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The influence of polystyrene and poly(ether imide) inserts with different roughness, on the activation of dendritic cells

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Abstract

Dendritic cells (DC) have a pivotal role during inflammation. DC efficiently present antigens to T cells and shape the subsequent immune response by the secretion of pro- or anti-inflammatory cytokines and by the expression of co-stimulatory molecules. They respond to "danger signals" such as microbial products or fragments from necrotic cells or tissues, but were also described to be reactive towards biomaterials. However, how mechanical and physical properties of the subjacent substrate influences the DC activation is currently poorly understood. In this study micro patterned inserts prepared from polystyrene (PS) as well as from poly (ether imide) (PEI) with three different roughness levels of i) R_q = 0.12 µm (PS) and 0.23 µm (PEI); ii) R_q = 3.47 µm (PS) and 3.92 µm (PEI); and iii) R_q = 22.16 µm (PS) and 22.65 µm (PEI) were analyzed for their capacity to influence the activation of human monocytes derived DC. Since the DC were directly cultured in the inserts, the effects of the testing material alone could be investigated and influences from additional culture dish material could be excluded.

The viability, the expression of the DC activation markers, and their cytokine/chemokine secretion were determined after the incubation with the different inserts *in vitro.*

Both the PS and the PEI inserts did not influence the survival of the DC and their expression of co-stimulatory molecules. The expression of inflammatory cytokines was not altered by the PEI and PS inserts. However, the secretion of chemokines such as CCL2, CCL3, and CCL4 was influenced by the different roughness levels, indicating that material roughness has the capacity to modulate the DC phenotype.

The data presented here will help to understand the interaction of DC with structured polymer surfaces. Biomaterial-induced immuno-modulatory effects mediated by DC may promote tissue regeneration or could potentially reduce inflammation caused by the implant material.

Keywords: Biomaterials, roughness, dendritic cells, cell culture devices

1. Introduction

The aim of material-mediated regenerative therapies is to reconstitute tissues, which lost their functionality due to degenerative diseases or severe injuries [32]. During this process biomaterials can support defined cell types, which subsequently guide the regeneration of the damaged tissues. Innovative biomaterials could improve existing therapies but may also support the development of completely new interventional therapeutic strategies [13].

Biomaterials are used to support the expansion or differentiation of distinct cell types. Physical and chemical properties of the biomaterials can influence cell growth or differentiation [30]. For example, copolymers, which show cell selectivity, were previously described [4]. Furthermore, stem cell growth and differentiation can be supported by the surface topography, surface chemistry as well as by the elastic modulus of the biomaterial [22]. Both examples highlight the potential of biomaterials to directly interact with distinct cell types, which eventually lead to the generation of functional tissues. However, cells, which indirectly support wound healing by secretion of soluble mediators or by direct cellular interaction, could also be suitable targets to develop biomaterial-mediated therapies or treatments. Recently, we could show that VEGF-A-producing monocytes are able to accelerate the formation of a functional confluent endothelial cell layer on a hydrophobic polymer surface [21].

DC are another cell type of the immune system, which are of therapeutic interest [38]. They are the most effective antigen presenting cells (APC) being able to initiate and shape T cell responses [23]. The antigens, captured from the periphery, are presented to T cells via major histo-compatibility complex (MHC) molecules expressed by DC. To mount an effective T cell response, DC need additionally to be activated via toll-like-receptors (TLR), which can be engaged by microbial products or fragments from necrotic cells [3]. The strength and type of the T cell response depends on the expression of co-stimulatory molecules on the DC surface such as CD40, CD80, and CD86, as well as on the expression of cytokines and chemokines such as IL-6, IL-12, or CCL2 [3]. An effective T cell response helps to clear infection and can mediate long term resistance to pathogens [34]. Furthermore, DC loaded with tumor antigens could be used to initiate an effective anti-cancer T cell response [25]. However, DC also instigate unwanted responses such as transplant rejection, allergy, and autoimmunity [33]. Therefore the manipulation of the DC function by biomaterials is attractive for improving therapeutic strategies. Enhancing the abilities of DC to mount effective immune response may improve vaccine efficacies against infectious disease, while the generation of tolerogenic DC might be beneficial for the treatment of allergies or autoimmune diseases such as rheumatoid

arthritis or multiple sclerosis [33, 34]. Biomaterials are also able to interact with DC and can induce their activation [2]. For example, it was recently shown that different polymethacrylates can induce the expression of co-stimulatory molecules as well as the expression of inflammatory cytokines [15].

In this study PEI and PS inserts with different roughness levels were investigated for their ability to influence the activation of DC. The insert system used in this study allows to analyze the effects of the different roughness levels on distinct cell types without the interference of an additional culture vessel material [12]. Recently, it could be shown that the inserts were cytocompatible with murine fibroblast, were free of endotoxin and other microbial contamination and did not activate innate immune mechanisms, indicating a very clean manufacturing in the injection moulding process [12, 28].

The different roughness levels were obtained by the use of differently structured cylinders, which allowed creating the different roughness levels without influencing the chemical composition of the material. It has been published that material topography can influence the behavior and growth of stem cells, osteoblasts, and fibroblasts [5, 8, 17]. For example, osteoblast formation was enhanced on rough clinical titanium (TI) surfaces obtained by chemical etching, indicating that rough materials may support the acceleration of bone formation [20]. Furthermore, the bone regeneration can be influenced by local inflammatory reactions, in which DC can play a critical role through secretion of cytokines or activation of T cells [36]. Indeed, it has recently been shown that DC changed their phenotype on rough TI surfaces and may thereby support the osteoclastogenesis [16]. Since polymer-based biomaterials can also be used as implants for bone regeneration, it was investigated in this study how different roughness levels influence the behavior of DC. We hypothesize that similarly to TI surfaces the different roughness levels of PEI and PS may induce a phenotypic change of the DC.

To investigate how the roughness levels influence the behavior of human monocytes-derived DC, the cells were incubated on the different PEI and PS inserts and their viability as well as their expression of co-stimulatory molecules was determined by flow cytometry. Furthermore, the cytokine and chemokine secretion levels were analyzed in the supernatants by a multiplex suspension assay.

2. Material and Methods

The study was performed in accordance with the ethical guidelines of the journal *Clinical Hemorheology and Microcirculation* [1].

2.1 Materials, Processing, and Surface characterization

PS (PS Type 158K, BASF, Germany) with an average molecular weight of M_n = 109.000 g·mol^{-1} determined by gel permeation chromatography with chloroform as eluent and PEI (ULTEM® 1000, General Electric, USA) with M_n = 17.000 g·mol^{-1} were used without any further purification.

Processing of the polymer inserts with a different micro structured bottom was performed by injection moulding using three different types of cylinders with a polished contact surface (R0), and two cylinders with micro structured surfaces (RI) and (RII) as previously described [28].

For surface characterization by optical profilometry, contact angle measurements and micromechanical testing, the bottom of the insert was detached using a water-cooled histological saw (Accutom-50, Struers, Willich, Germany).

Surface profiles of the inner bottom of the inserts were explored according to a method recently described [12] with an optical profilometer type MicoProf 200 (Fries Research & Technology GmbH, Bergisch Gladbach, Germany), equipped with a CWL 300 chromatic white-light sensor. The surface roughness (R_q) was analyzed by scanning a region from the border to the centre of the sample. The wettability of the samples was determined by measuring water contact angles (CA) using the captive bubble method as described in [12]. Microindentation measurements in the dry state were conducted with an atomic force microscope MFP-3D Bio-AFM (Asylum Research, Santa Barbara, CA, USA). The Young´s modulus was calculated by the Oliver-Pharr model [24] in a range from 20% to 80% at the force-distance curve.

Prior to biological testing and surface characterization of the polymeric inserts, all PS samples were sterilized by gas sterilization using ethylene oxide. PEI samples were sterilized via steam sterilization at 121 °C and a pressure of 2.0 bar for 20 min using a Systec Autoclav D – 65 (Systec GmbH, Wettenberg, Germany).

2.2 Generation of peripheral blood mononuclear cell (PBMC) derived DC

For the generation of human monocytes derived DC, PBMC were isolated from buffy coats by density gradient centrifugation as previously described [40]. In brief, monocytes were

purified from PBMC by depleting CD14 negative cells using the monocytes isolation Kit II (Milteniy Biotec, Bergisch-Gladbach, Germany). For all donors (n=4) the isolated CD14 positive monocytes had a purity above 95%. DC were generated from monocytes by the addition of 10 µg/mL interleukin (IL)-4 (Milteniy Biotec, Bergisch-Gladbach, Germany) and 100 µg/mL granulocyte macrophage - colony stimulating factor (GM-CSF) (R&D Systems, Minneapolis, USA).

After six days of cell culture, the differentiated DC were harvested and $1x10^6$ cells were seeded in the different inserts for 24 h with or without 1 µg/mL lipopolysaccharides (LPS) (LPS from *E.coli* O111:B4, Axxora GmbH, Lörrach, Germany). The differentiation of the CD14 positive monocytes into DC was checked by CD14 and CD209 expression and resulted routinely in more than 95% CD209-positive and CD14-negative cells.

2.3 Flow cytometry analysis for surface molecule expression

After exposure to the PEI and PS inserts, cells were harvested and washed with AutoMacs® washing buffer (Milteniy Biotec). The staining procedure was performed as described [40]. In brief, to avoid unspecific binding, Fc-receptors were blocked by incubating with FcR-Blocking Reagent (1:100, Milteniy Biotec) for 15 min on ice. After washing, cells were stained with anti-CD14-PC7 (clone RMO52, Beckman Coulter, Krefeld, Germany) and anti-CD209-APC (clone DCN47.5, Milteniy Biotec) to discriminate DC from monocytes. To determine the activation state of the DC, they were additionally stained either with anti-CD86-PE (clone 2331 (FUN-1)), anti-CD80-PE (clone L307.4), anti-CD83-PE (clone HB15), anti-HLA-ABC-PE (clone G46-2.6), anti-CD40-PE (clone HB14) and anti-HLA-DR-PE (clone AC122) antibodies, all purchased from Milteniy Biotec. Cells were stained for 10 min on ice and analyzed after a final washing step using a MACSQuant flow cytometer (Milteniy Biotec). Data were analyzed using FlowJo software (Tree Star, Ashland, USA).

2.4 Multiplex cytokine profiling of DC

Cell culture supernatants collected from the DC cultured on the PS and PEI inserts were stored at -20 °C and thawed prior cytokine analysis. The cytokine secretion was quantified using Bio-Plex suspension array systems (Bio-Plex 200®, BioRad, Germany) according to the manufacture's instructions. IL-1β, IL-6, IL-10, IL-12, and TNF-α bead assays were purchased from BioRad. The cytokine secretion was determined in duplicates from four individual donors.

2.5 Statistics

Data were statistically analyzed using GraphPad Prism (La Jolla, CA 92037, USA). For all samples mean values and the standard error of the mean are given. Multiple sample tests were performed using one-way analysis of variance (ANOVA). Bonferoni-adjusted p-values lower 0.05 are considered significant.

3. Results

3.1 Flow cytometric analysis of DC – gating strategy

The expression of activation markers by DC as well as their viability analysis was investigated by flow cytometry. Figure 1 shows the gating strategy, which was applied to all samples. First the cells were discriminated from debris (Fig. 1A) followed by doublet exclusion (Fig. 1B) [41]. The single cells were further analyzed for the expression of DC-sign, which is a marker for DC and DAPI incorporation to discriminate live from dead cells (Fig. 1C). The activation marker expression was subsequently analyzed on single live dendritic cells (Fig. 1D).

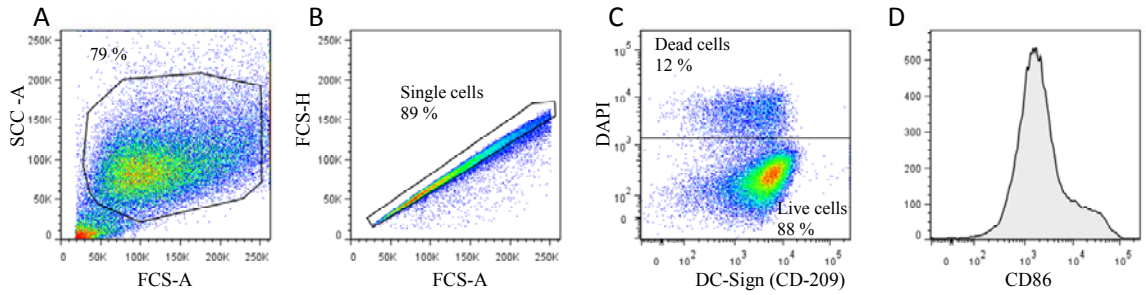

Fig. 1. Gating strategy of DC for the flow cytometric analysis. DC were cultured on the different PEI or PS inserts as well as on tissue culture plate (TCP) and analyzed by flow cytometry after 24 hours of incubation. **(A)** Scatter plot, which distinguished cell from debris. **(B)** FCS-H plotted against FCS-A to excluded cell doublets [41]. **(C)** DAPI vs DC-Sign was plotted to discriminate between live and dead dendritic cells. **(D)** Histogram showing the CD86 expression on single live DC. Representative dot plots are shown from untreated DC.

3.2 DC survival FACS

DC were cultured on PEI and PS inserts with three different roughness levels. The smoothest surface (R0) had a R_q value of 0.29 ± 0.07 µm for PS-R0 and 0.23 ± 0.07 µm for PEI-R0, the intermediate roughness (RI) was 3.47 ± 0.28 µm for PS-RI and 3.92 ± 0.20 µm for the PEI-RI inserts and the roughest surface (RII) was 22.16 ± 0.64 µm and 22.65 ± 0.80 µm for PS-RII and PEI-RII, respectively. R0 inserts had a surface roughness similarly to commercially available cell culture vessels and serve as reference. The intermediate surface roughness RI was previously described to influence the morphology and growth of L929 fibroblasts [12]. The highest surface roughness RII could support cell aggregation and might thereby support tissue formation or the generation of embryonic bodies from individual stem cells.

In order to investigate the survival of DC after contact to different PEI and PS inserts, the DC were analyzed by flow cytometry after 24 hours of cell culture as described previously [40]. Dead cells were distinguished from live cells by the incorporation of DAPI and gated accordingly (Fig. 1). The survival of the DC was influenced neither by the materials nor by

their different roughness levels (Fig. 2). Furthermore, the LPS treatment reduced the DC survival, which is described as physiological apoptosis to maintain self tolerance and prevent from autoimmune diseases [43]. However, the different PEI and PS inserts had no significant effect on the survival of LPS-activated DC.

3.3 DC activation marker expression

The activation status of DC can be determined by their expression of surface molecules such as CD80, CD86, CD40, HLA-DR and HLA-ABC as well as by the expression of the maturation marker CD83. In order to determine whether the PEI and PS inserts with their different roughness levels can influence the DC activation, the expression of surface molecules was determined after 24 hour of cell culture by flow cytometry.

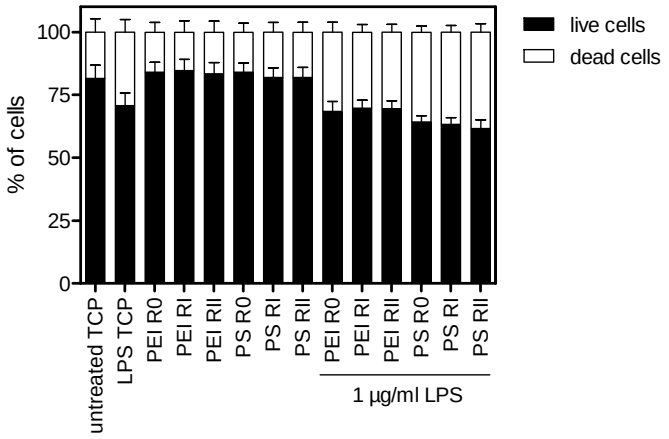

Fig. 2. Survival of DC after exposure to PEI and PS inserts. Monocyte-derived DC were cultured for 24 hours on the different PEI and PS inserts or on TCP. Viability was analyzed by flow cytometry using DAPI incorporation. Cells were gated as described in Fig. 1. Average of two independent experiments each with two Donors (n=4) is shown (mean ± SEM).

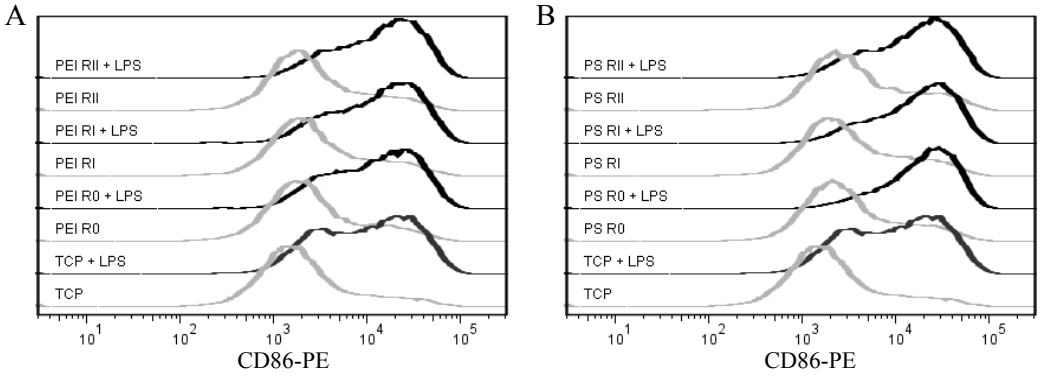

Fig. 3. CD86 Expression on DC cultured in PEI and PS inserts with different roughness. Monocyte-derived DC were cultured for 24 hours in the different PEI (A) and PS (B) inserts with or without 1 µg/mL LPS. Expression of CD86 was analyzed by flow cytometry. Dead cell were excluded from analysis by DAPI labelling. Cells were gated as described in Fig.1A. Representative histograms for four individual healthy donors are shown.

Histogram overlays of monocyte derived DC from one representative donor show that the CD86 expression after culturing DC on the different PEI and PS cups was not induced compared to TCP (Fig. 3). Furthermore, under inflammatory condition mimicked by the addition of LPS the surface expression of CD86 on DC was not altered by the PEI and PS inserts (Fig. 3).

The quantification of the flow cytometric data revealed that the expression of CD40, CD80, CD83, CD86, HLA-DR, and HLA-ABC was not altered by the PS and PEI inserts and was comparable to TCP (Fig. 4). The different roughness levels seem to have no influence on the activation of DC (Fig. 4). Although not significant, the LPS-induced expression of CD86 seems to be higher in DC cultured on PS compared to PEI (Fig. 4).

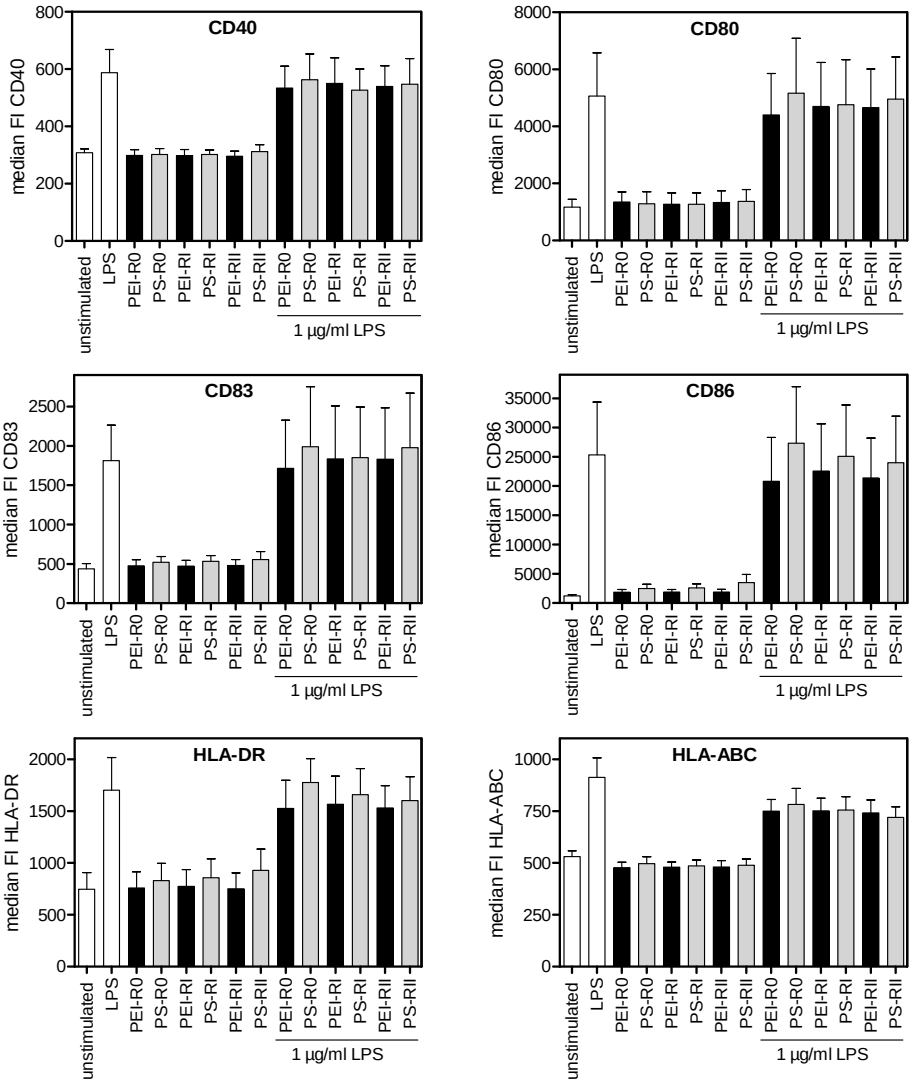

Fig. 4. Activation marker expression of monocytes derived DC from four individual donors. Monocyte derived DC were cultured for 24 hours in the different PEI (black bars) and PS (grey bars) inserts or in TCP (white bars), with or without 1 µg/mL LPS. Dead cells were excluded from analysis by DAPI labelling. Cells were gated as described in Fig. 1. The median fluorescence intensities (FI) of the

respective activation marker were plotted. Average of two independent experiments each with two healthy Donors (n=4) is shown (mean ± SEM).

3.4 Cytokine and chemokine expression by DC cultures on the different PEI and PS inserts

The cytokines released by DC are referred to as the 3rd signal for T cell activation and can direct the subsequent T cell response into the T_H1 or T_H2 bias [14]. To analyze the cytokines secreted by DC in response to the different insert, the cell culture supernatants were collected after 24 hours of incubation and a multiplex cytokine analysis was performed. The IL-6, IL-10, IL-12, and TNF-α production by DC was not induced by the PEI and by the PS inserts (Fig. 5). However, DC-derived IL-17 was significantly induced by both the PEI and PS inserts when compared to TCP (Fig. 5). Furthermore, it was investigated whether the LPS-induced DC cytokine release can be altered by the different inserts. The secretion of the pro-inflammatory cytokines IL-6, IL-12, IL-17, and TNF-α by LPS-activate DC was not substantially altered by the different PEI and PS inserts (Fig. 5).

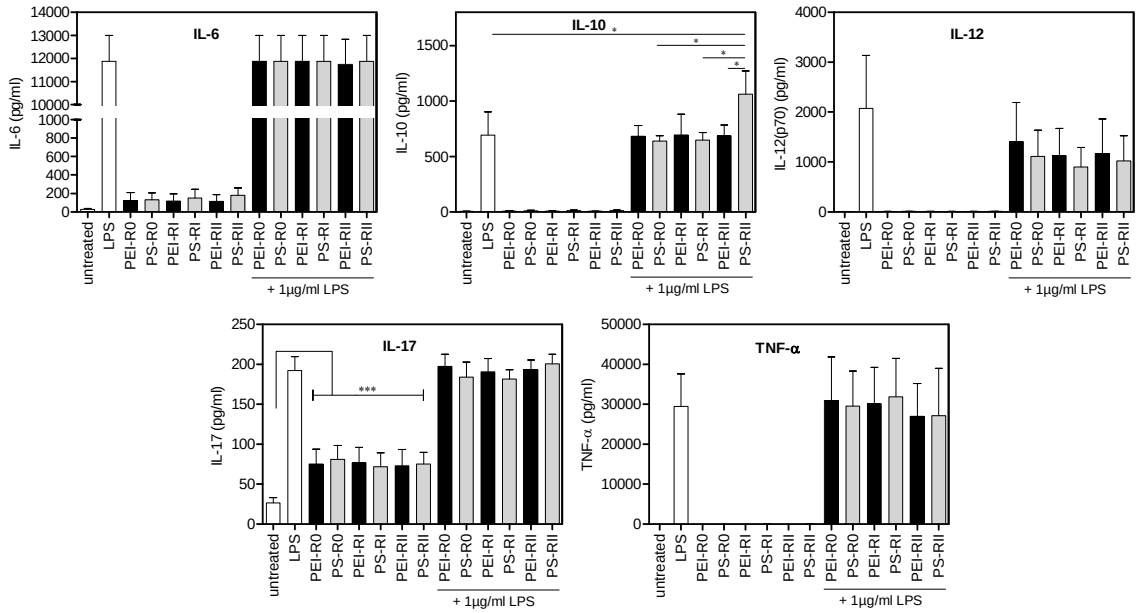

Fig. 5. Cytokine secretion by DC cultured on the different PEI and PS inserts. Monocyte-derived DC were cultured for 24 hours in the different PEI (black bars) and PS (grey bars) inserts or in TCP (white bars), with or without 1 µg/mL LPS. Cytokine secretion was detected using the BioPlex® multiplex system. Average of two independent experiments each with two healthy Donors (n=4) is shown (mean ± SEM). Results were statistically compared using the one-way Anova test for repeated-measures (*for $p<0.05$ and *** for $p<0.001$).

Remarkably, the secretion of anti-inflammatory IL-10 was significantly induced by the PS inserts with highest roughness level when compared to TCP or to the PS inserts with the smoother surfaces (Fig. 5).

Chemokines secreted by DC can attract other immune cells and regulate multiple cellular responses [29]. The secretion of CCL2, CCL3, and CCL4 was analyzed in supernatants of DC cultivated for 24 hour in the different inserts either without or with LPS to mimic inflammatory conditions (Fig. 6). All three chemokines were induced by both inserts materials. CCL2 was only slightly induced by the materials. However, with increasing roughness of the PS inserts the CCL2 production seems to increase systematically for both the untreated DC and the DC treated with LPS (Fig. 6). The expression of CCL3 and CCL4 increased proportionally with increasing roughness for DC cultured in PS inserts, but remained stable when DC were cultured in the PEI inserts. Furthermore, the LPS-induced production of CCL3 and CCL4 was not altered by the different inserts regardless of their roughness levels (Fig. 6).

4. Discussion

Biomaterials, which have the ability to replace functional tissues or which support cell growth as well as cell differentiation, have attracted great attention for various biomedical applications [31]. Examples for the development of advanced multifunctional materials are cardiovascular or bone implant materials, which can improve current treatments and may increase the live quality of many patients [6, 39]. Such newly developed materials have to be cytocompatible, especially with cells of the cardiovascular system or the musculoskeletal system, respectively [12, 27].

DC are an immune cell type, which are discussed to play a role in atherosclerotic diseases. They are present in non-diseased arteries and accumulate in atherosclerotic lesions, where they are co-localized with T cells [19]. Therefore, it is of interest how potential vascular implant materials interact with DC. Furthermore, DC play a key role in osteoimmunology and are associated with osteoclastogenesis as well as bone loss [36]. Thus, material-mediated activation of DC should be avoided to minimize possible adverse effects induced by the implants. However, a controlled DC activation or distinct DC phenotypes could support the regeneration of bone tissue [36].

Here, it was shown that DC did not upregulate co-stimulatory molecules when cultured on the PEI and PS inserts. These results indicate that the materials are free of microbial contaminations, since microbial products can activate DC via their TLR, which leads to the upregulation of co-stimulatory molecules [3]. Furthermore, the different roughness levels did not influence the DC activation neither under homeostatic conditions nor under inflammatory conditions simulated by the addition of LPS. However, it was previously reported that different roughness levels of clinical titanium surfaces induced the expression of co-stimulatory molecules on DC [16]. Interestingly, the different roughness levels of the polymeric PEI and PS surfaces, used in this study, showed no influence on the expression of co-stimulatory molecules by DC. Therefore, the combination of the material and roughness levels rather than the roughness levels alone seem to influence the behavior of DC. Hence, a general statement about the influence of material surface roughness on the expression of co-stimulatory molecules by DC can not be made. Other factors such as protein adsorption on different materials surfaces may also influence the activation of DC [35].

Activated DC are relatively short lived *in vitro* but also *in vivo*. The apoptotic cell death of DC is necessary to maintain self tolerance and to protect from autoimmune diseases, because mice with defective DC apoptosis displayed chronic lymphocyte activation and systemic autoimmune pathologies [7]. Viability study performed here showed that the survival of

monocytes-derived DC is not influenced of PEI and PS inserts with the different roughness levels. However, a biomaterial-mediated controlled and prolonged survival or activation of DC could be beneficial for several clinical applications. Biomaterials supporting DC cultures could help to expand tolerogenic DC, which could be used to minimize transplant rejection or to treat autoimmune diseases [33, 42].

Cytokines secreted by DC can guide the differentiation of T cells into distinct subsets such as T_H1, T_H2 or T_H17, but can also support a tolerogenic response [26]. Here, the different inserts did not induce secretion of IL-6, IL-10, IL-12, or TNF-α in DC. However the secretion of IL-17 was significantly induced at similar levels by all materials independently of their different roughness levels. IL-17 is a pro-inflammatory cytokine, which when produced by T cells (T_H17) is associated with several autoimmune diseases, but also with atherosclerosis and vascular inflammations [11, 37]. The levels of DC-derived IL-17 in response to the different inserts are moderate and may not have functional consequences, which should however be tested in future studies.

Treatment with LPS induced the secretion of IL-6, IL-12, IL-17, and TNF-α in DC, which was not altered when the DC were cultured in the different inserts. However, the secretion of IL-10 significantly increased on the PS inserts with the highest roughness level, while the respective PEI inserts showed IL-10 levels similar to TCP. IL-10 can be secreted by various immune cells and is able to suppress inflammatory reactions. For example, IL-10 secreted by B cells is able to suppress autoimmune reaction but also immune reaction against bacterial pathogens [18]. Since DC cultured on PS-RII also produce high levels of pro-inflammatory cytokines, it is not likely that they exert immune suppressive capacities.

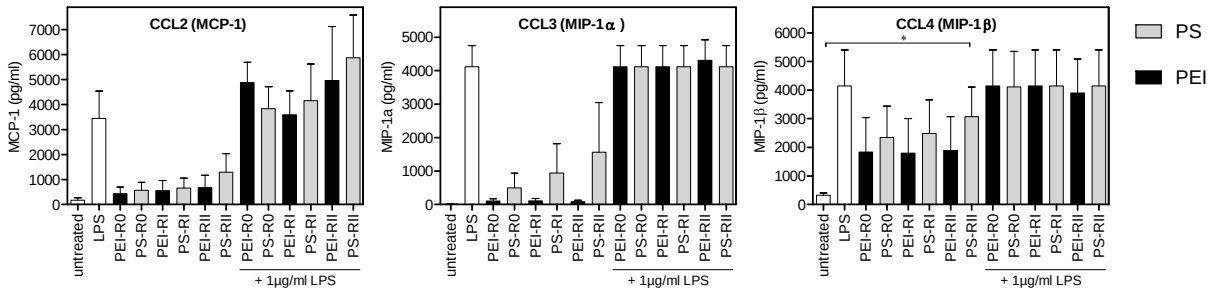

Fig. 6. Cytokine secretion by DC cultured on the different PEI and PS inserts. Monocyte-derived DC were cultured for 24 hours in the different PEI (black bars) and PS (grey bars) inserts or in TCP (white bars), with or without 1 µg/mL LPS. Chemokine secretion was detected using the BioPlex® multiplex system. Average of two independent experiments each with two healthy Donors (n=4) is shown (mean ± SEM). Results were statistically compared using the one-way Anova test for repeated-measures (*for $p<0.05$).

Besides cytokines, also chemokines produced by DC are capable of shaping T cell responses [9]. The expression of CCL2, CCL3, and CCL4 in response to the different materials was determined in this study. While CCL2 was only slightly induced by the different inserts, CCL4 was significantly higher expressed by DC cultured in the different inserts compared to TCP. Interestingly, the expression of CCL3 was only induced by the PS inserts and increased with increasing surface roughness, supporting the assumption that not only the roughness levels but also the material intrinsic properties influence the phenotype of DC. The signals and corresponding receptors, which induce this chemokine secretion as well as the functional consequences, remain to be elucidated in future studies. Recently, material-induced chemokine expression was described showing that different polymetacrylates can induce CCL2 [15]. However, in the same study the DC also showed an enhanced expression of co-stimulatory and pro-inflammatory cytokines in response to the materials, which could not be observed here. In contrast to the data described here, on clinical titanium surfaces in which the roughness was increased by sand blasting and acid etching, DC produced lower levels of CCL3 compared to the smooth titanium surface [16]. Therefore, the signals inducing chemokine expression by DC seem to be poorly understood and should be intensively characterized. The importance of chemokine secretion by DC is highlighted by a study showing that DC-derived CCL2 could induce a T_H2 polarization, since CCL2 deficient mice are unable to mount a T_H2 response, but show a stronger T_H1 response, which leads to a higher resistance against a *Leishmania major* infection [10]. How DC cultured on the different PEI and PS inserts influence the subsequent T cell response will be investigated in future studies.

5. Conclusion

Conclusively, the data presented in the study show that DC can be cultured in PEI and PS inserts with different micro-patterned surface roughness levels without influencing their viability. An effect of the different roughness levels could only be observed for chemokine secretion but not for the expression of co-stimulatory molecules and inflammatory cytokines, indicating that particular receptors, which are sensitive to material surfaces, can regulate the DC phenotype. The LPS-induced inflammatory cytokine response and the expression of co-stimulatory molecules by DC, which was induced by LPS, were not altered by the different inserts. The inserts system presented here can be used to systematically tailor surface characteristics and to analyze cell specific response as demonstrated here for DC.

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