New Models for Patient-specific Evaluation of the Effect of Biomaterials on Macrophages

Nienke Schmidt-Grotenhuis

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New Models for Patient-specific Evaluation of the Effect of Biomaterials on Macrophages

Nieuwe modellen voor patient-specifieke evaluatie van het effect van biomaterialen op macrofagen

Proefschrift

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	Prof.dr. N.D. Bouvy
Copromotor:	**Dr. Y.M. Bastiaansen-Jenniskens**

CONTENTS

CHAPTER 1

General introduction

Case report

A 42 year old female with a progressive swelling of the nasal tip presented herself at the outpatient clinic. The past decade, her nose and outer skin got infected several times. At the age of 25 she had minor nose surgery in which 'silicon based' fillers were injected in the nose for aesthetic purposes. During physical examination, a solid mass in the infratip and tip of her nose was palpable and visible, causing a downward rotation of the tip and a 'pseudo pollybeak deformation' (Figure 1). MRI showed a subcutaneous mass with low signal intensity on T2 sequence. An external rhinoplasty procedure was performed for a meticulous removal of the mass (Figure 1).

Microscopic evaluation with haematoxolin/eosin showed a chronic inflammation, suggesting a chronic foreign body reaction to the filler. Since inflammatory cells such as T-cells and macrophages are key players in the foreign body reaction [2], which will be explained later in detail, it was decided to further analyse the removed tissue. The tissue showed fibrosis and cell infiltrates with T-cells and macrophages. The tissue showed many empty round holes, likely the place where the filler has been. These holes were surrounded by giant cells (fused macrophages). Both pro- and anti-inflammatory macrophages were found close to the filler (Figure 2).

Biomaterials are seldom used in rhinoplasty due to a high complication rate as this case report shows. More research is needed to understand this reaction. The following chapters will give more inside in the foreign body reaction.

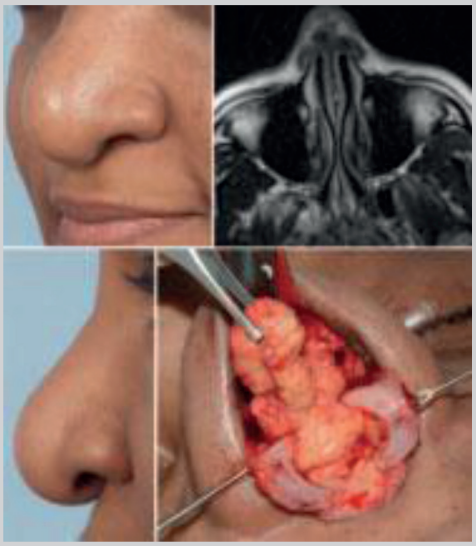

Figure 1 | Mass on tip of the nose. MRI showed a subcutaneous mass. Open technique rhinoplasty showing the mass.

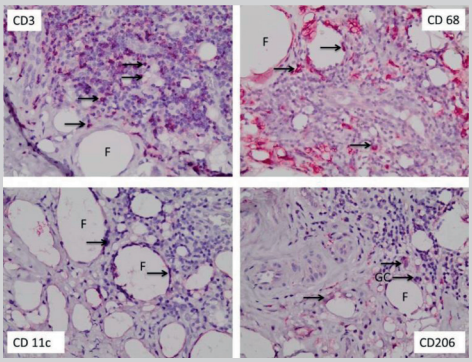

Figure 2 | Immunohistochemistry for macrophages and T-cells. T-cells (CD3), macrophages (CD68), pro-inflammatory macrophages (CD11c), anti-inflammatory macrophages (CD206).
Arrows indicate samples of positive staining, F = filler, GC = giant cell.

This patient was treated by FR Datema and PJFM Lohuis. A part of this case report was published in the Dutch Journal of Otorhinolaryngology [1].

A biomaterial is in the Merriam-Webster Dictionary defined as a natural or synthetic material that is suitable for introduction in living subjects and is often used in regenerative medicine to restore or replace tissue. In all fields of invasive medicine biomaterials play a role. In otorhinolaryngology, for example, biomaterials such as titanium are used to reconstruct the ossicular chain, cochlear implants to improve hearing and hyaluronic acid discs to reconstruct the tympanic membrane. Sometimes also biomaterials are used in rhinoplasty (nose surgery) or larynx surgery. Most biomaterials are implanted in vascular and general surgery where meshes are used for hernia repair, and vessels are repaired by vascular grafts and stents. There are many different types of biomaterials, each leading to a different reaction of the immune system after implantation. This wound healing reaction is named the foreign body reaction. The extend of this reaction depends on the type and design of the biomaterial [2-4].

STAGES OF WOUND HEALING

There are four stages in tissue response to biomaterials after implantation (foreign body reaction): Hemostasis, Inflammation, Proliferation and Remodelling [4,5]. Figure 3 shows a brief overview.

After implantation of a biomaterial, the first stage, hemostasis, immediately starts by adherence of proteins to the biomaterial and the formation of a blood clot. The type and design of the biomaterial can alter the attachment of proteins [2]. From the blood clot, all kinds of cytokines and chemokines are released leading to recruitment of inflammatory cells [2]. This leads to the second stage, inflammation, where neutrophils are the first inflammatory cells that arrive at the wound site [2]. They start cleaning the wound site from pathogens and dead cells. Then mast cells arrive releasing histamine, interleukin-4 (IL-4) and IL-13, thereby attracting monocytes which differentiate into macrophages [2]. Macrophages dominate the wound site after 2 days. These macrophages are mainly pro-inflammatory to promote more recruitment of inflammatory cells and phagocytosis of wound debris. After inflammation, the proliferation stage takes place, where fibroblasts or stem cells are attracted by cytokines produced by macrophages and T-cells. The fibroblasts proliferate and produce matrix creating granulation tissue. Also new blood vessels are formed (angiogenesis), which is stimulated by cytokines released from macrophages and T-cells. In the last stage, remodelling, macrophages orchestrate the breakdown and remodelling of matrix creating a new organized tissue [2,4-6].

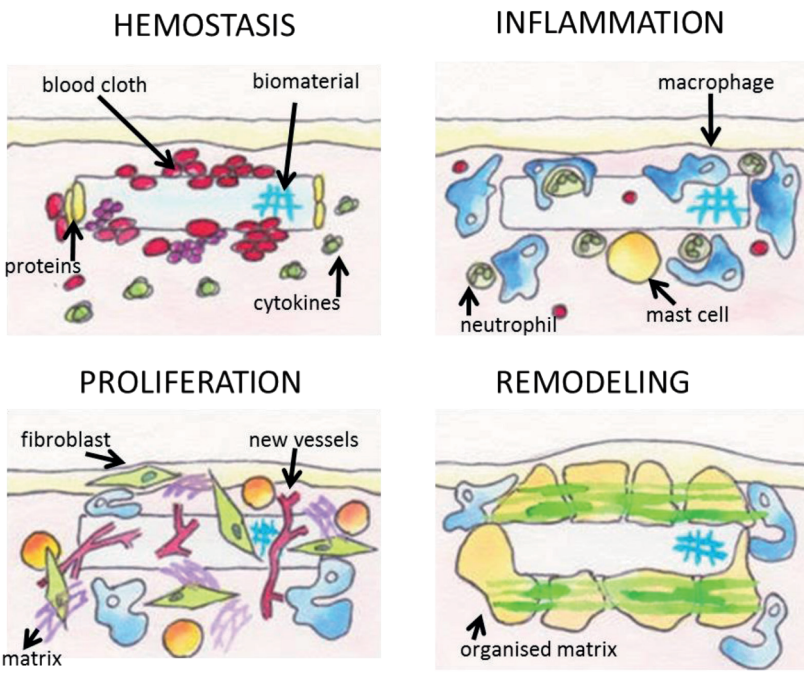

Figure 3 | Stages of wound healing, ©Greet Grotenhuis-Kramer.

Macrophages as key players

Macrophages orchestrate and conduct the foreign body reaction and are therefore key players. In our research we have focused on these cells. The word macrophage is derived from the Greek word for large (μακρος (makros)) and to eat (φαγειν (phagein)). This type of white blood cell is indeed a big eater since it engulfs dead cells, matrix, bacteria and much more, to clean up the wound site and to stimulate and orchestrate the immune system by secretion of factors [5]. Macrophages are resident in many tissues and organs and mostly derive from circulating monocytes which differentiate from hematopoietic stem cells in the bone marrow [5,6]. Macrophages can be roughly divided into two different subtypes: pro- and anti-inflammatory [5,8-10] (Figure 4). In response to the environment, macrophages can differentiate into these subtypes. Pro- inflammatory macrophages (M1 or classically activated) are the first cells to arrive at the wound site for attacking microbes, engulfing dead cells and thereby cleaning up the wound site. These macrophages are very important in host defence [5]. They recognize by pro-inflammatory factors such as interferon (IFN) γ and lipopolysaccharide (LPS) on their Toll-like receptors [6,8-10]. M1 macrophages produce many pro-inflammatory cytokines such as interleukin (IL)-6, tumour necrosis factor (TNF)-α and IL-1β, needed for attraction of more inflammatory cells [8-10]. Alternatively activated macrophages (M2) are a group of tissue repair /regulatory macrophages. These macrophages morphologically appear more round compared to M1 macrophages. The group of M2 macrophages can be subdivided in three types. M2a macrophages are stimulated by IL-4 and IL-13 and recruit eosinophils, basophils

and Th2 cells [8-10]. M2b macrophages are stimulated by immune complexes and work as an immune-regulatory type of macrophage [8-10]. Exposure to IL-10 will induce M2c macrophages which supress the immune response and help tissue remodelling. These macrophages are known as wound healing macrophages [8-10]. The M2 macrophages are conductors, orchestrating the foreign body reaction by stimulating proliferation of fibroblasts, stimulate angiogenesis by producing growth factors and remodelling. They also inhibit pro-inflammatory macrophages [7-10].

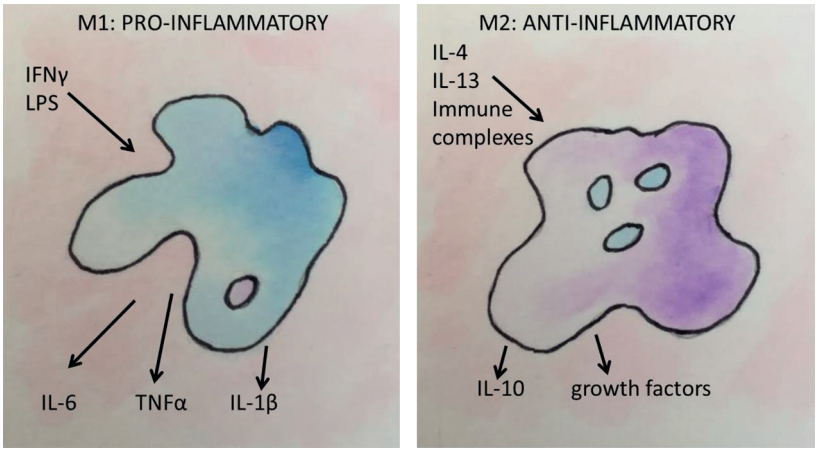

Figure 4 | Subtypes of macrophages © Greet Grotenhuis-Kramer.

Frustrated wound healing

After implantation not always an organized tissue is formed with resolution of inflammation. Sometimes chronic inflammation with excessive matrix production leads to complications as observed in the case report, humps, shrinking of biomaterials or chronic postoperative pain by pressure on nerves do occur. These complications occur in 14−52% of patients [11,12]. Normally during wound healing M1 macrophages are present from the first day on and dominate after two days [2,7]. When most of the debris and pathogens are removed, the macrophages change towards a more M2 phenotype. After implantation, macrophages will try to phagocytose the biomaterial. Single macrophages can phagocytose particles up to 5 µm [2,7]. When the biomaterial is bigger, macrophages will fuse under the influence of IL-4 and IL-13, becoming a giant cell. If the giant cell cannot phagocytose the biomaterial, it forms a capsule trying to protect the body from the biomaterial. In this capsule, they secrete many enzymes such as matrix metalloproteineases (MMPs) to try to resorb and degrade the biomaterial [7]. Under the influence of IL-4 giant cells release pro-fibrotic factors such as transforming growth factor (TGF)β and platelet-derived growth factor (PDGF) [7]. Although the mechanism is not completely understood, it is assumed that this will result in continued fibroblast activation and production of excessive extra-cellular matrix such as collagen, leading to fibrosis [7].

Biomaterials in a contaminated environment

A challenge for implantation of biomaterials is represented by a contaminated environment, such as bowel surgery or replacement of a previously infected mesh. Surgeons are reluctant to place biomaterials in a contaminated environment since the complication rate is higher and the biomaterial must sometimes be removed because of infection [13,14]. How the foreign body reaction differs from a sterile environment is not yet completely understood. It is expected that macrophages mainly are of the M1-phenotype, because the infection and bacteria need to be eliminated [9]. This will delay the foreign body reaction since M1-macrophages inhibit ingrowth of the biomaterial by producing matrix degrading enzymes [4,15]. Rhinoplasty (nose surgery) is also working in a field with a risk of contamination. Therefore very few biomaterials are used, and when used it often leads to complications as shown in the case report at the beginning of this chapter. In some cases, although, a biomaterial could be of great help to restore the function and cosmetics of the nose.

Stem cells in wound healing

Next to macrophages, another important cell in wound healing is represented by the mesenchymal stem cell. These stem cells can help rebuild tissue after trauma by differentiating into many types of cells like fibroblasts, adipose cells, cartilage, bone and so on. These multipotent stem cells are therefore a good cell source for tissue engineering [16]. Mesenchymal stem cells can be harvested from for instance bone marrow and adipose tissue. In adipose (fat) tissue many stem cells can be found, first described by Rodbell in 1964 [17]. Fat tissue is convenient since this tissue is often easily to harvest and culture [18-21]. Adipose derived mesenchymal stem cells (ASCs) are believed to be important in wound healing since these cells can rapidly migrate to the wound site, enhance angiogenesis and stimulate matrix remodelling [22-25]. Furthermore, ASCs can interfere with the immune system (have immunomodulatory capacities) and thereby influence the foreign body reaction [26].

AIMS AND OUTLINE OF THE THESIS

The foreign body reaction differs per biomaterial and per patient. Some patients have complications after implantation of a biomaterial and some have none with the same biomaterial. For clinical practice, it would be a great benefit to have a tailor-made model with patients own cells to test pre-operatively which biomaterial is best thereby reducing complication rates. Since most of the knowledge and use of biomaterials is in general surgery, we will focus our research in this field. The ultimate goal of our research is to develop a tailor-made *in vitro* model with human macrophages. Towards developing this model, the following aims are formulated:

1) to develop an *in vitro* model to study the effect of biomaterials on human macrophage polarisation
2) to investigate the influence of biomaterials on macrophage phenotype in an inflammatory environment and whether this is biomaterial-dependent

3) to investigate the influence of biomaterials on stem cells and macrophages together in an adjusted *in vitro* model

In the last decade, the role of macrophages in reaction to biomaterials is more and more investigated *in vivo* and *in vitro*.

In **Chapter 2** a review of the literature on polarisation of macrophages as response to biomaterials *in vitro* in a sterile environment is presented.

In **Chapter 3** a new model with human monocytes from peripheral blood in response to four different biomaterials and the outcome parameters of the model is described.

Another challenging field in reconstructive surgery is a contaminated environment. However, how biomaterials perform in this environment is not completely known. In **Chapter 4** an *in vivo* study is described in which in a contaminated environment in rats 7 different biomaterials are implanted. In this new model the complications like adhesions, incorporation and infection (Chapter 4a) and polarisation of macrophages (Chapter 4b) by immunohistochemistry is analysed.

In **Chapter 5** an adaptation of the *in vitro* model is reported. With pro-inflammatory cytokines a contaminated environment is mimicked to investigate the reaction of macrophages to biomaterials in more detail.

Another important factor in wound healing is the production of matrix. In **Chapter 6** the *in vitro* model is extended to a co-culture system with adipose tissue derived stem cells (ASCs) to investigate the influence of biomaterials on the interaction of stem cells and macrophages.

In **Chapter 7** the overarching themes of this thesis and proposed future research topics are discussed.

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CHAPTER 2

The effect of biomaterials used for tissue regeneration purposes on polarisation of macrophages

Boersema GSA, Grotenhuis N, Bayon Y, Lange JF, Bastiaansen-Jenniskens YM (2016) The effect of biomaterials used for tissue regeneration purposes on polarization of macrophages, BioResearch Open Access 5:1, 6–14, DOI: 10.1089/biores.2015.0041.

ABSTRACT

Activation of macrophages is critical in the acute phase of wound healing after implantation of surgical biomaterials. To understand the response of macrophages, they are often cultured *in vitro* on biomaterials. Since a wide range of biomaterials is currently used in the clinics, we undertook a systematic review of the macrophage polarization in response to these different surgical biomaterials *in vitro*. Beside the chemistry, material characteristics such as dimension, pore size, and surface topography are of great influence on the response of macrophages. The macrophage response also appears to depend on the differences in sterilization techniques that induce lasting biochemical changes or residues of chemicals and their byproducts used for sterilization. Regarding tissue-based biomaterials, macrophages on human or porcine dermis, strongly cross-linked by chemicals elicit in general a pro-inflammatory response with higher amounts of pro-inflammatory cytokines. Synthetic biomaterials such as polyethylene, polyethylene terephthalate (PET) + polyacrylamide (PAAm), PET + sodium salt of poly(acrylic acid) (PAANa), perfluoropolyether (PFPE) with large fibers, PEG-g-PA, and polydioxanone (PDO) always appear to elicit an anti-inflammatory response in macrophages, irrespective of origin of the macrophages, for example, buffy coats or full blood. In conclusion, in general *in vitro* models contribute to evaluate the foreign body reaction on surgical biomaterials. Although it is difficult to simulate complexity of host response elicited by biomaterials, after their surgical implantation, an *in vitro* model gives indications of the initial foreign body response and allows the comparison of this response between biomaterials.

INTRODUCTION

A wide range of biomaterials are used as implantable medical devices, notably for soft tissue repair. These materials have their own characteristics with regards to composition, mechanical strength, topography, porosity, and chemistry. Implantation of biomaterials is always associated with tissue damage, more or less important, according to the invasiveness of the surgical procedure, that is, surgical treatment of the disease and biomaterial delivery. Initially, the body response most often starts with blood coagulation followed by wound healing. This process is characterized by protein adsorption to the biomaterial, followed by recruitment of cells including macrophages already 60 min after implantation of the material. In response to the cytokines and chemokines produced by the macrophages, cells involved in wound healing are attracted [1]. The inflammatory response is very important following surgical tissue damage and material implantation, also called foreign body reaction.

Activation of macrophages is critical in the acute phase of wound healing [2,3]. Macrophages can be roughly divided into pro-inflammatory macrophages, also called M1 macrophages, and anti-inflammatory macrophages, also called M2 macrophages [4,5]. The balance between M1 and M2 plays a critical role in the phagocytosis of pathogens, the clearance of apoptotic cells and the healing and remodelling of injured tissues [6].

Almost immediately after implantation, macrophages are recruited to biomaterials. Depending on the biomaterial specific characteristics, these macrophages will determine the type and intensity of the host response [6,7]. The eventual success of an implantable medical device strongly depends on this response. The host response after implantation is inter alia guided by soluble factors such as cytokines and growth factors, as communication agents between cells, active in the wound healing process. Several studies point out the cytokine classification according to their role in the foreign body response [8-10]. These soluble factors are, among other cell types, produced by macrophages and play pivotal roles in wound healing and serve as useful markers of M1/M2 activation [7,10-12].

The pivotal role of macrophages in the wound healing process, including tissue repair or regeneration supported by biomaterials, is a strong incentive to interrogate the macrophage response, elicited by biomaterials, in well-defined *in vitro* conditions, with reasonable prediction of the complex foreign body reaction by using simplified single cell approaches. For this purpose, human monocyte-derived macrophages, human monocyte cell lines, mouse bone marrow-derived macrophages, and murine macrophage cell lines are used as culture models. In these models, it is examined whether biomaterials elicit a pro-inflammatory, anti-inflammatory, pro-wound healing, or an anti-wound healing response by macrophages. These models support the first step to analyse materials before use in the clinic. As nicely reviewed by Sridharan et al. [1] many different properties of the material influence the polarization of the macrophage, among others the mechanical properties, topography, and surface chemistry. Since many types of biomaterials are used in many different culture models with a large variety of read-out parameters, the purpose of this review was to provide an overview of which biomaterial leads to which response, in particular regarding the differentiation and activation of the macrophages and the associated production of soluble factors.

MATERIALS AND METHODS

Search methods

This systematic review was conducted in accordance to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines.

Search strategy and study selection

On the 29th of June 2015 a systematic literature search was performed using Medline, EMBASE, Cochrane, PUBMED, Google Scholar, and Web-of-Science libraries (Supplementary Appendix S1). There were no restrictions used during the search based on the publication year, publication language, and type of study. Two researchers (G.S.A.B. and N.G.) screened all titles and abstracts of the identified articles independently for their relevance. From all articles that possibly met the inclusion criteria, the full-text version was retrieved and assessed for inclusion. Disagreement was resolved by discussion or requesting advice from a third author (Y.M.B.J.).

An article was eligible for inclusion when it reported on macrophages and their response to biomaterials in an *in vitro* model. Presentations, reviews, and letters to the editor were not included. All references from the selected articles were screened for further possible inclusions.

Data extraction and analysis

The extracted data are presented in separate tables. The following information was retrieved from each study: first author, year of publication, culture model, biomaterial, and cytokine expression. A meta-analysis could not be performed due to the lack of sufficient comparative studies and the important variability of the *in vitro* macrophage models (e.g., cell origin and isolation procedure, culture conditions, markers).

RESULTS

Search

After the exclusion of 2904 duplicates we identified 4275 references. After screening the titles and abstracts, we excluded another 4169 articles. The other 106 articles were regarded relevant and evaluated as full text. After careful reviewing the full text, another 90 were excluded. In addition seven articles were included via references, resulting in 23 included articles (Figure 1).

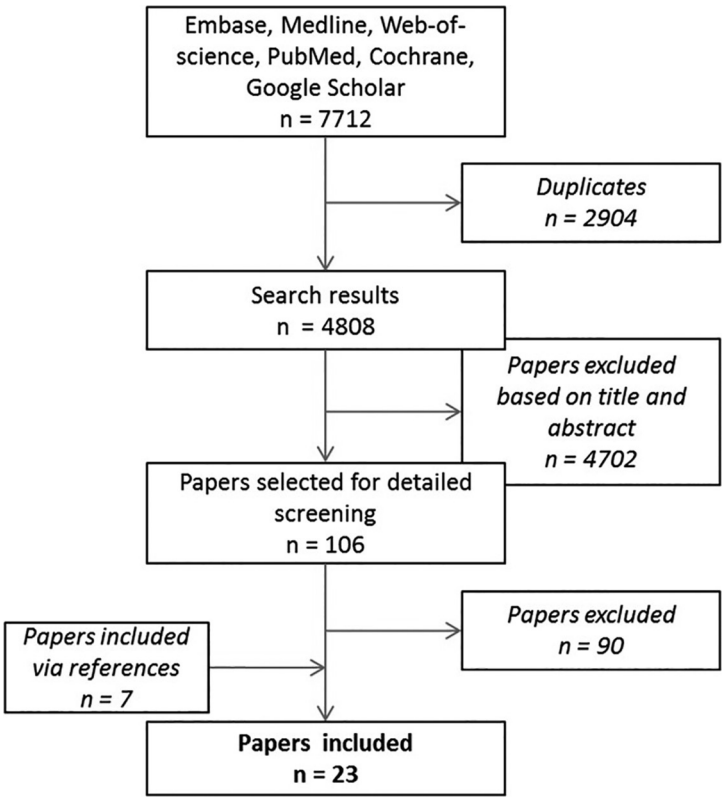

Figure 1 | Study selection for relevant articles.

Culture models/experimental conditions

All included studies cultured monocytes or macrophages on biomaterials. However, substantial differences were found in cell culture conditions between the studies. Monocytes isolated from a human buffy coat or human peripheral blood were used in 19/23 of the studies [7,8,11,13-28]. In the other four studies, one used monocytes derived from mouse bone marrow [4], one used the RAW 264.7 cell line (mouse leukaemia monocyte macrophage cell line) [9] and the other two used the THP-1 human monocyte cell line [10,29] (Table 1).

Table 1 | Included Studies Cultured Monocytes or Macrophages on Biomaterials, aTHP human leukemic monocyte. bRAW/J744 murine macrophage cell line.

Author	Year	Cells
Almeida et al. [13]	2014	Human buffy coat
Ballotta et al. [28]	2014	Human buffy coat
Bartneck et al.[14]	2010	Human peripheral blood
Bartneck et al. [15]	2012	Human peripheral blood
Bhardwaj et al. [16]	2001	Human buffy coat
Bhattacharjee et al. [17]	2013	Human peripheral blood
Bota et al. [18]	2010	Human peripheral blood
Brodbeck et al. [8]	2002	Human peripheral blood
DeFife et al. [19]	1995	Human peripheral blood
Fearing et al. [29]	2014	THP-1 cell line[a]
Garg et al. [4]	2013	Mouse bone marrow-derived Mφ
Gretzer et al. [20]	2003	Human buffy coat
Grotenhuis et al. [7]	2013	Human buffy coat
Jones et al. [21,31]	2007	Human peripheral blood
Oliveira et al. [22]	2012	Human buffy coat
Orenstein et al. [23]	2009	Human peripheral blood
Orenstein et al. [24,25]	2010	Human peripheral blood
Schachtrupp et al. [11]	2003	Human buffy coat
Schutte et al. [10]	2009	THP-1 cell line[a]
Spiller et al. [26]	2014	Human buffy coat
Van den Beucken et al. [9]	2007	RAW 264.7 & J744A.1[b]
Wagner et al. [27]	2003	Human peripheral blood

In most of the studies, no additional factors were added to the culture medium. However, some also added soluble factors to the media. Medium with lipopolysaccharides (LPS) was the most common, but media also contained LPS/interferon gamma (IFN-γ), interleukin (IL)-4, IL-4/IL-13, or monocyte chemotactic protein (MCP)-1/IL-6/IFN-γ. The culture time varied from 2 h to 14 days, but the majority cultured for 1, 3, 7, and/or 10 days.

Biomaterials

Biomaterials can be divided into three groups namely the nonbiodegradable polymers (synthetic), biodegradable polymers (synthetic), and biologic materials [30]. In total 35 different materials were used in the included articles (Table 2).

Table 2 | Reviewed Biomaterials and Their Predominant Reaction. This table shows results coming from different macrophage models, not necessarily equivalents. The results are adapted generally from one study.

Biomaterial	Predominant reaction of macrophages in contact with biomaterial	Low/high cytokine production	Refs.
PTFE	Mainly pro-inflammatory	High	10,15
ePTFE	Pro- and anti-inflammatory	High/high	9,17,25
PET	Mainly pro-inflammatory	High	6,7,20
PET + BDEDTC	Mainly pro-inflammatory	High	7,20
PET + BDEDTC + PAAm	Mainly anti-inflammatory	High	7,20
PET + BDEDTC + PAANa	Mainly anti-inflammatory	High	7,20
PET + BDEDTC + DMAPAAmMeI	Mainly pro-inflammatory	High	7,20
Parietex™ Composite	Pro- and anti-inflammatory	High/high	6
Polyethylene	Mainly anti-inflammatory	Low	9,18
Polyurethane	Pro- and anti-inflammatory	High/high	9,15,18
PFPE (small fibers)	Mainly pro-inflammatory	High	13
PFPE (large fibers)	Mainly anti-inflammatory	High	13
PP	Pro- and anti-inflammatory	Low/low	6,26
PP + polyglactin	Mainly pro-inflammatory	High	10
Poly(ethylene glycol):poly(acrylate)	Mainly anti-inflammatory	Low	27
Poly-d-lysine-PAH	Mainly pro-inflammatory	Low	8
Silicone	Pro- and anti-inflammatory	High/high	15
Polylactic acid	Pro- and anti-inflammatory	High/high	12
Poly(ethylene oxide)	Mainly pro-inflammatory	High	14
Bio-A	Mainly pro-inflammatory	Low	23
Polydioxanone	Mainly anti-inflammatory	High	3
Poly-e-caprolactone bisurea	Mainly anti-inflammatory	High	27
Poly(urethane urea)	Pro- and anti-inflammatory	Low/low	19
Collamend™	Mainly pro-inflammatory	High	24
Permacol™	Mainly pro-inflammatory/ pro- and anti-inflammatory	High/low	6,24
Allomax	Mainly pro-inflammatory	High	22,23
FlexHD	Mainly pro-inflammatory	High	22,23
Alloderm	Mainly pro-inflammatory	Low	22,23
Strattice™	Mainly pro-inflammatory	Low	24
Surgisis®	Mainly pro-inflammatory	Low	24
Collagen coating	Mainly pro-inflammatory	High	28
Ultrafoam	Mainly pro-inflammatory	Low	16,25
Silk	Mainly pro-inflammatory	High	16
Keratin	Pro- and anti-inflammatory	Low/high	28
Chitosan	Pro- and anti-inflammatory	Low/high	12,21

BDEDTC=poly(styrene-co-benzyl N,N-diethyldithiocarbamate); DMAPAAmMeI=methyl iodide of poly[3-(dimethylamino)propyl] acrylamide; ePTFE=expanded polytetrafluoroethylene; PAAm=polyacrylamide; PAANa=sodium salt of poly(acrylic acid); PFPE= perfluoropolyether; PET=polyethylene terephthalate; PP=polypropylene; PTFE=polytetrafluoroethylene.

Nonbiodegradable synthetic polymers

Expanded polytetrafluoroethylene (ePTFE) and polytetrafluoroethylene (PTFE) are commonly applied hernia mesh and vascular grafts materials. PTFE, also known as Teflon®, is naturally hydrophobic and nonporous. ePTFE is stretched and nano-porous and was introduced under the trademark GORE-TEX®, in 1969. Monocytes (precursors of macrophages) on PTFE produced low amounts of IL-1β and high amounts of tumor necrosis factor (TNF)-α and IL-6 in the first days of culture. IL-10 levels increased during culture time, it was mainly produced between culture day 2 and 6 [11,16]. After a culture time for 8–10 days the production of TNF-α and IL-10 decreased, while IL-8 increased after 8 days of culture [16]. Granulocyte-macrophage colony-stimulating factor (GM-CSF) was secreted during the whole culture time (1–10 days) [11,16,18]. Macrophages on PTFE also produced platelet-derived growth factor-BB, and matrix metalloproteinase 9 but vascular endothelial growth factor (VEGF) was undetectable [26]. Macrophages on ePTFE produced more pro-inflammatory cytokines (IL-1α, IL-1β, IL-6, and TNF-α) and chemokines (MCP-1, MIP1-β, and MCP-3) in association with an increase of the pore size of the material [18]. In contrast, immortalized human monocyte cell line (THP-1) cultured on ePTFE induced an anti-inflammatory and pro-wound healing profile characterized by a high IL-10 production in another study [10].

Current surgical applications of polyethylene terephthalate (PET) are surgical meshes, vascular grafts, heart valves, and sutures. Macrophages on PET produce predominantly pro-inflammatory cytokines, MCP-3, TNF-α, IL-6, IL-1β, MIP-1α,7,8,31 and pro-inflammatory chemokine IL-8 [31].

PET is also used in combination with different "coatings." These coatings affect biomaterial adherent monocyte/macrophage cytokine expression through modification of surface chemistry. Different coatings are used: PET + poly(styrene-co-benzyl N,N-diethyldithiocarbamate) (BDEDTC; hydrophobic), PET + BDEDTC + polyacrylamide (PAAm; hydrophilic and neutral), PET + BDEDTC + sodium salt of poly(acrylic acid) (PAANa; hydrophilic and anionic), PET + BDEDTC + methyl iodide of poly[3-(dimethylamino)propyl]acrylamide (DMAPAAmMeI; hydrophilic and cationic), and PET + absorbable, continuous and hydrophilic collagen film (Parietex™ Composite). Macrophages on PAAm and PAANa surfaces reacted anti-inflammatory with a higher IL-10 production and lower IL-8 production than when cultured in PET without coating during the culture time from day 3 till day 10 [8,31]. Monocytes adherent to PAAm produced the most IL-6, IL-1β, IL-10, IL-8, and MIP-1β at all time points, compared to the other coatings in combination with PET [31]. Macrophages cultured for 3 to 7 days, produced the highest concentrations of IL-1β on PAAm and least on BDEDTC. MIP-1β concentrations were greatest with PAANa at day 3. DMAPAAmMeI promoted a decrease of IL-10 and IL-1RA in macrophages, but it did not influence the expression levels of IL-1β, TNF-α, and IL-6 [7,29]. BDEDTC, PAAm, PAANa, and DMAPAAmMeI let the IL-1β, TNF-α, and IL-6 expression levels relatively unchanged at the end of culture time [8,31]. Parietex Composite (Covidien) induced high levels of pro-inflammatory and anti-inflammatory proteins [7].

Macrophages cultured on polyethylene (PE), with versatile use such as catheters and joint prosthesis, produced low amounts of cytokines in general but the balance was more toward anti-inflammatory and pro-wound healing cytokines [10,19]. Both THP-1 cell line monocytes/macrophages and macrophages isolated from human buffy coats cultured on polyurethane (PU), often used in blood contact applications, produced high levels of anti-inflammatory and pro-wound

healing cytokines [10,16]. Perfluoropolyether (PFPE) is a nondegradable homopolymer that shows chemical inertness, lipophobicity, and has very low surface energy [14]. This material was tested with different micro topographies and the effect on the response of macrophages. Different surface topographies resulted in different cytokine production by macrophages. An M1 surface marker, 27E10, had an enhanced expression in response to closely packed small fibers, comparable to when macrophages were stimulated with LPS. In contrast, macrophages cultured on PFPE with large fibers expressed the M2 surface marker CD163 the most. Large fibers also resulted in significantly the highest M2-M1 index based on macrophages surface markers [14].

Poly(propylene) (PP) is also commonly used mesh and suture materials in surgery. Both an anti-inflammatory reaction characterized by high levels of CCL-18 and IL1-RA among others and a pro-inflammatory reaction characterized by production of IL-8, IL-6, and IL-1β by macrophages seeded both for 24 h or 3 days on PP were observed [7,27]. When combined with polyglactin 910 materials (Vypro II®; Ethicon), monocyte/macrophages also released high amounts of TNF-α, IL-6, and low amounts of IL-10 after 5 days of culture, which indicates a pro-inflammatory response [11].

Poly(ethylene glycol):poly(acrylate) PEG-g-PA is also modified with cell adhesion promoting peptides (YRGDS and YEILDV, peptides recognized by integrins) to modulate the host cell response [27]. Culturing macrophages on PEG alone resulted in low production of TNF-α, IL-1β, IL-6, and IL-8. Macrophages on peptide modified PEG-g-PA produced even lower levels of TNF-α and IL-6 [27].

Poly-d-lysine (PDL) and poly(allylamine hydrochloride) (PAH), both synthetic polymers, were coated with DNA and seeded with two different cell line macrophages. All experiments showed decreased levels of TNF-α compared with the cultured polymers with LPS-stimulated murine macrophages (density of 1×105 cells/cm^2) [9]. The cytokine secretion of IL-1β, IL-10, and TGF-β1 was not different between macrophages cultured on PDL and PAH with or without LPS stimulation [9]. Monocytes on silicone cultured for 10 days produced high GM-CSF and IL-8 [16]. TNF-α and IL-10 were produced at high levels the first 2–6 days, where after the production decreased [16].

Biodegradable synthetic polymers

Synthetic biodegradable polymers were first used as biodegradable sutures in the 1960s. Synthetic biodegradable implants are mostly used in the clinic as soft/hard tissue reinforcement materials or temporary barriers/wound supports. Their purpose is to avoid a chronic foreign body reaction [32]. These polymeric biomaterials are based on lactic acid and glycolic acid, and other monomers, including dioxanone and trimethylene carbonate e-caprolactone as homopolymers and copolymers.

Polylactic acid (PLA) induces production of IL-6, IL-12/23, and IL-10, these cytokines are both pro-inflammatory and anti-inflammatory, it appeared like human monocytes cultured on PLA exhibited a heterogeneous profile [13].

Poly(D,L-lactide-co-glycolide) (PLGA) represents a major class of materials widely used in surgical applications and tissue engineering [15]. Bartneck et al. generated 3D nano-fibrous meshes in different porosities PLGA/sP(EO-stat-PO) and a 2D NCO-sP(EO-stat-PO) hydrogel. NCO-sP(EO-stat-PO) and sP(EO-stat-PO) are ethylene oxide-derived polymers, used for preventing unspecific protein adsorption and cell adhesion [15].

Macrophages on the 2D materials formed clusters with an elevated release of IL-1β and TNF-α. Macrophages produced more IL-8 and CCL-4 (proangiogenic chemokines) on the more covered 3D nanofibers PLGA/sP(EO-stat-PO) [15].

Macrophages seeded on a copolymer of glycolic acid and trimethylene carbonate, also known as GORE® BIO-A® Tissue Reinforcement (WL Gore Assoc), produced very low pro-inflammatory cytokine levels [24]. Polydioxanone (PDO) polymer is developed for biodegradable wound closure sutures. Bone marrow-derived macrophages were cultured on different PDO diameter fibers and pore sizes. An increase of the fiber/pore size resulted in an increased expression of anti-inflammatory and angiogenic markers as VEGF, TGF-β, and FGF2 [4].

The impact of mechanical cues on adherent monocytes on poly-e-caprolactone bisurea (PCL-U4U) was investigated. It has been demonstrated that strain affects macrophage response in terms of signaling and differentiation. Moderate strain (7%) elicits polarization toward a reparative M2 profile and enhance the expression of genes participating in the immune response [28].

Poly(urethane urea) elicited very small amounts of TNF-α and IL-10 [20].

Biologic materials

Biologic materials are either decellularized tissues such as human or porcine skin or porcine small intestine submucosa (SIS), or fabricated scaffolds or meshes made of natural molecules such as collagen, chitosan, silk, or keratin. The decellularized tissues can have additional chemical cross-links to alter the degradation speed [33].

After 7 days of culture CollaMend™ FM Implant (Bard/Davol), a moderately chemically cross-linked porcine dermis, mostly elicited a pro-inflammatory response in macrophages with high IL-1β, IL-6, IL-8, and VEGF production [25]. Macrophages on Permacol™ (Covidien), a slightly chemically cross-linked porcine dermal matrix, produced high IL-1β, IL-6, IL-8, and VEGF levels after 7 days of culture [25]. But in other settings, low levels of both pro-inflammatory and anti-inflammatory proteins after 3 days of culture, were released by macrophages, in the presence of Permacol [7]. There were no differences in culture method between the two studies. AlloMax™ Surgical Graft (Bard/Davol) and FlexHD® (Ethicon), nonchemically cross-linked decellularized dermis but of human instead of porcine origin, also induced mainly pro-inflammatory reactions with high IL-1β, IL-6, IL-8, and VEGF cytokine production [23,24]. AlloDerm® Regenerative Tissue Matrix (LifeCell) (nonchemically cross-linked decellularized human dermis) induced a lower pro-inflammatory response than the other decellularized human dermis, characterized by lower expression of IL-1β, IL-6, IL-8, and VEGF [23,24]. Macrophages seeded on the noncross-linked porcine dermis, Strattice™ (LifeCell), or on the noncross-linked porcine SIS, Cook® Biodesign® Surgisis® (Cook), produced low levels of IL-1β, IL-6, IL-8, and VEGF [25].

Macrophages cultured on collagen coatings expressed mostly M1 surface markers (CD86+) and express both M1 and M2 markers [29,34]. These macrophages produced also high levels of pro-inflammatory cytokines. Another collagen-based biomaterial is Avitene™ UltraFoam™ Collagen Sponge (Bard/Davol; bovine source collagen sponge). Macrophages cultured on this gel did not produce IL-1β, and IL-6 production was only seen at day 1 and was lower produced at day 3, indicating that the response of the macrophages was not pro-inflammatory [17].

Other noncommercial biopolymers have been investigated. Bhattacharjee et al. studied the macrophage responses against silk-fibroin and silk-sericin-based 2D films, and 3D silk-fibroin scaffolds [17]. These scaffolds are used for tissue engineering and drug delivery. The 3D fibroin scaffold induced gene expression of pro-inflammatory genes and accordingly the production of IL-1β and IL-6. Silk-sericin films also induced IL1-β gene expression [17].

Two other biologic biomaterials are keratin and chitosan. Keratin has been described for applications such as tissue regeneration, hemostasis, and wound healing. A low foreign body reaction against keratin was described characterized with predominantly M2 (CD206+) macrophages, high levels of IL-10, and low levels of IL-1β and IL-6 [29]. Chitosan (a natural polysaccharide composed of randomly distributed β-(1–4)-linked d-glucosamine and N-acetyl-d-glucosamine) induced an M2 phenotype in one study based on low TNF-α that decreased with time and high IL-10 and TGF-β1 levels cytokines [22]. In another study chitosan induces a predominant M1 response based on high production of TNF-α and IL-12/IL-23 and low expression of IL-6, especially in the 3D geometry [13]. Oliveira et al. cultured on chitosan films instead of 3D geometry [13].

DISCUSSION

Macrophages are key components of tissue repair and remodeling in wound healing. Their polarization appears to depend on the type of biomaterial and their characteristics. The release of a variety of cytokines and chemokines is decisive for the differentiation and activity of monocytes [35]. Here, we reviewed the macrophage response on different materials *in vitro* used in tissue repair and regeneration and provided an overview of commonly seen macrophage responses to these biomaterials.

Based on the literature review, we have shown that the dimensions of the cultured material is of great influence on the response of macrophages. This was (mostly) investigated in PFPE, ePTFE, chitosan, and PDO. The association was, however, different between increasing fiber/pore size and the polarization or release profile of macrophages. Two synthetic biomaterials showed the opposite effect of pore size. Bartneck et al. showed a higher pro-inflammatory effect when the pore size was smaller in PFPE [14]. Bota et al. saw a higher pro-inflammatory effect of macrophages cultured on ePTFE when the pores are larger [18]. Almeida et al. saw the same effect, on scaffolds based on chitosan, a biologic material [13]. In contrast, Garg et al. cultured macrophages on PDO, a synthetic biodegradable material, and they showed that large pores induced M2 phenotype and a decreased M1-marker expression. However, in this study, mouse bone marrow-derived macrophages were used instead of human macrophages. In an *in vivo* study with biodegradable pHEMA (2-hydroxyethyl methacrylate) hydrogel scaffolds it was also shown that pore size affect macrophage response. Pore size of 34 µm was shown to reduce fibrous encapsulation, however, more M1 cells were found than at those scaffolds with a larger pore size of 160 µm, this indicate that the initial M1 response is necessary [36].

As expected, macrophages on moderately chemically cross-linked human or porcine dermis responded in general pro-inflammatory with higher amounts of pro-inflammatory cytokines

than the macrophages cultured on nonchemically cross-linked or slightly chemically cross-linked materials. This was also seen in *in vivo* studies were Collamend™ FM Implant (Bard/Davol) induced a chronic foreign body response and downstream encapsulation [37,38]. This mainly pro-inflammatory response lead to chronic fibrosis [39]. Unfortunately, in all *in vitro* studies on these biologic materials, only investigated IL-1β, IL-6, IL-8, and VEGF, known for their mainly pro-inflammatory response, no anti-inflammatory cytokines were measured. A recent review presented that moderately to strongly cross-linked collagen materials can alter normal wound healing. In particular, residues of chemical cross-links in the material were associated with a M1 macrophage response, and inhibition of M2 macrophage polarization [33].

Chitosan, another biopolymer, showed a predominant M1 response with a very low IL-6 production [13]. The same effect was seen on the collagen gel; mainly pro-inflammatory cytokines were produced, but no production of IL-1β [17]. This can be considered a pleiotropic function of IL-6 and IL-1β. It is known that IL-6 can act either pro-inflammatory or anti-inflammatory, depending on the environment [40]. IL1-β is a key cytokine that is important for wound healing, activating and recruiting fibroblasts, resulting in expression of extracellular matrix components like collagen, elastin, and glycosaminoglycans [41-43].

Some materials induced different responses in different experiments such as acellular human dermis from different companies. This could be due to the differences in sterilization techniques that induce lasting biochemical changes or residues of the chemical used for sterilization; gamma radiation is used for AlloMax Surgical Graft (Bard Davol); FlexHD (Ethicon) is sterilized by detergents, disinfectants, and ethanol; and the sterilization process of AlloDerm Regenerative Tissue Matrix is proprietary. AlloDerm induced the least of the pro-inflammatory cytokines. Also, the methods of decellularization and processing of the materials were different, which can be an additional explanation for the different foreign body responses, notably explained by chemical residues, used for decellularization and fat removal.

Comparing all the responses of the different materials, it appears that polyethylene, PET + PAAm, PET + PAANa, PFPE (large fibers), PEG-g-PA, and PDO always elicited an anti-inflammatory response in macrophages, irrespective of origin of the macrophages.

In vitro testing of macrophage response to biomaterial can be an initial means of assaying biocompatibility. Macrophages are certainly great drivers of the acute inflammation reaction. Neutrophils (polymorphonuclear leukocytes [PMNs]) also characterize acute inflammatory response. Mast cell degranulation with histamine release and fibrinogen adsorption is also known to mediate acute inflammatory responses to implanted biomaterials [44,45]. For a complete *in vitro* model, these factors should also be taken into account. For example, Surgisis is known to strongly activate PMNs, particularly neutrophils [46]. Bryan et al. show a strong release of Reactive Oxygen Species by Surgisis versus Alloderm and Permacol, in animal models [47].

In general, *in vitro* models are useful in the first step to evaluate the foreign body reaction on surgical biomaterials. Although it is difficult to simulate the environment during a surgical procedure, an *in vitro* model gives an indication of the initial foreign body response even in an environment that simulates an infection by, for instance, addition of LPS. Grotenhuis et al. proved this by simulating a bacterial infection in their *in vitro* model, but the macrophage response remained biomaterial

dependent [48]. In this perspective it will be useful to test, for example, other surgical biomaterials like tissue adhesives that are used in the clinic.

Because of the complexity of host response to foreign body material it is difficult to predict the *in vivo* outcome from *in vitro* assays. Wolf et al. developed an in silico analysis by using an *in vitro* assay that characterized the dynamic inflammatory response of human monocyte-derived macrophages to biomaterials in combination with quasi-mechanistic analysis [49]. This approach can be used to better predict the *in vivo* response. More sophisticated systems, like multicellular approaches combining macrophages with fibroblasts, endothelial cells, and stem cells, aiming at recreating a better mimicking system, should certainly be useful for the in-depth investigation of the behavior of materials *in vivo* [50].

Simple models as single cell approaches should be used for screening approaches, enabling the direct comparison of materials. Macrophage models can gain even higher interest by including monocytes from specific patient groups, like obesity, which may react differently to materials.

CONCLUSION

With this review, we provided an overview of *in vitro* responses of macrophages to many different biomaterials. Some materials performed comparable in different studies and it appears clear which response these biomaterials elicit in macrophages. Other materials behaved differently in different culture setups. Therefore, all physical properties (e.g., stiffness, pore size, strain, topography, and surface chemistry) of the biomaterial must be announced, because these features can induce different macrophage behaviour [1,39]. Each step in cell culture is critical, the macrophage isolation, activation of the macrophage before culture or not, time duration of cell culture since it conditions the phenotype/differentiation of cells, and the type of culture medium, minimal changes in culture methods can cause the different outcome [2,35,51,52]. *In vitro* culture models using macrophages on biomaterials are a valuable addition to the development of new biomaterials. Based on this review there is, however, a need for standardized culture models and a systematic comparison to the *in vivo* response.

SUPPLEMENTARY MATERIAL

We used the following search strategy in the Embase, and the searching strategy was modified in other databases accordingly. ('tissue adhesive'/exp OR 'adhesive agent'/de OR 'surgical mesh'/de OR 'surgical equipment'/de OR 'tissue scaffold'/de OR biomaterial/de OR (adhesive* OR glue* OR bioglue* OR tachocomb* OR bucrilate* OR enbucrilate* OR cyanoacryl* OR mesh* OR 4DDOME OR AIGISRx OR AlloDerm OR AlloMax OR 'Bard Composix EX patch' OR 'BIO-A Tissue Reinforcement prosthesis' OR CollaMend OR DermaMatrix OR Dual-Mesh OR 'Evolution P3EM' OR FasLata OR FlexHD OR FortaGen OR 'IntePro Lite' OR InteXen OR NEOVEIL OR 'Optilene Mesh LP' OR 'Parietex composite' OR Pelvicol OR Pelvisoft OR Pelvitex OR PerFix OR 'Peri-Strips Dry' OR PeriGuard OR Permacol OR Physiomesh OR Strattice OR Surgisis OR TIGR OR Timesh OR 'TiMESH light' OR Tutomesh OR Tutopatch OR Ultrapro OR Ventralex OR Veritas OR Vivosorb OR Vypro OR X-Repair OR XenMatrix OR scaffold* OR biomaterial* OR biocompatib* OR Hemocompatib* OR Haemocompatib* OR resorbable OR (implant* NEAR/3 integrat*)):ab,ti) AND ('macrophage culture'/de OR 'monocyte culture'/de OR ((macrophage/exp OR 'macrophage activation'/de OR monocyte/de OR (macrophag* OR monocyte*):ab,ti) AND ('in vitro study'/exp OR monoculture/de OR ('in vitro' OR culture* OR monocultur*):ab,ti))) NOT ([Conference Abstract]/lim OR [Conference Paper]/lim OR [Review]/lim OR [Conference Review]/lim OR [Letter]/lim OR [Note]/lim OR [Editorial]/lim) AND [english]/lim

ABBREVIATIONS USED

BDEDTC	poly(styrene-co-benzyl N,N-diethyldithiocarbamate)
DMAPAAmMeI	methyl iodide of poly[3-(dimethylamino)propyl]acrylamide
ePTFE	expanded polytetrafluoroethylene
GM-CSF	granulocyte-macrophage colony-stimulating factor
IFN-γ	interferon gamma
IL	interleukin
LPS	lipopolysaccharides
MCP	monocyte chemotactic protein
PAAm	polyacrylamide
PAANa	sodium salt of poly(acrylic acid)
PAH	poly(allylamine hydrochloride)
PDL	poly-d-lysine
PDO	polydioxanone
PFPE	perfluoropolyether
PLA	polylactic acid
PLGA	poly(D,L-lactide-co-glycolide)
PMNs	polymorphonuclear leukocytes
PP	polypropylene
PTFE	polytetrafluoroethylene
SIS	small intestine submucosa
TNF	tumor necrosis factor
VEGF	vascular endothelial growth factor

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CHAPTER 3

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.

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 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.

MATERIALS AND METHODS

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 1000g 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 × 106 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).

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.

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.

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 TGGCCGTA TGCCGGTCACTGTTA, Rev ACTTGTGAGGTCACCGCCTTCCT; TNFα Fw GCCGCATCGCCGTCTCCTAC, Rev AGCGCTGAGTCGGTCACCCT (all Eurogentec). Relative gene expression was calculated using the 2−ΔCT method [24].

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 cyto-kines 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.

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 $p < 0.05$.

RESULTS

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 *tnfa* 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 (Figure 1).

After this confirmation of the pro-inflammatory (M1) and anti-inflammatory/repair (M2) subtype of macrophages we analysed 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 (Figure 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 (Figure 2B).

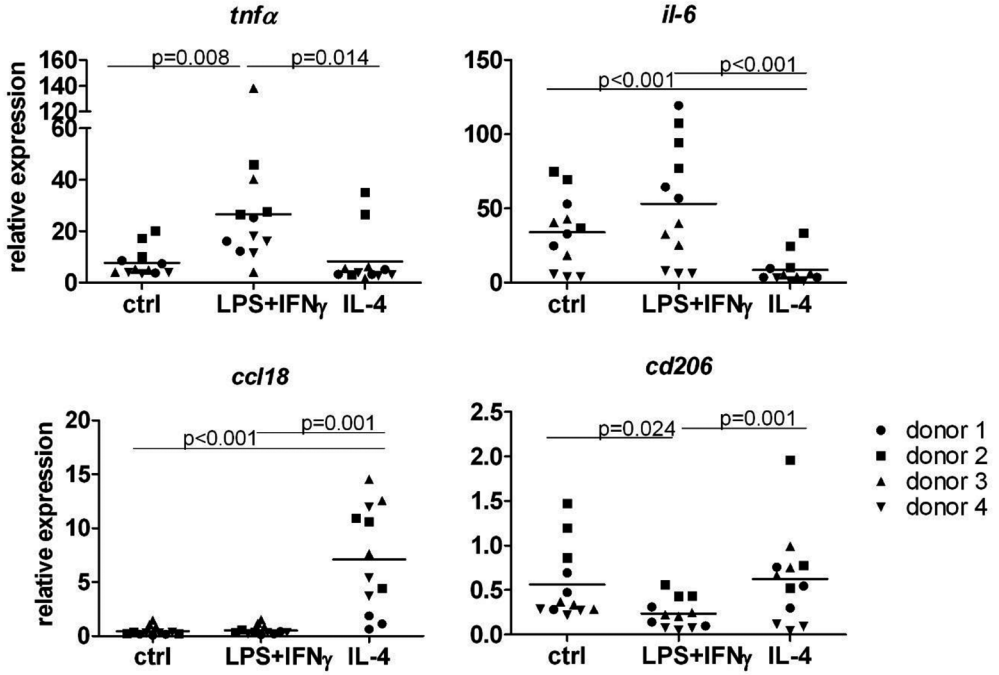

Figure 1 | Gene expression by stimulated macrophages. Macrophages were stimulated with LPS+IFNγ or IL-4 cultured for 1 day, n = 4 donors with samples in triplicate for each donor. tnfα and il-6 were used as M1 markers; ccl18 and cd206 as M2 markers. Gene expression was normalized for GAPDH.

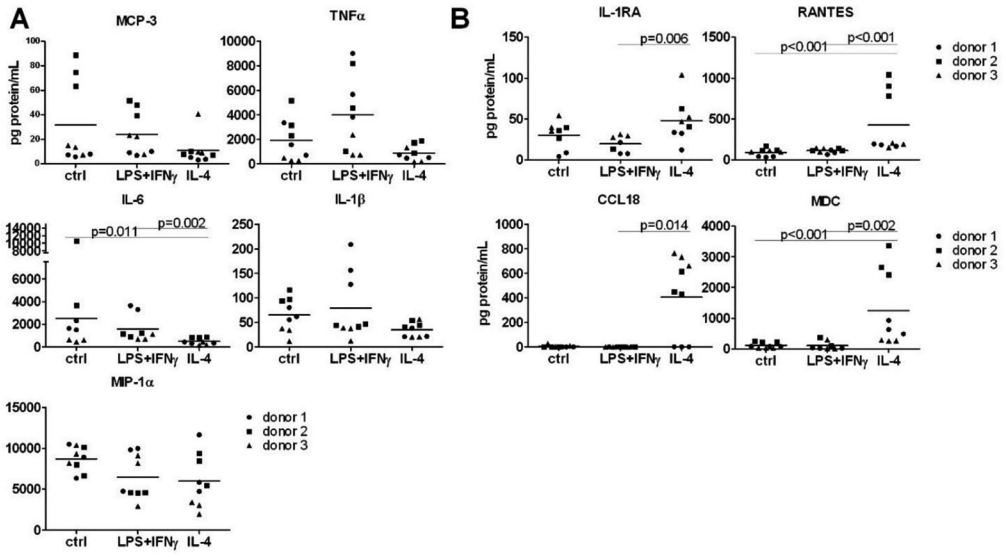

Figure 2 | Protein production of stimulated macrophages. Protein production of **A** | pro-inflammatory markers **B** | anti-inflammatory markers by macrophages in response to LPS+IFNγ or IL-4 stimulation after 3 days, n = 3 donors with samples in triplicate for each donor.

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 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 (Figure 3).

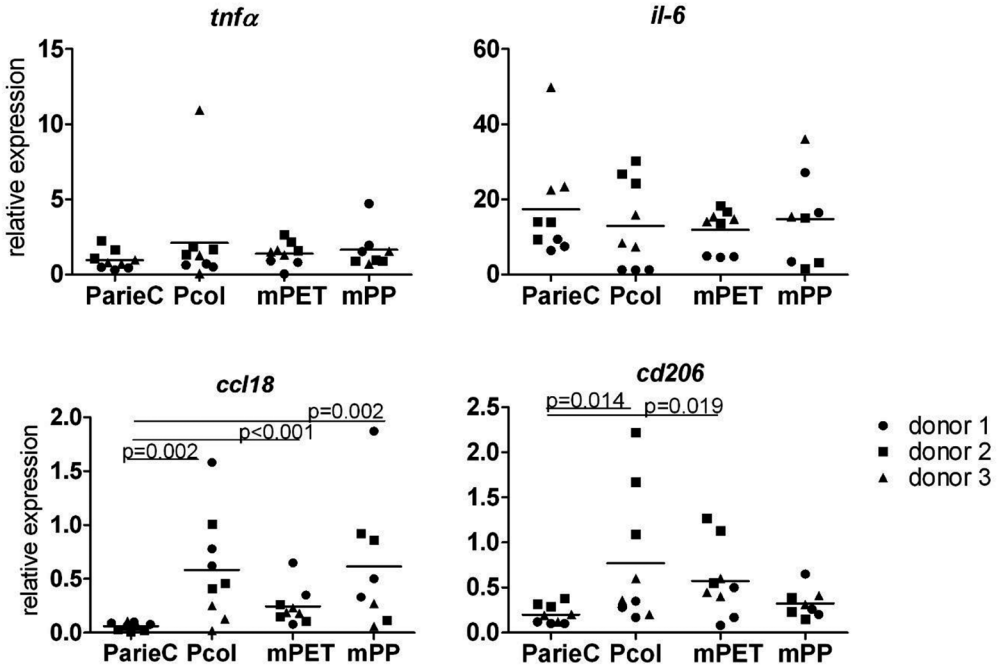

Figure 3 | Gene expression of macrophages seeded on biomaterials. Gene expression of macrophages seeded on biomaterials cultured for 1 day. n = 3 different donors, samples in triplicate. Gene expression was normalized for GAPDH.

ParieC = Parietex™ Composite; Pcol = Permacol™; mPET = multifilament polyethylene terephthalate; mPP = multifilament polypropylene.

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™ (Figure 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 (Figure 4C).

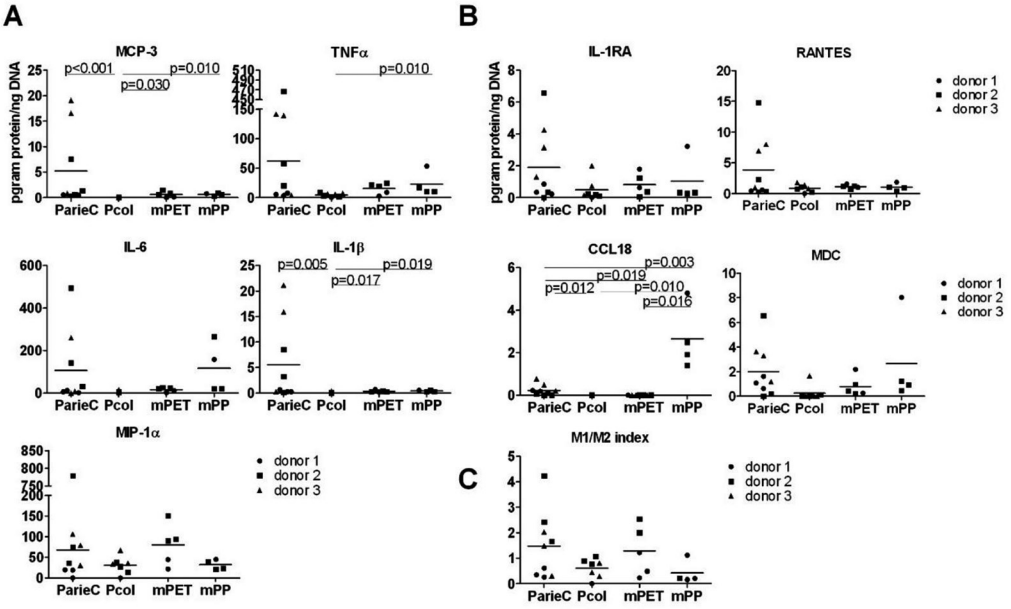

Figure 4 | Protein production by macrophages seeded on biomaterials. Protein production by macrophages seeded on biomaterials cultured for 3 days corrected for DNA. **A** | Pro-inflammatory markers **B** | anti-inflammatory markers **C** | M1/M2 index, n = 3 different donors, samples in triplicate. The 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.

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 reaction 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

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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 [28,29,30]. 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 favour 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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CHAPTER 4A

Experimental study on synthetic and biological mesh implantation in a contaminated environment

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ABSTRACT

Background

Implantation of meshes in a contaminated environment can be complicated by mesh infection and adhesion formation.

Methods

The caecal ligation and puncture model was used to induce peritonitis in 144 rats. Seven commercially available meshes were implanted intraperitoneally: six non-absorbable meshes, of which three had an absorbable coating, and one biological mesh. Mesh infection, intra-abdominal abscess formation, adhesion formation, incorporation and shrinkage were evaluated after 28 and 90 days. Histological examination with haematoxylin and eosin and picrosirius red staining was performed.

Results

No mesh infections occurred in Sepramesh®, Omyramesh® and Strattice®. One mesh infection occurred in Parietene® and Parietene Composite®. Significantly more mesh infections were found in C-Qur® (15 of 16; $P \leq 0.006$) and Dualmesh® (7 of 15; $P \leq 0.035$). Sepramesh® showed a significant increase in adhesion coverage from 12.5 per cent at 28 days to 60.0 per cent at 90 days ($P = 0.010$). At 90 days there was no significant difference between median adhesion coverage of Parietene Composite® (35.0 per cent), Omyramesh® (42.5 per cent), Sepramesh® (60.0 per cent) and Parietene® (72.5 per cent). After 90 days the adhesion coverage of Strattice® was 5.0 per cent, and incorporation (13.4 per cent) was significantly poorer than for other non-infected meshes ($P \leq 0.009$). Dualmesh® showed shrinkage of 63 per cent after 90 days.

Conclusion

Parietene Composite® and Omyramesh® performed well in a contaminated environment. Strattice® had little adhesion formation and no mesh infection, but poor incorporation. Some synthetic meshes can be as resistant to infection as biological meshes.

Surgical relevance

Surgeons are reluctant to use synthetic materials in contaminated environments owing to the risk of mesh infection. Mesh infection often necessitates removal of the mesh, leaving an abdominal wall deficit larger than the original hernia. Recently developed biological meshes are suggested to allow implantation in a contaminated environment. This experiment shows promising results regarding infection rate, incorporation and adhesion formation of certain synthetic meshes in a contaminated environment. Biological meshes showed no mesh infection and little adhesion formation. However, incorporation of biological meshes was poor, making the biomechanical strength of the repair questionable. In contaminated abdominal wall surgery one-stage repair might be performed with implantation of certain types of synthetic mesh.

INTRODUCTION

Mesh reinforcement during ventral hernia repair drastically reduces 10-year recurrence rates [1,2]. Non-absorbable synthetic materials are currently the most commonly used prosthesis for reinforcement of ventral hernias. Advantages of synthetic meshes are low recurrence rates, ease of use and relatively low costs. However, implantation of synthetic meshes can be complicated by mesh infection and adhesion formation. Mesh infection is a feared complication and reported in up to 16 per cent of patients after abdominal wall repair [3]. The risk of mesh infection is increased in a contaminated environment, which makes the use of synthetic mesh debatable [4]. Mesh infection after implantation often necessitates its removal, which leaves the patient with a contaminated field and an abdominal wall deficit that is often larger than the original hernia.

Macroporous meshes have been preferred because large pores permit infiltration of macrophages and allow rapid fibroplasia and angiogenesis, with reduced infiltration and growth of bacteria [5, 6]. The drawback of macroporous meshes is the increased risk of visceral adhesions to the site of the repair, with associated small bowel obstruction, pain, infertility and enterocutaneous fistula formation [5,7,8]. These adhesions arise as a result of fibrin deposition in the abdominal cavity, with subsequent formation of adhesions. The presence of contamination increases fibrin deposition, leading to an increased amount and tenacity of adhesions intra-abdominally and to the mesh [9]. In a clean environment antiadhesive coatings have proved to reduce adhesion formation to macroporous meshes [8,10,11].

The aim of the study was to compare commercially available synthetic and biological meshes in terms of infection rate, adhesion formation, incorporation and shrinkage after implantation in a contaminated environment.

METHODS

One hundred and forty-four male Wistar rats weighing 250–350 g were obtained from a licensed breeder (Harlan Laboratories, Boxmeer, The Netherlands). They were bred under specific pathogen-free conditions, kept under standard laboratory conditions in individually ventilated cages, and fed freely with standard rat chow and water throughout the experiment. The protocol of the experiment was approved by the Ethical Committee on Animal Experimentation of Erasmus University Rotterdam.

Peritonitis model

Rats were anaesthetized by isoflurane/oxygen inhalation and received buprenorphine analgesia (0,05 mg/kg subcutaneously). The abdomen was shaved and the skin disinfected with 70 per cent alcohol, after which the abdominal cavity was opened through a 3-cm midline incision. To induce peritonitis, a caecal ligation and puncture (CLP) model was used [12]. The caecum was carefully manipulated outside the abdominal cavity and ligated just distal to the ileocaecal valve with a monofilament non-absorbable suture (4/0 Ethilon®; Ethicon, Johnson & Johnson, Somerville, New

Jersey, USA), maintaining the continuity of the bowel. The caecum was punctured distally to the ligation with an 18-G needle. The fascia and skin were closed with a running absorbable suture (5/0 Safil®; B. Braun, Melsungen, Germany). After 24 h the abdomen was reopened, a culture swab was taken to confirm peritonitis, the necrotic caecum was resected and the abdominal cavity was rinsed with at least 20 ml phosphate-buffered saline at 37 °C. A sterile mesh, measuring 2·5 × 3 cm, was implanted intraperitoneally with three transmuscular non-absorbable sutures (5/0 Ethilon®) on both sides of the incision in all mesh groups. No mesh was implanted in the control group. After administration of gentamicin (6 mg/kg intramuscularly) the abdominal wall and skin were closed separately with a running absorbable suture (5/0 Safil®).

Implanted meshes

The rats were divided into eight groups, a control group that received no mesh and groups in which one of the following seven meshes was implanted intraperitoneally: non-cross-linked collagen (Strattice®; LifeCell, Branchburg, New Jersey, USA), polypropylene (Parietene®; Sofradim, Trevoux, France; part of Covidien, North Haven, Connecticut, USA), collagen–polyethyleneglycol–glycerol-coated polypropylene (Parietene Composite®; Sofradim), omega-3-fatty acid-coated polypropylene (C-Qur®; Atrium, Hudson, New York, USA), carboxymethylcellulose–sodium hyaluronate-coated polypropylene (Sepramesh®; Bard, New Providence, New Jersey, USA), expanded polytetrafluoroethylene (PTFE) (Dualmesh®; Gore, Flagstaff, Arizona, USA) and condensed PTFE (Omyramesh®; B. Braun).

Measurements

Half of the surviving animals were euthanised after 28 days and half after 90 days. The abdomen was shaved, disinfected and opened through a equation image-shaped incision extending laterally and caudally to the mesh. Directly after opening the abdomen, a swab of the abdominal cavity was taken for culture. Mesh infection was defined as the presence of abscesses of the mesh, and parts of the mesh were cultured for microbiological evaluation. Adhesions were scored using a grid placed over the mesh, dividing it into 30 equal squares. The tenacity of the adhesions was graded using the Zühlke score, a four-degree classification of adhesions based on histological and morphological criteria [13]. Pictures of the abdominal wall with mesh and any adhesions were taken with a 5.0-megapixel digital camera. The abdominal cavity was inspected for abscesses; when present, these were scored and cultured at four sites: the liver, abdominal wall, bowel and omentum [14]. Mesh incorporation was defined as the percentage of the mesh edge incorporated into the abdominal wall, taking into account any shrinkage. Shrinkage was defined as the relative loss of surface compared with the original size of the mesh, measured with a calliper. The animals were killed by cardiac cut. All measurements were carried out by two independent observers and disagreements reconciled by discussion.

Histological evaluation

At least two representative samples of macroscopically non-infected meshes with adjacent abdominal wall were excised by full-thickness (mesh and abdominal wall muscle) biopsy punches of 5 mm diameter. The samples were embedded in Tissue-Tek© (Sakura, Alphen, Rijn, The Netherlands) and immediately frozen in liquid nitrogen. Frozen sections of 6 μm were made using a cryostat (Leica; Davis Instruments, Vernon Hills, Illinois, USA). Sections were stained with either haematoxylin and eosin or picrosirius red (Direct Red 80; Fluka Chemie, Zwijndrecht, The Netherlands) [15]. Samples were assigned a random number before evaluation and scored by two observers blinded to the specific type of mesh. Fibrosis, lymphocyte infiltration and angiogenesis were scored macroscopically at 200× magnification using a light microscope (Olympus, Center Valley, Pennsylvania, USA). The following grading scale was used: 0, none present; 1, little; 2, moderate; and 3, extensive. The picrosirius red-stained sections were analysed for collagen and scored by means of the same scale for the presence of collagen around the mesh and abdominal wall.

Statistical analysis

Results are presented as median (interquartile range). Mesh infection, tenacity and percentage of adhesions, histological score, abscess formation, survival and weight were compared using Kruskal–Wallis, Mann–Whitney U, $\chi 2$ and Fisher's exact tests as the data did not show a normal distribution. If the overall test showed differences, pairwise tests were done to determine the groups causing the overall significance. Exact methods for significance were used when computational limits allowed these. All reported P values are two-sided and $P < 0.05$ was considered statistically significant. In view of the numbers, it was not possible to adjust the P values using Bonferroni's correction. Statistical analysis was performed using PSAW® statistical software package version 17 (IBM, Armonk, New York, USA).

RESULTS

During the first 2 days of the experiment 22 (15.3 per cent) of the 144 rats died. Necropsy was performed and septicaemia was found to be the cause of death in all rats (Table 1). On day 13 one rat in the C-Qur® group died from intestinal obstruction due to severe adherence of the bowel to the infected mesh. Abdominal cultures on day 1 confirmed bacterial contamination in all animals with Gram-positive (Enterococcus, Staphylococcus, Streptococcus) and Gram-negative (Escherichia coli and Proteus) micro-organisms. All animals exhibited symptoms of sepsis including apathetic behaviour, ocular exudates, piloerection, diarrhoea and weight loss. The maximum percentage weight loss varied between 11.1 and 14.2 per cent, and was more pronounced in the C-Qur® group ($P \leq 0.048$ compared with other groups).

Table 1 | Postoperative mortality and number of animals analysed at 28 and 90 days after surgery.

Group	Mesh material	No. of animals	Postoperative death	No. analysed 28 days	No. analysed 90 days
Control	No mesh	18	2	8	8
Strattice®	Non-cross-linked collagen	18	4	7	7
Parietene®	Polypropylene	18	2	8	8
Parietene Composite®	Collagen–polyethyleneglycol–glycerol-coated polypropylene	18	4	7	7
Sepramesh®	Carboxymethylcellulose–sodium hyaluronate-coated polypropylene	18	2	8	8
C-Qur®	Omega-3-fatty acid-coated polypropylene	18	2	8*	8
Dualmesh®	Expanded polytetrafluoroethylene	18	3	7	8
Omyramesh®	Condensed polytetrafluoroethylene	18	3	7	8
Total		**144**	**22**	**60**	**62**

* One rat in the C-Qur® group died after 13 days. The results for this rat were analysed together with those for rats killed after 28 days in the C-Qur® group.

Mesh infection

At the time of death macroscopic infection of the mesh was present in 24 (22.6 per cent) of 106 animals. The infection rate among C-Qur® meshes was high (15 of 16 rats) compared with all other meshes ($P \leq 0.006$) (Figure 1). Dualmesh® also showed a high infection rate (7 of 15 rats), significantly higher than all other groups apart from C-Qur® ($P \leq 0.035$). All infected meshes became large fibrotic pseudotumours. No additional mesh infection was discovered by microbiological culture of the meshes.

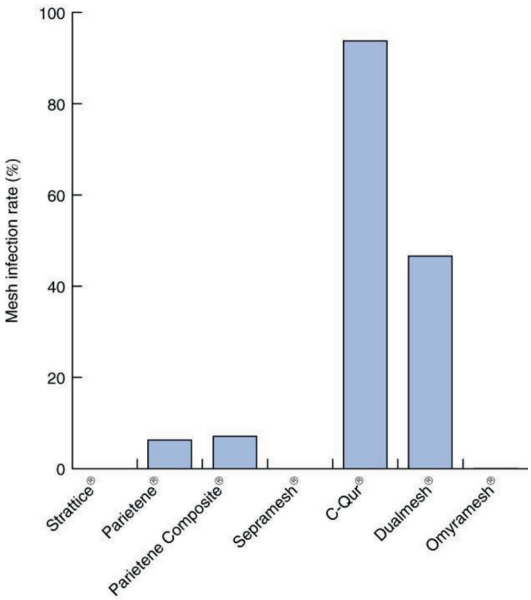

Figure 1 | Comparison of mesh infection rates (combined 28 and 90 days). Values are percentage of macroscopically infected meshes among surviving animals.

Abscesses

Intra-abdominal abscesses were found in 37 rats (62 per cent) after 28 days and 27 (44 per cent) after 90 days (P = 0.049). The majority of abscesses were located at the caecum or abdominal wall. There was no significant difference between groups in intra-abdominal abscesses (P = 0.482).

Adhesions

After 28 and 90 days the surfaces of all infected meshes were completely covered with adhesions. Owing to the high infection rate in C-Qur® and Dualmesh® the median adhesion coverage was 90–100 per cent (Figure 2). After 28 days significantly less adhesion to the mesh surface was found for Strattice® (median 10.0 (5.0–10.0) per cent) and Sepramesh® (12.5 (6.3–22.5) per cent) compared with all other meshes (P ≤ 0.004 and P ≤ 0.017 respectively). Median adhesion coverage was 45.0 per cent for Parietene Composite®, 52.5 per cent for Parietene® and 55.0 per cent for Omyramesh®.

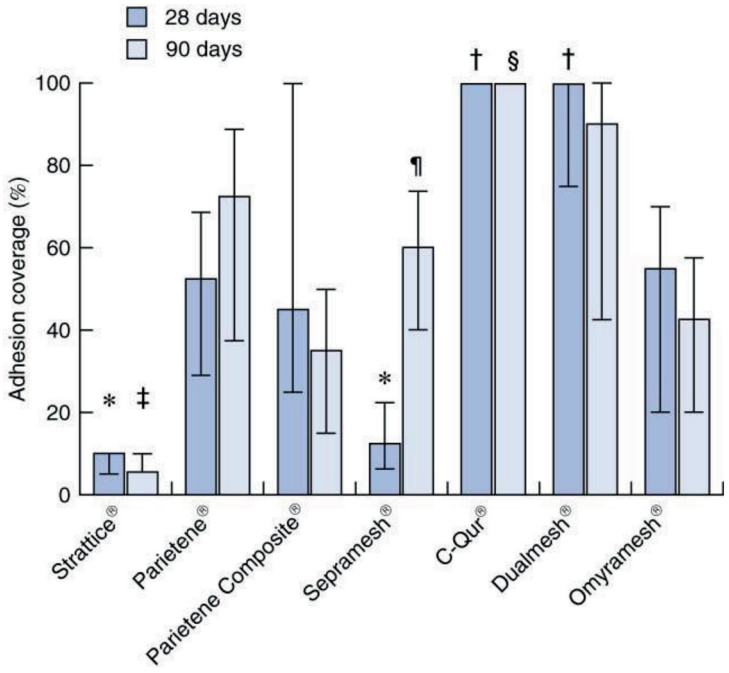

Figure 2 | Comparison of percentage of mesh adhesions at 28 and 90 days' follow-up. Values are median (interquartile range).

*P < 0·050 versus Parietene®, Parietene Composite®, C-Qur®, Dualmesh® and Omyramesh® at 28 days; †P < 0·050 versus Parietene® and Omyramesh® at 28 days; ‡P < 0·050 versus all other meshes at 90 days; §P < 0·050 versus Parietene®, Parietene Composite®, Sepramesh® and Omyramesh® at 90 days; ¶P = 0·010 versus Sepramesh® at 28 days (Mann–Whitney U test)

Sepramesh® showed an increase in adhesion formation from a median 12.5 of per cent at 28 days to 60.0 per cent at 90 days (P = 0.010). After 90 days Strattice® (5.0 (5.0–10.0) per cent) had significantly less adhesion coverage than the other meshes (P ≤ 0.003). At 90 days there was no

significant difference between median adhesion coverage of Parietene Composite® (35.0 per cent), Omyramesh® (42.5 per cent), Sepramesh® (60.0 per cent) and Parietene® (72.5 per cent).

Incorporation

After 28 and 90 days C-Qur® showed no or very little incorporation into the abdominal wall owing to the high rate of mesh infection (Figure 3). Strattice® showed a poor incorporation of 22.7 per cent at 28 days, which was lower than for Omyramesh® (47.1 per cent; P = 0.004), Parietene Composite® (42.5 per cent; P = 0.004) and Sepramesh® (35.6 per cent; P = 0.004).

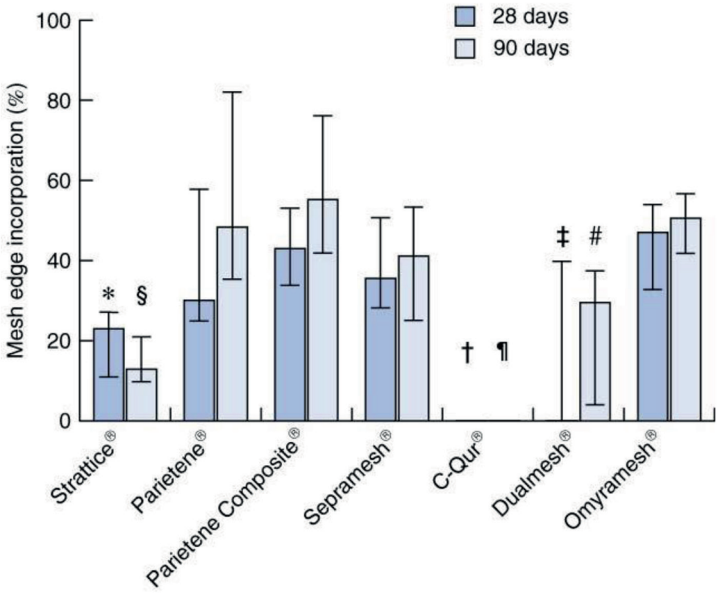

Figure 3 | Mesh edge incorporation at 28 and 90 days' follow-up. Values are median (interquartile range).

*P < 0·050 versus Parietene Composite®, Sepramesh®, C-Qur® and Omyramesh® at 28 days; †P < 0·050 versus Parietene®, Parietene Composite®, Sepramesh® and Omyramesh® at 28 days; ‡P < 0·050 versus Parietene Composite® and Omyramesh® at 28 days; §P < 0·050 versus Parietene®, Parietene Composite®, Sepramesh®, C-Qur® and Omyramesh® at 90 days; ¶P < 0·050 versus all other meshes at 90 days; #P < 0·050 versus Parietene®, Parietene Composite®, C-Qur® and Omyramesh® at 90 days (Mann–Whitney U test)

The incorporation of Strattice® was not improved after 90 days (median 13.4 per cent). This was significantly worse than the incorporation of Parietene Composite® (54.5 per cent; P = 0.003), Omyramesh® (50.4 per cent; P < 0.001), Parietene® (48.4 per cent; P = 0.009) and Sepramesh® (40.9 per cent; P = 0.002). At 90 days, Dualmesh® (29.4 per cent) was incorporated more poorly than Parietene® (P = 0.020), Parietene Composite® (P = 0.009) and Omyramesh® (P = 0.002).

Shrinkage

The shrinkage of C-Qur® could not be determined owing to the formation of large fibrotic pseudotumours in all but one of the meshes. The non-infected Dualmesh® showed the highest percentage loss of mesh surface, of 63 per cent after 90 days ($P \leq 0.012$ compared with other meshes). All other meshes had a median loss of mesh surface of between 0 and 10 per cent after 28 days. Strattice® showed a progressive median loss of surface from 0 per cent at 28 days to 23 per cent at 90 days ($P = 0.003$). After 90 days the purely synthetic Dualmesh®, Omyramesh® and Parietene® showed shrinkage of between 0 and 15 per cent. Parietene Composite® and Sepramesh® did not shrink after 90 days ($P \leq 0.026$ and $P \leq 0.014$ respectively compared with all other meshes).

Histology

Fibrosis was observed in all mesh-surrounding tissues. This was especially pronounced for the four polypropylene-based meshes and Omyramesh® (Figure S1, supporting information). Dualmesh® showed a clear encapsulation of the mesh, almost without cellular infiltration into it. A large number of vessels could be seen in the tissue surrounding Parietene Composite® and Omyramesh®. Because of wide intra-animal variation, no statistically difference was found for fibrosis, influx of lymphocytes, angiogenesis and collagen deposition (data not shown).

DISCUSSION

In this experimental contaminated environment, the collagen-coated polypropylene mesh Parietene Composite® and the condensed PTFE Omyramesh® had a low risk of infection, moderate adhesion formation and good incorporation. The biological Strattice® mesh did not become infected and showed remarkably little adhesion formation, but poor incorporation.

If a mesh is used in a contaminated environment, consensus exists that a biological collagen mesh or a synthetic macroporous, monofilament mesh may be advantageous [5,16-18]. Biological collagen meshes have been developed specifically for a contaminated environment and Strattice® did not show any mesh infection in this experiment. Biological meshes, particularly Strattice®, have shown improved clearance of bacteria, which decreases the possibility of infection and formation of adhesions [19]. A prospective multicentre study of contaminated ventral hernia repair with Strattice® reported a similar low infection rate with little need to remove the mesh [20].

The macroporous Parietene®, Parietene Composite®, Sepramesh® and Omyramesh® had a low risk of infection. Large pores allow admission of macrophages, fibroplasia and angiogenesis, which improves the ability to clear infection [5,6]. In this study, however, the macroporous C-Qur® mesh showed a high infection rate. This polypropylene mesh is coated with anti-inflammatory omega-3 fatty acids. In an experimental clean environment macrophages were scarcely present in the mesh after implantation [11,21]. It might be hypothesized that the anti-inflammatory properties of the omega-3 fatty acid coating have prevented macrophage penetration, although no clinical or experimental literature on the characteristics of omega-3 fatty acids in the presence of bacteria has yet been published.

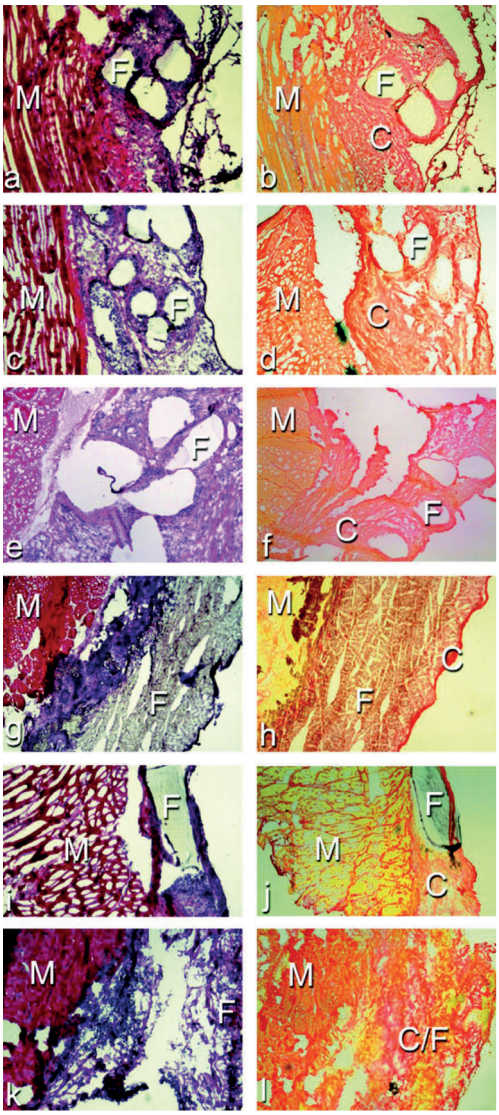

Figure S1 | Histological samples after 90 days: a,c,e,g,i haematoxylin and eosin staining and b,d,f,h,j picrosirius red staining of histological samples after 90 days (original magnification ×40). **a,b** | Polypropylene (Parietene®; Sofradim, Trevoux, France; part of Covidien, North Haven, Connecticut, USA); **c,d** | collagen–polyethyleneglycol–glycerol-coated polypropylene (Parietene Composite®; Sofradim); **e,f** | carboxymethylcellulose–sodium hyaluronate-coated polypropylene (Sepramesh®; Bard, New Providence, New Jersey, USA); **g,h** | expanded polytetrafluoroethylene (Dualmesh®; Gore, Flagstaff, Arizona, USA); **i,j** | condensed polytetrafluoroethylene (Omyramesh®; B. Braun, Melsungen, Germany); and **k,l** | non-cross-linked collagen mesh (Strattice®; LifeCell, Branchburg, New Jersey, USA). The purple and pink cells in the haematoxylin and eosin-stained sections are fibroblasts and lymphocytes. The synthetic fibres of the Parietene® **a,b** | Parietene Composite® **c,d** | Sepramesh® **e,f** | and Omyramesh® **i,j** | are surrounded with fibrotic tissue with newly formed collagen. Around Dualmesh® **g,h** | a cellular layer is observed, forming a capsule; cellular infiltration into the mesh is minimal. In the picrosirius red-stained section of the Strattice® mesh **l** | it is impossible to differentiate between the collagen of the mesh and newly formed collagen (C/F). **M** | abdominal wall muscle; **F** | mesh fibres, **C** | newly formed collagen layer

Dualmesh® showed a high infection rate, probably because of its partially microporous structure (smaller than 10 µm). The increased risk of infection after surgery with Dualmesh®, and the need to remove the prosthesis in case of infection, is notorious in the clinical situation [22-24]. Mesh infection is caused by infiltration and proliferation of bacteria within the pores and interstices of synthetic materials. Small pores prevent infiltration of immune cells and make microporous meshes more susceptible to infection [5,25]. Additionally, the hydrophobic visceral surface of Dualmesh® decreases adhesion of tissue cells, allowing bacteria a free passage to the implant surface [16].

Intra-abdominal adhesion and abscess formation are important causes of morbidity and mortality following contaminated abdominal surgery. During peritonitis fibrin is deposited in the abdominal

cavity, inducing adhesion formation and providing possible niduses for abscess formation9. Biological Strattice® mesh showed low adhesion formation after 90 days, confirming previous experimental results [26-28]. Sepramesh® showed a significant increase in adhesion formation between 28 and 90 days, implying that the cellulose–hyaluronate coating is absorbed before a neoperitoneal layer is formed. These results confirm that adhesion formation in the presence of mesh is not complete after 7 days [8,11]. The surface of Parietene Composite® and Omyramesh® were least covered with adhesions after 90 days. Low adhesion formation on the collagen-coated Parietene Composite® has been described in a clean environment [8,11]. The present results suggest that the collagen coating remains present until a neoperitoneum has formed, even in a contaminated environment. The low adhesion formation on Omyramesh® confirms experimental findings with this relatively new mesh in a clean environment [29,30]. The low adhesion formation might be explained by its smooth, monolayer, non-fibrous, macroporous structure. The plain polypropylene Parietene® mesh was largely covered with adhesions. Clinically, uncoated polypropylene meshes are known to induce severe adhesion formation with attachment of intestine to the mesh when implanted intraperitoneally [7,31]. In 21 per cent of patients with an intraperitoneal uncoated polypropylene mesh, adhesions made bowel resection necessary during re-exploration in one study [7].

The non-infected, partially microporous, expanded PTFE Dualmesh® had an alarmingly high shrinkage rate (median 63 per cent after 90 days). Such shrinkage has frequently been reported experimentally, but this does not seem to be correlated with a higher recurrence rate clinically [8,23,32]. A fibrous capsule surrounding the mesh was observed, almost without cellular infiltration into the mesh. Contraction of this capsule was probably the cause of shrinkage, which might have been more pronounced in the small meshes used in the present experiment compared with the much larger meshes used clinically. Of the macroporous meshes, the plain polypropylene Parietene® showed the most shrinkage (15 per cent after 90 days), confirming experimental results [32,33].

The biological Strattice® mesh had a 23 per cent loss of surface after 90 days, probably caused by collagenase activity. Premature weakening of the biomechanical properties of the scaffold combined with insufficient incorporation can possibly result in loss of the prosthesis and hernia recurrence [34]. Until evidence of biomechanical strength after hernia repair with biological meshes has been provided, synthetic meshes are preferred for primary repair.

Translation of experimental results to the clinical situation should be done with caution. However, the CLP model is suitable for studying the behaviour of synthetic and biological meshes experimentally in a contaminated environment. In this model, as in clinical infections, peritonitis arises from a complex interaction of the immune system with inflammatory, haemodynamic and biochemical alterations similar to human sepsis, with a consistent increase in cytokine levels [35-38]. Another advantage of this experimental model is the use of rats of the same age and sex, and specified pathogen-free bacterial status. This minimizes biological and microbiological variability, and makes it suitable for comparing characteristics of different meshes in a similar contaminated environment [38]. A limitation of the model is the size of the mesh and mesh pores in relation to the abdominal wall, which is different between rats and humans. This might lead to an overestimation of shrinkage. The meshes in this experiment were fixated with six sutures. In humans the number of fixation points in relation to the mesh size would be much higher. This might have influenced

incorporation, as described in previous experimental mesh studies [8,11]. Finally, the concentration of the antiadhesive coatings and its systemic effects during breakdown in this model might be different from the human situation.

The experimental results of synthetic mesh implantation in a contaminated environment make strict contraindication in humans questionable. Although there are no meshes without disadvantages, certain permanent synthetic meshes might be somewhat infection-resistant and therefore useful for permanent hernia repair in a contaminated environment.

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CHAPTER 4B

Mesh-specific inflammatory cell response in a contaminated environment

Grotenhuis N, Deerenberg EB, Mulder IM, Kops N, van Osch GJVM,
Bastiaansen-Jenniskens YM, Lange JF

ABSTRACT

Background

The use of meshes for abdominal hernia surgery in a contaminated environment is compromised due to a high risk of complications. Little is known about differences in the foreign body reaction between materials in contaminated environments. Therefore we compared the presence of macrophages and their attractors after implantation of different meshes in a contaminated environment *in vivo*.

Methods

28 and 90 days after implantation, biopsies of the abdominal wall with implanted meshes (Parietene™, Parietene Composite™, C-Qur™, Sepramesh™, Dualmesh® and Omyramesh®) were harvested from a peritonitis rat model. Biopsies were analysed with immunohistochemistry for macrophage markers CD68, iNOS, and CD206, and for T-cells with CD3. Toluidine-staining was used for mast cells.

Results

More CD3- and CD68-positive cells were found in samples with meshes than in the control group without a mesh. After 90 days, Parietene Composite™ and Sepramesh™ were surrounded by more iNOS-positive cells than the control group. C-Qur™ and Dualmesh® were surrounded by more CD206-positive cells than the control group at day 28. The M1/M2 ratio was low for all meshes.

Conclusions

Mesh-specific cellular responses are evident in a contaminated environment and therefore these data can help the surgeon to select suitable meshes for implantation.

INTRODUCTION

Meshes are occasionally used in a clean-contaminated or even in a contaminated environment, like fascial defects after bowel resection, near stomas or after removal of an infected mesh. Generally spoken, in clinical application the risk of complications like infection of the mesh is higher in a contaminated field and therefore surgeons are hesitant to use meshes in these cases [1,2]. The extent of the inflammatory response of the body, also known as foreign body reaction, depends on the type and consistency of the mesh [3-5]. Using an *in vitro* model, we have recently described mesh-dependent reactions of macrophages in a contaminated environment [6].

Many researchers investigate the foreign body reaction in a sterile environment. After implantation, all types of meshes used for abdominal wall hernia surgery induce a foreign body reaction. After implantation of the mesh, inflammatory cells, starting with neutrophils and mast cells are attracted to the wound site [3]. Mast cells attract macrophages to the wound site and the number and degranulation of mast cells is important for the extend of the foreign body reaction [3,4,7,8]. Besides mast cells, T-cells are also important attractors of macrophages [3 4,9]. After being recruited, macrophages will dominate the wound site [3,5]. Macrophage phenotypes can range between pro-inflammatory (M1) and repair/anti-inflammatory (M2). M1-macrophages produce pro-inflammatory factors such as interleukin (IL)-6, tumor necrosis factor (TNF)α and express inducible nitric oxide synthase (iNOS) [10]. M2-macrophages produce anti-inflammatory factors such as IL-1 receptor antagonist (IL-1RA), chemokines such as CCL18, and growth factors such as vascular endothelial growth factor (VEGF). M2-macrophages express among others the surface protein CD206, which is the mannose receptor important for recognition of pathogens [10].

How the foreign body reaction in a contaminated environment will depend on the type of material is not yet completely understood. In a contaminated environment, macrophages are expected to change mainly into the M1-phenotype because the infection and presence of bacteria needs to be eliminated [11]. M1-macrophages negatively influence incorporation of the mesh, by producing matrix degrading enzymes and inhibitors of extracellular matrix [5]. Van Putten et al. [12] found that the foreign body reaction against collagen discs is delayed in the presence of bacterial cell wall components. Whether the presence of bacterial components also delays the foreign body reaction against synthetic meshes is not known. Using an *in vitro* model, we have confirmed mesh-dependent reactions of macrophages in a contaminated environment [6]. Previously we studied the *in vivo* behavior of seven commercially available meshes (1 biological and 6 synthetic meshes) in a contaminated environment in rats and found differences in mesh infection, adhesions and incorporation of the biomaterial [13]. In this experiment polypropylene was used, a mesh often used in patients and also polypropylene-based meshes with a hydrophilic collagen-coating, omega 3-fatty acid-coating, and a hyaluronate-carboxymethylcellulose coating, which are described to have a lower complication rate in a clean environment [14]. Expanded (microporous) and condensed (macroporous) expanded polytetrafluoroethylene (PTFE) meshes were also included. Expanded PTFE has a high infection risk due to the small micropores whereas condensed PTFE is believed to have a good outcome in a contaminated environment due to its macroporous structure [15,16].

In this study, the cellular immune responses to different synthetic meshes in a contaminated environment *in vivo* are compared in more detail. As macrophages are the key players in the foreign body reaction, the presence of T-cells and mast cells as macrophage attractors and the phenotypes of macrophages with immunohistochemistry are investigated. This knowledge can help the surgeon to choose the best materials to use in an environment with high risk of contamination.

MATERIALS AND METHODS

Contaminated model *in vivo*

The rat experiment protocol is according to the Animal Research: Reporting In Vivo (ARRIVE) guidelines and was approved by the Ethical Committee on Animal Experimentation of Erasmus University Rotterdam, the Netherlands (EMC 2075-105-10-03). We used samples of an earlier presented study in which in 144 (8 groups, 9 rats per group, two time points) male Wistar rats (Harlan Laboratories, Boxmeer, the Netherlands) weighing 250–350 grams a contaminated environment was created by caecum ligation and puncture [13]. Briefly, the caecum was ligated just distally to the ileocaecal valve maintaining the continuity of the bowel and punctured distally to the ligation with an 18-G needle leading to leakage of fecal fluids with bacteria into the abdominal cavity to induce peritonitis. After 24 hours the abdomen was re-opened and peritonitis was confirmed by microbiological culture, resulting in a contaminated wound. One of the following meshes (2.5 x 3 cm) was implanted intraperitoneally with 6 transmuscular nonabsorbable sutures (5/0 Ethilon, Johnson & Johnson: New Brunswick, New Jersey, United States): Parietene™ (polypropylene (PP), Covidien-Sofradim Production, Trevoux, France), Parietene Composite™ (PP with an one-sided absorbable, hydrophilic collagen-coating, Covidien- Sofradim Production, Trevoux, France), C-Qur™, (PP with omega 3-fatty acid-coating and triglycerides, Atrium, Hudson, New York, USA), Sepramesh™ (PP, with a hyaluronate-carboxymethylcellulose coating, Bard, New Providence, New Jersey, USA), Dualmesh® (expanded polytetrafluoroethylene (PTFE), Gore, Flagstaff, Arizona, USA), Omyramesh® (condensed PTFE, B Braun, Melsungen, Germany) or Strattice™ (collagen derived from porcine skin, LifeCell, Branchburg, New Jersey, USA). A control group was included following completely the same protocol, only no mesh was implanted after re-opening the abdomen. After implantation, all rats received one dose of gentamicin (6 mg/kg) intramuscularly. Two to four rats per group died from sepsis [13].

Harvesting

At 28 days and 90 days after implantation of the materials, the animals were euthanized by cardiac cut and a swab was taken to culture bacteria; C-Qur™ resulted in 95% (15 out of 16) of the samples positive for bacteria, Dualmesh® 50% (7 out of 15) and Parietene™ and Parietene Composite™ both 5% (1 out of 15), in the other groups no infections were found [13]. One biopsy per animal was taken from the incorporated mesh with surrounding tissue. In the rats without a biomaterial a biopsy of the abdominal wall was taken at the same place where in the other rats the mesh was implanted. In some animals, incorporation of the material was insufficient and because there was no adjacent

tissue, no biopsy could be taken. For Strattice™ at both time points, Sepramesh™ at day 28 and C-Qur™ and Omyramesh® at day 90 only 1 or 2 samples could be taken because of insufficient incorporation and therefore these conditions were excluded for analysis. Biopsies were snap-frozen in Tissue-Tek© (Sakura, Alphen, Rijn, The Netherlands) with liquid nitrogen and stored at -80 °C till sectioning. Sections of 6 µm were cut on a cryostat (Leica; Davis Instruments, Vernon Hills, Illinois, USA) and stored at -80 °C.

Staining

Immunohistochemistry

Frozen sections were defrosted and fixed in acetone. After fixation sections were washed in PBS and incubated with 10% normal goat serum (Sigma-Aldrich, St Louis, MO, USA) to block non-specific binding. After incubation sections were washed with phosphate buffered saline and incubated with primary antibodies against CD206 (2.5 µg/mL, Abcam, 64693, Cambridge, UK), iNOS (2 µg/mL, Abcam, 15323), CD3 (1:100, Abcam, 16669), CD68 (5 ug/ml, Acris Antibodies GmbH, BM 4000, Herford, Germany). We choose the antibiodies based on literature [4,10,22]. Irrelevant IgG was used as a negative control. Link biotinylated goat-anti-mouse (Biogenex, HK-325-UM, Fremont, CA, USA) was used at a second antibody, Label streptavidin-AP (Biogenex, HK-321-UK) as a tertiary antibody with neu-fuchsin as substrate. Sections were counterstained with hematoxylin (Sigma). Lung and spleen tissue were used as a positive controls. Sections were mounted with vectamount (Vector Laboratories, Burlingame, CA).

Toluidine blue (mast cells)

Sections were defrosted and fixated in acetone. After washing in demineralised water the sections were placed in a toluidine blue solution (1% Toluidin blue (Fluka (Sigma), 89640) in 50% isopropanol and 50% demineralised water) for 30 minutes at 37 °C. Sections were washed for 1 minute in pure isopropanol. Sections were air-dried and mounted with vectamount (Vector Laboratories).

Analyses

Stained sections were analysed by light microscopy (Olympus, Tokyo, Japan). Per staining, sections were blinded and the number of cells in 5 areas at the interface of the mesh and tissue was ranked. In the case of the control group, cells were counted subcutaneously, at the place where in the other groups the mesh was implanted. Samples were ranked based on the number of positive cells, ranks were ranging from 1 to 58 (due to a total of 58 analysed samples). Control group day 28: 8 samples, day 90: 7 samples. Parietene™ 8 and 5 samples respectively, Parietene Composite™ 5 and 4 samples, C-qur™ day 28: 5 samples, Sepramesh™ day 90: 4 samples, Dualmesh© day 28: 3 samples, day 90: 4 samples, Omyramesh© day 28: 5 samples. Ranking was performed by two independent observers (NG and NK). The ranking of one observer was compared with the ranking of the other observer. If there was a difference in ranking per sample of more than 15, the samples were analysed again. After that, the mean ranking per sample was calculated from the ranking of one observer and the other observer. Then the samples were unblinded and were used for further analysis. The number of iNOS-positive cells was divided by the number of CD206-positive cells leading to an M1/M2 ratio.

The natural logarithm of this ratio was calculated for visualization. Data is presented as box plots with medians and whiskers showing the interquartile range.

Statistics

The medians of the groups were compared with a Kruskal-Wallis test (independent samples median test) and Mann-Whitney in SPSS (IBM Corp. Released 2011. IBM SPSS Statistics for Windows, Version 20.0. Armonk, NY: IBM Corp.) False Discovery Rate was used for mathematical correction by multiple comparisons. $P < 0.05$ was considered statistically significant.

RESULTS

The number of mast cells and T-cells were analysed in the tissue adjacent to the meshes, since these two cells are the main attractors of macrophages. We found no significant differences in the numbers of mast cells between the biomaterials or compared to the control group (Figure 1A). All meshes had more CD3-positive T-cells at day 28 than the control group ($P = \text{or} < 0.03$). There were also mesh-dependent differences: Parietene™ was surrounded by less CD3-positive cells than Dualmesh® ($P = 0.03$) and Parietene Composite™ was surrounded by less CD3-positive cells than Omyramesh® ($P = 0.03$). After 90 days still all samples with meshes contained more CD3-positive cells than the control group ($P = 0.03$) (Figure 1B).

To investigate the total number of attracted macrophages, samples were stained for CD68 as a general macrophage marker. After 28 days more macrophages were found adjacent in the groups with a mesh than in the control group ($P = \text{or} < 0.015$). The same finding was still observed after 90 days, but this was only statistically significant for Sepramesh™ ($P = 0.02$) and Dualmesh© ($P = 0.02$) (Figure 2).

To investigate how the different meshes influence the macrophage phenotype, we stained the samples with antibodies against iNOS for M1-macrophages and with antibodies against CD206 for M2-macrophages. At day 28 we did not find significant differences between the conditions, however after 90 days, Parietene Composite™ and Sepramesh™ were surrounded by significantly more iNOS-positive cells than the control group ($P = 0.03$). There were no statistically significant differences between the meshes (Figure 3a). After 28 days we found more CD206-positive cells surrounding C-Qur™ and Dualmesh® than in the control group ($P = 0.045$). After 90 days, no significant differences were observed (Figure 3b). To determine for each mesh whether it induces a more pro- or anti-inflammatory reaction the M1/M2 ratio was calculated based on iNOS positive and CD206-positive cells (Figure 3c). All meshes except Parietene Composite™ after 90 days, had a negative mean ratio, indicative for a predominant M2, or anti-inflammatory, reaction. However no statistically significant differences in M1/M2 ratios were observed between the meshes.

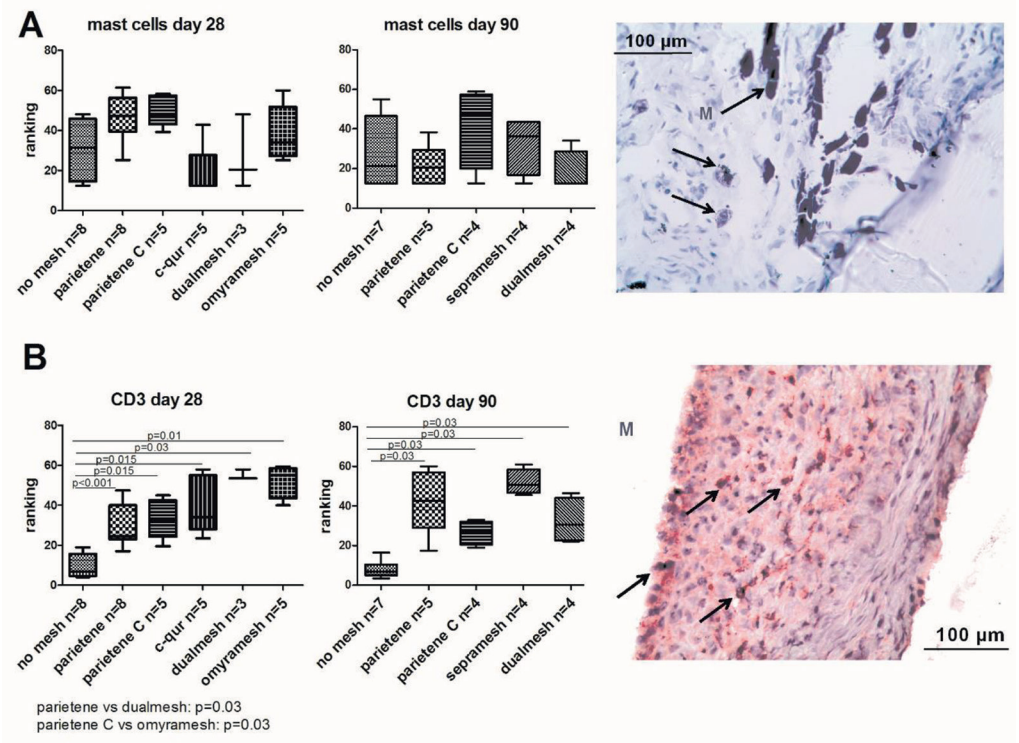

Figure 1 A | Analysis of the presence of mast cells at day 28 and day 90 after implantation of a mesh. Graphs show the mean rank per type of mesh, numbers behind the groups indicate sample size. An example of the toluidine staining is shown in which positive cells are indicated by arrows. M indicates mesh. **B** | Analysis of the presence of T-cells with antibodies against CD3 after 28 and 90 days. An example of the CD3 staining is shown in which positive cells are indicated by arrows. M indicates mesh. Graphs show the mean rank per type of mesh, P-values are indicated in the graphs, numbers behind the groups indicate sample size

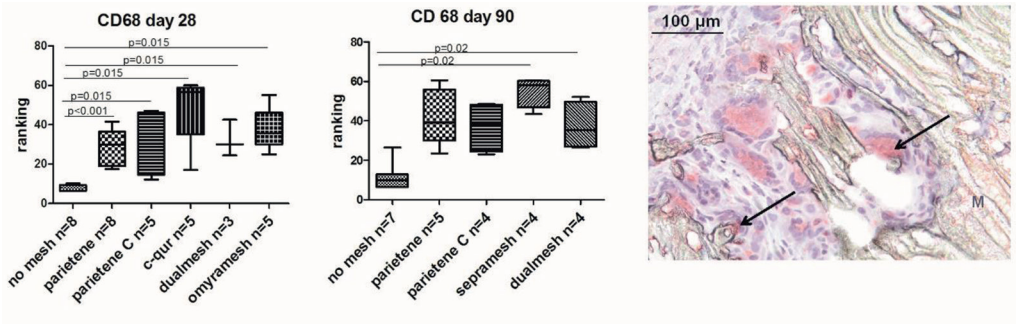

Figure 2 | Analysis and ranking for the presence of CD68-positive macrophages 28 and 90 days after implantation of a mesh. An example of CD68-positive cells is shown in which positive cells are indicated by arrows. M indicates mesh. Graphs show the mean rank per type of mesh, P-values are indicated in the graphs, numbers behind the groups indicate sample size

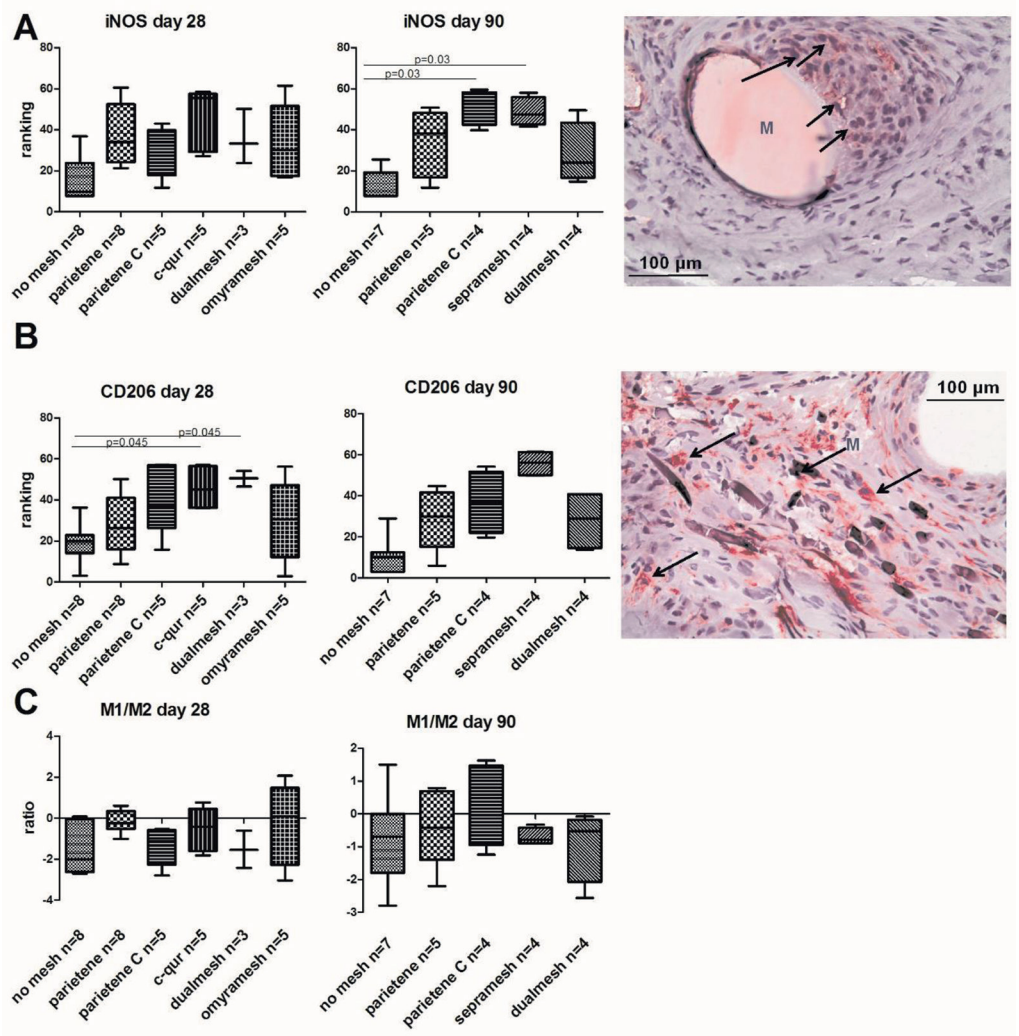

Figure 3 A | Analysis and ranking for the presence of iNOS-positive M1 macrophages 28 and 90 days after implantation of a mesh. An example of iNOS-positive cells is shown in which positive cells are indicated by arrows. M indicates mesh. Graphs show the mean rank per type of mesh, P-values are indicated in the graphs, numbers behind the groups indicate sample size. **B |** Analysis and ranking for the presence of CD206-positive macrophages 28 and 90 days after implantation of a mesh. An example of CD206-positive cells is shown in which positive cells are indicated by arrows. M indicates mesh. Graphs show the mean rank per type of mesh, P-values are indicated in the graphs, numbers behind the groups indicate sample size. **C |** The M1/M2 ratio based on the number of iNOS- positive cells divided by the CD206- positive cells, the natural logarithm of this ratio was calculated for visualisation. P-values are indicated in the graph, numbers behind the groups indicate sample size

DISCUSSION

Surgeons often hesitate to use biomaterials in a contaminated environment, like fascial defects after bowel resection, near stomas or after removal of an infected mesh. Nowadays, most used biomaterials in this environment are biologic materials, which are very expensive compared to synthetic biomaterials. However, a critical review describes that there is not enough evidence to state that biologic biomaterials perform better than synthetic biomaterials [1]. Therefore a close look to synthetic biomaterials in a contaminated environment is needed.

Little is known about the mesh-specific phenotypes and presence of macrophages after implantation of a mesh in a contaminated environment. In this study, different meshes were implanted in a rat model in a contaminated environment. The attractors of macrophages, namely T-cells and mast cells, and the different phenotypes of macrophages were analysed. In these experiments mesh-specific cellular responses were seen. All meshes induced the influx of T-cells and macrophages, still present after 90 days compared with the control group without a mesh. High levels of T-cells and macrophages indicate a chronic inflammatory reaction when meshes were implanted in a contaminated environment [3,4].

Both PTFE-meshes were surrounded by the most T-cells whereas the polypropylene biomaterials Parietene™ and Parietene Composite™ had the lowest number of T-cells. The latter is indicative for resolution of the inflammatory reaction, possibly leading to a fibrotic reaction for Parietene™ which is often seen *in vivo*. This macroscopically represents in a firm incorporation and shrinking of this mesh suggesting fibrosis [3,13,17,18]. Parietene Composite™ performed well macroscopically with a low amount of adhesions and a low percentage of infection in a contaminated environment [13], most likely due to the collagen layer which is known to reduce adhesions [14].

We found high numbers of CD206-positive and iNOS-positive macrophages around C-Qur™- and Dualmesh®-samples after 28 days, indicative for a chronic inflammation reaction. Indeed macroscopically these meshes had the highest infection rate and a bad incorporation in the abdominal wall [13]. This might be explained by the presence of endotoxins released by bacteria during the infection, which are known to delay the foreign body reaction [12]. Dualmesh® is a partially microporous mesh with a higher risk of infection than PP and polyethylene [15,19]. This can be explained by the small pore size allowing bacteria to infiltrate when macrophages cannot [20]. Also small pores induce a M1 pro-inflammatory reaction, known to induce tissue turnover and thereby negatively influencing incorporation of meshes in the abdominal wall [5,21]. This was macroscopically confirmed [13]. Higher numbers of M2 macrophages are associated with a better outcome in wound healing than with a predominant M1-reaction [22,23]. We found high levels of CD206(M2)- positive cells around Parietene Composite™ and Sepramesh™ which are meshes known for a good biocompatibility *in vivo* with low adhesion formation [14]. C-Qur™ is coated with triglycerides and Omega 3-fatty acids. Cardiovascular research showed that triglycerides can enhance an inflammatory response in endothelial cells. Whether this is also the case in the foreign body reaction is not investigated, however this can be a possible explanation for the found chronic inflammation reaction [24].

We expected more distinguished differences between the meshes regarding the M1/M2 ratio, however macrophages are a heterogeneous population of cells, M1 and M2 being two extremes in the spectrum [25,26]. Subtle differences in this ratio might have been missed. Due to poor incorporation of some of the meshes we did not have equal group sizes leading to a lower probability of finding significant differences. Sepramesh™ at day 28, C-Qur™ and Omyramesh® at day 90, and Strattice™ at both timepoints had a very low sample size due to no ingrowth in the surrounding tissues which made it impossible to draw conclusions. Therefore these meshes for these time points were not included in our analysis. No differences were found for mast cells. This is likely due to the time point of analysis for we did our first analysis 28 days after implantation. The amount and presence of mast cells is indicative for an acute inflammatory reaction [7,27] and therefore differences could not be detected in these experiments. Future studies with increased sample numbers and time points are needed to obtain more insight in the precise foreign body reaction and thereby the different performances of meshes in a contaminated environment.

For surgery in an environment at risk of contamination, the choice of a specific mesh is important. More insight in mesh-dependent cellular immune responses can help surgeons choose between the various commercially available meshes for implantation in a contaminated environment.

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CHAPTER 5

In vitro model to study the biomaterial-dependent reaction of macrophages in an inflammatory environment

Grotenhuis N, Vd Toom HF, Kops N, Bayon Y, Deerenberg EB, Mulder IM, van Osch GJ, Lange JF, Bastiaansen-Jenniskens YM. *In vitro* model to study the biomaterial-dependent reaction of macrophages in an inflammatory environment. Br J Surg. 2014 Jul;101(8):983-92. doi: 10.1002/bjs.9523. Epub 2014 May 19.

ABSTRACT

Background

Macrophages play an important role in the reaction to biomaterials, which sometimes have to be used in a surgical field at risk of contamination. The macrophage phenotype in reaction to biomaterials in an inflammatory environment was evaluated in both an *in vivo* and *in vitro* setting.

Methods

In the *in vivo* setting, polypropylene (PP) biomaterial was implanted for 28 days in the contaminated abdominal wall of rats, and upon removal analysed by routine histology as well as immunohistochemistry for CD68 (marker for macrophages), inducible nitric oxide synthase (iNOS – a marker for pro-inflammatory M1 macrophages) and CD206 (marker for anti-inflammatory M2 macrophages). For the *in vitro* model, human peripheral blood monocytes were cultured for 3 days on biomaterials made from PP, collagen (COL), polyethylene terephthalate (PET) and PET coated with collagen (PET+COL). These experiments were performed both with and without lipopolysaccharide and interferon γ stimulation. Secretion of both M1- and M2-related proteins was measured, and a relative M1/M2 index was calculated.

Results

In vivo, iNOS- and CD206-positive cells were found around the fibres of the implanted PP biomaterial. *In vitro*, macrophages on both PP and COL biomaterial had a relatively low M1/M2 index. Macrophages on the PET biomaterial had a high M1/M2 index, with the highest increase of M1 cytokines in an inflammatory environment. Macrophages on the PET+COL biomaterial also had a high M1/M2 index.

Conclusion

Macrophages in an inflammatory environment *in vitro* still react in a biomaterial-dependent manner. This model can help to select biomaterials that are tolerated best in a surgical environment at risk of contamination.

Surgical relevance

Biomaterials in an environment at risk of contamination are often not tolerated owing to a high risk of postoperative infection, which may ultimately lead to removal of the biomaterial. An *in vitro* model with primary human macrophages was used to provide insight into the acute reaction of macrophages to a biomaterial in an inflammatory environment simulated with lipopolysaccharide and interferon γ. The reaction of macrophages in such an inflammatory environment was still biomaterial-dependent. This *in vitro* model can be used to study the reaction of macrophages to different biomaterials in an inflammatory environment in more detail, and thereby help to select biomaterials that are tolerated best in a surgical environment at risk of contamination.

INTRODUCTION

Biomaterials are used widely in reparative and regenerative medicine. However, in an environment at risk of contamination, surgeons are reluctant to use biomaterials owing to a higher risk of complications. A feared postoperative complication of biomaterial implantation is infection of the biomaterial and surrounding tissue by bacteria, reported in up to 16 per cent of patients [1]. The risk of infection is even higher in some circumstances, such as in surgery of the gastrointestinal tract or nasal cavity, as well as in the presence of peritonitis. The risk of infection also depends on the type of biomaterial, such as its configuration, hydrophobicity and whether it is made from monofilament or multifilament [1-3]. All biomaterials elicit a foreign body reaction, and the degree of this reaction varies depending on the nature of the biomaterials. At present, the foreign body reaction in an environment with a high risk of contamination is not well characterized.

Macrophages play a pivotal role in the foreign body reaction [1,4,5]. The phenotype of the macrophages can change in response to environmental factors, giving rise to different populations of macrophages with distinct functions, which can force the foreign body reaction into tolerance of the biomaterial or into a state of inflammation. Classically activated macrophages, or M1 macrophages, have been characterized and described most thoroughly. They propagate pro-inflammatory responses by producing cytokines such as interleukin (IL) 1β, tumour necrosis factor (TNF) α and IL-6 [6-8]. Another macrophage phenotype is represented by the alternatively activated macrophages, referred to as M2 macrophages. These cells can arise when exposed to IL-4 or immune complexes. They express scavenger receptors and IL-1 receptor antagonist (IL-1RA). M2 macrophages also produce IL-10 and chemokines, such as CCL18 and macrophage-derived chemokine (MDC, or CCL22) [6-8], and are able to produce growth factors, thus promoting angiogenesis and tissue repair [6]. During wound healing, M1 macrophages are normally present from day 1, and accumulate and dominate the wound site after 2–3 days. After cleaning the wound site by phagocytosis, macrophages change towards an M2 phenotype. Persistent inflammation can cause an imbalance of M1 to M2 macrophages and lead to fibrosis. Synthetic biomaterials can induce the formation of fibrous wound healing tissue within 2–4 weeks. Macrophages cannot phagocytose this synthetic biomaterial, leading to the formation of giant cells situated at the biomaterial surface [9].

In a contaminated environment macrophages adapt to an M1 phenotype [10], needed for control of the acute infection by phagocytosis. However, prolonged M1 phenotype of macrophages can lead to tissue damage, and may compromise the integration of the material in the body by the release of inflammatory cytokines [9]. Therefore, the foreign body reaction is altered in a contaminated environment.

New biomaterials should be developed for use in an environment where the risk of contamination of the biomaterial is high. Biological materials, such as collagen-based biomaterials processed from human or porcine dermis, are thought to be tolerated in an environment at high risk of contamination and have a low postoperative complication rate [11,12]. Biomaterials with low actual surface area, such as monofilament biomaterials, were well tolerated in a contaminated field in an experimental study [2], and in several clinical studies have been associated with fewer postoperative infections [13,14]. However, there is no consensus yet, and only a few comparative studies [13,

14] are available. In a recent study [15] employing an experimental rat model, the foreign body reaction in rats was biomaterial-dependent in a contaminated environment. Some biomaterials had poor incorporation into the abdominal wall with a high infection rate, whereas others, such as monofilament polypropylene (PP) biomaterials, had good incorporation into the abdominal wall and a low inflammatory reaction [15].

The aim of this study was to investigate the reaction of macrophages to biomaterials in an environment at risk of contamination. First, the phenotype of macrophages surrounding a monofilament polypropylene biomaterial was analysed *in vivo*, as this material has been shown previously to induce the mildest foreign body reaction [15]. Second, the macrophage phenotype and reaction were characterized in more detail in an *in vitro* model. In this model bacterial contamination was simulated, thereby permitting comparison of the macrophage reaction in a contaminated and a clean environment. Contamination was simulated using a combination of lipopolysaccharide (LPS) and interferon (IFN) γ, and the macrophage reaction was studied by measuring a panel of proteins indicative of the macrophage phenotype.

METHODS

Rat peritonitis model and tissue collection

The protocol of the rat experiment was approved by the Ethical Committee on Animal Experimentation of Erasmus University Rotterdam, The Netherlands, and is in accordance with the Animal Research: Reporting *In Vivo* Experiments (ARRIVE) guidelines. A contaminated environment was created by the caecal ligature puncture model, in which the caecum is punctured to provide leakage of faecal fluid into the abdominal cavity, thus causing peritonitis. After 24 h the abdominal cavity was reopened, peritonitis was confirmed by microbiological culture, and a monofilament PP biomaterial (Parietene™; Covidien – Sofradim Production, Trévoux, France) was placed intraperitoneally in four rats [15]. 28 days after implantation, a sample of the abdominal wall with the incorporated biomaterial was harvested using biopsy punches (5 mm diameter). As controls, abdominal walls from rats with peritonitis, but with no biomaterial, were collected. All tissue samples were fixed in 4 per cent formalin and embedded in paraffin.

Histology and immunohistochemistry

Tissue sections were cut and stained with haematoxylin and eosin in accordance with standard procedures. To identify macrophage types, immunohistochemical staining was carried out with the following antibodies: CD68, a general macrophage marker; CD206, a marker for M2 macrophages [8], and inducible nitric oxide synthase (iNOS) as a marker for M1 macrophages [9]. Briefly, paraffin sections were dewaxed and, to block the sections for aspecific binding, the sections were pretreated with heat-mediated antigen retrieval solution (Target Retrieval Solution; Dako, Glostrup, Denmark) at 90 °C for 20 min. Sections were incubated with CD68 (1 : 100; Acris, Herford, Germany), CD206 (1 : 100) or iNOS (1 : 50) (both Abcam, Cambridge, UK) antibodies for 60 min, and subsequently incubated with link and label (Concentrated MultiLink® and Concentrated HRP Label (peroxidase-

conjugated streptavidin); BioGenex, Fremont, California, USA); 3,3′-diaminobenzidine was used as substrate. Sections were dried overnight and mounted with VectaMount™ (Vector Laboratories, Burlingame, California, USA). Matching irrelevant isotype antibodies were used as negative controls, and tissues known to contain the specific markers were employed as positive controls. Sections were also Gram-stained to visualize potential bacteria. All slides were analysed with an Olympus BX50 light microscope (Olympus, Tokyo, Japan).

Monocyte isolation

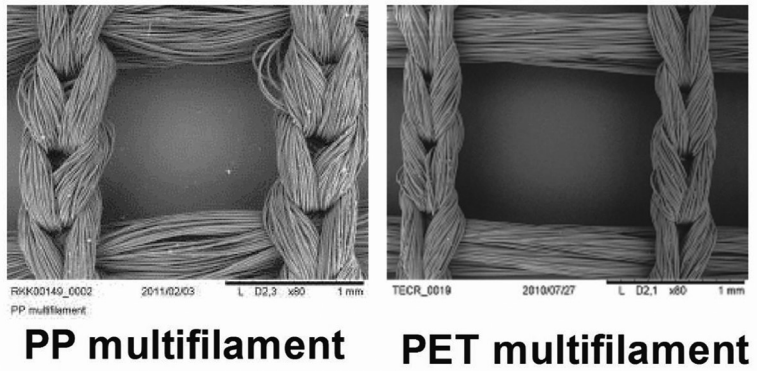
Ficoll density gradient (Ficoll-Paque™ PLUS; GE Healthcare, St Giles, UK) was used to isolate monocytes from the buffy coat of four healthy donors (men and women aged 25–65 years). All buffy coats were obtained from the blood bank (Sanquin, Rotterdam, The Netherlands). Some 30 ml of buffy coat diluted 1 : 5 with 0.1 per cent bovine serum albumin (BSA) in phosphate-buffered saline (PBS) was layered on 15 ml Ficoll. After 15 min centrifugation at 1000g with no brake, the interphase band containing peripheral blood mononuclear cells was aspirated and washed in PBS/BSA 0·5 per cent 2 mmol/l EDTA and labelled with 100 µl anti-CD14+ magnetic beads (CD14 microbeads human, MACS Separation columns LS and MidiMACS™ Separator; all from Miltenyi Biotec, Bergisch Gladbach, Germany), and isolated according to the manufacturer's guidelines. This positive selection of monocytes will not activate the cells [16]. Purity of the isolation was assessed by fluorescence-activated cell sorting (FACS) analysis, in which 1×106 monocytes were incubated for 15 min at room temperature with the following antibodies: FITC-conjugated CD14 and peridinin chlorophyll protein complex (PerCP)-conjugated CD45 (all BD Biosciences, Franklin Lakes, New Jersey, USA). After incubation, cells were washed in PBS/BSA 0.1 per cent and FACS analysis was performed with CellQuest™ Pro on a FACSCalibur™ (both BD Biosciences); the purity of the freshly isolated CD14+ monocytes was above 95 per cent (data not shown). In the case of donors 1, 2 and 4, the yield of monocytes was not sufficient to allow testing of all four biomaterials in the experiments.

Culturing macrophages on biomaterials

Four different biomaterials were chosen to study macrophage response in relation to the biomaterial (all from Covidien – Sofradim Production): a multifilament PP biomaterial (Parietene™), hydrophobic with a contact angle of 95°; a collagen-based material (COL) (Permacol™), processed from porcine skin and cross-linked with hexamethylene di-isocyanate; a multifilament polyethylene terephthalate (PET) biomaterial, hydrophilic with a contact angle of 80.9°; and a multifilament PET biomaterial with an absorbable, continuous and hydrophilic collagen film on one of its sides (PET + COL) (Parietex™ Composite). The PET and PP biomaterials have a similar weave (Figure S1, supporting information).

The materials were cut into 1.5×1.5-cm pieces with a sterile scalpel. Before cell seeding, materials were incubated in 100 per cent non-heat-inactivated fetal calf serum (FCS) (Lonza, Verviers, Belgium) for 2 h to provide protein attachment. Freshly isolated monocytes were adjusted to a concentration of 0.7×10^6 cells/ml in a total volume of 25 ml in a 50-ml PP tube (Falcon™; Becton, Dickinson, Franklin Lakes, New Jersey, USA). Twelve samples were incubated per 25 ml for 2 h at 37°C. Subsequently, samples were placed in a 24-well non-adherent plate (NUNC™, non-treated multiplate; Thermo Scientific, Rochester, New York, USA) and cultured for 3 days in serum-free X-VIVO™ 15 medium with

20 per cent FCS (Lonza). To simulate an inflammatory environment caused by bacterial infection, macrophages on biomaterials were cultured with 10 ng/ml LPS (Sigma-Aldrich, St Louis, Missouri, USA) and 1 ng/ml recombinant human IFN-γ (PeproTech, Rocky Hill, New Jersey, USA), and compared with macrophages on the same materials without simulation. The medium was refreshed after 48 h of culturing, and after a further 24 h in culture the supernatant was harvested for protein analysis.



Supplementary Figure 1 | Detailed picture of wave pattern of PET and PP.
Left: polypropylene multifilament. Right polyethylene multifilament.

Analysis of the production of inflammatory and anti-inflammatory cytokines

Proteins were measured in 25 µl cell culture supernatant using a multiplex system (Millipore, Billerica, Massachusetts, USA) [17]. IL-1β, IL-6, TNF-α, monocyte chemotactic protein (MCP) 3 and macrophage inflammatory protein (MIP) 1α, IL-1RA, RANTES (regulated on activation, normal T cell expressed and secreted, or CCL5), and MDC (CCL22) were measured according to manufacturer recommendations. The CCL18 DuoSet® enzyme-linked immunosorbent assay (R&D Systems, Minneapolis, Minnesota, USA) was used to analyse CCL18 in 100 µl cell culture supernatant according to the manufacturer's instructions. These nine proteins were selected based on previous experiments, where the read-out parameters were chosen after stimulation of macrophages towards either the M1 or M2 phenotype [17].

To correct for the numbers of macrophages on the different biomaterials, the cells were lysed in 0.1 per cent Triton in PBS (Sigma-Aldrich) and samples were frozen at -80 °C before being analysed with a CyQUANT® cell proliferation assay kit (Invitrogen, Carlsbad, California, USA). DNA content was measured according to the manufacturer's recommendation.

Statistical analysis

The *in vitro* experiments were performed in triplicate with four different monocyte donors. All data are presented as scatterdot plots, with each dot representing an individual sample. The mean of the four donors is indicated by a line in the graphs. When evaluating the effect of an inflammatory

environment, the data are presented as the ratio of the LPS/IFN-γ-stimulated condition versus the non-stimulated condition for each biomaterial. To calculate the ratio between LPS/IFN-γ-stimulated samples and non-stimulated samples, the stimulated samples were divided by the mean of the non-stimulated samples per donor. To compare the effect of the four biomaterials on the macrophage phenotype in an inflammatory environment, a relative M1/M2 index for each material was determined by calculating for each cytokine the percentage of production relative to the mean production on the four materials. This was followed by taking the mean of the percentages of the M1 cytokines (MIP-1α, TNF-α, MCP-3, IL-1β, IL-6) divided by the mean percentages of the M2 cytokines (MDC, RANTES, IL-1RA and CCL18) per sample. Groups were compared in SPSS® for Windows® version 20.0 (IBM, Armonk, New York, USA) using the Kruskal–Wallis test (independent samples median test) and Mann–Whitney U test, because the data were not normally distributed. Correlation between proteins was analysed by Spearman correlation. The Bonferroni correction was used. Differences were considered statistically significant when $P < 0.050$.

RESULTS

Macrophage phenotype *in vivo*

The PP biomaterial was well integrated in the surrounding tissues 28 days after implantation into the contaminated abdominal wall of rats. On histological examination, all samples displayed dense tissue surrounding the fibres of the biomaterial, with many multinucleated CD206-positive giant cells. iNOS- and CD206-positive cells were also observed in this dense layer. In addition, many blood vessels were observed in the connective tissue surrounding the biomaterial (Figure 1). To investigate the influence of a biomaterial, samples of abdominal wall tissue from control rats with contamination but without implanted biomaterial were also stained with haematoxylin and eosin, and for CD68, CD206 and iNOS, at 28 days. These samples had no infiltration of lymphocytes and only a few macrophages, some of which were iNOS- or CD206-positive (Figure 1).

Biomaterial-dependent effect on macrophage phenotype in an *in vitro* model

LPS and IFN-γ were chosen to simulate bacterial infection in the *in vitro* model. LPS is a bacterial wall fragment and IFN-γ is known to activate the immune system and macrophages following bacterial infection [18]. To investigate how macrophages react on biomaterials in this simulated inflammatory environment *in vitro*, production of IL-1β, IL-6, TNF-α, MCP-3, MIP-1α, IL-1RA, RANTES, MDC and CCL18 was measured. The production of these proteins in an inflammatory environment was compared with that in a non-stimulated environment. Although the inflammatory environment increased the production of most pro-inflammatory proteins by macrophages, there were still differences in relation to the tested biomaterials. Macrophages on PET biomaterial induced the biggest increase in pro-inflammatory proteins (Figure 2).

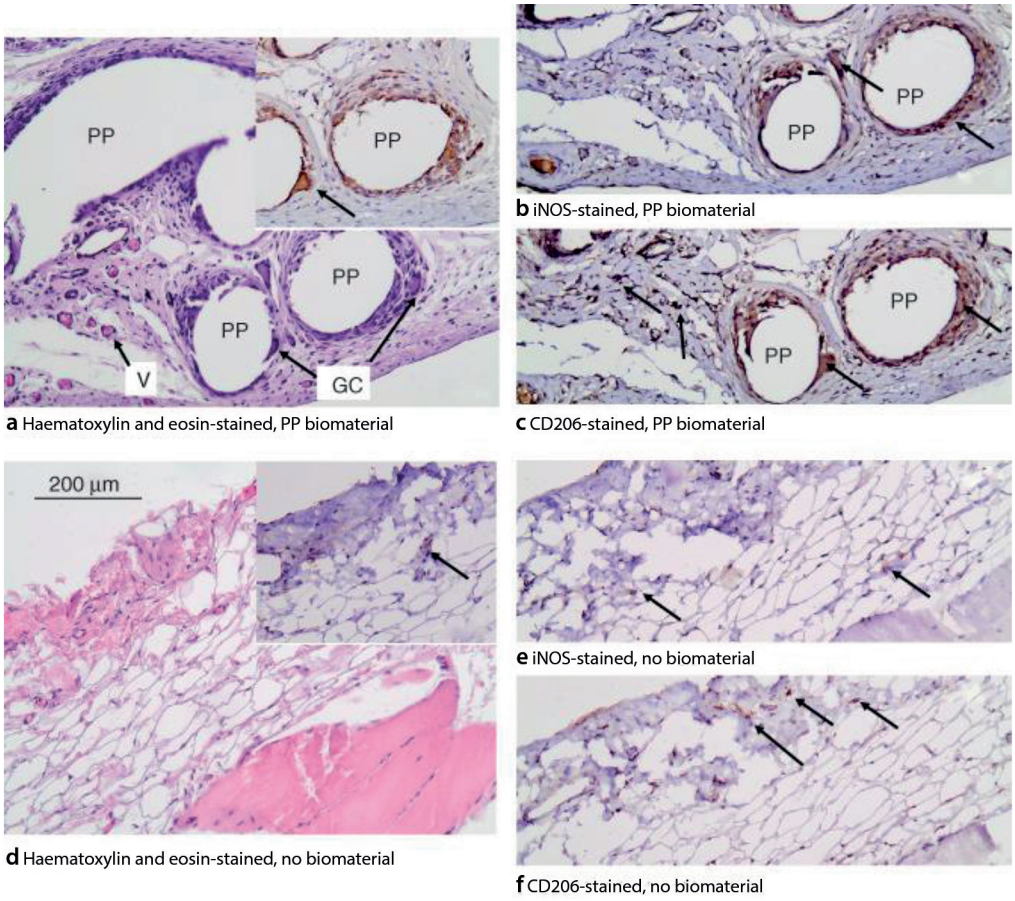


Figure 1 a,d | Haematoxylin and eosin (CD68 shown in inset), b,e inducible nitric oxide synthase (iNOS) and **c,f** | CD206 staining 28 days after implantation of polypropylene (PP) in a contaminated environment in the rat. CD68-, iNOS- and CD206-positive macrophages can be seen surrounding the PP fibres. **a–c** | PP biomaterial from a contaminated abdominal wall. **d–f** | Abdominal wall without biomaterial from the same model. Representative sections and samples are shown. Brown colour represents positive staining; arrows indicate positive cells. GC, giant cell; V, vessels. (Original magnification ×200)

The stimulated versus non-stimulated ratio for anti-inflammatory proteins was approximately 1, indicating no increase in the production of these proteins in an inflammatory environment, except for RANTES, which was produced in greater amounts by macrophages on PET biomaterial in an inflammatory environment (Figure 3).

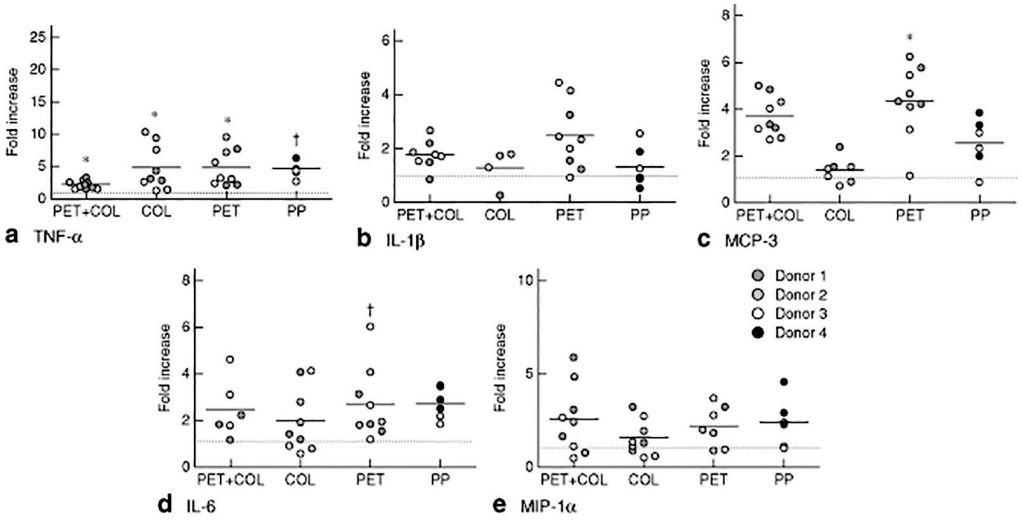


Figure 2 | Production of pro-inflammatory cytokines by macrophages seeded on different biomaterials in an inflammatory (as induced by lipopolysaccharide/interferon γ) compared with a non-stimulated environment after 3 days of culture. **a** | Tumour necrosis factor (TNF) α, **b** | interleukin (IL) 1β, **c** | monocyte chemotactic protein (MCP) 3; **d** | IL-6, **e** | macrophage inflammatory protein (MIP) 1α. The dotted line indicates the basal level of expression, where there is no difference between stimulated and non-stimulated environments, and the bars denote the mean value. Monocytes from a total of four donors were divided over the different biomaterials in triplicate samples. Cells from all donors could not be tested on every biomaterial owing to a low yield of monocytes. Protein production was corrected for DNA before comparison of stimulated and non-stimulated environments.

PET+COL = polyethylene terephthalate with a collagen coating; COL = collagen; PET = polyethylene terephthalate; PP = polypropylene. *P < 0·001, †P < 0·050 (Kruskal–Wallis and Mann–Whitney U tests), indicating a significant increase in pro-inflammatory cytokines compared with baseline values

To compare the reaction of macrophages on the four different biomaterials in an inflammatory environment, the total amount of protein corrected for DNA is shown (Figure 4 and Figure S2, supporting information). The greatest induction of pro-inflammatory cytokines TNF-α, IL-1β, MCP-3 and MIP-1α was induced by macrophages on PET + COL biomaterial in the inflammatory environment. The lowest induction of pro-inflammatory and anti-inflammatory cytokine production was seen on the COL biomaterial (Figure 4).

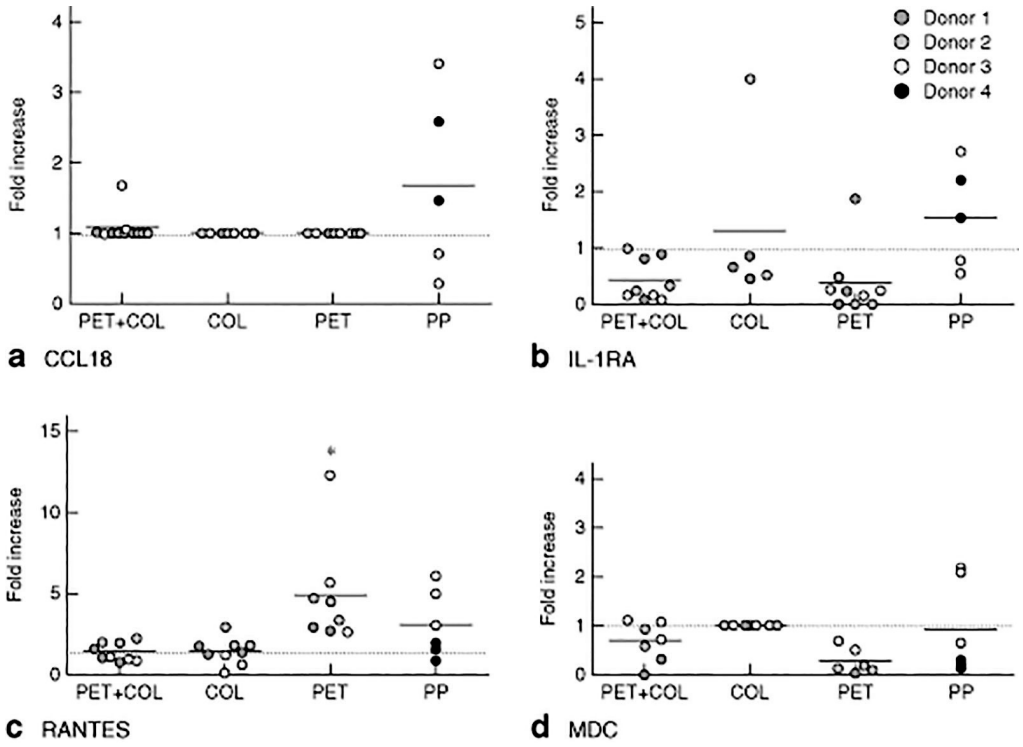


Figure 3 | Production of anti-inflammatory cytokines by macrophages seeded on different biomaterials in an inflammatory environment (as induced by lipopolysaccharide/interferon γ) compared with a non-stimulated environment after 3 days of culture. **a** | CCL18, **b** | interleukin 1 receptor antagonist (IL-1RA), **c** | RANTES (regulated on activation, normal T cell expressed and secreted), **d** | macrophage-derived chemokine (MDC). The dotted line indicates the basal level of expression, where there is no difference between stimulated and non-stimulated environments, and the bars denote the mean value. Monocytes from a total of four donors were divided over the different biomaterials in triplicate samples. Cells from all donors could not be tested on every biomaterial owing to a low yield of monocytes. Protein production was corrected for DNA before comparison of stimulated and non-stimulated environments.

PET+COL = polyethylene terephthalate with a collagen coating; COL = collagen; PET = polyethylene terephthalate; PP = polypropylene. *P < 0·050 (Kruskal–Wallis and Mann–Whitney U tests), indicating a significant increase in anti-inflammatory cytokines compared with baseline values.

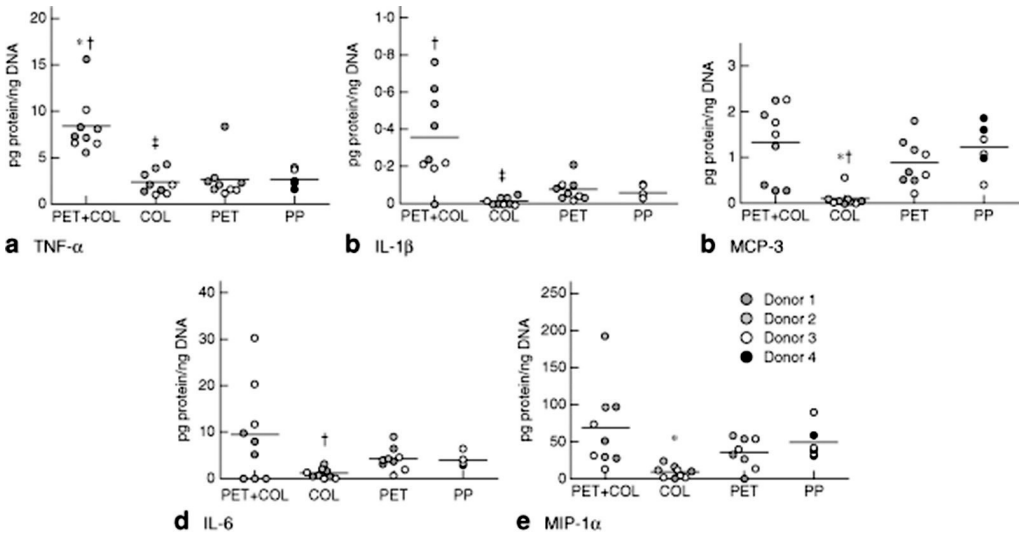
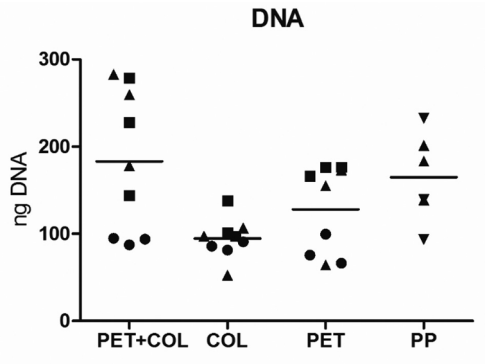


Figure 4 | Comparison of secretion of pro-inflammatory cytokines by macrophages seeded on different biomaterials on the third day of culture with lipopolysaccharide/interferon γ, corrected for DNA. **a** | Tumour necrosis factor (TNF) α, **b** | interleukin (IL) 1β, **c** | monocyte chemotactic protein (MCP) 3; **d** | IL-6, **e** | macrophage inflammatory protein (MIP) 1α. Monocytes from a total of four donors were divided over the different biomaterials in triplicate samples. Cells from all donors could not be tested on every biomaterial owing to a low yield of monocytes.

PET+COL = polyethylene terephthalate with a collagen coating; COL = collagen; PET, polyethylene terephthalate; PP = polypropylene. **a** *P < 0·001 (PET+COL versus COL), †P < 0·050 (PET+COL versus PET), ‡P < 0.050 (COL versus PP); **b** †P < 0·050 (PET+COL versus COL and PET), ‡P < 0·050 (COL versus PET and PP); **c** *P < 0·001 (COL versus PET+COL and PET), †P < 0·050 (COL versus PP); **d** †P < 0·050 (COL versus PET and PP); **e** *P < 0·001 (COL versus PET+COL and PP) (Kruskal–Wallis and Mann–Whitney U tests)



Supplementary Figure 2 | DNA of macrophages cultured on biomaterials. DNA (ng) of macrophages seeded on different biomaterials at day 3 of culture with LPS/IFNγ. n = 4 monocyte donors with samples in triplicate. Due to the yield of the monocytes not all biomaterials could be seeded except for donor 3.

PET+COL = polyethylene terephthalate with a collagen coating; COL = collagen; PET = polyethylene terephthalate; PP = polypropylene

Macrophages on PP biomaterial produced significantly more CCL18 and MDC than macrophages on other biomaterials, with the exception of MDC on PET + COL biomaterial. Macrophages on PET + COL biomaterial induced a significantly higher RANTES production compared with macrophages on COL (Figure 5). Macrophages on PP and COL biomaterial had the lowest M1/M2 index, whereas macrophages on PET and PET + COL biomaterials had the highest M1/M2 index in the inflammatory environment (Figure 5).

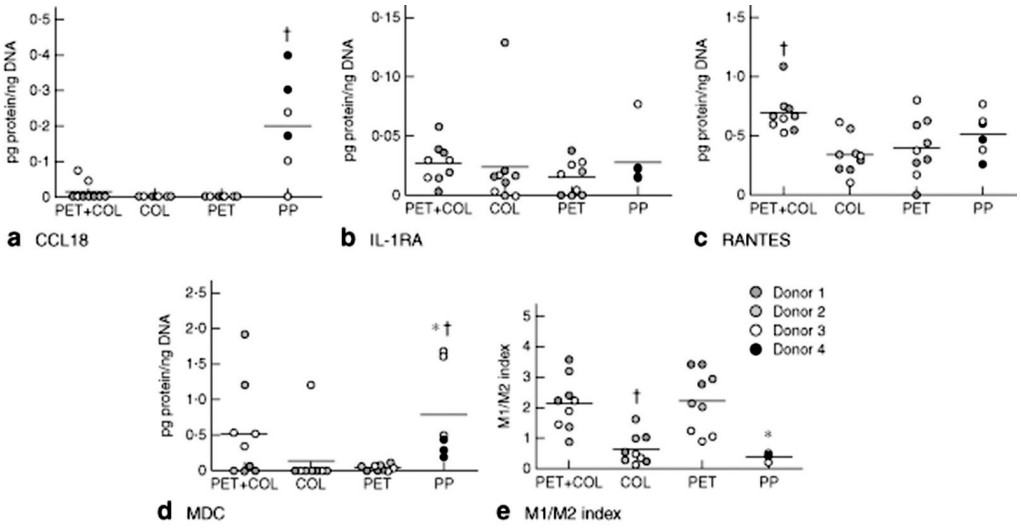


Figure 5 | Comparison of secretion of anti-inflammatory cytokines by macrophages seeded on different biomaterials at the third day of culture with lipopolysaccharide/interferon γ, corrected for DNA. **a** | CCL18, **b** | interleukin 1 receptor antagonist (IL-1RA), **c** | RANTES (regulated on activation, normal T cell expressed and secreted), **d** | macrophage-derived chemokine (MDC); **e** | M1/M2 macrophage index. Monocytes from a total of four donors were divided over the different biomaterials in triplicate samples. Cells from all donors could not be tested on every biomaterial owing to a low yield of monocytes. The M1/M2 index for each sample was calculated as the percentage of the mean for each cytokine. The mean of M1 cytokines (macrophage inflammatory protein 1α, tumour necrosis factor α, monocyte chemotactic protein 3, interleukin (IL) 1β, IL-6) was divided by the mean of M2 cytokines (MDC, RANTES, IL-1RA and CCL18).

PET+COL = polyethylene terephthalate with a collagen coating; COL = collagen; PET, polyethylene terephthalate; PP = polypropylene. **a** | †$P < 0{\cdot}050$ (PP versus all other biomaterials); **c** | †$P < 0{\cdot}050$ (PET+COL versus COL); **d** | *$P < 0{\cdot}001$ (PP versus PET), †$P < 0{\cdot}050$ (PP versus COL); **e** | *$P < 0{\cdot}001$ (PP versus PET+COL and PET), †$P < 0{\cdot}050$ (COL versus PET+COL and PET) (Kruskal–Wallis and Mann–Whitney U tests)

Taking all the samples together, after correction for multiple testing, significant correlations with $P < 0.050$ were found between MCP-3 and MDC ($rs = 0.80$), IL-6 and IL-1β ($rs = 0.59$), MIP-1α and MCP-3 ($rs = 0.64$), MIP-1α and IL-1β ($rs = 0.60$), TNF-α and IL-1β ($rs = 0.59$), and MIP-1α and RANTES ($rs = 0.72$).

DISCUSSION

As tolerance to biomaterials in surgical areas at risk of postoperative contamination is not understood completely, surgeons are reluctant to use biomaterials in these circumstances. Biomaterials should be explored for safer use in surgical environments prone to the development of postoperative infection. Macrophages are key players in the foreign body reaction, thus influencing the fate of biomaterials. In the present study the effect of biomaterials on macrophage phenotypes in an experimental model of postoperative contamination in rats, and in an *in vitro* model of inflammation, were studied.

Implantation of the monofilament PP biomaterial in a contaminated environment in the rat *in vivo* [15] revealed that PP fibres became surrounded by a small layer of dense tissue with many macrophages and other leucocytes. Compared with a contaminated abdominal wall without PP, which by day 28 displayed only a few inflammatory cells, the implanted PP mesh appeared to extend the postoperative inflammatory reaction. No residual bacteria were observed on Gram staining (data not shown), in agreement with previous results of negative microbiological cultures of the biomaterial 28 days after implantation [15]. This means that the extended inflammatory reaction is not caused by the presence of bacteria. The macrophages surrounding the PP mesh displayed mainly an M2 phenotype, which is associated with tissue repair and angiogenesis, thus indicating a remodelling phase of wound healing [10]. In earlier *in vivo* rat studies, monofilament PP biomaterial evoked an anti-inflammatory/fibrotic reaction with formation of fibrotic tissue around the mesh fibres, a low infection rate, and good incorporation into the abdominal wall, in both a contaminated [15] and a sterile [19-21] environment.

For the *in vitro* analysis, the M1/M2 index was calculated to summarize the effects of a biomaterial on macrophages. However, it should be appreciated that dividing macrophages into either M1 or M2 phenotypes is a simplification, as several intermediate states exist [22]. In the *in vitro* inflammatory environment, macrophages on the multifilament PP biomaterial induced the expression of anti-inflammatory proteins at a higher rate than the other biomaterials tested, thus resulting in a low M1/M2 index. The low M1/M2 index in the case of PP is caused mainly by a high protein production of CCL18, which is known for its association with fibrosis [23].

Macrophages on the COL biomaterial produced a relatively low amount of pro-inflammatory and anti-inflammatory cytokines, indicative of a mild reaction to the biomaterial. A mild foreign body reaction against collagen-based biomaterial has also been observed *in vivo* by others [11,12,14,24].

Macrophages on the PET biomaterial had a relatively high M1/M2 index in the *in vitro* model, indicating a predominantly pro-inflammatory reaction of macrophages. PET and PP biomaterials are knitted according to similar weaves, resulting in comparable surfaces (Figure S1, supporting information). The difference *in vitro* is thus mainly in the contact angle/hydrophobicity, and therefore the pro-inflammatory reaction; thus the high M1/M2 index can be caused partly by the polymer type itself.

The PET + COL composite biomaterial tested is the mesh type generally preferred for intraperitoneal hernia surgery, as it minimizes the formation of postoperative tissue adhesions [25,26]. A high M1/M2 index was found for PET + COL biomaterial, indicating a high pro-inflammatory reaction

in an inflammatory environment. In fact, this material evoked the highest absolute production of pro-inflammatory cytokines. This acute reaction can be explained by phagocytic activity of macrophages, trying to break down and digest the thin collagen layer [27]. A pro-inflammatory reaction was induced by the macrophages on PET + COL, even in a non-stimulated environment. When this environment was compared with an inflammatory environment *in vitro*, only a slight further increase in pro-inflammatory protein production was observed. This indicates that the PET + COL material itself has a great influence on the reaction of macrophages.

In a previous study [17], the M1/M2 index in a sterile environment was analysed *in vitro*. Most interestingly, the present data indicate that the macrophage response remains biomaterial-specific even in an environment with simulated contamination. When comparing sterile and contaminated environments, the largest differences were observed for TNF-α production. TNF-α is an acute-phase protein, and reacts quickly in the present *in vitro* system. However, this does not indicate that the fourfold increases in MCP-3 or the threefold increases in IL-6 are less relevant, as these factors might have a different potency or kinetics.

In vivo there is a great difference between multifilament and monofilament biomaterials, as the former allow more cells to attach and fill the biomaterial. Monofilament biomaterials are less prone to infection because they provide fewer niches for bacterial infiltration [2,3]. In the present study, monofilament biomaterials were not tested in the *in vitro* system owing to the low number of macrophages attaching to these in comparison with multifilament biomaterials.

The variation between macrophages isolated from different donors is not unexpected because it is known from clinical practice that patients respond differently to biomaterials. However, variations between the samples from one donor were also observed, which can be explained by the fact that monocytes are a heterogeneous population with different sensitivities to biomaterials or cytokines. However, taken together, distinct differences in macrophage reactions to biomaterials were observed.

The present study describes the very acute reaction to biomaterials, with analysis after 3 days of culture. The acute reaction is indicative of the subsequent outcome. It is obvious that the *in vivo* conditions are more complex than the *in vitro* situation. Most importantly, this study shows that an *in vitro* model system can be used to evaluate and simulate the foreign body reaction in an inflammatory environment, which can aid in selecting and developing new biomaterials that are well tolerated under conditions with a high risk of postoperative biomaterial infection.

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CHAPTER 6

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/cm^2 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/cm^2 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 $C_6H_8O_7$, 0.88 M NaAc ·3H_2O, 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 $2^{-\Delta CT}$ method.

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).

Gene	Fw	Rev	Probe
Reverence gene: Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)	GTCAACGGATTTGGTC GTATTGGG	TGCCATGGGTGGAATC ATATTGG	FAM-CGCCCAATACGACCAAATCCGTT GAC-TAMRA
procollagen-Lysine, 2-Oxoglutarate 5-Dioxygenase (PLOD2)	CCCTCCGATCAGAGAT GATT	AATGTTTCCGGAGTAG GGGAGTCTTTTT	FAM-CGTGCGCGTGATAAACTGGATCCT GATATGGCTTCTTCGCACG-Dabcyl
α-smooth muscle actin *(ASMA)*	CGTTGCCCCTGAAGAG CAT	CCGCCTGGATAGCCAC ATACA	
Collagen type 1 (*COL1A1*)	CAGCCGCTTCACCTAC AGC	TTTTGTATTCAATCACT GTCTTGCC	
Prostaglandin-Endoperoxide Synthase 2 (*PTGS2*)	AATGGGGTGATGAGCA G TTGTTC	GGATGCCAGTGATAGA GGGTGTTA	
matrix metalloprotease (*MMP1)*	CTCAATTTCACTTCTGT TTTCTG	CATCTCTGTCGGCAAA TTCGT	FAM-CGTGCCAAAGCCTTTCAACTCTGG AGCAATGTCACGGCACG-Dabcyl
transforming growth factor 1 (TGFB1)	GTGACAGCAGGGATAA CATACTG	CATGAATGGTGGCCAG GTC	
Interleukin-6 (IL-6)	TCGAGCCCACCGGGA ACGAA	GCAGGGAAGGCAGCA GGCAA	
Interleukin-10 (IL-10)	CCTGGAGGAGGTGAT GCCCCA	GACAGCGCCGTAGCCT CAGC	
Chemokine Ligand 18 (CCL18)	GCACCATGGCCCTCT GCTCC	GGGCACTGGGGGCTG GTTTC	
Mannose receptor (CD206)	TGGCCGTATGCCGGTC ACTGTTA	ACTTGTGAGGTCACCG CCTTCCT	

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 (Figure 1A). MCM stimulated the collagen deposition and the amount of DNA of ASCs (Figure 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].

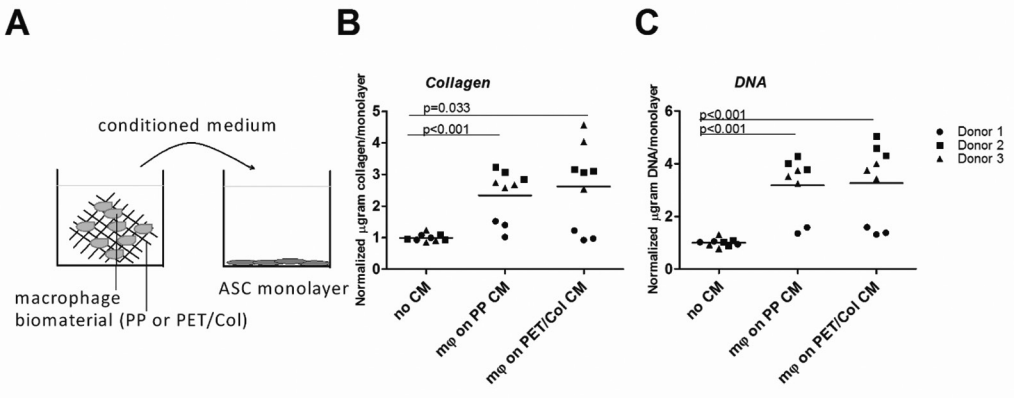


Figure 1 | Collagen production and proliferation of ASCs. **A** | Schematic representation of the culture setup in which ASCs in monolayer were stimulated with MCM for 7 days. **B** | Amount of collagen in microgram per monolayer of ASCs stimulated with or without MCM. **C** | Microgram of DNA per monolayer 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 (Figure 2).

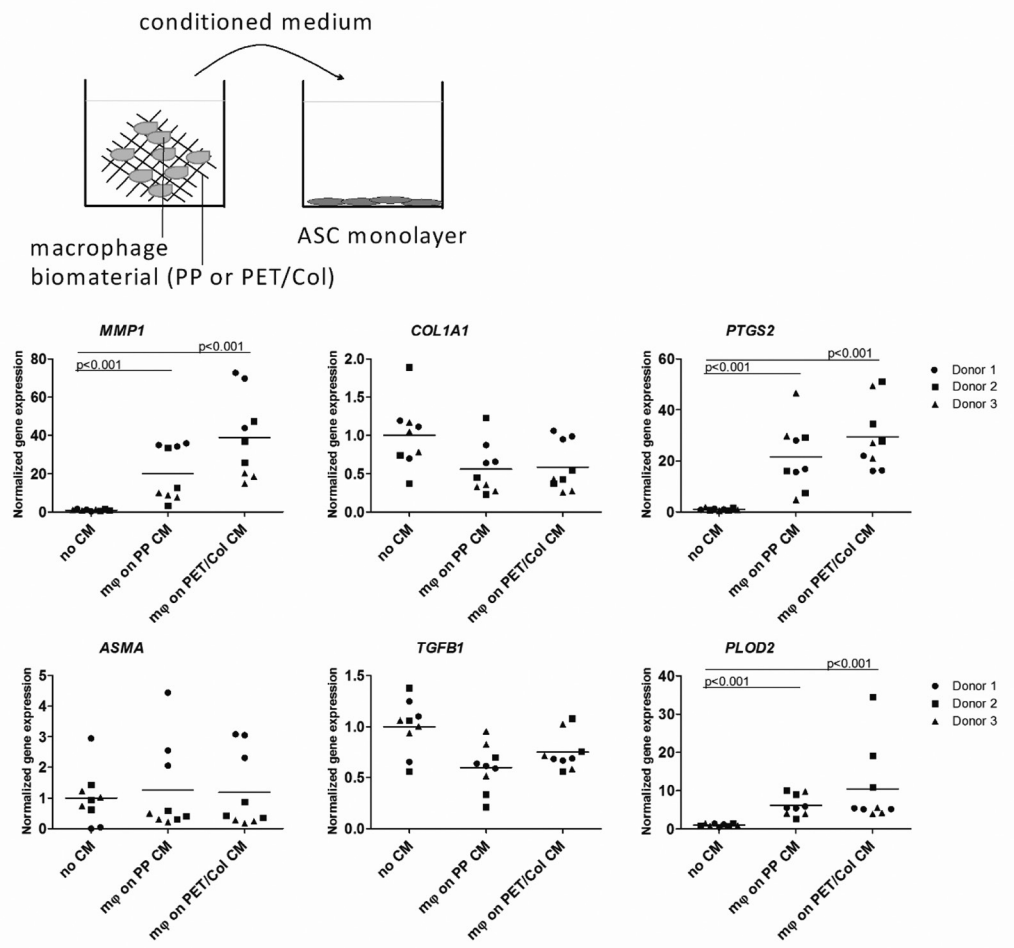


Figure 2 | Gene expression by ASCs stimulated with CM. Gene expression of ASCs cultured in monolayer with or without MCM of macrophages cultured on biomaterials (PP and PET/Col). The gene expression was normalized to the average of the control condition without MCM within each separate ASC experiment.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

6

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 (Figure 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 (Figure 3).

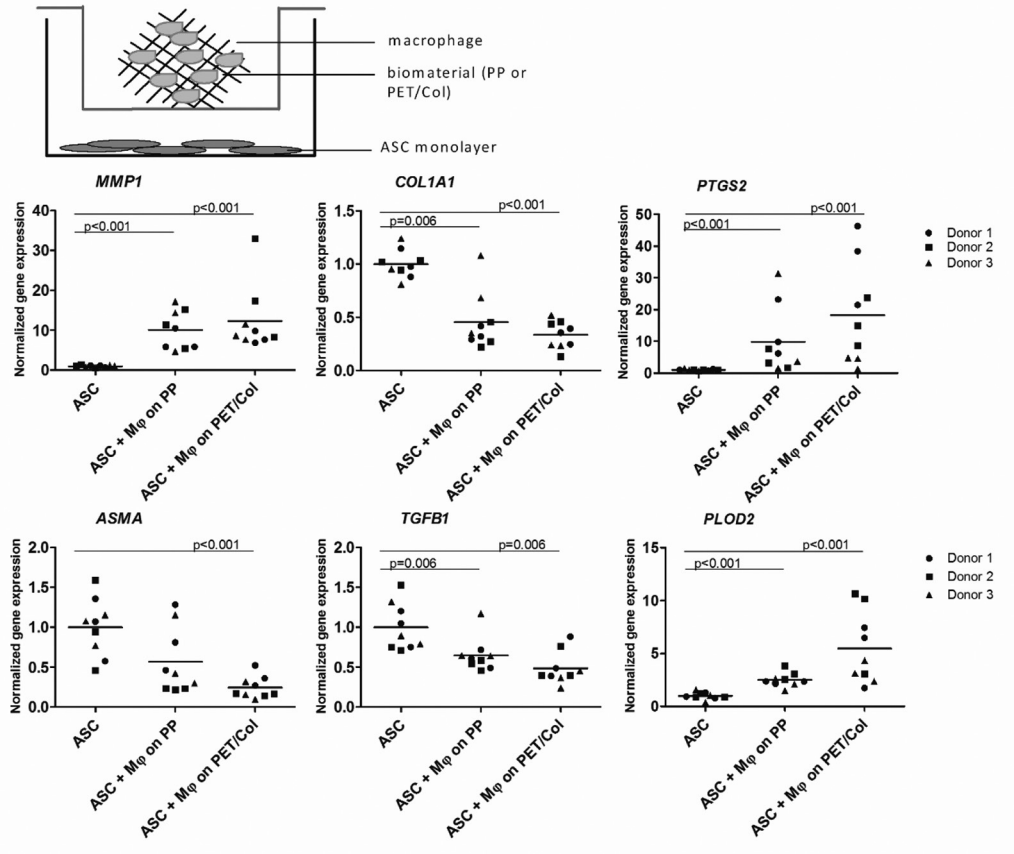


Figure 3 | Gene expression of ASCs in monolayer cocultured with macrophages. Gene expression of ASCs in monolayer cocultured with macrophages on biomaterials (PP and PET/Col) in a transwell system. The average gene expression of ASCs without macrophages was set to 1 for each ASC donor. Experiments were 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 (Figure 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 (Figure 4).

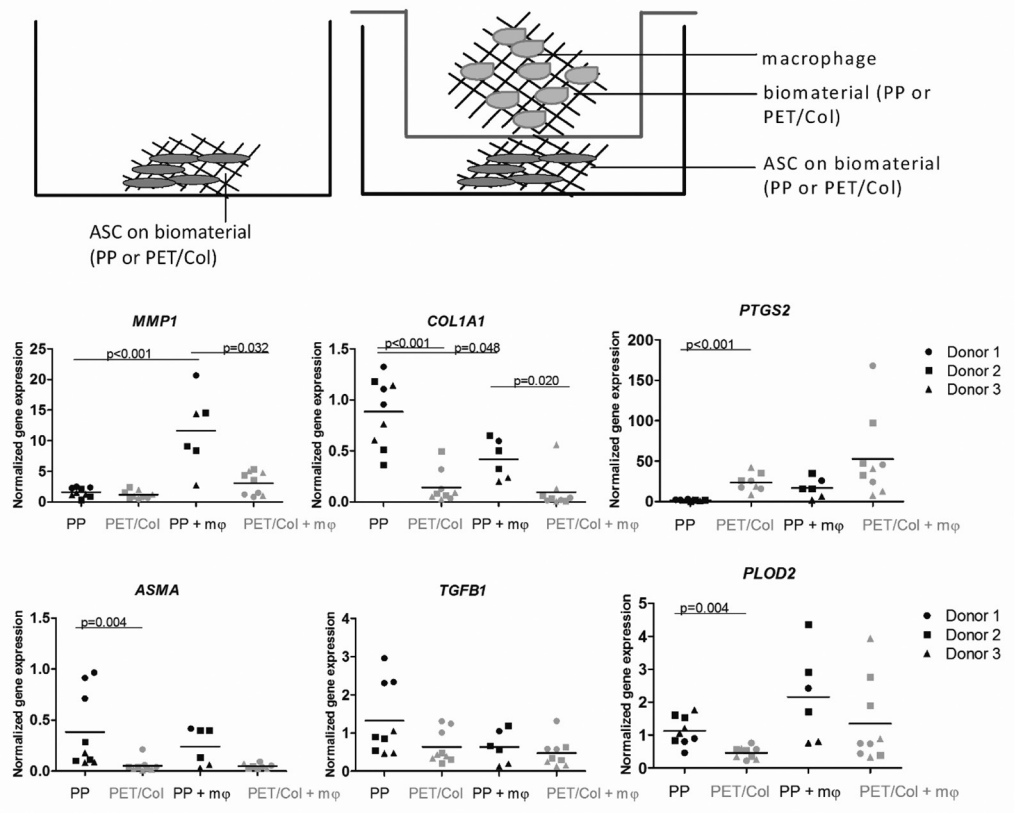
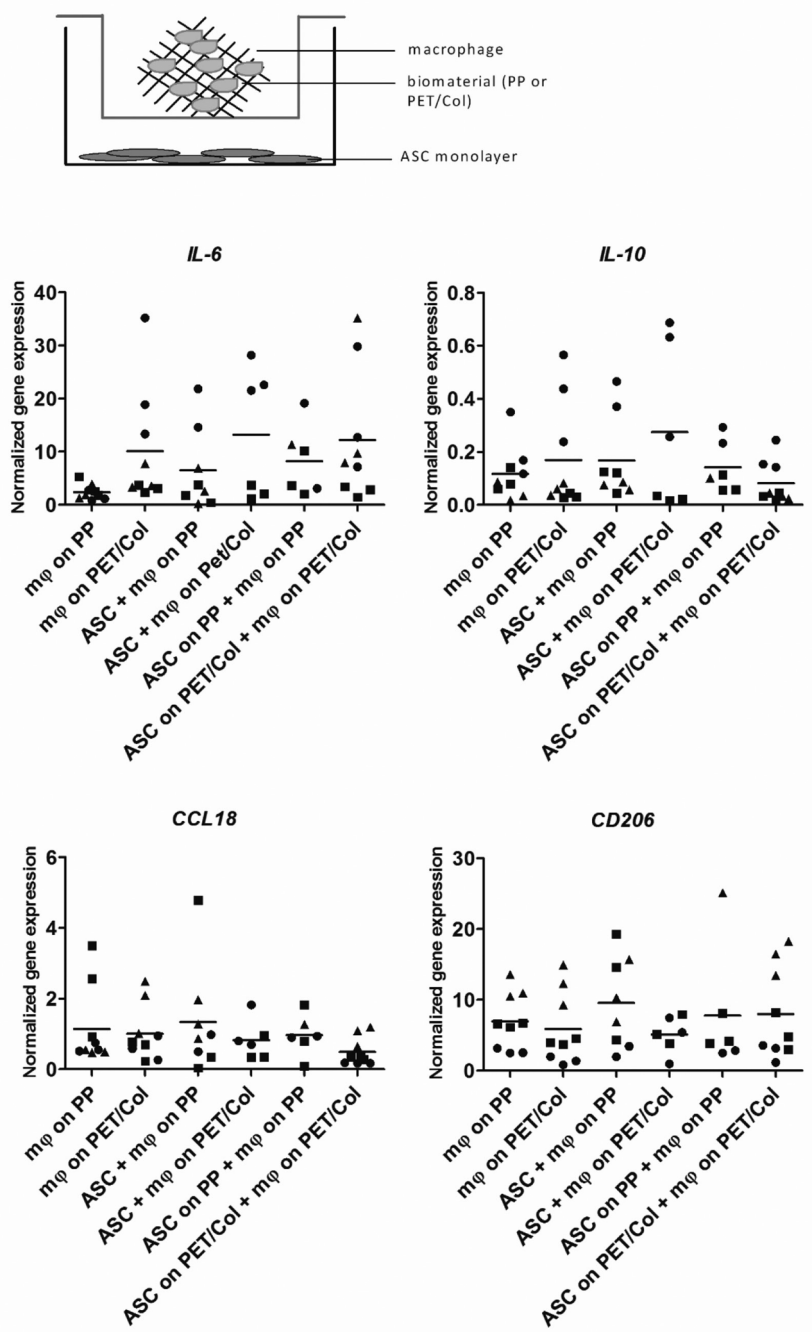


Figure 4 | The influence of biomaterials on ASCs. The effect of biomaterials on the gene expression of MMP1, COL1A1, PTGS2, ASMA, TGFB1, and PLOD2 by ASCs with or without the presence of macrophages on the same biomaterial.

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 Figure 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 (Figure 5).

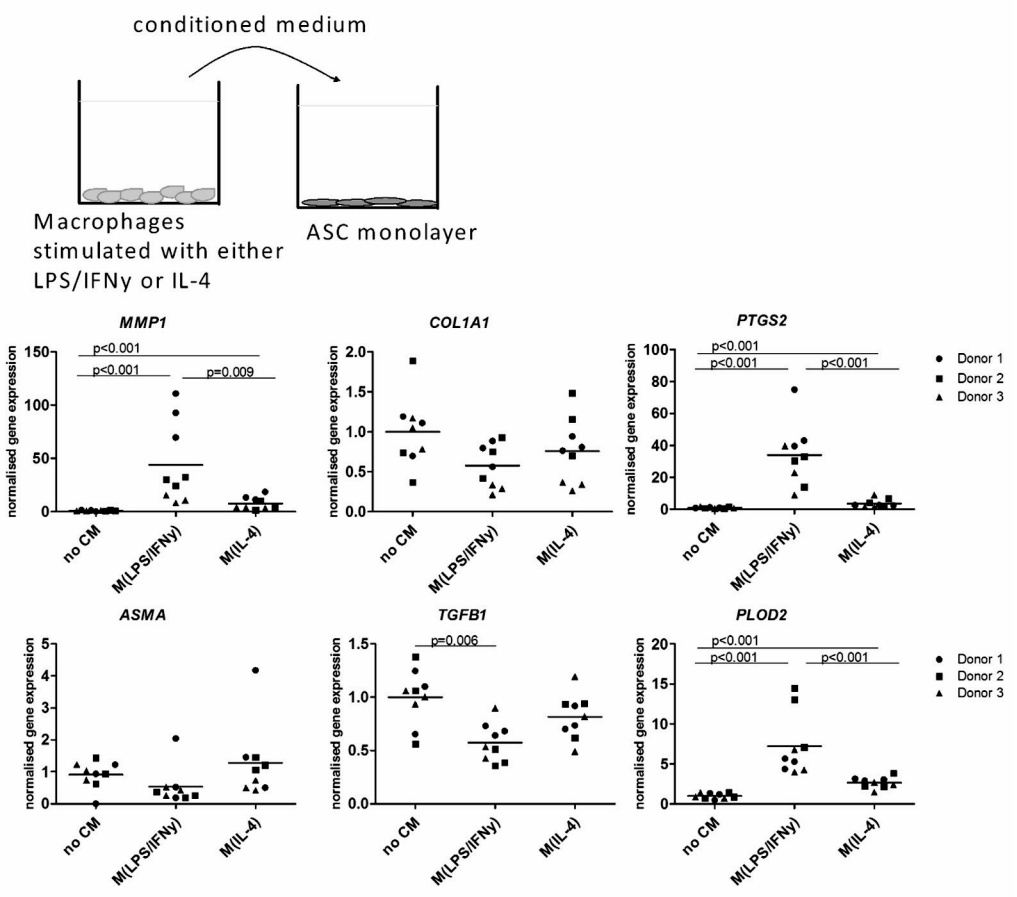


Figure 5 | Gene expression of ASCs with MCM. Gene expression of ASCs in monolayer stimulated with or without M(LPS/IFNγ) or M(IL-4)-CM. The average gene expression of ASCs without MCM was set to 1 for each ASC donor. Experiments were performed in triplicate for three ASC donors.

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, inclu ding 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 non-healthy 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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CHAPTER 7

General discussion

THE QUEST FOR TAILOR-MADE SELECTION OF BIOMATERIALS

Wound healing is a complex cascade of processes, especially when biomaterials are involved. In this reaction, also called the foreign body reaction, the type and consistency of biomaterials will alter the immune response [1,2]. Much research has been performed to investigate the differences between biomaterials and for instance it is found that lightweight mesh is preferable over heavyweight mesh for abdominal wall hernia surgery in terms of scarring and patient complaints [3,4]. However since there are hundreds of different biomaterials, it is difficult to state which biomaterial is the best. Besides the consistency of the biomaterial and the type of tissue it is implanted in, another important factor is the patient. Until now we do not fully understand why some patients have complications after implantation of a biomaterial and others have none with the same biomaterial. Some risk factors for impaired wound healing are found such as smoking, aging, obesity and diabetes [5]. For diabetes and obesity literature shows that macrophages are mainly of a pro-inflammatory subtype, known to negatively influence wound healing [1,6]. For clinical practice, it would be a great benefit to have a tailor-made model with patients own cells to test which biomaterial is best thereby reducing complication rates.

New *in vitro* models

In this thesis, the first steps are described for this tailor-made treatment. Since macrophages are the conductors of the immune system after implantation of a biomaterial, in our research these cells were used to investigate the wound healing process when biomaterials are involved. In Chapter 2 many different studies with many different biomaterials were reviewed. Since the biomaterials were studied in different culture settings, it was difficult to compare the effect of different biomaterials. Therefore a culture model was developed in which macrophages on four different biomaterials were cultured. As such, distinguishing differences between the reaction of macrophages to these biomaterials were found (Chapter 3). An *in vitro* model is a simplification of the foreign body reaction. However when the knowledge of what is known from these biomaterials *in vivo* is integrated, this model can predict the foreign body reaction. A big advantage of this new culture model is the short time of culture. In 3 days culture and a few days to analyse the results, the answer to the question: 'Which biomaterial is best for this patient' might already be given. For a complete comparison it would be very interesting to use macrophages from patients scheduled for surgery and culture these in our culture model. Also cells of patients with complications can be compared with patients without complications to see whether a difference in our culture model can be found. Previously we used buffy coats (prepared from 500 mL of blood) to isolate monocytes, however, this amount of blood is probably too much asked for of patients. The first goal was to downscale the protocol in which our lab recently succeeded by Boersema et al. [7]. They used only 30mL of peripheral blood instead and was still able to isolate sufficient monocytes to test biomaterials.

Since contamination cannot be completely prevented in all surgical fields, our model was also adapted to predict the reaction of the body to biomaterials in a field with a high risk of contamination (Chapter 4). Contamination was mimicked by the use of interferon (IFN)γ and lipopolysaccharide (LPS). LPS is a main component of the membrane of gram negative bacteria like Escherichia coli,

Salmonella and Pseudomonas. In bowel surgery, most pathogens are gram negative. In nose surgery, some of the postoperative infections are gram negative (E. coli, Klebsiella), some are gram positive (*Staphylococcus aureus*) [8]. For the future it would be interesting to culture with 'real' bacteria, gram positive and gram negative like S. aureus and E. coli. These two bacteria are known from literature to induce a strong M1 polarisation [9]. It would be possible then to compare if these bacteria induce the same reactions in macrophages in combination with biomaterials, as in our inflammatory model. To have more insight in the wound healing process, cells that can produce matrix which is necessary to close the wound were added to our culture model. For this purpose adipose-derived stem cells (ASCs) were added, since these cells are known to migrate to the wound site and are either able to accelerate wound healing by secretion of factors or by differentiation into fibroblasts and produce matrix to repair the defect [10,11]. We found distinguishing differences between the effects of biomaterials on gene expression and cytokine production of macrophages and ASCs. Some biomaterials induced a predominantly pro-inflammatory and others induced a predominantly anti-inflammatory reaction. The question arose: which type or balance between M1 and M2 macrophages is optimal?

M1 or M2, that's the question (12)

From literature, it is known that every foreign body reaction will start with a solemnly M1 i.e. pro-inflammatory reaction, since these macrophages start with cleaning up the wound site by phagocytosis. By production of pro-inflammatory cytokines and chemokines, many different cell types are attracted to the wound site [1,6,13]. After the first inflammation reaction the macrophages change to an anti-inflammatory subtype to conduct the wound healing [6]. When this reaction is out of balance device failure or complication does occur. When macrophages stay at the M1 polarisation, poor incorporation of the biomaterial and impaired wound repair will be the result [6,14]. This reaction is often found in chronic ulcers in diabetic patients and in patients with chronic inflammatory diseases such as rheumatoid arthritis, Crohn's disease and asthma [6,14]. However, when M1 macrophages are depleted and the reaction is of a solemnly M2 type, wound healing will have delayed granulation tissue formation and delayed wound closing [6,14]. This means that both types of macrophages are necessary and a good balance is needed. To make it even more complex, M2 macrophages can also induce fibrosis by myofibroblast differentiation and by producing TGFβ leading to an increased collagen production [14]. M1 macrophages sometimes help organizing scar tissue, and therefore reduce fibrosis [14]. In our own co-culture (Chapter 6) it was indeed apparent that cytokines from M2 macrophages induced gene-expression of TGFβ1 and ASMA (alpha smooth muscle actin) in ASCs, both associated with fibrosis [15,16]. This means that the answer to the question: "M1 or M2" is not black and white and indeed a balance of these two subtypes is needed to conduct the wound healing cascade, starting with a balance more towards M1 and after a few days the balance must shift towards M2. In our culture model cells were cultured for 3 days; the wound healing cascade, however, can take sometimes several weeks. Still we found differences compatible with the *in vivo* outcome. Macrophages normally arrive at day 2 or 3 at the wound site. Macrophages were used in our culture model, so our starting point in the wound healing cascade is not at time point zero. Furthermore, in culture there might be less cell debris than at the wound site, since only

living cells were attached to the biomaterial. This is might accelerate the M2 transition. Therefore in only 3 days the foreign body reaction can already be predicted.

Modulation of macrophages to interfere with the wound healing

A potential way to improve wound healing might be modulation of macrophages when influenced by biomaterials. All synthetic (non-degradable) biomaterials induce mainly a M1 reaction since macrophages are constantly triggered by the foreign body, trying to engulf the biomaterial. During the last decades, synthetic biomaterials are altered to help transit this reaction. For instance small pores will induce more M2 macrophages, than with a non-porous biomaterial [1,6].

Another possibility is the addition of stem cells, these cells being known to induce a more M2 subtype of macrophages when co-cultured [17]. Also in pre-clinical studies, it is described that stem cells will promote the M1 to M2 transition in for instance brain and cardiac injury [6,18,19]. However, the use of stem cells in clinical medicine is still controversial since we do not know the long term effects and possible carcinogenic differentiation.

In vitro, macrophages can be forced into an anti-inflammatory type by adding for instance IL-4 (Chapter 2). This is not directly applicable for systemic administration, since this can enhance an inflammation reaction and IL-4 is described to be important in the pathogenesis of asthma and metastasis [20,21]. Interference with other cytokines can alter wound healing, like blockages of cytokines IL-1β, IL-17 and TNFα in diabetic mice accelerate skin wound healing [13]. However since we do not know all the effects of these cytokines in the human body, it is difficult to use it in a clinical setting.

Another possibility to modulate macrophage behaviour is the use of drugs, already used in patients (for other purposes) such as Haloperidol and Risperidone which are used for mental disorders. They are described in literature to stimulate macrophages to produce pro-inflammatory cytokines, which can be useful to promote ingrowth of the biomaterial, since a solemnly M2 reaction can delay wound closure [6,13,22]. Some drugs can also suppress the inflammation reaction. Recently our group published an article on the reaction of macrophages to dexamethasone, rapamycine and pravastatin [23]. With dexamethasone suppression of the inflammation reaction was found. *In vivo* it is also described to influence wound healing, by suppression of the inflammation reaction [24], so in clinical practise dexamethasone can maybe be used to supress a chronic inflammation reaction. Rapamycine is commonly used to prevent rejection of transplanted organs, meaning a very strong drug suppressing the immune system, so this is also not favourable to use in healthy patients. Pravastatin, a relatively save drug to reduce cholesterol enhanced the production of IL-10 and is therefore likely to promote M2 macrophages. It would be interesting to investigate this in a clinical setting since many patients use statins. Every drug, however, has side-effects. Our group also investigated the possibility of modulation of macrophages when attached to a biomaterial [25] and found that some drugs did modulate macrophages on all different types of biomaterials, and some only effected one or two different types. This means that the type of biomaterial is of strong influence, which we also find in our culture models. Therefore more and more research is performed to investigate the possibility for local administration or even incorporate it in a biomaterial such as the addition of antibiotics or for instance diclofenac [26,27].

The use of biomaterials in otorhinolaryngology

In otorhinolaryngology different biomaterials are being used. In ear surgery, titanium prostheses are used to reconstruct the ossicular chain. Most of these biomaterials perform well and only a few complications are found. Failure of these implants is sometimes due to the formation of fibrous tissue, preventing the prosthesis to move freely and thereby limiting the transfer of sound vibration in 2%–37% of patients [28]. In rhinoplasty almost no biomaterials are used due to a high risk of complications like infection, extrusion and movement [29]. The nose is not a sterile working field because it has direct connections to air. Most of the time cartilage (septum, ear of rib) is used in rhinoplasty, however due to donor site morbidity and lengthening of the surgery sometimes biomaterials are used. In Asia biomaterials are more frequently used than in Europe. Mostly used are silicone, expanded polytetrafluoroethylene (ePTFE) , porous high-density polyethylene (pHDPE) and sometimes acellular human dermis [29]. After silicone implantation, a high complication rate up to 36% is described [30]. In the case report in the Introduction such a complication was described, which resulted in extensive fibrous tissue formation and chronic inflammation, even years after implantation. ePTFE is described with a low complication rate (2−3.7%, 0,38% infection) [30,31]. In Chapter 4 using ePTFE (Dualmesh®) in a rat peritonitis model, we found, however, infection rates as high as 50%. Rhinoplasty is a surgical field with a risk of contamination and therefore not completely comparable with our peritonitis model where contamination is always present, but still this biomaterial had a higher percentage of infection than other biomaterials used in this study. Histologically we found more T-cells and macrophages, indicative for a chronic inflammation reaction [12,32]. Based on our research, this biomaterial would not be a good candidate for rhinoplasty. Acellular human dermis is sometimes used in rhinoplasty, even though it can give bulkiness and it is not as stable as cartilage [30]. In our culture model with simulated inflammation (Chapter 5), we found the lowest production of pro- and anti-inflammatory cytokines than with the other biomaterials, indicative for a mild foreign body reaction against acellular dermis. In hernia surgery, biologic biomaterials seem not to perform better than synthetic biomaterials and infection rates of 15.9% are found [33,34]. Taken all together, this means that there is still much research to do to find the best biomaterial for each application like for instance rhinoplasty. Maybe biomaterials can be improved by coating with extracellular matrix components to improve incorporation of the biomaterial, like is done with ParietexTM Composite. This material, also used in our *in vitro* model, is never used in rhinoplasty. Biomaterials with antibodies to polarise the macrophages or drugs to prevent infections or reduce inflammation might also be a topic to focus on in future research [27,35]. Our culture model with simulated inflammatory can help testing newly developed biomaterials.

The search of the best tailor-made biomaterial has just started and we have made the first steps in this quest by developing an *in vitro* model with human macrophages in which we can measure biomaterial-dependent differences. Already in the near future, it is pivotal to investigate which M1/M2 ratio and which reaction of adipose-derived stem cells is needed to get the best outcome *in vivo*. Furthermore, since biomaterial research is performed by many different groups, it would be beneficial to coordinate all surgeons working in the biomaterial field to register complications leading to a research databank. This will provide much knowledge helping to develop better materials and preventing complications.

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Summary

Biomaterials are often used in many fields of medicine to restore or replace tissue. These biomaterials always elicit a reaction of the immune system, called the foreign body reaction, which can lead to complications in patients and failure of the device. Macrophages are key players in this reaction [1]. Because the foreign body reaction depends on the type and consistency of biomaterials [2-4] but also on the patient itself, a tailor-made model will be of great help to assess the best treatment. Therefore the ultimate aim of our research was to develop a tailor-made model.

Much research has already been performed on macrophages and biomaterials, therefore we started with a literature research of what is already known. First a systematic review of *in vitro* models describing the macrophage polarisation (pro- (M1) or anti-inflammatory (M2)) in response to different biomaterials was performed (**Chapter 2**). It was found that many factors are influencing this polarisation such as chemistry, pore size and surface topography. Also sterilisation and chemically crosslinking will alter the macrophage polarisation. However, since many different culture conditions were used, it was difficult to compare the biomaterials.

Since we eventually aimed for a tailor-made model, the development of an *in vitro* model with human isolated macrophages from blood was initiated (**Chapter 3**). First, distinguishing genes and cytokines for polarisation were determined. These read-out parameters were used for investigating the influence of four different biomaterials on macrophage polarisation; the model showed biomaterial-dependent differences. Macrophages on polypropylene had a phenotype comparable to M2, while macrophages on polyethylene terephthalate and on a combined biomaterial Parietex™ Composite (polyethylene terephthalate and collagen) had a phenotype similar to M1. Macrophages on a collagen biomaterial (Permacol™) produced a low amount of proteins and therefore did not have a clear phenotype. This model can be useful in the future to predict the *in vivo* outcome of biomaterials.

Most research is performed in a sterile environment. However some anatomical locations in the human body are not sterile, like in bowel surgery or rhinoplasty as described in the case report in the introduction. The use of biomaterials in these fields has an increased risk of complications, such as infection [5]. In **Chapter 4a** an *in vivo* animal model was used in which a contaminated environment was created by puncture of the bowel, creating a peritonitis to compare the performance of different biomaterials. Six different synthetic and one biological biomaterial were implanted in the abdominal wall. Significant differences in infection rate and incorporation between materials were found. Most infections occurred in C-Qur™ and Dualmesh®. The incorporation of the biological mesh (Strattice®) was less than the other synthetic biomaterials, however this mesh was never infected. Dualmesh® showed the most shrinkage. In **Chapter 4b** samples of the previous study were used to analyse the subtype of macrophages. Parietene Composite™ and Sepramesh™ induced more iNOS-positive cells (M1 polarisation) and C-Qur™ and Dualmesh® were surrounded by more CD206-positive cells (M2 polarisation), finding biomaterial-dependent differences in this *in vivo* rat model.

The biomaterial-dependent polarisation of macrophages in a contaminated environment in the rat study inspired to modify the culture model developed in **Chapter 3**. Inflammatory cytokines (LPS

and IFNγ) were added to our *in vitro* model in **Chapter 5**, to mimic an inflammatory environment. Polypropylene again stimulated M2 polarisation and Parietex™ Composite and polyethylene terephthalate stimulated an M1 reaction. Despite inflammation, macrophages still behaved biomaterial-dependent.

Another important factor in tissue regeneration is the production of matrix, our *in vitro* model in **Chapter 6** was extended to a co-culture with adipose tissue derived stem cells (ASCs). The cytokines produced by macrophages when cultured on biomaterials, increased proliferation of ASCs and collagen deposition. Co-culture of macrophages and ASCs demonstrated biomaterial-dependent differences in gene-expression of wound healing genes.

In conclusion, this thesis describes the development of three *in vitro* models with human macrophages in different conditions where biomaterial-dependent differences can be found. This confirms that the type of biomaterial is very important in the foreign body reaction and for patient outcome. These models can be useful to study the biological mechanisms of foreign body reactions as well as to evaluate newly developed, modified or functionalized biomaterials. Moreover, this work represents the first step to a tailor-made model that in the future can preoperatively predict the foreign body reaction in a specific patient and therefore help to select the best biomaterial per patient.

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Nederlandse samenvatting

Biomaterialen worden in verschillende medische specialismen gebruikt om weefseldefecten te herstellen. Omdat deze materialen lichaamsvreemd zijn, reageert het lichaam met een afweerreactie, ook wel vreemdlichaamreactie genoemd. Deze reactie kan complicaties bij patiënten veroorzaken en ervoor zorgen dat het biomateriaal niet goed functioneert. Macrofagen zijn de dirigenten in deze reactie, zij regelen het wondgenezingsproces. Biomaterialen zijn al jaren op de markt en er is dan ook al veel onderzoek naar gedaan. Omdat de reactie tussen patiënten en de reactie per biomateriaal verschillend is, zou het helpen over een patiënt-specifiek model te beschikken om per patiënt te kunnen testen wat het beste biomateriaal is.

In **Hoofdstuk 2** wordt een literatuurstudie van de bestaande *in vitro* modellen gepresenteerd. Omdat macrofagen de dirigenten zijn van de vreemdlichaamreactie is gekeken naar *in vitro* modellen, die de invloed van biomaterialen op de polarisatie van macrofagen (pro-(M1) of anti-inflammatoir (M2)) beschrijven. Veel factoren zijn van invloed op deze polarisatie zoals poriegrootte, oppervlaktekarakteristieken en materiaaltype. Ook de manier van steriliseren en versterken van het materiaal blijken van grote invloed. Omdat elk model andere kweekomstandigheden kent, bleek het lastig de materialen met elkaar te vergelijken.

Daarom werd er een *in vitro* model opgezet met humane macrofagen geoogst uit bloed. In **Hoofdstuk 3** wordt dit model beschreven waarin specifieke genen en eiwitten gevonden werden om te differentiëren tussen M1 en M2 macrofagen. Deze "markers" werden gebruikt om de reactie van macrofagen op biomaterialen te onderzoeken. In ons model zijn vier verschillende materialen gebruikt: polypropyleen, polyethyleentereftalaat, een gecombineerd biomateriaal: polyethyleentereftalaat met collageen (Parietex™ Composite) en een puur collageen biomateriaal (Permacol™). Op basis van onze markers, hadden macrofagen op polypropyleen een M2 profiel, de macrofagen op polyethyleentereftalaat een M1 profiel. Permacol™ toonde slechts een beperkte reactie van de macrofagen en polariseerden niet duidelijk.

Het meeste onderzoek, tot op heden, is in een steriel milieu uitgevoerd. Echter niet alle anatomische onderdelen van het menselijk lichaam zijn steriel, zoals bijvoorbeeld de darm- en de neuschirurgie. Hoe hier de vreemdlichaamreactie verloopt is nog niet helemaal duidelijk. Wel worden er meer complicaties gezien waarvan een voorbeeld is gegeven in het case-report in de introductie. In **Hoofdstuk 4a** is een *in vivo* model beschreven, waarin in ratten een gecontamineerd milieu werd gecreëerd door een darmlekkage en buikvliesontsteking te veroorzaken. Vervolgens werden zeven verschillende biomaterialen geïmplanteerd. Na 28 en 90 dagen werd onderzocht hoe vaak infectie voorkwam en hoe de biomaterialen waren ingegroeid en/of gekrompen. Ook werd gekeken naar verklevingen in de buikholte door het biomateriaal. Hierbij werden duidelijke verschillen tussen de biomaterialen gevonden. Het doel van het onderzoek beschreven in **Hoofdstuk 4b** was om te onderzoeken of er in deze ratstudie verschillen te vinden zouden zijn met betrekking tot de macrofaagpolarisatie. Om die reden zijn in de biopten, genomen van de geïmplanteerde materialen, algemene macrofagen (CD68) en de subtypes: iNOS (M1) en CD206 (M2) aangetoond. In dit *in vivo* model werd een biomateriaal-afhankelijke reactie van macrofagen gevonden.

Omdat het ratmodel een duidelijk biomateriaal-afhankelijk verschil liet zien met betrekking tot polarisatie van macrofagen, is in **Hoofdstuk 5** het *in vitro* model aangepast om materialen in een gecontamineerd/inflammatoir milieu te kunnen onderzoeken. Door het toevoegen van inflammatoire cytokines (lipopolysacharide en interferon gamma) werd een inflammatoir milieu nagebootst. Zelfs in een inflammatoir milieu werd gevonden dat de biomaterialen een biomateriaal-afhankelijke polarisatie van de macrofagen induceren.

Omdat bindweefsel-producerende cellen ook heel belangrijk zijn in de reparatie van weefsels, is in **Hoofdstuk 6** het *in vitro* model gemodificeerd, waarbij naast macrofagen ook stamcellen werden gekweekt. Ook hier waren duidelijke verschillen in stimulatie door een biomateriaal waarneembaar. Met dit model is het mogelijk om de interactie tussen macrofagen en stamcellen te onderzoeken. Hierbij bleken biomaterialen op beide soorten cellen een grote invloed hebben, waarbij bovendien biomateriaal-afhankelijk effecten werden gezien.

Concluderend werden in alle beschreven kweekmodellen biomateriaal-afhankelijke polarisaties van macrofagen gezien. Dit bewijst dat de keuze voor een bepaald type biomateriaal erg belangrijk is voor de patiënt. Dit model helpt bij de keuze en kan ook nieuw ontwikkelde materialen evalueren. Hiermee zet dit onderzoek de eerste stappen naar een preoperatief toe te passen model om te bepalen welk biomateriaal het beste is voor welke patiënt.

Addendum

List of abbreviations

Summary of PhD training and teaching

List of publications

Curriculum Vitae

Dankwoord

List of abbreviations

ACS	adipose-derived mesenchymal stem cells
ASMA	α-smooth muscle actin
BDEDTC	poly(styrene-co-benzyl N,N-diethyldithiocarbamate
CCL-18	chemokine ligand 18
CD(x)	cluster of differentiation
CLP	caecal ligation and puncture model
CM	conditioned medium
COL	collagen
COX	cyclo-oxygenase
DMAPAAmMel	methyl iodide of poly[3-(dimethylamino)propyl]acrylamide
FCS	fetal calf serum
FGF	fibroblast growth factor
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GM-CSF	granulocyte-macrophage colony-stimulating factor
IL-(x)(RA)	interleukin-(x) (receptor antagonist)
IFNγ	interferon gamma
iNOS	inducible nitric oxide synthase
LPS	lipopolysaccharide
M1	pro-inflammatory macrophage
M2	anti-inflammatory macrophage
MCP	monocyte chemotactic protein
MDC	macrophage-derived chemokine
MIP	macrophage inflammatory protein
MMP	matrix metalloproteinase
MSC	mesenchymal stem cells
PAAm	polyacrylamide
PAANa	poly(acrylic acid)
PAH	poly(allylamine hydrochloride)
PCL-(U4U)	poly-e-caprolactone bisurea
PDGF	platelet-derived growth factor
PDO	polydioxanone
PDL	poly-d-lysine
PE	polyethylene
PEG-g-PA	perfluoropolyether with large fibers
PET	polyethylene terephthalate
pHEMA	2-hydroxyethyl methacrylate
(m)PP	(multifilament)polypropylene
PFPE	perfluoropolyether

PLA	polylactic acid
PLGA	poly(D,L-lactide-co-glycolide)
PLOD2	procollagen-lysine,2-oxoglutarate 5-dioxygenase 2
PMN	polymorphonuclear leukocytes
(e)PTFE	(expanded) polytetrafluorethylene
PTGS2	prostaglandin-endoperoxide synthase 2
PU	polyurethane
RANTES	regulated and normal T-cell expressed and secreted
SIS	small intestine submucosa (biological mesh)
THP-1	monocyte cell line
TGFβ	transforming growth factor beta
Th2	T-helper 2
TNFα	tumor necrosis factor alpha
VEGF	vascular endothelial growth factor

Summary of PhD training and teaching

Name PhD student: **Nienke Schmidt-Grotenhuis**
Erasmus MC Department: Otorhinolaryngology and General Surgery
Research School: Molecular Medicine

PhD period: 2010-2017
Promotor(s): GJVM van Osch, JF Lange
Supervisor: YM Bastiaansen-Jenniskens

1 | PhD training

	Year	Workload (ECTS)
Courses		
– Biomedical Scientific English writing course (Molmed)	2011	4
– Introduction to Data Analysis (NIHES)	2010	2
Presentations		2
– Science day Otorhinolaryngology	2014	
– Science day (Stafdag) Surgery	2011 & 2012	
– Research group meeting Orthopaedics/Otorhinolaryngology (multiple times)	2010-2013	
	2010-2013	
– CTCR research group (multiple times)	2010-2016	
– Otorhinolaryngology department research meeting (multiple times)	2011 & 2012	
– Molmed PhD-day (2 times, poster presentation)	2012, 2014	
– Scientific meeting of Dutch society for ENT	2010-2013	
– Journal club meeting		
(Inter)national conferences-poster presentation		
– COST-Nambio: Biomaterial-dependent reaction of macrophages in conditions stimulating an inflammatory environment	2012	5
(Inter)national conferences-podium presentation		5
– TERMIS Granada, Spain: Differentiation of macrophages into pro-or anti-inflammatory/repair subtype in culture	2011	
– NBTE Lunteren, the Netherlands: A culture model to analyse the acute biomaterial-dependent reaction of macrophages	2011	
– TERMIS Vienna, Austria: Biomaterial-dependent reaction of macrophages in conditions simulating an inflammatory environment	2012	
– NBTE Lunteren, the Netherlands: The reaction of macrophages to biomaterials in an inflammatory environment	2012	
– European Hernia Society congress, Rotterdam, the Netherlands: The acute response of macrophages to biomaterials, as a predictor for late outcome.	2012	
– Inflammaday, Covidien, Lyon, France: Biomaterial dependent reaction of macrophages in conditions simulating an inflammatory environment	2012	

Other		
– Heidelberg visit/workshop culturing and isolation of monocytes with podium presentation, 3 days visit	2012	1

2 | Teaching

	Year	Workload (Hours/ ECTS)
Lecturing		5
– Basic ENT for interns (multiple times)	2016	
– Cancer in ENT for interns (multiple times)	2015-2016	
– Acute ENT for ER-nurses (multiple times)	2012-2016	
– Cancer in ENT for nurses	2015	
– ENT surgery for anaesthesiology nurses	2016	
Supervising Master's theses		
– Hylke vd Toom, medical student, the inflammatory *in vitro* model, 20 weeks	2011-2012	4
– Samantha de Witte, stem cells and macrophages, 40 weeks	2013	8
– Jovita Schoffelmeer, technical medicine student, 10 weeks	2013	2

List of publications

1) N.Grotenhuis, S.F. de Witte, G.J. van Osch, Y. Bayon, J.F. Lange, Y.M. Bastiaansen-Jenniskens. Biomaterials Influence Macrophage-Mesenchymal Stem Cell Interaction *In Vitro*. Tissue Eng Part A. 2016 Sep;22(17-18):1098-107. doi: 10.1089/ten.TEA.2016.0162.
2) N. Grotenhuis, J.P. Koopman, J.M. Kruyt, L.K. Rothuizen, R. Knevel, H. Blom. Implementation of the hyaluronic acid fat graft myringoplasty technique, pitfalls and lessons learned. Acta Otolaryngol. 2017 May 29:1-4. doi: 10.1080/00016489.2017.1330556.
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4) N. Fay, M.L. de Vries-van Melle, J. Lehmann, W. Wei, N. Grotenhuis, E. Farell, P.M. van der Kraan, J.M. Murphy, Y.M. Bastiaansen-Jenniskens. G.J.V.M. van Osch. Human osteoarthritic synovium impacts chondrogenic differentiation of mesenchymal stem cells via macrophage polarisation state. Osteoarthritis Cartilage. 2014 Aug;22(8):1167-75. doi: 10.1016/j.joca.2014.05.021. Epub 2014 Jun 7.
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Curriculum Vitae

Nienke Grotenhuis werd geboren op 2 februari 1985 in Enter, Overijssel. Ze groeide op in Maarssenbroek en verhuisde op zesjarige leeftijd naar Drenthe waar zij op Protestantse Basisschool 'de Bron' te Nijensleek haar basisonderwijs volgde. Nadien doorliep ze het gymnasium op de Regionale Scholen Gemeenschap te Steenwijk en behaalde in 1997 haar diploma. Ze begon aansluitend met Geneeskunde bij de Rijksuniversiteit te Leiden. Tijdens haar studie was Nienke actief binnen studentenverening LSKO 'Collegium Musicum' als praeses. In haar tweede jaar begon ze met onderzoek binnen de afdeling neonatologie naar pulsoximeters. In het vierde jaar na afronding van haar vakken ging ze voor een wetenschappelijke stage voor 9 maanden naar Yokohama, Japan, onder Prof.dr. H. Kawamato en Prof.dr. W. van Ewijk, waar ze onderzoek deed naar het afweersysteem. Na deze stage doorliep ze haar co-schappen en ontving ze in maart 2010 haar artsexamen. Tijdens een extra coschap KNO in Zwolle raakte Nienke geïnteresseerd in de KNO en na haar semi-arts stage in het LUMC wist ze zeker dat ze KNO-arts wilde worden. In mei 2010 startte ze onder leiding van Prof. dr. G.J.V.M. Van Osch, Prof. dr. J.F. Lange en dr. Y.M. Bastiaansen-Jenniskens haar promotietraject in het Erasmus MC te Rotterdam, wat resulteerde in dit proefschrift. In september 2013 startte ze als AIOS KNO in het Erasmus MC bij Prof. dr. R.J. Baatenburg de Jong, dr. R.M.L. Poublon en dr. R.M. Metselaar. Haar perifere opleiding doorliep ze in het HAGA ziekenhuis bij dr. H.M. Blom en drs. J.P. Koopman en in het Reinier de Graaf Gasthuis te Delft bij drs. F.A.W. Peek en dr. H.C. Hafkamp.

Nienke is in 2014 getrouwd met Marc Schmidt en op 5 januari 2017 werd hun dochter Lilian geboren.

Dankwoord

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