In vitro modulation of the behavior of adhering macrophages by medications is biomaterial-dependent

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Lizette Utomo
Geesien S.A. Boersema
Yves Bayon
Johan F. Lange
Gerjo J.V.M. van Osch
Yvonne M. Bastiaansen-Jenniskens

ABSTRACT

After implantation of a biomaterial, an inflammatory response involving macrophages is induced. The behavior of macrophages depends on their phenotype and by directing macrophage polarization, unwanted effects may be avoided. In this study, the possibility to modulate the behavior of macrophages activated by biomaterials, was assessed in an *in vitro* model. Primary human monocytes were seeded on polyethylene terephthalate, polypropylene and polylactic acid yarns, and treated with medications frequently used by patients: rapamycin, dexamethasone, celecoxib or pravastatin. Modulation of the adhering macrophages with rapamycin resulted in a generally pro-inflammatory effect. Dexamethasone caused an overall anti-inflammatory effect on the macrophages cultured on either material, while celecoxib only affected macrophages adhering to polyethylene terephthalate and polylactic acid. Pravastatin increased pro-inflammatory genes of macrophages cultured on polypropylene and polylactic acid. Pairwise comparison revealed that macrophages adhering to polylactic acid seemed to be more susceptible to phenotype modulation than when adhering to polypropylene or polyethylene terephthalate. This data shows that macrophages activated by the biomaterials can be modulated, yet the degree of the modulatory capacity depends on the type of material. Combined, this model provides insights into the possibility of using a medication in combination with a biomaterial to direct macrophage behavior and thereby possibly avoiding unwanted effects after implantation.

INTRODUCTION

Biomaterials are commonly used in orthopedics, gynecology, cardiology, reconstructive surgery and general surgery. After implantation, the host immune system will interact with materials by blood-material interaction, matrix formation, acute inflammation, chronic inflammation, granulated tissue development, and a foreign body reaction that is eventually followed by fibrosis[57]. Adverse effects due to implantation include material-induced inflammation, excessive fibrosis, excessive coagulation and infection[58]. Macrophages are members of the mononuclear phagocyte system that include tissue macrophages, circulating monocytes and precursor monocytes in the bone marrow[198]. They are pivotal in the initial response following biomaterial implantation. Macrophages can adhere to the material and become activated and acquire a phenotype ranging from pro-inflammatory to anti-inflammatory[47]. *In vitro*, pro-inflammatory (M1 or classically activated) macrophages develop when monocytes are simultaneously subjected to interferon-γ (IFN-γ) and lipopolysaccharide (LPS) or tumor necrosis factor alpha (TNFα)[45]. Anti-inflammatory (M2 or alternatively activated) macrophages can be further divided into subtypes depending on the stimuli. One of the subtypes develops when exposed to IL-4 or IL-13, also referred to as 'M2a', and the 'M2c' subtype develops when stimulated with IL-10[38,59].

In the field of orthopedics, polyethylene is the most commonly used polymer for metal-on-plastic prosthesis for total hip arthroplasty in the United States[199]. Although improvements of these polymers (e.g., ultra-high-molecular-weight polyethylene (UHMWPE)) have increased durability, wear debris accumulates in the joint space and interact with the resident tissue cells. Macrophages are key players that become activated and initiate immune responses due to exposure to wear debris[200,201]. They have been shown to play an important role in particle wear-induced synovitis, osteolysis[202,203] and aseptic loosening of hip prostheses[204,205], which are major causes of failure of total hip replacements and account for 30-75% of all revision surgeries[206-209]. In other fields, macrophages are in many cases the underlying initiators or driving forces that control the progression of numerous inflammation mediated diseases[25,210,211]. Studies have shown *in vitro* that monocyte/macrophages can adhere to biomaterials *in vitro*, become activated, and secrete proteins in a pro-inflammatory or anti-inflammatory manner, as reviewed by Boersema, *et al*.[193]. Previously, we have shown that the behavior of different macrophage phenotypes can be modulated using compounds[68]. These compounds are members of commonly used medication groups that are in literature described to have effects on inflammatory cells or inflammation in general[129-131,135,136,138,143,148,212,213]. Since these medications were able to modulate different macrophage phenotypes, and their effect was dependent on the phenotype, we hypothesized that these medications can also modulate the phenotype of macrophages activated by biomaterials. The possibility

to modulate adhering macrophages can eventually be used to influence the foreign body reaction after implantation of a biomaterial. Therefore, the aim of this study was to investigate *in vitro* as a proof of concept whether the behavior of human monocyte-derived macrophages activated by a biomaterial can be modulated by rapamycin, dexamethasone, celecoxib or pravastatin, to direct macrophage polarization. To assess this, human monocyte-derived macrophages were seeded on three commonly used biomaterials: polypropylene, polyethylene terephthalate and polylactic acid followed by treatment of the medications. Gene expression analysis and protein production were used as parameters to assess the effects of the medications on the macrophages.

MATERIALS AND METHODS

Monocyte isolation and seeding on biomaterials

Monocytes were isolated from 13 buffy coats (male donors, 52±14 years) purchased from the national blood bank (Sanquin Blood bank, Amsterdam, the Netherlands). $CD14^+$ monocytes were acquired from the buffy coats by density gradient separation using Ficoll Paque Plus (GE Healthcare Life Sciences, Chicago, USA) followed by magnetic activated cell sorting (MACS) using human CD14 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) as described previously[43]. Prior to seeding, the biomaterials were pre-conditioned with 100% non-heat-inactivated fetal calf serum (FCS; Lonza, Verviers, Belgium) for 2h at 37°C to improve attachment conditions for the monocytes due to the presence of complement. For each experiment, the monocytes were pooled from at least two donors. The monocytes were seeded for 2h at 37°C by rotation at 20 rpm (VWR, Pennsylvania, USA) on monofilament polypropylene (ρ=0.9 g/cm^3; ø=0.10 mm; *l*=8 cm), polyethylene terephthalate (ρ=1.34 g/cm^3; ø=0.09 mm; *l*=8.8 cm,) or polylactic acid (ρ=1.25 g/cm^3; ø=0.10 mm; *l*=8 cm) yarns (Medtronic-Sofradim, Trévoux, France). The length of the yarns was adjusted for the density and volume of the materials. Each yarn was exposed to 850,000 $CD14^+$ monocytes in a cell suspension of 500,000 monocytes/mL. The materials with adhering monocytes were cultured for 3d in 48 well-plates in 250 µL culture medium (X-VIVO-15 (Lonza, Verviers, Belgium) supplemented with 20% heat-inactivated FCS, 50 µg/mL gentamicin (Gibco, Carlsbad, USA) and 1.5 µg/mL amphotericin B (Fungizone; Gibco)) at 37°C and 5% CO_2 to allow differentiation into activated macrophages. A concentration of 20% FCS was chosen based on pilot experiments and previous studies, where this culture condition was more favorable in terms of macrophage viability and differentiation capacity into different phenotypes *in vitro*[5,60,68,69].

Modulation of macrophages adhering to biomaterials

After 3d of differentiation in response to the biomaterial, the medium was removed and replaced with medium containing 1 or 10 ng/mL rapamycin (R&D Systems), or 10^{-8} or 10^{-7} M dexamethasone (Sigma-Aldrich), or 0.5 or 5 µM pravastatin (Sigma-Aldrich), or 0.1 or 1 µM celecoxib (Sigma-Aldrich). Concentrations were chosen based on literature136,138-[140] and previous work where macrophages cultured in monolayers were treated with these medications[68]. Dimethyl sulfoxide (DMSO) was used as a vehicle for dexamethasone and rapamycin and the final DMSO concentration in the cultures was <0.01%. To only take into account the adhering macrophages to the biomaterials, the materials were transferred to new well plates after 2d of culture and the medium containing medications was refreshed. After an additional 24 hours, the medium was harvested, centrifuged at 300 x *g*, aliquoted and stored at -80ºC until protein measurement. The yarns were either stored in PBS/0.1% Triton X-100 (Sigma-Aldrich) at -20ºC for measuring the number of adhering cells or in TRIzol Reagent (Invitrogen, Carlsbad, USA) at -80ºC until processing for gene expression analysis. The timeline for this experiment is based on pilot studies and our previous work [43,60,68] where we have seen that after 3d of culture and 3d modulation, macrophages cultured in monolayers can polarize and be modulated towards specific phenotypes, and present a distinct gene expression and protein profile. Per material, all experiments were conducted independently three times (n=3 in triplicate) with pooled monocytes from at least two buffy coats per experiment.

Quantification of IL-6, CCL18 and sCD163 protein production by macrophages cultured on biomaterials

The concentrations of proteins released in the cell culture supernatants were quantified using enzyme-linked immunosorbent assays (ELISAs). Interleukin 6 (human IL-6 ELISA development kit; PeproTech), Chemokine (C-C motif) ligand 18 (human CCL18 DuoSet ELISA; R&D Systems) and soluble cluster of differentiation 163 (Human sCD163 DuoSet ELISA; R&D Systems) were measured according to the manufacturer's instructions. These proteins were chosen based on previous studies[43,60,68] as they are able to indicate the phenotype of the cultured macrophages. For technical reasons, medium was diluted at least 1:1. To normalize the cytokine production for the number of macrophages adhering to the biomaterials, the cell numbers were determined in the cell suspensions in which the materials were collected. For the standard, $CD14^+$ cells were cultured in monolayers in X-VIVO/20% heat-inactivated FCS in Nunc UpCell thermo reversible culture plates (Thermo Fisher Scientific). The cells were non-enzymatically detached from the plates by reducing the temperature to 32°C and the macrophages were collected in PBS/0.1% Triton X-100 to be used as a standard. CyQUANT GR dye (Invitrogen, Carlsbad, USA) was added to the cell suspensions according to the manufacturer's instructions and

the fluorescence was measured at 480 nm excitation and 520 nm emission with a Spectramax Gemini (Molecular Devices, Sunnyvale, USA).

Gene expression analysis of adhering macrophages

The mRNA of the macrophages attached to the yarns was isolated using TRIzol Reagent according to manufacturer's instructions. Quantification of total extracted mRNA was determined using a NanoDrop ND-8000 spectrophotometer (Thermo Fisher Scientific Inc., Waltham, USA) at 260/280 nm. Complementary DNA (cDNA) was synthesized using the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, USA) according to manufacturer's instructions. Finally, PCR analysis was conducted with a Bio-Rad CFX96 Real-Time PCR Detection System using TaqMan® Universal PCR Master Mix (Applied Biosystems, Carlsbad, USA) or qPCR™ Mastermix Plus for SYBGR® Green I (Eurogentec, Liege, Belgium). Expression of Interleukin-1B (*IL1B*; Fw: CCCTAAACAGATGAAGTGCTCCTT; Rev: GTAGCTGGATGCCGCCAT), *IL6* (Fw: TCGAGCCCACCGGGAACGAA; Rev: GCAGGGAAGGCAGCAGGCAA), Tumour necrosis factor-α (*TNFA*; Fw: GCCGCATCGCCGTCTCCTAC; Rev: AGCGCTGAGTCGGTCACCCT), chemokine (C-C motif) ligand 18 (*CCL18*; Fw: GCACCATGGCCCTCTGCTCC; Rev: GGGCACTGGGGGCTGGTTTC), Mannose receptor, C type 1/CD206 (*MRC1/CD206*; Fw: TGGCCGTATGCCGGTCACTGTTA; Rev: ACTTGTGAGGTCACCGCCTTCCT) and cluster of differentiation 163 (*CD163*; Fw: GTTGGCCATTTTCGTCGCATT; Rev: CTCTCCTCTTGAGGAAACTGCAA) were assessed, as they can be used to discriminate between different macrophage phenotypes [43,45,68]. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*; Fw: CAACGGATTTGGTCGTATTGGG; Rev: TGCCATGGGTGGAATCATATTGG; Probe: GGCGCCCCAACCAGCC), Beta-actin (*ACTB*; Fw: CCTGGCACCCAGCACAAT; Rev: GGACAGCGAGGCCAGGAT), Beta-2-microglobulin (*B2M*; Fw: TGCTCGCGCTACTCTCTCTTT; Rev: TCTGCTGGATGACGTGAGTAAAC) and hypoxanthine phosphoribosyltransferase 1 (*HPRT1;* Fw: TATGGACAGGACTGAACGTCTTG; Rev: CACACAGAGGGCTACAATGTG; Probe: AGATGTGATGAAGGAGATGGGAGGCCA) were all tested as housekeepers for primary human monocyte-derived macrophages. *GAPDH* was found to be the most stable using geNorm software (Biogazelle NV, Zwijnaarde, Belgium)[214] (data not shown) and was further used as normalization of the genes of interest. The amplification efficiency of all primers was between 0.9 and 1.1 and the relative expression was determined by the $2^{-\Delta CT}$ formula.

Statistics

MS Excel 2013 and PASW Statistics 22.0 (SPSS Inc. Chicago, USA) were used for statistical analysis. To take into account donor variations, a mixed linear model

without log-transformation was used followed by a Bonferroni's *post hoc* comparisons test to compare gene expression and protein levels between a compound treatment and the untreated control. Medication treatment of macrophages per material (e.g., macrophages cultured on polypropylene treated with rapamycin) were defined as fixed factors in the model, and the individual experiments were considered as random factors, using n=9 per condition in the model. For calculation purposes, when protein levels were undetectable in the assay, samples were assigned the detection limit of the ELISA assay as provided by the manufacturer (IL-6: < 23.44 pg/mL; CCL18: <7.8 pg/mL and sCD163: <156 pg/mL) and corrected for the lowest used dilution factor in the assay. Initial differences between materials (i.e., number of adhering cells and protein production) were evaluated using one-way ANOVA after log-transformation, followed by a Bonferroni's post hoc test. To assess which material favored phenotype modulation, the protein levels were normalized to untreated controls, log-transformed, and pair wised compared using one-way ANOVA followed by a Bonferroni's *post hoc* test to correct for multiple comparisons. Differences were considered statistically significant for p<0.05. All data displayed in the graphs are non-log-transformed data points.

RESULTS

IL-6 and CCL18 are secreted by adherent macrophages in response to biomaterials

The number of macrophages that adhered to the biomaterials differed between materials. Without modulation, more macrophages adhered to polyethylene terephthalate (58584 ± 19951 cells) than to polypropylene (26312 ± 10781 cells), but not more than polylactic acid (36648 ± 13958 cells) (Supplementary Figure S7.1A). Based on our previous studies, IL-6 protein production can be considered as a marker for pro-inflammatory M1/M(IFNγ+TNFα) macrophages in this culture system, CCL18 as marker for tissue repair M2a/M(IL-4), and sCD163 protein production as marker for anti-inflammatory M2c/M(IL-10)[43,60,68]. Without modulation by the medications, IL-6 and CCL18 were produced by the macrophages adhering to polyethylene terephthalate, polypropylene, and polylactic acid. IL-6 protein levels of macrophages adhering to polyethylene terephthalate were slightly lower than of macrophages adhering to polypropylene. sCD163 was, with the exception of one polylactic acid sample, not detected in the medium of macrophages adhering to any of the materials (Supplementary Figure 7.1B).

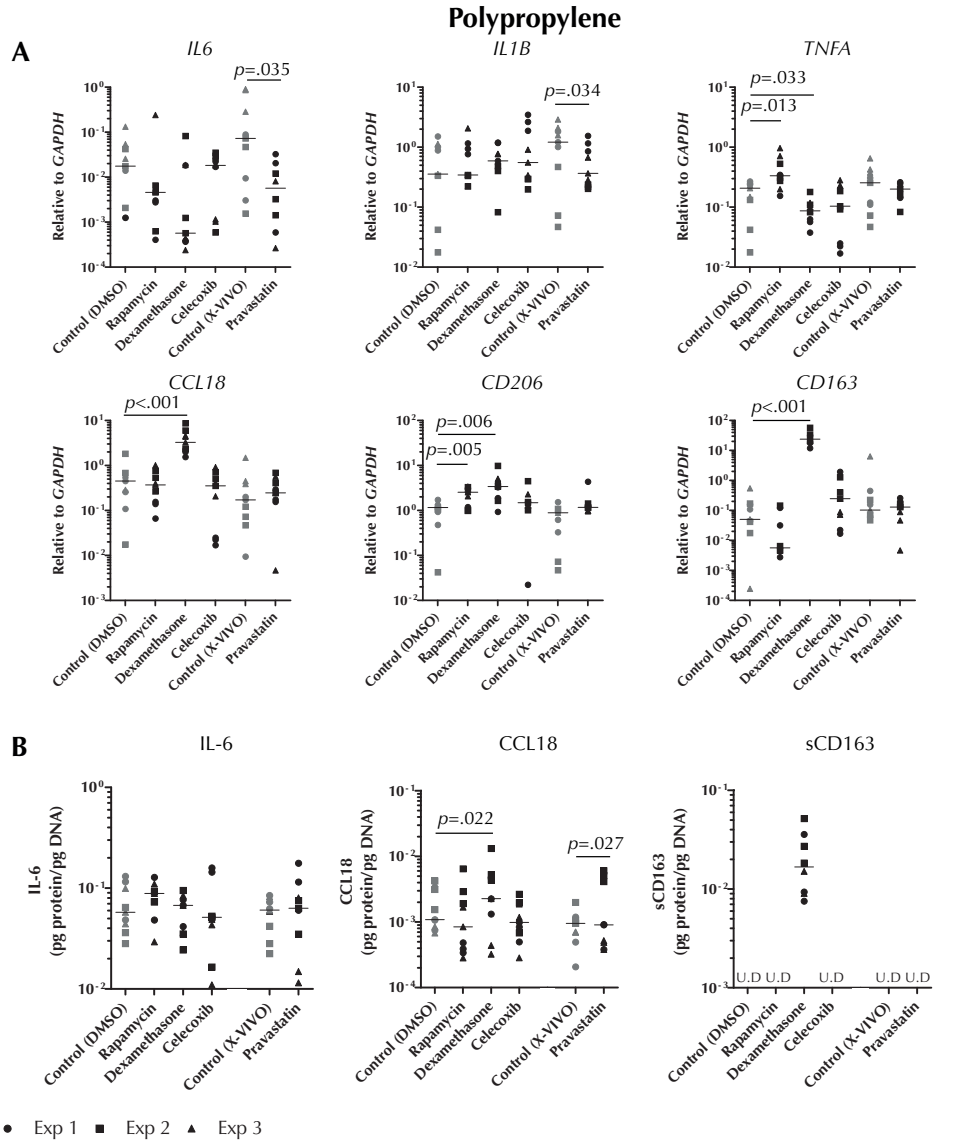


Figure 7.1: Modulation of macrophages adhering to polypropylene. (**A**) Gene expression levels and (**B**) protein levels of macrophages adhering to polypropylene after modulation with rapamycin, dexamethasone, celecoxib and pravastatin. Vehicle treated conditions are indicated by grey symbols, where 0.01% DMSO was used as vehicle for rapamycin, dexamethasone and celecoxib, and X-VIVO medium was used as vehicle control for pravastatin. Statistical evaluation was conducted with a linear mixed model followed by a Bonferroni's *post hoc* test. No statistical analysis was conducted for sCD163 since the protein was not detectable in the untreated control conditions. The data is presented as dot plots including the grand means for n=3 independent experiments with three samples per experimental condition. Abbreviations: *IL6*: Interleukin 6, *IL1B*: Interleukin 1β; *TNFA*: Tumor Necrosis Factor-α; *CCL18*: C-C motif chemokine ligand 18; *CD206*: cluster of differentiation 206; *CD163*: cluster of differentiation 163.

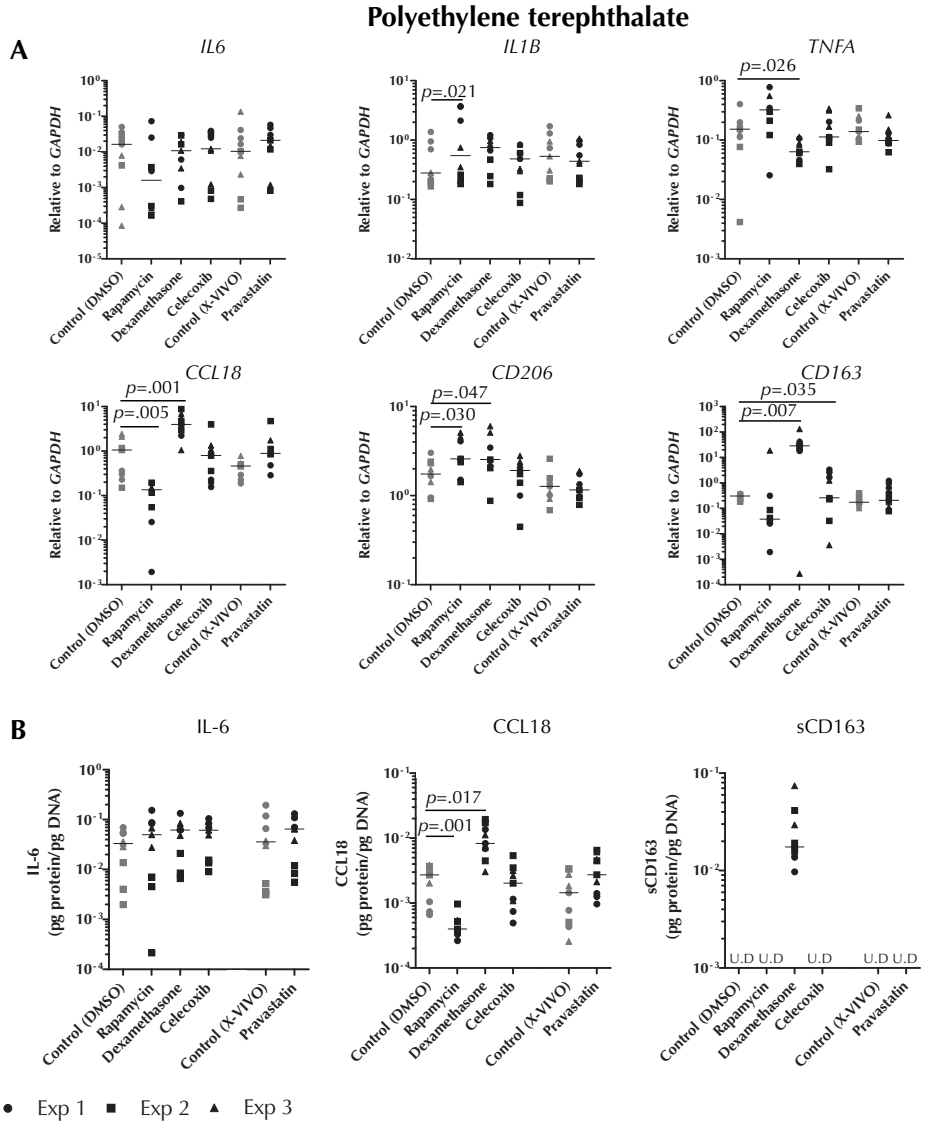
Gene expression and protein secretion profiles of biomaterial-adhering macrophages can be modulated with commonly used medications

In pilot studies, concentrations of 1, 10 and 100 ng/mL rapamycin, 10^{-8}M, 10^{-7}M, and 10^{-6}M dexamethasone, and 0.5, 5, and 50 μM pravastatin were cultured on macrophages in monolayers. As the highest concentrations did not affect the macrophages differently than the lowest two in these monolayer cultures (data not shown), only the two lowest were used for the current experiments to modulate macrophages adhering to biomaterials. Since then the concentrations of 10 ng/mL rapamycin, 10^{-7} M dexamethasone, 5 μM pravastatin and 1 μM celecoxib exerted extremely minor differences compared to a ten times lower concentration (Supplementary figure S7.2), only the lowest concentrations of the medications were further used in this study.

To assess whether the modulation by the medications initiated processes related to macrophage phenotype polarization, expression of genes encoding for pro-inflammatory proteins (*IL6*, *IL1B* and *TNFA*) and genes encoding for anti-inflammatory proteins (*CCL18*, *CD206* and *CD163*) were measured. Rapamycin, dexamethasone and pravastatin were all able to alter the gene expression profiles of macrophages adhering to polypropylene. This was indicated by increased expression levels of *TNFA* and *CD206* after treatment with rapamycin compared to the expression levels of macrophages adhering to polypropylene without treatment. Dexamethasone lowered *TNFA* expression, and increased *CCL18, CD206,* and *CD163* expression, whereas pravastatin lowered *IL6* and *IL1B* compared to the untreated macrophages adhering to polypropylene (Figure 7.1A. On protein level, IL-6 remained unchanged in response to any of the medication treatments. CCL18 production of macrophages adhering to polypropylene was significantly higher when treated with dexamethasone and pravastatin than of the untreated macrophages. sCD163 was undetectable, except after treatment with dexamethasone (Figure 7.1B).

Of the macrophages adhering to polyethylene terephthalate, treatment with rapamycin caused higher expression of *IL1B* and *CD206* than in the untreated controls and lower expression of *CCL18*. Dexamethasone lowered *TNFA* while *CCL18, CD206* and *CD163* expression was higher than the expression of the untreated controls. Celecoxib increased *CD163* expression (Figure 7.2A). IL-6 protein production was not significantly affected by any of the treatments. CCL18 production was reduced after treatment with rapamycin, while dexamethasone caused higher CCL18 production than in the untreated controls. sCD163 was only detectable when the macrophages were treated with dexamethasone (Figure 7.2B).

On gene expression level of macrophages adhering to polylactic acid, rapamycin treatment resulted in lower expression levels of *IL6*, *CCL18* and *CD163*, while *TNFA* and *CD206* expression levels were higher. Dexamethasone reduced *IL6*, *TNFA,* and simultaneously increased *CCL18*, *CD206,* and *CD163*. Expression of *CD206* was higher



● Exp 1 ■ Exp 2 ▲ Exp 3

Figure 7.2: Modulation of macrophages adhering to polyethylene terephthalate. (**A**) Gene expression levels and (**B**) protein levels of macrophages adhering to polypropylene after modulation with rapamycin, dexamethasone, celecoxib and pravastatin. Vehicle treated conditions are indicated by grey symbols, where 0.01% DMSO was used as vehicle for rapamycin, dexamethasone and celecoxib, and X-VIVO medium was used as vehicle control for pravastatin. Statistical evaluation was conducted with a linear mixed model followed by a Bonferroni's *post hoc* test. No statistical analysis was conducted for sCD163 since the protein was not detectable in the untreated control conditions. The data is presented as dot plots including the grand means for n=3 independent experiments with three samples per experimental condition.

after the macrophages were treated with celecoxib and expression of *IL1B* was higher after treatment with pravastatin (Figure 7.3A). On protein level, none of the medications affected IL-6 or CCL18 production of the macrophages adhering to polylactic acid, while sCD163 was undetectable in the majority of the control samples, though it was detectable after the adhering macrophages were treated with dexamethasone (Figure 7.3B).

Polylactic acid-activated macrophages seem more susceptible to phenotype modulation than macrophages adhering to polypropylene or polyethylene terephthalate

To compare the biomaterial-dependent modulation of the macrophages, gene expression levels of *IL6*, *IL1B*, *TNFA*, *CCL18*, *CD206* and *CD163*, and protein production of IL-6, CCL18 and sCD163 were normalized to the untreated controls and pairwise compared between materials. On gene expression level, the relative change in *IL6* expression levels of macrophages adhering to polylactic in response to dexamethasone, was lower than when the macrophages were adhering to polyethylene terephthalate, while the change in *IL1B* in response to pravastatin was higher in polylactic acid-adhering macrophages than when adhering to polypropylene (Supplementary Table S7.1). The relative change in *CD206* expression in response to rapamycin was higher when macrophages adhered to polylactic acid than to polyethylene terephthalate (Supplementary Table S7.2). The relative change in expression of *CCL18* of macrophages that adhered to polypropylene and were modulated with rapamycin, was higher than when macrophages adhered to polyethylene terephthalate or polylactic acid. The change in expression of *CD206* in response to pravastatin was higher of macrophages adhering to polypropylene than when adhering to polyethylene terephthalate (Supplementary Table S7.2). The change in IL-6 protein production by macrophages as a result of celecoxib and pravastatin treatment, was significantly higher when adhering to polyethylene terephthalate than when adhering to polypropylene or polylactic acid. The relative change in sCD163 after modulation with dexamethasone was lower when macrophages were adhering to polypropylene than when the macrophages were adhering to polyethylene terephthalate or polylactic acid. The relative change in CCL18 protein production as a result of the modulation, was only higher when macrophages were cultured on polyethylene terephthalate than when cultured on polylactic acid (Table 7.1).

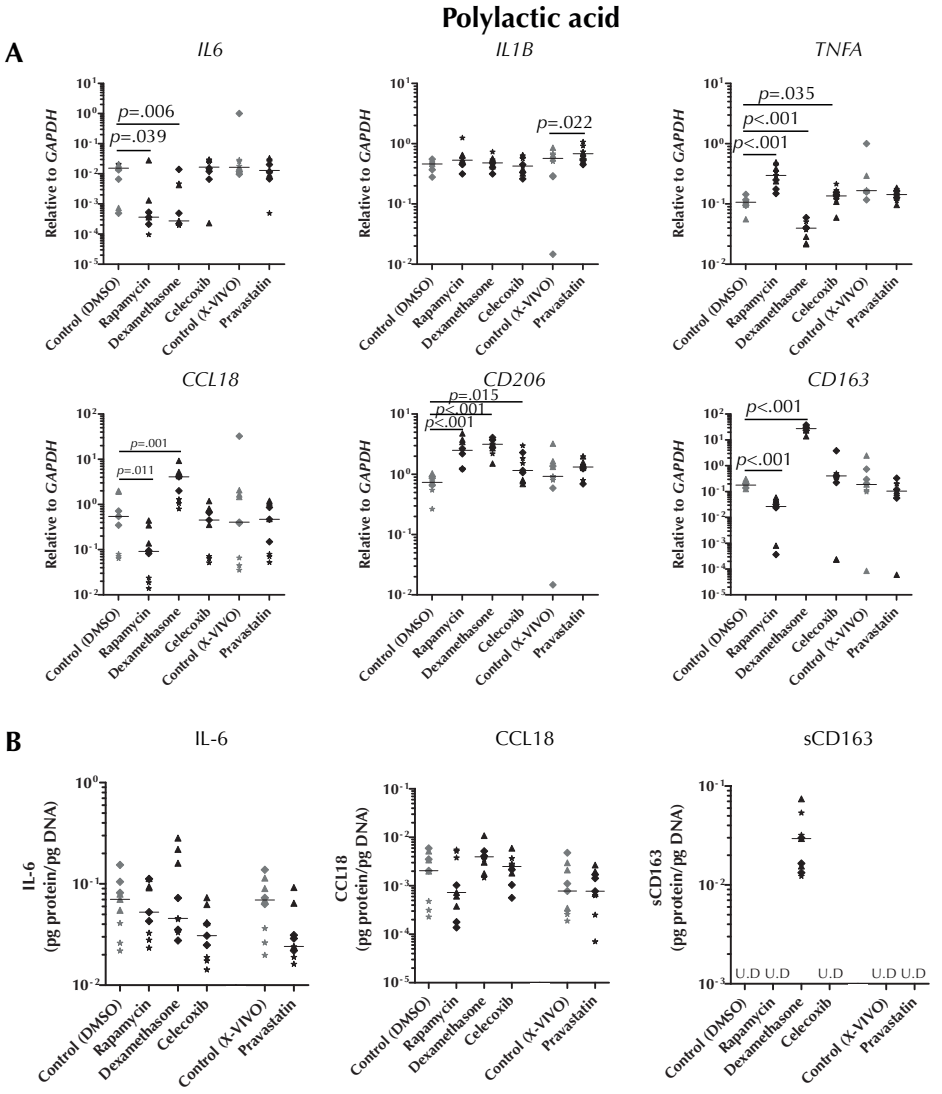


Figure 7.3: Modulation of macrophages adhering to polylactic acid. (**A**) Gene expression levels and (**B**) protein levels of macrophages adhering to polypropylene after modulation with rapamycin, dexamethasone, celecoxib and pravastatin. Vehicle treated conditions are indicated by grey symbols, where 0.01% DMSO was used as vehicle for rapamycin, dexamethasone and celecoxib, and X-VIVO medium was used as vehicle control for pravastatin. Statistical evaluation was conducted with a linear mixed model followed by a Bonferroni's *post hoc* test. No statistical analysis was conducted for sCD163 since the protein was not detectable in the majority of the samples of the untreated control conditions. The data is presented as dot plots including the grand means for n=3 independent experiments with three samples per experimental condition.

Table 7.1: Pairwise comparison of IL-6, CCL18 and sCD163 protein production normalized to untreated controls of modulated macrophages adhering to biomaterial. Values denote the mean difference (group A-group B) of the protein production normalized to the untreated controls. Statistical evaluation was conducted with one-way ANOVA tests after log-transformation followed by Bonferroni's *post hoc* tests. For sCD163, absolute protein levels (in pg/mL) that were uncorrected for cell numbers were used for normalization since sCD163 was not detected in the control samples. Statistical significant values are presented in bold.

	polypropylene[a] vs polyethylene terepthalate[b]			polypropylene[a] vs polylactic acid[b]			polyethylene terepthalate[a] vs polylactic acid[b]		
	IL-6	CCL18	sCD163	IL-6	CCL18	sCD163	IL-6	CCL18	sCD163
Rapamycin	0.372 *p*=0.935	1.011 *p* =0.600		0.167 *p* =1.000	-0.281 *p* =1.000		-0.204 *p* =1.000	-1.292 *p* =0.315	
Dexamethasone	0.092 *p* =1.000	-0.373 *p* =1.000	**-1.023** ***p*=0.040**	0.378 *p* =0.683	0.257 *p* =1.000	**-1.461** ***p*=0.001**	0.286 *p* =1.000	0.630 *p* =0.967	-0.438 *p* =0.699
Celecoxib	**-1.099** ***p* =0.012**	-0.540 *p* =1.000		0.287 *p* =1.000	-0.932 *p* =0.271		**1.387** ***p* =0.002**	-0.393 *p* =1.000	
Pravastatin	-0.690 *p* =0.290	-0.666 *p* =1.000		0.729 *p* =0.241	1.343 *p* =0.205		**1.419** ***p* =0.005**	**2.009** ***p*=0.027**	

DISCUSSION

In this study we have shown as proof of concept that the gene expression and protein production profile of macrophages activated by the biomaterials polypropylene, polyethylene terephthalate and polylactic acid, can be modulated *in vitro* by medications that are commonly used by patients. This modulation capacity seems to depend on the type of biomaterial that is responsible for the initial activation of the macrophages. This knowledge may be applied by the possibility to utilize commonly used medications in combination with a biomaterial to direct macrophage behavior and thereby avoiding unwanted effects after implantation.

In our *in vitro* model, three types of biomaterials were investigated that represent frequently used materials. To limit the parameters that may influence macrophage polarization and to be able to study the effect of the material property, the biomaterials were all braided into a yarn in the same manner. However, due to differences in diameter of the individual fibers, the topography between the materials was not identical. In this study, and as well as our previous work[215], we found very minimal differences between materials regarding initial activation of the macrophages, which is in contradiction with conducted studies by others as reviewed elsewhere[193]. This discrepancy does indicate the importance of not only material composition and property, but architecture as well on macrophage polarization. Interestingly, even though the three materials did not cause differences in initial secretion levels of IL-6, CCL18, and sCD163, and very minimal differences in IL-6 levels prior to modulation, the modulatory response of the macrophages were biomaterial dependent.

Treating the adhering macrophages with rapamycin, an immunosuppressive drug, caused a generally pro-inflammatory effect, due to suppression of genes encoding for anti-inflammatory proteins and simultaneously enhancing genes encoding for pro-inflammatory proteins. The stimulating effect of rapamycin on pro-inflammatory macrophages might suggest its use for controlling severe fibrotic reactions. On the other hand, treating the macrophages adhering to any of the materials with dexamethasone, a corticosteroid, always resulted in increased expression levels of anti-inflammatory genes, while levels for pro-inflammatory genes remained unaffected or were slightly lower. This shows that dexamethasone specifically enhances the performance of anti-inflammatory macrophages, rather than suppressing pro-inflammatory macrophages. On protein level, the effect of dexamethasone was clearly seen by the fact that only dexamethasone treatment caused detectable levels of sCD163 produced by the macrophages cultured on either material, and CCL18 was additionally higher when the macrophages were cultured on polyethylene terephthalate. This is a striking finding, as chronic systemic steroid use may complicate wound healing[216], likely due to inhibition of specific factors secreted by dermal fibroblasts such as keratinocyte growth factor[217]. The fact that in

our previous study[68], the function of tissue repair macrophages (i.e., M(IL-4) and M(IL-10)) were significantly enhanced *in vitro*, indicates a specific advantageous action of dexamethasone on macrophages. In-depth research of its use for controlling severe inflammatory reactions after biomaterial implantation may be of clinical importance. Both the overall pro-inflammatory effect of rapamycin and the anti-inflammatory effect of dexamethasone on the adhering macrophages, were expected and in concordance with our previous work where we modulated macrophages of specific subtypes in conventional monolayer cultures[68] and generally emphasizes the use of glucocorticoids for macrophage polarization[47,73]. Celecoxib, a selective COX-2 inhibitor, did not clearly affect the expression levels of the genes of interest of the macrophages that were cultured on any of the selected materials. On protein level, celecoxib did not significantly affect IL-6, CCL18 or sCD163 production of the macrophages adhering to any of the materials. Though when comparing the relative protein production to that of the untreated controls, celecoxib treated macrophages adhering to polyethylene terephthalate produced more IL-6 than when adhering to polyethylene terephthalate or polylactic acid, suggesting that the protein secretion and the modulatory potential of celecoxib is dependent on the type of biomaterials that activated the macrophages initially. Pravastatin, a cholesterol synthesis inhibitor used by patients at risk of cardiovascular disease, lowered expression levels of pro-inflammatory genes of macrophages cultured on polypropylene, but did not affect any of the genes of interest of macrophages cultured on polyethylene terephthalate and polylactic acid. Protein levels of CCL18 of the macrophages increased in response to pravastatin when cultured on polypropylene, and had the tendency to increase when cultured on polyethylene terephthalate, suggesting an overall anti-inflammatory effect of pravastatin. This effect was partially expected, since statins have been shown to exert many pleiotropic effects, among which anti-inflammatory effects[155,156,218]. Atorvastatin reduced expression of *CXCR7*, a chemokine receptor involved in macrophage migration, in the human THP-1 cell line[156] and induced expression of *CD206*, *IL10* and *CCL18* in human monocyte derived macrophages[155]. Striking though, is that in our previous study[68], pravastatin only affected protein secretion levels of sCD163 when macrophages were cultured in a monolayer, while in the current study, CCL18 protein levels were increased after treatment. This again confirms that the type of biomaterial determines the protein secretion profile and thus the capacity to modulate this profile.

After pairwise comparison of both the gene expression and protein production relative to the controls per material, we could not determine a very clear conclusion that one of the three tested biomaterials was more sensitive to allow phenotype modulation of adhering macrophages. Yet, macrophages adhering to polylactic acid appeared to have the tendency to be more susceptible to phenotype modulation, as the relative change in gene expression levels and protein levels of the genes and proteins of interest, were more often affected, in either direction, by the medications when

the macrophages where adhering to polylactic acid then when they were adhering to polypropylene or polyethylene terephthalate. In some cases, gene expression levels and protein levels do not correspond with each other, likely due to post-translational processing of the proteins. By measuring the gene expression levels, we acquire an indication of an array of proteins that may be produced, but it remains an indication[219,220] and should therefore be carefully interpreted. Furthermore, when we treated the adhering macrophages with a ten times higher concentration of the medications, only minor differences between concentrations were found. This could either be due to non-specific actions of the medications, or on the other hand, emphasize the sensitivity of macrophages to become activated after exposure to a minimal stimulus.

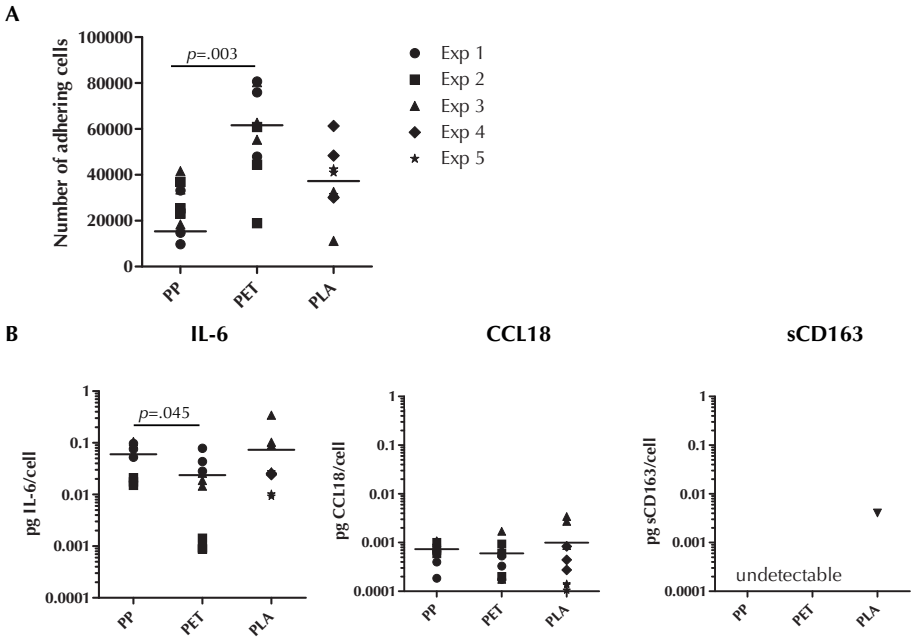
Today, understanding modulation of macrophage polarization in response to biomaterials has become of increased interest, since macrophages significantly contribute to the response of the host to the material. Various ingenious methods to alter macrophage polarization from an engineering point of view have been proposed, such as altering surface properties of the material itself[221]. These methods may seem promising but require alterations of the material itself which may result in different outcome and advantageous properties of the material may have to be sacrificed. The possibility to modulate biomaterial-adhering macrophages using medications, as we have shown in the current study, opens up potential applications to direct adverse effects after implantation of a material. Modulating the phenotype of these macrophages can be conducted by combining the medication with a biomaterial that acts as a controlled release system for local macrophage modulation[222,223] as systemic use of the medication could have a general impact on wound healing. Moreover, in terms of clinical application, the results of this study suggest that differences in the responses of macrophages can be expected by patients who are chronically using medications that belong to the medication groups that were evaluated in this study. This knowledge could eventually contribute to the choice of drug prescriptions when biomaterials are involved, and thus might provide a small step towards future personalized medicine. *In vivo* experiments should be conducted to determine whether macrophage modulation would affect the foreign body response after implantation and favors wound healing.

CONCLUSION

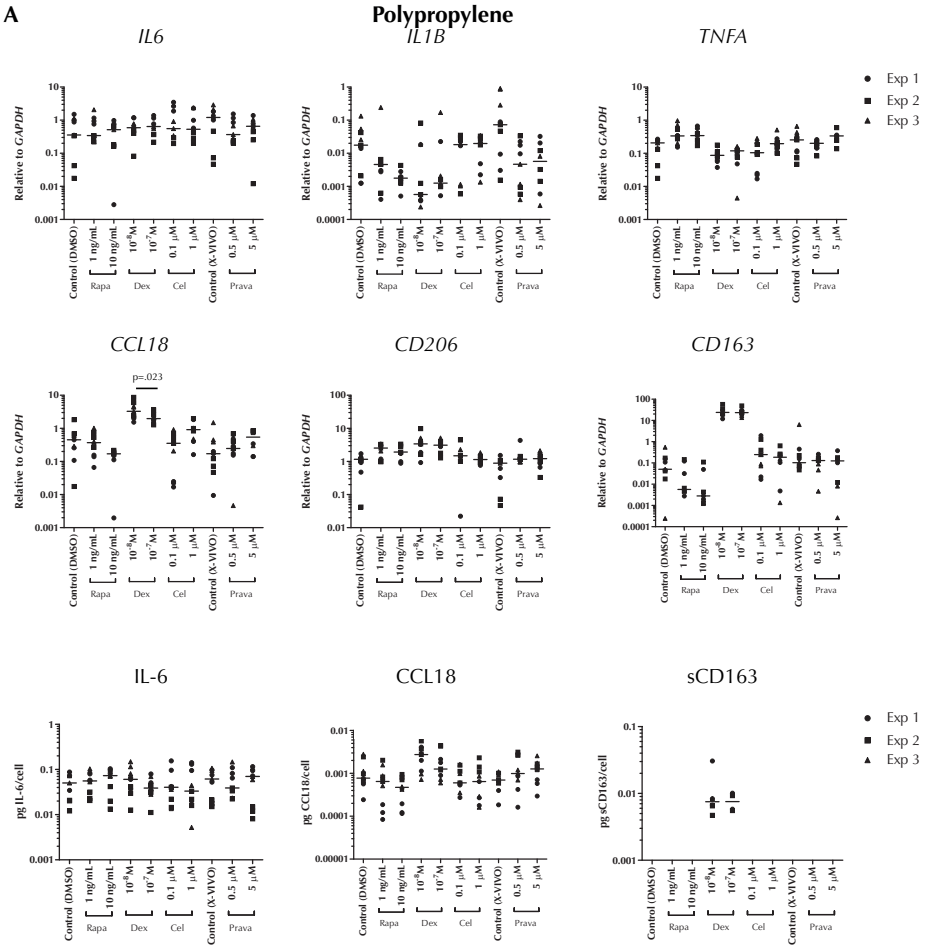
To conclude, the phenotype of macrophages activated by polypropylene, polyethylene terephthalate and polylactic acid can be modulated using commonly used medications. Modulation of macrophages activated by other biomaterials than used in the current study, may be possible as well. The degree of the modulatory capacity however depends on the type of biomaterial that causes initial activation. Combined, this knowledge may provide insights into the possibility of using a medication in combination with a certain

biomaterial to direct macrophage behavior and thereby possibly avoiding unwanted effects after implantation of a biomaterial.

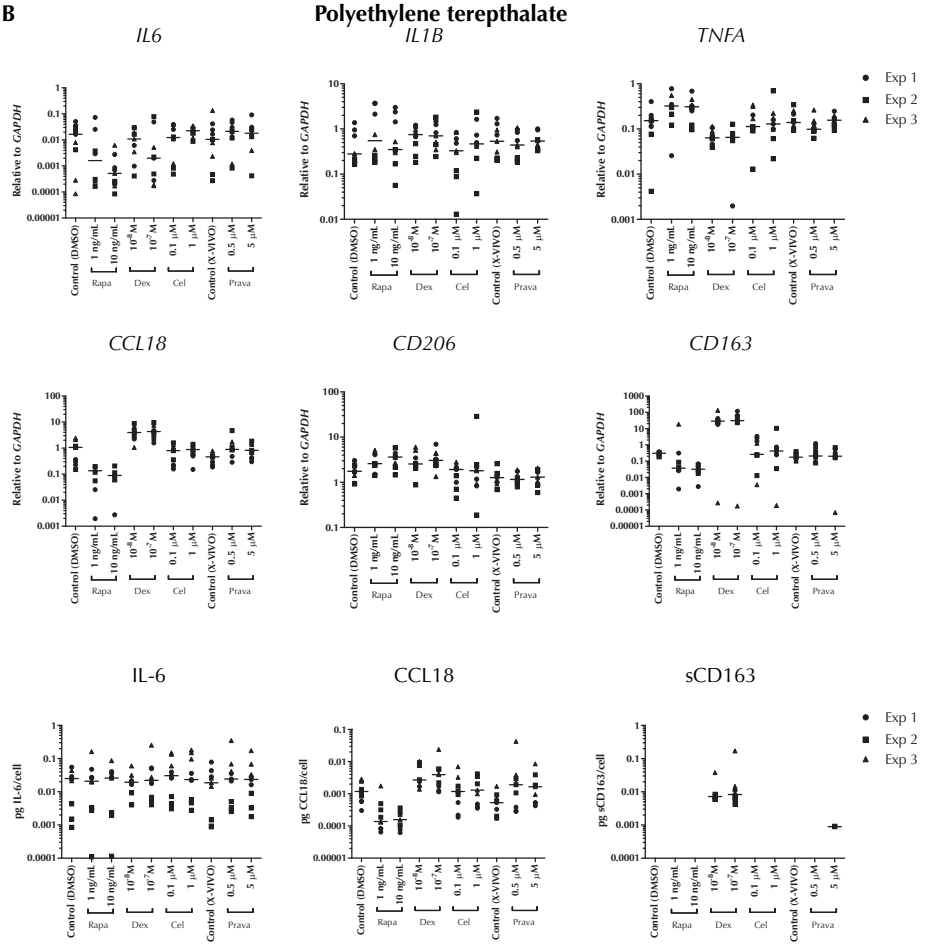
SUPPLEMENTARY DATA



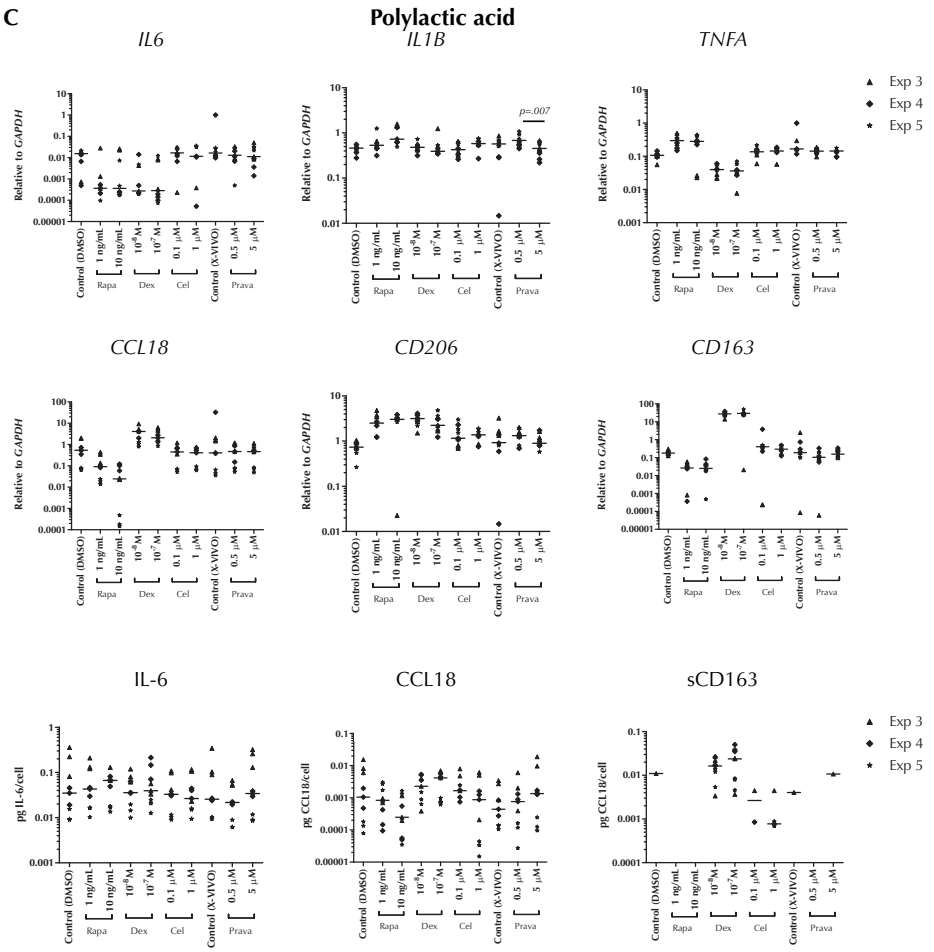
Supplementary Figure S7.1: (**A**) The number of adhering macrophages on polypropylene, polyethylene terephthalate and polylactic acid after seeding followed by 6d of culture without modulation. (**B**) Protein secretion of macrophages without modulation in response to the biomaterials. Data shown as dot plots including the grand means for n=3 experiments per material. Statistical evaluation was conducted with one-way ANOVA tests after log-transformation followed by Bonferroni's *post hoc* tests. Abbreviations: PP: polypropylene; PET: polyethylene terephthalate; PLA: polylactic acid; IL-6: interleukin-6; CCL18: C-C motif chemokine ligand 18; sCD163: soluble cluster of differentiation 163.



Supplementary Figure S7.2: Gene expression levels and protein levels of macrophages adhering to **(A)** polypropylene, **(B)** polyethylene terephthalate, and **(C)** polylactic acid. Two concentrations of medications were used in the experiments to modulate the biomaterial-adhering macrophages, yet showing minimal differences between concentrations. Statistical evaluation was conducted with a linear mixed model transformation followed by a Bonferroni's *post hoc* test. P-values are only given for statistically significant differences between concentrations of the medications. Abbreviations: Rapa: rapamycin; Dex: dexamethasone; Cel: celecoxib; Prava: pravastatin. Continued on next pages



Supplementary Figure S7.2 (continued): Gene expression levels and protein levels of macrophages adhering to **(A)** polypropylene, **(B)** polyethylene terephthalate, and **(C)** polylactic acid. Two concentrations of medications were used in the experiments to modulate the biomaterial-adhering macrophages, yet showing minimal differences between concentrations. Statistical evaluation was conducted with a linear mixed model transformation followed by a Bonferroni's *post hoc* test. P-values are only given for statistically significant differences between concentrations of the medications. Abbreviations: Rapa: rapamycin; Dex: dexamethasone; Cel: celecoxib; Prava: pravastatin.



Supplementary Figure S7.2 (continued): Gene expression levels and protein levels of macrophages adhering to **(A)** polypropylene, **(B)** polyethylene terephthalate, and **(C)** polylactic acid. Two concentrations of medications were used in the experiments to modulate the biomaterial-adhering macrophages, yet showing minimal differences between concentrations. Statistical evaluation was conducted with a linear mixed model transformation followed by a Bonferroni's *post hoc* test. P-values are only given for statistically significant differences between concentrations of the medications. Abbreviations: Rapa: rapamycin; Dex: dexamethasone; Cel: celecoxib; Prava: pravastatin.

Supplementary Table S7.1: pairwise comparison of expression levels of pro-inflammatory M1 genes normalized to untreated controls of modulated macrophages adhering to biomaterials. Statistical evaluation was conducted with one-way ANOVA tests after log-transformation followed by Bonferroni's *post hoc* tests. Values denote the mean difference (group A-group B) of the gene expression levels normalized to the untreated controls. Statistical significant values are presented in bold.

	polypropylene[a] vs polyethylene terephthalate[b]			polypropylene[a] vs polylactic acid[b]			polyethylene terephthalate[a] vs polylactic acid[b]		
	IL6	*IL1B*	*TNFA*	*IL6*	*IL1B*	*TNFA*	*IL6*	*IL1B*	*TNFA*
Rapamycin	0.569 *p*=1.000	-0.460 *p*=0.346	0.393 *p*=1.000	1.426 *p*=0.227	-0.067 *p*=1.000	-0.119 *p*=1.000	0.857 *p*=0.868	0.393 *p*=0.526	-0.512 *p*=0.726
Dexamethasone	-1.265 *p*=0.570	-0.297 *p*=0.732	0.187 *p*=1.000	1.414 *p*=0.510	0.133 *p*=1.000	0.510 *p*=0.329	**2.678** ***p*=0.035**	0.431 *p*=0.289	0.323 *p*=0.908
Celecoxib	-0.365 *p*=1.000	0.896 *p*=0.059	-0.362 *p*=1.000	0.448 *p*=1.000	0.819 *p*=0.094	-0.769 *p*=0.333	0.813 *p*=1.000	-0.077 *p*=1.000	-0.405 *p*=1.000
pravastatin	-1.910 *p*=0.179	-0.382 *p*=0.539	0.194 *p*=1.000	-0.730 *p*=1.000	**-0.839** ***p*=0.017**	-0.223 *p*=1.000	1.180 *p*=0.610	-0.457 *p*=0.332	-0.418 *p*=0.261

Supplementary Table S7.2: pairwise comparison of expression levels of anti-inflammatory M2 genes normalized to untreated controls of modulated macrophages adhering to biomaterials. Statistical evaluation was conducted with one-way ANOVA tests after log-transformation followed by Bonferroni's *post hoc* tests. Values denote the mean difference (group A-group B) of the gene expression levels normalized to the untreated controls. Statistical significant values are presented in bold.

	polypropylene[a] vs polyethylene terepthalate[b]			polypropylene[a] vs polylactic acid[b]			polyethylene terepthalate[a] vs polylactic acid[b]		
	CCL18	*CD206*	*CD163*	*CCL18*	*CD206*	*CD163*	*CCL18*	*CD206*	*CD163*
Rapamycin	**1.842** ***p*<0.001**	0.279 *p*=0.683	-0.982 *p*=0.834	**1.157** ***p*=0.014**	-0.491 *p*=0.104	0.605 *p*=1.000	-0.685 *p*=0.257	**-0.770** ***p*=0.007**	0.3777 *p*=1.000
Dexamethasone	0.330 *p*=1.000	0.761 *p*=0.104	0.522 *p*=0.171	-0.006 *p*=1.000	-0.004 *p*=1.000	0.237 *p*=1.000	-0.330 *p*=1.000	-0.765 *p*=0.101	-0.285 *p*=0.855
Celecoxib	-0.757 *p*=0.171	0.219 *p*=1.000	0.412 *p*=1.000	-0.804 *p*=0.133	-0.251 *p*=1.000	-0.260 *p*=1.000	-0.047 *p*=1.000	-0.470 *p*=1.000	-0.672 *p*=1.000
pravastatin	-0.732 *p*=0.689	**0.759** ***p*=0.023**	-1.182 *p*=0.218	0.379 *p*=1.000	0.345 *p*=0.592	0.040 *p*=1.000	1.110 *p*=0.221	-0.414 *p*=0.374	1.222 *p*=0.193