Hofbauer cells of M2a, M2b and M2c polarization may regulate feto-placental angiogenesis

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Abstract

The human placenta comprises a special type of tissue macrophages, the Hofbauer cells (HBC), which exhibit M2 macrophage phenotype. Several subtypes of M2-polarized macrophages (M2a, M2b and M2c) exist in almost all tissues. Macrophage polarization depends on the way of macrophage activation and leads to the expression of specific cell surface markers and the acquisition of specific functions, including tissue remodeling and the promotion of angiogenesis. The placenta is a highly vascularized and rapidly growing organ, suggesting a role of HBC in feto-placental angiogenesis. We here aimed to characterize the specific polarization and phenotype of HBC and investigated the role of HBC in feto-placental angiogenesis. Therefore, HBC were isolated from third trimester placentas and their phenotype was determined by the presence of cell surface markers (FACS analysis) and secretion of cytokines (ELISA). HBC conditioned medium (CM) was analyzed for pro-angiogenic factors, and the effect of HBC CM on angiogenesis, proliferation and chemoattraction of isolated primary feto-placental endothelial cells (fpEC) was determined *in vitro*. Our results revealed that isolated HBC possess an M2 polarization, with M2a, M2b and M2c characteristics. HBC secreted the pro-angiogenic molecules VEGF and FGF2. Furthermore, HBC CM stimulated the *in vitro* angiogenesis of fpEC. However, compared with control medium, chemoattraction of fpEC toward HBC CM was reduced. Proliferation of fpEC was not affected by HBC CM. These findings demonstrate a paracrine regulation of feto-placental angiogenesis by HBC *in vitro*. Based on our collective results, we propose that the changes in HBC number or phenotype may affect feto-placental angiogenesis.

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Introduction

Successful pregnancy requires appropriate development and adaption of the feto-placental vascular system. Feto-placental vessel formation initially occurs by vasculogenesis in the early first trimester of pregnancy when endothelial progenitor cells form a primitive vascular network. This is followed by angiogenesis that pursues up to the end of pregnancy (Kaufmann *et al.* 2004). The process of angiogenesis is balanced by many factors: secreted soluble molecules, hypoxia, shear stress, components of the extracellular matrix (ECM) and direct cell–cell interaction with distinct cell types, such as pericytes, fibroblasts and tissue resident macrophages (Carmeliet 2000, Simons 2005, Adams & Alitalo 2007).

Macrophages play versatile roles in many tissues. The variety in their functions is reflected by a heterogeneous profile of phenotypes, which depends on their polarity, that is state of activation. The manifestation of macrophage activation status is determined by the surrounding milieu which they sense (Stout & Suttles 2004, Roszer 2015).

Two distinct states of macrophage polarization have been defined: M1 macrophages are activated classically, that is by interferone γ and LPS, and promote inflammatory processes. M2 macrophages are activated alternatively, that is by interleukins, and promote ECM construction, cell proliferation and angiogenesis (Mantovani *et al.* 2004, Jetten *et al.* 2014, Martinez & Gordon 2014). M2 macrophages can be further discriminated depending on their phenotype and functional properties. M2a macrophages are activated by IL4/IL13 and possess tissue repair and immunoregulating features. Stimulation by immune complexes favors the activation of the M2b phenotype, which supports humoral immunity and allergic reactions. M2c macrophages are stimulated by IL10 or glucocorticoids and induce anti-inflammatory reactions by remodeling of the ECM and suppression of immunity (Martinez *et al.* 2008).

The feto-placental vasculature is encompassed by the villus stroma, which contains different cell types such as mesenchymal cells, fibroblasts, myofibroblasts, pericytes and tissue macrophages (Challier *et al.* 1995, Demir *et al.* 1997, Abumaree *et al.* 2013).

These tissue macrophages, termed Hofbauer cells (HBC), are abundant in the villous stroma and represent M2 phenotype (Joerink *et al.* 2011, Sisino *et al.* 2013, Tang *et al.* 2013a,b, Young *et al.* 2015). The role of HBC in the placenta is not yet clearly understood, but due to their location and paracrine abilities, they are thought to play a role in early placental development, placental immunology, as well as development and maturation of the placental mesenchyme throughout pregnancy (Khan *et al.* 2000, Anteby *et al.* 2005, Seval *et al.* 2007, Ingman *et al.* 2010, Young *et al.* 2015). Moreover, the fact that HBC produce and secrete vascular endothelium growth factor (VEGF) (Ahmed *et al.* 1995, Cooper *et al.* 1995, Khan *et al.* 2000) reinforces the assumption that similar to macrophages in other tissues, HBC may regulate angiogenesis in the foeto-placental vasculature (Demir *et al.* 2004, Seval *et al.* 2007). Despite the importance of placental vascular function during pregnancy, there are still gaps in our knowledge about the molecular pathways that control vessel development.

Since tissue macrophages are key regulators of angiogenesis, our objective was to better understand whether and how HBC affect foeto-placental endothelial cell (fpEC) angiogenesis in late pregnancy. Therefore, we isolated primary HBC and determined their activation status. Then, we investigated how HBC-derived factors influence network formation, proliferation, chemoattracted migration and survival of isolated primary fpEC.

Materials and methods

Ethical approval

The study was approved by the local ethics committee, and all women gave written informed consent (27-265 ex 14/15).

Tissue collection

Placentas were obtained after uncomplicated vaginal delivery or cesarean section between gestational weeks 37 and 41. Subjects' characteristic of placental donors for cell isolations are shown in Table 1.

Immunofluorescence

For colocalization of markers for HBC, TB and fpEC sections (5µm) were cut and placed on Superfrost Plus slides (Menzel, Braunschweig, Germany). Sections were deparaffinized in xylene and rehydrated through a series of graded alcohol. Heat-induced antigen retrieval was performed in epitope retrieval solution at pH 9 (Leica Biosystems Newcastle Ltd, Newcastle, UK). Then, slides were boiled in a pressure cooker for 7 min at 120°C and allowed to cool down for 20 min before being rinsed in wash buffer (tris-buffered saline, 0.05% Tween 20 (TBS/T), pH 7.4). All further steps were performed at room temperature in a dark, humidified chamber. Slides were incubated with UltraVision Hydrogen Peroxidase Block (Thermo Scientific) for 5 min and rinsed in TBS/T three times before applying blocking solution (TBS/T with 10% goat serum; Thermo Fisher) and 6% BSA (Sigma-Aldrich) for 30 min. After three washings, primary antibodies were applied (rabbit anti-CD34; 1:500, Abcam), mouse anti-CD163 (1:25, Thermo Scientific) and anti-CK7-FITC (1:50, USBiological, Swampscott, MA, USA) diluted in antibody diluent with Background Reducing Components (Dako). After three washings in TBS/T, slides were incubated for 30 min with fluorescent-labeled secondary antibodies (DyLight 633 goat anti-mouse IgG; 1:100, Thermo Fisher) and CY3 goat anti-rabbit IgG (1:25, Abcam). Afterward, slides were stained with 4,6-diamidino-2-phenylindole dihydrochloride (DAPI; diluted 1:2000 in PBS; Invitrogen) for 10 min, rinsed in deionized water, air dried and mounted with ProLong Gold antifade reagent (Invitrogen). Sections were assessed with a Leica DM 6000B microscope and photographed using an Olympus DP 72 Camera (Leica Microsystems, Wetzlar, Germany).

Isolation of primary fpEC

Primary fpEC were isolated following a standard protocol (Lang *et al.* 2003). Briefly, chorionic arteries were dissected and endothelial cells isolated by perfusion with a collagenase/dispase (Roche) solution. Cells were resuspended in endothelial basal medium (EBM, Lonza, Portsmouth, NH, USA) supplemented with the EGM-MV BulletKit (Lonza) containing gentamicin/amphotericin, hydrocortisone, human epidermal growth factor (EGF), bovine brain extract and fetal bovine serum (FBS) (Thermo Scientific), and plated on culture plates precoated with 1% gelatin (Sigma-Aldrich). All cell preparations were subjected to immunocytochemical characterization for identity and

Table 1 Subjects' characteristics of placental donors for cell isolations.

	Feto-placental endothelial cells	Hofbauer cells for characterization	Hofbauer cells for functional assays
Number of isolations (n)	6	10	5
Maternal age (years)	31.7±7.3	32.6±5.9	33.0±5.0
Prepregnancy BMI	25.5±5.4	22.8±3.5	22.1±3.0
Postgravida BMI	30.7±4.8	27.4±4.0	26.4±4.6
Gestational age (weeks)	39.1±1.3	39.7±1.4	39.0±2.2
Placental weight (g)	679.1±249.1	614.0±269.4	697.1±315.9
Fetal weight (g)	3487±775	3350±410	3592±763
Fetal sex (m/f)	3/3	5/5	3/2
Mode of delivery (vaginal/C-section)	3/3	5/5	3/2

purity. Isolated fpEC were grown at 37°C and 21% oxygen, and used up to passage ten.

Isolation of placental macrophages (HBC)

For isolation of HBC, the protocol developed by Tang and coworkers (Tang *et al.* 2011) was modified. For characterization (Dil-Ac-LDL uptake, FACS analysis, ELISA and immunocytochemistry), HBC isolations from 10 different placentas were used. After this, five further isolations were used to collect CM for functional assays. Villous tissue (60–100 g) was dissected from membranes and large vessels, finely minced and stored overnight at 4°C in phosphate-buffered saline (PBS). The next day, the tissue was subjected to three sequential enzymatic digestion steps containing 0.25% trypsin (Gibco), 0.2% DNase I (Roche), 25 mM HEPES (Roth, Karlsruhe, Germany), 2 mM CaCl₂ and 0.8 mM MgSO₄ in Hanks' balanced salt solution (HBSS, Gibco) at 37°C. The first digestion step was performed for 15 min in 150 mL digestion solution and the subsequent two steps for 30 min each in 150 and 200 mL of digestion solution, respectively. After each step, tissue was washed with PBS. The last washing ensued through gauze and a 100 µm sieve. Then, tissue was further digested with collagenase A (1 mg/mL)/DNase I (0.2 mg/mL) in RPMI-1640 (Gibco) containing 25 mM HEPES, 5% FBS and 1% antibiotic–antimycotic for 1 h at 37°C. The cell suspension was filtered through gauze and a 70 µm sieve and centrifuged at 4°C and 300 g for 10 min. Cells were resuspended in RPMI medium and loaded onto a discontinuous Percoll gradient ranging from 70 to 10% and centrifuged without break. The gradient layer containing the cells was taken off and after extensive washing with PBS, cells were immunopurified by negative selection using sequential treatment with anti-EGFR (clone 528, Cat#MS-609-P0, Thermo Scientific) and anti-CD10 (clone MEM-78, Cat#ab659, Abcam) conjugated to magnetic beads (Tang *et al.* 2011). Remaining cells were counted and plated at 37°C and 21% oxygen in macrophage medium (ScienCell, Carlsbad, CA, USA). One gram of tissue gave between 1.2 and 2.9×10^6 of immunopurified cells. After 1 h of culture, nonattached cells were removed and the remaining highly adhesive macrophages were further cultured. Immediate quality control of isolated HBC using CD90 antibodies for FACS analysis revealed that $2 \pm 1.5\%$ of cells were fibroblasts.

Culture of HBC on different ECMs

In order to identify optimal culture conditions for HBC, cell adhesion on different ECMs was tested. Tissue culture polystyrene, gelatin and two major ECM components of the placenta, that is fibronectin and collagen I (Cao *et al.* 2003), were used. Therefore, culture plates were coated for 1 h at 37°C with 1% gelatin, with 3 µg/cm² rat collagen I (Cultrex, R&D Systems) or for 30 min at 37°C with 1 µg/cm² of bovine fibronectin (Cultrex, R&D Systems). Before plating of HBC (1.5×10^6 cells/mL), the coated wells were washed with PBS. After 1 h, cells were washed with PBS to remove nonadhered cells. HBC were monitored by a Zeiss Cell Observer microscope with an AxioCam HRm camera and an A-Plan 5×/0.12 Ph0 objective (Carl Zeiss Imaging Solutions GmbH)

for 5 days. Since best attachment of HBC was observed using tissue culture polystyrene and fibronectin, cells were grown under this condition for all further experiments (Supplementary Fig. 1, see section on supplementary data given at the end of this article).

Dil-Ac-LDL uptake

Fluorescence-labeled acetylated low-density lipoprotein (Dil-Ac-LDL) (Biomedical Technologies, Stoughton, MA, USA) was used to indicate the presence of M2 marker and LDL-binding protein CD36 (Mandal *et al.* 2011) and thus to determine purity and viability of isolated HBC (Goldstein *et al.* 1979). Dil-Ac-LDL was added in a final concentration of 10 µg/mL to the culture medium. After incubation for 4 h at 37°C, cells were washed with PBS and Dil-Ac-LDL uptake was observed and quantified by a Zeiss Cell Observer microscope with an AxioCam HRm camera and an A-Plan 5×/0.12 Ph0 objective using AxioVision software (Carl Zeiss Imaging Solutions GmbH). FpEC were also capable of ingesting fluorescent-conjugated ac-LDL, but in a much lower extent, while placental trophoblasts did not incorporate ac-LDL (Supplementary Fig. 2).

Flow cytometry

HBC-specific surface markers were determined by flow cytometry. Four days after isolation, cells were detached by gently scraping them (Costar, New York, NY, USA) in HBSS. Cell suspension was centrifuged with 300 g at 4°C for 5 min. Then, Fc receptors were blocked by incubation with 3% FBS/HBSS for 10 min at 4°C. PE-conjugated mouse anti-human CD45 (Cat#555483, BD Biosciences, Franklin Lakes, NJ, USA), V450-conjugated mouse anti-human CD80 (Cat#560442, BD), APC-conjugated mouse anti-human CD163 (Cat#333609, BioLegend), V450-conjugated mouse anti-human CD11b (Cat#560481, BD), PerCP-Cy5.5-conjugated mouse anti-human CD209 (#558263, BD), V450-conjugated mouse anti-human CD86 (Cat#560367, BD) and isotype-matched controls (Cat#345816, Cat#561504, Cat#345818, BD) were added and incubated for 30 min at 4°C. For identification of potential contaminating fibroblasts, PE anti-human CD90 (Cat#328110, BioLegend) was used. Afterward, cells were washed twice with PBS and resuspended in 150 µL PBS for analysis.

After setting the forward scatter threshold as to exclude cell debris, 10,000 events were collected using FACS LSRII and Diva software (BD). The results were analyzed using FlowJo software (Tree Star, Ashland, OR, USA). The percentage of positive cells was based on comparison with the isotype-matched control antibody, for which gating was set at 1%.

Immunocytochemistry

Cells (250,000 cells per 1.7 cm² chamber) were grown on chamber slides and fixed with ice-cold acetone (Merck) for 5 min. The presence of HLA-DR, CD206 and CD14 was detected using Ultra Vision HRP Polymer (Thermo Scientific). Slides were washed in PBS pH 7.5 for 5 min. Nonspecific binding sites were blocked with UV block (Lab vision) for 10 min. Subsequently, the primary antibodies rabbit

anti-CD206 (#NBP1-90020PEP, Novus Biologicals, Littleton, CO, USA), rabbit anti-human HLA-DR (#ab92511, Abcam) and mouse anti-human CD14 (#MS-1080, Thermo Scientific) diluted in Dako antibody diluent were applied for 30 min. After three washings in PBS, primary antibody enhancer was applied and incubated for 10 min. Following further washings in PBS, the slides were incubated with HRP polymer for 15 min and washed again. Then, chromogenic reaction was started by the addition of peroxidase-compatible chromogen (Thermo Scientific). After washing in aqua dest, Hemalaun (Sigma) solution was used for nuclear counterstaining. Slides incubated with rabbit- or mouse-unspecific immunoglobulin fractions (Rabbit and mouse IgG1 negative control, Dako) in the same concentration as the primary antibody served as negative controls.

Conditioned medium

Freshly isolated HBC ($1.5 \times 10^6/\text{mL}$) were seeded in macrophage medium with supplements (Sciencell Research Laboratories, San Diego, CA, USA) for 24 h. Thereafter, medium was changed to DMEM/EBM (DE, 1:1) with 7.5% FBS for another 48 h of incubation. This CM was aspirated and centrifuged for 5 min with 300g to remove dead cells and cell debris. CM was aliquoted and stored at -80°C . CM from at least two different isolations was pooled and used for functional experiments. As control medium, DMEM/EBM with 7.5% FBS was incubated at the same conditions, but without cells.

ELISA and multiplex immunoassay

IL1RN, IL6, TNFA and PLGF concentrations in CM were measured using immunoassays (Peprotech, Vienna, Austria) according to the manufacturer's instructions. Secretion of FGF2 and VEGF from HBC was assessed by multiplex immunoassay on beads (Aimplex, YSL Bioprocess Development Co, Pomona, CA, USA). HBC ($n=5$) were cultured up to 4 days, CM taken every 24 h. The multiplex experiment was carried out according to the manufacturer's instructions. Bead signals were quantified using a FACS Calibur instrument (BD Biosciences) and FlowCytomixPro software (eBioscience) was used for the calculation of standard curves and sample concentrations.

In vitro network formation assay

FpEC (1×10^4) were resuspended in 100 μL CM and plated on growth factor-reduced Matrigel. Nonconditioned DE medium was used for control conditions. Formation of tube-like structures was followed for 24 h by a Zeiss Cell Observer microscope with an AxioCam HRm camera and an A-Plan 5x/0.12 Ph0 objective using the software AxioVision (Carl Zeiss Imaging Solutions GmbH). Total tube length, branching points and number of meshes was quantified after 3, 6, 12 and 24 h using ImageJ software (NIH) with the AngioJ-Matrigel assay plugin, kindly provided by Diego Guidolin (Department of Human Anatomy and Physiology, Section of Anatomy, University of Padova, Italy) (Lassance *et al.* 2013). Total tube length and the number of branching points were used as representative parameter for quantification.

Chemoattraction assay

Chemoattraction of fpEC toward the CM was observed using a 96-well chemotaxis microplate system (Neuro Probe Inc, Warwick, UK). After serum starvation for 3 h, fpEC (1×10^4 cells/well) were placed in the upper part of the chemotaxis system, which was separated from the lower well by a fibronectin-coated polycarbonate filter with 8 μm pores. Cells were allowed to migrate toward chemoattractants to the lower well (CM) for 4 h at 37°C . DE medium supplemented with 7.5% FBS (EGM-MV BulletKit, Lonza) was used as control. The upper surface of the filter was wiped clean of nonmigrating cells. Cells were fixed with 4% formaldehyde and stained with DAPI (Invitrogen). DAPI staining on the microplate was quantified by a Zeiss Axioplan fluorescence microscope and a 10x objective using the AxioVision software (Carl Zeiss Imaging Solutions GmbH). Pictures ($n=35$) were taken from each filter well; out of these, seven pictures were randomly selected and analyzed using DotCount v1.2 (online provided by Martin Reuter, MIT).

Proliferation assay

Proliferation of fpEC was assessed after cell treatment with CM using the BrdU ELISA kit (Cyclex, Japan) according to the manufacturer's recommendations. FpEC (6×10^3 cells/well) were seeded in a 96-well plate. After 24 h, the medium was replaced by CM and cells were incubated for another 24 h. Subsequently, BrdU was added to a final concentration of 10 μM and incubated for 2 h. Cells were fixed, denaturated and incubated with

Figure 1 Immunofluorescent staining of a third trimester placental villus. The cross section of the villus is bordered by the trophoblast (TB) layer (CK7, green, green arrowhead). Inside the villus, fetal-placental endothelial cells (fpEC) line the fetal-placental vessels (CD34, white, white arrowhead). Hofbauer cells (HBC) are evenly distributed, but often close to fetal-placental vessels (CD163, red, red arrowhead). A representative image taken from three individual placentas is shown. Original magnification: 200x, scale bar = 50 μm . The upper insert shows cultured primary fpEC that have a polygonal shape, grown in loose arrangements and exhibit the classical cobblestone-like appearance. The lower insert shows cultured HBC, which exhibit a pleiomorphic phenotype with many vacuoles. Original magnification of the inserts: 200x, scale bars = 100 μm .

Figure 2 Characterization of HBC by flow cytometry and immunocytochemistry. Isolated HBC were cultured for 4 days before staining for flow cytometry analysis. HBC were stained for the general macrophage marker CD45 (A), and for markers of macrophage subtypes, that is CD80 (B), CD163 (C), CD11b (D), CD209 (E) and CD86 (F). The histograms are representatives of independent experiments. CD45: $n=4$; CD80: $n=7$; CD163: $n=9$; CD11b: $n=4$; CD209: $n=6$; CD86: $n=4$. Dotted lines represent unstained cells, continuous lines represent positively stained cells. Immunocytochemistry for HLA-DR (G), CD206 (H) and CD14 (I) was performed after 5 days of culture. Photographs are representative of independent experiments of $n=4$ different isolations. Inserts show negative controls incubated with unspecific IgG of the same isotype as the specific antibody.

anti-BrdU antibody. Absorbance was measured at 450/540 nm using the FluoSTAR Optima 413 spectrofluorometer (BMG Lab Technologies, Ortenberg, Germany).

LDH assay

Cytotoxicity of CM on fpEC was tested by the measurement of released lactate dehydrogenase (LDH, Takara, Japan) according to the manufacturer's instructions. 6×10^3 cells per well were seeded in a 96-well plate and incubated with CM for 24 h. Absorbance was measured at 490/650 nm using the spectroMax 250 molecular devices microplate reader (MWG-Biotech, Ebersberg, Germany).

Statistical analysis

Data are expressed as mean $\pm$ S.E.M. Statistical differences were assessed by Student's *t*-test (Shapiro–Wilk test for normal distribution). Statistical analysis used Sigma Plot 13.0 software and a *P*-value of less than 0.05 was considered as significant. The number of independent experiments is provided in the figure legends.

Results

HBC often localized close to feto-placental vessels

In order to identify and to localize HBC in human third trimester placentas, placental cross sections were stained with the cell-type-specific markers

cytokeratin 7 (CK7) for trophoblast (TB), CD34 for fpEC and CD163 for HBC. The immunofluorescent staining shows the placental villus framed by the TB layer (CK7, green) with the feto-placental vessels inside the stroma (CD34, white). HBC (CD163, red) are distributed in the villous stroma, sometimes in close proximity to the basal membrane of the trophoblast layer. Moreover, HBC are often in close proximity to feto-placental vessels, suggesting an interaction with endothelial cells (Fig. 1). Inserts show isolated and cultured primary HBC and fpEC, respectively.

Primary HBC represent a heterogeneous cell population with M2 characteristics

Several studies demonstrated that HBC provide M2 characteristics (Joerink *et al.* 2011, Sisino *et al.* 2013, Tang *et al.* 2013a,b, Young *et al.* 2015). We wanted to further identify the specific subtype of HBC polarization in normal pregnancy. Therefore, we analyzed the expression of surface markers for macrophage phenotypes (CD11b (Tarique *et al.* 2015), CD80, CD86, CD163 and CD209 (Orme & Mohan 2012, Roszer 2015, Gensel & Zhang 2015)) using flow cytometry, and measured the secretion of interleukin 1 receptor antagonist (IL1RN), tumor necrosis factor α (TNF α) and interleukin 6 (IL6) by HBC using ELISA. The expression of the common leukocyte antigen CD45 (Moore *et al.* 2013), which has been

Figure 3 Secretion of IL1RN, TNFA and IL6 by HBC. Cytokines were measured by ELISA up to 96 h after isolation. In the nonconditioned control medium, IL1RN, TNFA and IL6 concentrations were below the detection limit of the ELISAs. Conditioned media of $n=5$ independent HBC isolations were analyzed.

shown on placental macrophages *in situ* (Bockle *et al.* 2008), was used as a general marker for flow cytometry (Fig. 2A). Almost no staining ($2 \pm 1.3\%$) was observed when CD80, a marker for M1 polarization, was used. Absence of the M1 marker was paralleled by positive staining of cells for CD163 ($95 \pm 5\%$) and CD11b ($84 \pm 11\%$), both M2 markers, by the majority of cells (Fig. 2B, C and D). Moreover, the ability to uptake Dil-Ac-LDL by visually all HBC (Supplementary Fig. 2) indicates the presence of M2 marker and LDL-binding protein CD36 (Mandal *et al.* 2011). Further analysis of markers for M2 subtypes revealed $12 \pm 9\%$ of HBC positive for the M2a marker CD209 (Fig. 2E). The presence of CD209 was paralleled by the secretion of IL1RN (Fig. 3A), which also indicates M2a polarization (Bai *et al.* 2015). $31 \pm 30\%$ of HBC were positive for the M2b marker CD86 (Fig. 2F), which was paralleled by the secretion of TNFA and IL6 (Fig. 3B and C), two M2b macrophage-specific cytokines (Orme & Mohan 2012, Roszer 2015, Gensel & Zhang 2015). Immunocytochemistry revealed positive staining for the M2a/M2b marker HLA-DR (major histocompatibility complex, class II, DR) (Mantovani *et al.* 2004, Orme & Mohan 2012), the M2a/M2c marker CD206 (Mannose Receptor C, Type 1) (Orme & Mohan 2012) and the M2c marker CD14 (Lolmede *et al.* 2009, Orme & Mohan 2012) (Fig. 2G, H and I). Surface markers and cytokines used to characterize HBC phenotypes are summarized in Table 2.

Secretion of pro-angiogenic factors by HBC

Since M2 macrophages have the capacity to regulate vessel development in other tissues, we determined

Table 2 Macrophage markers and cytokines used in this study to characterize HBC phenotype.

	M1	M2	M2a	M2b	M2c
Surface molecules	CD80 ⁽¹⁾	CD11b⁽¹⁾	CD163⁽¹⁾	CD163⁽¹⁾	CD163⁽¹⁾
		CD36⁽³⁾	CD206⁽²⁾		CD206⁽²⁾
			HLA-DR⁽²⁾	HLA-DR⁽²⁾	
			CD209⁽¹⁾	CD86⁽¹⁾	CD14⁽²⁾
Cytokines			IL1RN⁽⁴⁾	TNFA⁽⁴⁾	
				IL6⁽⁴⁾	

Superscript numbers indicate the method of analysis: ⁽¹⁾FACS, ⁽²⁾immunocytochemistry, ⁽³⁾dil-ac-LDL uptake, ⁽⁴⁾ELISA. Expressed surface proteins and secreted cytokines are printed in bold.

Figure 4 Secretion of VEGF and FGF2 by HBC. VEGF (A) and FGF2 (B) were measured by multiplex immunoassay up to 96 h after isolation. In the nonconditioned control medium, VEGF and FGF2 were below the detection limit of the assay. Conditioned media of $n=5$ independent HBC isolations were analyzed.

whether HBC ($n=5$) secrete pro-angiogenic factors. Thus, levels of VEGF, FGF2 and PLGF as key factors to stimulate angiogenesis were determined in HBC CM by multiplex immunoassay and ELISA, respectively. VEGF and FGF2 were identified in HBC CM (Fig. 4), whereas the PLGF level could not be detected by the assay and was below 30 pg/mL (data not shown).

Paracrine effects of HBC on fpEC angiogenesis

The presence of pro-angiogenic factors in HBC CM suggests a stimulatory effect of HBC-derived paracrine factors on placental angiogenesis. Therefore, we analyzed the impact of HBC CM on network formation of primary fpEC. Indeed, CM of HBC induced the network formation of fpEC, increased the length of the network by $26 \pm 12\%$ ($P=0.018$) and the number of branching

Figure 5 HBC conditioned medium stimulates network formation of fpEC. CM from HBC ($n=5$, pooled in two different stocks) was used for network formation assays with fpEC. (A) Representative phase contrast light microscopic images show network formation of fpEC after 12 h on growth factor-reduced Matrigel. (B) Conditioned medium of HBC increased the length of network and the number of branching points of fpEC. Original magnification: 50 $\times$; scale bar = 500 μ m. Data are given as mean $\pm$ s.e.m. Statistical analysis used the mean of the triplicates of the number of individual biological replicates of fpEC ($n=6$). Control M (control medium): medium without cells. *indicates $P<0.05$.

Figure 6 HBC conditioned medium affects fpEC functions. CM from HBC ($n=5$ pooled in two different stocks) was used for functional assays with fpEC. (A and B) CM of HBC stimulated proliferation of fpEC by trend and had no effect on viability. Chemoattracted migration of fpEC toward the HBC CM was reduced. Data are given as mean $\pm$ S.E.M. Statistical analysis used the mean of the triplicates of the number of individual biological replicates of fpEC ($n=6$). Control M (control medium): medium without cells.

points by $15 \pm 9\%$ ($P=0.048$) as compared with control medium (Fig. 5).

The angiogenic event comprehends different processes, and, besides network formation, includes chemoattraction, proliferation and survival of fpEC. We investigated the paracrine effect of HBC on these fpEC functions. Proliferation of fpEC was slightly increased by HBC CM, but this did not reach significance (Fig. 6A). Interestingly, chemoattraction of fpEC to the CM was reduced by $28 \pm 12\%$ ($P=0.037$) when compared with nonconditioned control medium (Fig. 6C). HBC CM did not affect LDH release from fpEC (Fig. 6B) indicating that the decrease in chemoattraction did not result from reduced cell survival and viability.

Discussion

This review investigated the effect of isolated placental tissue macrophages on angiogenic processes of fpEC. Key findings were that HBC displayed M2a, M2b and M2c polarization. Moreover, paracrine factors secreted by HBC modulated angiogenesis-related processes in fpEC, that is the stimulation of network formation and the reduction of chemoattracted migration.

In line with previous studies, the absence of CD80 on HBC indicates an M2 polarization of macrophages (Joerink *et al.* 2011, Sisino *et al.* 2013, Tang *et al.* 2013a,b, Young *et al.* 2015). Moreover, we determined the protein expression of markers for M2 subpopulations. Based on these markers ascribed to different human macrophage phenotypes in recent literature (Orme & Mohan 2012, Ambarus *et al.* 2012, Roszer 2015, Gensel & Zhang 2015, Zhang *et al.* 2015), we revealed that HBC represent a macrophage phenotype that cannot be readily classified into one M2 macrophage subset phenotype, as M2a-, M2b- and M2c-specific proteins and markers were present. This indicates the presence of all three M2 macrophage subtypes in the placenta. Furthermore, fetal macrophages may represent a unique polarization pattern (Stouch *et al.* 2014) and hence, not suit the above polarization classification.

The observed heterogeneity of HBC, however, is probably not only a result of the influencing

microenvironment. Moreover, the origin of HBC may prime their polarization. There is evidence that HBC appear even before the establishment of the fetal circulation in the first trimester. This implies that subsets of HBC derive from mesenchymal cells, whereas later in pregnancy, it is supposed that HBC arise from recruited fetal monocytes (Castellucci *et al.* 1987, Kim *et al.* 2008). Considering that HBC derive from different progenitor cells, it may be possible that differentiation is a programmed process and only secondary determined by the environment (Roszer 2015).

The presence of M2a, M2b and M2c macrophages in the placenta implies that regenerative and tissue remodeling function, but also pro- and anti-immunological functions are present in normal HBC population. As HBC display a potentially pro-angiogenic phenotype, we analyzed the secretion of angiogenesis-related factors of HBC in the CM and identified VEGF and FGF2 secreted by HBC, while PLGF was not detected. TNFA, a further factor secreted by HBC was shown to possess not only pro-inflammatory but also pro-angiogenic features (Fajardo *et al.* 1992). Furthermore, we tested the paracrine effect of HBC CM on primary fpEC. In fact, HBC CM increased network formation, and stimulated proliferation by trend, that may be related to the secretion of the pro-angiogenic factors. A role of FGF2 in the stimulation of angiogenesis in Matrigel plugs by M2 macrophages in mice was already suggested (Jetten *et al.* 2014). Interestingly, chemotactic migration of fpEC was impaired by HBC CM, but without affecting cytotoxicity. There are several possibilities how HBC-derived molecules may affect fpEC migration. On one hand, HBC may secrete factors that limit migration such as migration-inhibiting factor (MIF), or reduce MMP secretion, whereas on the other hand, HBC-derived molecules may affect the expression of fpEC integrins or receptors influencing chemotactic migration of fpEC.

Whether the paracrine effect of HBC on fpEC is specific for the placenta or a common feature of M2 macrophages on endothelial cells remains speculative. Since both macrophages and endothelial cells are well known for their organ-specific heterogeneity (Regan & Aird 2012, Giorgio 2013), the observed paracrine effect may well be specific for the placenta. However, the ability to reduce migration of endothelial cells toward the macrophage and thus the regulation of angiogenesis without altering the direction of vascular growth may occur also in other situations and organs than in the placenta.

Given a role of HBC in feto-placental angiogenesis, conditions and pathologies altering macrophage abundance and polarization may affect this process. In fact, a shift in HBC phenotype was observed in placentas exposed to maternal Type 1 diabetes (Sisino *et al.* 2013), although this study analyzed only a very limited number of samples. Also, maternal obesity is

associated with an increase in placental macrophages (Challier *et al.* 2008). For both conditions, analysis of the feto-placental vascular tree revealed altered morphology with increased vascular growth (Jirkovska *et al.* 2012, Loardi *et al.* 2015). Also pre-eclampsia was associated with reduced numbers of HBC (Tang *et al.* 2013a,b), but results are conflicting about whether or not pre-eclampsia is associated with reduced or unaltered placental vascularity (Mayhew *et al.* 2004, Srinivasan *et al.* 2014). Whether fetal growth restriction, a pathology hallmarked by reduced feto-placental angiogenesis (Mayhew *et al.* 2004), is also associated with an altered number of HBC remains to be studied.

Strength of the study is the use of isolated human primary cells: HBC CM was applied on fpEC from late pregnancy. However, this represents also the limitation of the study, since some aspects of placental angiogenesis may be more important for placental development in earlier stages of pregnancy. Because methods for the isolation of HBC and fpEC from early pregnancy with high yield and purity have not been established so far, we had to restrict our study to the third trimester.

In summary, primary isolated HBC represent an M2-activated phenotype with diverse, that is M2a, M2b and M2c subtypes, and secrete pro-angiogenic and mitogenic molecules and survival factors for endothelial cells. These characteristics are in fact referred to the tissue remodeling macrophages and thus HBC are likely to support feto-placental angiogenesis. This was verified by our *in vitro* experiments showing that HBC CM stimulates 2D network formation of primary fpEC.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/REP-16-0159>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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