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Cellular response of blood-borne immune cells to PEEU fiber meshes

Janita A. Maring[1#], Matthias Becker[4#], Wing Tai Tung[1,2#], Christof Stamm[1,4,5,6*], Nan Ma[1, 3*], Andreas Lendlein[1,2,3*]

1 Institute of Active Polymers and Berlin-Brandenburg Centre for Regenerative Therapies, Helmholtz-Zentrum Hereon, Teltow, Germany

2 Institute of Biochemistry and Biology, University of Potsdam, Potsdam, Germany

3 Institute of Chemistry and Biochemistry, Free University of Berlin, Berlin, Germany

4 Deutsches Herzzentrum Berlin, Berlin, Germany

5 German Centre for Cardiovascular Research, Partner Site Berlin, Berlin, Germany

6 Berlin Institute of Health Center for Regenerative Therapies, Charité – Universitätsmedizin Berlin, Berlin, Germany

* To whom correspondence should be addressed: Prof. Dr. Christof Stamm, Prof. Dr. Nan Ma, Prof. Dr. Andreas Lendlein, Helmholtz-Zentrum Hereon, 14513 Teltow, Kantstraße 55, Germany
Email: christof.stamm@hereon.de; nan.ma@hereon.de; andreas.lendlein@hereon.de

# These authors contributed equally to this work.

Abstract

Background: Polymeric materials have been widely used as artificial grafts in cardiovascular applications. These polymeric implants can elicit a detrimental innate and adaptive immune response after interacting with peripheral blood. A surface modification with components from extracellular matrices (ECM) may minimize the activation of immune cells from peripheral blood. The aim of this study is to compare the cellular response of blood-born immune cells to the fiber meshes from polyesteretherurethane (PEEUm) and PEEUm with ECM coating (PEEUm+E). **Materials and Methods:** Electrospun PEEUm were used as-is or coated with human cardiac ECM. Different immune cells were isolated form human peripheral blood. Cytokine release profile from naïve and activated monocytes was assessed. Macrophage polarization and T cell proliferation, as indication of immune response were evaluated. **Results:** There was no increase in cytokine release (IL-6, TNF-α, and IL-10) from activated monocytes, macrophages and mononuclear cells on PEEUm; neither upon culturing on PEEUm+E. Naïve monocytes showed increased levels of IL-6 and TNF-α, which were not present on PEEUm+E. There was no difference on monocyte derived macrophage polarization towards pro-inflammatory M1 or anti-inflammatory M2 on PEEUm and PEEUm+E. Moreover, T cell proliferation was not increased upon interacting with PEEUm directly. **Conclusion:** As PEEUm only elicits a minimal response from naïve monocytes but not from monocytes, peripheral blood mononuclear cells (PBMCs) or T cells, the slight improvement in response to PEEUm+E might not justify the additional effort of coating with a human ECM.

Keywords: immune response; cardiovascular; polymer; ECM; cytokine

Introduction

Polymeric biomaterials hold great promise as implantable cardiovascular devices, given their versatile multifunctionality as well as the high standard of fabrication ensuring quality required for medical applications [1]. For those reasons, synthetic polymer materials have occupied the cardiovascular implant landscape including vascular grafts and cardiovascular stents. There are many requirements, which polymeric materials have to fulfil as cardiovascular graft [2,3]. Among those requirements, their immunological compatibility plays a central role [3]. The immunological response, particular the innate immune response, not only has a strong involvement in unwanted thrombosis and inflammatory response, but ultimately dictates the outcome of implants [4]. Any triggered immune response to vascular grafts by the blood born immune cells, such as monocytes, macrophages or T cells, has the potential of further activating clotting cascade and thrombosis [5]. Therefore, it is important to extensively examine the immune response of these blood born immune cells to the polymeric biomaterials before their intended clinical application.

There are different cellular components interacting with peripheral blood contacting polymer implants, including neutrophils, monocytes and other innate immune cells, which are responsible for cytokine release and mobilization of other immune cells [6]. Among those cells, monocytes are classified as classic monocytes ($CD14^{++}/CD16^{-}$), intermediate monocytes ($CD14^{++}/CD16^{+}$) and non-classical monocytes ($CD14^{DIM}/CD16^{++}$) [7]. These monocytes differ in their cytokine secretion profile, differentiation potential and functionality [7]. Classical and intermediate monocytes secrete higher levels of TNF-α, with the classic monocytes also secreting higher IL-6 and IL-1β [8]. Further, macrophage derived from monocyte precursors undergo specific differentiation depending on the local tissue environment. M1 macrophage, which is referred to as ‘classically activated’ phenotype, modulates pro-inflammatory response by producing reactive oxygen species (ROS), secreting hydrolytic enzymes and promoting helper T cells response [9]. A prolonged M1 presence in response to implants leads to a severe foreign body response, granuloma and fibrous encapsulation, resulting in chronic inflammatory events [10,11]. M2 phenotype of macrophages, as ‘alternatively activated’ macrophages, plays a major role in suppression of inflammatory immune reactions by secreting anti-inflammatory cytokines and tissue remodelling, which greatly improves the integration of the implants and enabling it to fulfil their intended function [12]. Monocytes and macrophages can act as antigen presenting cells to T cells, thereby modulating the inflammatory response [13]. Subsequently, CD4 positive helper T cells are the main producers of cytokines in the chronic phase. T cells

have also been proposed to aid in giant cell formation [14] and the presence of T cells increased the amount of adherent cells to polyether urethane [15]. Even though an exaggerated and chronic immune response is detrimental, a mild innate immune response is shown to be beneficial for the regenerative process after implantation [16,17]. In case of a properly regulated response, a transition to the regenerative phase can be promoted by the polarization of M1 to M2 macrophages [16].

Polyesteretherurethanes (PEEU) are multiblock copolymers consisting of poly(ε-caprolactone) (PCL) and poly(p-dioxanone) (PPDO) segments, which are connected through *L*-lysine based diurethane junction units [18]. They are prepared from dihydroxy-terminated telechelics, which are obtained by ring-opening polymerization initiated by a low molecular weight diol [19], and diisocyanates. As thermoplastics they can be processed from the melt or from solution, which is an advantage compared to covalently crosslinked block copolymers [20]. Fibrous scaffold from PEEU are explored for vascular stent applications [21]. By varying the weight ratio between PCL and PPDO segments, the mechanical properties could be tailored for its intended purpose [21]. It was reported PEEU has a strong pro-angiogenesis function [22]. In particular, PEEU with 70 wt% of PPDO can promote more tubing formation of endothelial cells than other PEEU with lower PPDO weight content [23].

The early phase of the foreign body response occurring after device implantation is strongly influenced by the biomaterial surface forming the interface to the biological environment. In this context the hydrophobicity of the device surface plays a role in the immune response [13]. Also surface charges at a substrate surface can activate the signalling cascade of the immune system influencing the balance between immunological and regeneration processes [13,24]. One possibility to modulate the initial response to synthetic implant surface, is masking the interface with components of native extracellular matrices (ECM). Native ECM consists of a highly complex macromolecular network of proteins and polysaccharides, which provide structural anchorage points and biochemical cues for surrounding cells [25]. It was reported that the immobilisation of type I collagen facilitates osteoconduction and integration of implants [26]. For the cardiovascular implants, coating of substrate with ECM component obtained from the decellularized vascular tissue can reduce the intrinsic immunogenicity and is particularly favorable for the endothelialization [27]. ECM coating on a polymer scaffold surface can combine the benefit of reducing immune response [28], augmenting the initial protein adsorption and promote endothelialization and tissue growth [27]. In this study, we examined the *in vitro* cell response of different human immune cells from human peripheral blood to

electrospun PEEU fiber meshes (PEEUm) and PEEUm with ECM (PEEUm+E) from human cardiac tissue.

Materials and methods

Tissue Source and ECM processing

Left ventricular myocardium was collected from explanted hearts after informed consent from patients who underwent heart transplantation for end-stage dilated cardiomyopathy. The study protocol conformed to the ethical principles outlined in the Declaration of Helsinki. Tissue collection was approved by the Institutional Review Board and ethics committee of Charité—Universitätsmedizin Berlin (EA4/028/12). Human myocardium was harvested under sterile conditions and processed as described before [29]. The final concentration of the ECM hydrogel was set to 8 mg/mL with 1× PBS and stored at 4 °C for a maximum of 24 h before use.

Preparation of electrospun fiber meshes

Polyesteretherurethane (PEEU) with a 30:70 weight ratio between PCL and PPDO segments was obtained as described in [30]. L-lysine diisocyanate was used as to form the diurethane junction units between PCL and PPDO segments. The electrospinning setup (Linari Engineering, Italy) consists of a voltage supply, a syringe pump, and a rotatory drum collector. Electrospinning was performed in a homemade transparent plastic chamber with tubing connected to air source for humidity control. The PEEU solution (11 wt% in HFIP) was filtered with a glass fiber syringe filter of 1 μm pore size before electrospinning. A 19-guage blunt tip needle was connected to the tubing and syringe containing the PEEU solution. The flow rate of PEEU solution supply for electrospinning was 1.77 ml/h. The tip to collector distance was kept at 25 cm, with a voltage supply of 10 – 15 kV and under a humidity of 20% to produce stable electrospinning, and the fibers deposited on the grounded drum collector rotating at 5 rpm for 4 h, forming a sheet of PEEUm.

Coating of PEEUm

PEEUm were cut with sterile scalpel into approximately 0.5 cm x 0.5 cm squares. One PEEUm square was placed into each well of a 48-well plate and held in place by rings cut from silicone tubing (Ismatec, Wertheim, Germany) providing an inner diameter equal to 96-well format. The samples were washed with PBS and, in case of ECM coating, completely coated by adding 150 μL/cm² ECM solution followed by drying for 48 h at 37 °C in a non-humidified incubator. After two gentle washes with PBS, the PEEUm+E were used for experiments.

Mechanical Tensile Testing

Uniaxial tensile tests were performed in a wet state as described [28] under a BOSE testing bench (BOSE ElectroForce® TestBench, TA Instruments, New Castle, DE, USA). Sample length (between the clamps) was set to 1 cm, thickness and width were individually measured using a micrometer (precision: ±0.001 µm) and a caliper (precision: ±0.01 mm) (Mitutoyo Corporation, Kawasaki, Japan). Crosshead speed was set to 0.05 mm/s and pulling up to 0.9 strain. Elastic moduli were calculated as the slope of initial linear region presented on the stress over strain curve. Maximum stress was obtained as the maximum load. The measurements on fiber meshes was performed till the strain reached 110%.

Scanning Electron Microscopy (SEM)

Sample preparation was performed as described [28]. Briefly, samples were washed twice in PBS and fixed with 2.5% grade I glutaraldehyde (Sigma-Aldrich) at room temperature followed by drying dehydration using rising Ethanol (Carl-Roth) concentrations. Ethanol was removed by two incubations in hexamethyldisilazane (Sigma-Aldrich) and samples were air-dried overnight under a fume hood followed by gold sputter coating (JFC-1200 Fine Coater; JEOL, Tokyo, Japan). Finally, samples were imaged using the JCM 6000 benchtop SEM (JEOL) in high vacuum mode at 10 kV. Fiber diameter was determined by using Image J software (NIH, USA) on the SEM images.

Cell isolation and culture

Human immune cells (monocytes, macrophages and peripheral blood mononuclear cells (PBMCs)) were cultured in VLE RPMI 1640 Medium (Biochrom), 10% hAB Serum (Sigma-Aldrich) 100 U/mL penicillin, 100 µg/mL streptomycin and 2 mM L-Glutamine (Gibco). PMBCs were isolated from Buffy Coat (bought from Deutsches Rotes Kreuz with approval by the Institutional Review Board and ethics committee of Charité—Universitätsmedizin Berlin; EA1/372/16) using Biocoll (Biochrom) density gradient. Monocytes were isolated from PMBCs using the $CD14^+$ Magnetic Cell Separation kit (Miltenyi Biotec, Bergisch Gladbach, Germany). Macrophages were differentiated from isolated monocytes by a 7 days incubation in culture medium with 50 ng/mL M-CSF (Miltenyi Biotec) and collected with a cell scraper.

Monocytes–Cytokine Secretion

To determine cytokine secretion, 100,000 monocytes isolated from PBMCs by Ficoll density gradient medium centrifuge were cultured onto PEEUm and PEEUm+E and incubated for 24 h at 37 °C. For the positive control, medium was supplemented with 200 ng/mL Lipopolysaccharide (LPS). Supernatants were collected and cytokine secretion was detected

using Human ELISA MAX™ Deluxe Kits (Thermo-Fisher) for IL-6, IL-10 and TNF-α following the manufacturer's protocol.

Macrophage Polarization

To investigate macrophage polarization, 100,000 macrophages were cultured for 48 h on PEEUm, PEEUm+E or cell culture dishes. To induce polarization towards M1, the culture medium was supplemented with 20 ng/mL INF-γ +100 ng/mL LPS. For M2a polarization, 20 ng/mL IL-4 and for M2c 20 ng/mL IL-10 was added. After 2 days of culture, supernatants were collected for detection of cytokine secretion via ELISA using Human ELISA MAX™ Deluxe Kits (Thermo-Fisher) for IL-6, IL-10 and TNF-α following the manufacturer's protocol. Cells were harvested using Accutase (Innovative Cell Technologies, San Diego, CA, USA) and analyzed for polarization marker expression by flow cytometry analysis using described staining protocol [28]. All groups were normalized to M0.

PBMCs Cytokine Secretion and T cells Proliferation

Determination of T cells proliferation was performed using the CFSE labelling procedure from the Total Cytotoxicity & Apoptosis Detection Kit (Biomol, Hamburg, Germany). Briefly, PBMCs (provided by Karen Bieback; Heidelberg) were adjusted to 1×10^7 cells in 1 mL assay buffer, washed twice in assay buffer and a labelled 200 µL CFSE working solution was added to 1.8 mL cell suspension in assay buffer. The labelled suspension was incubated for 15 min at room temperature. The reaction was stopped by adding medium followed by two washing steps with assay buffer. Finally, cells were suspended in the medium and 150,000 PBMCs were cultured on PEEUm and PEEUm+E for 5 days. The positive control medium was supplemented with 5 µg/mL Phytohaemagglutinin (PHA). Subsequently, cells were harvested and analysed for proliferation of $CD3^+$, $CD3CD4^+$ and $CD3CD8^+$ T cells proliferation by flow cytometry using described staining protocol [28]. Supernatants were collected and cytokine secretion was detected using Human ELISA MAX™ Deluxe Kits (Thermo-Fisher) for IL-6, IL-10 and TNF-α following the manufacturer's protocol.

Statistics

Data are shown as mean ± SD. Comparisons passed normality and equal variance testing before the significance was tested. Differences between more than two groups were determined by one-way ANOVA with Bonferroni *t*-test for multiple comparisons. For two-group comparisons, a two-tailed Student's t-test was performed if the normality test was passed. The Mann-Whitney test was performed if non-normality was detected. Changes over time were tested by two-way

ANOVA with Bonferroni's correction. GraphPad Prism v. 5.03 (GraphPad, La Jolla, CA, USA) was used for data analysis and plotting. A p-value of $p < 0.05$ was considered significant.

Results

Characterisation of PEEUm and PEEUm+E

PEEU with a PCL to PPDO weight ratio of 30:70 was selected as the substrate material [18]. PEEUm is an elastic fiber mesh with an elongation at break around 330% and maximum stress of 2.8 MPa at room temperature [18]. After the ECM coating process, a layer of ECM with thickness around 3 μm masked the fiber morphology of PEEU fiber meshes, as shown in Figure 1a & 1b. There was no significant difference in fiber mesh elastic modulus between PEEUm and PEEUm+E (Table 1). The values determined by tensile tests characterize the macroscopic elastic properties, which could be related to handling during surgery and the blood pressure on the fiber mesh stent after implantation as vascular graft. Table 1 contains the data, which were determined for the stress at 110% elongation. This result indicates that the maximum stress as well as the elongation at break will be higher.

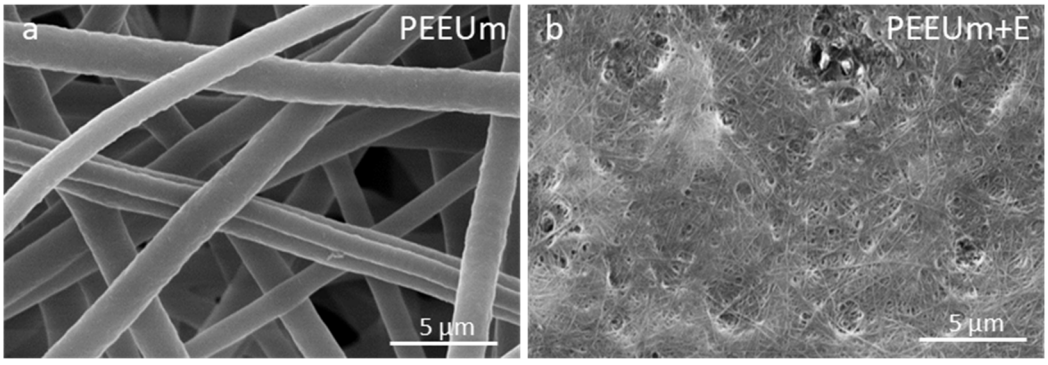

Figure 1. Morphological characterisation of (a) PEEUm and (b) PEEUm+E by SEM.

Table 1. Mechanical properties determined by tensile tests at room temperature and fiber diameter of PEEUm and PEEUm+E

	E-modulus (MPa)	Stress at ε = 110% (MPa)	Fiber diameter (μm)
PEEUm	3.99 ± 0.45	1.51 ± 0.18	1.49 ± 0.40
PEEUm+E	4.03 ± 0.33	1.54 ± 0.13	N.A.

Cytokine secretion of naïve monocytes

In order to examine the innate immune response to PEEUm, we investigated reaction of monocytes in terms of cytokine secretion profile to both PEEUm and PEEUm+E. There was no significant difference in the levels of IL-10 (Figure 2C) from naïve monocytes. There was also no significant difference in the secretion of IL-6 (Figure 2D), TNF-α (Figure 2E) or IL-10

(Figure 2F) from the activated monocytes on PEEUm or PEEUm+E. An increased secretion of IL-6 and TNF-α from naïve monocytes was observed, when cultured on PEEUm alone compared to PEEUm+E (Figure 2A and 2B).

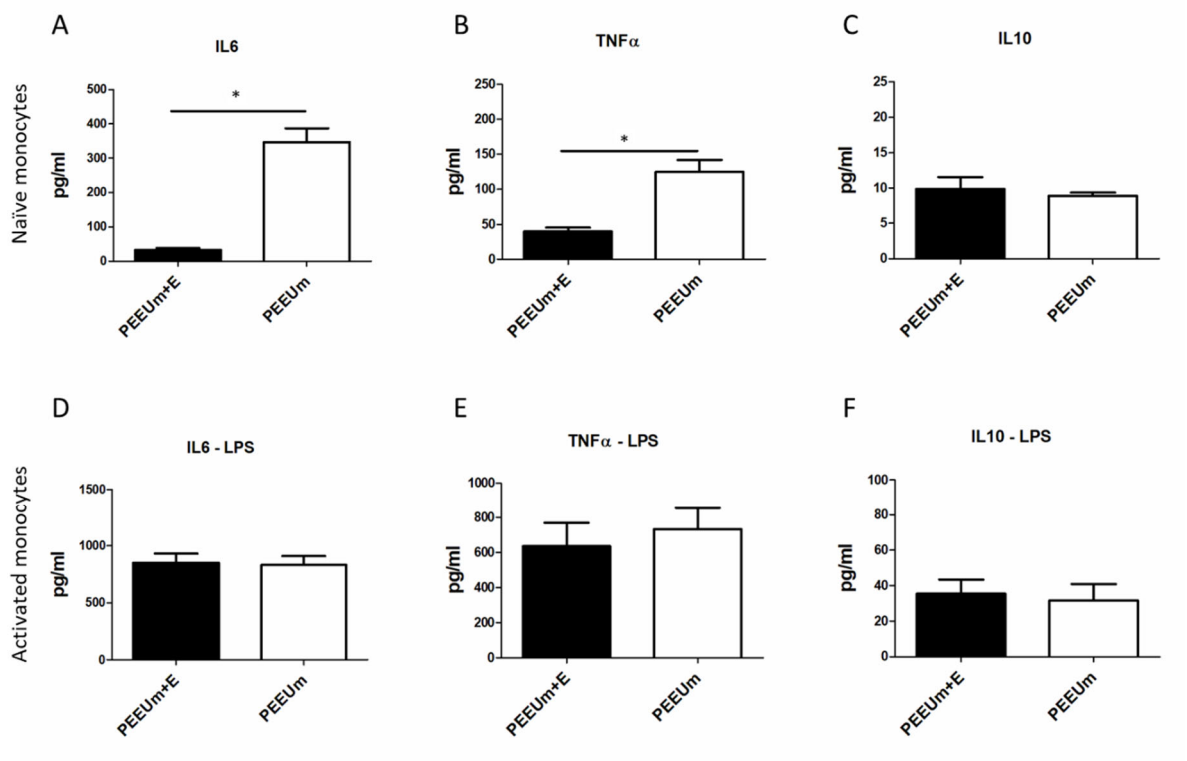

Figure 2. Secretion of cytokines from naïve (A-C) and activated (D-F) monocytes. Secretion of IL-6 (A) and TNF-α (B) by naïve monocytes was increased on PEEUm alone, not on PEEUm+E. (C) IL-10 secretion shows no difference. Activated monocytes displayed no difference in secretion of IL-6 (D), TNF-α (E), or IL-10 (F).

Macrophage polarization

The differentiation of monocytes to M1 or M2 macrophages greatly influences the overall inflammatory response towards polymeric implants and subsequently influences the regenerative process. Therefore, we compared the polarisation of macrophage cultured on PEEUm and PEEUm+E as well as the cytokine secretion. Upregulation of human leukocyte antigen DR isotype (HLA-DR) (Figure 3A) and CD80 (Figure 3B) was seen in M1 polarisation controls, but not in the macrophages cultured on PEEUm or PEEUm+E. Moreover, there was no upregulation of CD206 (Figure 3C) and CD163 (Figure 3D) expression when cultured on PEEUm or PEEUm+E, indicating that the tested polymeric substrate did not induce polarisation of macrophages towards either M2a or M2c phenotype, respectively.

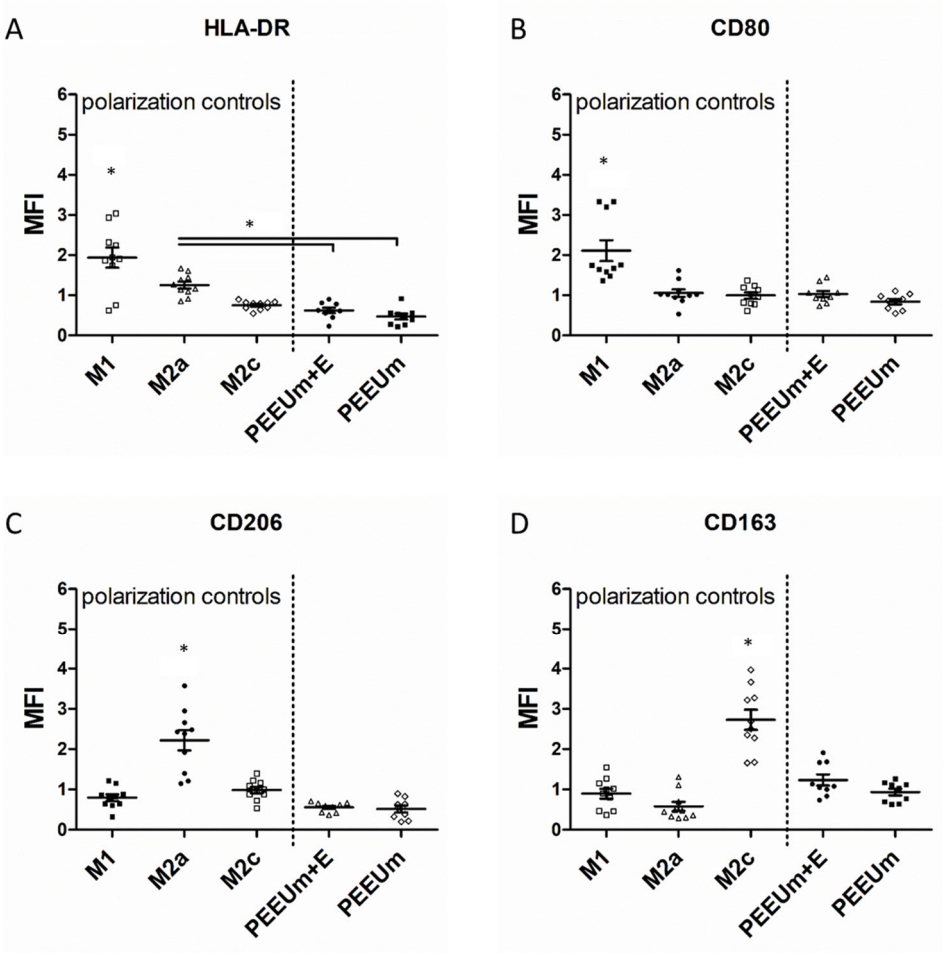

Figure 3. Flow cytometry analysis of macrophage polarisation on PEEUm and PEEUm+E. A) HLA-DR was increased on M1 and slightly on M2a macrophages, no increase when cultured on PEEUm or PEEUm+E. B) CD80 was increased on M1 postive control, M1 polarisation was found on PEEUm and PEEUm+E cells. C) CD206 expression was increased on M2a control, not on macrophages from PEEUm or PEEUm+E. D) An increased CD163 was seen only in M2c positive controls and not in PEEUm or PEEUm+E groups.

Next, the cytokine secretion of macrophages cultured on PEEUm and PEEUm+E was investigated. While the increased secretion of IL6 (Figure 4A), TNF-α (Figure 4B) and IL10 (Figure 4C) was found in the M1 polarisation control, no upregulation was detected in macrophages on PEEUm or PEEUm+E. Altogether, these results demonstrated that PEEUm+E did not affect macrophage polarisation towards an M1 nor M2 phenotype.

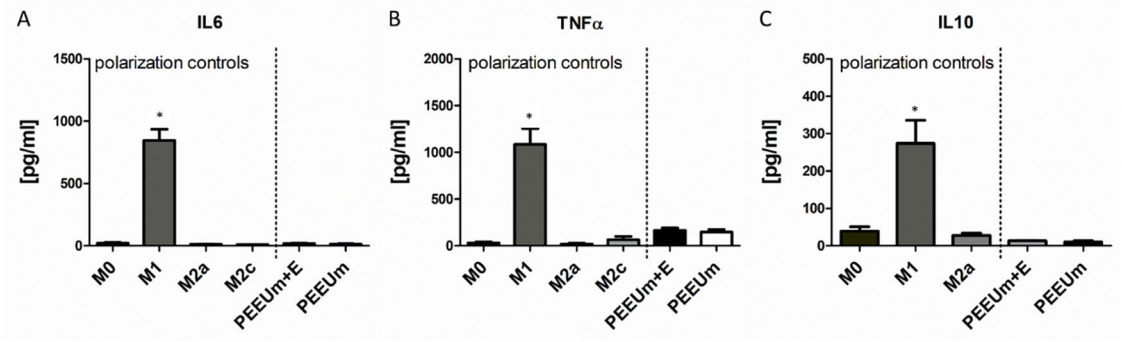

Figure 4. Cytokine secretion of macrophages on PEEU. A) IL-6 level was increased in M1 control only. B) TNF-α secretion was increased in M1 controls, not in macrophages cultured on PEEUm or PEEUm+E. C) IL-10 secretion was not affected by culture on PEEUm or PEEUm+E.

PBMCs cytokine secretion

In order to study the effect of PEEUm and PEEUm+E on other immune cells, we studied the cytokine secretion of PBMCs. PBMCs refer to any peripheral blood cell having a round nucleus, consisting of T cells, B cells, NK cells and monocytes [31]. PHA is a most frequently used mitogen of PBMCs, which can elicit a strong cytokine release [32]. Activation of PBMCs with PHA as positive control, resulted in an increased secretion of IL-6, which was not detected on either PEEUm or PEEUm+E (Figure 5A). The level of TNF-α secretion was also not affected by culture on PEEUm and was only slightly increased in the presence of PHA (Figure 5B). PHA, as the positive control, also induced IL-10 release from PBMCs. There was a similar and low level of IL-10 secretion from PBMCs on both PEEUm and PEEUm+E (Figure 5C). Thus, there was no stimulation effect on PBMCs' cytokine secretion of both PEEUm and PEEUm+E.

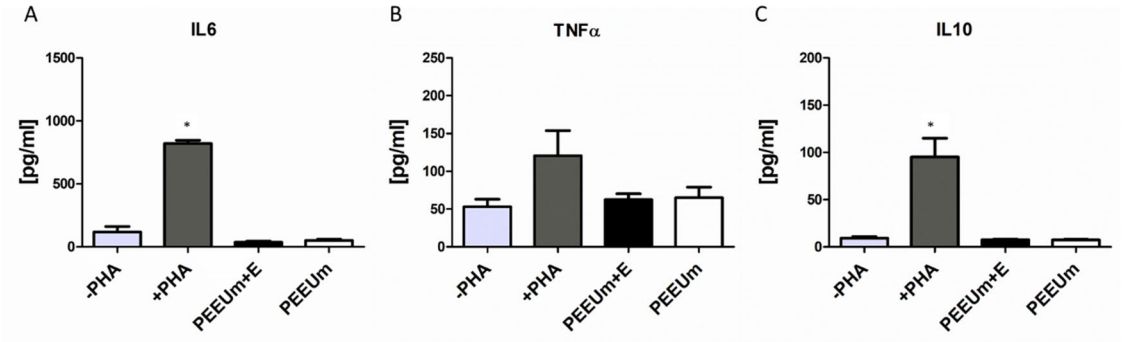

Figure 5 PBMCs cytokine secretion on PEEUm and PEEUm+E. A) IL-6 secretion was not increased on PEEUm or PEEUm+E. B) TNF-α secretion was not affected. C) IL-10 secretion was not increased upon culture on PEEUm or PEEUm+E.

T cells proliferation

The interaction of T cells with macrophage plays a central role in chronic inflammatory response towards polymeric biomaterials [33]. In order to investigate T cell proliferation on PEEUm or PEEUm+E, $CD3^+$ T cells were isolated from PBMCs and cultured on PEEUm or PEEUm+E. Flow cytometry analysis of $CD4^+$ T cell subset showed no detectable proliferation on PEEUm, while the positive control with PHA activation did indeed show remarkable T cells proliferation (Figure 6A). Similarly, there is no detectable activation of $CD8^+$ T cells upon culture on PEEUm or PEEUm+E (Figure 6B). This shows that PEEU does not induce proliferation of $CD4^+$ and $CD8^+$ T cells.

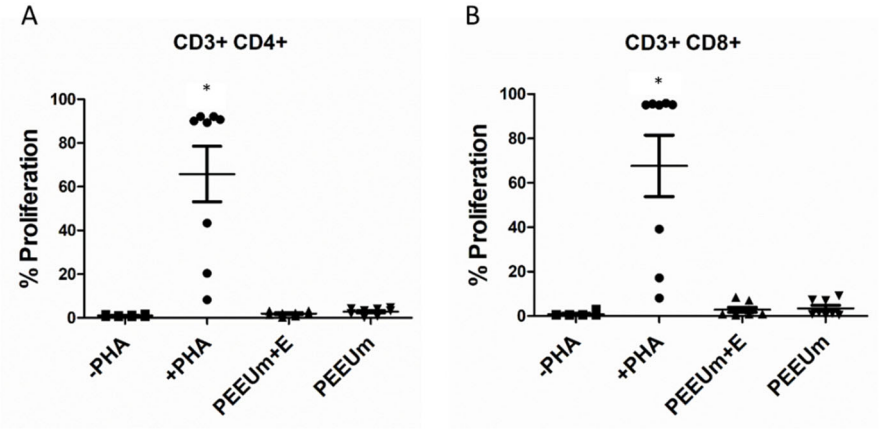

Figure 6 T cells proliferation on PEEUm or PEEUm+E. A) $CD4^+$ T cells did not show increased proliferation on PEEUm or PEEUm+E. B) Proliferation of $CD8^+$ T cells was not increased by PEEUm or PEEUm+E.

Discussion

The induction of adverse immune reactions from cardiovascular implants may lead to excessive inflammation, thrombosis, and impairment of healing [2,3]. Therefore, it is important to design and fabricate a polymeric substrate eliciting a controllable inflammatory-immune response. To advance toward this goal, we have performed an *in vitro* immunogenic assessment with different types of immune cells present in human PBMCs in response to PEEUm and PEEUm+E. Since using PEEU alone presents a fully synthetic surface to the biological environment, this may pose a risk by inducing a pro-inflammatory response. Therefore we studied in parallel whether equipping the substrate surface with components from human ECM

could modulate the biological response since ECM is involved in a plethora of biological processes.

Peripheral blood is directly in contact with a cardiovascular implant upon implantation in the recipient [34]. In the peripheral blood, PBMCs including monocytes are the primary cells of the innate immune system to interact with the cardiovascular implants. Upon contact with the PEEU material, we found that naïve monocytes increased the secretion of IL-6 and TNF-α. Interestingly, this increase was completely abolished when the PEEUm was coated with ECM. These results indicate that the surface of PEEU substrate may elicit the secretion of some pro-inflammatory cytokines from naïve monocytes, but not all pro-inflammatory cytokines. The potential reason may be due to the fact that ECM treatment could mask the surface and influence protein adsorption [35], which could consequently influence the secretion of naïve monocytes.

Despite of this increased secretion of two cytokines by naïve monocytes in response to PEEUm, the secretion profile from PBMCs to PEEUm was not altered with and without coating. The possible explanation for the discrepancy is that PBMCs encompass a heterogeneous cell population comprising lymphocytes, monocyte and dendritic cells [36,37], which are critical components of both the innate and adaptive immune system. Among all the cell population in PBMCs, the frequency of monocyte in peripheral blood is only 5-10 percent [38,39]. The main function of naïve monocyte in PBMCs is not to produce pro-inflammatory cytokines but to coordinate the repair of a damaged endothelium [40]. The small differences in the secretion profile of naïve monocyte did not lead to the alternation of secretion of total cell population in PBMS. The level of IL-6, TNF-α and IL-10 from PBMCs is a general indicator of the material compatibility and integration process of implants [41]. Therefore, the PEEUm fulfilled the general requirement of PBMCs material compatibility.

Previous studies have shown that PCL fiber meshes induced a pro-inflammatory reaction in macrophages when randomly aligned thicker fibers increased macrophage infiltration and activation [42,43]. Similar results were observed for PLA membranes, which induced macrophage proliferation and inflammation *in vitro* and *in vivo* [44]. Here, we did not observe any indication of macrophage polarisation towards either M1 or M2, based on cytokine release or cell surface markers. These results demonstrated that electrospun PEEUm did not induce an acute inflammatory response in macrophages.

An adaptive immune response is initiated when an antigen-presenting cell (APC), such as macrophage, provides both antigen and co-stimulatory molecules (e.g.CD28) to a naive T cell. Further, the activation of macrophages attracts T cells to the site of implantation, which

expanded locally [45]. Therefore, T cells are mainly responsible for the adaptive immune response, maintaining a chronic inflammation. T cells response to a polymeric substrate is an important factor for its immunocompatibility. Smith et al. showed that polymers with different blends ratio of PPDO and elastin (ELAS), from 100:0 to 0:100, are immunosuppressive for T cells proliferation, except PPDO by itself (100:0) did not exert this effect [46]. In our study, when T cells were cultured upon PEEUm or PEEUm+E, no proliferation of $CD4^+$ or $CD8^+$ T cells was observed, suggesting their good integration potential as cardiovascular implants.

Conclusion

Altogether, our results show that PEEUm only elicits a minimum immune response in vitro from naïve monocytes, while both PEEUm and PEEUm+E do not elicit an immune response *in vitro* from monocytes, macrophages, PBMCs or T cells. A longterm *in vivo* study should be performed before a definite conclusion regarding the immunocompatibility can be made.

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