Monocyte/macrophage cytokine activity regulates vascular smooth muscle cell function within a degradable polyurethane scaffold

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A B S T R A C T
Tissue engineering strategies rely on the ability to promote cell proliferation and migration into porous biomaterial constructs, as well as to support specific phenotypic states of the cells in vitro. The present study investigated the use of released factors from monocytes and their derived macrophages (MDM) and the mechanism by which they regulate vascular smooth muscle cell (VSMC) response to a VSMC–monocyte co-culture system within a porous degradable polyurethane (D-PHI) scaffold. VSMCs cultured in monocyte/MDM-conditioned medium (MCM), generated from the culture of monocytes/MDM on D-PHI scaffolds for up to 28 days, similarly affected VSMC contractile marker expression, growth and three-dimensional migration when compared to direct VSMC–monocyte co-culture. Monocyte chemotactic protein-1 (MCP-1) and interleukin-6 (IL-6) were identified as two cytokines present in MCM, at concentrations that have previously been shown to influence VSMC phenotype. VSMCs cultured alone on D-PHI scaffolds and exposed to MCP-1 (5 ng ml⁻¹) or IL-6 (1 ng ml⁻¹) for 7 days experienced a suppression in contractile marker expression (with MCP-1) and increased growth (with MCP-1) compared to no cytokine medium supplementation. These effects were also observed in VSMC–monocyte co-culture on D-PHI. Neutralization of IL-6, but not MCP-1, was subsequently shown to decrease VSMC growth and enhance calponin expression for VSMC–monocyte co-cultures on D-PHI scaffolds for 7 days, implying that IL-6 mediates VSMC response in monocyte–VSMC co-cultures. This study highlights the use of monocytes and their derived macrophages in conjunction with immunomodulatory biomaterials, such as D-PHI, as agents for regulating VSMC response, and demonstrates the importance of monocyte/MDM-released factors, such as IL-6 in particular, in this process.

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1. Introduction

Small diameter vascular grafts (<6 mm internal diameter) are prone to failure due to complications arising from thrombogenesis and neointimal hyperplasia (associated with vascular smooth muscle cell (VSMC) migration and proliferation). VSMCs can shift between contractile and synthetic phenotypes depending on the stimuli to which the VSMCs are exposed [1]. For strategies related to the in vitro development of tissue-engineered vascular grafts, the synthetic phenotype is initially desirable as it is associated with VSMC proliferation and matrix synthesis, which leads to a faster maturation of the vascular tissue in vitro [1,2]. Accelerating the rate of cell and tissue growth into biomaterials intended for use as tissue engineered constructs is important because, typically, in vitro strategies to develop a vascular graft with sufficient cellular and mechanical properties have required prolonged culture times (>4 weeks) [2,3]. These extended culture times increase the risk of bacterial contamination, introduce labor-intensive costs for the culture practices and ultimately increase the wait time for a patient to receive treatment if the graft is being prepared using an autologous cell source.

A commonly used strategy to promote a desired VSMC phenotypic state involves the use of growth factor stimulation [1,4–6]. Transforming growth factor-β1 (TGF-β1) is one example of a biological factor that is used for such purposes and has been able to promote the contractile VSMC phenotype, which is characterized by the expression of α-smooth muscle actin (α-SMA), calponin and smooth muscle myosin heavy chain (SMMHC) [5,7,8]. Moreover, TGF-β1 has been shown to support an increase in matrix production, such as collagen and elastin [9]. Despite these effects, TGF-β1 exposure is also associated with the inhibition of cell proliferation [4,6], hindering the necessary expansion of VSMCs during
the in vitro development of tissue engineered vessels. In contrast to TGF-β1, growth factors that can be used to promote the synthetic VSMC phenotype include platelet-derived growth factor-BB (PDGF-BB), epidermal growth factor (EGF) and fibroblast growth factor (FGF), all of which promote the growth of VSMCs [1]. Despite their promise, the use of exogenous growth factors can add significant cost and time to the production of a tissue engineered graft, particularly if its supplementation is required over long culture times. Furthermore, the issue of endotoxin contamination associated with the use of recombinant proteins [10] can ultimately lead to impaired in vivo wound healing responses [11] in addition to affecting the phenotype of cells cultured in vitro [12,13].

An alternative strategy for modulating VSMC phenotype involves the use of monocytes and monocyte-derived macrophages (MDM). These are early cell types present following biomaterial implantation [14], and depending on their phenotypic state they can be positive contributors to tissue regeneration and remodeling [15,16]. Monocytes can easily and relatively non-invasively be isolated from the mononuclear fraction of peripheral blood and can act as an endogenous source of stimulatory cytokines and growth factors, which can be used in place of growth factor medium supplementation. The ability of monocytes and MDM to provide stimuli to induce a desired phenotype has also been used in several stem cell differentiation strategies, such as promoting osteoblast formation from mesenchymal stem cells (MSCs) [17], as well as regulating their growth [18]. These studies highlight the successful use of monocytes in modulating the phenotypic state of other cells. Such strategies require the use of a biomaterial that supports a particular MDM state, which in the case of vascular tissue engineering is one that is supportive of VSMC growth and function, whether it is through direct cell–cell interactions or the release of particular cytokines and growth factors [19–22].

The phenotype of monocytes and MDM is heterogeneous and can vary depending on different environmental stimuli, both in vitro and in vivo [23]. Biomaterial surface properties [24,25] and the adsorbed protein layer [26,27] are two important factors influencing the monocyte's activation state. Biomaterials that have been designed to elicit a desirable activation state of the monocyte/MDM can harness the potential of these cells to act as stimulatory agents for other cell types through the release of appropriate cytokines and growth factors. It has been shown that the M2 (anti-inflammatory) macrophage state is associated with the promotion of VSMC proliferation [28] and that supernatants derived from M2 vs. M1 (or classically activated, pro-inflammatory) macrophages support enhanced migration of C2C12 myoblasts [15].

Monocyte/MDM dysfunction with respect to their activation in healthy tissue has been associated with adverse outcomes. Co-culture systems with monocytes/MDM that are induced into a pro-wound healing, anti-inflammatory state by a biomaterial thus hold promising potential in tissue engineering [15,16]. With particular regard to vascular tissue engineering, monocytes have successfully been shown to increase graft patency when pre-seeded on grafts composed of a non-woven poly-glycolic acid mesh with a 50:50 copolymer sealant of poly-e-caprolactone–l-lactide [29]. A critical aspect of the monocyte's role in this process is thought to occur through the release of secreted factors, of which MCP-1 was shown to be of particular importance. MCP-1 aids in the recruitment of host monocytes following implantation, which are then able to contribute to the regeneration of vascular tissue [30].

Previous work with a degradable polar hydrophobic ionic polyurethane (D-PHI) has illustrated the ability of this material to support a two-dimensional VSMC–monocyte co-culture system for prolonged culture times with limited inflammatory cytokine production [31] as well as being able to support tissue infiltration with minimal inflammation relative to PLGA when implanted subcutaneously in rats [32]. The objective of the present study was to investigate the effect of monocyte/MDM-released factors on VSMC response within three-dimensional (3-D), porous D-PHI scaffolds with regards to growth, 3-D migration and contractile marker expression, and to identify key proteins that may contribute to specific effects on VSMC phenotype observed in a VSMC–monocyte co-culture system on D-PHI scaffolds. These factors were compared to the observations seen within the co-culture in order to assess the relative role of monocyte/MDM-released factors vs. cell–cell interactions are primarily responsible for the observed VSMC activity in monocyte–VSMC co-culture on 3-D D-PHI scaffolds.

2. Materials and methods

2.1. D-PHI scaffold preparation

D-PHI scaffolds were prepared using previously established methods [33,34]. Briefly, a divinyl oligomer (DVO), methacrylic acid (MAA) and methyl methacrylate (MMA) were mixed in a 1:5:15 M ratio along with the initiator benzoyl peroxide (0.032 mol mol−1 vinyl group) and 10 wt.% polyethylene glycol and 65 wt.% sodium bicarbonate as porogens. The resulting mixture was packed into Teflon molds and allowed to cure for 24 h at 110 °C. Porogen leaching was performed by 14 × 2 h periods of sonication with ddH2O, with water changed between each sonication period. This process resulted in a porous scaffold with 79 ± 3% porosity [33]. Scaffolds used for cell culture were in the form of 6.5 mm diameter discs with a thickness of ~1 mm, such that they fit within the wells of a 96-well plate.

2.2. Culture of vascular smooth muscle cells

SMGMM−2 coronary artery smooth muscle cells (Lonza, CC-2583) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. Cells were obtained from Lonza at passage 3 and were further passaged and used between passage 7 and 9 with at least one passage occurring between thawing and seeding. For experiments where VSMCs were cultured alone on D-PHI scaffolds, 100,000 VSMCs (100 μl of a 1,000,000 VSMCs ml−1 suspension) were seeded per scaffold in 50:50 RPMI–1640:DMEM medium, containing 10% FBS, 1% penicillin/streptomycin and 0.35 mM l-glutamine.

2.3. Isolation and culture of monocytes

Monocytes were isolated from whole human peripheral blood obtained from healthy volunteers (University of Toronto ethics approval #22203). Approximately 100 ml of blood was collected into ethylenediaminetetraacetic acid (EDTA)–containing Vacutainers (Becton Dickenson, Toronto, Canada). The blood was layered onto Histopaque 1077 (Sigma) and the blood components separated by centrifugation to isolate the mononuclear cell fraction. A series of washes was performed, after which cells were re-suspended in 50:50 RPMI–1640:DMEM medium, containing 10% FBS, 1% penicillin/streptomycin and 0.35 mM l-glutamine and counted. For monocyte-only cultures on D-PHI scaffolds, an estimated 200,000 monocytes were seeded per scaffold (100 μl of a 1,000,000 cell ml−1 solution, of which 20% were previously shown to be monocytes, with mostly monocytes remaining after performing a medium change 2 h post-seeding, and with 95% of the cells previously shown to be CD68+ [monocytes and MDM] after 72 h, with only 3% staining positive for CD3 [T-cells]) [35,36].
2.4. Comparison of monocyte/MDM-conditioned medium (MCM) to direct co-culture

VSMCs were co-cultured with monocytes on D-PHI scaffolds in a 1:2 ratio (100,000 VSMCs and 200,000 monocytes per scaffold) by combining VSMC and monocyte suspensions such that 100 μl of 50:50 RPMI-1640:DMEM medium (with 10% FBS, 1% penicillin/streptomycin, and 0.35 mM L-glutamine) contained 100,000 VSMCs and 200,000 monocytes, which was then seeded onto D-PHI scaffolds. Co-cultures were maintained in the 50:50 RPMI-1640:DMEM medium for the duration of the experiment. To generate MCM, supernatants from monocyte/MDM-only cultures were collected during each medium change and centrifuged for 5 min at 1400 rpm to remove any non-soluble components. The resulting supernatant was used to culture VSMC-seeded D-PHI scaffolds (100,000 VSMCs per scaffold). This supernatant was retrieved from monocyte-only cultures on D-PHI scaffolds that were run in parallel with co-culture vs. VSMC+MCM experiments, such that VSMCs cultured in MCM on a given day were exposed to the monocyte/MDM released factors obtained from monocyte-only cultures on the very same day of culture.

2.5. Effects of cytokines on VSMC response in monoculture and in co-culture with monocytes

To evaluate the effect of specific cytokines on VSMCs, VSMCs were first seeded on D-PHI scaffolds (100,000 VSMCs per scaffold) and left for 24 h to allow initial cell attachment. After 24 h, monocyte chemotactic protein-1 (MCP-1, 5 ng ml⁻¹, Peprotech), interleukin-6 (IL-6, 1 ng ml⁻¹, Peprotech) or granulocyte macrophage-colony stimulating factor (GM-CSF, 0.25 ng ml⁻¹, Peprotech) were supplemented into culture medium and replenished with medium changes every 48 h. To assess the role of MCP-1 and IL-6 in VSMC–monocyte co-culture on D-PHI scaffolds, antibodies for MCP-1 (Human CCL2/JE/MCP-1 antibody monoclonal mouse IgG1, 4.5 μg ml⁻¹, R&D systems) or IL-6 (Human IL-6 antibody monoclonal mouse IgG1, 1 μg ml⁻¹, R&D systems) were supplemented into culture medium after allowing an initial 24 h period for cell attachment post-seeding (100,000 VSMCs and 200,000 monocytes per scaffold). Antibodies were supplemented with each medium change every 48 h.

2.6. DNA mass quantification

Scaffolds were removed from 96-well plates, placed in micro-centrifuge tubes and rinsed twice with phosphate buffered saline (PBS). The scaffolds were then mixed using a needle, and 300 μl of lysis buffer (100 mM Tris–HCl, 100 mM NaCl, 25 mM EDTA (TNE), 0.1% SDS, pH 8.0) was subsequently added. Samples were vortexed briefly and incubated at 65 °C for 60 min with intermittent vortexing. To quantify the DNA mass extracted from the scaffolds, 10 μl of the lysates were loaded into black flat-bottom 96-well plates containing 1 × TNE solution with Hoechst 33258 DNA stain. Fluorescence was read using an FL600 Microplate Fluorescence Reader with excitation and emission wavelengths of 360 and 460 nm, respectively. To determine the amount of DNA associated with a given fluorescence intensity, sample values were compared to a standard curve prepared from DNA standards of calf thymus DNA (Sigma).

2.7. Immunoblotting

Whole cell lysates (as obtained in Sec. 2.6) were quantified for protein concentration using a modified Bradford assay (Bio-Rad Protein Assay Dye Reagent) using a bovine serum albumin (BSA) standard (Bio-Rad). Volumes equivalent to 2 μg of total protein were separated by SDS–PAGE using a Protein II Cell System (Bio-Rad) by loading onto a 5% stacking and 12% separating gel. Proteins were then transferred to nitrocellulose membranes for 90 min at 130 V, washed twice for 10 min in Tris-buffered saline-Tween 20 (TBST; 10 mM Tris, 0.1 M NaCl, 0.1% Tween-20, pH 7.5), and blocked for 1 h with 5% non-fat dry skim milk in TBST. Membranes were subsequently incubated with primary antibody solutions for α-SMA (mouse monoclonal to α-SMA, Abcam, 1:2000 in 5% BSA in TBST) or calponin (rabbit monoclonal [EP798Y] to calponin, Abcam, 1:10,000 in 5% BSA in TBST) overnight at 4 °C. Primary antibodies were also used for GAPDH (mouse monoclonal to GAPDH clone 6C5, Millipore, 1:2000 in 5% milk in TBST) or β-actin (beta-actin [13E5] rabbit mAb, Cell Signaling Technology, 1:1000 in 5% BSA in TBST) to serve as loading controls. Membranes were subsequently washed eight times for 5 min in TBST, incubated with horseradish peroxidase conjugated secondary antibodies (1:2000 goat anti-rabbit IgG in 5% milk in TBST, BioShop or 1:2000 goat anti-mouse IgG in 5% milk in TBST, Thermo Scientific) for 1 h, followed by eight more 5 min washes. Blots were visualized with a chemiluminescence detection kit (Pierce) with images obtained using a ChemiDoc™ XR Imager and densitometric analysis performed using Quantity One software (Bio-Rad).

2.8. Histology

At 1, 7, 14 and 28 day time points, scaffolds were washed twice with PBS (pH 7.4) and subsequently fixed with a 4% paraformaldehyde solution on ice for 20 min. Samples were then stored in 15% sucrose at 4 °C overnight, followed by 30% sucrose until scaffolds were paraffin-embedded. 20 μm sections of paraffin-embedded samples were stained with hematoxylin and eosin (H&E). For each condition, 18 sections were taken and analyzed to ensure images shown were representative of each condition.

2.9. Boyden chamber assay

Cell migration was assessed using a 96-well BME Cell Invasion Assay (Trevigen). 24 h prior to starting the migration assay, VSMCs were serum-starved (DMEM supplemented with 0.5% FBS) to allow for receptor expression. Cells were subsequently harvested and resuspended in 0.5% FBS supplemented DMEM. 10,000 VSMCs were added to the top chamber of each well, with 150 μl of medium supplemented with MCP-1 (5 ng ml⁻¹), IL-6 (1 ng ml⁻¹) or GM-CSF (0.25 ng ml⁻¹) added to the bottom chamber. Cells were then incubated for 18 h, with 0.5% FBS and 10% FBS containing medium used as negative and positive controls, respectively. Migrated cells were then dissociated from the bottom of the membrane, stained with calcein-AM and fluorescence intensity was read using a microplate fluorescence reader, with excitation and emission wavelengths of 485 and 520 nm, respectively.

2.10. Cytokine antibody array

An antibody array (RayBio Human Cytokine Antibody Array G Series 3) was purchased from RayBiotech Inc. and performed according to the manufacturer’s instructions. Sample releasates were obtained from monocyte-only seeded D-PHI scaffolds at 1 and 4 and days post-seeding, with 24 h given for cytokine accumulation in the medium prior to collection. As a negative control, medium supplemented with 10% FBS was used to determine background intensity contributed from cytokines in non-monocyte/MDM conditioned medium. The intensity readings obtained for each marker were normalized to the positive control contained within the array, such that each value is a fraction of the maximum possible signal intensity.
2.11. ELISAs

Enzyme-linked immunosorbent assays (ELISAs) were performed for human MCP-1, IL-6, GM-CSF and IL-13 as per protocols provided with the kits purchased from the manufacturer (eBio-science). Supernatants were collected from samples 24 h post-medium change, such that cytokines were allowed to accumulate in culture medium for 24 h prior to collection.

2.12. Statistical analysis

Statistical analysis was performed using SPSS Statistics 17.0 (SPSS Inc., Chicago, IL) by analysis of variance (ANOVA) or an independent samples t-test where appropriate, with statistical significance reported for $P < 0.05$. A minimum of three blood donors were used for all experiments involving monocytes.

3. Results and discussion

The objective of the present study was to investigate the interplay between monocyte/MDM-released factors on VSMC–monocyte co-cultures when grown in D-PHI scaffolds in order to assess their potential role in regulating VSMC growth, 3-D migration, and contractile marker expression, and to identify some of the key proteins released by monocytes/MDM influencing the observed VSMC response on D-PHI scaffolds.

3.1. Monocyte/MDM-released factors regulate VSMC response similar to direct co-culture on D-PHI scaffolds

In order to isolate the effects of monocyte/MDM-released factors from the effects due to cell–cell contact between VSMCs and monocytes as they undergo a phenotypic change to macrophages, VSMCs were cultured in the presence of MCM. MCM was generated from monocyte-only seeded D-PHI scaffolds that were run in parallel with all experiments. This allowed for the isolation of monocyte/MDM-released factors produced at the same time point as a given VSMC culture condition. This protocol is important since monocyte phenotype will change over time on a biomaterial [15] and this will alter the make-up of the conditioned medium, and therefore is anticipated to affect VSMC response differently. VSMC growth (DNA mass quantification) and contractile marker expression ($\alpha$-SMA, calponin) were compared between VSMCs cultured in MCM to those cultured in regular medium and VSMCs in co-culture with monocytes/MDM.

Co-culturing monocytes/MDM with VSMCs was shown to increase DNA from day 7 onwards when compared to the sum of DNA from individual VSMC (V) and monocyte (M) monocultures on D-PHI scaffolds. The total DNA in co-culture is compared to the sum of the two monocultures, both when VSMCs are cultured in regular medium (V) and when they are cultured in the presence of MCM (V+MCM). Data represent the mean ± SE, $n = 9$, with three distinct donors used for samples containing monocytes. (B) Comparison of DNA mass for VSMCs cultured on D-PHI scaffolds in the presence of MCM or in regular medium. Data represent the mean ± SE, $n = 9$, with three distinct donors used for samples containing monocytes. $^* P < 0.05$.

![Fig. 1. DNA mass quantification for (A) VSMC–monocyte co-culture (C) samples compared to the sum of individual VSMC (V) and monocyte (M) monocultures on D-PHI scaffolds. The total DNA in co-culture is compared to the sum of the two monocultures, both when VSMCs are cultured in regular medium (V) and when they are cultured in the presence of MCM (V+MCM). Data represent the mean ± SE, $n = 9$, with three distinct donors used for samples containing monocytes. (B) Comparison of DNA mass for VSMCs cultured on D-PHI scaffolds in the presence of MCM or in regular medium. Data represent the mean ± SE, $n = 9$, with three distinct donors used for samples containing monocytes. $^* P < 0.05$.](image-url)
microenvironments [23]. It should be noted that over the 28 days of culture, MDM number was shown to decrease while VSMC number increased in monoculture (Fig. 1), suggesting that by day 28 the 1:2 VSMC:monocyte had changed to contain more VSMCs relative to monocytes.

A suppression of the contractile phenotypic markers α-SMA and calponin was observed in VSMC–monocyte co-culture over time and in particular between day 1 and day 28 of culture, in contrast to the similar expression of these markers at day 1 and day 28 when VSMCs were in monoculture on D-PHI scaffolds (Fig. 2). MCM had a similar effect on VSMC calponin expression as was observed for direct co-culture, suppressing levels below those measured after initial seeding, while there was no suppressive effect observed between day 1 and day 28 for the expression of α-SMA. This may suggest that the biological factors regulating calponin and α-SMA expression are not the same for the cells cultured on D-PHI, and that while the former is potentially mediated by the proteins released from monocytes/MDM, the latter may be more affected by direct co-culture of the two cell types on D-PHI scaffolds.

Since the loss of contractile phenotypic marker expression and an enhanced proliferative phenotype is also associated with cell migration [1], sections of D-PHI scaffolds were stained with H&E in order to visualize the extent of VSMC infiltration into the porous D-PHI scaffolds (Fig. 3). By later time points (>14 days), there was a notable increase in the presence of VSMCs within the middle portion of the D-PHI scaffolds, which was an effect also observed with MCM. In contrast, VSMCs cultured in regular medium formed a monolayer on the surface with little to no cellular penetration into the scaffold observed by day 14 and beyond.

3.2. Factors released by monocytes/MDM during culture on D-PHI scaffolds

To provide initial insight into the proteins present within MCM that may be contributing to the regulation of VSMC phenotype and cell migration, a cytokine antibody array was used to screen 42 potential markers (Fig. 4). As a control, 10% FBS containing medium was also screened to determine background contributions from regular medium. Of the identified cytokines and growth factors, several stood out for their role in regulating VSMC growth and migration, while others provided important insight into the shifting activation state of the monocytes when cultured on D-PHI scaffolds. IL-1β, a pro-inflammatory cytokine involved in the acute immune response [39], was detected at day 1 but was no longer present by day 7. Furthermore, IL-13, a cytokine that is used to polarize macrophages towards the M2, or anti-inflammatory, state [40], was not detected at day 1 but was upregulated at day 7. These findings provide further support for the ability of D-PHI to promote a monocyte/MDM activation state that is anti-inflammatory.

The ability of a biomaterial to support a phenotypic transition from a pro-inflammatory to pro-wound healing macrophage/MDM
enhanced fibrotic scarring \[42–44\]. Furthermore, persistence of healing, while excessive levels have been associated with of these cytokines has been associated with impaired wound healing, while excessive levels have been associated with a different secretory profile (i.e. IL-10, IL-1ra) [46].

Cytokines detected by the array screen that have been shown to promote VSMC migration and proliferation included MCP-1 and IL-6 [47–51]. In addition, GM-CSF was of interest for its known role in promoting extracellular matrix production [52]. ELISAs were performed to determine the concentration of these cytokines in MCM and their temporal dependence. MCP-1, IL-6, GM-CSF and IL-13 all showed different temporal trends in their concentrations (Fig. 5). The concentration ranges measured for MCP-1 (5–25 ng ml\(^{-1}\)) and IL-6 (1–10 ng ml\(^{-1}\)) include values that have been shown in the literature to influence both VSMC proliferation and migration [47,50,51], making these two cytokines attractive candidates for investigating the regulation of VSMC phenotype observed in VSMC–monocyte co-culture on D-PHI scaffolds, and reflected in part by the presence of monocyte/MDM-released factors in MCM (Figs. 1–3).

As shown in Fig. 4, IL-1β levels decreased over 7 days of culture for monocyte-seeded D-PHI scaffolds. Similarly, the pro-inflammatory cytokine IL-6 showed its highest concentration of 10 ng ml\(^{-1}\) at day 1, but dropped by an order of magnitude to 1 ng ml\(^{-1}\) over two weeks of culture (Fig. 5). MCP-1 is also important as its release from bone marrow mononuclear cell pre-seeded vascular grafts has been shown to be critical in the recruitment of host monocytes that initiate remodeling within the region of the implanted biomaterial [30]. It has also been suggested that macrophages and MCP-1 play a key role in tissue reparative processes with respect to skeletal muscle regeneration [53,54]. The ability of bone-marrow-derived cells (BMDCs) to mediate vascular growth and tissue healing has also been reported to require the production of MCP-1 [55].

3.3. Response of VSMCs to cytokine stimulation

In order to test the ability of MCP-1, IL-6 and GM-CSF to affect VSMC growth and contractile marker expression, VSMCs were cultured on D-PHI scaffolds and exposed to MCP-1 (5 ng ml\(^{-1}\)), IL-6 (1 ng ml\(^{-1}\)) or GM-CSF (0.25 ng ml\(^{-1}\)) for 7 days since the stimulatory effects of co-culture and MCM were apparent in this time frame (Figs. 1 and 2). These particular concentrations were chosen since they were concentrations assayed to be present in VSMC+MCM experiments (Fig. 5). After 7 days of culture on D-PHI scaffolds, MCP-1 had a modest but significant positive effect on VSMC growth and contractile marker expression. MCP-1 had a modest but significant positive effect on VSMC growth and contractile marker expression. MCP-1 had a modest but significant positive effect on DNA mass (22 ± 7% increase vs. regular medium), while IL-6 (+6.3 ± 5.1%) and GM-CSF (+12 ± 9%) did not have a significant effect on growth over this time period (Fig. 6). With regards to contractile marker expression, IL-6 had a suppressive effect on both α-SMA (74 ± 9% of regular medium) and calponin (69 ± 6% of regular medium), whereas only calponin was significantly affected by MCP-1 (67 ± 10% of regular medium) (Fig. 7). GM-CSF did not have any effect on either α-SMA or calponin expression.

Because MCM and co-culture also promoted VSMC migration, the impact of MCP-1, IL-6, and GM-CSF on migration was tested in a Boyden chamber assay. However, none of the cytokines had a significant effect on migration (data not shown). MCP-1 has previously been shown to have a stimulatory effect on rabbit VSMC migration in doses ranging from 5–20 ng ml\(^{-1}\) [47,49]. MCP-1 has already been studied with respect to VSMC proliferation, where inhibition of MCP-1 reduced the proliferation of human SMCs explanted from saphenous veins [48], whereas the proliferation of rat VSMCs has been shown not to be affected by MCP-1 [49]. This difference in response to MCP-1 between human and rat over time in vivo is important since during normal wound healing processes there are dynamic changes in the monocyte/MDM phenotype that are characteristic of different wound healing stages [23]. Pro-inflammatory cytokines such as IL-6, IL-1β and TNF-α initiate the wound healing process by promoting the recruitment of inflammatory cells to the wound area and acting as mitogens for fibroblasts and SMCs [41]. The absence or presence of low levels of these cytokines has been associated with impaired wound healing, while excessive levels have been associated with enhanced fibrotic scarring [42–44]. Furthermore, persistence of pro-inflammatory cytokine levels at the wound site promotes a chronic inflammatory phase that is detrimental to the processes of wound repair and tissue regeneration [45]. There is thus a desired shift towards pro-wound healing and anti-inflammatory cytokine production to facilitate the wound healing process. The presence of different cytokines will in part be dependent on the activation state of the macrophage, with M1 macrophages associated with the release of pro-inflammatory mediators (i.e. TNF, IL-12, IL-1, IL-6), while alternatively activated macrophages are associated with a different secretory profile (i.e. IL-10, IL-1ra) [46].
VSMCs may explain the lack of migration seen in response to MCP-1 in the present study, while an increase in proliferation in response to MCP-1 was observed. This is similar to other studies with human VSMCs [48], but contrasted to studies with rat VSMCs [49]. Similar to MCP-1, IL-6 has been shown to support the migration of VSMCs isolated from rat [51] or mouse aortas [56]. IL-6 has also been shown to support proliferation of both mouse [56] and human VSMCs [50]. While none of these studies have looked
specifically at VSMC contractile marker expression, it would be expected based on the enhanced proliferative and migratory state of VSMCs exposed to MCP-1 and IL-6, indicative of the synthetic phenotype, that these proteins would also suppress contractile marker expression [57].

3.4. Inhibition of IL-6 and MCP-1 in VSMC–monocyte co-culture

Since MCP-1 and/or IL-6, but not GM-CSF, were shown to be capable of influencing VSMC proliferation (Fig. 6) and contractile marker expression (Fig. 7) in monoculture on D-PHI scaffolds, the specific effect of MCP-1 and IL-6 in VSMC–monocyte co-culture on D-PHI was investigated. VSMCs and monocytes were co-cultured on D-PHI scaffolds for 7 days with or without the presence of antibodies for MCP-1 or IL-6. Neutralization of IL-6, but not MCP-1, was shown to have a suppressive effect on DNA mass (Fig. 8) as well as a stimulatory effect on calponin, but not α-SMA, expression (Fig. 9). This suggests that IL-6, but not MCP-1, regulates VSMC phenotype with respect to the aforementioned outcomes when in co-culture with monocytes/MDM on D-PHI scaffolds. Furthermore, while calponin expression was increased following neutralization of IL-6, there was no effect on α-SMA, which was similarly not affected by MCM (Fig. 2).

The relationship of calponin and IL-6 vs. the absence of IL-6 association with α-SMA expression suggests different regulation mechanisms. One possible explanation for this difference is that α-SMA expression requires direct cell–cell contact interactions be-
between VSMCs and monocytes/MDM, as occurs in direct co-culture. Direct VSMC–monocyte contact has been reported to occur through interactions with β1 and β2 integrins, and that these interactions are increased in the presence of growth factors such as PDGF-BB [58]. Furthermore, the regulation of other effects by monocytes/macrophages on VSMCs, such as MMP production, have been shown to have dependence on both released factors as well as cell–cell contact [22,59]. Another possibility is that while MCM was used to approximate the presence of cytokines and growth factors being released by monocytes in co-culture, it is also anticipated that the presence of VSMCs will alter the products being released by monocytes/MDM over time. The factors that are more important in the regulation of α-SMA may only be present under direct co-culture conditions. Finally, calponin is more specifically associated with smooth muscle tissues, while α-SMA can be expressed by a number of other cells and is less specific to the contractile VSMC phenotype [60,61]. Calponin is also typically detected later in the maturation of SMCs during development, while α-SMA is detected earlier on, suggesting that calponin is a marker of a higher SMC differentiation stage [62]. For this reason, it would be expected that calponin expression may be affected more significantly by IL-6 alone, while α-SMA down regulation may occur in the presence of multiple stimulatory cytokines and growth factors, rather than only exposure to IL-6.

IL-6 promoted a shift in VSMCs to a synthetic phenotype, characterized by enhanced VSMC proliferation and migration, when VSMCs were co-cultured with monocytes. Depending on the cytokine profile being released from the monocytes/MDM, which is known to be biomaterial dependent [24,31,63,64], different effects will be expected in monocyte co-culture systems. Furthermore, it should also be noted that while the current study indicated IL-6 as being key to regulating VSMC response in the present culture system, VSMCs sourced from different locations in the vasculature are known to respond differently to cytokine and growth factor stimulation [65]. Therefore, the specific cytokines predominantly responsible for inducing effects on VSMCs may differ depending on the source of VSMCs and the specifics of the culture system used. Several studies have looked at the effect of polarizing macrophages towards the M1 vs. M2 activation state and using the conditioned medium from these cells to determine their differential effects [15,28]. In particular, it has been shown that the M2, or the “wound healing macrophage”, produces cytokines and growth factors that promote enhanced proliferation of VSMCS [28] and greater migration of myoblasts [15] when compared to M1 polarized macrophages. Therefore, depending on the particular goals of the co-culture system, a biomaterial that supports different macrophage polarization will be required. In the present study, both the cytokine profile (decreasing IL-6 and IL-1β), increasing IL-13) and the enhanced proliferation and migration of VSMCs (associated with an M2 secretory profile [28]) are indicative of a hypothesized shift towards a more M2, or anti-inflammatory state for monocytes cultured on D-PHI scaffolds in vitro.

4. Conclusions

The present study investigated the contribution of monocyte/MDM-released factors after exposure of the cells to D-PHI on the stimulatory effects of monocyte co-culture on VSMC migration and proliferation, as well as the suppressive effects on contractile marker expression. Similar to direct co-culture, monocyte/MDM-released factors were observed to increase VSMC penetration into porous D-PHI scaffolds, enhance growth, and suppress calponin expression. IL-6 and MCP-1 were identified as two potential markers contributing to this response, and were confirmed to affect proliferation and/or contractile marker expression of VSMCs cultured alone on D-PHI scaffolds and exposed to concentrations of these proteins relevant to the culture system. In co-culture, however, only IL-6 and not MCP-1 was shown to be linked to enhanced proliferation of VSMCs and suppression of calponin, but not α-SMA expression. This study highlights the use of monocytes/MDM as stimulatory agents in combination with biomaterial strategies for directing VSMC response, and that in the present system involving the degradable polyurethane D-PHI, IL-6 is an important contributor to this process.

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Appendix A. Figures with essential colour discrimination

Certain figures in this article, particularly Fig. 2, are difficult to interpret in black and white. The full colour images can be found in the online version, at http://dx.doi.org/10.1016/j.actbio.2013.12.022.

References
