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Citation for published version (APA):

Bonito, V., de Kort, B., Bouten, C., & Smits, A. I. P. M. (2019). Cyclic Strain Affects Macrophage Cytokine Secretion and Extracellular Matrix Turnover in Electrospun Scaffolds. *Tissue engineering. Part A*, 25(17-18), 1310-1325. <https://doi.org/10.1089/ten.TEA.2018.0306>

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DOI:
[10.1089/ten.TEA.2018.0306](https://doi.org/10.1089/ten.TEA.2018.0306)

Document status and date:
Published: 01/09/2019

Document Version:
Publisher's PDF, also known as Version of Record (includes final page, issue and volume numbers)

Please check the document version of this publication:

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ORIGINAL ARTICLE

Cyclic Strain Affects Macrophage Cytokine Secretion and Extracellular Matrix Turnover in Electrospun Scaffolds

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Controlling macrophage behavior has become a high-potential target strategy for regenerative therapies, such as *in situ* tissue engineering (TE). *In situ* TE is an approach, in which acellular resorbable synthetic scaffolds are used, to induce endogenous tissue regeneration. However, little is known regarding the effect of the biomechanical environment on the macrophage response to a scaffold. Therefore, the aim of this study was to assess the effect of cyclic strains (0%, 8%, and 14% strain) on primary human macrophage polarization in electrospun scaffolds with two different fiber diameters in the micrometer range (4 μ m or 13 μ m). High strains led to a proinflammatory profile in terms of gene expression, expression of surface proteins, and cytokine secretion. These results were consistent for scaffolds with small and large fiber diameters, indicating that the effect of cyclic strain was not affected by the different scaffold microstructures. Notably, macrophages were identified as direct contributors of early secretion of extracellular matrix proteins, including elastin, which was deposited in a strain-dependent manner. These findings are instrumental for the rational design of scaffolds for *in situ* TE and underline that immunomodulatory scaffolds for biomechanically loaded applications should be mechanically tailored, for example, in terms of stiffness and compliance, to support a desirable proregenerative macrophage phenotype.

Keywords: immunomodulation, *in situ* tissue engineering, tissue regeneration, macrophage polarization, biomechanics, extracellular matrix

Impact Statement

Electrospun elastomeric scaffolds are being used for a variety of *in situ* tissue engineering applications, in which biomechanical loads play a dominant *in vivo* role, such as cardiovascular replacements (e.g., heart valve and blood vessel prostheses) and pelvic floor reconstruction. The findings of this study underline that immunomodulatory scaffolds for biomechanically loaded applications should be mechanically tailored, for example, in terms of stiffness and compliance, to support a desirable proregenerative macrophage phenotype. Moreover, this research contributes to the general understanding of pathophysiological macrophage phenotypes in cyclically strained tissues (e.g., atherosclerotic plaques), and their role in tissue regeneration and degeneration.

Introduction

MACROPHAGES ARE ESSENTIAL cellular mediators of tissue formation, controlling the deposition, and remodeling of extracellular matrix (ECM) in various physiological and pathological processes, such as wound healing and fibrosis.^{1,2} Macrophages have been long investigated for their direct involvement in tissue regeneration.³ To illustrate, in several studies, macrophage depletion caused delayed reepithelialization, decreased collagen deposition, and impaired healing.^{4–9} Strikingly, in regenerating species such

as salamanders and zebrafish, depletion of the endogenous macrophage population resulted in failure of growth after limb or tail fin amputation.^{10,11} The directing roles of macrophages for tissue regeneration are dependent on the macrophage polarization state.^{12,13} As such, the modulation of macrophage polarization has become a target strategy for new regenerative therapies, such as *in situ* tissue engineering (TE).

In situ TE has been applied to render living replacement tissues by implanting cell-free substrates intended for cell infiltration and repopulation *in vivo*.^{14,15} Foundational principle of the *in situ* TE approach is to restore the native

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tissue structure and function by modulating the host inflammatory response to the implanted biomaterial.³ Instrumental to this approach is the use of readily available engineered scaffolds, which are designed to promote host cell repopulation and *in situ* tissue remodeling. In brief, upon implantation, there is a rapid influx of blood granulocytes and monocytes into the scaffolds that subsequently differentiate into macrophages. It is hypothesized that macrophages secrete cytokines and growth factors to attract additional immune cells as well as tissue producing cells, originating from surrounding tissues and/or circulating progenitor cells, to produce and remodel *de novo* ECM.^{3,16}

According to the canonical classification, polarized macrophages exist in the form of classical M1 macrophages and alternative M2 macrophages, which represent two extremes of a dynamically changing range of macrophage polarization states.¹⁷ Based on their phenotypes, macrophages have multiple functions in both inhibiting or promoting cell proliferation and tissue repair.^{13,18,19} Typically, M1-type macrophages release cytokines that inhibit the proliferation of surrounding cells and damage contiguous tissue, and M2-type macrophages release cytokines that promote the proliferation of contiguous cells, and ECM formation.²⁰

Macrophages are known to display remarkable plasticity and phenotypic changes in response to changes in the cellular microenvironment, such as biochemical stimuli. In the case of *in situ* TE, the local environment provided by the scaffold has profound effects on the elicited inflammatory response and on host cells colonization. Monocyte-to-macrophage differentiation and the consequent macrophage phenotypic profile are affected, among others, by scaffold microstructural parameters, such as the fiber diameter and pore size.^{21,22}

Moreover, previous work has suggested that the macrophage biomechanical environment may play an important role in dictating macrophage behavior. This is particularly relevant for *in situ* TE of load-bearing tissues, such as heart valve and blood vessel replacements¹⁻³ and meshes for pelvic floor reconstruction or hernia repair.⁴ To illustrate, straining of monolayers of monocytes/macrophages on two-dimensional (2D) membranes was shown to contribute to monocyte-to-macrophage differentiation^{23,24}; to upregulate the early response gene *IL8* and the immediate early gene-1 (*IEX1*),²⁴ and the *c-fos* and *c-jun*, possibly in a process mediated by proinflammatory cytokines; and to selectively augment related cytokines, matrix metalloproteinases (MMP-1, MMP-3), and scavenger receptors.²³

In three-dimensional (3D) electrospun environments, macrophage polarization was demonstrated to be strain dependent as well, with an optimal magnitude of cyclic strain (7%) promoting the presence of reparative M2-like macrophages and allowing for early synthesis of ECM components such as sulfated glycosaminoglycan (sGAG) and hydroxyprolines (HYPs).²⁵ Finally, in the study of Dziki *et al.*, macrophages subjected to 10% strain for 5 h upregulated the M2-like marker Fizz1 in both 2D and 3D environments.²⁶

While significant attention has been given to the investigation of macrophage polarization and early tissue deposition in response to scaffold microstructural cues, relatively little is known about the mechanoresponsiveness of macrophages and the combined effects of microstructural and biomechanical cues in 3D scaffolds. Therefore, the aim of the present study was to investigate the combined effects of

cyclic strain and fiber diameter on human mononuclear cell infiltration, monocyte-to-macrophage differentiation, and macrophage polarization, in terms of phenotypic changes, expression of pro- and anti-inflammatory cytokines and early matrix deposition *in vitro*. A supramolecular elastomer were used as the scaffold material, representing a class of materials that has previously demonstrated to be suitable for *in situ* TE purposes for various load-bearing applications (e.g.,²⁷⁻³⁰).

Materials and Methods

Experimental layout

Scaffold strips with two different fiber diameters, that is, $3.6 \pm 0.5 \mu\text{m}$ (small fiber) and $13.1 \pm 4.2 \mu\text{m}$ (large fiber), were seeded with human peripheral blood mononuclear cells (hPBMCs) and either cultured statically (0% group), or subjected to low (8%) or high (14%) strain, which represent strains within the range of physiological circumferential strain levels of small diameter arteries.¹⁸ For each fiber diameter and strain condition, experiments were conducted for up to 7 days with three samples per group and repeated for three different donors to account for inter-donor variations (total $n=9$). After 4 or 7 days of culture, cell phenotype was evaluated in terms of gene expression (qPCR), cell surface markers (immunofluorescence), and protein secretion (multiplex ELISA), as well as deposition of ECM components.

Scaffold preparation

Scaffold fabrication via electrospinning. Chain-extended ureidopyrimidinone (UPy)-modified polycaprolactone (CE-UPy-PCL) was provided by SupraPolix (Eindhoven, The Netherlands) and used as a base material to electrospin the fibrous scaffolds. Specifically, CE-UPy-PCL (15%, w/v) was dissolved in a solution of amylene-stabilized chloroform (CHCl_3 ; Sigma) and hexafluoroisopropanol (HFIP; Sigma) (CHCl_3 :HFIP 80:20 v/v or 60:40 v/v for large and small fiber diameter, respectively) and stirred overnight at room temperature. Using a syringe pump, the polymer solution was brought into the electrospinning system (EC-CLI, IME, Technologies, Geldrop, The Netherlands), where it was dispersed from a 14 G needle. The solution flowed at $15 \mu\text{L}/\text{min}$ or $25 \mu\text{L}/\text{min}$ for large and small fiber diameter, respectively, in presence of 15 kV voltage, at a constant temperature of 23°C and a relative humidity of 30%.

Randomly oriented fibers were collected on the cylindrical drum (ϕ 26 mm) rotating at 100 rpm, with an applied voltage of -4 kV, and at a distance from the nozzle ranging from 13 cm to 17 cm. Afterward, annealing was performed to the electrospun meshes at 37°C for 1 week to eliminate solvent remnants and to avoid polymer shrinkage.

Scaffold characterization. Scaffold thickness was measured using a digital microscope (VHX-500FE, Keyence, Mechelen, Belgium) at three different regions of the electrospun mesh, averaging 15 positions per region. The scaffold fiber diameter was measured using a scanning electron microscope (SEM; Quanta 600F, GEI, Eindhoven). Images were taken for three different pieces of the mesh, at five different positions per region, averaging 10 fibers per

region. Values for scaffold thickness and fiber diameter were both expressed as a mean with standard deviation.

Biaxial tensile tests were performed with BioTester 5000 (CellScale, Canada) to evaluate scaffold mechanical performance and isotropy within the range of applied deformations (strain 0–14%). Constant strain rate of 1.6% per second was applied for strains up to 50% on a square patch. To simulate the experimental conditions, patches were prewet overnight and immersed during the biaxial tests in PBS at 37°C.

Cycles of straining, resting, and recovering were repeated five times on samples from three different electrospun sheets for each fiber diameter. Cauchy stresses for the last cycle of each measurement were averaged over the three samples for each fiber diameter and plotted as a function of the strain.

Scaffold seeding and culturing

Cell isolation and seeding. Human peripheral blood buffy coats were obtained from healthy, anonymized volunteers (six donors in total, (coincidentally) all male, ages 21–63; three donors for scaffolds with small fiber diameter and three donors for scaffolds with large fiber diameter) that provided written informed consents, which were approved by the Sanquin Research Institutional Medical Ethics Committee (Sanquin, Nijmegen, the Netherlands), in accordance with the Dutch regulations and the 1964 Declaration of Helsinki standards. Buffy coats were diluted in 0.6% (w/v) sodium citrate in PBS, after which the peripheral mononuclear cell fraction was isolated using density gradient centrifugation on iso-osmotic medium with a density of 1.077 g/mL (Lymphoprep, Axis-Shield, Oslo, Norway).

After washing, hPBMCs were resuspended in freezing medium consisting of RPMI, supplemented with 20% (v/v) Fetal Bovine Serum (FBS; Greiner Bio one, Frickenhausen, Germany) and 10% (v/v) Dimethyl Sulfoxide (DMSO; Merck Millipore, Amsterdam, the Netherlands), and cryopreserved in the vapor phase of liquid nitrogen. Before use, cells were rapidly thawed, centrifuged, and the supernatant was aspirated to remove any DMSO.

Scaffold strips preparation and cell seeding. Scaffold strips (25 × 5 mm) were cut out of the electrospun meshes and their outer ends were glued on noncoated BioFlex culture plates (Flexcell® International Corporation, NC) with Silastic MDX4-4210 (Dow Corning, MI). After silicon glue

polymerization, the scaffold strips were sterilized by incubation with 70% ethanol, followed by 5 min UV sterilization. Scaffold strips were washed twice with sterile PBS for 30 min and incubated overnight with RPMI 1640 medium (Gibco, Grand Island, NY), supplemented with 20% FBS and 1% Penicillin/Streptomycin (Lonza, Basel, Switzerland) to allow for protein adsorption. Cells were seeded on the scaffold strips with a density ranging from 1.2×10^6 to 1.6×10^6 hPBMCs/mm³, using fibrin as a cell carrier.³¹ The culturing medium applied to the seeded scaffold strips was supplemented with vitamin C (0.25 mg/mL L-ascorbic acid 2-phosphate; Sigma) and refreshed every 2 days.

Application of cyclic straining. Immediately after seeding, hPBMCs seeded strips were subjected to cyclic strains of moderate (8%) and high (14%) magnitude at a frequency of 0.8 Hz with Flexcell FX-5000™ Tension System (Flexcell® International Corporation) for up to 7 days. Strain distribution and maximum strain within a single strip were analyzed via digital imaging to correlate the applied strain to the actual scaffold deformation, as previously described.³² Images at 60 frames/sec of randomly dotted strips strained at 8% or 14% were collected with a high speed camera (MotionScope, M5C; IDT, Tallahassee) and subsequently analyzed with ARAMIS DIC software (Gom mbH, Braunschweig, Germany).

Analyses

Immunofluorescence. Whole mount immunofluorescent stainings were performed at days 4 and 7. Scaffold strips were washed twice with sterile PBS, fixated with formalin (3.7% formaldehyde in PBS; Sigma) overnight, and stored in PBS at 4°C until staining. Samples were cut in three pieces, washed with PBS, and permeabilized for 10 min with 0.5% Triton X-100 (Merck, Schipholrijk, The Netherlands). Afterward, the scaffold pieces were washed two times 5 min with PBS. Blocking was performed to reduce nonspecific binding, applying 5% goat serum or horse serum in PBS.

For each scaffold piece, markers specific for macrophage polarization and ECM formation were used (Table 1). The primary antibodies were dissolved in PBS with 5% goat serum or horse serum and incubated overnight at 4°C. Afterward, the scaffold pieces were washed three times 5 min with PBS, stained with the secondary antibodies in PBS for 1 h, and stored in PBS until analysis. Z stacks were

TABLE 1. ANTIBODIES FOR IMMUNOFLUORESCENCE ANALYSIS

Readout	Time point	Marker	Function	Primary antibody	Secondary antibody
Macrophage polarization	Day 4	CD86	Pro- and Anti-inflammatory	Mouse anti-human IgG1 (Abcam)	Goat anti-mouse IgG1 A647 (Molecular Probes)
		CCR7	Proinflammatory	Rabbit anti-human IgG (Cell applications)	Goat anti-rabbit IgG A555 (Molecular Probes)
		CD163	Anti-inflammatory	Mouse anti-human IgG2a (LS BioSciences)	Goat anti-mouse IgG A488 (Molecular Probes)
ECM formation	Day 7	ColII	Collagen deposition	Mouse anti-human IgG1 (Sigma-Aldrich)	Goat anti-mouse IgG1 A555 (Molecular Probes)
		ColIII	Collagen deposition	Mouse anti-human IgG1 (Sigma-Aldrich)	Goat anti-mouse IgG1 A555 (Molecular Probes)
		ELN	Elastin deposition	Rabbit anti-human IgG (Abcam)	Goat anti-rabbit IgG1 A647 (Molecular Probes)

TABLE 2. QUANTITATIVE POLYMERASE CHAIN REACTION PRIMERS FOR MACROPHAGE PHENOTYPE AND EXTRACELLULAR MATRIX FORMATION

Readout	Primer	Symbol	Amplicon size (bp)	Accession number	Primer sequence [5'-3']
Macrophage polarization	Interleukin 6	<i>IL6</i>	45	NM_000600	FW:ACTCACCTCTTCAGAACGAATTG RV:GTCGAGGATGTACCGAATTGT
	Tumor necrosis factor alpha	<i>TNFA</i>	91	NM_000594	FW:GAGCCCAAGCCTGGTATG RV:CGGGCCGATTGATCTCAGC
	C-C motif chemokine receptor 7	<i>CCR7</i>	58	NM_001838	FW:AAGCCTGGTTCCTCCCTATC RV:ATGGTCTTGAGCCTCTTGAATA
	Monocyte chemotactic protein1	<i>MCP1</i>	190	NM_002982	FW:CAGCAGATGCAATCAATGCC RV:TGGAATCCTGAACCCACTTCT
	Cluster of differentiation 68	<i>CD68</i> var1	70	NM_001251.2	FW:CACTGGGCAGAGAAACT RV:TTACACCACTGTCCACCTC
	Transforming growth factor beta	<i>TGFβ1</i>	57	NM_000660	FW:GCAACAATCTCTGGCGATACCTC RV:AGTTCTCTCCGTGGAGCTGAAG
	Cluster of differentiation 163	<i>CD163</i>	72	NM_004244	FW:CACTATGAAGAAAGCCAAAATTACCT RV:AGAGAGAAAGTCCGAATCAGAGA
	Interleukin 10	<i>IL10</i>	112	NM_000572	FW:GACTTAAAGGTTACCTGGGTG RV:TCACATGCGCCTTGATGCTG
	Cluster of differentiation 200R1	<i>CD200R1</i> var4	73	NM_170780.2	FW:GAGCAATGGCACAGTGACTGT RV:GTGGCAGGTACGGTACAGA
	Matrix metalloproteinase 2	<i>MMP2</i>	174	NM_001127891	FW:ATGACAGCTGCACCACTGAG RV:ATTGTTGCCACGGAAGTG
	Matrix metalloproteinase 9	<i>MMP9</i>	224	NM_004994	FW:TGGGGGGCAACTCGGC RV:GGAATGATCTAAGCCCAAG
	Collagen type 1	<i>Col I</i>	112	NM_000088.3	FW:TGGCTCTCTGTTGTAACAAG RV:GCCAGGGAGACCGTTGAG
	Collagen type 3	<i>Col III</i>	140	NM_000090	FW:ATCTTGTGTCAGTCTATGC RV:TGGAAATTCTGGGTGGG
	Smooth muscle actin	<i>ACTA2</i> (αSMA)	134	NM_001613.1	FW:CGTGTGCCCCCTGAAGAGCAT RV:ACCGCCTGGATAGCCACATACA
	Elastin	<i>ELN</i>	146	NM_000501	FW:CTGGAATTGGAGGCATCG RV:TCCTGGGACACCAACTAC
ECM production & remodeling	Lysyl oxidase	<i>LOX</i>	184	NM_002317.3	FW:CTGGCTGTTATGATAC RV:GAGGCATACGCATGATG
	Fibrillin 1	<i>Fibr1</i>	124	NM_00138	FW:TGTTGGTTTGTGAAGATATTG RV:GTGGAAGGTGAAGCGGTAG
	Fibrillin 2	<i>Fibr2</i>	126	NM_001999	FW:ATCCCTGTGAGATGTGTC RV:TTCTCTCTGGCATATCC
	Fibullin 5	<i>Fibu5</i>	103	NM_006329.3	FW:ATCTGTGTCACACTCGTCCA RV:AGCTCCAAACTATATCCACGA

TABLE 3. PROTEINS ANALYZED VIA MULTIPLEX ENZYME-LINKED IMMUNOSORBENT ASSAY

<i>Protein</i>	<i>Symbol</i>	<i>Function</i>
Monocyte chemoattractant protein 1	MCP-1	Proinflammatory factor; chemotactic for monocytes and undifferentiated macrophages.
Tumor necrosis factor α	TNF- α	Proinflammatory factor; regulator of apoptotic cell death.
Interleukin 6	IL-6	Proinflammatory factor.
Interleukin 1 β	IL-1 β	Proinflammatory factor; regulator of cell proliferation, differentiation, and apoptotic cell death.
Stromal cell-derived factor 1/Chemokine (C-X-C) ligand 12	SDF-1/CXCL12	Proinflammatory factor; regulator of immune cell recruitment.
Monokine induced by gamma interferon/Chemokine (C-X-C) ligand 9	MIG/CXCL9	Proinflammatory factor; IFN- γ induced T-cell chemoattractant; marker of adverse cardiovascular remodeling.
Interleukin 10	IL-10	Anti-inflammatory factor; Inhibitor of TNF- α , IFN- γ , IL-1 β
Transforming growth factor β 1	TGF- β 1	Anti-inflammatory factor; downregulation of inflammatory cytokine in monocytes and macrophages; MMP-9-dependent polarization.
Alternative macrophage polarization-associated CC chemokine -1/Chemokine (C-C motif) ligand 18	AMAC-1/CCL18	Anti-inflammatory factor produced by monocytes and macrophages; upregulated in presence of T-helper 2 favoring cytokines IL-10, IL-4, and IL-13.
Matrix Metalloproteinase 1	MMP-1	Interstitial collagenase involved in breakdown of interstitial Col I, Col II, and Col III.
Matrix Metalloproteinase 9	MMP-9	Type IV collagenase involved in ECM breakdown and remodeling.

taken at three random positions of the scaffolds with a confocal microscope (Leica TCS SP5X; Leica microsystems CMS GmbH, Mannheim, Germany) to look at cell distribution and cell morphology within the scaffold strips. In addition, images (63 $\times$ magnification) were taken at five random positions on the same plane to visualize specific macrophage phenotypes and early signs of ECM formation.

Gene expression. After 4 days of culture, seeded scaffold strips were cut out of the biaxial Flexcell plate, snap frozen, and stored at -80°C . Two scaffold strips of the same condition were then pooled together in a Nalgene cryogenic vial (Sigma), containing four RNA-free metal beads, and disrupted using a dismembrator (Sartorius, Göttingen, Germany) for 30 s at 3000 rpm for three times. The cells were then lysed by adding 600 μL RLT-buffer with β -mercaptoethanol (Sigma). RNA was isolated using the Qiagen RNeasy kit (Qiagen, Venlo, Netherlands) according to manufacturer's protocols. Purity and quantity of the RNA was determined with a spectrophotometer (Nanodrop, ND-1000, Isogen Life science, IJsselstein) and also with fluorometric quantitation (Qubit; ThermoFisher scientific).

From 100 ng of RNA, cDNA was made in 20 μL reaction volume, containing dNTPs (Invitrogen), random primers (Promega, Madison), Superscript 4 reverse transcriptase (Invitrogen), 5 $\times$ first-strand buffer (Invitrogen), and RNA-free water. A Thermal Cycler (C1000 Touch, Bio-rad, Hercules, CA) was used for the cDNA synthesis using the following heating steps: 65°C for 5 min, 23°C for 10 min (during which the SSIV was added), 50°C for 30 min, 80°C for 10 min, and finally 12°C for cooling down. The samples were then checked for contaminations by performing PCR with a primer of the housekeeping gene Glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The cDNA samples were stored at -20°C until qPCR was performed.

Primers of genes involved in macrophage polarization, tissue formation, and breakdown (Table 2) were, together with SYBR Green Supermix (Bio-Rad) and ddH₂O, used for real-time qPCR (CFX384 Touch Real-Time PCR Detection System; Bio-Rad). GAPDH and β -actin were first tested as reference genes, and GAPDH was determined to be the most stable for the test conditions. cDNA of uncultured hPBMCs of each donor was also implemented in the analysis for normalization. All samples were diluted 20 $\times$ and analyzed in duplicates. The following thermal protocol was used: 95°C for 3 min, a cycle of each 95°C for 20 s, 60°C for 20 s and 72°C for 30 s repeated 40 times, 95°C for 1 min, 65°C for 1 min, and finally a melting curve measurement.

The $\Delta\Delta\text{Ct}$ - or Livak method was used to analyze the data. Gene expression for the samples was normalized to the geometric mean of both reference genes and, afterward, to the control hPBMCs. Moreover, to obtain a relative M1/M2 index, the percentage of the mean expression per gene was calculated, followed by dividing the mean percentage of M1 genes and proinflammatory cytokines (C-C chemokine receptor 7 [CCR7], interleukin 6 [IL6], tumor necrosis factor- α [TNF- α], monocyte chemotactic protein 1 [MCP1]) by the mean expression of M2 genes, and anti-inflammatory cytokines (cell surface transmembrane glycoprotein 200 receptor 1 [CD200R1], cluster of differentiation 163 [CD163], interleukin 10 [IL10], and transforming growth factor- β 1 [TGF β 1])²⁰ per sample, as described elsewhere.³³

Cytokine secretion. Incubation medium of the scaffold strips was collected after 4 and 7 days of culture, snap frozen, and stored at -80°C until analysis. Multiplex ELISA was performed at the Multiplex core facility of the Laboratory for Translational Immunology of the University Medical Center Utrecht, The Netherlands, as described elsewhere.³⁴

Proteins characteristic for the macrophage phenotype and matrix remodeling were analyzed (Table 3). The concentrations were normalized to the number of cells present in the scaffold, which was quantified via counting of DAPI-stained cells. In brief, five representative images with $20\times$ magnification were taken for each scaffold, and the mean amount of attached cells was calculated.

For each cytokine, the normalized concentrations were plotted as percentages of average secretion over the three donors. Moreover, to obtain a relative M1/M2 index, the percentage of the mean production per cytokine was calculated, followed by dividing the mean percentage of the detected M1 cytokines (IL-6, TNF- α , interleukin 1 beta [IL-1 β], chemokine [C-X-C motif] ligand 9 [CXCL-9]) by the

mean percentage of the detected M2 cytokines [TGF- β 1, IL-10, chemokine (C-C motif) ligand 18 (CCL-18), matrix metalloproteinase 9 (MMP-9)] per sample, for both days 4 and 7, as previously described.³³

Quantitative tissue assays. Scaffold strips sacrificed after 7 days of culture were used for the determination of the sGAG, HYP as a measure for collagen, and soluble α -elastin (α -ELN) content. Before the assays, the scaffold strips were lyophilized and the dry weight was measured. Afterward, scaffolds were snap frozen and dismembrated three times 30 s at 3000 rpm. Scaffold strips were digested with a digestion buffer containing 100 mM phosphate buffer (500 mM),

FIG. 1. SEM images of electrospun, isotropic CE-UPy-PCL scaffolds with small fiber diameter ($\varnothing = 3.6 \pm 0.5 \mu\text{m}$) and large fiber diameter ($\varnothing = 13.1 \pm 4.2 \mu\text{m}$) (Scale bar: $500 \mu\text{m}$, $100 \mu\text{m}$ [insets]) (A, B). Averaged ($n = 3$) Cauchy X- and Y-stress as a function of the strain obtained with biaxial tensile test, indicating absence of plastic deformation and isotropic behavior up to 50% strain or 20% strain for scaffolds with small and large fibers, respectively (C, D). Digital image correlation-determined local strains in scaffolds subjected to low strains (8%, left) or high strains (14%, right) with the Flexcell system (E). CE-UPy-PCL, chain-extended ureidopyrimidinone-modified polycaprolactone. Color images are available online.

5 mM L-cystein, 5 mM ethylenediaminetetraacetic acid (EDTA), and Milli-Q. The obtained scaffold powder was dissolved in 500 μ L digestion buffer, supplemented with 140 μ g/mL of papain from papaya latex (Sigma), and digested overnight at 60°C. Both HYP and GAG assays were performed on this solution, as described elsewhere.^{35,36}

Following tissue extraction via oxalic acid, α -ELN was quantified via Fastin™ Elastin Assay (Biocolor Ltd., Northern Ireland, United Kingdom) according to manufacturer's instructions. GAG, HYP, and α -ELN content were normalized for the DNA content. The DNA content was measured using the Qubit® dsDNA BR (Broad-Range) Assay Kit (ThermoFisher Scientific), and the final amount was normalized for the dry weight of the scaffolds.

Statistical analysis

All data from qPCR, protein analyses, and biochemical analyses are expressed as dot plots with the grand mean of samples from all donors per group. Normality of data was verified via the Kolmogorov–Smirnov test. For qPCR, the data were logarithmically transformed before statistical analysis. Statistical differences were analyzed with one-way ANOVA, followed by Bonferroni's multiple comparison test. All data were analyzed using Prism software 5.0 (GraphPad Software, La Jolla, CA), and differences were considered significant for p -values <0.05. *, **, and *** denote significant differences for p -values <0.05, 0.01, and 0.001, respectively.

FIG. 2. Immunostainings of hPBMCs-derived macrophages. DAPI staining (blue in merge image) showed cellular infiltration throughout both microarchitectures, while CD86 (white in merge image) proved monocyte to macrophage differentiation, with predominance of CCR7⁺ M1 macrophages (red in merge image) compared to CD163⁺ M2 macrophages (yellow in merge image) with increasing strain levels. Autofluorescence of the scaffold fibers is visible, predominantly in the blue channel. Scale bars, 50 μ m. hPBMC, human peripheral blood mononuclear cell. Color images are available online.

Results

Scaffold characterization and strain validation

Electrospun CE-UPy-PCL sheets with an average thickness of $227 \pm 11 \mu\text{m}$ were obtained, with scaffold fibers of $13.1 \pm 4.2 \mu\text{m}$ or $3.6 \pm 0.5 \mu\text{m}$ (Fig. 1A, B). Mechanical testing of the scaffold showed absence of plastic deformation and no differences between x and y direction up to 20% cyclic strain or 50% strain for scaffold with small and large fibers, respectively (Fig. 1C, D). Cyclic strain applied to the scaffold with the Flexcell Tension System induced strains between 5.8% and 9.9% for the intermediate strain level and between 11% and 16.5% for the high strain level (Fig. 1E). The two strain sets were indicated as 8% and 14% group, respectively.

Cyclic strains dictate macrophage phenotype

Immunofluorescent DAPI staining revealed cell infiltration and distribution throughout the scaffolds for both large and small fiber diameters, and for all the strain conditions applied (Fig. 2).

A substantial fraction of cells exhibited the macrophage marker CD86, with no detectable differences between different fiber diameters and strain levels. Cell surface markers for the proinflammatory profile M1 (CCR7) and immunomodulatory and tissue remodeling profile M2 (CD163) revealed

differential macrophage polarization among the different groups over time. For both scaffolds with small and large fiber diameter, similar amounts of M1 phenotype and M2 phenotypes were observed in presence of 0% and 8% strain, with frequent colocalization of CCR7 and CD163 on a single cell. In addition, regardless of the fiber diameter, a predominance of M1 cells was observed for scaffolds subjected to 14% strain.

qPCR results for hPBMCs cultured up to 4 days showed strain-dependent expression of anti- and proinflammatory genes and ECM-related genes, regardless of the fiber diameter of the scaffold (Figs. 3 and 4). Expression of the pan-macrophage marker *CD68* was similar for scaffolds with small fiber diameter (Fig. 3), while for scaffolds with large fiber diameter, a decreasing, yet not significant, trend was observed for high strain levels (Fig. 4). The M1 marker *CCR7* showed highest expression for scaffolds with small fiber diameter cultured in static conditions, with downregulation in presence of either low or high strain levels. Differently, scaffolds with large fiber diameter gave similar expression of *CCR7*, regardless of the strain applied. For the small fiber diameter scaffolds, the expression of the M2 markers, *CD200R1* and *CD163*, was significantly lower for both low and high strain levels, compared to static. A similar trend was observed for the large fiber diameter scaffolds. Reversely, the expression of the proinflammatory genes *IL6* and *MCP1* tended to increase with increasing strain levels in the large fiber diameter scaffolds,

FIG. 3. qPCR results for hPBMCs cultured on small diameter fiber scaffolds for 4 days. Genes for pro- and anti-inflammatory macrophages, that is, *CD68*, *CCR7*, *CD200R1*, *CD163*, pro- and anti-inflammatory cytokines, that is, *MCP1*, *IL6*, *IL10*, and genes involved in early matrix deposition, that is, *MMP2*, *MMP9*, *TGFβ1*, and *ACTA2* were selected. Overall, an upregulation and downregulation of the majority of the proinflammatory genes and anti-inflammatory genes, respectively, was observed for increasing strain levels. The calculated M1/M2 ratio was significantly increased with strains.

FIG. 4. qPCR results for hPBMCs cultured on large diameter fiber scaffolds for 4 days. Overall, the majority of the proinflammatory genes tended to increase with strain, while the expression of anti-inflammatory genes tended to decrease with strain. Gene expression levels displayed pronounced donor-to-donor variability.

while expression of these genes tended to be highest in the low-strain group for small fiber diameter scaffolds. The proinflammatory marker *TNF α* showed similar expression levels for all the experimental conditions and groups (data not shown). The expression of the anti-inflammatory genes *IL10* and *TGF β 1* revealed a decreasing trend with increasing strain for small diameter fibers, while the expression of these genes was relatively stable for large diameter fibers.

The calculated M1/M2 ratio significantly increased for scaffolds with small fiber diameters exposed to increasing strain levels, while a strain-dependent increasing trend was observed for scaffolds with large fiber diameter.

With respect to ECM-related genes, hPBMCs cultured on both scaffolds with large and small fiber diameters showed limited to nondetectable expression of collagen type I (*Col I*) and collagen type III (*Col III*), *ELN*, lysyl oxidase (*LOX*), fibrillin 1 (*FBN1*) and fibrillin 2 (*FBN2*), and fibulin 5 (*FBLN5*) (data not shown), regardless of the strain applied. *MMP2* and *MMP9* showed similar behavior with a slight, yet not significant, increase in expression for low-strain levels for scaffolds with small fiber diameter and an increasing trend for *MMP9* expression with strain for the large fiber diameter scaffolds. A decreasing trend was observed for *ACTA2* (α SMA) expression with increasing strain levels for both scaffold types.

High strains promote proinflammatory cytokine secretion

At day 4, the proinflammatory cytokines IL-6, TNF- α , and IL-1 β showed similar trends, with a strain-dictated increase in secretion levels for both scaffolds with small and large fiber diameters (Figs. 5 and 6). Macrophages secreted comparable amounts of CXCL-9 when cultured on scaffolds with small fiber diameter statically or under strain, while a downregulating trend was observed for increasing strain levels applied to scaffolds with large fiber diameter. The anti-inflammatory cytokines TGF- β 1 and IL-10 showed moderate upregulation for both scaffold types for increasing strain levels. MMP-9 secretion tended to decrease with strain for small diameter scaffolds and CCL-18 secretion tended to decrease with strain for large fiber diameter scaffolds in particular.

At day 7, the quantification of the proinflammatory cytokines IL-6 and TNF- α gave comparable outcomes on scaffolds with small fiber diameter for all the strain conditions (Fig. 5). However, for scaffolds with large fiber diameter, a moderate downregulation was observed for both cytokines in the presence of strain (Fig. 6). IL-1 β secretion tended to increase with strain for both scaffold types, while CXCL-9 secretion tended to decrease with low strain or with

FIG. 5. Percentage of average secretion of pro- and anti-inflammatory cytokines and M1/M2 ratio for the small fiber diameter scaffold group. Concentrations were normalized for the total amount of attached cells for each group at day 4 and the total DNA content at day 4. Overall, a moderate upregulation and downregulation of proinflammatory cytokines and anti-inflammatory cytokines, respectively, was observed for increasing strain levels. The resulting M1/M2 ratio tended to increase with increasing strains.

low and high strain for scaffolds with small and large fibers, respectively. At day 7, the anti-inflammatory cytokines IL-10 and CCL-18 showed a strain-dependent downregulation for small fiber diameter scaffolds, while TGF-β1 and MMP-9 secretion was relatively constant. For large fibers, all anti-inflammatory proteins showed a decreasing trend with increasing strains, which was statically significant for CCL-18 and MMP-9.

Overall, the M1/M2 ratio calculated on the basis of the secretion levels of pro- and anti-inflammatory cytokines tended to show a moderate increase for increasing strain levels for both scaffold types.

Macrophages regulate ECM turnover in a strain-dependent manner

DNA measurements revealed a significant decrease of the amount of cells for increasing strain levels in scaffolds with small fiber diameter (Fig. 7). Similarly, the DNA content for scaffolds with large fiber diameter showed a decreasing trend for scaffolds subjected to 8% strain, when compared to the static control. Overall, hPBMCs cultured on both scaffold topographies, regardless of the strain applied, deposited detectable amounts of soluble α-ELN and sGAG. α-ELN production tended to be inhibited or moderately down-

regulated by increasing strain levels for scaffolds with small and large fiber diameters, respectively (Fig. 7). The highest sGAG amounts were observed with high or low strain levels in scaffolds with small and large fiber diameters, respectively (Fig. 7). HYP was undetectable for all the experimental conditions (data not shown).

At day 4, MMP-1 secretion levels increased with increasing strain levels for both scaffold types, with a statistically significant increase in the large fiber diameter high strain group (Fig. 7). At day 7, MMP-1 showed a slight decrease in large fiber diameter scaffolds with high strains, while secretion remained relatively unaffected in the small fiber diameter scaffolds. TIMP-1 secretion was hardly affected by strain for the small fiber diameter scaffolds, while TIMP-1 expression tended to decrease with strain in large fiber diameter scaffolds at day 7.

Collagen immunostainings showed decreasing amount of collagen type I for increasing strain levels on scaffolds with small fiber diameter and very limited amounts on scaffolds with large fiber diameter, regardless of the applied strain (Fig. 8). Similar amounts of Collagen type III were detected for all the strain levels and for scaffolds with small and large fiber diameter. The staining for elastin revealed strain-dependent deposition of elastin along the polymer fibers in scaffolds with small fiber diameter, with

FIG. 6. Percentage of average secretion of pro- and anti-inflammatory cytokines and M1/M2 ratio for the large fiber diameter scaffold group. Concentrations were normalized for the total amount of attached cells for each group at day 7 and the total DNA content at day 7. Overall, a moderate upregulation and downregulation of proinflammatory cytokines and anti-inflammatory cytokines, respectively, was observed for increasing strain levels. The resulting M1/M2 ratio tended to increase with increasing strains, at day 7 in particular.

the highest amounts detected for statically cultured scaffolds and lower amounts for scaffolds subjected to either 8% or 14% deformation. Very limited amounts of elastin were detected on scaffolds with large fiber diameter, among which statically cultured scaffolds showed the highest deposition when compared with scaffolds strained up to 8% or 14% (Fig. 8).

Discussion

The goal of this study was to investigate the effect of cyclic strains (0%, 8%, and 14% strain) on primary human macrophage polarization in 3D electrospun scaffolds with various fiber diameters in the micrometer range. The polarization profile of hPBMCs-derived macrophages, their resulting cytokine/chemokine profile, and their direct contribution to ECM synthesis were investigated. It was demonstrated that, when compared to high strain levels, moderate strain levels promote a favorable host response, which is characterized by the preservation of a reparative macrophage phenotype and a cytokine secretion panel skewed toward an anti-inflammatory profile.

The sensitivity of several cell types to cyclic strain is well acknowledged. For example, cyclic strain was identified as the predominant regulator of the proliferation, cytoplasmic

organization, and differentiation of smooth muscle cells³⁷ and progenitor cells, such as endothelial progenitor cells and mesenchymal stem cells.^{38,39} Physiological strains have a profound effect on the synthesis and organization of the fibrous ECM and specifically on the maturation of elastic fibers.^{40,41} However, macrophage responsiveness to mechanical loading is still poorly understood. Previously, it was shown that dynamic uniaxial or biaxial mechanical perturbation can influence cytoskeletal rearrangement and changes in the orientation of a macrophage-like cell line.^{42–44} Moreover, it was demonstrated that strain affects actin polymerization and, therefore, elasticity and phagocytosis functionality of murine macrophages.⁴⁵ In line with the results described in this study, Ballotta *et al.* observed that macrophage polarization in 3D scaffolds is dictated by strain amplitudes, with moderate amounts of biaxial strain (7%) contributing to a more anti-inflammatory M2 macrophage phenotype when compared to high strains (12%), although the readout parameters to characterize macrophage phenotype in that study were limited.²⁵

Given the complexity in characterizing macrophage phenotypes, here, we opted for a more elaborate readout panel. Importantly, the strain-dictated macrophage polarization observed via immunostainings was confirmed at both

FIG. 7. Quantitative analysis of DNA, α -ELN, and sGAG (*top panel*) at day 7, and percentage of average secretion of MMP-1 and TIMP-1 at day 4 and 7 as determined via multiplex-ELISA (*bottom panel*). DNA content was normalized for the scaffold dry weight, while α -ELN and sGAG were normalized for the measured DNA. Results show significant downregulation of DNA content for strained scaffolds with small fiber diameter, when compared to static controls and, overall, limited cellular viability on scaffolds with large fiber diameter. The α -ELN assay showed a decreasing trend for increasing strain levels in the small fiber diameter scaffolds and limited secretion in the large fiber diameter group. sGAG deposition tended to decrease with low strain for small fiber diameter scaffolds, while sGAG deposition was significantly increased for large fiber diameter scaffolds at low strains. MMP-1 and TIMP-1 concentrations were normalized for the total amount of attached cells for each group at day 4 and for the total DNA content at day 7. Overall a strain-dependent upregulation was observed at day 4 for MMP-1 on both scaffold types and for TIMP-1 on small fiber scaffolds. Reversely, at day 7, strain-dependent downregulation was observed for MMP-1 on scaffolds with large fibers. sGAG, sulfated glycosaminoglycan.

gene and protein level for both scaffold types. Although the use of primary human immune cells led to a relatively large inter-donor variability, leading to limited statistically significant differences, all readout parameters demonstrate consistent trends for all donors. The exact intracellular mechanisms behind the mechanoreponse of macrophages remain to be elucidated. However, McWhorter *et al.* demonstrated that M2 macrophage polarization can be induced by imposing an elongated cell shape, using murine macrophages on 2D printed micropatterns. Interestingly, shape-induced macrophage was demonstrated to be governed by a distinct actin-dependent polarization pathway, different from the biochemical polarization pathway.⁴⁶

Scaffold topography and, specifically, scaffold fiber diameter have previously been demonstrated to have an impact on macrophage polarization, as we extensively reviewed somewhere else.³ In this study, however, overall, no apparent differences were detected in terms of macrophage polarization, gene expression, and protein secretion for macrophages cultured on scaffolds with either fiber diameter, either in static conditions or under strain. Therefore, these results suggest

that variations in the large μ m-range do not greatly influence macrophage polarization, in apparent contrast with previous findings.

Previously, Wang *et al.* reported an increased presence of RAW264.7-derived M2 macrophages for PCL scaffolds with fiber diameters of 5.59 μ m when compared to scaffolds with 0.69 μ m fiber diameter, both *in vitro* and *in vivo*.⁴¹ Similarly, Garg *et al.* demonstrated a positive correlation between increasing fiber diameter and expression of M2 markers when culturing mouse bone marrow-derived macrophages on polydioxanone scaffolds with fiber diameter of either 0.35 μ m or 2.8 μ m *in vitro*.⁴² Given the variety of materials, cell sources and fiber diameters used, a direct comparison of the results of this study with previous findings is difficult. Here, we specifically opted for relatively large fiber diameters as these correlate to large pore sizes, which allow for complete cell infiltration throughout the scaffold.^{47,48} Moreover, this study was conducted with primary human cells, representing a more clinically relevant cell population, as opposed to murine macrophages.

FIG. 8. Immunostainings of Col I (left), Col III, and ELN (right). For small fiber scaffolds, Col I (red in merge image) was primarily deposited in static conditions or in presence of low strain, while Col III (red in merge image) was largely deposited for all the strain conditions applied. ELN stainings (green in merge image) showed higher deposition for scaffolds with small fiber diameter in static conditions, and lower, comparable amounts for increasing strain levels. Autofluorescence of the scaffold fibers is visible, predominantly in the white channel. Scale bar, 30 μ m. Color images are available online.

Beside the paradigmatic role of macrophages in regulating tissue production from other tissue-cell types (e.g., vascular smooth muscle cells, fibroblasts),⁴⁹ recent studies have shed new light on the direct contribution of macrophages themselves to the deposition of new ECM components. Previous studies have demonstrated that macrophages express all known collagen and collagen-related mRNAs, suggesting their capability of producing both collagen *in vitro* and *in vivo*.^{50,51} In addition, it was demonstrated that macrophages secrete collagen type VI, depending upon their stage of differentiation, polarization state, and cell density.⁵⁰

However, it was shown that the deposited collagen type VI modulates cell-cell and cell-matrix interactions, rather than contributing to the native ECM. In addition, deposition of collagen VIII and VI was proved to be dependent on the polarization state of macrophages, with lipopolysaccharide and interferon gamma (IFN- γ) activated proinflammatory macrophages giving the lowest amounts of collagen VIII,⁵² and TGF- β 1 activated reparative macrophages promoting the deposition of collagen VI.⁵⁰ However, to date, the ability of macrophages to deposit other collagen types, for example, collagen type I or collagen type III, as well as phenotype-dependent secretion activities remain less investigated.

As reported by Ballotta *et al.*, hPBMC-derived macrophages were shown to synthesize ECM components within a week from seeding. Importantly, similar amounts of collagen type III, sGAG, and HYP were measured for all the strain levels applied, possibly suggesting that ECM synthesis by macrophages is a process independent of polarization profile.²⁵ Similarly, in our study, immunostainings of collagen type III showed equal distribution for the different strain levels applied. In addition, for scaffolds with small fiber diameter, limited amounts of collagen type I were observed for static conditions and under low strain levels, while a moderate downregulation was observed in presence of high strain levels.

Importantly, in this study, macrophages were also shown to deposit significant amounts of soluble α -elastin, a protein that is often referenced as the missing link for successful tissue regeneration in many reported articles on cardiovascular TE.^{49,53} Tropoelastin production by human macrophages was previously reported in the context of human arterial disease, presumably contributing to disordered elastogenesis in atherosclerotic plaques and abdominal aortic aneurysms.⁵⁴ In this study, elastin synthesis at day 7 was proved to be strain dependent, with the highest deposition

observed under either static conditions or low strain levels, and downregulation in presence of high strain levels, which was distinctly evident for hPBMCs cultured on scaffolds with small fiber diameter. In line with these results, a previous study showed that monocytes cultured in 3D in presence of 10% strain deposited lower amounts elastin when compared with the static controls.⁴⁹

Taken together, these findings support the notion of a more direct role of macrophages in the deposition and turnover of ECM components, including elastin. Although we may speculate that macrophages do not necessarily deposit mature fibrous ECM, secretion of ECM components by the macrophages may contribute either directly (by being incorporated into fibrous tissue) or indirectly (by coating/priming the scaffold) to the new tissue that is formed by secondary cells, such as (myo)fibroblasts.

The present study has some limitations. For a complete characterization of the combined effects of scaffold topography and hemodynamic loads in terms of immunomodulation, other important physical stimuli might be taken into account, namely biomaterial porosity, fiber alignment, surface chemistry, and biofunctionalization, which were previously proved to influence macrophage polarization.^{21,22,55,56} In addition, the role of physiological shear stresses induced by flow *in vivo* could be considered, given its acknowledged influence on monocyte attachment, differentiation, and signaling, as reported elsewhere.^{57,58} Moreover, even though macrophages have long been identified as crucial mediators of the inflammatory response and, as such, as determinant of the long-term host response on a biomaterial,^{3,59} tackling of solely macrophage polarization remains a rather simplistic approach, which, as such, does not fully represent the complexity of the biomaterial-driven response occurring *in vivo*.

Conclusion

To conclude, the results of this study demonstrate that mechanical loads acting on tissue-engineered constructs heavily influence the polarization of macrophages, their capability to secrete pro- and anti-inflammatory cytokines, and the deposition of ECM components such as soluble α -ELN, and Col I and Col III. Importantly, the strain-dictated modulation of the inflammatory response is determined to be a highly robust effect, minimally affected by micrometer changes in the fiber diameter of the electrospun scaffolds. The findings of this study underline that elastomeric scaffolds for *in situ* TE for biomechanically loaded applications should be mechanically tailored, for example, in terms of stiffness and compliance, to support a desirable proregenerative macrophage phenotype.

Acknowledgments

This research is part of the ImaValve Project, which received funding from the European Union's Seventh Framework Programme for research, technological development, and demonstration under grant agreement no 604514. Emily Lurier is gratefully acknowledged for her help with the statistical analysis of the qPCR results.

Disclosure Statement

No competing financial interests exist.

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Received: October 25, 2018

Accepted: December 18, 2018

Online Publication Date: February 27, 2019