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Chapter 5

Hydrocortisone and M-CSF promote in vitro education of pro-angiogenic monocytes

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ABSTRACT

Circulating angiogenic cells (CACs) and M2 macrophages promote neovascularization by secretion of paracrine factors. We identified which in vitro conditions are required to induce monocyte differentiation towards CACs and to analyze their relation with monocyte subsets. Human blood monocyte subsets (classical, intermediate and non-classical monocytes) all differentiate to CACs when cultured in endothelial growth medium conditions, as judged by their adherence to fibronectin, uptake of DiI-Ac-LDL, binding to Ulex-Lectin, and expression of VEGFR-2 and Tie2 (angiopoietin receptor). From the medium components essential for CAC differentiation, we determined that specifically hydrocortisone induced Tie2 and VEGFR-2 surface protein expression. In contrast to endothelial growth factors, treatment with monocyte growth factor M-CSF increased the number of CACs by at least 3-fold. Both hydrocortisone and M-CSF left the capacity of CACs to stimulate endothelial tube formation in vitro intact. CACs shared a significant number of differentiation markers with M2 macrophages, but their pro-angiogenic capacity may be superior as judged by higher >10-fold mRNA expression levels of pro-angiogenic growth factors. In conclusion, hydrocortisone induces a pro-angiogenic phenotype of CACs, while M-CSF expands their numbers without compromising their functionality in vitro. These observations may improve cell-based therapies to enhance vascularization of ischemic tissues.

INTRODUCTION

Ischemic heart disease (IHD) is the most common cause of death in the Western world. IHD due to coronary artery atherosclerosis has been an increasing problem in recent years[1]. Coronary artery atherosclerosis may lead to acute myocardial infarction (AMI) which is characterized by the interruption of blood flow, causing myocardial loss in downstream regions of the heart and ventricular remodeling[2]. To prevent loss of myocardial tissue, animal experiments and clinical trials have been designed to test the potential of myeloid cell transplantation for treatment of IHD. The option to reduce infarct size by promoting coronary angiogenesis/arteriogenesis through circulating angiogenic cells (CACs) has gained worldwide interest. Angiogenesis refers to the growth of new vessels from the pre-existing vasculature, whereas arteriogenesis occurs by outward remodeling of pre-existing inter-arterial connections into true collateral arteries[1].

CACs were previously defined as early endothelial progenitor cells (eEPCs) based on their endothelial phenotypic characteristics and the time of their appearance during *in vitro* culture. Typically, CACs emerge from peripheral blood mononuclear cells after 4 to 7 days of *in vitro* culture under endothelial cell growth conditions. The characterization of CACs is based on adherence to fibronectin, binding to lectins from Ulex europaeus, and the take up of Dil-labeled acetylated low-density lipoprotein (Dil-Ac-LDL)[3]. CACs possess the phenotype of monocytes/macrophages, display a spindle-shaped morphology and express marker proteins of the endothelial cell lineage[3-11], such as the angiopoietin receptor (Tie2), and the receptor for vascular endothelial growth factor (VEGFR-2/KDR/Flk-1 or CD309). CACs were shown to home to vessels but do not become incorporated in the endothelium[12-15]. Instead, CACs are thought to provide paracrine signals (angiogenic cytokines, chemokines, EC growth factors, and metalloproteinases) to adjacent endothelial cells and tissue[5,7,13-16], thus promoting angiogenesis *in vitro* and *vivo*[14-16]. CACs originate from monocytes, and can be mobilized and recruited from the bone marrow by cytokines, chemokines, growth factors, hormones, drugs or other cues after tissue ischemia[13,17,18]. CACs express the monocytic marker CD14[5,7] and their transcriptome is similar to monocytes but distinct from endothelial cells, while late endothelial outgrowth cells (EOC) or endothelial colony forming cells (ECFC) from peripheral blood mononuclear cells (PBMNC) resemble mature endothelial cells and are not bone marrow-derived[19] (reviewed in[20,21]). However, the subtype of the originating monocyte for CACs is unknown.

Human circulating monocyte subsets have been defined based on the differential expression of surface markers; classical monocytes ($CD14^{++}/CD16^{-}$), intermediate monocytes ($CD14^{++}/CD16^{+}$) and non-classical monocytes $CD14^{+}/CD16^{+}$[22,23]. Classical monocytes express the chemokines receptors CCR1 and CCR2 and migrate towards MCP-1/CCL2 and MIP-1α/CCL3, while non-classical monocytes patrol the blood vessels through an LFA1-dependent crawling behaviour along the endothelial cells[24], express higher levels of CX3CR1 and show efficient

transendothelial migration in response to fractalkine[25]. Both $CD16^+$ and $CD16^-$ monocytes migrate towards SDF-1/CXCL12, although $CD16^+$ cells migrate more efficiently. It has been suggested that classical monocytes, after their release from the bone marrow, develop to non-classical monocytes through an intermediate stage[22,23,26]. Indeed, fate mapping studies on the murine correlate of classical monocytes ($CX3CR1^{int}Ly6C^{high}$ monocytes)[27] indicated that these cells are the progenitors of the circulating $CX3CR1^{high}Ly6C^{low}$ cells, the murine counterpart of non-classical monocytes[28]. Not only under steady state conditions[28], but also during inflammation this differentiation pathway is apparent, as it was recently demonstrated that inflammatory conditions induce an accumulation of peripheral blood $Ly6C^{high}$ monocytes in the affected tissue, which then locally differentiate into $Ly6C^{low}$ non-classical cells during the reparative phase[29].

In humans, approximately half of the non-classical fraction of circulating $CD16^+$ monocytes is positive for Tie2, the angiopoietin receptor. It is this fraction that promotes angiogenesis *in vivo*[30], indicating that a minority of the monocytes is responsible for angiogenesis. However, it is not known which monocyte subtype can be instructed to become pro-angiogenic *in vitro*, during specific culture conditions, or *in vivo*. Neither is it known which factors are responsible for this differentiation.

Several reports have demonstrated the therapeutic potential of *ex vivo* cultured circulating myeloid cells which improved blood flow to ischemic tissues in the experimental animal hind limb ischemia model[6,7]. In myocardial infarction patients, bone marrow-derived progenitor cells have been used in clinical trials, resulting in improvement of cardiac function[31,32]. Administration of *ex vivo* cultured peripheral blood mononuclear cell-derived CACs has shown its long term safety after acute myocardial infarction[33]. Importantly, transplantation of human $CD14^+$ monocytes/macrophages without *ex vivo* pre-conditioning is less effective in improving neovascularization than CACs, in the ischemic hind limb in nude mice[6]. Thus, isolation, *ex vivo* expansion and administration of CACs represent a new clinical strategy for treating ischemic heart disease in which stimulation of neovascularization is essential. Like CACs, M2 macrophages originate from monocytes, promote vasculogenesis, and show a related proteome and transcriptome[16,21]. M2 macrophages are generated by monocyte stimulation with IL-4, are anti-inflammatory, are involved in tissue repair and remodeling, and promote both angiogenesis[34] and arteriogenesis[35].

Currently, CACs are generated from mononuclear cells after culture in endothelial growth medium (EGM)[3,17] and this *in vitro* "education" contributes to their enhanced promotion of neovascularization. It is not clear which of the pro-angiogenic factors present in EGM, which was originally used to stimulate endothelial precursor cells, contribute to functional differentiation of CACs. Glucocorticosteroids, in particular hydrocortisone, are commonly used for CAC culture[6], but no reports explain its relevance or mode of action in CAC differentiation. Neither the relevance of endothelial growth factors in CAC expansion has

been studied in detail, nor the potential role for M-CSF, a hematopoietic growth factor stimulating survival, proliferation and differentiation of cells of the monocyte/macrophage lineage[36,37]. In this study we set out to define the potential factors contributing to the pro-angiogenic activity of CACs and their numbers, which can therapeutically be used for augmentation of neovascularization.

METHODS

Isolation of human monocyte subsets by cell sorting

Peripheral blood mononuclear cells (PBMCs) were isolated from healthy volunteer buffy coats (Sanquin, Amsterdam, The Netherlands), using Ficoll density gradient centrifugation (Lymphoprep™, Axis-Shield, Oslo, Norway). For the isolation of monocytes we performed a second density gradient centrifugation step using PBMCs overlayed on Percoll solution (GE Healthcare Life Sciences, Uppsala, Sweden). After centrifugation, monocytes were collected, washed in wash buffer containing 1% heat-inactivated fetal bovine serum (FBS; Lonza, Breda, The Netherlands) and 1 mM EDTA (Merck, Darmstadt, Germany) in phosphate buffered saline (PBS), and adjusted to a concentration of 1 x 10^6/ml in wash buffer. For purification of $CD14^{++}CD16^-CD56^-$ (classical), $CD14^{++}CD16^+CD56^-$ (intermediate), $CD14^+CD16^+CD56^-$ (non-classical) monocytes, 100 x 10^6 monocytes were incubated with FITC-labeled mouse anti-human CD14 (1:100; Immunotools, Friesoythe, Germany), APC-labeled mouse anti-human CD16 (1:100; Immunotools), and PE-labeled mouse anti-human CD56 (1:100; Immunotools) antibodies for one hour at dark in 4°C on a rotating shaker. After incubation, cells were washed in wash buffer, and resuspended in 2 mM EDTA in endothelial growth medium (EGM) containing endothelial basal medium (EBM; Lonza) supplemented with 20% heat-inactivated FBS and 1% penicillin/streptomycin (Lonza) in the presence of EGM SingleQuots: bovine brain extract (BBE; 1:252), human epidermal growth factor (hEGF; 1:1007), and hydrocortisone (HC; 1:1007; Lonza). Positive/negative cells were sorted using MoFlo XDP cell sorter (Beckman Coulter, Woerden, The Netherlands). After sorting, the cells were washed in PBS, seeded to a concentration of 1 x 10^6/ml on human fibronectin-coated (10 µg/ml; Sigma-Aldrich, Zwijndrecht, The Netherlands) 96 well tissue-culture plates (Greiner Bio-One, Alphen a/d Rijn, The Netherlands) in EGM, and cultured at 37°C with 5% CO_2. After three days of culture, cells were washed carefully with pre-warmed PBS (37°C) to remove non-adherent cells, and fresh EGM medium was added. Adherent cells at day 4 were stained for Dil-Ac-LDL/Lectin. Total PBMCs and monocytes were used as control cells.

CAC isolation and culture

CACs were obtained as previously described[3]. Briefly, PBMCs and monocytes were isolated from healthy volunteer buffy coats using Ficoll and Percoll density gradient centrifugation,

respectively. After centrifugation, PBMCs and monocytes were collected and washed in PBS containing 1% citrate (Sigma-Aldrich) at 200 x g for 10 minutes to remove platelets. Isolated PBMCs or monocytes were subsequently resuspended to a concentration of 8 x 10^6/ml or 1 x 10^6/ml, respectively in different growth medium consisting of EBM supplemented with 20% heat-inactivated FBS and 1% penicillin/streptomycin in the presence or absence of EGM SingleQuots: BBE (1:252), hEGF (1:1007), and/or HC (1:1007). Unless indicated otherwise, CACs were also cultured in the presence of varying concentrations of HC (Sigma-Aldrich) or dexamethasone (DXM; Sigma-Aldrich) as indicated, recombinant human M-CSF (100 ng/ml; ebioscience, Viena, Austria), or combinations in EBM containing 20% heat-inactivated FBS and 1% penicillin/streptomycin. To compare CAC culture medium with other media, PBMCs or monocytes were also cultured in standard culture media for monocytic cells i.e. RPMI 1640 (Invitrogen, Bleiswijk, The Netherlands) containing 20% heat-inactivated FBS, 1% penicillin/streptomycin, and 2 mM L-glutamine (Lonza). Cells were then seeded in tissue-culture plates (Greiner Bio-One) in the presence or absence of human fibronectin coating (10 µg/ml; Sigma-Aldrich), and cultured at 37°C with 5% CO_2. After three days of culture, all non-adherent cells were removed by washing with pre-warmed PBS (37°C), and fresh growth medium was added. Adherent cells between day 4-7 of culture were used for DiI-Ac-LDL/Lectin staining, flow cytometry, RT-PCR, or *in vitro* tube formation assay.

DiI-Ac-LDL/Lectin staining and CAC calculation

CACs cultured for 4-5 days in different treatment media were incubated with 2.4 µg/ml DiI-labeled ac-LDL (Tebu-Bio BV; Heerhugowaard, The Netherlands) and 10 µg/ml fluorescein isothiocyanate (FITC)-labeled *Ulex europaeus* agglutinin type I lectin (Ulex-Lectin; Sigma-Aldrich) for one hour at 37°C with 5% CO_2. After one hour, cells were fixed with 4% paraformaldehyde (PFA) in HBSS (Invitrogen) for 30 minutes at room temperature (RT). Then, the cell nuclei were counterstained with 5 µg/ml of Hoechst (Invitrogen) for 30 minutes at RT. Cells were viewed and photographed at 20x magnification using an inverted fluorescence microscope (Nikon Eclipse TE300, Tokio, Japan). Fibronectin-adherent cells double positive for DiI-Ac-LDL and lectin staining were considered as CACs, and counted in five randomly selected fields using WCIF Image J software (National Institutes of Health, Bethesda, Maryland, USA).

Flow cytometry

Flow cytometric analysis of the surface expression of Tie2, KDR/VEGFR-2 and CXCR4 in PBMCs and monocytes cultured under different growth conditions or fresh cells was performed as indicated. CACs or macrophages were detached using 1 mM EDTA or 4 mg/ml lidocaine hydrochloride monohydrate in PBS, respectively by incubating the cells at 37°C with 5% CO_2 for 10 minutes. Before labeling with the primary antibodies, cells were

washed in 0.5% bovine serum albumin (BSA; Roche Diagnostics, Almere, The Netherlands) in PBS and blocked with 10% normal mouse serum in 0.5% BSA/PBS for 10 minutes at 4°C. After blocking, cells were incubated for 30 minutes at RT with the fluorescent monoclonal antibodies: mouse FITC-conjugated anti-human CD14 (Immunotools), APC-conjugated anti-human CD16 (Immunotools), Alexa-fluor 647 anti-human VEGFR-2/KDR (Biolegend, San Diego, CA, USA), PE-conjugated anti-human Tie2 (R&D Systems, Abingdon, UK), and APC-conjugated anti-human CXCR4 (Biolegend). Fluorescent isotype-matched antibodies (Immunotools) were used as negative controls. After labeling, the cells were washed and resuspended in 0.5% BSA/PBS and analyzed on a Cyan ADP High Performance Research Flow Cytometer (Beckman Coulter, Woerden, The Netherlands). The data were analyzed using the Summit V4.3 (Dako, Fort Collins, CO, USA) software. The results are expressed as fold change of mean fluorescence intensity (MFI) relative to EBM or EGM samples.

In vitro polarization of human monocytes to M2 macrophages

Human monocytes were resuspended to a concentration of 1 x 10^6/ml in DMEM complete medium (Invitrogen) containing 5% heat-inactivated human AB serum (Sanquin), 1% penicillin/streptomycin and 2 mM L-glutamine, and differentiated into M0 macrophages by culturing them for five days in 94 mm petri dishes (Greiner Bio-One) at 37°C with 5% CO_2. After five days of culture, the adherent M0 macrophages were harvested with 4 mg/ml lydocaine hydrochloride monohydrate (Sigma-Aldrich) in PBS at 37°C with 5% CO_2 for 10 minutes. M0 macrophages were seeded at a density of 1 x 10^6/ml in tissue-culture plates (Greiner Bio-One) in DMEM complete medium and incubated with 10 ng/ml rhIL-4 (Immunotools) for differentiation into alternatively activated M2 macrophages for 48 hours. M0 macrophages were cultured for the same time period in DMEM complete medium alone. Both macrophage subtypes were used for further experiments as indicated.

RNA isolation, cDNA synthesis, quantitative real-time PCR

Total RNA was isolated from CACs and macrophage subtypes using RNeasy mini kit (Qiagen, Venlo, The Netherlands) according to the manufacturer's recommendations, including a DNase I (Qiagen) digestion step. RNA samples were concentrated by SpeedVac for 30 minutes. RNA purity and concentration was measured using NanoDrop ND-1000 Spectrophotometer (Thermo Scientific, Breda, The Netherlands). cDNA was synthesized from 500 ng total RNA per sample using RevertAid™ H Minus First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's recommendations. Quantitative real-time polymerase chain reaction (PCR) was performed in an ABI PRISM 7900HT system (Applied Biosystems, Foster City, CA, USA) with the following primers (Invitrogen) designed by Primer Express version 2.0 (Applied Biosystems):

Human genes	Primer sequences (5'-3')
IL-10	Forward: GAGGCTACGGCGCTGTCAT
	Reverse: CCACGGCCTTGCTCTTGTT
CD206	Forward: CCTGTGCATTCCCGTTCAA
	Reverse: TCGTGCAATCTGCGTACCAC
CCL18	Forward: CCAGGTGTCATCCTCCTAACCA
	Reverse: GCCCTCGCAGCTTCCA
CXCL12	Forward: GATGCCCATGCCGATTCTT
	Reverse: GTTCTTCAGCCGGGCTACAAT
IL-8	Forward: TGAGAGTGGACCACACTGCG
	Reverse: TCTCCACAACCCTCTGCACC
MCP-1	Forward: ATCTCAGTGCAGAGGCTCGC
	Reverse: GCACAGATCTCCTTGGCCAC
VEGF-B	Forward: AGAGCTCAACCCAGACACCTG
	Reverse: TCTGAAAAGCCATGTGTCACC
HGF	Forward: GCAAGAAAACAATGCCTCTGG
	Reverse: GAGGTCAAATTCATGGCCAA
PDGF-B	Forward: GCAGCAGCTTCAGAGACCAAC
	Reverse: ATCGGCAGGAGAGTGTGTGG
PDGF-C	Forward: CCACGAGGTCCTTCAGTTGAG
	Reverse: CGTCGGTGAGTGATTTGTGC
CCND1	Forward: AGATCATCCGCAAACACGC
	Reverse: GCGGATTGGAAATGAACTTCA
CCNA2	Forward: CACCACAGCATGCACAACAGT
	Reverse: GGTTGAGGAGAGAAACACCATGAT
CCNB1	Forward: TCCAGTTATGCAGCACCTGG
	Reverse: TGCTTCGATGTGGCATACTTG
GAPDH	Forward: GCCAGCCGAGCCACATC
	Reverse: TGACCAGGCGCCCAATAC

Briefly, in a 10 µl reaction volume, 4 µl of diluted cDNA, 5 µl SYBR Green PCR Master Mix (Applied Biosystems), and 0.5 uM of each gene-specific primers were mixed. Gene expression levels were calculated using an arbitrary standard curve and normalized to the human housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Gene expression levels were expressed as a fold change relative to untreated EGM samples unless otherwise indicated.

In vitro tube formation assay

Human fibrin matrices were prepared in the wells of 96-wells plates by addition of a 100 µl solution of a mixture of 0.05 U/ml thrombin and 2 mg/ml plasma-derived fibrinogen (Kordia, Leiden, The Netherlands) in M199 medium (Lonza) as previously described[38]. After preparation of the matrices, confluent human foreskin microvascular endothelial cell (MVEC) monolayers were detached, concentrated and seeded on the fibrin matrices at a confluent density of ~27.500 cells/cm^2 and cultured for 16 hours in M199 supplemented with 10% HS, 10% NBCS, and penicillin/streptomycin at 37°C in 5% CO_2. Then the MVECs were stimulated with 10 ng/ml TNF-α (Sigma-Aldrich) and 25 ng/ml VEGF165 (Invitrogen) to initiate the tube formation. 24 hours after stimulation, the medium was replaced with serum-containing M199 medium supplemented with TNF-α and VEGF165 in the absence or presence of day 5 CACs (5% of the number of seeded HMVECs) that had been cultured in EBM, EBM with HC (1 µM), EBM with M-CSF (100 ng/ml) or combinations thereof in EBM, and EGM (1 µM HC, 252x diluted BBE, 1007x diluted hEGF). CACs were washed twice before use in the tube forming assay. After 48 hours, the combined cell populations were restimulated with M199 medium containing TNF-α and VEGF165. Three days after the last stimulation, the experiment was terminated by fixing the plates with pre-warmed (37°C) 2% PFA (pH 7.4) in PBS for two hours at RT. Tubular structures of HMVECs in the 3D fibrin matrix were photographed at 2.5x magnification by phase-contrast microscopy (Olympus-CK2 microscope), and the total length of tube-like structures was quantified using Optimas image analysis v. 6.5 software. Four microscopic fields per well were used to calculate the total length of the tube-like structures, expressed as mm/cm^2.

Statistical Analysis

Intergroup comparisons were performed using Student's t-test (paired, two-sided, for normally distributed data) or Wilcoxon test (for non-Gaussian data). Multiple groups were compared using one-way ANOVA and Tukey's multiple comparisons post hoc test (for normally distributed data) or Kruskal-Wallis with Dunn's multiple comparisons test (for non-Gaussian data). Differences were considered statistically significant if probability values (P) were less than 0.05. All experiments were performed with at least three independent donors unless otherwise indicated, and results are presented as mean ± standard error of the mean (SEM). All statistics were performed with Graphpad Prism version 6.0.

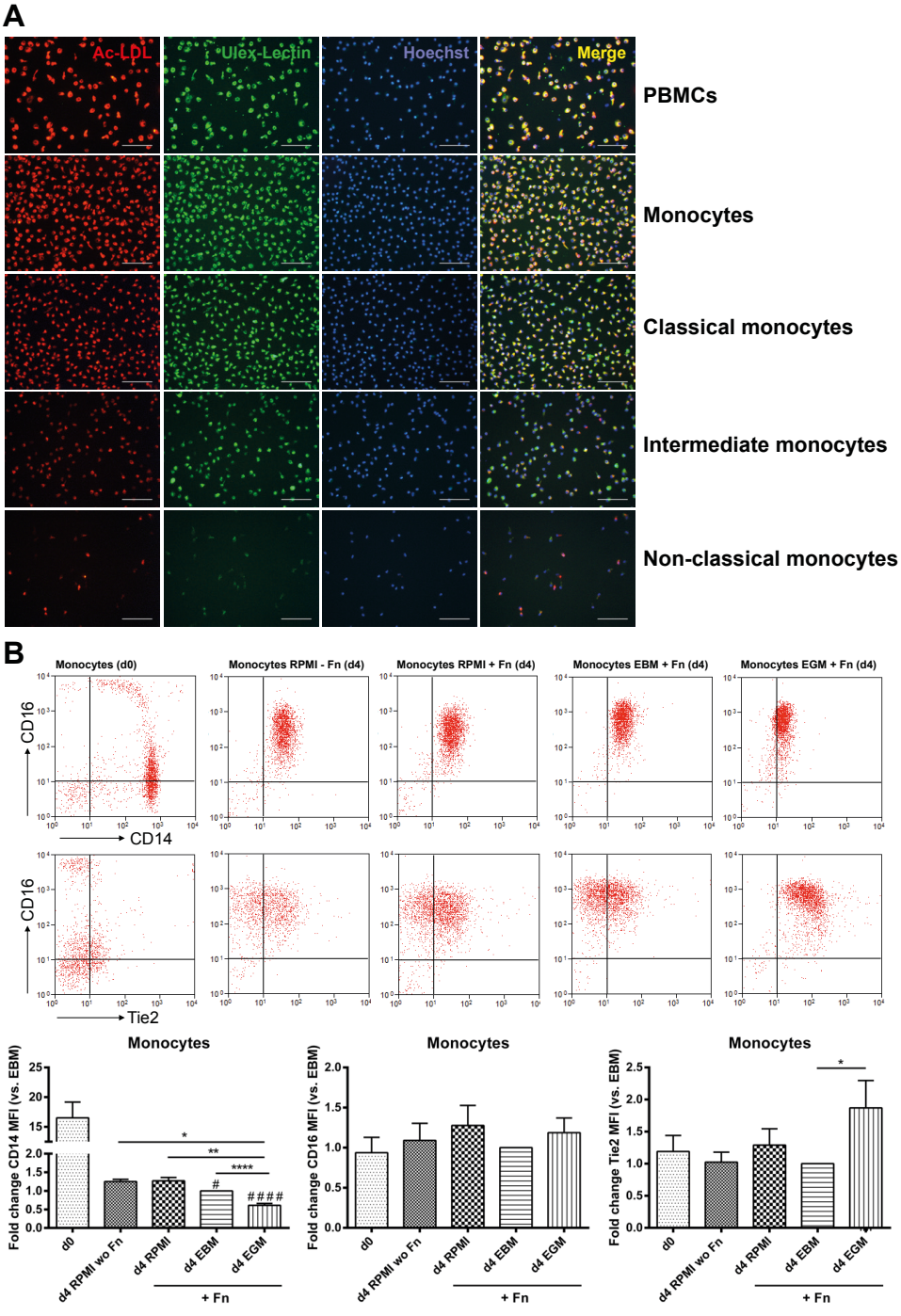

A
Ac-LDL
Ulex-Lectin
Hoechst
Merge
PBMCs
Monocytes
Classical monocytes
Intermediate monocytes
Non-classical monocytes
B
Monocytes (d0)
Monocytes RPMI - Fn (d4)
Monocytes RPMI + Fn (d4)
Monocytes EBM + Fn (d4)
Monocytes EGM + Fn (d4)
CD16
CD14
Tie2
Monocytes
Fold change CD14 MFI (vs. EBM)
Fold change CD16 MFI (vs. EBM)
Fold change Tie2 MFI (vs. EBM)
d0
d4 RPMI wo Fn
d4 RPMI
d4 EBM
d4 EGM
+ Fn

Figure 1. Differentiation and characterization of CACs from monocyte subsets. Classical ($CD14^{++}CD16^{-}CD56^{-}$), intermediate ($CD14^{++}CD16^{+}CD56^{-}$), and non-classical ($CD14^{+}CD16^{+}CD56^{-}$) human monocyte subsets were sorted, and cultured in endothelial growth medium (EGM) on fibronectin (Fn) for 4 days at 37°C in 5% CO_2 (A). EGM contains bovine brain extract (BBE; 252x diluted), human epidermal growth factor (hEGF; 1007x diluted), and hydrocortisone (HC; 1007x diluted). CACs were stained for uptake of Dil-Ac-LDL (red), binding of Ulex-Lectin (green), Hoechst (blue; cell nucleus), and photographed using an inverted fluorescence microscope at 20x magnification. Total PBMCs and monocytes were used as control cells. Results are representative images from two independent experiments. Scale bar represents 100 µm (A). Monocytes were cultured under different angiogenic growth conditions consisting of RPMI without (-) Fn, RPMI with (+) Fn, endothelial basal medium (EBM) with Fn, and EGM (1007x diluted HC, 252x diluted BBE, 1007x diluted hEGF) with Fn for 4 days at 37°C in 5% CO_2 (B). All live cells were gated in a forward scatter/sideward scatter dot plot, and the surface expression of CD14, CD16 and Tie2 was measured by flow cytometry on the gated cells. Fresh monocytes were used as control cells. Representative dot plots of $CD14^{+}CD16^{+}$ and $CD16^{+}Tie2^{+}$ are shown (B). Expression levels (in mean fluorescence intensity; MFI) in bar graphs are presented as fold change relative to EBM condition. Data are shown as mean ± SEM from at least 3 independent experiments. *$P < 0.05$, **$P < 0.01$, ****$P < 0.0001$, as indicated. # $P<0.05$, #### $P < 0.0001$, compared with day 0.

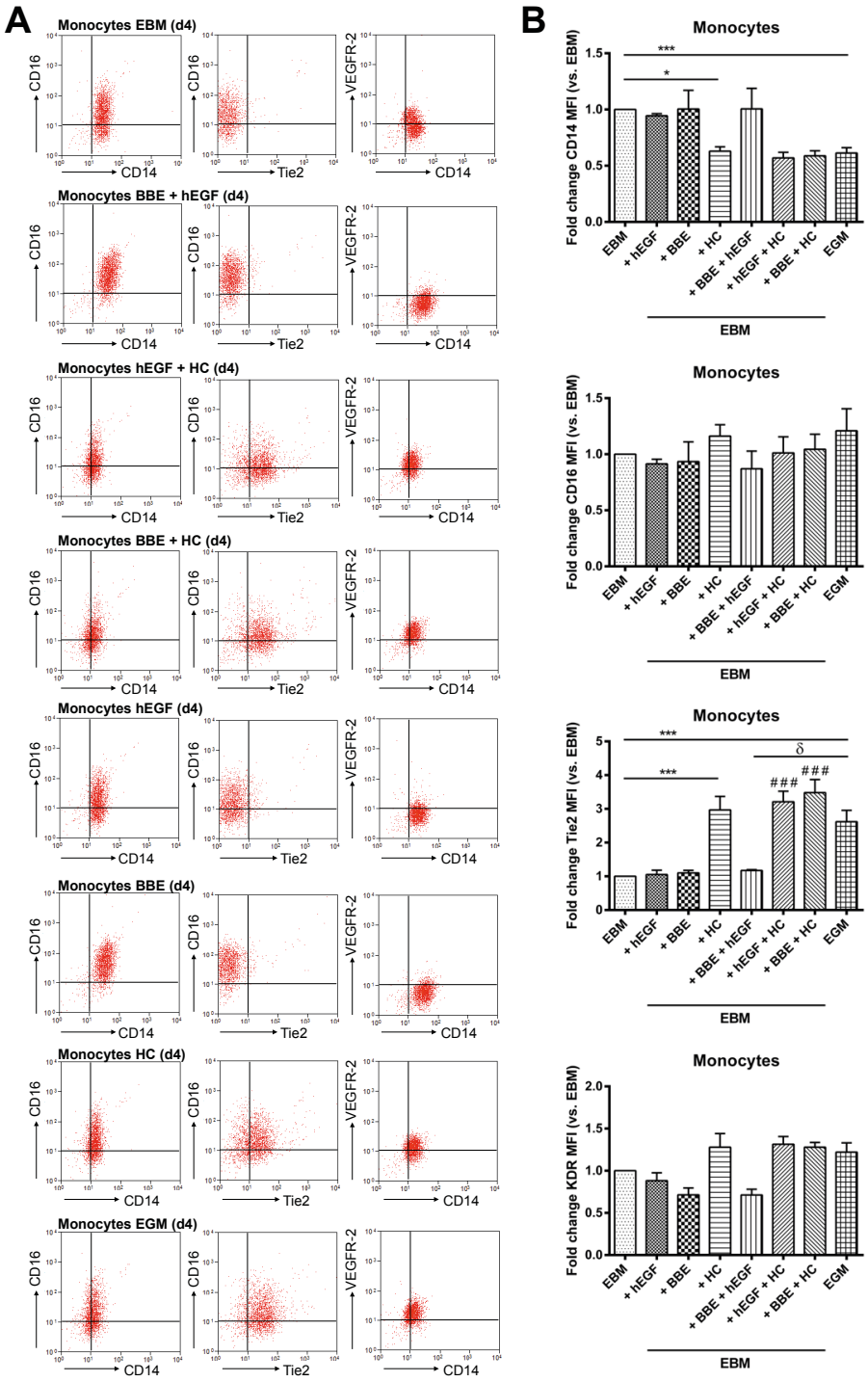
RESULTS

Differentiation of CACs from monocyte subsets

It is not known which circulating monocyte subset can differentiate to CAC *in vitro*, but it has been suggested that only a minor fraction of circulating monocytes ($CD14^{++}/CD16^{+}/Tie2^{+}$) can be considered pro-angiogenic[30]. To test this we sorted peripheral blood monocytes into classical ($CD14^{++}CD16^{-}CD56^{-}$), intermediate ($C14^{++}CD16^{+}CD56^{-}$), and non-classical ($CD14^{+}CD16^{+}CD56^{-}$) monocytes, and cultured them in EGM on fibronectin-coated plates for 4 days. The sorting strategy is shown in supplementary Fig. S1. CAC differentiation was assessed by immunofluorescence staining for the uptake of Dil-Ac-LDL and binding of Ulex-Lectin as it has been shown to be the phenotypical characteristics shared by all types of endothelial progenitors and CACs. After four days of differentiation, all three monocyte subsets adhered to fibronectin and obtained the spindle-shaped CAC morphology. In addition, all three monocyte subsets were also able to take up Dil-Ac-LDL and to bind Ulex-Lectin, although fewer non-classical monocytes seemed to differentiate to CACs (37% of classical monocytes, n=2). These results implicate that not a particular subtype, but all three human monocyte subsets are able to differentiate to CACs with the main characteristics of the pro-angiogenic phenotype (Fig. 1A).

In vitro education increases the expression of Tie2 in monocytes

Next, the effect of *in vitro* education on the pro-angiogenic marker expression on monocytes was determined. Therefore, we cultured human PBMCs and monocytes for 4 days in different treatment groups to evaluate the influence of fibronectin coating (fn) and EGM on surface expression of pro-angiogenic monocyte markers such as CD16 and Tie2[30,39]. As a control freshly isolated monocytes or PBMCs were used (Fig. 1B and Supplementary Fig. S2).


A
Monocytes EBM (d4)
Monocytes BBE + hEGF (d4)
Monocytes hEGF + HC (d4)
Monocytes BBE + HC (d4)
Monocytes hEGF (d4)
Monocytes BBE (d4)
Monocytes HC (d4)
Monocytes EGM (d4)
CD16
CD14
Tie2
VEGFR-2
B
Monocytes
Fold change CD14 MFI (vs. EBM)
Fold change CD16 MFI (vs. EBM)
Fold change Tie2 MFI (vs. EBM)
Fold change KDR MFI (vs. EBM)
EBM
+ hEGF
+ BBE
+ HC
+ BBE + hEGF
+ hEGF + HC
+ BBE + HC
EGM

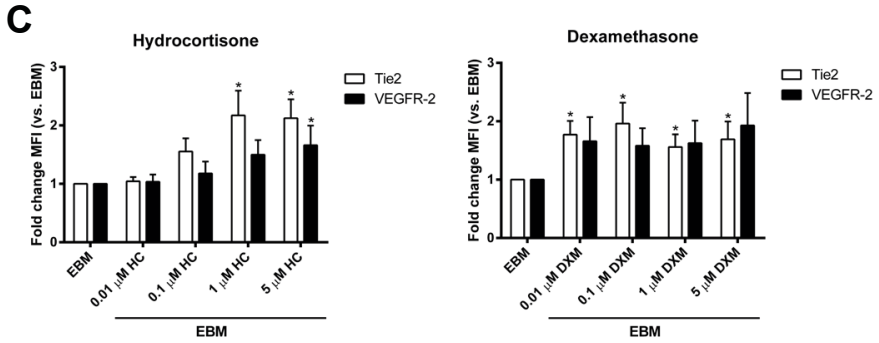


Figure 2. Hydrocortisone in endothelial growth medium reduces the expression of CD14 and increases the expression of Tie2 in monocytes. Monocytes were cultured under different growth conditions consisting of EBM + BBE + hEGF; EBM + hEGF + HC; EBM + BBE + HC or EBM + hEGF; EBM + BBE; EBM + HC on Fn for 4 days at 37°C in 5% CO_2 (A, B). All live cells were gated in a forward scatter/sideward scatter dot plot, and the surface expression of CD14, CD16, Tie2 and KDR/VEGFR-2 was measured by flow cytometry on the gated cells. Cells were also cultured in EBM and EGM to determine the minimum and maximum expression levels, respectively. Representative dot plots of $CD14^+CD16^+$, $CD16^+Tie2^+$ and $CD14^+VEGFR\text{-}2^+$ are shown (A). Expression levels (in MFI) in bar graphs are presented as fold change relative to EBM condition (B). Data are shown as mean ± SEM from at least 3 independent experiments. **P* < 0.05, ****P* < 0.001, compared with EBM. ### *P* < 0.001, EBM + hEGF vs. EBM + hEGF + HC, EBM + BBE vs. EBM + BBE + HC. δ *P* < 0.05, EBM + BBE + hEGF vs. EGM. Monocytes were cultured in the presence or absence of varying concentrations HC and dexamethasone (DXM) in EBM on Fn. After 5 days of culture, surface expression of Tie2 and KDR/VEGFR-2 was measured by flow cytometry (C). Expression levels (in MFI) in bar graphs are presented as fold change relative to EBM condition (set at 1). Data are shown as mean ± SEM from at least 3 independent experiments. **P* < 0.05, compared with EBM.

The expression of CD14 was reduced under all culture conditions, consistent with differentiation of monocytes (Fig. 1B). EGM/fn induced the strongest downregulation of CD14, suggesting a higher degree of differentiation. EGM-cultured monocytes displayed increased expression of Tie2 when compared with cells cultured in EBM medium, while EGM had no effect on CD16 expression. Fibronectin coating did not influence surface marker expression. Altogether, these data indicate that EGM may increase the pro-angiogenic activity of monocytes. The same results were obtained for PBMCs (Supplementary Fig. S2).

Hydrocortisone is specifically responsible for *in vitro* monocyte education

To determine which pro-angiogenic factor in EGM medium gives rise to pro-angiogenic receptor expression in CACs, we first tested different combinations of the compounds present in EGM medium (Fig. 2A and B) on monocytes. The results showed that combinations which included hydrocortisone induced Tie2 expression and that neither hEGF nor BBE were capable to induce CAC differentiation. To confirm that hydrocortisone was the responsible factor, cells were also treated with hEGF, BBE or hydrocortisone alone. Only hydrocortisone increased the expression of Tie2 (Fig. 2A and B), indicating that hydrocortisone is contributing to monocyte education. The same results were obtained for PBMCs (Supplementary Fig. S3).

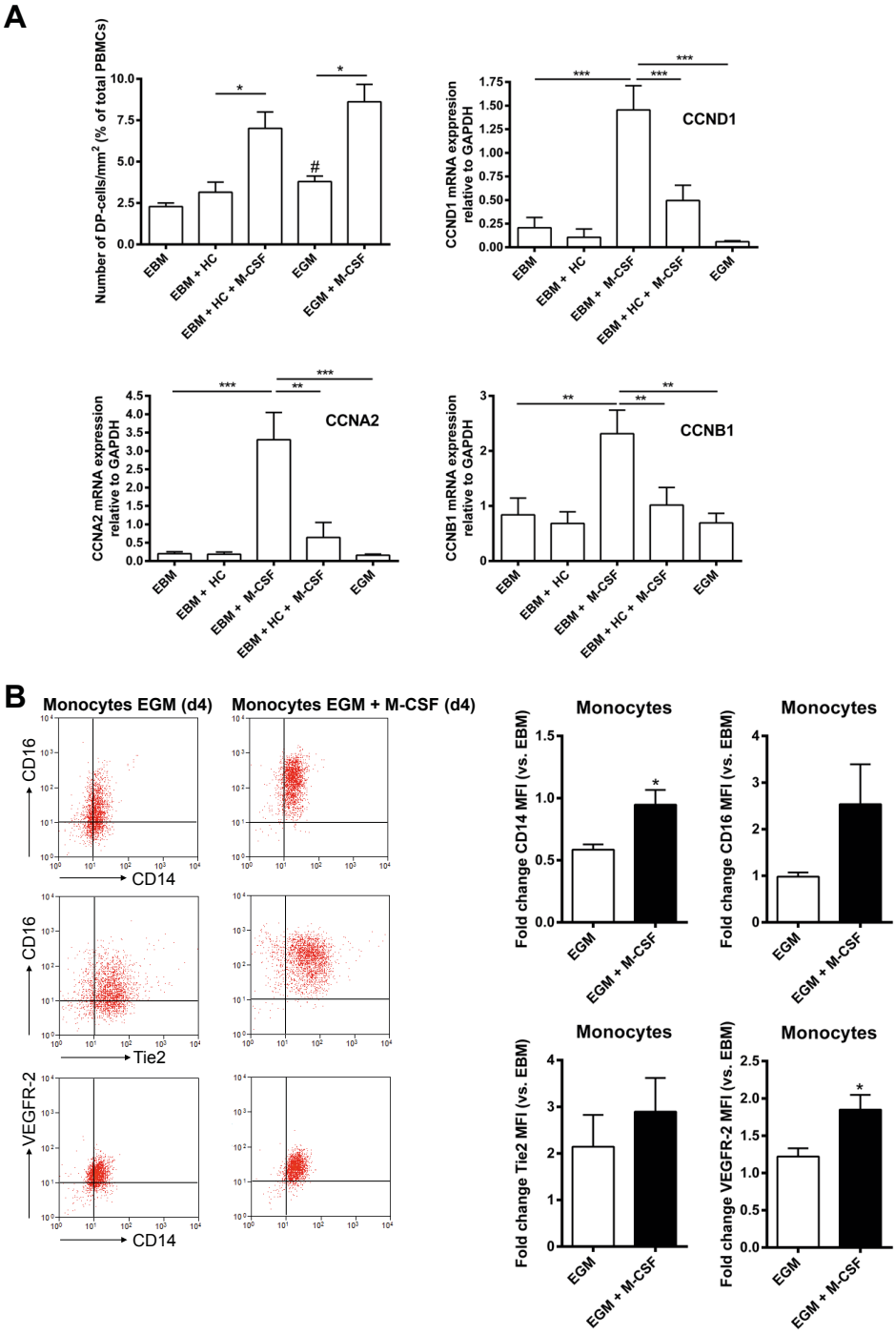
Furthermore, no significant effect of hydrocortisone, hEGF, or BBE was observed on CD16 expression (Fig. 2B). Interestingly, only hydrocortisone seems to be necessary to maintain KDR/VEGFR-2 expression, while it represents the sole factor responsible for the EGM-mediated reduction in surface expression levels of CD14 (Fig. 2B).

The concentration of hydrocortisone derived from the single quots in EGM (Fig. 2A and B) is unknown. Therefore, we next investigated the dose-response relationships of hydrocortisone, and a related glucocorticosteroid, dexamethasone, which is described to be a more potent steroid than hydrocortisone[40] for both Tie2 and KDR/VEGFR-2 expression (Fig. 2C). Both glucocorticosteroids enhanced the expression of Tie2, while the observed increase in KDR/VEGFR-2 only reached statistical significance after stimulation with hydrocortisone. The maximal expression of Tie2 was reached at 0.01 μM for dexamethasone and at 1 μM for hydrocortisone. Thus, this concentration of hydrocortisone was used in the next experiments.

M-CSF expands CAC numbers but does not reduce their pro-angiogenic repertoire

To develop a new strategy to increase the number of CACs derived from peripheral blood monocytes, we additionally used macrophage colony stimulating factor (M-CSF). Mononuclear cells cultured with M-CSF showed morphological changes (Supplementary Fig. S4A) and over 3-fold increased number of double positive cells compared to either EBM + hydrocortisone or EGM alone (Fig. 3A). To confirm that M-CSF indeed increased proliferation of CACs as described previously for monocytes[41], we quantified the induced expression of 3 cell cycle genes; CCND1 (G1 phase progression), CCNA2 (S phase progression) and CCNB1 (G2 to M-phase transition, indicative for cell division) to determine active cell cycle progression. All three genes were strongly upregulated by M-CSF, while hydrocortisone partially prevented the increase in cell cycle genes in CACs derived from monocytes (Fig. 3A). In addition, M-CSF enhanced the expression of KDR/VEGFR-2 in EGM-cultured monocytes (Fig. 3B) and in hydrocortisone-treated cells (Fig. 3C), but did not affect the expression of Tie2, analogous to the results with EGM-cultured monocytes. The same results were obtained for PBMCs (Supplementary Fig. S4B).

Altogether, these data indicate that M-CSF increases the number of CACs by inducing proliferation as well as their expression of KDR/VEGFR-2, while leaving the glucocorticosteroid-induced Tie2 expression unaffected.


A
Number of DP-cells/mm² (% of total PBMCs)
EBM
EBM + HC
EBM + HC + M-CSF
EGM
EGM + M-CSF
CCND1 mRNA exppresion relative to GAPDH
CCND1
CCNA2 mRNA expression relative to GAPDH
CCNA2
CCNB1 mRNA expression relative to GAPDH
CCNB1
B
Monocytes EGM (d4)
Monocytes EGM + M-CSF (d4)
CD16
CD14
Tie2
VEGFR-2
Monocytes
Fold change CD14 MFI (vs. EBM)
Fold change CD16 MFI (vs. EBM)
Fold change Tie2 MFI (vs. EBM)
Fold change VEGFR-2 MFI (vs. EBM)

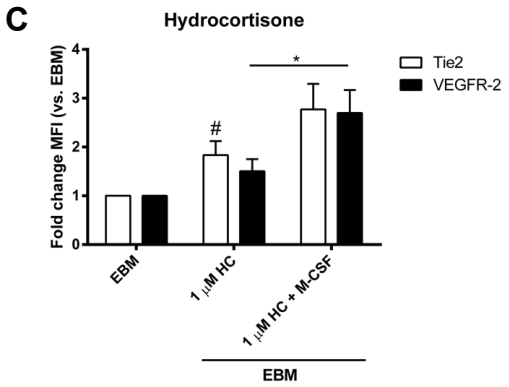


Figure 3. M-CSF enhances the number of CACs, increases cell cycle gene expression, and induces surface expression of VEGFR-2. PBMCs were differentiated into CACs by culturing in EBM, EBM + HC (1007x diluted), EBM + HC + M-CSF (100 ng/ml), EGM (1007x diluted HC, 252x diluted BBE, 1007x diluted hEGF), or EGM + M-CSF on Fn for 5 days at 37°C in 5% CO_2. CACs were stained for uptake of DiI-Ac-LDL, binding of Ulex-Lectin, Hoechst, and the number of double-positive (DP) CACs for uptake of DiI-Ac-LDL and binding of Ulex-Lectin was counted (A). Cell numbers are presented as percentage of totally seeded PBMCs and presented as mean ± SEM from at least 3 independent experiments. Cell cycle genes were quantified in after monocytes were differentiated into CACs by culturing in EBM, EBM + HC (1 μM), EBM + M-CSF (100 ng/ml), EBM + HC + M-CSF, or EGM (1 μM HC, 252x diluted BBE, 1007x diluted hEGF) on Fn for 5 days at 37°C in 5% CO_2. Cell cycle genes were quantified by real-time PCR and are expressed as the mean ± SEM of 4 experiments. **P* < 0.05, ***P* < 0.01, ****P* < 0.001. #*P* < 0.05 EBM vs. EGM. In panel B, monocytes were cultured in EGM (1007x diluted HC, 252x diluted BBE, 1007x diluted hEGF) on Fn for 4 days at 37°C in 5% CO_2, in the presence or absence of M-CSF (100 ng/ml). All live cells were gated in a forward scatter/sideward scatter dot plot, and the surface expression of CD14, CD16, Tie2 and KDR/VEGFR-2 was measured by flow cytometry on the gated cells. Representative dot plots of $CD14^+CD16^+$, $CD16^+Tie2^+$ and $CD14^+KDR^+$ are shown. Expression levels (in MFI) in bar graphs are presented as fold change relative to EBM condition. Data are shown as mean ± SEM from at least 3 independent experiments. **P* < 0.05, compared with EGM. In panel C, monocytes were cultured in the presence or absence of HC (1 μM) in EBM on Fn for 5 days at 37°C in 5% CO_2, without or with M-CSF (100 ng/ml). Thereafter, the surface expression of Tie2 and KDR/VEGFR-2 was measured by flow cytometry. Expression levels (in MFI) in bar graphs are presented as fold change relative to EBM condition. Data are shown as mean ± SEM from at least 3 independent experiments. #*P* < 0.05, compared with EBM. **P* < 0.05, EBM + HC vs. EBM + HC + M-CSF.

Glucocorticosteroids and M-CSF induce an M2 macrophage-like marker expression in CACs

M2 macrophages have been described to resemble CACs in terms of gene- and protein expression levels[16,21]. As shown in Figure 4, we observed that surface protein expression levels of the pro-angiogenic markers Tie2, KDR/VEGFR-2 and CXCR4 on M2 macrophages (and undifferentiated M0 macrophages) do not differ from CACs. Still, M-CSF increased the surface expression of KDR/VEGFR-2 and CXCR4 on glucocorticosteroid-cultured CACs to levels exceeding the expression levels on M2 macrophages.

To determine whether M2 marker transcripts were altered by glucocorticosteroids and M-CSF, we measured gene expression levels of IL-10, CD206, and CCL18[16] using real-time PCR (Fig. 5A), and compared M0 and M2 macrophages to EBM- and EGM-cultured monocytes.

Glucocorticosteroids were sufficient to induce the expression of IL-10 and CD206 in monocytes, with even higher expression levels in glucocorticosteroid-derived and EGM-derived CACs compared to M2 macrophages. Expression levels were comparable between glucocorticosteroid-derived and EGM-derived CACs, implicating that hEGF and BBE present in EGM medium are not necessary for M2-like differentiation of CACs. In contrast, the expression of the M2 chemokine, CCL18, was not induced in both glucocorticosteroid-derived and EGM-derived CACs. M-CSF treatment maintained the M2-like phenotype in glucocorticosteroid-derived CACs. Altogether, these data indicate that glucocorticosteroids are capable to induce CACs with an M2-like phenotype, and M-CSF has no major influence on this phenotype.

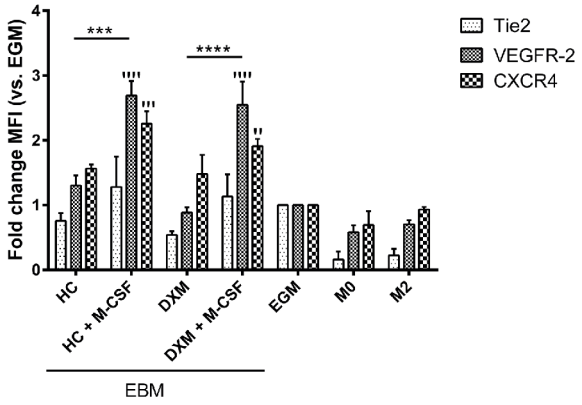


Figure 4. M-CSF increases KDR/VEGFR-2 and CXCR4 to levels exceeding expression levels of M2 macrophages. Monocytes were differentiated into CACs by culturing with glucocorticosteroids (HC; DXM; 1 μM) in EBM, in the presence or absence of M-CSF (100 ng/ml), or EGM on Fn for 7 days at 37°C in 5% CO_2. Monocytes were also differentiated into M0 macrophages by culturing in DMEM complete medium without additional stimuli for 7 days. For M2 differentiation, M0 macrophages were stimulated with rhIL4 (10 ng/ml) at day 5 for 48 hours. At day 7, the surface expression of Tie2, KDR/VEGFR-2 and CXCR4 was measured by flow cytometry. Expression levels (in MFI) in bar graphs are presented as fold change relative to EGM condition (set at 1). Data are shown as mean ± SEM from 3 independent experiments. ''$P < 0.01$, '''$P < 0.001$, ''''$P < 0.0001$, compared with M2. ***$P < 0.001$, ****$P < 0.0001$, EBM + glucocorticosteroid vs. EBM + glucocorticosteroid + M-CSF.

Glucocorticosteroids and M-CSF work in concert to induce pro-angiogenic paracrine factors superior to M2 macrophages

Next, we determined the pro-angiogenic cytokine/chemokine/growth factor expression by real-time PCR in CACs, M0- and M2 macrophages (Fig. 5B). Glucocorticosteroids reduced the expression levels of the pro-inflammatory chemokine IL-8 in EBM-cultured monocytes. M-CSF further reduced the glucocorticosteroid-inhibited expression of IL-8, but enhanced MCP-1 expression. Interestingly, pro-angiogenic growth factors CXCL12, VEGF-B, HGF,

PDGF-B and PDGF-C were expressed at least 10-fold higher in CACs than M2 macrophages, which was not affected by either glucocorticosteroid. Altogether, these data suggest that glucocorticosteroids and M-CSF are of minor importance for paracrine factor production in CACs, but that the expression levels of all paracrine factors in CACs is in most cases (except CCL18) much higher than their expression levels in M2 macrophages.

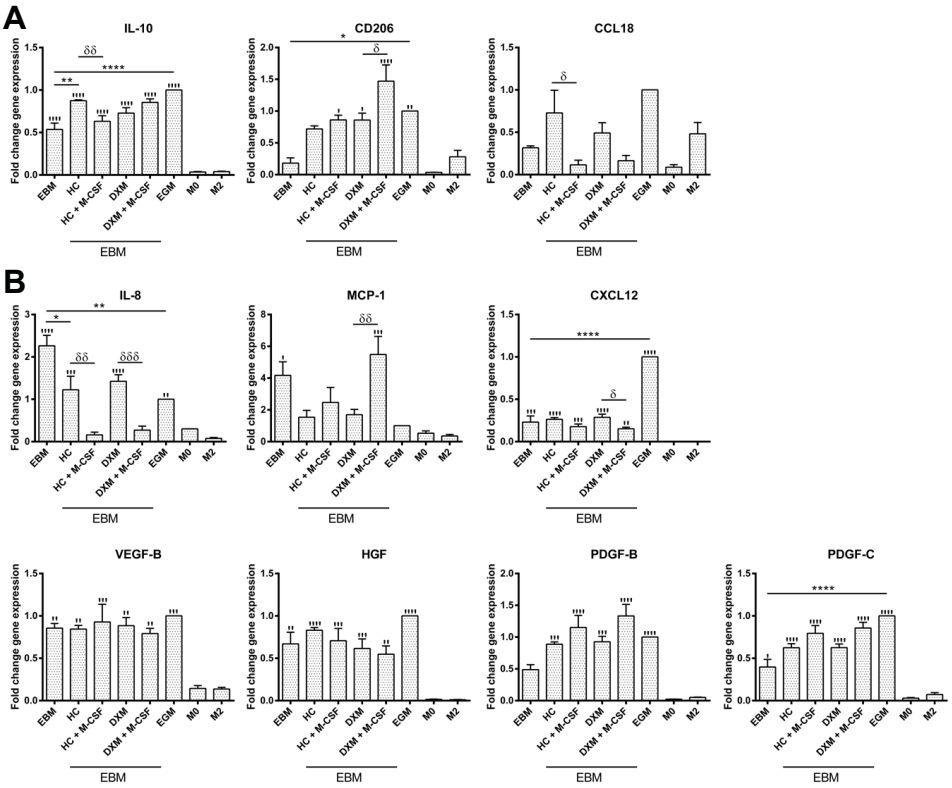


Figure 5. Glucocorticosteroids and M-CSF induce an M2 phenotype and a more pronounced pro-angiogenic signature in CACs as compared to M2 macrophages. Monocytes were differentiated into CACs by culturing in the presence or absence of glucocorticosteroids (HC; DXM; 1 μM) in EBM, without or with M-CSF (100 ng/ml), or EGM on Fn for 7 days at 37°C in 5% CO_2. Monocytes were also differentiated into M0 macrophages by culturing in DMEM complete medium without additional stimuli for 7 days. For M2 differentiation, M0 macrophages were stimulated with rhIL4 (10 ng/ml) at day 5 for 48 hours. At day 7, CACs and macrophage subtypes were analyzed for the indicated M2 markers (A) or paracrine factors (B) by RT-PCR. Gene expression levels are presented as fold change relative to EGM condition (set at 1). Data are shown as mean ± SEM from at least 3 independent experiments. '$P < 0.05$, ''$P < 0.01$, '''$P < 0.001$, ''''$P < 0.0001$, compared with M2. *$P < 0.05$, **$P < 0.01$, ****$P < 0.0001$, compared with EBM. δ $P < 0.05$, δδ $P < 0.01$, δδδ $P < 0.001$, EBM + glucocorticosteroid vs. EBM + glucocorticosteroid + M-CSF.

Hydrocortisone and M-CSF does not affect the functional capacity of CACs in an *in vitro* angiogenesis assay

To evaluate the functional consequences of hydrocortisone and M-CSF treatment of CACs we used our established *in vitro* tube-forming assay, comprised of primary microvascular endothelial cells (MVECs) grown on a fibrin matrix[38] as shown in Figure 6. The addition of CACs to the MVECs increased their organization in tube-like structures. The addition of hydrocortisone during monocyte differentiation did not affect the potential of CACs to support tube-like structure formation. We showed that M-CSF lead to increased numbers of CACs during differentiation from mononuclear cells (Fig. 3A). When added in equal numbers, however, the stimulating effects of these M-CSF-expanded cells were equal to CACs grown in the absence of M-CSF. This indicates that M-CSF does not attenuate the supportive potential of CACs.

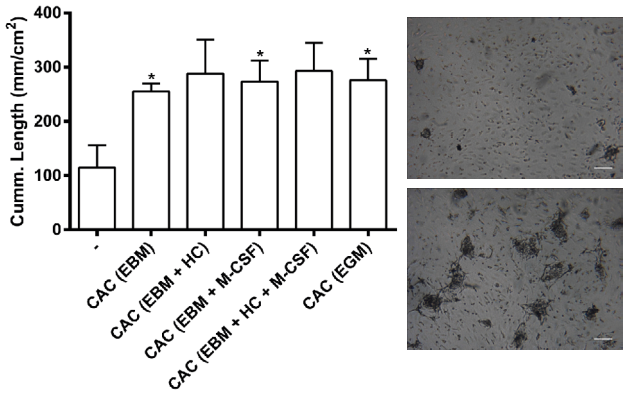


Figure 6. CACs cultured in endothelial basal medium induce endothelial cell tube formation, which is unaffected by hydrocortisone and M-CSF. Monocytes were differentiated into CACs by culturing in EBM, EBM + HC (1 μM), EBM + M-CSF (100 ng/ml), EBM + HC + M-CSF, or EGM (1 μM HC, 252x diluted BBE, 1007x diluted hEGF) on Fn for 5 days at 37°C in 5% CO_2. Total length of endothelial cell tube formation was measured in the presence or absence of different CACs. Results are expressed as mm/cm^2 and presented as mean ± SEM from 3 experiments. *$P < 0.05$ without CAC vs. with CAC. Scale bar represents 200 μm.

DISCUSSION

There is increasing evidence that monocytes can differentiate into CACs under angiogenic conditions. These cells, previously defined as EPCs, adhere to the extracellular matrix protein fibronectin, start to express endothelial markers and display a spindle-shaped morphology after a 7 day culture period in endothelial growth medium (EGM)[3-11]. These *ex vivo* cultured CACs accelerate neovascularization in a murine model of hind limb ischemia[6,7]. It has been suggested that only a minor subset of circulating human monocytes, expressing CD16 and

Tie2, provides help during angiogenesis *in vivo*[30]. Which of the circulating monocyte subset is able to differentiate to CAC *in vitro* was currently unknown.

In the present study, we have demonstrated that all three human monocyte subsets are able to differentiate to CACs. $CD14^{++}CD16^{-}$ (classical), $CD14^{++}CD16^{+}$ (intermediate), and $CD14^{+}CD16^{+}$ (non-classical) monocytes cultured in EGM adhered to fibronectin and were able to endocytose DiI-Ac-LDL and to bind Ulex-Lectin. Importantly, differentiated CACs highly expressed the endothelial angiopoietin receptor Tie2, which is crucial for neovascularization. Tie2-deficiency leads to reduced recovery of perfusion in a mice model of hind limb ischemia, indicating the importance of the Tie2 pathway in spontaneous neovascularization in response to ischemia[42]. The importance of Tie2 expression on monocyte-derived cells is demonstrated by cell-specific Tie2 knockdown in TEMs (Tie2 expressing macrophages), which impaired restoration of blood flow in a mouse model of hind limb ischemia, whereas delivery of murine bone marrow-derived macrophages overexpressing Tie2 rescued hind limb ischemia[43]. Angiopoietin-2 (Ang-2, a ligand for Tie2) acts as a chemoattractant for $Tie2^{+}$ monocytes[44] and is upregulated by endothelial cells at sites of vessel remodeling in ischemic tissue. Ang-2 was shown to be involved in restoration of blood flow after arterial occlusion *in vivo* by inducing arteriogenesis and the recruitment of inflammatory cells to the ischemic tissue[45]. Furthermore, exposure of TEMs to Ang-2 enhances their pro-angiogenic activity in a tumor model using genetically modified mice with Ang-2 overexpression specifically in endothelial cells. This resulted in tumors with more vascularization and higher numbers of infiltrated TEMs than wild-type (WT) mice[46].

As Tie2 expression is important for angiogenesis, it is of interest to know which of the pro-angiogenic factors present in commercially available EGM contribute to enhanced Tie2 expression and to neovascularization by CACs. To address this question, we have tested each compound supplemented in EGM alone or in combination, for their ability to increase the expression of Tie2 on monocytes differentiating to CACs. Surprisingly, our results show that hydrocortisone, known for its anti-inflammatory and immunosuppressive actions[47,48], was responsible for the increased expression of Tie2, while the endothelial growth factors hEGF and BBE did not influence Tie2 expression. Furthermore, we observed that binding of the cells to fibronectin was not essential for the enhanced expression of Tie2 in monocytes. Altogether, these results suggest that glucocorticosteroids play a major role in CAC differentiation and enhance their pro-angiogenic activity.

Additionally, we showed that M-CSF treatment increased the numbers of CACs at least 3-fold by stimulating their proliferation, concomitant with an increase of several cell cycle genes. This is in line with the fact that M-CSF is a hematopoietic growth factor stimulating the survival, differentiation and proliferation of cells of the monocyte/macrophage lineage[36,37]. Furthermore, M-CSF accelerates neovascularization of ischemic hind limbs by stimulating the mobilization of EPCs from the bone marrow[49]. Injection of *in vitro* cultured lineage

negative bone marrow-derived cells with M-CSF for two days improves angiogenesis in the hind limb ischemia model[50]. The *in vivo* relevance was recently demonstrated, by showing that endothelial cells produce M-CSF which induces the expansion of M2-like macrophages from hematopoietic progenitor cells by endothelial cell contact. These macrophages have enhanced expression of Tie2 and CD206/Mrc1, and increase tumor growth and angiogenesis *in vivo*[51]. M-CSF did not affect the capacity of CACs to support tube-like structure formation of endothelial cells in the *in vitro* angiogenesis assay. Thus, M-CSF is instrumental in increasing the numbers of CACs *in vitro*, without affecting the functional capacities. The positive effect we observed of M-CSF on the expression levels of the pro-angiogenic marker KDR/VEGFR-2 on CACs may improve their angiogenic capacity *in vivo*, in ischemic tissues where local expression of VEGF induces the ligand for CXCR4, SDF-1/CXCL12 in perivascular myofibroblasts, required for the recruitment of CACs[13].

We previously performed a transcription factor binding site analysis of the genes expressed in CACs (and M2 macrophages), which revealed that Ets family transcription factors (Ets-1, Ets-2, Elf-1) are involved in the regulation of the CAC transcriptome[21]. The requirement for Ets factors in myeloid cells during angiogenesis is demonstrated by conditional deletion of Ets-2 in tumor associated macrophages (TAM), which results in diminished angiogenesis and tumor growth[52]. The authors established that Ets-2 inhibits expression of anti-angiogenic genes in TAM. M-CSF could contribute to the angiogenic capacity of CACs, because it is an important activator of Ets-2 in monocytes/macrophages and is required for their survival[53]. All together, M-CSF may thus contribute to angiogenesis through several mechanisms, including cell recruitment, survival, proliferation, and the inhibition of anti-angiogenic gene expression.

M-CSF-cultured monocytes, CACs and M2 macrophages show an overlapping transcriptome[21,54], while glucocorticosteroids have also been associated with M2-like macrophage polarization, as they inhibit pro-inflammatory cytokine production and increase the expression of the M2 marker CD206/mannose receptor[55,56]. Because of these similarities we compared the functional gene expression profile of CACs with that of M2 macrophages induced by the unrelated cytokine IL-4. Overall, the expression levels of paracrine pro-angiogenic (growth) factors that stimulate the expansion of endothelial cells and smooth muscle cells are > 10-fold higher in CACs when compared to M2 macrophages (IL-8, VEGF-B, HGF, PDGF-B, PDGF-C and CXCL12). Glucocorticosteroids are responsible for the M2 phenotype in CACs, but are not involved in the higher expression of paracrine factors by CACs.

In conclusion, culture of monocytes with glucocorticosteroids in combination with M-CSF enhances *in vitro* differentiation of CACs leading to increased numbers of CACs with upregulated surface expression of Tie2 and KDR/VEGFR-2, all important receptors during angiogenesis[13,46,57]. CACs display M2 phenotype characteristics, but are distinct from M2

macrophages in terms of a far superior paracrine growth factor expression. The present study demonstrates a new effective method to accelerate CAC differentiation from peripheral blood monocytes *in vitro*, which may have a great potential for cell-based therapy in clinical applications.

Acknowledgements

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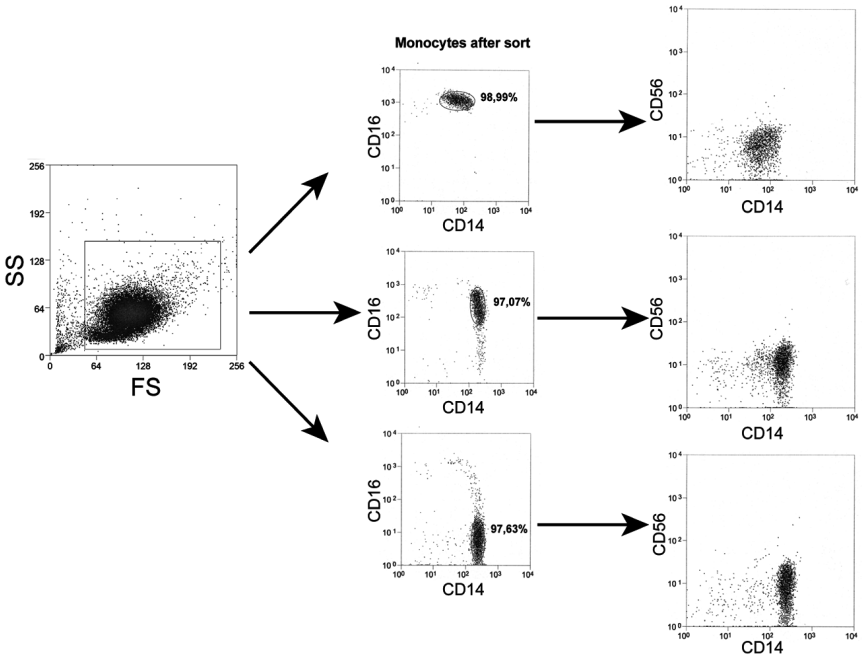
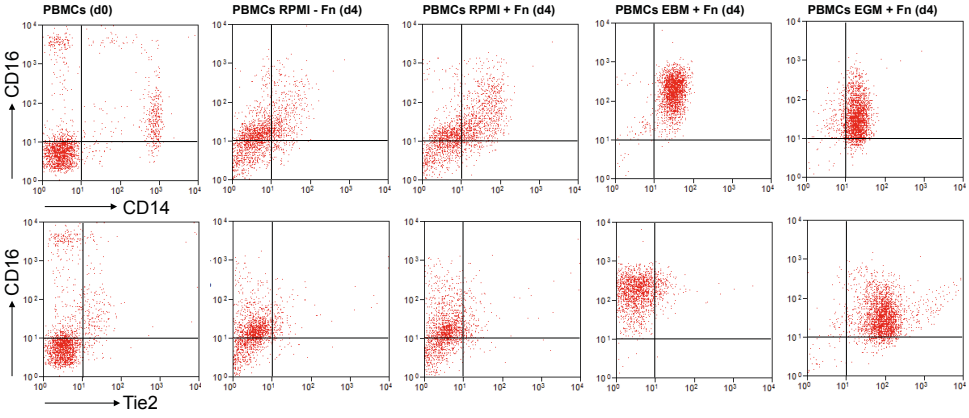
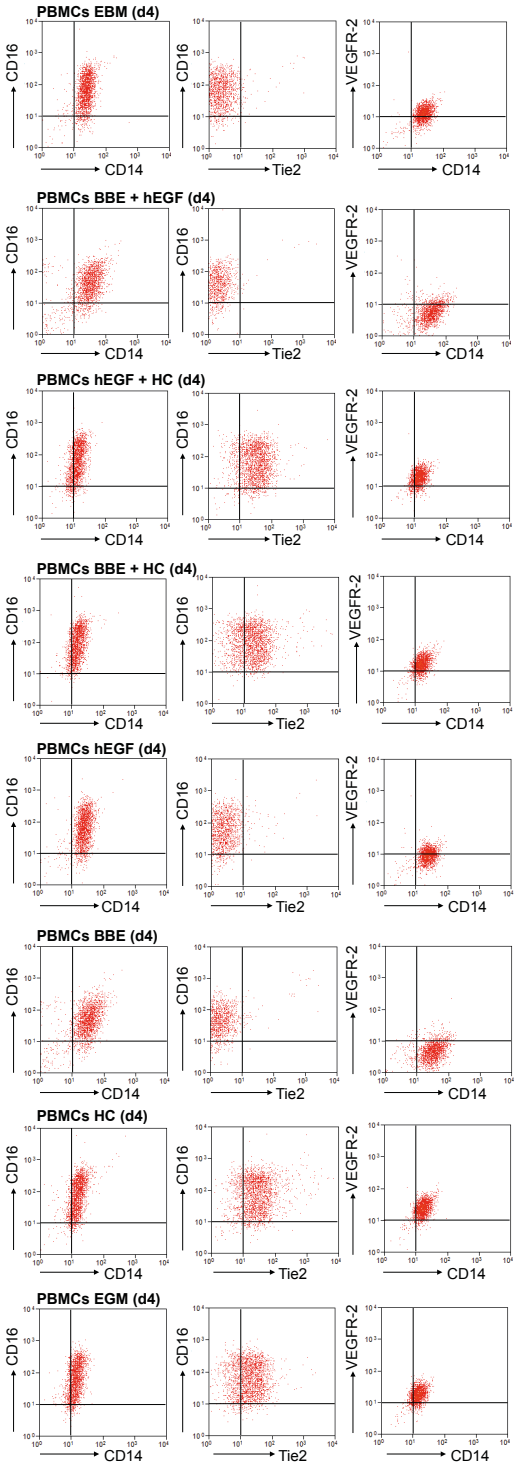
SUPPLEMENTARY INFORMATION



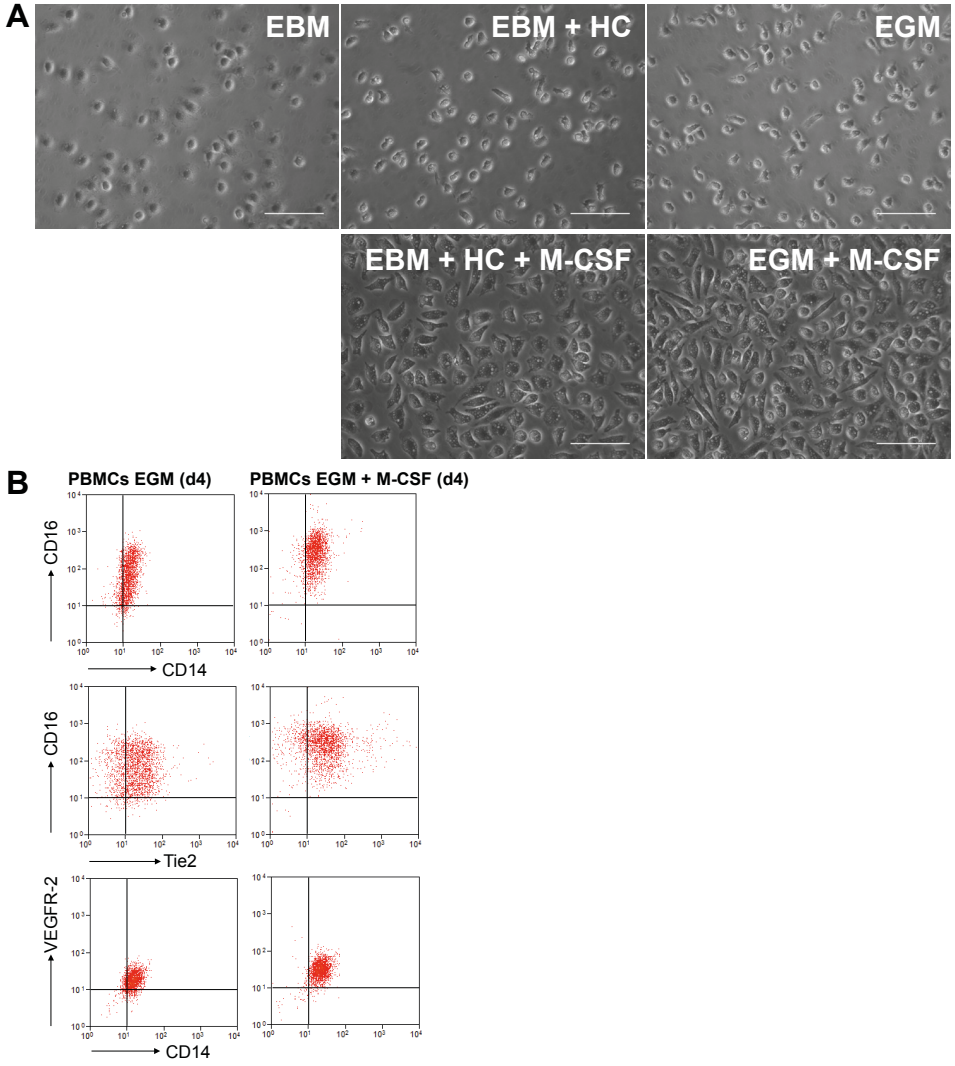
Supplementary Figure S1. Sorting strategy of human monocyte subsets. Total monocytes were sorted in classical ($CD14^{++}CD16^{-}CD56^{-}$), intermediate ($CD14^{++}CD16^{+}CD56^{-}$), and non-classical ($CD14^{+}CD16^{+}CD56^{-}$) subsets. Reanalysis of the three monocyte subsets is shown.



Supplementary Figure S2. PBMCs cultured under angiogenic condition have reduced expression of CD14 and increased expression of Tie2. PBMCs were cultured under different growth conditions consisting of RPMI without (-) Fn, RPMI with (+) Fn, EBM with Fn, and EGM (1007x diluted HC, 252x diluted BBE, 1007x diluted hEGF) with Fn for 4 days at 37°C in 5% CO_2. All live cells were gated in a forward scatter/sideward scatter dot plot, and the surface expression of CD14, CD16 and Tie2 was measured by flow cytometry on the gated cells. Fresh PBMCs were used as control cells. Representative dot plots of $CD14^{+}CD16^{+}$ and $CD16^{+}Tie2^{+}$ are shown.



Supplementary Figure S3. Hydrocortisone in endothelial growth medium reduces the expression of CD14 and increases the expression of Tie2 in PBMCs. PBMCs were cultured under different growth conditions consisting of EBM + BBE + hEGF; EBM + hEGF + HC; EBM + BBE + HC or EBM + hEGF; EBM + BBE; EBM + HC on Fn for 4 days at 37°C in 5% CO_2. All live cells were gated in a forward scatter/sideward scatter dot plot, and the surface expression of CD14, CD16, Tie2 and KDR/VEGFR-2 was measured by flow cytometry on the gated cells. Cells were also cultured in EBM and EGM to determine the minimum and maximum expression levels, respectively. Representative dot plots of $CD14^+CD16^+$, $CD16^+Tie2^+$ and $CD14^+KDR^+$ are shown.



Supplementary Figure S4. M-CSF induces morphological changes in CACs derived by hydrocortisone and surface expression of CD14, CD16, and KDR/VEGFR-2. PBMCs were differentiated into CACs by culturing in EBM, EBM + HC (1007x diluted), EBM + HC + M-CSF (100 ng/ml), EGM (1007x diluted HC, 252x diluted BBE, 1007x diluted hEGF), or EGM + M-CSF on Fn for 5 days at 37°C in 5% CO_2. CACs were photographed at 20x magnification using an inverted microscope. CACs cultured with M-CSF are larger, and more spindle-shaped than without M-CSF. Scale bar represents 100 µm (A). PBMCs were cultured in EGM (1007x diluted HC, 252x diluted BBE, 1007x diluted hEGF) on Fn for 4 days at 37°C in 5% CO_2, in the presence or absence of M-CSF (100 ng/ml). All live cells were gated in a forward scatter/sideward scatter dot plot, and the surface expression of CD14, CD16, Tie2 and KDR was measured by flow cytometry on the gated cells. Representative dot plots of $CD14^+CD16^+$, $CD16^+Tie2^+$ and $CD14^+KDR^+$ are shown (B).