Biomaterials 33 (2012) 4136–4146

Contents lists available at SciVerse ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials

Inducing healing-like human primary macrophage phenotypes by 3D hydrogel coated nanofibres

Matthias Bartneck [a,*,1], Karl-Heinz Heffels [b,c,1], Yu Pan [d], Manfred Bovi [e], Gabriele Zwadlo-Klarwasser [a,f,2], Jürgen Groll [c,**,2]

[a] Interdisciplinary Center for Clinical Research, Medical Faculty, RWTH Aachen, Pauwelsstr. 30, 52074 Aachen, Germany
[b] DWI e.V. and Institute of Technical and Macromolecular Chemistry, RWTH Aachen, Forckenbeckstr. 50, 52056 Aachen, Germany
[c] Department of Functional Materials in Medicine and Dentistry, University Hospital Würzburg, Pleicherwall 2, 97070 Würzburg, Germany
[d] Biomedical Engineering, Biointerface Laboratory, RWTH Aachen, Pauwelsstr. 30, 52074 Aachen, Germany
[e] Electron Microscopic Facility, Medical Faculty, RWTH Aachen, Pauwelsstr. 30, 52074 Aachen, Germany
[f] Department of Dermatology, Medical Faculty, RWTH Aachen, Pauwelsstr. 30, 52074 Aachen, Germany

ARTICLE INFO

Article history:
Received 4 January 2012
Accepted 27 February 2012
Available online 13 March 2012

Keywords:
Monocyte
Macrophage
Cytokine
Angiogenesis
SEM

ABSTRACT

Immune cells are present in the blood and in resident tissues, and the nature of their reaction towards biomaterials is decisive for materials success or failure. Macrophages may for example be classically activated to trigger inflammation (M1), or alternatively activated which supports healing and vascularisation (M2). Here, we have generated 3D nanofibrous meshes in different porosities and precisely controlled surface chemistries comprising PLGA, hydrogel-coated protein repellant and protein repellant endowed with the bioactive peptide sequences GRGDS or GLF. We also prepared 2D substrates with corresponding surface chemistry for a systematic evaluation of primary human macrophage adhesion, migration, transcriptome expression, cytokine release and surface marker expression. Our data show that material morphology is a powerful means in biomaterial design to influence immune cell response. Flat substrates lead to an increased number of M2 classified $CD163^{+}$ macrophages. However, these M2 cells released large amounts of pro-inflammatory cytokines. In contrast, 3D nanofibres with corresponding surface chemistry yielded M1 classified $27E10^{+}$ macrophages with a significantly increased release of pro-angiogenic chemokines and angiogenesis related molecules and a strong decrease of pro-inflammatory cytokines. We thus suggest that, for macrophages in contact with biomaterials, cytokine release is taken as main criterion instead of surface-markers for macrophage classifications.

1. Introduction

The success of implants in living tissues strongly depends on the response of the immune system. For classical biomaterials, prevention of foreign body response and passive acceptance by the body have for long been regarded as criteria for biocompatibility. However, with the rise of tissue engineering, three-dimensional (3D) scaffold structures and increasingly complex surface bioactivation have tremendously gained importance. With the aim to regenerate tissue, critical size defects and ultimately organs, passive integration and eventually fibrous capsule formation is no longer acceptable, and active effects of materials such as support of angiogenesis are increasingly important. It has recently been reported that three-dimensional cell culture scaffolds significantly affect cell behaviour, for example, 3D PLGA meshes were shown to improve the differentiation and function of stem cell-derived hepatocytes compared to monolayer culture controls [1]. However, the role of 3D material morphology and the effects of bioactivating motifs on the response of human innate immune cells are still largely unknown.

Macrophages are the most important cellular component of the innate immune system and are almost omnipresent in the body. The long term response to foreign materials, hence biomaterial acceptance, strongly depends on macrophage response, and the surface chemistry of materials is classically believed to be decisive [2]. Topographical properties of microstructures have however recently been found to fundamentally affect macrophage response as well [3,4]. Human macrophage subsets are classically identified by their expression of function-associated antigens such as 27E10 that is expressed by pro-inflammatory macrophages (M1) as well

* Corresponding author. Tel.: +49 2418089198; fax: +49 2418080124.
** Corresponding author.
E-mail address: mbartneck@ukaachen.de (M. Bartneck).
[1] Both authors contributed equally to the work.
[2] Shared senior authors.

0142-9612/$ – see front matter © 2012 Elsevier Ltd. All rights reserved.
doi:10.1016/j.biomaterials.2012.02.050

as by monocytes [5]. M1 express enzymes to degrade the extracellular matrix (ECM) [6,7] as well as polymeric scaffolds [8]. Alternatively activated macrophages (M2) support healing processes and remodelling of the ECM [7,9] and their presence is generally associated with good implant healing [10]. Important markers that are used to characterise human M2 subsets are scavenger receptors such as CD163 and stabilin-1. CD163 codes for a receptor that scavenges haemoglobin whereas Stabilin-1 is a homeostatic scavenger receptor which expression depends on the presence of glucocorticoids [11]. M2 may also express the factor XIIIA (FXIIIA) that catalyses cross-linking of the major fibroblast-associated glycoprotein fibronectin [12]. The scavenger receptor with collagenous structure (MARCO) is involved in the binding of unopsonised environmental particles [13] and also limits inflammation [14]. There are several different sub-types of co-existing M1 and M2 [7] and furthermore, it has been shown that under pathological conditions, macrophages can exhibit both M1 and M2 profiles at the same time [15].

The sensitivity of cells towards their local environment demands scaffold designs for tissue engineering that optimally mimic the natural extracellular matrix (ECM). Electrospun nanofibres have gained great interest during the last decade especially as substrates for both tissue engineering and regenerative medicine [16,17]. Biodegradable poly esters such as poly ε-caprolacton, poly lactide, and poly(D,L-lactide-co-glycolide) (PLGA) represent a major class of materials widely used in tissue engineering [18] and are US Food and Drug Administration (FDA) approved for human therapy [19]. Poly(ethylene oxide) (PEO), another FDA approved polymer [18], is known to prevent unspecific protein adsorption as well as cell adhesion and is largely used for biomaterials surface modification in combination with functionalization possibilities to tune a specific interaction with proteins or cells. We have reported the use of isocyanate functional star shaped molecules based on PEO (NCO-sP(EO-*stat*-PO)) as coating system to create dense and covalently attached PEO layers on biomaterials with the possibility to functionalise these layers with bioactive molecules such as peptides, proteins or aptamers [20]. Recently, we showed the transfer of this system to 3D biodegradable nanofibrous meshes based on PLGA with corresponding control over surface chemistry via NCO-sP(EO-*stat*-PO) as functional additive for electrospinning [21].

Here, we present a systematic and detailed study using NCO-sP(EO-*stat*-PO) surface modified PLGA fibres as well as flat substrates with and without covalently bound peptide sequences to study the adhesion, migration, surface antigen expression, and cytokine release of primary human macrophages. We discuss the effects of changing material morphology with a constant sP(EO-*stat*-PO)-based surface chemistry, compare these PLGA/sP(EO-*stat*-PO) fibres to pure PLGA fibres and investigate for the effects of coupling bioactive molecules to both flat 2D and 3D substrates on the response of macrophages. Gene expression profiling is currently one of the most efficient methods to gather new information in unexplored fields of research. Therefore, the expression of 28,869 human genes was studied to examine the roles of a 3D material morphology compared to flat structures with identical surface chemistry. In addition to the characterization of macrophage function associated surface marker expression studies, eleven different cytokines as well as myeloperoxidase (MPO) were quantified using a multiplex screening system. Additionally, the release of the soluble CD163 receptor was monitored by enzyme-linked immunosorbent assay (ELISA) (R&D systems, Minneapolis, MN, USA). Our results show stronger effects of morphology on macrophage behaviour than surface chemistry, and the data suggest that cytokine release should be regarded as major criterion for immune cell response to biomaterials rather than surface antigen markers.

2. Materials and methods

Unless overwise stated all chemicals were purchased from Sigma–Aldrich, Taufkirchen, Germany.

2.1. Electrospinning of nanofibres

2.1.1. PLGA solution

PLGA (RG 504, Boehringer Ingelheim, Germany) was dissolved in a mixture of 90/10 (v/v) acetone and dimethyl sulfoxide (DMSO) at 25 wt% under mechanical stirring.

2.1.2. NCO-sP(EO-stat-PO)/PLGA solution

NCO-sP(EO-*stat*-PO) was dissolved in a mixture of 90/10 (v/v) acetone and DMSO at 5 wt% under mechanical stirring. 25 wt% PLGA (RG 504, Boehringer Ingelheim, Germany) was added to the prepolymer solution and mixed with a magnetic stirrer.

2.1.3. NCO-sP(EO-stat-PO)/PLGA/peptide solution

The peptide sequences GRGDS or GLF (Bachem AG, Bubendorf, Switzerland) were dissolved in water at 10 wt% and diluted with a five-fold amount of DMSO. The dispersion was added to NCO-sP(EO-*stat*-PO) and stirred for 10 min. Finally, acetone was added resulting in a solvent mixture of 90/10/2 (v/v/v) of acetone – DMSO – water. Dispersions were immediately used for electrospinning.

2.1.4. Spinning parameters

Polymer solutions were fed at 1.0 mL per hour to a flat-tip stainless steel spinneret connected to a high-voltage power supply. A KNH34 high voltage generator (Eltex, Freiburg, Germany) was utilised to charge the solutions at 20 kV while the collector remained grounded. The fibres were collected on coated glass cover slips in a distance of 180 mm.

2.2. Surface preparation

Glass cover slips with a diameter of 15 mm were activated using UV/O_3-treatment and silanised immediately. The cover slips were placed into a mixture of 60 mL dry toluene and 0.2 mL N-[3-(trimethoxysilyl)-propyl]-ethylene diamine for 2 h, followed by washing with dry toluene and storage in dry toluene. The silanised cover slips were dried in a N_2-stream and immediately used for spin coating with a solution of 1 wt% NCO-sP(EO-*stat*-PO) in water and tetrahydrofuran (THF) 90/10 (v/v). NCO-sP(EO-*stat*-PO) coated glass cover slips were fixed on a conducting surface and used as targets for electrospinning as described earlier in detail [22].

2.3. Calculation of fibre density on substrates

Besides parameters such as molecular composition and functionality the reactivity of electrospun fibres as well as their influence on cells is determined by the amount of reactive groups and hence also depending on the amount of fibres. Quantitative results from ELISA, fluorescence measurements and degradation will only be comparable with the corresponding fibre surface or mass. We investigated electrospun fibres on coated glass substrates due to the easier processing and quantification in comparison to a non woven mesh. With the help of scanning electron microscopy (SEM), pictures of thin spun fibres were taken and the fibre diameter was determined, according to recent studies [23]. For further details, see supporting section 1.

2.4. Contact angle measurement

The contact angle at an interface is determined by the equilibration of the present interface clamping. There is a proportional relation between the contact angle and the hydrophobicity of a material. The sessile drop method was performed to determine the contact angle. Droplets of water (2 μL) were deposited on the surfaces and the contour of the droplets was captured by a digital camera. Contact angles were calculated from three independent experiments.

2.5. Endotoxin testing

The detection of endotoxin contaminations in material samples is required to exclude the activation of macrophages by bacterial products in particular by the endotoxin LPS. To this effect the materials were incubated for 24 h in phosphate-buffered saline (PBS) and the content of endotoxin was measured using a chromogenic limulus amebocyte lysate (LAL) test (Lonza, Walkersville, MD, USA).

2.6. Generation of autologous human serum

To obtain serum from venous whole blood samples, autologous serum was generated by 1 h clotting of blood at 37 °C and collected after 10 min of centrifugation at 2000 g. Heat inactivation was performed for 60 min at 57 °C and filtration

was done before using (0.2 μm). Serum was used directly or kept at 4 °C for up to two weeks.

2.7. Isolation and purity control of human blood cells

2.7.1. Isolation of peripheral blood mononuclear cells

Cells were isolated from healthy volunteers (the local ethics commission gave ethical approval for this study, and informed consent was obtained from all participants). To isolate peripheral blood mononuclear cells (PBMC) from heparanised venous whole blood of healthy volunteers or purchased single donor buffy coats (Institute for Transfusion Medicine, University Hospital Aachen, Germany), these were diluted in an identical volume of PBS and density gradient centrifugation using Ficoll Paque (Pharmacia, Erlangen, Germany) was performed (40 min, 900 g, no brake). Thrombocytes were removed by washing with PBS without calcium or magnesium.

2.7.2. Isolation of monocytes

Monocytes were isolated from PBMC using the Dynal® Monocyte Negative Isolation Kit (Invitrogen, Carlsbad, CA), according to the manufacturer instructions. The kit depletes non-monocytic cells (T cells, B cells, NK cells, dendritic cells, erythrocytes, granulocytes and macrophages) and results in a purity of 98%.

2.7.3. Generation of monocyte-derived macrophages

In vitro, macrophages can be derived from blood by culturing monocytes with serum containing medium [24]. Therefore, monocytes were cultured for seven days in bacterial grade petri dishes in medium supplemented with 5% autologous serum. To obtain alternatively activated macrophages, monocytes were stimulated with 10^{-7} M Dexamethasone (Dex) or with 20 ng/mL recombinant human interleukin 4 (IL4) after culture start for seven days of culture. Stimulation with 1 μg/mL lipopolysaccharide (LPS) was performed 24 h before harvesting the cells using a silicone rubber.

2.7.4. Analysis of cell population purity

The purity of all immune cell populations was assessed using the DiffQuik kit.

2.8. Scanning electron microscopy and time lapse microscopy

Scanning electron microscopy (SEM) was used for the investigation of the cell and fibre morphology. SEM images of gold sputtered samples using an SCD 500 (Leica Microsystems, Vienna, Austria) were taken with an ESEM XL 30 FEG (FEI, Eindhoven, The Netherlands) in high vacuum mode. For the time lapse studies, a DMI6000B microscope equipped with a DFC 360FX camera was used (Leica Microsystems, Vienna, Austria). To keep the incubation temperature at 37 °C, the Incubation System for Microscopes (Tokai Hit, Fujinomiya-shi, Japan) was used. To analyse movies, the LAS AF software was used (Leica Microsystems, Vienna, Austria).

2.9. Migration and fibre degradation studies

Cells were defined as rapidly migrating in time lapse studies when they moved a distance of more than 120 μm per hour. To compare the effects of hydrolysis to the cell-mediated degradation of nanofibres, the fibres were incubated in RPMI1640 medium containing 5% human serum in the absence or presence of macrophages. The degree of fibre degradation was defined by the area covered with fibres based on the same equation used above to calculate fibre density.

2.10. Flow cytometry and fluorescence microscopy

After seven days of culture, macrophages were detached by putting the plates for 20 min at 4 °C and scraping of the cells using a silicone rubber. For fluorescence microscopy, PBS including magnesium and calcium was used (to keep cells attached). Otherwise conditions were identical to flow cytometry. To stain cell nuclei, incubation of cells with 10 μg 4′,6-diamidino-2-phenylindole (DAPI) per mL PBS was performed for 15 min and excess dye was removed by washing three times with PBS.

2.11. Cytokine and protein detection

On the high-throughput scale, cytokines can be quantified using multiplex bead assays, microparticles that are labelled with cytokine directed antibodies with different sizes and fluorescent labels [25]. The release of 11 cytokines (CCL2, CCL3, CCL4, CSF-3, CXCL8, CXCL9, IL1β, IL6, IL10, IL12p70, and TNFα) as well as of MPO into the culture medium was measured using the FlowCytomix® system (Bender Medsystems, Vienna, Austria). A commercial ELISA was performed to quantify the level of soluble CD163 in cell culture supernatant (R&D systems, Minneapolis, MN, USA).

2.12. RNA isolation, microarray analysis and real-time PCR

RNA was isolated using the RNeasy Mini Kit (QIAGEN, Hilden, Germany). The GeneChip® Human Gene 1.0 ST Array was used according to the instructions of the manufacturer. For Real-Time PCR analysis, RNA was reverse transcribed into cDNA using the High Capacity RNA to cDNA Kit (Applied Biosystems, California, USA). Real-Time PCR was done using a 7300 Real Time PCR System (Applied Biosystems, California, USA). To generate data, (CT) values were generated automatically and relative quantification was related to the endogenous control gene beta-action which was found to be the most suitable house-keeping gene. Gene expression was considered up or down-regulated if the $\log_2$ values between the reference and the sample were higher than one (greater than twofold increase) or lower than one (greater than twofold decrease), respectively.

2.13. Analysis of gene expression groups and hierarchical clustering

The internet database DAVID was used to characterise gene groups [26]. All gene ontology (GO) terms were used to classify the genes, using medium classification stringency and an enrichment score of one or larger. Hierarchical clustering analysis (HCA) was performed using average linkage as agglomeration rule and Euclidean distance. The clustering data are presented as dendrogram, a colour code corresponds to the expression of each gene: black colour indicates that the transcript of the corresponding gene is not regulated, green colouring reflects that a gene is down-regulated and red colour indicates up-regulation [27].

2.14. Statistical analysis

Statistical analysis was performed using Graph Pad Prism 4.0. One-way analysis of variance with Bonferroni's post test was performed to test significance of data. p values below 0.05 were considered as statistically significant.

3. Results and discussion

Hydrogels based on sP(EO-*stat*-PO) have been shown to minimise protein adsorption and are considered as cell-repulsive for numerous cell lines, human primary cells and progenitor cells [20]. The attachment of many cell types to these materials requires a modification of the material with adhesion signals such as ECM derived peptide sequences. With the lately achieved transfer of this coating system to 3D nanofibres it is now possible to generate 2D and 3D materials with identical surface chemistry for studies on cellular adhesion, morphology, or inflammatory reactions. The core intention of this study is to compare 2D substrates to 3D mesh-like structures via increasing fibre densities to elucidate the effects on human primary macrophage response. The final aim is to identify criteria that enable to define biomaterial design criteria to direct immune cell reactions.

3.1. Substrate and nanofibre generation

Clinically applied bioresorbable polyester poly(lactid-*co*-glycolid) (PLGA) and the protein adsorption minimizing hydrogel coating sP(EO-*stat*-PO) were chosen as base systems. We generated sP(EO-*stat*-PO)-coated hydrogels and PLGA coated 2D substrates as well as 3D nanofibre networks with corresponding surface chemistry as described before [20]. Two different types of fibres and three levels of covering density resulting in 2D structures and 3D networks with different porosity were prepared for systematic comparison. The sP(EO-*stat*-PO) based hydrogel coatings were hydrophilic with contact angles (CA) $<40°$ whereas PLGA coated substrates were more hydrophobic exhibiting a CA of $>70°$. Both tendencies were pronounced on three dimensional fibrous structures. While PLGA fibre meshes were hydrophobic and exhibited a contact angle of 120°, sP(EO-*stat*-PO) surface modified PLGA fibres were too hydrophilic for CA determination, with water droplets sinking into the mesh within seconds after deposition. The morphology of these scaffolds was characterised based on the coverage with fibres as described before [23].

The mean coverage of expected fibres in the plane of their network was defined as $\bar{c}$ (see supporting section 1 for details). Nomenclature of the substrates was performed according to the mean coverage: plain sP(EO-*stat*-PO) coated substrates, substrates

with immobilised fibres exhibiting $\bar{c} = 0.15$ corresponding to the lowest coverage with nanofibres, a medium level of coverage $\bar{c} = 0.70$ that can be described as three-dimensional fibres with a medium pore size of 100 μm and those exhibiting a coverage of 1.90, three-dimensional fibres with the highest level of coverage that results in a low pore size of 20 μm. The standard deviation of $\bar{c}$ was below 15% for each substrate.

3.2. Cellular response to material morphology

In contrast to flat PLGA or tissue culture polystyrene (TCPS) plates, we observed that on flat sP(EO-*stat*-PO) substrates monocyte attachment was considerably inhibited reflected by the formation of clusters most pronounced in the first 12 h consisting of up to several hundreds of cells (Fig. 1A,B,H, Supplementary Video 1). After three days, the cell clusters on flat sP(EO-*stat*-PO) started to resolve (Fig. 1C), while the migration of single macrophages (mostly into a single direction) was significantly elevated (Fig. 1I, Supplementary Video 2). On TCPS control material, and similarly, on PLGA coated substrates, monocytes rather migrated circularly, when encountering another cell, shifting left or right (Supplementary Video 3).

Supplementary data related to this article can be found online at doi:10.1016/j.biomaterials.2011.11.087.

Monocyte clustering was reduced in the presence of sP(EO-*stat*-PO) coated nanofibres and the sizes of the cell clusters reflected by the number of cells forming a single cluster decreased with increasing nanofibre covering density (Supplementary Fig. S1). A high density of nanofibres inhibited cluster formation and also caused a significant decrease of the migratory activity of macrophages (Fig. 1D–F, H, and Supplementary Video 2). Interestingly, PLGA-based nanofibres had similar effects like the sP(EO-*stat*-PO) coated fibres on macrophage behaviour. In summary, we observed a delayed attachment and an increased migratory activity of monocytes on the sP(EO-*stat*-PO) coated flat materials in contrast to TCPS and PLGA, whereas all nanofibres significantly reduced monocyte migration. Together with monocyte clustering these results suggest that, in agreement with findings reported earlier for other human primary cell types [20], also monocytes cannot adhere promptly on the sP(EO-*stat*-PO) surface especially in 2D.

Organization of monocytes in cell clusters and the dependence of their migration on material morphology of a biomaterial is a new observation. We explain this behaviour as a reaction of the cells to the non-adhesive nature of the protein repellant sP(EO-*stat*-PO)

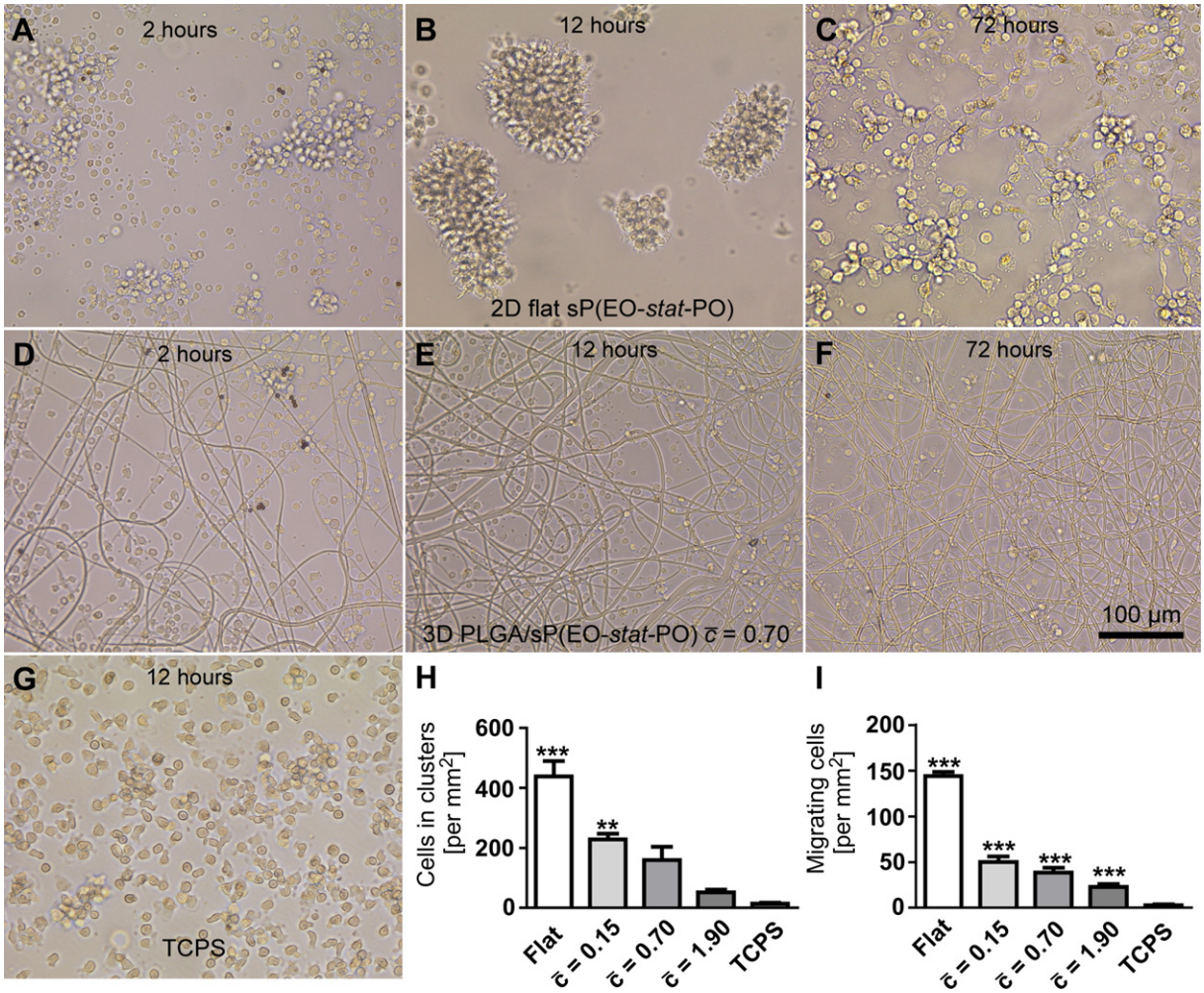

Fig. 1. Monocyte behaviour on protein repellant materials. Monocyte clusters on flat sP(EO-*stat*-PO) coated substrates after 2 (A) and 12 h (B), that start to resolve after approximately 72 h (C). Monocytes on substrates with sP(EO-*stat*-PO) coated nanofibres (D–F), and on tissue culture treated polystyrene (TCPS) (G). Statistical summary of monocyte cluster formation in the first 12 h (H) and migratory activity on 2D sP(EO-*stat*-PO) coated substrates after 72 h (I) as observed in time lapse videos. Significant differences related to TCPS control $^{*}P < 0.05$, $^{**}P < 0.005$, $^{***}P < 0.001$ (one way ANOVA). Data represent mean values ($n = 8$).

surface. With enhanced formation of cell–cell clusters and enhanced migration on 2D substrates, the cells are seeking for possibilities to adhere. Monocyte clustering represents a mechanism of multicellular organization on biomaterials that might also occur *in vivo* as monocytes form cell clusters together with T cells [28].

Macrophages form *in vitro* after the maturation of monocytes as late as after three days. A cellular reaction to implanted biomedical devices *in vivo* might therefore also be inhibited for as long as three days, as only by then macrophages will have developed. Accordingly, after seven days of cell culture, mature macrophages formed dense cell layers on sP(EO-*stat*-PO) coated substrates and the migratory activity was similarly low on all materials (Supplementary Video 4). However, the cellular morphology was strongly affected by the morphology of the material: on flat substrates, macrophages covered a large area and appeared larger as compared to cells attached to nanofibres. Fibres ($\bar{c}$ = 0.15 and 0.7) further induced a cellular alignment, and densely spun fibres ($\bar{c}$ = 1.9) triggered a fusion of the cells with the fibres (Fig. 2B). Human primary fibroblasts, which were studied as control cells, did neither attach to the 2D substrates nor to the 3D nanofibres, not even after seven days of culture (data not shown), in accordance with previous findings [22]. Hence, macrophages show a significantly different adhesion behaviour than other cell types, and the reactions of macrophages to biomaterials can rather be altered by designing different material morphologies than by different surface chemistry.

Supplementary data related to this article can be found online at doi:10.1016/j.biomaterials.2011.11.087.

3.3. Nanofibre degradation

Since macrophages are capable of degrading foreign body material [29] we compared macrophage mediated degradation to cell free hydrolytic degradation of PLGA and PLGA/sP(EO-*stat*-PO) fibres under physiological conditions in culture medium. We found that all fibres were rapidly degraded by macrophages within a few days up to a week, and that PLGA fibres were much more rapidly degraded by macrophages than PLGA/sP(EO-*stat*-PO) fibres (Fig. 3). In contrast, the cell free hydrolytic cleavage of ester linkages in the polymer backbone

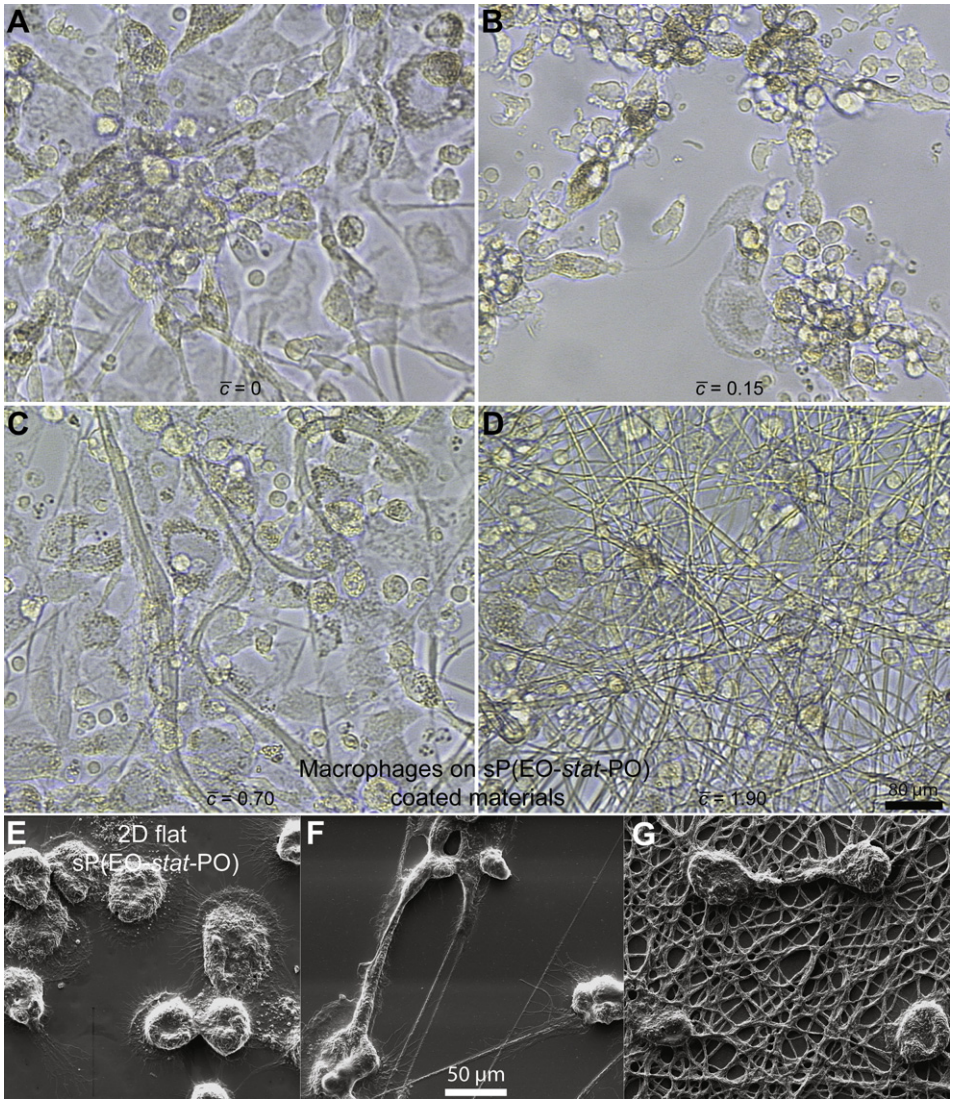

Fig. 2. Macrophage shape alteration by material morphology. Representative micrographs of macrophages cultured on 2D sP(EO-stat-PO) coated substrates (A, E), on substrates with additional covering densities with sP(EO-*stat*-PO) coated nanofibres (B–D,F,G) using optical microscopy (A–D) and scanning electron microscopy (E–G).

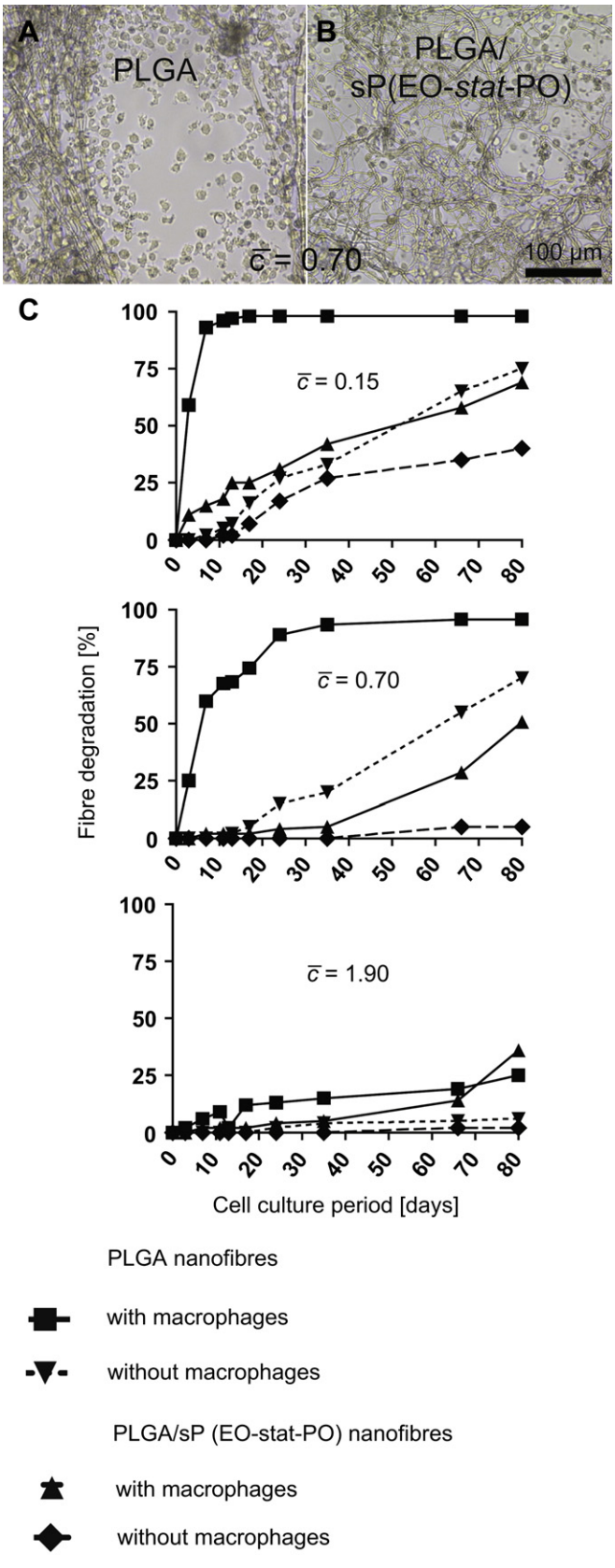

Fig. 3. Degradation of nanofibres by macrophages. Representative micrographs of macrophages cultured on PLGA (A) or PLGA/sP(EO-*stat*-PO) (B) based nanofibres after seven days of culture. PLGA fibres were more rapidly degraded that PLGA/sP(EO-*stat*-PO)-based fibres (C).

of PLGA [30] proceeds much slower over several weeks and is thus of minor relevance in the presence of macrophages.

3.4. Molecular response to material morphology

The long term immune response of the human body to biomaterials and therefore the outcome of the inflammatory reaction is determined by macrophages. To study the effects of material morphology on macrophage phenotype reflected by the expression of function-associated surface antigens, we examined the surface expression of 27E10 (M1, pro-inflammatory) and of CD163 (M2, anti-inflammatory). Fluorescence microscopic analysis showed that the macrophages that were attached to the flat 2D sP(EO-*stat*-PO)-coated substrates expressed CD163 (Fig. 4A) but those attached to 3D PLGA/sP(EO-*stat*-PO)-based nanofibres strongly expressed 27E10 (Fig. 4B). Flow cytometry revealed that the 2D flat sP(EO-*stat*-PO) hydrogels led to a significant increase in the number of macrophages expressing CD163, an even larger number of cells than induced by the glucocorticoid Dexamethasone (Dex), an inducer of CD163 expression by M2 cells [31]. Contrarily, 2D flat PLGA substrates stimulated the inflammatory $27E10^{+}$ M1 phenotype (Fig. 4C). Interestingly, substrate attached peptides expected to influence macrophage activation did not alter CD163 and 27E10 expression significantly (Fig. 4C–D).

In contrast to the 2D hydrogel surfaces, nanofibres significantly reduced CD163 expression whereas in parallel, 27E10 expression proportionally increased with nanofibre coverage. Notably, fibres enhanced the number of 27E10 expressing cells more than the positive control LPS, representative flow cytometric scatter plots demonstrate the strong alterations in 27E10 expression (Fig. 4E). Modifications of nanofibres with the peptides RGD and GLF again had, similar to 2D substrates, a negligible effect on macrophage activation with respect to 27E10 and CD163 expression (Supplementary Fig. S2).

These results show that 2D substrates exert opposite effects to 3D fibres with respect to the activation and phenotype of macrophages. The sP(EO-*stat*-PO) coated materials appear to induce alternative activation of macrophages reflected by CD163 expression, thus a phenotype associated with healing properties [32]. We could further show that the additional peptide modifications of materials did not affect the cell response of monocytes and macrophages significantly as anticipated from other studies [20,33], neither attached to fibres nor to sP(EO-*stat*-PO)-coated substrates.

3.5. Release of inflammatory mediators

For further characterization of the macrophage subpopulations on 2D and 3D environments we monitored the release of 11 inflammation relevant cytokines as well as of the inflammation related enzyme myeloperoxidase (MPO) and the anti-inflammatory soluble receptor of CD163, sCD163 [34]. Due to the rapid degradation of pure PLGA fibres in cell culture and the various functionalization possibilities of the more stable PLGA/sP(EO-*stat*-PO) fibres [22] (Fig. 3) we turned our focus on the latter fibre type in the different densities introduced before, immobilised to sP(EO-*stat*-PO) coated substrates.

Analysis of the cytokine release profiles shows that they can be grouped into three classes: those not being affected by substrate morphology such as MPO, the anti-inflammatory sCD163 (Fig. 5), and the cytokines IL10, IL12 (Supplementary Fig. S3), those showing a nonlinear dependency with surface coverage of fibres from 2D to 3D and those clearly being preferentially released either on flat or on 3D substrates.

On 2D flat substrates, $CD163^{+}$ macrophages released pro-inflammatory cytokines, a finding so far only reported for

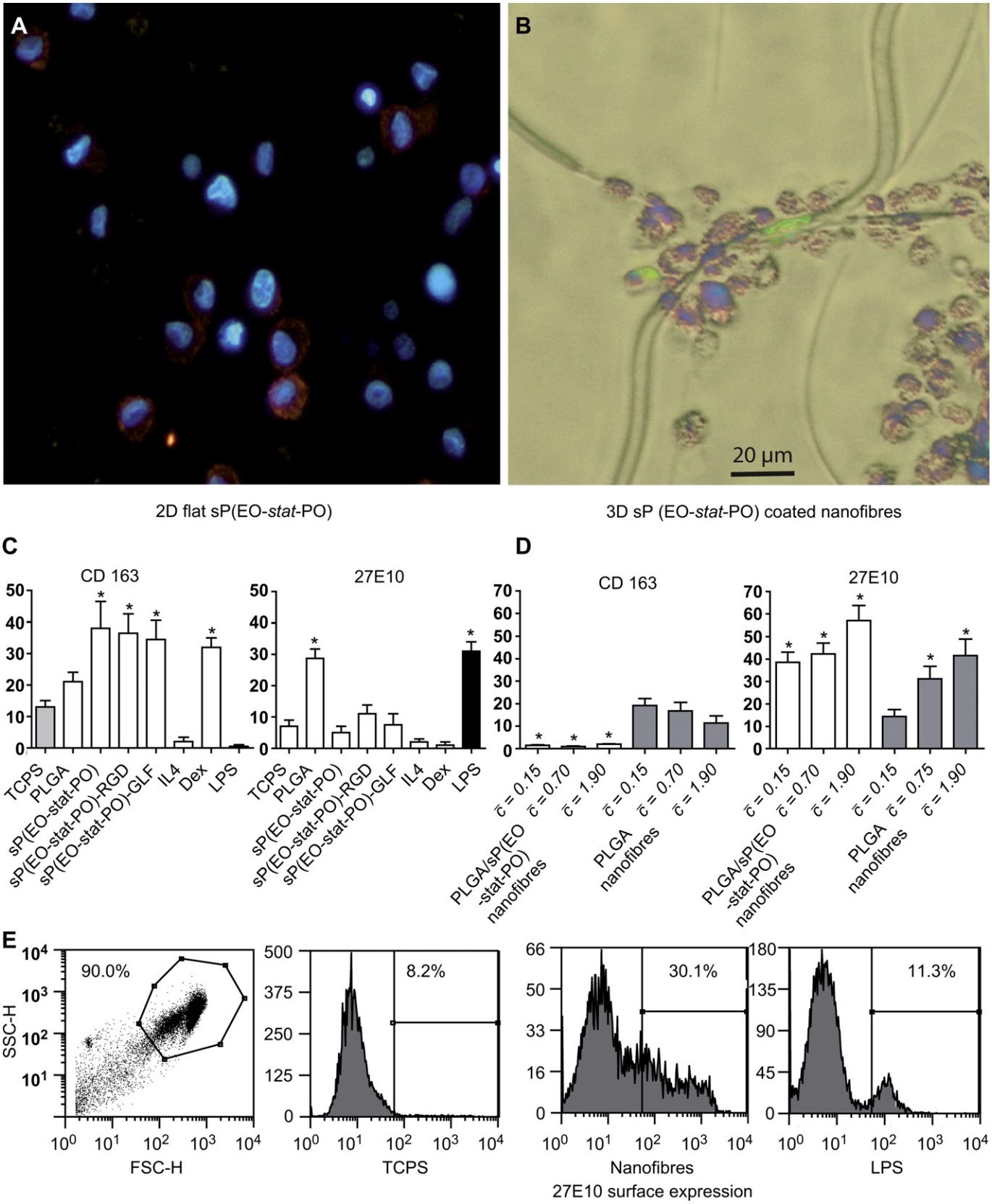

Fig. 4. Material morphology dependent polarization of macrophages. Fluorescence microscopic co-staining of macrophages after culture on 2D sP(EO-*stat*-PO) coated substrates (A) and with additional electrospun nanofibres (B) with CD163 (red), 27E10 (green) and of nuclei using 4′,6-diamidine-2-phenyl indole (blue). Statistical summary of flow cytometric analysis of macrophages after seven days of culture on different substrates and nanofibres including control material (tissue culture polystyrene, TCPS), treatment for M1 (Lipopolysaccharide, LPS) as well as for M2 polarization (Dexamethasone, Dex). Scatterplot of macrophages and histograms of 27E10 expression of macrophages on tissue culture treated polystyrene (TCPS), after culture on 3D nanofibres and after treatment with LPS (E). $^{*}P < 0.05$ (one way ANOVA). Data represent mean values ($n = 4$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

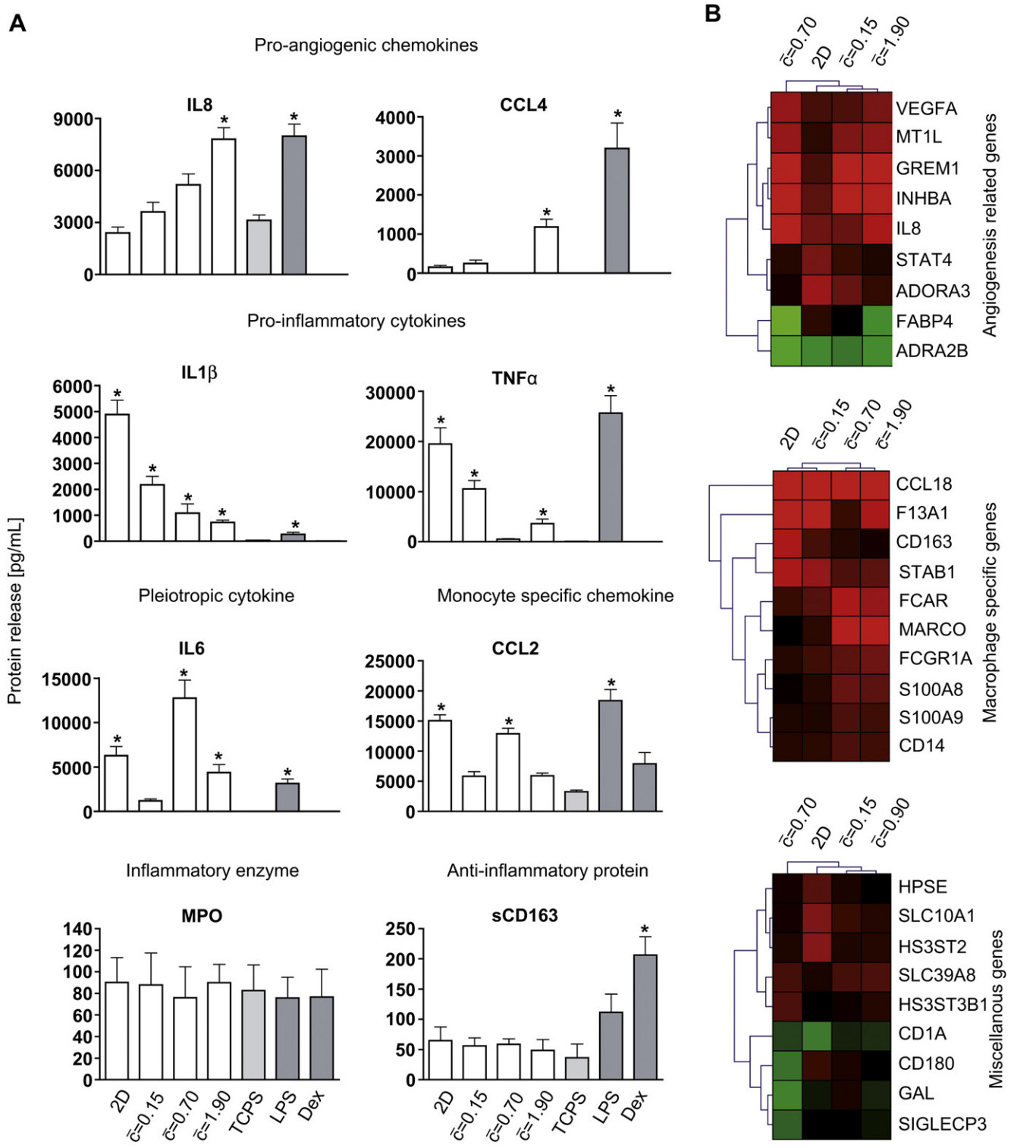

Fig. 5. Macrophage response to 2D and 3D environments. Release of cytokines was strongly altered by material morphology represented by flat 2D sP(EO-*stat*-PO) substrates and by 3D PLGA/sP(EO-*stat*-PO) nanofibres whereas other protein classes were rather unaffected. Tissue culture treated polystyrene (TCPS) was used as control, bacterial products (lipopolysaccharides, LPS) as positive control for cytokine induction, and Dexamethasone (Dex) as control for alternative stimulation (A). Gene expression profiling revealed angiogenesis related genes, macrophage specific and miscellaneous genes to be regulated by material morphology, expression normalized on TCPS (B). $^*P < 0.05$ (one way ANOVA). Data represent mean values ($n = 4$).

macrophages in the human dermis of patients suffering from psoriasis [35]. As the surfaces did not trigger release of soluble CD163, we suggest that this induction of CD163 expression depends on a different molecular mechanism than that of glucocorticoids. This receptor has many different functions: in addition for the scavenging of haemoglobin derivative it acts on the adhesion of monocytes to endothelial cells, it participates in tissue regeneration and functions in tolerance [34]. Elevated release of IL1β and TNFα

due to culture on 2D flat PEO-based hydrogels is in accordance to previous studies [36].

IL8 release increased gradually with nanofibre coverage. Interleukin 8 (IL8, CXCL8) is a potent pro-angiogenic chemokine that up-regulates VEGF mRNA and protein levels in endothelial cells [37]. CCL4 release was specifically induced by the 3D fibres with the highest coverage exhibiting $\bar{c} = 1.90$. In comparison to the flat hydrogel, the release of the pro-inflammatory cytokines IL1β and TNFα was markedly reduced by nanofibres, but the secretion of the pro-angiogenic chemokines IL8 and CCL4 was stimulated. These results clearly indicate that a transformation from 2D to 3D with identical surface chemistry may support the release of pro-angiogenic and reduce that of pro-inflammatory cytokines by macrophages.

As examples for cytokines with nonlinear behaviour, release of the pleiotropic interleukin-6 (IL6) and of the chemokine (C-C motif) ligand 2 (CCL2) were most significantly enhanced by fibres that exhibited $\bar{c} = 0.7$ in comparison to lower and higher fibre density. Also CXCL9, CCL3 and CSF3 showed a similar behaviour (Supplementary Fig. S3). It was shown that CCL2 and IL6 promote the survival of human integrin alpha M (CD11b) expressing monocytes and induce M2-type macrophage polarization [38]. The regulation of various pro-angiogenic cytokines might therefore be related to the three-dimensional surrounding of the cells, and an optimal scaffold density or porosity may maximally induce M2 activation of macrophages and trigger release of anti-inflammatory and pro-angiogenic cytokines.

3.6. Gene expression profiling

To evaluate the comprehensive effects of material morphology on macrophage response, we performed gene expression profiling using microarrays. We found that 475 genes were differentially regulated (more than two-fold change) by 2D versus 3D sP(EO-*stat*-PO) coated materials. Three-dimensional nanofibres exhibiting the highest surface coverage of 1.9 led to the most pronounced effects on macrophage gene expression by up-regulating 212 genes, similar to 210 genes at 0.7 coverage, including several genes involved in angiogenesis (Supplementary Fig. 4A). Interestingly, intermediate fibre coverage of 0.7 led to the largest number of downregulated genes (Supplementary Fig. 4B).

The gene expression data were further subjected to hierarchical clustering analysis and selected genes, according to specific cell functions, are displain as dendrograms (Fig. 5B). The vascular endothelial growth factor (VEGF) - vascular endothelial growth factor receptor (VEGFR) axis probably represents the most important fundament of angiogenesis and transcripts of several genes interacting with VEGF or VEGFR were found to be upregulated by 3D nanofibres, such as several metallothioneins, specifically metallothionein 1L, gremlin-1 (GREM1), an agonist of the vascular endothelial growth factor (VEGFR2) [39]. Metallothioneins are antioxidant proteins with cell-protective effects [40]. Inhibin beta, A (INHBA) is an ECM-inducing gene recently only known to be expressed by fibroblasts during the regulation of wound healing [41]. Even VEGFA was directly altered, it is also known to be expressed by mononuclear cells *in vivo* [39]. These data strongly suggest a pro-angiogenic and inflammation limiting activity of 3D materials (Fig. 5B, top dendrogram).

Several well-known molecules that are used for macrophage classification were regulated by nanofibres (Fig. 5B, intermediate dendrogram). The expression of several different scavenger receptors such as CD163 and STAB-1 appear as false positive indicators for an anti-inflammatory activity as the cells released tremendous amounts of pro-inflammatory cytokines (Fig. 5A). Contrarily, the scavenger receptor MARCO that is known to limit inflammation [14] was induced by 3D fibres (coverage 0.7 and 1.9). Nanofibres further enhanced transcript abundance of two Fc receptors that are termed Fc fragment of IgA (FCAR), Fc fragment of IgG, and high affinity receptor Ia (CD64) (FCGR1A). As Fc receptors are responsible for uptake of pathogens and materials [42] and their expression correlates with phagocytic activity [43], their regulation by nanofibres suggests an involvement into fibre degradation (Fig. 5B, intermediate dendrogram).

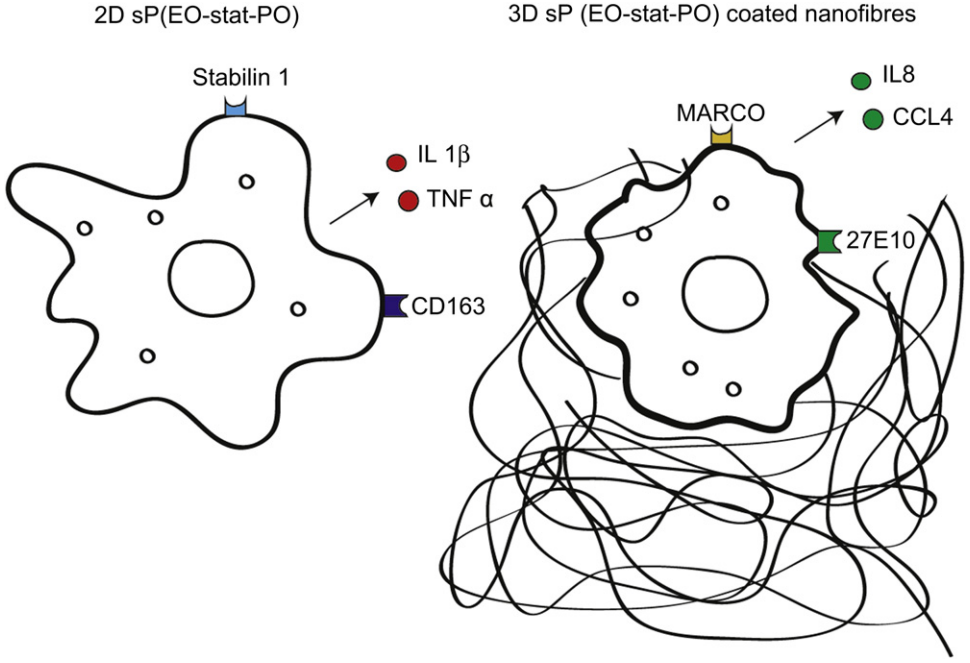

Fig. 6. Macrophage response to 2D and 3D environments. Cartoon reflecting the most important functional differences of macrophages cultured on flat 2D sP(EO-*stat*-PO) substrates or in 3D nanofibres.

Many other interesting genes were regulated by material morphology. Among these were different solute carriers, especially the solute carrier 39A8 (SLC39A8) that is known to be involved into zinc transportation [44] appears to be induced by 3D nanomaterials whereas the opposite is the case for SLC10A1 that was up-regulated by fibres with 0.7 coverage. The regulation of the heparin associated molecules heparanase (HPSE), heparan sulphate sulfotransferase (HS3ST2), and heparan sulphate sulfotransferase (HS3ST3B1) suggest that material morphology might also affect coagulation [45]. Interestingly, galanin (GAL) is expressed by nerve fibres [46] (Fig. 5B, bottom dendrogram).

The expression of selected genes was measured using quantitative Real-Time PCR as an additional technique and confirmed the results from microarray analysis (Supplementary Table S1).

3.7. Comprehensive discussion of macrophage material interaction

General trends and significant main differences in macrophage characteristics on 2D versus 3D substrates are summarised in Fig. 6. Macrophages on 2D sP(EO-*stat*-PO) expressed an anti-inflammatory phenotype although releasing pro-inflammatory cytokines. Contrarily, macrophages cultured on 3D fibres produce pro-angiogenic mediators essential in wound healing, despite their expression of pro-inflammatory M1 markers, with some indication for an ideal porosity that maximally favours release of anti-inflammatory cytokines and pro-angiogenic signals. This is of particular relevance for scaffolds designed for tissue engineering, because macrophages are decisive in the healing process, and are stimulating angiogenesis through the release of angiogenic molecules [47]. Chemokines that are released by macrophages in large amounts were shown to interact directly (CXCL9) [48] or indirectly (IL8) [37] with VEGF and thereby with angiogenesis [48]. The data suggest that also in humans *in vivo* there might be a subset of MARCO expressing macrophages that is active in regenerating and remodelling tissue.

Although our study was purely performed *in vitro*, we believe that our results do possess a high relevance for *in vivo* effects, as it was shown before that primary monocytes and macrophages show very similar behaviour *in vitro* and *in vivo* [49]. Therefore, we are confident that the correlation between substrate morphology and the induction of the release of important angiogenesis related molecules is of utmost importance for biomaterials development.

Furthermore, our data demonstrate that conventional macrophage phenotype classification via surface marker expression may be misleading for biomaterials development. Especially scavenger receptors have a broad variety of biological activities [11,34] and their expression by macrophages should therefore not be the only criterion for a proper assessment of the inflammatory potential of a biomaterial. We have shown that depending on material characteristics, M1 classified macrophages may release anti-inflammatory and pro-angiogenic signals, while M2 classified macrophages may release pro-inflammatory cytokines. As cytokines also act on various other cell types [50] and even tissues [51] we conclude that this criterion is of higher importance for biomaterial fate than direct surface marker expression of macrophages and we suggest to consider cytokine release as the most decisive factor in evaluating the macrophage response to biomaterials.

4. Conclusions

We conclude that in our study with primary human monocytes and macrophages derived thereof, material morphology (2D versus fibrous 3D) has a much stronger effect than alteration of surface chemistry (adhesive versus non-adhesive versus non-adhesive decorated with adhesion mediating (RGD) or specifically macrophage activating (GLF) peptide sequences) on monocyte adhesion and migration as well as on macrophage phenotype and inflammatory mediator profile. These basic but systematic studies help to understand the alteration of attachment and migration of monocytes and link it to the following polarization of macrophages on materials. Our data emphasise that macrophage expression of function-associated surface molecules, inflammatory mediator release, and angiogenic profile significantly differs between 2D and 3D materials. This study further demonstrates that conventional macrophage phenotype classification via surface marker expression may be misleading for judgement of biomaterials, and that cytokine release should rather be used as criterion in this context.

Future macrophage based studies should further elucidate the effects of a three-dimensional surrounding on the response of the cells and the hypothetically impaired relevance of *in vitro* studies that are performed in controlled 3D environments.

Acknowledgements

This study was supported by the DFG research training group “Biointerface” (GRK1035) and the Interdisciplinary Centre for Clinical Research (IZKF Aachen) within the faculty of medicine at the RWTH Aachen.

Appendix. Supplementary material

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.biomaterials.2012.02.050.

References

[1] Li J, Tao R, Wu W, Cao H, Xin J, Li J, et al. 3D PLGA scaffolds improve differentiation and function of bone marrow mesenchymal stem cell-derived hepatocytes. Stem Cells Dev 2010;19(9):1427–36.
[2] Brodbeck WG, Voskerician G, Ziats NP, Nakayama Y, Matsuda T, Anderson JM. In vivo leukocyte cytokine mRNA responses to biomaterials are dependent on surface chemistry. J Biomed Mater Res A 2003;64(2):320–9.
[3] Bartneck M, Schulte VA, Paul NE, Diez M, Lensen MC, Zwadlo-Klarwasser G. Induction of specific macrophage subtypes by defined micro-patterned structures. Acta Biomater 2010;6(10):3864–72.
[4] Paul NE, Skazik C, Harwardt M, Bartneck M, Denecke B, Klee D, et al. Topographical control of human macrophages by a regularly microstructured polyvinylidene fluoride surface. Biomaterials 2008;29(30):4056–64.
[5] Zwadlo G, Schlegel R, Sorg C. A monoclonal antibody to a subset of human monocytes found only in the peripheral blood and inflammatory tissues. J Immunol 1986;137(2):512–8.
[6] Katsuda S, Kaji T. Atherosclerosis and extracellular matrix. J Atheroscler Thromb 2003;10(5):267–74.
[7] Martinez FO, Sica A, Mantovani A, Locati M. Macrophage activation and polarization. Front Biosci 2008;13:453–61.
[8] van Apeldoorn AA, van Manen HJ, Bezemer JM, de Bruijn JD, van Blitterswijk CA, Otto C. Raman imaging of PLGA microsphere degradation inside macrophages. J Am Chem Soc 2004;126(41):13226–7.
[9] Gratchev A, Kzhyshkowska J, Utikal J, Goerdt S. Interleukin-4 and dexamethasone counterregulate extracellular matrix remodelling and phagocytosis in type-2 macrophages. Scand J Immunol 2005;61(1):10–7.
[10] Badylak SF, Valentin JE, Ravindra AK, McCabe GP, Stewart-Akers AM. Macrophage phenotype as a determinant of biologic scaffold remodeling. Tissue Eng Part A 2008;14(11):1835–42.
[11] Kzhyshkowska J, Gratchev A, Goerdt S. Stabilin-1, a homeostatic scavenger receptor with multiple functions. J Cell Mol Med 2006;10(3):635–49.
[12] Keski-Oja J, Mosher DF, Vaheri A. Cross-linking of a major fibroblast surface-associated glycoprotein (fibronectin) catalyzed by blood coagulation factor XIII. Cell 1976;9(1):29–35.
[13] Palecanda A, Paulauskis J, Al-Mutairi E, Imrich A, Qin G, Suzuki H, et al. Role of the scavenger receptor MARCO in alveolar macrophage binding of unopsonized environmental particles. J Exp Med 1999;189(9):1497–506.
[14] Arredouani MS, Franco F, Imrich A, Fedulov A, Lu X, Perkins D, et al. Scavenger receptors SR-AI/II and MARCO limit pulmonary dendritic cell migration and allergic airway inflammation. J Immunol 2007;178(9):5912–20.
[15] Pettersen JS, Fuentes-Duculan J, Suarez-Farinas M, Pierson KC, Pitts-Kiefer A, Fan L, et al. Tumor-associated macrophages in the cutaneous SCC microenvironment are heterogeneously activated. J Invest Dermatol 2011;131(6):1322–30.

[16] Greiner A, WJ H. Electrospinning: a fascinating method for the preparation of ultrathin fibres. Angew Chem Int Ed Engl 2007;46(30):5670–703.
[17] Huang Z-M, Zhang Y-Z, Kotaki M, Ramakrishna S. A review on polymer nanofibres by electrospinning and their applications in nanocomposites. Comp Sci Tech 2003;63(15):2223–53.
[18] Deng M, Uhrich KE. Effects of in vitro degradation on properties of poly(DL-lactide-co-glycolide) pertinent to its biological performance. J Mater Sci Mater Med 2002;13(11):1091–6.
[19] MedWatch safety alerts for human medical products [database on the internet]. U.S. Food and Drug Administration [cited January 4, 2012]. Available from: http://www.fda.gov/Safety/MedWatch/SafetyInformation/default.htm; 2012.
[20] Grafahrend D, Heffels KH, Beer MV, Gasteier P, Moller M, Boehm G, et al. Degradable polyester scaffolds with controlled surface chemistry combining minimal protein adsorption with specific bioactivation. Nat Mater 2011;10(1):67–73.
[21] Kleinman HK, Philp D, Hoffman MP. Role of the extracellular matrix in morphogenesis. Curr Opin Biotechnol 2003;14(5):526–32.
[22] Grafahrend D, Calvet JL, Klinkhammer K, Salber J, Dalton PD, Moller M, et al. Control of protein adsorption on functionalized electrospun fibers. Biotechnol Bioeng 2008;101(3):609–21.
[23] Eichhorn SJ, Aampson WW. Statistical geometry of pores and statistics of porous nanofibrous assemblies. J R Soc Interface 2005;2(4):309–18.
[24] Traber MG, Kayden HJ. Low density lipoprotein receptor activity in human monocyte-derived macrophages and its relation to atheromatous lesions. Proc Natl Acad Sci U S A 1980;77(9):5466–70.
[25] Fu Q, Zhu J, Van Eyk JE. Comparison of multiplex immunoassay platforms. Clin Chem 2010;56(2):314–8.
[26] Dennis Jr G, Sherman BT, Hosack DA, Yang J, Gao W, Lane HC, et al. DAVID: database for annotation, visualization, and integrated discovery. Genome Biol 2003;4(5):P3.
[27] Sturn A, Quackenbush J, Trajanoski Z. Genesis: cluster analysis of microarray data. Bioinformatics 2002;18(1):207–8.
[28] Green DM, Trial J, Birdsall HH. TNF-alpha released by comigrating monocytes promotes transendothelial migration of activated lymphocytes. J Immunol 1998;161(5):2481–9.
[29] Su SH, Nguyen KT, Satasiya P, Greilich PE, Tang L, Eberhart RC. Curcumin impregnation improves the mechanical properties and reduces the inflammatory response associated with poly(L-lactic acid) fiber. J Biomater Sci Polym Ed 2005;16(3):353–70.
[30] Park TG. Degradation of poly(lactic-co-glycolic acid) microspheres: effect of copolymer composition. Biomaterials 1995;16(15):1123–30.
[31] Hogger P, Dreier J, Droste A, Buck F, Sorg C. Identification of the integral membrane protein RM3/1 on human monocytes as a glucocorticoid-inducible member of the scavenger receptor cysteine-rich family (CD163). J Immunol 1998;161(4):1883–90.
[32] Pechkovsky DV, Prasse A, Kollert F, Engel KM, Dentler J, Luttmann W, et al. Alternatively activated alveolar macrophages in pulmonary fibrosis-mediator production and intracellular signal transduction. Clin Immunol 2010;137(1):89–101.
[33] Williams DF. The role of short synthetic adhesion peptides in regenerative medicine; the debate. Biomaterials 2011;32(18):4195–7.
[34] Onofre G, Kolackova M, Jankovicova K, Krejsek J. Scavenger receptor CD163 and its biological functions. Acta Med (Hradec Kralove) 2009;52(2):57–61.
[35] Fuentes-Duculan J, Suarez-Farinas M, Zaba LC, Nograles KE, Pierson KC, Mitsui H, et al. A subpopulation of CD163-positive macrophages is classically activated in psoriasis. J Invest Dermatol 2010;130(10):2412–22.
[36] Schmidt DR, Kao WJ. Monocyte activation in response to polyethylene glycol hydrogels grafted with RGD and PHSRN separated by interpositional spacers of various lengths. J Biomed Mater Res A 2007;83(3):617–25.
[37] Martin D, Galisteo R, Gutkind JS. CXCL8/IL8 stimulates vascular endothelial growth factor (VEGF) expression and the autocrine activation of VEGFR2 in endothelial cells by activating NFkappaB through the CBM (Carma3/Bcl10/Malt1) complex. J Biol Chem 2009;284(10):6038–42.
[38] Roca H, Varsos ZS, Sud S, Craig MJ, Ying C, Pienta KJ. CCL2 and interleukin-6 promote survival of human CD11b+ peripheral blood mononuclear cells and induce M2-type macrophage polarization. J Biol Chem 2009;284(49):34342–54.
[39] Mitola S, Ravelli C, Moroni E, Salvi V, Leali D, Ballmer-Hofer K, et al. Gremlin is a novel agonist of the major proangiogenic receptor VEGFR2. Blood 2010;116(18):3677–80.
[40] Zbinden S, Wang J, Adenika R, Schmidt M, Tilan JU, Najafi AH, et al. Metallothionein enhances angiogenesis and arteriogenesis by modulating smooth muscle cell and macrophage function. Arterioscler Thromb Vasc Biol 2010;30(3):477–82.
[41] Seifert O, Bayat A, Geffers R, Dienus K, Buer J, Lofgren S, et al. Identification of unique gene expression patterns within different lesional sites of keloids. Wound Repair Regen 2008;16(2):254–65.
[42] Mori T, Murakami M, Okumura M, Kadosawa T, Uede T, Fujinaga T. Mechanism of macrophage activation by chitin derivatives. J Vet Med Sci 2005;67(1):51–6.
[43] Leidi M, Gotti E, Bologna L, Miranda E, Rimoldi M, Sica A, et al. M2 macrophages phagocytose rituximab-opsonized leukemic targets more efficiently than m1 cells in vitro. J Immunol 2009;182(7):4415–22.
[44] Wang B, Schneider SN, Dragin N, Girijashanker K, Dalton TP, He L, et al. Enhanced cadmium-induced testicular necrosis and renal proximal tubule damage caused by gene-dose increase in a Slc39a8-transgenic mouse line. Am J Physiol Cell Physiol 2007;292(4):C1523–35.
[45] Liu J, Pedersen LC. Anticoagulant heparan sulfate: structural specificity and biosynthesis. Appl Microbiol Biotechnol 2007;74(2):263–72.
[46] Gai WP, Blumbergs PC, Geffen LB, Blessing WW. Galanin-containing fibers innervate substance P-containing neurons in the pedunculopontine tegmental nucleus in humans. Brain Res 1993;618(1):135–41.
[47] Rosenkilde MM, Schwartz TW. The chemokine system – a major regulator of angiogenesis in health and disease. Apmis 2004;112(7–8):481–95.
[48] Sahin H, Borkham-Kamphorst E, Kuppe C, Moreno Zaldivar M, Grouls C, Nellen A, et al. The chemokine Cxcl9 attenuates liver fibrosis-associated angiogenesis in mice. Hepatology, doi: 10.1002/hep.25545, in press.
[49] Schwarzer E, De Matteis F, Giribaldi G, Ulliers D, Valente E, Arese P. Hemozoin stability and dormant induction of heme oxygenase in hemozoin-fed human monocytes. Mol Biochem Parasitol 1999;100(1):61–72.
[50] Zimmermann HW, Seidler S, Nattermann J, Gassler N, Hellerbrand C, Zernecke A, et al. Functional contribution of elevated circulating and hepatic non-classical CD14CD16 monocytes to inflammation and human liver fibrosis. PLoS One 2010;5(6):e11049.
[51] Mullarky IK, Szaba FM, Berggren KN, Kummer LW, Wilhelm LB, Parent MA, et al. Tumor necrosis factor alpha and gamma interferon, but not hemorrhage or pathogen burden, dictate levels of protective fibrin deposition during infection. Infect Immun 2006;74(2):1181–8.