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Macrophage differentiation and polarization on a decellularized pericardial biomaterial★

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Abstract

The monocyte-derived macrophage (MDM), present at biomaterial implantations, can increase, decrease or redirect the inflammatory and subsequent wound healing process associated with the presence of a biomaterial. Understanding MDM responses to biomaterials is important for improved prediction and design of biomaterials for tissue engineering. This study analyzed the direct differentiation of monocytes on intact, native collagen. Human monocytes were differentiated on decellularized bovine pericardium (DBP), polydimethylsiloxane (PDMS) or polystyrene (TCPS) for 14 d. MDMs on all surfaces released high amounts of MMP-9 compared to MMP-2 and relatively little MMP-1. MDMs differentiated on DBP released more MMP-2, but less acid phosphatase activity. MDMs on all three surfaces released low amounts of cytokines, although substrate differences were found: MDMs on DBP released higher amounts of IL-6, IL-8, and MCP-1 but lower amounts of IL-10 and IL-1ra. This research provides evidence that MDMs on decellularized matrices may not be stimulated towards an activated, inflammatory phenotype, supporting the potential of decellularized matrices for tissue engineering. This study also demonstrated that the differentiation surface affects MDM phenotype and therefore study design of macrophage interactions with biomaterials should scrutinize the specific macrophage culture method utilized and its effects on macrophage phenotype.

Keywords

Macrophage; Acid phosphatase; Collagen; Matrix metalloproteinase; Cytokine; Biocompatibility

1. Introduction

An increasing number of devices are being designed from biological scaffolds derived from decellularized tissues [1]. There are three principal reasons for this increase in popularity [2]. First, the native matrix architecture (e.g. intact collagen molecules and glycosaminoglycans GAGs) may provide structural and chemical cues for cell interactions

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[3]. Second, these protein scaffolds generally retain their mechanical properties post-decellularization, which should enable them to withstand long-term function [2]. Third, and possibly most important from the aspect of regeneration, these scaffolds can be remodeled by cellular enzymes present in all human hosts, which make them an ideal “regenerative” matrix [1]. Although decellularized matrices are being used in clinical trials, they still represent a relatively new biomaterial whose biological performance is still uncertain and not well understood [4]. These materials hold the potential for becoming an excellent tissue-engineering scaffold, however this potential has not yet been fully realized. Despite many studies that have examined the response of these decellularized scaffolds in vivo, it is still not possible to truly predict the biocompatibility of one material over another [3]. This is likely due to the complexity of inflammatory and wound-healing responses to these materials in animals, which make it difficult to elucidate the exact mechanism(s) causing the response(s). An alternative to in vivo studies is to begin with a simpler host interaction: that is, the monocyte-derived macrophage (MDM) interaction with decellularized tissues. The knowledge generated from such in vitro studies will then help to design more sophisticated and enlightening in vivo studies.

The MDM response to a biomaterial can be an important determinant to the biological performance due to the multifunctional roles that MDMs play in the body: sentinels of host defense, initiators of tissue/matrix destruction and recruiters for wound healing [5]. On the biomaterial surface, MDMs secrete cytokines and chemical mediators that will subsequently direct the inflammatory and wound healing response to that biomaterial [6]. Recent studies in the field of biomaterials have concluded that the ‘long-term remodeling outcome’ as described by Badylak [7] is dependent on the phenotype of the MDM population at that site. The recent separation of in vivo macrophages into two phenotypic and functional polarizations based on cytokine release and surface markers, M1 and M2 [8], may provide a framework by which the biological performance of biomaterials can be more clearly characterized, at least in terms of MDM interaction.

With regards to decellularized biomaterials, MDM phenotypic characterization is also important for understanding the effect of the cell on the scaffold itself. These materials are especially susceptible to the enzymes that activated MDMs can release into the extracellular milieu such as matrix metalloproteinases (MMPs). Controlled degradation of these biomaterials is necessary for their biocompatibility as the remodeling rate of the scaffold must not exceed the regeneration rate of the tissue matrix.

There have been a limited number of in vitro studies that have looked at the response of MDMs or macrophage-like cells on decellularized scaffolds [9–12]. Previous studies of DBP have investigated the in vitro response of U937 cells and MDMs that were trypsinized and reseeded on the material in an attempt to characterize the macrophage response to these matrices [11,12]. These studies found that both cell types were less activated on DBP as determined by enzyme release and cell morphology, when compared to two synthetic polymer controls. To obtain applicable information from in vitro studies, the type of cell and the culturing must replicate the in vivo situation as closely as possible. While it is understood that the biomaterial surface can have a profound effect on the macrophage response, it is not clear how the biomaterial surface affects and/or directs the differentiation of monocytes to macrophages.

In this study, decellularized bovine pericardium (DBP) was used as a prototypical extracellular matrix scaffold due to the extensive use of bovine pericardium (albeit glutaraldehyde cross-linked) in cardiac repair, guided tissue regeneration and heart valve replacements [13]. Human monocytes were directly differentiated onto DBP and the phenotype of the MDM was compared to the MDM response on two polymer controls,

polydimethylsiloxane (PDMS) and tissue-culture polystyrene (TCPS); controls that are recognized as standards for low reactivity (PDMS) [14] and cell culture (TCPS). In addition to microscopic methods, cytokine and enzyme analysis were used for macrophage characterization.

2. Materials and methods

Unless otherwise described, all reagents were purchased from Sigma–Aldrich (Oakville, ON, Canada) and volume/volume percentages are used instead of molar concentrations, where applicable.

2.1. Preparation of surfaces

2.1.1. Polymer materials—16-mm diameter discs were cut from 0.02” sheets of medical grade PDMS (Specialty Manufacturing Inc, Saginaw, MI, USA). The discs were soaked in 70% ethanol and rinsed in sterile phosphate buffered saline (PBS) (without Ca^{2+} or Mg^{2+}) prior to use. Falcon brand tissue-culture polystyrene 24-well plates were used exclusively for TCPS surfaces, although sterile polystyrene coverslips (Sarstedt, Newton, NC) were used for microscopy experiments.

2.1.2. Decellularization—The complete decellularization of pericardium collected from young steers (24–30 months) has been described previously [11]. Briefly, 35 mm × 35 mm samples were exposed to a hypotonic solution for 36 h at 4 °C followed by a 24 h rinse in a 1% Triton X-100 solution. Samples were immersed in a 37 °C DNase/RNase solution for 1 h, then rinsed again for 24 h in 1% Triton X-100 at room temperature. To ensure the removal of any residual detergents, samples were soaked in sterile PBS for 48 h at room temperature. Each sample of DBP was stored in a sterile bottle filled with PBS containing 1% antibiotic solution (10,000 U/mL penicillin, 10,000 mg/mL streptomycin) at 4 °C for no longer than 4 months. DBP was cut into 16-mm diameter discs once needed.

Once prepared, all discs were placed into a 24-well plate and held in place by sterilized PDMS tubing (Fisher Scientific, Ottawa, ON) as described previously [11]. DBP was placed into wells with the fibrous layer down (and mesothelial surface up). Three discs of each substrate were used as no cell controls and three discs of each substrate were seeded with cells.

2.2. MDM cell culture

Whole blood was collected from healthy volunteers and monocytes were isolated via Histopaque 1077 gradient centrifugation as previously described [15,16]. This research protocol was approved by the Research Ethics Committee at the University of Ottawa Heart Institute, Ottawa, ON Canada (HI Protocol number 2005979-01H, valid until February 18, 2010). The mononuclear fraction, which consisted of both monocytes (~20%) and lymphocytes, was seeded directly onto the three surfaces (DBP, PDMS and TCPS) at a density of $\sim 6 \times 10^6$ cells/mL. Cells were cultured in RPMI-1640 supplemented with 10% fetal bovine serum, 0.68 mM L-glutamine, 2% antibiotic and 1% (250 µg/mL) amphotericin B for 14 days at 5% CO_2 /100% humidity. Culture medium was replaced after 2 h, 24 h, 48 h and then 3 times a week as previously described [17]. Cells were lysed in cold PBS (containing 0.05% Triton X-100 and 10 mM EDTA) for 1 h at 3 d, 7 d or 14 d of differentiation and immediately analyzed for DNA using Hoechst 33258 dye, as described previously [11]. At 14 d, additional samples were fixed for confocal and scanning electron microscopy (SEM) as detailed below. Lysates that were analyzed for gelatinase activity also contained 4.17% protease inhibitor cocktail (P8340).

2.3. Real-time polymerase chain reaction

RNA was isolated from cell lysates using the TRIzol[®] Plus RNA Purification System (Invitrogen, Burlington, ON), according to manufacturer's instructions. Briefly, cells were lysed directly in their wells with 1 mL of TRIzol Reagent[®] and pipetted to fully disrupt the cells. DBP and PDMS discs were collected with the TRIzol lysates and all lysates were frozen at -80°C for at least 24 h. Once thawed, substrate discs were removed and lysates were incubated for 5 min at room temperature. 200 μL of chloroform were added to each lysate prior to brief mixing and centrifugation at 12,000 g for 15 min at 4°C . The RNA-containing aqueous phase was collected and mixed with an equal amount of 70% ethanol. Then RNA was purified using ethanol wash solutions through spin columns. The purified RNA was collected in RNase-free distilled water and stored at -80°C prior to use.

Total RNA (0.2–0.4 μg) was reverse transcribed using Affinityscript[™] Reverse Transcriptase (Stratagene, Cedar Creek, TX) with 12.5 ng/ μL random hexamers and 1.0 mM deoxynucleoside-5'-triphosphates according to the supplier's instructions. Specifically samples were incubated for 5 min at 65°C to disrupt any secondary structure of the RNA, followed by rapid cooling and annealing of random primers at 25°C for 10 min. Reverse transcription proceeded at 42°C for 60 min and the enzyme reaction was heat inactivated at 95°C for 5 min. One microliter of the cDNA product was amplified in duplicate by quantitative-polymerase chain reaction (QPCR) using 2.5 nM of each primer pair (Table 1) in a total volume of 20 μL with Brilliant SYBr Green QPCR Master Mix (Stratagene) using a Stratagene MX3000p thermocycler. The cDNA was amplified using the following protocol: a 10 min hot start at 95°C followed by 40 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 18 s and elongation at 72°C for 30 s. One single melt cycle completed the protocol (95°C for 60 s, 60°C for 30s, ramp and hold at 95°C). The formation of a single gene product was verified by melting curves and separation of the final QPCR product on 2.5% 0.5 $\times$ TAE (Tris-acetate-ethylene diamine tetraacetic acid) agarose gels. The cycle threshold (C_T) values were calculated by MXPro-Mx3000P (Stratagene) software. Relative gene expression was normalized to cyclophilin A (CYCA) expression following the $2^{-\Delta\Delta C_T}$ protocol as described by Livak et al. [18]. Gene expression on TCPS was used as the reference to which the gene expression on DBP and PDMS surfaces were compared.

2.4. Immunoblotting

Proteins from cell lysates were separated by SDS-PAGE using pre-cast 4–15% linear gradient polyacrylamide gels (Bio-Rad Laboratories, Mississauga, ON). Lysate was loaded based on a standard amount of DNA. Gels were run at 100 V for ~1.5 h and were transferred onto nitrocellulose membranes (Bio-Rad) overnight at 4°C at 35 V. Membranes were probed for vinculin, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), monocyte-specific esterase (MSE) and CD68, as previously described [19]. Briefly, membranes were blocked with 5% nonfat dry milk in tris-buffered saline (TBS: 10 mmol/L Tris, 0.1 mol/L NaCl, pH 7.5) for 1 h prior to incubation with antibodies in tris-buffered saline with Tween (TBST: TBS + 0.1% Tween-20). Each membrane was incubated for 1 h with monoclonal mouse anti-human vinculin (1:2000, 5% milk), polyclonal rabbit anti-porcine esterase (1:1000, 1% milk) (Rockland Immunochemicals Inc, Gilbertsville, PA — reacts with MSE), monoclonal mouse anti-human GAPDH (Millipore International, Billerica, MA) (1:10 000, 5% milk) or monoclonal mouse anti-human CD68 (Santa Cruz Biotechnology, Santa Cruz, CA) (1:200, 5% milk) then rinsed with TBST. The membrane was incubated with a secondary antibody: IRDye 800CW-conjugated goat anti-mouse (CD68, GAPDH, vinculin) (1:20 000, 5% milk) or IRDye 700DX-conjugated goat anti-rabbit (MSE) (1:17 000, 5% milk) (Rockland). Membranes were imaged on an Odyssey near-infrared imaging system (LI-COR Biosciences, Lincoln, NE) at excitation wavelengths of 800 and 700 nm. Bands of

protein were quantified using Quantity One® (Bio-Rad, version 4.6.1) and expressed relative to the average density units on the TCPS surface.

2.5. Acid phosphatase activity

To assess differences in lysosomal acid phosphatase activity throughout differentiation, lysates and releasates were assayed at all three time points: 3 d, 7 d and 14 d. Enzyme activities were measured using a modification of the protocol described previously by Ariganello et al. [11]. Briefly, the activities were determined spectrophotometrically by the production of p-nitrophenol from the hydrolysis of p-nitrophenylphosphate. Each sample was compared to a standard curve prepared using acid phosphatase enzyme and normalized to a standard DNA content (10 µg DNA). The acid phosphatase activity is reported in units (a unit is defined as the release of 1 nmol of p-nitrophenol per minute at 37 °C).

2.6. Matrix metalloproteinase activity and gelatinase activity

To determine if MDMs contained and released different amounts of MMP-1 on the three surfaces, cell lysates and releasates were analyzed for MMP-1. MMP-1 was quantified using a human MMP-1 ELISA (enzyme-linked immunosorbent assay) kit (RayBiotech Inc., Norcross GA) according to the protocol provided by the supplier; specifically 100 µL of releasate and 50 µL of lysate were used for quantification.

To investigate differences in MMP-2 and MMP-9 activity after differentiation, cell lysate containing protease inhibitors was separated by SDS-PAGE using pre-cast 10% polyacrylamide gels containing 0.1% gelatin (Bio-Rad). Lysate was loaded based on a standard amount of DNA and gels were run at 90 V for 90 min under non-reducing conditions. Gels were washed in 2.5% Triton X-100 then incubated in substrate buffer (50mM Tris buffer, 5mM CaCl₂, 200mM NaCl, pH 7.5) for at least 12 h. Gels were stained with 0.5% Coomassie Brilliant Blue (Bio-Rad) in water containing methanol (40%) and glacial acetic acid (10%) for 45 min and destained with the water containing methanol (40%) and glacial acetic acid (10%) for 2 h. Gels were photographed on a transilluminator and densitometry was performed with Gel-Pro Analysis Software (MediaCybernetics, Silver Spring, MD).

2.7. Confocal microscopy

To compare cell spreading and multi-nucleation after differentiation, MDMs were double-stained for filamentous actin and nuclear material after 14 d culture on each surface as previously described [11]. Briefly, each disc with adherent MDMs was fixed in 4% paraformaldehyde and incubated in rhodamine phalloidin (1:150) and DRAQ5™ (Cedarlane Laboratories, Hornby, ON) (1:1000) at room temperature and viewed on an Olympus 1 × 80 inverted laser scanning confocal microscope. An Olympus 100× oil immersion objective with a numerical aperture of 1.4 was used with Fluoview FV1000 software (version 1.4.1.5). Rhodamine phalloidin and DRAQ5™ fluorochromes were excited with a helium/neon laser at 543 nm and 633 nm, respectively.

2.8. Scanning electron microscopy

MDMs were fixed and prepared for SEM after 14 d culture on each surface as previously described [11]. Briefly, discs were fixed for 2 h in 2.5% glutaraldehyde in PBS and then rinsed. Samples were dehydrated in increasing concentrations of ethanol then critical-point dried and sputter-coated with a gold-palladium mixture. Samples were imaged using a Hitachi S-4700 FEG SEM at an accelerating voltage of 7 kV and current of 10 µA.

2.9. Extracellular cytokine release

Cell lysate and releasate samples were shipped to Millipore (St. Charles, MO) for cytokine analysis. The samples were analyzed with a Lincplex assay cytokine panel. Sample concentrations (pg/mL) were determined from median fluorescence intensities compared to a 5-parameter logistic standard curve for each cytokine. Twenty-nine different cytokines were assayed: IL-1 α , IL-1 β , IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12(p40), IL-12(p70), IL-13, IL-15, IL-17, TNF- α , TGF- α , RANTES, epidermal growth factor (EGF), eotaxin, fractalkine, granulocyte colony stimulating factor (G-CSF), IFN- γ , GM-CSF, interferon-inducible protein-10 (IP-10), sCD40L, MIP-1 α , MIP-1 β , and MCP-1. The limit of quantification for each cytokine was set at 20 pg/mL, as used by Schutte et al. [20].

2.10. Statistical analysis

All data were analyzed using a one-way ANOVA at an overall significance level of 0.05, with post-hoc tests Bonferroni-adjusted for multiple comparisons. Dunnett post-hoc tests were used to identify differences between the surfaces using DBP as the reference. At least three separate experiments were performed, with three samples for each of the three substrates ($n = 9$). In tables, data are presented as mean $\pm$ standard error of the mean. All statistical analyses were carried out using JMP software (SAS, version 5.0.1.2). All data presented in graphs, with the exception of the cytokine data, were normalized to the values of MDMs on TCPS for the 14 d time point. Therefore, the values on TCPS have a defined value of 1 or 100%. Data presented in tables represent raw data prior to normalization.

3. Results

3.1. Cell attachment

DNA content was used as a measure of cell viability and attachment since viability has been previously shown to correlate with the amount of DNA in biomaterial-adherent MDMs [21]. During the course of differentiation, no difference in cell attachment between substrates was found at either 3 d or 7 d, however, the DNA content was lower on DBP at 14 d compared to the PDMS surface (Table 2). A decrease in DNA content over time, from 3 d to 14 d, was seen on all three surfaces.

3.2. Gene expression

Significant differences between the MDMs on the three surfaces were detected for five out of the eight genes measured as shown in Fig. 1. Cells differentiated on DBP for 14 d expressed lower amounts of CD11b, TIMP-2 and MMP-9 than cells differentiated on TCPS. Relative to the DBP surface, cells differentiated on TCPS expressed less TIMP-1 and MMP-2 ($p < 0.08$, $n = 6$), while cells on PDMS expressed less MMP-1. No difference in gene expression of CD68 or CD86 could be detected between MDMs on the three surfaces.

3.3. Intracellular protein content

Differences in protein content in the MDMs on the three surfaces were identified only at 14 d for MSE as shown in Fig. 2. Cells differentiated on TCPS at 14 d contained higher MSE compared to DBP, although no difference in MSE over time could be detected for any surface. Cell lysates were analyzed for CD68 at all three time points, however the protein was only detected at 7 d and 14 d (Fig. 2). At both time points, no differences in CD68 protein could be detected across the surfaces, which correlated with the gene expression data at 14 d. Additionally, an increase in CD68 with time was found only for the TCPS surface.

3.4. Enzyme activity

Cells on all three surfaces released acid phosphatase activity even after 3 days of differentiation and a substrate-dependent difference in acid phosphatase activity could be detected by this time both intracellularly and extracellularly (Fig. 3). Specifically at 3 d, cells on DBP released and contained lower acid phosphatase activity than cells on TCPS. At 14 d, cells on DBP contained and released less acid phosphatase activity relative to both PDMS and TCPS. For each surface, no difference in intracellular acid phosphatase activity with differentiation time was found, however, there was an increase in *extracellular* acid phosphatase activity with differentiation time from MDMs on both DBP and PDMS surfaces.

Cells on all three surfaces released MMP-1 (DBP: $5.1 \times 10^2 \pm 2.1 \times 10^2$, PDMS: $6.6 \times 10^2 \pm 2.0 \times 10^2$, TCPS: $4.6 \times 10^2 \pm 1.1 \times 10^2$, all in units of pg MMP-1/ μ g DNA). However, due to the high variability in the amount of released protein, no significant differences in the MMP-1 released by MDMs on the three surfaces could be detected. The amount of *intracellular* MMP-1 in the MDMs on all surfaces was generally below the detection limits of the kit (<8 pg/mL).

Cells on all three surfaces released both MMP-2 and MMP-9 (Table 3). For every surface, MDMs released significantly more active MMP-9 compared to MMP-2. The amount of intracellular MMP-2 was highly variable in MDMs on all three surfaces. No detectable differences in the amount of intracellular MMP-2 or MMP-9 activity were found between the MDMs on the three surfaces. Despite this, MDMs cultured on DBP *released* greater amounts of active MMP-2 compared to either polymer control. This differential expression of active MMP-2 mirrors what was found with MMP-2 gene expression.

3.5. MDM morphology

Cells differentiated onto the decellularized matrix did not appear to penetrate the matrix even after 14 d of culture. Additionally, the surface of the scaffold was not visibly degraded or modified. Cells cultured on DBP had a distinctive morphology compared to MDMs on PDMS and TCPS. While cytoskeletal staining of cells on the two flat polymer surfaces showed MDMs that were highly spread onto the material surfaces (Fig. 4B,C), the cells on the DBP tended to maintain a smaller footprint and often displayed an elongated morphology (Fig. 4A,D). Additionally, cells on the PDMS and TCPS surfaces often displayed punctate-actin expression on the ventral surface of the cell, a feature which could not be visualized in MDMs on DBP. Cells on DBP were more likely to contain a single nucleus, while cells on the two control surfaces were often multinucleated, containing two or three nuclei per cell.

Additional differences were discovered with SEM. The surface of cells on DBP was often decorated with surface blebs (spherical protrusions) (Fig. 5A,B) as well as the microvilli which were also present on the cell surface on PDMS and TCPS, though to a lesser degree (Fig. 5C,D). One final difference seen with SEM was that cells on the two control polymers frequently extended numerous filopodia onto the polymer surface. By contrast, cells on DBP displayed these cytoplasmic extensions less regularly, and these extensions were often much finer projections. SEM images also allow a greater exploration of the decellularized matrix; as shown in previous studies, the collagen network exposed after decellularization was quite tight, with pores that are orders of magnitude smaller than the dimensions of an MDM [11].

3.6. Extracellular cytokine analysis

Significant differences between substrates were identified for 12 cytokines (See Table 4). Only 9 of those cytokines were released at quantities greater than 20 pg/mL and those

cytokines are shown in Fig. 6. By contrast, eotaxin, IL-12p70, fractalkine, VEGF and IL-1 β could not be detected reproducibly in the cell supernatants. Only three cytokines were released at the nanogram level: IL-8, IL-1ra and MCP-1. Cells differentiated on DBP released greater amounts of the inflammatory cytokines IL-6, IL-8 and MCP-1 but lower amounts of MIP-1 β , TNF- α , GM-CSF and sCD40L compared to the polymer controls. The cells on DBP also released less of the anti-inflammatory cytokines: IL-10 and IL-1ra.

4. Discussion

In a previous study, MDM interactions with biomaterials were examined using monocytes that had been differentiated to MDMs while adherent to TCPS. The resulting MDMs were then trypsinized prior to reseeding onto the biomaterial of interest [16]. Although this method of monocyte differentiation does model a more activated cell type [16], it poorly replicates any influence the biomaterial may have on the differentiation process and the MDM phenotype expressed. The current study used the macrophage model of direct differentiation described by Dinnes et al. [17]. This model is possibly more relevant to the in vivo situation than the trypsinized MDM model since it is monocytes, not differentiated macrophages, that are recruited to a biomaterial implantation site.

In this study, biomaterial surfaces (decellularized pericardium and two control polymers) were investigated for their ability to *directly* induce the differentiation of monocytes, without the addition of exogenous growth factors or cytokines. In this way, importance was placed on how the material surface stimulated the release and/or expression of cytokines and enzymes, without confounding variables and/or 'priming' of the monocyte towards a specific macrophage differentiation pathway. Many studies, for example, have utilized the addition of IL-4, IL-10 or GM-CSF to induce the formation of foreign body giant cells [22,23]. By contrast, this study focused on the ability of the *material* to induce these cellular changes.

Similar to the work reported by Dinnes et al. [17], the current study demonstrated that the differentiation surface does affect the functional phenotype of the resulting MDM as shown by differences detected both during and after differentiation. These differences included gene expression, active enzyme release, extracellular cytokine release, chemokine release and cell morphology. The similar gene expression and protein expression of the macrophage marker CD68 on all three surfaces demonstrated that the monocytes there have equally differentiated into macrophages by 14 d (Fig. 5-2 and 3) [24]. Therefore the differences seen in the response of these MDMs to each surface represented a difference in differentiated macrophage phenotype not a difference in cell type per se.

The first difference observed between the MDMs on the three substrates was in the gene expression profiles. Cells on the three surfaces displayed differential expression of CD11b with cells on the TCPS surface exhibiting the highest expression (Fig. 5-2). CD11b (or α_M) is complement receptor 3, which binds complement protein fragment, C3b [25,26]. The differential expression of CD11b may indicate that the mechanisms by which MDMs attach to each surface is different; specifically the TCPS surface may be activating complement to a greater degree than the DBP surface. Such activation of complement by polystyrene surfaces has been previously demonstrated [27].

Gene expression analysis also found a difference in MMP transcripts in MDMs across the surfaces. The high gene expression of the gelatinase MMP-9 on TCPS did not correlate well with the MMP-9 activity (Fig. 5-5), which was equivalent across the substrates. This difference may be due to the fact that gene expression only measures the inactive MMP zymogen transcript while zymography measures only the protein that was successfully

synthesized and uninhibited by TIMPs [28]. Higher expression of the collagenase MMP-1 in MDMs on the DBP surface was expected due to the predominance of non-denatured collagen in the scaffold. Therefore it was surprising that the release of MMP-1 protein (ELISA detected both pro- and active forms) was equivalent in the MDMs on all three surfaces. Initially, the MMP-1 protein results appeared contradictory to the idea that MDMs on DBP are less activated. However, upon reviewing the literature, it is apparent that the amount of MMP-1 released into the media from MDMs on all three surfaces was quite low. The quantity of released MMP-1 from MDMs in this study was $\sim 10\times$ less than for MDMs stimulated by LPS [29,30] and almost $1000\times$ less than for 6 d differentiated MDMs exposed to C-reactive protein [30]. Therefore, it appeared that none of these three biomaterial surfaces were great activators of MMP-1 release when compared to the stimulation produced by these pro-inflammatory mediators. Additional indirect evidence of low, active MMP-1 release may be the lack of cell penetration into the collagen matrix as seen in the SEM images (Fig. 5-7). The relatively dense collagen network would require local collagen disruption for MDMs to migrate into the matrix, however, even after 14 d on the surface, no cells were found to penetrate the scaffold—nor was there evidence of local matrix damage.

The release of both MMP-2 and MMP-9 on all surfaces does not necessarily imply a degradative response to the biomaterial surface because gelatinases play important roles in both immune and inflammatory responses (by processing cytokines and chemokines) in addition to matrix or protein proteolysis [31–33]. More specifically, a recent study of M-CSF differentiated macrophages demonstrated that MMP-9 secretion might be a marker of wound healing MDMs [34]. The study by Lolmede et al. [34] found that MMP-9 was secreted only from MDMs that were polarized towards a wound healing phenotype (cells previously exposed to IL-4 or IL-10), but not MDMs polarized towards an inflammatory phenotype (cells previously exposed to LPS). Interestingly, MDMs released equivalent amounts of MMP-2 independent of their phenotype.

Protein and enzyme content also confirmed cell response differences between the three surfaces, with cells on the DBP consistently releasing and producing less enzyme. After 14 d, cells on DBP contained less MSE (Fig. 5-3) and less acid phosphatase activity, and released less acid phosphatase activity (Fig. 5-4). MSE is an enzyme that has been associated with certain pathological conditions and diseases, including acute leukemia [35], and may be a marker for inflammation [36]. The lower expression of MSE in MDMs on the DBP, which mirrors the lower gelatinase ratio, was also demonstrated in previous studies using trypsinized MDMs or U937 cells [11,12]. Acid phosphatase was measured as a representative lysosomal enzyme and it has previously been described as a marker for the innate immune response and cellular activation [37]. The decreased amount of released acid phosphatase activity from MDMs on the DBP surface may imply that these cells are less activated because activated MDMs show increased secretion of lysosomal acid hydrolases [38]. The lower amount of acid phosphatase activity contained in and released from MDMs on the DBP compared to cells on the polymer controls correlated well with previous studies of trypsinized MDMs. Compared to a previous study in which macrophages were trypsinized prior to reseeding onto each substrate, the ratio of extracellular to intracellular acid phosphatase activity was much higher in the trypsinized MDMs, which supports the idea that trypsinization activates MDMs [16].

The differences that occurred as a result of the biomaterial surface induced differentiation were evident morphologically as well. Cell attachment and/or spreading was quite distinct between the DBP and the two polymer controls. Macrophage spreading in response to specific soluble agents has been classically considered a marker of cell activation [39], however the significance of cell spreading in this study was difficult to interpret due to the different mechanisms by which MDMs would be expected to attach to a three-dimensional

matrix compared to a smooth, relatively flat polymer material. The decrease in multi-nucleation seen in the cells differentiated on the DBP surface was more revealing. Differentiation on the collagen surface produced cells that had a lower propensity to fuse into multinucleated cells and the cells did not display the unique punctate-actin expression seen on the two smooth control surfaces. Multi-nucleation is generally accepted to be a sign of macrophage activation in the foreign body response to biomaterials [23]. The sharp differences in the cell shape and surface attachment prompted the quantification of the cytoskeletal protein vinculin via immunoblotting; however, no difference between the surfaces could be detected. The absence of these structures in MDMs on DBP is intriguing. These morphological observations (decreased spreading and decreased multi-nucleation) together provide greater evidence that the surface of the pericardium led to MDMs that appeared to be less pro-inflammatory and less degradative.

The final differences that were found in this study were in extracellular chemokine and cytokine release. Substrate-specific differences were identified, however, in general the cytokine release was quite low. Of all the cytokines measured only three had a concentration within the ng/mL range: IL-8 (all three surfaces), MCP-1 (DBP) and IL-1ra (control surfaces). Both IL-8 and MCP-1 have previously been shown to be released in high amounts from biomaterial-adherent MDMs while IL-1ra release was increased in the presence of C3a and C5a [40]. In addition, the gene expression of all three of these mediators is increased after exposure to amphotericin B, which was used in the culture media [41]. While this could possibly explain why these enzymes were elevated in general, it does not explain the differential release on each substrate.

The concentration of cytokines seen in the present study was considered to be low because (i) similar studies have shown higher amounts of cytokines released (on the order of ng/mL) and (ii) studies that utilized the addition of cytokines to elicit a response required significantly higher concentrations than those found in this study. A study by Schutte et al. [20] looked at cytokine release from THP-1 cells after PMA differentiation (an alternative model of macrophage activation and differentiation). Compared to the cytokine expression from the PMA-treated THP-1 cells, the MDMs generated in this direct differentiation model consistently released lower levels of all cytokines. Another in vitro study similarly looked at cytokine release during the course of differentiation of human monocytes onto various polymers [42]. The MDMs on all surfaces in that study released greater amounts of cytokines and chemokines than were seen here in MDMs on DBP. And, as noted above, in vitro studies that utilized cytokines to modulate cellular behaviour (such as IL-4 or GM-CSF) used concentrations that are 5–100× greater than what was released in this current study [43]. However it is difficult to ascertain, in general, whether statistically different levels of cytokine released in vitro have physiological significance and whether cytokine levels used to stimulate cell behaviour in vitro are similar to the concentration required in vivo.

In vivo studies may provide some insight into physiologically relevant cytokine levels. Two studies have investigated the physiological levels of cytokines present within an implanted hollow biomaterial tube [44,45]. Unfortunately, the outcomes obtained in those in vivo studies were constrained by the protocol employed: the subcutaneous implantation of the tube resulted (not surprisingly) in encapsulation. As a result it is unclear whether these outcomes (the cytokine concentrations) are representative of a clinical situation, or are artefacts of the methodology. That being said, the cytokine levels obtained in those two in vivo studies were of a similar order of magnitude to our results; however it is difficult to directly compare these values due to differences in clearance rates and effective volumes between that work and the present in vitro study.

In our study, the phenotype of macrophages on DBP cannot be uniquely described in terms of classically/alternatively-activated or M1/M2. The lack of IL-12 release from MDMs on any surface makes it unlikely that the MDMs are displaying a phenotype similar to classically-activated (M1) macrophages by 14 d. In vivo studies of an implanted acellular ECM (small intestinal submucosa) have shown that such a material triggers a Th2 response and ‘activates’ MDMs towards an M2 phenotype, although only one marker of the M2 phenotype was used (CD163) [7,46]. The monoculture of MDMs in this present study may have precluded the development of M2-like characteristics due to the lack of signals from lymphocytes: cells that are, at least transiently, present at an implant site.

5. Conclusions

This study analyzed the direct differentiation of monocytes on intact, native collagen. Using this in vitro model, it was found that DBP induces the direct differentiation of monocytes to macrophages. Additionally, the differentiation of monocytes was different on this natural scaffold than on control, synthetic polymers; however, the MDMs’, subsequent responses were mild. The decellularized bovine pericardium did not elicit the differentiation of an ‘activated’ or pro-inflammatory macrophage. This study therefore provides support for the use of decellularized matrices as a useful scaffold for tissue replacement and regeneration.

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Appendix

Figures with essential colour discrimination. Fig. 4 in this article has parts that are difficult to interpret in black and white. The full colour images can be found in the on-line version, at [doi:10.1016/j.biomaterials.2010.09.004](https://doi.org/10.1016/j.biomaterials.2010.09.004).

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Fig. 1.

Gene expression after 14 d differentiation on three surfaces. mRNA was expressed as a fold change in the gene expression of cells cultured on TCPS. All genes were normalized to the housekeeping gene cyclophilin A. An asterisk (*) denotes a significant difference from DBP. A double asterisk (**) denotes a significant difference ($p < 0.08$ overall). DBP surface (white bars), PDMS surface (light grey bars), TCPS surface (dark grey bars).

Fig. 2. Intracellular protein content during differentiation time course. Intensity values of MSE and CD68 protein are expressed as a percentage of the protein intensity of cells cultured on TCPS at 14 d. An asterisk (*) denotes a significant difference from DBP. Representative blots are shown below each graph.

Fig. 3. Acid phosphatase activity during differentiation time course. Intracellular and extracellular acid phosphatase activity values (units of activity) normalized to 10 µg DNA from MDMs on each surface. Within each time point, an asterisk (*) denotes a significant difference from DBP at ($p < 0.05$). Within each substrate, † denotes a significant difference from 3 d ($p < 0.05$).

Fig. 4.

Laser scanning confocal images of MDMs after 14 d differentiation on three surfaces. Filamentous actin was visualized by rhodamine phalloidin (pseudocoloured red), nuclei were visualized by DRAQ5™ (pseudocoloured blue). MDMs were cultured on (A, D) DBP, (B, E) PDMS and (C, F) TCPS. Scale bars = 20 μm . Punctate-actin expression could be seen on the ventral surface on MDMs on (E) PDMS and (F) TCPS.

Fig. 5. SEM images of MDMs after 14 d differentiation on three surfaces. Representative images of cells differentiated on DBP, PDMS and TCPS for 14 d. Cells cultured on the DBP surface were less likely to extend filipodia and did not spread or flatten as much as cells on the two polymer controls. Scale bars = 20 μm (DBP) and 30 μm (PDMS, TCPS).

Fig. 6.

Extracellular cytokines after 14 d differentiation on three surfaces. A) Pro-inflammatory cytokines released in greater amounts from MDMs on the DBP surface. B) Pro-inflammatory cytokines released in lower amounts from MDMs on DBP. C) Anti-inflammatory cytokines released in lower amounts from MDMs on DBP. Bars displaying an asterisk (*) denote a significant difference from DBP ($p < 0.05$).

Table 1

Quantitative PCR Primer Sequences.

Gene	GenBank Accession No.	PCR Primer Sequences (5' to 3')	PCR Product Size
Integrin α_M CD11b	NM_000632.3	Fw: cacatgacttcggcggatga Rv: gctgcgttattggcttcacc	174 bp
CD68	NM_001251.2	Fw: gctggctgtgcttttctcg Rv: gtcaccgtgaaggatggca	111 bp
CD86	NM_175862.3	Fw: gcggcctcgcaactcttata Rv: tctctttcttggtctgttcactctc	75 bp
CD163	NM_004244.4	Fw: cctcagaaaattcccatgagtc Rv: tcttaaaggctgaactgagtgg	157 bp
CYCA	NM_021130.3	Fw: ttcatctgcactccaagac Rv: tcgagttgtccacagtcagc	158 bp
MMP-1	NM_002421	Fw: tctgggtgtgtgtgtcca Rv: gcctcccatcattcttcaggtt	114 bp
MMP-2	NM_004530	Fw: agggcacatcctatgacagc Rv: attgttgcccaggaaagtg	186 bp
MMP-9	NM_004994	Fw: catttcgacgatgacgagttgt Rv: cgggtgtagagtctctcgc	230 bp
RPII	NM_004805	Fw: gcggatgaggatgcaatatga Rv: accaagccttctcgtcaaaata	110 bp
TIMP-1	NM_003254.2	Fw: aatccgacctcgtcatcag Rv: gttgtgggacctgtggaagt	195 bp
TIMP-2	NM_003255.4	Fw: gtagtgatcaggccaaagc Rv: ggcccttctgcaatgagat	222 bp

RPII: Ribosomal Polymerase II; CYCA: Cyclophilin A, Fw: forward; Rv: reverse.

Table 2

DNA content on biomaterial surfaces during differentiation.

Time Point	3 d	7 d	14 d
DBP	9.67 ± 0.47	8.10 ± 0.89	3.80 ± 0.40 ^a
PDMS	10.50 ± 0.48	8.46 ± 0.92	5.82 ± 0.59 ^{a,b}
TCPS	10.49 ± 0.62	9.7 ± 1.1	6.03 ± 0.58 ^a

DNA content on each surface. Values are expressed as µg of DNA.

DBP: Decellularized Bovine Pericardium; PDMS: Polydimethylsiloxane; TCPS: Tissue Culture Polystyrene. Within each surface:

^aDenotes a significant difference from 3 d.^bDenotes a significant difference from DBP at 14 d.

Table 3

Gelatinase activity after 14 d differentiation on each surface.

	MMP-2	MMP-9
Lysate		
DBP	1.8 ± 1.8	240 ± 100 ^a
PDMS	1.7 ± 1.7	300 ± 91 ^a
TCPS	8.3 ± 6.0	157 ± 54 ^a
Releasate		
DBP	11.6 ± 1.3	900 ± 120 ^a
PDMS	6.77 ± 0.64 *	71.6 ± 5.0 ^a
TCPS	5.9 ± 1.1 *	67.5 ± 4.7 ^a

Densitometry values obtained from zymography. (*Units of 10³ Relative Density Units*). No differences were found between substrates for intracellular MMP activity.

Values displaying an asterisk (*) are significantly different from DBP.

DBP: Decellularized Bovine Pericardium; PDMS: Polydimethylsiloxane; TCPS: Tissue Culture Polystyrene.

^a cells on each surface contained and released more MMP-9 than MMP-2.

Table 4

Cytokine release in releasate (pg/mL/10 µg DNA).

	DBP	PDMS	TCPS
“Pro-inflammatory”			
IL-1α	27.0 ± 7.8	15.1 ± 2.8	10.6 ± 1.4
IL-6	5.2 × 10 ² ± 1.7 × 10 ²	41 ± 24 *	4.9 ± 1.1 *
IL-8 ^a	>35.4 × 10 ² ± 6.3 × 10 ²	12.0 × 10 ² ± 1.2 × 10 ² *	11.9 × 10 ² ± 1.5 × 10 ² *
IFN-γ	18.7 ± 9.2	64 ± 12	49 ± 14
TNF-α	17.5 ± 5.3	61.7 ± 8.6 *	29.1 ± 4.4
GM-CSF	64 ± 28	181 ± 22 *	137 ± 30
sCD40-L	14.4 ± 5.9	28.9 ± 3.2	39.8 ± 5.6 *
G-CSF	4.9 × 10 ² ± 2.3 × 10 ²	2.23 ± 0.68	1.90 ± 0.43 **
“Anti-inflammatory”			
IL-10	26 ± 13	18.3 ± 3.4	79 ± 20 *
IL-13	10.4 ± 5.6	30 ± 11	24.8 ± 9.7
TGF-α	2.1 ± 0.84	2.8 ± 0.55	5.1 ± 1.0
IL-1ra	169 ± 93	11.2 × 10 ² ± 1.2 × 10 ² *	10.2 × 10 ² ± 1.1 × 10 ² *
Chemokines			
IP-10	4.1 × 10 ² ± 2.4 × 10 ²	121 ± 30	73.0 ± 9.9
MIP-1α	144 ± 59	65 ± 21	53 ± 16
MIP-1β	77 ± 19	300 ± 97 *	309 ± 56 *
MCP-1	14.0 × 10 ² ± 2.6 × 10 ²	2.7 × 10 ² ± 1.3 × 10 ² *	98 ± 38 *
Low Expression (<20 pg/mL)			
IL-1β	0.47 ± 0.35	ND	ND
IL-2	0.75 ± 0.38	0.42 ± 0.19	0.30 ± 0.16
IL-4	ND	0.52 ± 0.52	2.7 ± 0.65 *
IL-5	ND	0.51 ± 0.35	0.047 ± 0.047
IL-7	3.04 ± 0.63	1.49 ± 0.13 *	1.19 ± 0.11 *
IL-12p40	ND	4.1 ± 1.9	2.1 ± 1.1
IL-15	0.31 ± 0.15	1.02 ± 0.15 *	0.96 ± 0.14 *
IL-17	0.41 ± 0.24	0.38 ± 0.24	1.0 ± 0.10
EGF	0.74 ± 0.26	0.77 ± 0.17	0.22 ± 0.10
Eotaxin	ND	ND	ND
IL-12p70	ND	ND	ND
Fractalkine	ND	ND	ND
VEGF	ND	ND	ND

ND: Not detected.

Values are mean ± SEM.

Data displaying an asterisk (*) are significantly different from DBP ($p < 0.05$), while ** denotes a difference from DBP at the ($p < 0.065$) level only).

^aThe concentration of cytokine released from cells on DBP was slightly above the detection limits of the assay, thus the value is reported as being greater than the detection limit.