1. Introduction

The use of bioresorbable replacements (e.g., cell-free scaffolds) designed to direct tissue regeneration at the locus of implantation has emerged as a promising strategy to create replacements that are living and adaptive.[1,2] This technology has the potential to not only reduce the clinical demand for autologous living blood vessel replacements but also bypass the challenges associated with synthetic, nondegradable vascular substitutes. The regenerative response is initiated by the infiltration of immune cells, e.g., macrophages, followed by the attraction, migration, and distribution of tissue-producing cells that deposit de novo extracellular matrix (ECM) in the first weeks after implantation.[3] Ideally, the deposited tissue is remodeled, guided by physiological hemodynamic loads, to resemble a tissue with native-like structural and functional properties, while the scaffold slowly degrades.[4,5]

Despite encouraging in vivo proof-of-concept studies,[5,6] early stenosis and aneurysm formation remain prevalent complications in situ vascular tissue engineering.[7–9] These complications are often attributed to a mismatch between scaffold properties (e.g., microstructure, geometry, and mechanical properties) and mechanical loading, resulting in aberrant inflammation and pathological tissue deposition. It is therefore important to attain a more fundamental understanding of the cell and tissue responses to the chemophysical microenvironment during in situ tissue regeneration.

One major process that plays a critical role for successful in situ regeneration is the host immune response. While the immune response is essential for the initiation and guidance of wound healing, it can also contribute to adverse tissue deposition (e.g., fibrosis) and scaffold failure (e.g., accelerated degradation) in conditions of chronic inflammation.[10] Particularly, due to its phenotypic and functional plasticity, the monocyte-derived macrophage has been identified as the commanding cellular player in the initial immune response,
driving biomaterial degradation as well as tissue formation and remodeling.[10–12,14] The macrophage “prepares the ground”—by secreting cytokines and degrading the scaffold—for colonizing cells (e.g., infiltrating mature fibroblasts, smooth muscle cells, as well as stem/progenitor cells) to produce a new tissue.[17] Thus, the interplay between macrophages and tissue-producing cells within the scaffold environment will largely determine the functionality of the resulting neotissue. 

Cocultures of macrophages and tissue-producing cells revealed that paracrine signaling between these cell types promote cellular recruitment, attachment, migration, and distribution throughout porous scaffolds.[10–12,14] Moreover, paracrine factors secreted by different macrophage phenotypes differentially regulate fibroblast and smooth muscle cell behavior (e.g., proliferation and phenotype).[13,15,18] Multiple macrophage phenotypes have been identified, spanning a spectrum with the classical proinflammatory (M1) and the alternative anti-inflammatory (M2) phenotypes at the extremes.[19] Paracrine factors secreted by M2 macrophages (e.g., TGF-β, PDGF) are known to stimulate fibroblast proliferation and collagen formation, whereas factors secreted by M1 macrophages (e.g., TNF-α, MMP-7) give rise to a more proinflammatory fibroblast with tissue degrading as well as profibrotic properties.[13,15] Of note, fibroblast behavior can be reversed by reversing or removing the paracrine signals, emphasizing fibroblast plasticity.[11]

The phenotypic state of the macrophage is ambiguous, often displaying both pro- and anti-inflammatory features simultaneously, and depends on the phase of tissue repair and the biochemical cues from the cellular environment it is exposed to,[20–22] In addition, while the exact (intra)cellular mechanisms remain elusive, it is increasingly acknowledged that macrophages are mechanosensitive and adjust their function in response to both physical and mechanical cues.[23–25] For example, it has been reported that lower strains (8%) permit macrophage polarization toward a reparative M2 profile in 3D electrospun scaffolds, whereas higher strains (12%) promote a more proinflammatory M1 profile, both in terms of cell phenotype and cytokine secretion.[26,27] Moreover, Battiston et al. demonstrated increased collagen deposition in cyclically-stretched scaffolds seeded with monocytes and SMCs.[10] In addition to mechanical stretching, the relation between flow-induced shear stress and cell behavior has been the topic of multiple studies.[28–31] These studies have provided insights into the effect of either shear stress or cyclic stretch on various cell types individually, especially on 2D substrates.[32,33]

However, knowledge on the relative contribution of shear stress and cyclic stretch to tissue regeneration in a more physiologically relevant 3D environment remains elusive. To address this knowledge gap, we recently employed a human in vitro model that mimics the early phase of the in situ scaffold environment in which the endothelial lining has not been formed yet. We used this model to study the individual and combined roles of these mechanical cues on biomaterial-activated macrophages and their downstream effects on human vena saphena derived fibroblasts and myofibroblasts (referred to as (myo) fibroblasts).[38] The results showed that shear stress enhanced macrophage secretion of both pro- and anti-inflammatory cytokines. Downstream, these effects resulted in the activation of tissue formation- and remodeling-related genes in (myo) fibroblasts. In this study, we only looked at the secretome of the macrophages under the influence of hemodynamics and investigated what effects the paracrine factors of these cells have on (myo)fibroblast activation. These are important insights into the initial macrophage-dominated inflammatory phase of the in situ tissue engineering process. However, as in the proliferative phase of wound healing, the formation and remodeling of new matrix deposition during in situ tissue engineering is governed by the continuous cross-talk between macrophages and (myo) fibroblasts, macrophages and (myo)fibroblasts should be cocultured together for a longer culture period.

In this study, motivated to improve the long-term clinical performance of in situ tissue engineered vascular grafts, we further employed our human in vitro model, as a mimic of several critical aspect of the proliferative phase in in situ tissue engineering (Figure 1A), to identify the mutual roles of physiological levels of shear stress and cyclic stretch on macrophage/fibroblast-mediated neotissue formation. Using our recently developed bioreactor,[34] we reveal that these loads differently regulate immune profiles, matrix growth, and matrix remodeling in cocultures of (myo)fibroblasts and primary monocytes in electrospun vascular polycaprolactone bis-urea (PCL-BU) scaffolds. Whereas shear stress abrogates stretch-induced matrix growth and stimulates collagenous remodeling, cyclic stretch inhibits shear stress-driven secretion of proinflammatory cytokines (MCP-1 and IL-6) during our 20 d long cell culture. Moreover, both loads cumulatively affect anti-inflammatory IL-10 secretion and (myo)fibroblast phenotype. Together, the data highlight the importance of considering the effects of both cyclic stretch and shear stress during the early phases postimplantation.

2. Results and Discussion

2.1. Characterization of Scaffolds and Hemodynamic Loads

PCL-BU was chosen as a biomaterial as this supramolecular elastomer has shown its potential for in situ cardiovascular TE applications.[35,36] PCL-BU was processed using electrospinning into vascular scaffolds with an inner diameter of 3 mm and wall thickness of 200 μm (Figure 1B). The resulting scaffolds, with a fiber diameter of 4.58 ± 0.34 μm, showed a highly porous and isotropic microstructure, allowing for cell infiltration (Figure 1C). Biaxial tensile testing revealed that the material behaves linearly (Figure 1D) within the applied loading regime (up to 1.08 stretch), but is slightly stiffer in the axial direction (3.6 ± 0.9 MPa) in comparison to in the circumferential direction (3.0 ± 0.4 MPa). The scaffolds were seeded with a 2:1 mixture of human primary monocytes and (myo)fibroblasts[10,11] using fibrin as a cell carrier (Figure 1E), and cultured for 20 d under hemodynamic loads typically present in in situ cardiovascular TE applications,[17,2] i.e., shear stress (1.11 ± 0.08 Pa, Figure 1F), cyclic stretch (1.06 ± 0.02 at 0.5 Hz, Figure 1G), or a combination of both in our bioreactor as monitored over the course of the experiment. In short, each scaffold construct was mounted around impermeable silicone tubing and centered in a glass tube. The resulting annular channel was perfused with medium at a constant pressure gradient to apply shear...
stress and the silicone tubing was cyclically pressurized up to a constant pressure at 0.5 Hz to apply circumferential stretch (Figure 1H). How these macroscopic loads translate exactly to local, cell-perceived forces depends on many variables (e.g., ligands present, scaffold fiber diameter, and orientation [38]) and should be subject of future studies. For more information regarding the application and quantification of hemodynamic loads, the reader is referred to van Haaften et al. [34] Since the scaffold constructs were mounted around impermeable silicone tubing for the application of circumferential stretch, transmural flows are assumed to be negligible.

2.2. Cyclic Stretch Promotes (myo)Fibroblast Proliferation

After 3 days of coculture, (myo)fibroblasts and macrophages were homogeneously distributed in the scaffold (Figure 2A, B). We then assessed whether coculture of macrophages and
(myo)fibroblasts for 20 d in the vascular scaffolds resulted in matrix deposition. Indeed, SEM analysis revealed an increasing presence of cells and ECM deposition in the scaffolds from day 3 to day 20 in all sample groups (Figure 2C), with the highest increase in overall tissue mass for the cyclic stretch group (Figure 2D). Correspondingly, elevated DNA levels were found in the presence of cyclic stretch at day 20 (Figure 2E), confirming that cyclic stretch stimulates cell proliferation. In contrast, shear stress alone or in combination with cyclic stretch were not found to lead to increased DNA levels.

To identify which cell population is responsible for this overall proliferation, we stained the cells with KI67. KI67 seemed to colocalize only with vimentin-positive (myo)fibroblasts, which adopted an elongated spindle-shaped morphology.
Combined with shear stress (Figure 3C–E). These changes in the anti-inflammatory protein IL-10, especially when in the presence of proinflammatory MCP-1 and IL-6, together with a concomitant upregulation of these cytokines were relatively low compared to other cytokines. To what extent these specific cytokine quantities contributed to the variations seen on tissue level should be subject of further investigation.

To examine in more detail the cellular response to the applied hemodynamic loading, we normalized the total protein secretion levels to the DNA content at day 20 (Figure 3B). Cyclic stretch contributed to a clear reduction of proinflammatory cytokines (MCP-1 and IL-6), together with a concomitant upregulation of the anti-inflammatory protein IL-10. More specifically, TNF-α was present in all mechanically loaded samples at day 3 but not at day 20, and IL-10 levels slightly decreased with time, but remained stable and was highest when both loads were combined. However, it should be noted that the concentrations of these two cytokines were relatively low compared to other cytokines. To what extent these specific cytokine quantities contributed to the variations seen on tissue level should be subject of further investigation.

2.3. Loading Regimes Differentially Affect Cell Inflammatory Response and Phenotype

To characterize the inflammatory environment in the vascular constructs under different regimes of hemodynamic loading, we examined the cytokine secretion profiles in the medium (Table S1, Supporting Information). No obvious overall difference between different loading regimes at day 3 was observed, except for the secretion of MMP-9, which was clearly enhanced with cyclic stretch (Figure 3A). All cytokine secretion levels increased with longer culture, except for the proinflammatory protein TNF-α and the anti-inflammatory IL-10. More specifically, TNF-α was present in all mechanically loaded samples at day 3 but not at day 20, and IL-10 levels slightly decreased with time, but remained stable and was highest when both loads were combined. However, it should be noted that the concentrations of these two cytokines were relatively low compared to other cytokines. To what extent these specific cytokine quantities contributed to the variations seen on tissue level should be subject of further investigation.

To examine in more detail the cellular response to the applied hemodynamic loading, we normalized the total protein secretion levels to the DNA content at day 20 in the medium (Figure 3B). Cyclic stretch contributed to a clear reduction of proinflammatory MCP-1 and IL-6, together with a concomitant upregulation of the anti-inflammatory protein IL-10, especially when combined with shear stress (Figure 3C–E). These changes strongly suggest that cyclic stretching tunes and prevents excessive inflammation by promoting an anti-inflammatory environment. Based on our previous study, we expected that the contribution of macrophages via direct mechanosensing would lead to increased levels of these cytokines in the presence of hemodynamic loading, especially when both loads are combined. The discrepancy we find for MCP-1 and IL-6 indicate that (myo)fibroblasts and juxtacrine interactions between both cell types must have contributed to these secretion profiles as well. This finding corroborates with previous studies that showed that moderate stretches can promote an anti-inflammatory environment, both via direct mechanosensing and via indirect paracrine signaling.

Furthermore, we found that the secretion of TGF-β1 and CTGF was reduced with combined loads, implying a suppressed tissue-stimulatory environment (e.g., to produce collagen) compared to no or single loads. In contrast, MMP-1 and TIMP-1 secretion was increased with combined loads, implying an enhanced tissue-remodeling environment (e.g., to remodel collagen). The findings of TGF-β1 and MMP-1 are largely in line with the mechanoresponse of macrophages, indicating that both cell types respond similarly to the hemo-dynamic loads with regard to cytokines related to growth and remodeling.

We next examined whether these loading-dependent inflammatory responses modulate the (myo)fibroblast phenotype. Differences in the gene expressions representing (myo)fibroblast phenotypic markers became most apparent at day 20 (Figure 3F, G). Especially for αSMA, a strong increase in expression was observed from day 3 to day 20. Interestingly, at day 20, both cyclic stretch and shear stress application contributed to an upregulation of S100A4, a marker for activated fibroblasts that has been recently related to inflammation, and a downregulation of the contractile markers αSMA and calponin in a synergistic manner, suggesting that mechanical loading, in either or combined form, reduces cell contractility (Figure 3G). The (synergistic) downregulation of αSMA due to cyclic stretch and shear stress could also be confirmed at the protein level (Figure 3H). Intriguingly, the reduction in αSMA expression in the mechanically loaded samples was confined to the scaffold. Nevertheless, the expression of smoothelin, another specific marker related to cell contractility, as well as vimentin, was significantly enhanced in the presence of cyclic stretch and to a lower extent in the presence of shear stress.

Both calponin and αSMA have been found to be regulated via cytokines (e.g., MCP-1 and IL-6) as well as loading regime (e.g., shear stress). As such, we detect paradoxical load-dependent changes in phenotype marker expressions that are likely to be a combined effect of direct mechanotransduction (i.e., (myo)fibroblast response to cyclic stretch and superficial/interstitial shear stress) and the indirect, cell-mediated cytokine profiles in the medium.

2.4. Shear Stress Dampens Cyclic-Stretch-Induced Matrix Deposition and Enhances MMP-1/TIMP-1-Mediated Remodeling

Cyclic stretch, depending on the magnitude and duration, is known to stimulate matrix formation. Since the (myo)fibroblast phenotype was affected by the applied loading regime, we hypothesized that the loads applied in this study also affect vascular regeneration by modulating matrix growth and remodeling. We found that, in general, the gene expression of growth and remodeling markers increased over time, as indicated by the yellow colors in the heat maps at day 20 compared to day 3 (Figure 4A). Most strikingly, at day 20, cyclic stretch stimulated the expression of several markers related to the production of collagenous matrix (collagen I, collagen III), elastic matrix (elastin, fibrillin I), and GAGs (versican), but not in the presence of shear stress (Figure 4A,B). Thus, shear stress seemed to have a damping effect on stretch-induced matrix growth. This is particularly intriguing because shear stress has been previously suggested to be a regulating factor in matrix deposition by inhibiting cell proliferation and neointimal thickening under elevated levels of shear stress.
Figure 3. Inflammatory environment and (myo)fibroblast phenotype in the vascular construct. Color maps of the A) total cytokine secretion at day 3 ($n = 4$ per group) and day 20 ($n \geq 5$ per group) and B) cytokine secretion at day 20 normalized to DNA content (scales are normalized for each cytokine; TNF-α was only detected in the mechanical loaded groups at day 3 ($n \geq 2$); n.d., not detected). C–E) Boxplots for a selection of cytokines at day 20 normalized to total DNA content (see Figures S1A,B and S2, Supporting Information for the boxplots of all cytokines). F,G) Relative gene expressions of (myo)fibroblast-specific phenotypic markers at day 3 ($n \geq 3$ per group, except for calponin ($n = 2$ in combined group) and day 20 ($n \geq 4$ per group, except for calponin $n = 3$ in cyclic stretch group)). H) Cross-sections stained for αSMA (green) and DAPI (blue) at day 20. # measured at or below the detection limit. (p-values are calculated using Kruskal-Wallis test with a Dunn’s multiple comparison test, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

Abbreviations: metallopeptidase inhibitor (TIMP), matrix metalloproteinase (MMP), interleukin (IL), monocyte chemoattractant protein 1 (MCP-1), transforming growth factor beta 1 (TGF-β1), connective tissue growth factor (CTGF), tumor necrosis factor alpha (TNF-α), platelet derived growth factor (PDGF), S100 calcium binding protein A4 (S100A4), Alpha Smooth Muscle Actin (αSMA or ACTA2), calponin 1 (CNN1), smoothelin (SMTN), vimentin (VIM), static (ST), cyclic stretch (CS), shear stress (SS).
In terms of matrix remodeling, we found elevated levels of gene and protein expressions of MMPs and TIMPs upon loading compared to the static controls at day 20. Whereas shear stress stimulated MMP-1 gene expression and MMP-1 and TIMP-1 protein secretion, cyclic stretch independently stimulated the expression of MMP-2 and TIMP-2. MMP-9, on the other hand, was only minimally secreted and no clear differences between loading conditions were found (Figure 4A,C–E). Together, these observations suggest that there is more effective tissue remodeling over a period of 20 d when both loads are applied. Although comparable observations in literature are found on gene and protein levels when either shear stress or stretch is applied,[54–59] these data should be interpreted with caution as they represent only snapshots of the remodeling process without discriminating between latent and active forms of the proteases. Further insight into the activity of the secreted proteases can be provided by zymography,[60] which we additionally performed to test for MMP-2 and MMP-9 activity (i.e., using gelatin zymography, see Figure S1C–E, Supporting Information). These data confirm that the secreted proteases are active and thus able to remodel the newly formed matrix.

2.5. Shear Stress Abrogates Cyclic-Stretch-Induced Matrix Stiffening

The long-term clinical performance of our resorbable vascular scaffolds is largely determined by the structural and compositional properties of this newly formed matrix. Thus, we next sought to identify the effect of hemodynamic loading on the organization of the deposited matrix after 20 d of cell culture. At the superficial tissue layer (i.e., up to a depth of 25 µm into the newly formed matrix, largely free from the influence of the scaffold fibers), cyclic stretch and shear stress were found to promote the (myo)fibroblasts and collagen to align in a right-handed helical structure with an angle of around 60° with respect to the circumferential axis (Figure 5A–D,G). To make a direct comparison across all loading conditions, we plotted the average collagen and actin distributions in a single figure (Figure 5E,F). Cyclic stretch was found to result in a strong alignment of the actin fibers, and to a lesser extent in the presence of shear stress (Figure 5F). A similar but less pronounced effect was found in the collagen structures (Figure 5E). In contrast, inside the scaffold, cells and matrix organization consistently followed the scaffold fibers (Figure S4, Supporting Information).
Information). In line with the earlier findings of de Jonge et al.,[3] these results demonstrate the importance of the contact-guiding cue provided by the microfibrous scaffold and the impact of both loads on late-stage cell and matrix reorientation (i.e., when the scaffold has degraded). The exact reorientation response to the applied loading regime, however, also depends on the tissue constraints, dimensionality (2D vs. 3D), and the magnitude and type (laminar vs. oscillatory) of the loads.[31,61–64]

It is tempting to speculate that the hemodynamic-loading-dependent organization of the newly formed matrix is responsible for changes in the construct mechanical properties. It should be noted, however, that the superficial tissue layer is likely too thin to markedly affect the mechanical performance of the overall construct, which is a superposition of the relative scaffold and matrix properties. Therefore, we also analyzed the compositional properties of the matrix in the constructs. Quantification of sample thickness from the tissue sections indicated that the presence of cyclic stretch slightly, but not significantly, led to the formation of a thicker construct after 20 d of culture (Figure 6A). Collagen staining and HYP quantification at day 20 further revealed that loading regime influenced collagen composition, both in terms of the type, rate of collagen birefringence, and the amount of the collagen present in the construct (Figure 6B,D–F). Cyclic stretch stimulated the formation of more numerous, thicker and potentially more mature fibers of collagen type I, but this effect was overruled by the dampening effect of shear stress, in direct correspondence with our earlier finding at the gene expression level (Figure 4B). In contrast to collagen, the formation of GAGs was independent of the applied loading regime (Figure 6C,G).

The effects of hemodynamic loading on the matrix growth and remodeling over a period of 20 d are reflected in the mechanical properties of the constructs, which are important for maintaining vascular integrity during in situ regeneration. Biaxial tensile tests revealed that the cultured constructs exhibited some degree of nonlinear behavior at day 20 in both the axial and circumferential directions and were slightly stiffer in the axial direction (Figure 6H,I). This is in contrast to the largely isotropic structure and mechanical properties of the bare scaffolds (3.6 ± 0.9 and 3.0 ± 0.4 MPa in axial and circumferential direction respectively). Cyclic stretch resulted in the stiffest construct (7.7 ± 2.1 and 7.2 ± 2.2 MPa in Figure 6H,I). Interestingly, the presence of shear stress abrogates this stretch-induced stiffening in both directions, leading to tissue stiffness that is almost indistinguishable from that of the bare scaffold (4.7 ± 1.8 and 3.5 ± 0.8 MPa in Figure 6H,I). In our previous study,[18] it was shown that the scaffold structural and chemical properties are unaffected by the application of shear stress and/or cyclic stretch. Together with the notion that negligible (cell-driven) scaffold degradation was observed, it is expected that the scaffold mechanical properties remained constant throughout the culture period.
Taken together, we observed more collagen and more predominant collagen type I in the cyclically stretched samples, but negligible differences in lysyl oxidase (LOX) expression (a catalyzer of covalent fiber crosslinking important for the stabilization of collagen and elastin networks) in comparison to the other experimental groups at day 20 (Figure 4A). Based on these findings, we conclude that the increased axial and circumferential stiffness in the cyclically stretched samples should be attributed to differences in the matrix composition (i.e., quantity and type). Where cyclic stretch stimulated the formation of a stiffer matrix, containing more and predominantly collagen type I, shear stress stimulated the deposition of collagen type III and the secretion of MMP-1 and TIMP-1 during the 20 d of culture. Our observations therefore indicate that shear stress plays a key regulating role in the progression of tissue growth and remodeling, especially in the presence of cyclic stretch.

2.6. Future Perspectives

This study aids in understanding the mechanobiological processes that underlie early material-driven tissue regeneration in situ. We opted for a physiological coculture with the inherent difficulty that the cell ratio varied with time, due to (myo)fibroblast proliferation and a (relative) reduction in the macrophage number (e.g., as a result of apoptosis and potential...
transdifferentiation), complicating the direct translation of our observations to an in vivo situation. It would be interesting to explore the impact of temporal variations in resident cell populations and, given that our model enables the study of primary human cells, investigate potential donor-to-donor variability by using cells from specific donor cohorts.

The (myo)fibroblasts that were used in our model are a consistent vascular cell source known to play a considerable role in tissue deposition and remodeling, with the ability to accommodate to extreme loading conditions.\[65,66\] Nevertheless, as these cells originate from the venous circulation, they may have been “overstressed” as they are not “primed” to arterial loading conditions.\[37,67\] Potential overstressing of the cells could have contributed to the more synthetic phenotype that we observed in the presence of hemodynamic loads, with only subtle differences between the applied loading regimes. It will therefore be instructive to repeat and prolong the experiments with human cells from various origins (e.g., arteries, bone marrow) to elucidate the time dependent impact of hemodynamic loading on various cell populations.

3. Conclusion

In summary, by employing a dynamic 3D human in vitro model to investigate material-driven in situ tissue engineering, we successfully discriminated the distinct and combined effects of cyclic stretch and shear stress during the proliferative phase of this process (Scheme 1). Most importantly, during this early phase, shear stress can be recognized as a stabilizing factor in cyclic stretch-induced matrix deposition, highlighting that both hemodynamic loads should be considered in the design of resorbable vascular grafts. Our approach paves the way for future research, in which this study should be extended to unravel the underlying principles of late-stage vascular tissue homeostasis, for example, by focusing on the underlying mechanisms of the mechanoresponse, the individual and combined responses of cells (e.g., other immune and progenitor cells) to variable hemodynamic loading regimes, as well as the changes that occur after endothelialization. This knowledge is critical to identify optimal scaffold designs that improve the long-term clinical performance of in situ tissue engineered blood vessels.

4. Experimental Section

**Scaffold Preparation:** Isotropic 3 mm (inner diameter) scaffolds were electrospun from polymer solutions containing 15% (w/w) bisurea-modified poly-ε-caprolactone (PCL-BU, SyMO-Chem, Eindhoven, the Netherlands) and 85% (w/w) chloroform (Sigma; 372978). The solutions were delivered at 40 µL min\(^{-1}\) via a charged needle (16 kV) on a negatively charged, rotating mandrel (−1 kV, 500 rpm, 3 mm diameter) in a climate-controlled cabinet (23 °C and 30% humidity, IME Technologies, Geldrop, the Netherlands). The distance between the needle and the mandrel was kept constant at 16 cm. The resulting scaffolds were dried overnight under vacuum to remove solvent remnants and placed over silicone tubing (2.8 mm outer diameter) as previously described.\[34\] The scaffold microarchitecture (i.e., fiber morphology, diameter, and organization) was assessed from scanning electron microscopy (SEM) images (Quanta 600F; FEI, Hillsboro, OR) and the scaffold mechanical properties were characterized from biaxial stress–strain curves (CellScale Biomaterial Testing, Waterloo, Canada) as described below. Prior to cell seeding, the silicone-mounted scaffolds were sterilized by UV exposure (30 min per side), prewetted with water, and incubated overnight at 37 °C in complete medium (1:1 Roswell Park Memorial Institute-1640: advanced Dulbecco’s modified Eagle (RPMI-1640:a-DMEM) medium (Gibco; ref A10491 and ref 124910) with 10% fetal bovine serum (FBS, Greiner, Alphen aan den Rijn, the Netherlands), 1% penicillin/streptomycin (P/S, Lonza, Basel, Switzerland; DE17-602E),

**Scheme 1.** Graphical synopsis of the main findings of the study. A) Cyclic stretch stimulates matrix growth and cell proliferation, and inhibits shear stress-driven secretion of pro-inflammatory cytokines (MCP-1 and IL-6), while both loads contribute to the secretion of MMP-1 and anti-inflammatory IL-10. B) At the tissue level, shear stress (τ) abrogates stretch-induced matrix growth and stimulates, especially in the presence of cyclic stretch (λ), collagenous remodeling, resulting in a matrix with directed collagen fibers.
0.5% GlutaMax ( Gibco; ref 35050), and 0.25 mg mL\(^{-1}\) -l-ascorbic acid 2-phosphate (Sigma; A8960) to allow for protein adsorption.

**Human Vena Saphena (HVSC) Cell Culture:** HVSCs were isolated from a single human donor after coronary by-pass surgery conforming to well-established protocols,\(^{[48]}\) in accordance to the Dutch advice for secondary-use material. The cells were cultured in complete a-DMEM (10% FBS, 1% GlutaMax, and 1% P/S) in a standard culture incubator (37 °C, 5% CO\(_2\)) and passed at 80% confluence. Media was refreshed every 3–4 d. For experiments, cells at passage 7 were used. Cell phenotype was assessed via qPCR.

**Peripheral Blood Mononuclear Cell Isolation and Monocyte Purification:** Human peripheral blood mononuclear cells (hPBMCs) were isolated from two different buffy coats of healthy donors (purchased from Sanquin Blood Supply Foundation, Nijmegen, the Netherlands) using density gradient centrifugation on iso-osmotic medium (density: 1.077 g mL\(^{-1}\), Lymphoprep, Stemmcell Technologies, Köln, Germany).

In short, buffy coats were diluted in 0.6% sodium citrate (Sigma-Aldrich, C7254) in phosphate-buffered saline (PBS), after which the solutions were carefully layered on top of an iso-osmotic medium (density: 1.077 g mL\(^{-1}\), Lymphoprep, Axis Shield). The layered samples were centrifuged (21000 rpm for 30 min) to extract the plasma and the erythrocytes from the white blood cell fraction. The hPBMCs were washed, resuspended in freezing medium (RPMI-1640 supplemented with 20% FBS, 1% P/S, and 0.1% dimethyl sulfoxide (Merck Millipore)), and cryopreserved in liquid nitrogen until further use. For each donor, hPBMCs were characterized via flow cytometry (Guava easyCyte 6HT, Merck Millipore; Table S2, Supporting Information). Prior to cell seeding, the hPBMCs were thawed, resuspended in complete RPMI medium (10% FBS, 1% P/S), and counted. The monocyte-cell fraction was isolated from the hPBMC fraction via negative selection using the MACS pan-monocyte isolation kit (Miltenyi Biotec) according to the supplier’s instructions. Monocyte purity (i.e., lymphocyte contamination expressed as the CD3/CD14 ratio), and the classical (CD14+ CD16), non-classical (CD14+, CD16+), and intermediate (CD14+, CD16+) monocytes were quantified via flow cytometry (Table S2, Supporting Information).\(^{[69,70]}\)

**Cell Seeding and In Vitro Culture:** Human monocytes and HVSCs were seeded in a 2:1 ratio using bovine fibron as a cell carrier.\(^{[71]}\) Prior to seeding, medium was removed from the scaffold. To ensure uniform seeding, a suspension of bovine fibrinogen (10 mg mL\(^{-1}\) bovine thrombin (10 IU mL\(^{-1}\)), bovine albumin (BSA) in PBS (10% FBS, 1% P/S), and 0.25 mg mL\(^{-1}\) Poly-L-lysine (Sigma; P8630), bovine thrombin (10 IU mL\(^{-1}\), Sigma; T4648), and cells (30x10\(^{6}\) monocytes cm\(^{-3}\) and 15x10\(^6\) HVSCs cm\(^{-3}\)) was carefully dripped and absorbed over the long axis at two opposing sides into the prewetted scaffold. Fibron was allowed to polymerize for 60 min in an incubator (37 °C, 5% CO\(_2\)). Cell distribution 3 d after in vitro culture was evaluated from samples containing human monocytes and HVSCs that were, respectively, Cell Tracker Orange (Invitrogen Molecular Probes, C34551) and Cell Tracker Green (Invitrogen Molecular Probes, C2925) prior to seeding. The whole-mounts were visualized using a confocal laser scanning microscope (Leica TCS SP5X with a 40×/0.95 HC PL Fluotar lens).

**Gene Expression Analysis:** Prior to RNA extraction, samples were reduced to a fine powder with a micro-disembrator and RLT buffer containing β-mercaptoethanol (Sigma; M3348) was added to further lyse the samples. Successively, RNA was extracted with the Qiagen RNeasy kit according to the manufacturer’s description. A 30 min DNase incubation step (Qiagen; 74106) was performed to exclude genomic DNA contamination. RNA quantity and purity, and RNA integrity were assessed using, respectively, a spectrophotometer (NanoDrop, ND-1000, Isogen Life Science, the Netherlands) and gel electrophoresis. cDNA was synthesized in a thermal cycler (C1000 Touch, Bio-Rad) using a reaction solution consisting of RNA (100 or 200 ng) supplemented with 1 μL random primers (50 ng μL\(^{-1}\), Promega, C1181), 1 μL dNTPs (10 × 10\(^{-3}\) M, Invitrogen), 2 μL 0.1 M DTT, 4 μL 5 × first strand buffer, 1 μL M-MLV Reverse Transcriptase (200 U μL\(^{-1}\), Invitrogen, 28025-013, Breda, the Netherlands), and RNase-free ultrapure water (ddH\(_2\)O) up to a final volume of 11 μL. The reaction solution was exposed to the following thermal protocol: 65 °C (5 min), on ice (2 min) while adding the enzyme mixture, 37 °C (2 min), 25 °C (1 min), 37 °C (5 min), and 70 °C (15 min). Genomic contamination in the cDNA was assessed with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers, conventional PCR, and gel electrophoresis.
After this check, qPCR was performed employing the gene-specific primer sequences listed in Table S4 (Supporting Information). GAPDH was identified as most stable and therefore selected as reference gene. Gene expression levels involved in inflammatory processes, cell phenotype, tissue formation, and remodeling were evaluated by adding 500 × 10^{-6} µL primer mix, 5 µL SYBR Green Supermix (Bio-Rad; 170-8886), and an additional 1.75 µL ddH2O to 3 µL cDNA (30× or 100× diluted). Solutions were exposed to the following thermal protocol: 95 °C (3 min), 40 cycles of 95 °C (20 s), 60 °C (20 s), 72 °C (30 s), 95 °C (1 min), and 65 °C (1 min), completed with a melting curve measurement. C\(_{\text{t}}\) values were normalized for the reference gene and the 2\(^{-}\Delta\Delta\text{Ct}\) formula was applied to calculate relative expression levels.[79]

ELISA: Secreted cytokines were quantified to obtain insight in the generated early (immune) profiles of the different experimental conditions (Table S1, Supporting Information). Cytokines were quantified at the Multiplex core facility of the Laboratory for Translational Immunology of the University Medical Centre Utrecht (UMCU), the Netherlands, using a multiplex immunoassay built upon Luminex technology. In short, supernatants were incubated (1 h) with antibody-bound MagPlex microspheres (Bio-Rad, Hercules, CA), succeeded by an incubation step with biotinylated antibodies (1 h), and a subsequent incubation with phycoerythrin-conjugated streptavidin (10 min), which was diluted in high performance ELISA (HPE) buffer (Sanquin). Data acquisition was executed using a FLEXMAP 3D system controlled with xPONENT 4.1 software (Luminex, Austin, TX), and analyzed by fitting a 5-parametric curve with Bio-Plex Manager software (version 6.1.1., Biorad).

Biochemical Assays: The engineered tissue constructs collected at day 20 were lyophilized, weighed, and reduced to a fine powder prior to determination of glycosaminoglycan (GAG), hydroxyproline (HYP), and DNA content. To obtain the power, the constructs, together with 3 mm Ø beads (Sartorius, Goettingen, Germany), were added to Naalgene cryogenic vials (Thermo Scientific; 5000-0012), frozen in liquid nitrogen, and disrupted with a micro-dismembrator (Sartorius, 2 × 30 s at 3000 rpm). Subsequently, 500 µL of digestion buffer (100 × 10^{-3} M phosphate buffer (pH = 6.5), 5 × 10^{-3} M L-cysteine (C-1276), 5 × 10^{-4} M ethylene-di-amine-tetra-acetic acid (EDTA, ED2SS), and 140 µg mL\(^{-1}\) papain (P4762), all from Sigma-Aldrich) was added, and the suspensions were transferred to new Eppendorf tubes for overnight digestion (16 h at 60 °C). Following digestion, samples were vortexed and centrifuged at 12000 rpm for 10 min to precipitate scaffold remnants. The supernatant was used for DNA, GAG, and HYP quantification.

DNA content was quantified employing the Qubit dsDNA BR assay kit (Life Technologies, Carlsbad, California, USA) and the Qubit fluorometer (Life Technologies) according to the manufacturer’s description and corrected for total dry mass. GAG and HYP content were determined as indicators of ECM formation. GAG quantities were determined using a modified dimethyl methylene blue (DMMB) assay,[80] Shark chondroitin sulfate (Sigma; C4348) was included as a standard. In short, 40 µL of the supernatants and prepared standards were transferred (in duplicate) to a 96-well plate. Subsequently, DMMB solution (150 µL) was added and absorbance (540 nm) was measured using a microplate reader (Synergy HTX; Biotek). To quantify HYP content, as a measure of mature collagen, digested samples were first hydrolyzed using 16 m sodium hydroxide (Merk; B1438798). Then, HYP content was quantified with a Choloramin-T assay, including trans-4-hydroxyproline as a reference (Sigma; H5334).[81] GAG and HYP values were normalized to total dry mass to assess overall tissue formation.

Mechanical Analysis: The mechanical properties of bare scaffolds and constructs at day 20 were quantified using a biaxial tensile setup (CellScale Biomaterial Testing, Waterlo, Canada). Tests were performed in PBS at 37 °C. After 10 uniaxial stretch cycles (at a stretch magnitude of 1.10) in the two orthogonal directions (i.e., axial and circumferential), the samples were equibiaxially stretched at a rate of 100% min\(^{-1}\) while recording forces with a 1500 mN load cell. Graphite particles sprayed onto the sample’s surface facilitated optical strain analysis. Sample thickness was measured with a digital microscope (Keyence VHX-500FE).

Stress–stretch curves were derived from the force and displacement measurements, assuming incompressibility and plane-stress conditions. Elastic moduli were computed as the slope of the stress–stretch curves at 10% strain as a measure of stiffness.

Statistical Analysis: All data are reported as mean ± standard deviation unless stated otherwise. In addition, ELISA and qPCR data are displayed as heat maps using Heatmapper, a freely available web tool.[79] Here, each cytokine/gene expression is transformed into a z-score (i.e., the number of standard deviations from the mean expression) and converted to a color map. To test for significant differences, the nonparametric Kruskal-Wallis test with a Dunn’s multiple comparison test was performed using Prism (GraphPad, La Jolla, CA, USA). Prior to the statistical analysis, C\(_{\text{t}}\) values from the qPCR analysis were logarithmically transformed. Differences were considered significant for p-values <0.05.

Supporting Information
Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest
The authors declare no conflict of interest.

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