluble collagen fragments released from the collagen film directly after seeding of the monocytes, which is also seen *in vivo* [33,34]. The M1/M2 index in favor of M1 also corresponds to the less fibrotic reaction seen *in vivo* [34,35]. This association of macrophage phenotype *in vitro* and *in vivo* data of foreign body reaction indicates that our *in vitro* model based on human macrophages is representative.

To our knowledge, this is the first model with human primary macrophages that is well characterized and provides the possibility to study the differentiation of macrophages into different subtypes as a response to biomaterials. This *in vitro* model has two particular

advantages over those previously used: it is an easy and fast way to evaluate the response of macrophages to different biomaterials compared to animal models and it uses human freshly isolated macrophages making the results translatable to the human *in vivo* situation.

To conclude, for analysis of the reaction of human primary macrophages to biomaterials, we selected a panel of genes and proteins based on literature and own experiments that can be applied to discriminate between pro-inflammatory and anti-inflammatory/repair macrophages. Indeed, different biomaterials, widely used in surgeries with their specific responses, resulted in different inflammatory responses *in vitro*. This indicates that this culture model is suitable to evaluate macrophage responses to biomaterials. It can provide more insight in the interaction of macrophages with other cells such as fibroblasts, neutrophils and lymphocytes, and helps to find ways to interfere with the foreign body reaction.

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