Macrophages and fibroblasts
Ploeger, Diana

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HUMAN MACROPHAGES PRIMED WITH ANGIGENIC FACTORS SHOW DYNAMIC PLASTICITY, IRRESPECTIVE OF EXTRACELLULAR MATRIX COMPONENTS

Diana T.A. Ploeger, Sander M. van Putten, Jasper A. Koerts, Marja J.A. van Luyn, Martin C. Harmsen

Department of Pathology and Medical Biology, Medical Biology Section, University Medical Center Groningen, University of Groningen, Hanzeplein 1, 9713 GZ Groningen, The Netherlands.
ABSTRACT

Macrophages are important in inflammation as well as in tissue repair processes. They can be activated by various stimuli and classified into two major groups: M1 (classically activated) or M2 (alternatively activated). Inflammation, angiogenesis and matrix remodeling play a major role in tissue repair. Here, we investigate the combined influence of a pro-angiogenic microenvironment and specific extracellular matrix (ECM) components or tissue culture polystyrene (TCPS) on the dynamics of human macrophage polarization. We established that human angiogenically primed macrophages cultured on different ECM components exhibit an M2-like polarization. These M2-like macrophages polarized to M1 and M2 macrophages with classical (LPS and IFNγ) stimuli and alternative (IL4 and IL13) stimuli, respectively. Moreover, these M1 and M2 (primary) polarized macrophages rapidly underwent a secondary (re)polarization to M2 and M1 with conditioned media from M2 and M1 primary polarized macrophages respectively. In these initial priming and later (re)polarization processes the soluble factors had a dominant and orchestrating role, while the type of ECM (collagen I, fibronectin, versus tissue culture polystyrene) did not play a crucial role on the polarization of macrophages.
INTRODUCTION

Macrophages play a central role in tissue repair and inflammation, for instance after myocardial infarction (Mosser and Edwards 2008; Mahdavian Delavary et al. 2011; van Amerongen et al. 2007). Macrophages are derived from circulating blood monocytes. Before their differentiation to macrophages, monocytes have to transmigrate the activated endothelium of blood vessels, which is facilitated by chemotactic stimuli from the underlying tissue (Gordon and Taylor 2005; Mosser and Edwards 2008). After transmigration, the differentiation of monocytes into macrophages is mediated by the microenvironment, in particular by the exposure to the extracellular matrix (ECM) and inflammatory cytokines and growth factors (Bauer et al. 2000; Eierman et al. 1989; Gordon 2003). Newly formed macrophages may remain resident as tissue macrophages or participate in innate immune reactions that range from regulation of inflammation to remodeling of the ECM and from phagocytosis of bacteria and cellular debris to regulation of angiogenesis (David Dong et al. 2009; Gordon and Taylor 2005; Hellingman et al. 2011). Many of these processes involve the secretion of regulatory molecules such as cytokines, chemokines, growth factors and proteolytic enzymes. This multiplicity of functions requires the macrophages to be versatile and flexible, i.e. they should readily adapt to changes in the microenvironment.

Indeed, several macrophage subsets have been described that may explain the diversity of biological activities of macrophages (Gordon and Taylor 2005; Mosser and Edwards 2008; Murray and Wynn 2011). Macrophage subsets are classified in two major groups denoted as M1 (classically activated) and M2 (alternatively activated) macrophages. Both types of macrophages participate in tissue repair and remodeling. M1 macrophages are pro-inflammatory and secrete a host of pro-inflammatory cytokines and chemokines. M2 macrophages modulate inflammation and contribute to wound healing, arteriogenesis and angiogenesis, but also to adverse processes like atherosclerosis (David Dong et al. 2009; Heilmann et al. 2002; Brochériou et al. 2011). Macrophages can be polarized in vitro to M1 macrophages by stimulation with interferon gamma (INFγ), tumor necrosis factor-α (TNFα), and/or pathogen-associated molecules such as lipopolysaccharides (LPS) or combinations (Popova et al. 2010; Wang et al. 2010). Polarization to M2 macrophages is achieved by stimulation with interleukins IL4, IL13, IL10, transforming growth factor-β (TGF-β) or glucocorticoids (GC) or combinations of these molecules (Gordon 2007; Gratchev et al. 2005; Martinez et al. 2009).

Interestingly, macrophage polarization shows a high and dynamic plasticity, in vitro murine bone marrow derived macrophages and peritoneal macrophages change their secretion pattern of cytokines and chemokines several times in responses to changes in the cytokine environment (Stout et al. 2005). Human M1 macrophages can repolarize in vitro to M2 macrophages and vice versa, using INFγ and LPS or IL4 and IL10 as a secondary stimulus (Gratchev et al. 2006). In vivo, it has been shown that both tumor-infiltrating macrophages and tumor-associated macrophages, which seemed chronically polarized in function by the tumor environment, could still rapidly adapt their functional profile after interleukin-12 treatment (Watkins et al. 2007).

During transmigration and subsequent migration in the underlying tissue, monocytes and macrophages are continuously in contact with the ECM. This contact
with the ECM affects macrophage functionality, since macrophages matured on ECM components, such as collagens and fibronectin, more efficiently phagocytes bacteria than macrophages cultured on tissue culture polystyrene (Esendagli et al. 2009; Hanson et al. 2011; Brown 1986; Newman and Tucci 1990). The nature of the ECM, such as the amino acid sequence, posttranslational modifications, 3D structure and stiffness, will influence the differentiation of monocytes to macrophages by as yet largely unknown mechanisms (Philippeaux et al. 2009). Similarly, it is unknown whether specific ECM components influence the polarization of macrophages towards an M1 or M2 and by which mechanisms.

In this in vitro study we set out to investigate the combined influence of a pro-angiogenic microenvironment and ECM components on the dynamics of human macrophage polarization. We investigated the in vitro response of angiogenically primed human macrophages to classical or alternative polarization signals. Such conditions mimic, at least in part, the angiogenic microenvironment seen during tissue repair and remodeling. Here we describe (1) the polarization state of human primary macrophages cultured under angiogenic conditions on tissue culture polystyrene (TCPS), collagen I (COL I), and fibronectin (FN), (2) the polarization of macrophages under angiogenic conditions to M1 and M2 by means of stimulation with LPS/INFγ or IL4/IL13 and (3) the reversal in polarization of M2 to M1 and M1 to M2 by stimulation with conditioned medium from M1 or M2 under angiogenic conditions.

MATERIAL AND METHODS

CELL ISOLATION

Human peripheral blood from healthy donors was drawn by venapuncture in heparin-coated tubes. The mononuclear cell (MNC) fraction was isolated by density-gradient centrifugation using Lymphoprep (Axis- Shield, Oslo, Norway) according to manufacturer’s protocol. Briefly, blood was diluted three times with phosphate buffered saline (PBS), 0.5% Fetal Bovine Serum (FBS; Invitrogen/Gibco) and 2 mM EDTA (Merck, Darmstadt, Germany), pH 7.4 (isolation buffer). This mixture (30 ml) was layered over 20 ml of Lymphoprep and centrifuged at 500 x g for 30 min. The residual erythrocytes were lysed on ice (10 min) in 155 mM NH₄Cl, 10 mM KHCO₃, 0.1 mM EDTA (pH 7.4) and the suspension was centrifuged at 300 x g at 4 °C for 10 min after which the supernatant was discarded and the pellet gently resuspended in isolation buffer. MNCs were counted using a Coulter Counter and gently resuspended in angiogenic medium based on RPMI medium (Lonza, Basel, Switzerland) containing 2 mM L-glutamine (Sigma-Aldrich, St. Louis, USA), 5 U/ml Heparin (Leo Pharma, Denmark), 1% penicillin/streptomycin (Sigma-Aldrich, St. Louis, USA), 50 µg/ml crude ECGF suspension, 20% FBS, 1 ng/ml VEGFa and 10 ng/ml bFGF (both Peprotech, NJ, USA).

COATING OF CULTURE PLATES

Tissue culture polystyrene plates (TCPS; Corning Incorporated, NY, USA) were coated
with 1 μg/cm² of fibronectin (FN; Harbor Bio-products, Norwood, USA), or collagen type I (Col I; BD Biosciences, Bedford, USA), incubated at 37 °C for 1 h and washed with PBS.

MACROPHAGE CELL CULTURE AND STIMULATION

Immediately after isolation and counting, the cell suspension was seeded at a concentration of 250.000 cells/cm² onto FN, Col I precoated plates or on bare TCPS. Cells were cultured at 37 °C with 5% CO₂. Cells were refed on day 3 and day 5 and non-attached cells were removed by washing at day 7. At day 10, the adherent cells (macrophages) were washed and stimulated in the angiogenic culture medium with classical stimuli (1) 1 μg/ml LPS (Sigma-Aldrich, St. Louis, USA) and 10 ng/ml IFNγ (Peprotech, Rocky Hill, USA) or alternative stimuli (2) 10 ng/ml IL4 and 10 ng/ml IL13 (both Peprotech) or (3) no stimulation (control) at 37 °C for 24 h. After 24 h the culture supernatants of the different conditions were used for enzyme-linked immunosorbent assays as described below. The cells were lysed in RLT buffer and RNA was isolated as described below.

RE-POLARIZATION OF M1 OR M2 MACROPHAGES BY CONDITIONED MEDIUM

After isolation macrophages were cultured and stimulated as described above. After 24 h of stimulation the differentially polarized macrophages were washed and cultured in angiogenic medium for 4 h in order to create conditioned medium. After 4 h the medium conditioned by M1 macrophages was used to stimulate M2 macrophages for 24 h, and vice versa. Subsequently, the polarization of the stimulated macrophages was determined by quantitative RT-PCR (qRT-PCR).

RNA ISOLATION AND CDNA SYNTHESIS

At day 11, i.e. after stimulation of the macrophages, total RNA was isolated from the cells using the Rneasy Micro Kit (Qiagen Inc., CA, USA), in accordance to the manufacturer’s protocol. In short, a lysate was made in buffer containing 1% 2-mercaptoethanol and diluted with an equal volume of 70% ethanol. RNA was collected on an RNA binding column by centrifugation. Inadvertent DNA contamination was removed by incubation of the column with a DNase I solution at 25 °C for 15 min. Next, the RNA-binding column was washed twice and subsequently the RNA was eluted with 14 μl Elution Buffer. Concentration and purity of the RNA were determined by UV spectrophotometry (NanoDrop Technologies, Wilmington, NC). For quantitative RT-PCR analysis, total RNA was reverse transcribed using the First Strand cDNA synthesis kit (Fermentas UAB, Lithuania). In summary, 1 μg of total RNA was diluted in a final reaction volume of 20 μl containing random hexamer primer (0.5 μg), RiboLock™ Ribonuclease Inhibitor (20 U), 1 mM dNTP mix, RevertAidTM M-MuLV Reverse Transcriptase (200 U) and incubated at 42 °C for 1 h. The reverse transcription reaction was terminated by heating the mixture to 70 °C for 10 min, after which the samples were placed on ice. The samples were stored at -80 °C until used for qRT-PCR experiments.
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QUANTITATIVE RT-PCR (QRT-PCR)

Quantification of gene expression was performed using qRT-PCR analysis. qRT-PCR was performed in a final reaction volume of 10 μl, consisting of 1x Sybr green Supermix (Bio-Rad, Hercules, USA), 6 μM forward primer, 6 μM reverse primer (Table 1) and 5 ng cDNA. Reactions were performed at 95 °C for 15 sec, 60 °C for 30 sec, 72 °C for 30 sec, for 40 cycles in a TaqMan ABI7900HT cycler (Applied Biosystems, CA, USA). Analysis of the data was performed using Science Detection Software 2.2.2.

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Forward Sequence</th>
<th>Reverse Sequence</th>
</tr>
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<tbody>
<tr>
<td>CD14</td>
<td>5'-AGCTAAGGACCTCTGAGAGAC-3'</td>
<td>5'-AGTTGTGGCTGAAGCTTAGG-3'</td>
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<tr>
<td>CD68</td>
<td>5'-GTCACACCTGACCTGCTC-3'</td>
<td>5'-CACTGGGACGAGAAGATT-3'</td>
</tr>
<tr>
<td>MCP1</td>
<td>5'-GGCTGAGCTAACCAACAA-3'</td>
<td>5'-ATGGTGACCTGCTGAT-3'</td>
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<tr>
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<td>5'-AGCCCATGCTGAGAAGAC-3'</td>
<td>5'-ATGCAATGCAAGAATT-3'</td>
</tr>
<tr>
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<td>5'-TTACCTGTGCTCGTGTTGAA-3'</td>
<td>5'-CTTTGGTTAAATTTTGGATCT-3'</td>
</tr>
<tr>
<td>CCL3</td>
<td>5'-CAGACATGACAGCTCCAC-3'</td>
<td>5'-ATGCTGACGGGCAAGCT-3'</td>
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<tr>
<td>IL6</td>
<td>5'-AATTGCGCTGATAGAAATCAT-3'</td>
<td>5'-AGGAACTGATGACAGAATT-3'</td>
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<td>CD163</td>
<td>5'-CAATGCTGCTCCTATAT-3'</td>
<td>5'-CAACATTGTCCTGATACT-3'</td>
</tr>
<tr>
<td>CCL20</td>
<td>5'-CAACATGGAAGATGGACT-3'</td>
<td>5'-ACATTGTCCTGATGGACT-3'</td>
</tr>
<tr>
<td>MGL1</td>
<td>5'-AGGTTCTCAAGCAGAAGGC-3'</td>
<td>5'-AGGTTGCGCTCCGTGACT-3'</td>
</tr>
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</table>

Table 1 Overview of primers used for qRT-PCR analysis

ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

CCL3 (also known as macrophage inflammatory protein-1α (MIP-1α)) and interleukin 6 (IL6) content in cell culture supernatant were measured using the DuoSet® ELISA Development kit (R&D Systems, Minneapolis, USA) in accordance to the manufacturer’s protocol. Briefly, 96 wells plates (#9018, Corning, Amsterdam, The Netherlands) were coated with 0.5 μg/ml Capture Antibody and incubated overnight at room temperature. After incubation the plates were washed and blocked with 2% bovine serum albumin (BSA), 0.05% Tween-20 (Sigma-Aldrich) in PBS for 1 h. After washing the plates were incubated with diluted sample or matched standards for 2 h. The detection was performed using matched biotin conjugated antibodies followed by streptavidin-polyhorseradish peroxidase (Sanquin, Amsterdam, The Netherlands). The color reaction was done with tetramethylbenzidine (TMB; Roth, Karlsruhe, Germany) containing H₂O₂ and stopped with 2 N H₂SO₄. The optical density was measured using a microplate reader (VERSA max, Molecular DevicesInc., CA, USA). The detection limit for IL6 and CCL3 was 7.8 pg/ml.

IMMUNOHISTOCHEMISTRY

At day 11 cells were washed twice with PBS and fixed in 2% PFA at room temperature for 15 minutes. Fixed cells were incubated with mouse-anti-human CD68 (Dako, Glosstrup, Denmark) (1:100) diluted in 1% bovine serum albumin (BSA) (Sanquin) /2% normal goat serum (NGS)/PBS for 1 h. After three washes with PBS, cells were incubated with biotinylated rabbit-anti-mouse F(ab’)2 (Dako) (1:100) diluted in 1% BSA/2% normal human serum (NHS)/PBS for 30 min. Then the cells were washed three times with PBS and incubated with streptavidine-HRP (Dako) (1:400) in PBS for 30 min. The color development was performed with 3-amino-9-ethylcarbazole (AEC, Sigma-Aldrich)
and the cells were counterstained with Mayers haematoxylin (Fluka Chemie, Buchs, Switzerland).

TRANSMISSION ELECTRON MICROSCOPY

After 10 days of culture, non-adhered cells were removed by PBS washes. Next, the adhered cells were fixed with 2% glutaraldehyde (GA, TAAB Laboratories, Aldermastron, UK) at 4 °C for 24 h. Then, cells were washed with PBS and 6.8% sucrose (pH 7.4), and postfixed at 4 °C in 1% osmiumtetroxide (OsO₄) dissolved in 1.5% potassium hexacyanoferrate(II) trihydrate (Merck, Darmstadt, Germany) in 0.1 M PBS. Thereafter, cells were washed with distilled water, dehydrated through a grades series of ethanol, and embedded in EPON 812 (Serva Feinbiochemica, Heidelberg, Germany). Ultrathin sections of approximately 80 nm were cut on a Sorvall microtome (Sorvall, Newton, CT) and contrasted with uranyl acetate and lead citrate. Sections were evaluated in a Philips 201 TEM (Philips, Eindhoven, The Netherlands) operated at 60 kV.

STATISTICAL ANALYSES

All data are represented as means ± standard error of the mean of at least three independent experiments and were analyzed by one-way ANOVA followed by Tukey's post hoc analysis using Graph-Pad Prism Version 4 for Macintosh (GraphPad Software, Inc., La Jolla, CA, USA). Values of P < 0.05 were considered to be statistically significant.
CHAPTER 3

RESULTS

GENERAL FEATURES OF ANGIOGENICALLY-PRIMED MACROPHAGES

The mononuclear cell (MNC) fraction as isolated by density-gradient centrifugation using Lymphoprep was seeded onto FN or COL I coated plates and cultured in angiogenic medium; noncoated TCPS was used as a control. Cell adherence on FN, COL I and TCPS was similar (data not shown). The cell morphology on the different substrata was also comparable (Fig. 1A). Irrespective of coating, adhered cells presented as either rounded or spindle-shaped cells. Adhered cells were predominantly CD68 positive indicating that adhered cells had differentiated to macrophages (Fig. 1B). On an ultrastructural level the cells revealed macrophage characteristics such as indented nuclei with less condense chromatin, one or more large nucleoli, well-developed mitochondria and cell membranes with microvilli (Fig. 1C).

Expression of relevant genes of angiogenically-primed macrophages cultured on FN, COL I or TCPS was done by qRT-PCR after 11 days (Fig. 2). All macrophages showed, relative to GAPDH, a low gene expression of the monocyte marker CD14, which indicates monocytes to macrophage differentiation (Daigneault, et al. 2010), high expression of macrophage marker CD68, as well as monocyte chemotactic protein-1 (MCP1), which corroborates their phenotype. Expression levels of the pro-inflammatory mediators IL1B and CCL3 were similar in macrophages cultured on FN, COL I or TCPS. The expression of CD163 and CD206, which are known to be upregulated by M2 macrophages, was comparable on FN, COL I and TCPS. Although the expression levels of CD14, CD68, CD163 and CD206 were not statistically different, the expression of these genes all tended to be higher on FN and COL I compared to TCPS. The relatively high expression of CD163 and CD206 indicates that the angiogenically-primed macrophages had polarized to a M2-like phenotype.

GENE EXPRESSION PATTERNS AFTER MACROPHAGE STIMULATION

Because macrophages cultured under angiogenic conditions on different substrata did not show statistically differences in gene expression, we investigated whether these angiogenically-primed macrophages were able to respond to LPS/IFNγ or IL4/IL13, which induce M1 (classically activated) or M2 (alternatively activated) macrophage polarization, respectively. Irrespective of coating, the classical or alternative stimulations did not affect cell adhesion nor altered the morphology of the macrophages (Fig. 1D).

The expression of CD68 was not affected by classical or alternative stimulation on the different substrata (Fig 3). Macrophages stimulated with LPS/IFNγ cultured on FN showed a significantly higher MCP1 expression compared to TCPS and IL4/IL13 stimulated macrophages cultured on FN. In contrast, MCP1 expression of M1 polarized macrophages on COL I and TCPS was similar to M2 polarized and unstimulated macrophages (Fig. 3).

After stimulation with LPS/IFNγ and on TCPS and ECM coated plates, angiogenically-primed macrophages showed an upregulated gene expression of IL1B, CCL3 and IL6 compared to IL4/IL13-stimulated and unstimulated macrophages (Fig.3).
MACROPHAGE PLASTICITY, IRRESPECTIVE OF ECM COMPONENTS

Figure 1: General features of angiogenically-primed macrophages.
A) Monocytes are cultured under angiogenic conditions on ECM components (FN and COL I and on TCPS as control) for 10 days; Adhered cells on FN, COL I and TCPS formed rounded and spindle shaped cells. No differences are seen in growth and morphology between the three substrates. B) Monocytes differentiated into macrophages as shown by the expression of CD68. C) At ultra structural level these cells show macrophage characteristics; indented nucleoli with less condensed chromatin and a large nucleolus (representing transcriptional activity), cell membranes with microvilli and in their cytoplasm well developed mitochondria. D) 10 days culture of macrophages 24 hours stimulated with LPS/IFNγ or IL4/IL13; No differences in adhesion and morphology of cells were observed. Original magnifications: 100x (A, D), 200x (B), 4000x (C)

This indicates that macrophages cultured under angiogenic conditions were able to polarize towards M1 macrophages upon activation with LPS/IFNγ. After classical activation macrophages cultured on ECM components showed a higher expression of IL1B compared to macrophages cultured on TCPS. CCL3 and IL6 expression was highest in macrophages cultured on COL I compared to TCPS. Although on all three substrates macrophages polarized towards M1 after LPS/IFNγ stimulation, both FN and COL I promoted a more pronounced M1 polarization in terms of marker gene expression.

After stimulation of angiogenically-primed macrophages with IL4/IL13 the gene expression of macrophage galactose N-acetyl-galactosamine specific Lectin-1 (MGL1) was upregulated compared to unstimulated and M1 macrophages on all three substrates. After stimulation with IL4/IL13 the gene expression of CD163 and CD206 cultured on FN, COL I and TCPS were similar compared to unstimulated macrophages, but higher compared to classically stimulated macrophages. As stated before, unstimulated angiogenically-primed macrophages had a relatively high expression of CD163 and CD206, indicating that macrophages cultured under angiogenic conditions had acquired an “intermediate” M2 polarization.
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CHAPTER 3

CYTOKINE AND CHEMOKINE PRODUCTION AFTER MACROPHAGE STIMULATION

Differences in gene expression between angiogenically-primed macrophages cultured on FN, COL I and TCPS were only observed after LPS/IFNγ stimulation (Fig. 3). In contrast to gene expression levels, similar secretion levels of CCL3 and IL6 were detected between LPS/IFNγ-stimulated macrophages cultured on FN, COL I or TCPS (Fig. 4). As was observed for gene expression levels, macrophages stimulated with LPS/IFNγ secreted significantly more CCL3 protein and IL6 protein compared to IL4/IL13-stimulated and unstimulated macrophages (Fig. 4).

RE-POLARIZATION OF MACROPHAGES BY CONDITIONED MEDIA

Macrophages can be re-polarized by secondary classical or alternative stimuli [4]. We examined the possibility of re-polarization of angiogenically-primed macrophages by conditioned medium (CM). The expression of IL1B, CCL3 and IL6 by M1 macrophages was abolished by treatment with CM from M2 macrophages (Fig. 5A). Moreover, this treatment induced M2 marker genes, such as MGL1, CD163 and CD206 (Fig. 5A). These results indicate that M1 macrophages could be re-polarized by CM from M2 macrophages within 24 hours.
Figure 3: Gene expression analysis of angiogenically-primed macrophages after M1 or M2 polarization. After stimulation with LPS/IFNγ and IL4/IL13 no differences are seen in CD68 expression in macrophages cultured on ECM coated and TCPS plates. Macrophages cultured on FN stimulated with LPS/IFNγ show higher gene expression of MCP1 compared to TCPS and IL4/IL13 stimulated cells. After stimulation with LPS/IFNγ angiogenically-primed macrophages polarized towards M1 macrophages as shown by upregulation of IL1B, CCL3 and IL6. Macrophages stimulated with IL4/IL13 upregulate MGL1. No differences in CD163 and CD206 are found between unstimulated cells and IL4/IL13 stimulated macrophages. After stimulation with LPS/IFNγ, CD163 and CD206 are downregulated, which indicates that unstimulated angiogenically-primed macrophages are polarized towards a M2-like macrophage (n=5). * p<0.05, Difference between FN, COL I and TCPS in LPS and IFNγ stimulated macrophages, ** p< 0.01, ***p<0.001. † p<0.05, Difference between LPS/IFNγ and IL4/IL13 stimulated macrophages corresponding to the same coating, ‡ p<0.05, Difference between stimulated and unstimulated macrophages corresponding to the same coating. ^ p<0.01, ^^^ p<0.001, Difference between IL4/IL13 and LPS/IFNγ stimulated macrophages corresponding to the same coating.
M2 macrophages significantly upregulated the pro-inflammatory genes IL1B and IL6 after treatment with CM from M1 macrophages. CCL3 expression remained similar after treatment whereas MGL1 expression was significantly downregulated. Both CD163 and CD206 tended to be downregulated after treatment of M2 macrophages with CM from M1. These results indicate that the M2 macrophages quickly and almost completely repolarized to M1 macrophages after stimulation with M1 macrophages CM within 24 h.

Figure 4: Protein analysis of angiogenically-primed macrophages after M1 or M2 polarization. Macrophages stimulated with LPS/IFNγ secreted significantly more CCL3 and IL6 compared to IL4/IL13 stimulated and unstimulated macrophages. No differences in secreted CCL3 and IL6 are seen in LPS/IFNγ stimulated macrophages cultured on FN, COL I and TCPS (n=5). ‡‡‡ p<0.001, Difference between LPS/IFNγ and IL4/IL13 stimulated macrophages corresponding to the same coating. ### p<0.001, Difference between LPS/IFNγ stimulated and unstimulated macrophages corresponding to the same coating.

DISCUSSION

The main findings of our study are: firstly, that angiogenically-primed human peripheral blood mononuclear cells differentiated into M2-like polarized macrophages. Secondly, that these M2 macrophages rapidly polarized to M1 macrophages after stimulation with LPS/INFγ overnight. Similarly, their M2 features were further enhanced by stimulation with IL4 and IL13 overnight. Thirdly, induced M1 respectively M2 polarization could be reversed by treatment with conditioned media from M2 respectively M1 macrophages. Finally, the extracellular matrix compounds fibronectin or collagen I did not play a primary role in the dynamic switches of macrophage polarization.

In general, macrophages facilitate the removal of apoptotic and necrotic cells and coordinate tissue repair, including angiogenesis and scar formation. These processes are well-separated during e.g. myocardial healing: clearance of cellular debris precedes tissue remodeling. Our current *in vitro* results corroborate and help explain our previous findings in experimental animal models for tissue repair and remodeling. Previously, we showed that macrophages are pivotal in effective myocardial wound healing after myocardial infarction in mice (van Amerongen, *et al.* 2007).
Figure 5: Gene expression analysis after re-polarization of M1 or M2 polarization of angiogenically-primed macrophages.

M1 macrophages that were stimulated with CM from M2 macrophages showed downregulation of M1 genes (IL1B, CCL3 and IL6) and upregulation of M2 genes (MGL1 and CD206) (A).

M2 macrophages that were stimulated with CM from M1 show upregulation of M1 genes (IL1B and IL6) and downregulation of M2 genes (MGL1, CD163 and CD206) (B) (n=3). * p<0.05, Significant difference between M1 and re-stimulated M1 macrophages with CM from M2 macrophages, corresponding to the same coating. ** p<0.01, ***p<0.001. # p<0.05, Significant difference between M2 and re-stimulated M2 macrophages with CM from M1 macrophages, corresponding to the same coating. ## p<0.01, ### p<0.001
When macrophage infiltration was prevented, macrophage-depleted hearts showed non-resorbed cell debris but also less granulation tissue, along with reduced vascularization and scarring. Thus, it would seem likely that infiltrated macrophages in the myocardial infarction model first polarized towards M1 macrophages in order to start both inflammation and the removal of apoptotic and necrotic cells. After which these M1 macrophages may have re-polarized towards a M2 macrophage by functional adaptivity, through changes in the microenvironment (Stout, et al. 2004). In our current in vitro model we showed that angiogenically-primed human M1 or M2 had a high plasticity and could be polarized and re-polarized by conditioned medium from M1 or M2 macrophages.

In vivo, macrophage re-polarization might also occur as was shown in a mouse model for atherosclerosis (Khallou-Laschet, et al. 2010). In their model the authors showed that early plaques in ApoE knockout mice contained predominantly M2 macrophages. Remarkably, during aging the M2 switched to M1 macrophages, which augmented plaque growth. This M2 to M1 switch was accomplished by microenvironmental changes, which were replicated in vitro. This indicates that macrophages play a central role in the development and progression in atherosclerosis. Harel-Adar and colleagues also confirmed that macrophages are plastic, in vivo. They showed that an intramyocardial injection of phosphatidylserine-presenting liposomes (which mimic apoptotic cells) in rodents induces, after myocardial infarction, macrophages to secrete anti-inflammatory cytokines one day earlier than without treatment (Harel-Adar, et al. 2011). This suggest that re-polarization of M1 macrophages towards M2 macrophages can lead to better tissue repair, less scar formation and prevention of heart dilatation in vivo.

In summary, we have shown that angiogenically-primed human macrophages underwent classical and alternative activation in vitro. These M1 and M2 macrophages could be re-polarized by conditioned medium from M1 and M2 macrophages as a secondary stimulus. The type of substrate (COL I, FN, TCPS) did not play a primary role during macrophage polarization. We conclude that primary human macrophages cultured in an angiogenic microenvironment harbour a highly dynamic plasticity. This in vitro model for macrophage polarization can be further explored in studies that investigate the role of human macrophage dynamics in tissue repair.

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REFERENCES


MACROPHAGE PLASTICITY, IRRESPECTIVE OF ECM COMPONENTS