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# Strain-dependent modulation of macrophage polarization within scaffolds

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ABSTRACT

Implanted synthetic substrates for the regeneration of cardiovascular tissues are exposed to mechanical forces that induce local deformation. Circulating inflammatory cells, actively participating in the healing process, will be subjected to strain once recruited. We investigated the effect of deformation on human peripheral blood mononuclear cells (hPBMCs) adherent onto a scaffold, with respect to macrophage polarization towards an inflammatory (M1) and reparative (M2) phenotype and to early tissue formation. HPBMCs were seeded onto poly-ε-caprolactone bisurea strips and subjected to 0%, 7% and 12% cyclic strain for up to one week. After 1 day, cells subjected to 7% deformation showed upregulated expression of pro and anti-inflammatory chemokines, such as MCP-1 and IL10. Immunostaining revealed presence of inflammatory macrophages in all groups, while immunoregulatory macrophages were detected mainly in the 0 and 7% groups and increased significantly over time. Biochemical assays indicated deposition of sulphated glycosaminoglycans and collagen after 7 days in both strained and unstrained samples. These results suggest that 7% cyclic strain applied to hPBMCs adherent on a scaffold modulates their polarization towards reparative macrophages and allows for early synthesis of extracellular matrix components, required to promote further cell adhesion and proliferation and to bind immunoregulatory cytokines.



## 1. Introduction

A clinically relevant concept of cardiovascular regenerative medicine proposes to implant synthetic scaffolds seeded with autologous cells, entrusting the host response to promote successful integration of the construct. This method was effectively applied by Shinoka et al. to create tissue engineered vascular grafts (TEVG) with bone marrow mononuclear cells (BM-MNCs) for the treatment of congenital defects in children [1–3]. The contribution of the cellular component to tissue regeneration in vivo was further investigated, suggesting that pre-seeded cells play a pivotal role as mediators for tissue remodeling via paracrine mechanisms [4]. Among the numerous cytokines involved in the process, monocyte chemotactic protein 1 (MCP-1) secreted from cells upon implantation was identified by Roh et al. as a key player in early monocytic infiltration and subsequent TEVG remodeling. More specifically, a favorable neo tissue formation could be elicited by a relatively rapid release of the MCP-1 from the scaffold. In addition, early deposition of native-like extracellular matrix by seeded cells might promote host cell adhesion and proliferation and bind important immunoregulatory cytokines, such as interleukin-10 [5].

Another factor that determines the integration of the construct and the remodeling outcome is represented by the early interaction between the biomaterial and circulating cells. Shortly after implantation, scaffolds are extensively infiltrated by immune cells, which can modulate this inflammatory response via paracrine and autocrine signaling. Among them, macrophages were shown to be actively involved in the resolution of the inflammation, due to their ability to shift from a pro-inflammatory polarization state (M1) towards a reparative and homeostatic profile (M2) [6,7]. In particular, the delicate balance between M1 and M2 phenotypes might

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represent the key to functional regeneration in vivo [8]. Initially, the host response evoked at the implant site implies inflow of M1 macrophages, involved in tissue reorganization and in further recruitment of leukocytes via release of chemokines, such as MCP-1. At later stages, a predominance of M2 phenotype was shown to promote resolution of the inflammation and to result in successful remodelling of the construct [9].

The influence of substrate composition and topography over M1/M2 polarization was demonstrated, addressing the type of scaffold material, fiber diameter and pore size as parameters able to impact macrophage phenotypic profile [10–12]. We hypothesize that mechanical cues may additionally alter the evoked response. It is known that forces exerted on cells can directly affect their phenotype and their underlining genomic profile through mechanotransduction, causing the activation and inhibition of numerous pathways [13,14]. In vivo, synthetic substrates for the replacement of load-bearing structures, such as heart valves, are exposed to mechanical forces that result in local deformation, and thereby in the stimulation of pre-seeded and infiltrating cells.

The aim of the present study is to investigate the effect of mechanical strain on cells involved in the early immune response, in terms of phenotype polarization, expression of pro- and anti-inflammatory cytokines and matrix deposition. To obtain a mechanistic insight into this process, a pre-seeded electrospun scaffold was subjected to increasing strain levels. As scaffold material, a custom-made thermoplastic elastomer, i.e. poly-ε-caprolactone bisurea, PCL-U4U, was used [15]. Human mononuclear cells from peripheral blood (hPBMCs) were selected as cell source. They are representative of the circulating mononuclear population activated by the biomaterial implant, and might therefore serve as an *in vitro* model to comprehend the response to strain by infiltrated host cells [16]. In addition, hPBMCs are easier to isolate than bone marrow mononuclear cells (BM-MNCs) and may, therefore, provide a clinically interesting alternative for one-step interventions [17].

## 2. Materials and methods

### 2.1. Experimental layout

Scaffold strips seeded with human peripheral blood mononuclear cells (hPBMCs) were subjected to mechanical loading in two series of experiments. In the first series, the strips were subjected to moderate strain (7%), while, in the second series, high deformations (12%) were applied. Experiments of each series were conducted for up to 7 days and repeated 3 times. In all experiments, groups of scaffold strips seeded with hPBMCs (0% group) and with human vena saphena cells (hVSCs, control group) were cultured statically. For each experiment, hPBMCs were freshly isolated from different donors, whereas hVSCs were obtained from a single donor and included to account for inter-experimental differences.

Groups of 3 strips were sacrificed after 1, 2, 4, and 7 days. At every time point, part of each strip was snap-frozen and stored at −80 °C for gene expression analysis or biochemical assays, and the remainder was fixed in 3.7% formaldehyde solution (Sigma, St. Louis, MO, USA) for staining.

### 2.2. Scaffold preparation

#### 2.2.1. Scaffold fabrication

A thermoplastic elastomer based on a poly-ε-caprolactone PCL2000 soft block and a 1,4-bis-ureido butane hard block (PCL-U4U) was supplied by SyMO-Chem (Eindhoven, the Netherlands), and this material was processed into microfibers organized in a porous isotropic scaffold. Specifically, a solution of 12.5% PCL-U4U was prepared dissolving the polymer in amylene stabilized chloroform ($CHCl_3$; Sigma) with 1% methanol. After being stirred overnight, the polymer solution was dispensed with the aid of a syringe pump (PHD 22/2000, Harvard Apparatus, Holliston, MA) to a moving 14G needle placed at 15 cm distance from the collecting mandrel ($\varnothing = 29$ mm, length = 100 mm) of an electrospinning device (EC-CLI, IME Technologies, Geldrop, The Netherlands). The solution flowed at 25 μl/min in presence of 14 kV voltage, at constant temperature of 23 °C and 30% relative humidity. A coaxial flow of $CHCl_3$ saturated air was applied around the needle to prevent excessive solvent evaporation [18]. Randomly oriented fibers were collected on the cylindrical drum rotating at 100 rpm to obtain electrospun scaffolds, which were kept under vacuum overnight to eliminate solvent remnants.

#### 2.2.2. Scaffold characterization

For each scaffold, thickness and fiber diameter were measured with a digital microscope (VHX-500FE, Keyence, Mechelen, Belgium) and a scanning electron microscope (SEM; Quanta 600F, FEI, Eindhoven, The Netherlands) respectively. Measurements are expressed as mean ± 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 0–12%). Constant strain rate of 1.6% per second was applied for strains up to 35% on a square patch. In order to simulate the experimental conditions, biaxial tests were performed on patches pre-seeded with fibrin gel and immersed in distilled water at 37 °C. Cycles of stretching, resting and recovering were repeated 5 times on samples from 3 different electrospun sheets. Cauchy stresses and strains calculated for the last cycle of each measurement were averaged over the three samples and plotted with the relative standard deviations.

### 2.3. Scaffold seeding and culture

#### 2.3.1. Scaffold strips preparation

For each experiment, scaffold strips of 25 × 5 mm ($n = 36$) were cut out of 2 spun sheets and glued on their outer ends in wells of sterile non-coated BioFlex culture plates (Flexcell® International Corporation, NC, USA) with Silastic MDX4-4210 (Dow Corning, Michigan, USA). After silicon glue polymerization, sterilization was performed by incubation with 70% ethanol, followed by 2 washing steps of 30 min in sterile phosphate buffered saline (PBS). Strips for seeding with hPBMC ($n = 24$) were incubated overnight in RPMI 1640 medium (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; Greiner Bio one, Frickenhausen, Germany) and 1% Penicillin/Streptomicin (p/s, Lonza, Basel, Switzerland). Strips for seeding with hVSCs ($n = 12$), were incubated overnight in advanced Dulbecco's Modified Eagle Medium (DMEM, Invitrogen, Carlsbad, USA) supplemented with 10% FBS, 1% p/s and 1% GlutaMax (Invitrogen).

#### 2.3.2. Cells isolation and seeding

HPBMCs were isolated from fresh buffy coats obtained by 6 healthy donors after informed consent (Sanquin Blood Supply Foundation, Nijmegen, The Netherlands). The buffy coat was mixed with citrate buffer (6 g Sodium Citrate in 1000 ml phosphate buffer solution, Sigma) and mononuclear cells were isolated by centrifugation on an isosmotic medium with a density of 1.077 g/ml (Lymphoprep™, Axis-Shield, Scotland). For each experiment, cells from a single donor were seeded at a density of $4 \times 10^5$ cells/mm$^3$ onto the PCL-U4U strips using fibrin gel as a cell carrier [19]. Human vascular-derived cells harvested from saphena magna vein (hVSCs) and previously characterized as myofibroblasts were used as control [20]. After being cultured for 7 days with advanced DMEM medium supplemented with 10% FBS, 1% p/s, 1% GlutaMax, cells were suspended in fibrin gel and seeded onto the PCL-U4U strips at a density of $1.5 \times 10^4$ cells/mm$^3$. hVSCs were used at passage 7 in all experiments.

#### 2.3.3. Application of cyclic loading

Immediately after seeding, hPBMCs seeded strips were subjected to cyclic stretch of moderate (7%) and high (12%) magnitude at frequency of 0.8 Hz with Flexcell® FX-5000™ Tension System (Flexcell® International Corporation, NC, USA) for up to 7 days. RPMI and DMEM enriched media prepared for strip incubation were used for hPBMCs and hVSCs groups respectively, additionally supplemented with L-ascorbic acid 2-phosphate (0.25 mg/ml; Sigma) and replaced every 3 days.

Strain distribution within a single strip was analyzed via digital imaging to correlate the applied stretch to the actual scaffold deformation, as previously described by Boerboom et al. [21]. Images at 60 frames/sec of randomly dotted strips strained at 7% or 12% were collected with a high speed camera (MotionScope, M5C; IDT, Tallahassee, USA) and subsequently analyzed with ARAMIS DIC software (Gom mbH, Braunschweig, Germany).

### 2.4. Analyses

#### 2.4.1. Cells distribution

Cells distribution within the scaffold was evaluated 1 and 7 days after seeding. Slices of formaldehyde-fixed seeded scaffolds with a thickness of 10 μm were cut out of strips embedded in Tissue-Tek® O.C.T™ Compound (Sakura Finetek Europe B.V., Alphen aan den Rijn, The Netherlands) with a cryotome (Thermo Scientific™ HM 550 Cryostats, Thermo Fisher Scientific Inc, Waltham, MA). Cells nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI, Sigma) and examined with fluorescent microscopy (Axiovert 200M, Zeiss, Göttingen, Germany) with a 10× objective.

#### 2.4.2. Gene expression

Seeded scaffolds, sacrificed after 1 and 2 days of straining and stored at −80 °C were placed in Nalgene® cryogenic vials (Sigma) containing RNA-free metal beads and disrupted with a microdismembrator (Sartorius, Göttingen, Germany) 3 times for 30 s at 3000 rpm. RLT buffer with β-mercaptoethanol (Sigma) was added to lysate the cells and RNA was subsequently isolated with Qiagen RNeasy kit (Qiagen, Venlo, The Netherlands) according to manufacturer's instructions. RNA quantity and purity were determined with a spectrophotometer (NanoDrop®, ND-1000, Isogen Life Science, Ijsselstein, The Netherlands). cDNA was synthesized starting from 125 ng RNA in a 25 μl reaction volume consisting of random primers (Promega,