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Control of leucocyte differentiation from embryonic stem cells upon vasculogenesis and confrontation with tumour tissue

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Abstract

Embryonic stem (ES) cells spontaneously differentiate capillary-like structures as well as leucocytes such as monocytes/macrophages, neutrophils, natural killer (NK) cells and cytototoxic T lymphocytes. The interplay between vasculogenesis and leucocyte differentiation as well as the population of tumour tissues with ES cell-derived leucocytes and endothelial cells is, however, not sufficiently specified. In the present study, gene expression of the cell surface markers CD68 and CD14 (expressed on monocytes and macrophages), Mac-1 (CD11b) (expressed on granulocytes, monocytes and NK cells) and CD16 (expressed on neutrophils) was investigated in murine CGR8 ES cells in relation to the endothelial cell markers CD31 and vascular endothelial (VE)-cadherin. Expression of leucocyte markers increased from day 7–8 of cell culture on. Furthermore, addition of macrophage colony-stimulating factor to the cell culture medium resulted in a threefold increase in the number of $CD68^+$ monocytes/macrophages. Treatment of embryoid bodies with lipopolysaccharide (LPS) up-regulated CD14 thus suggesting functionality of the CD14 LPS receptor. Differentiation of vascular structures positive for CD31 and VE-cadherin preceded leucocyte differentiation by 2 days (*i.e.* from day 5–6 on) suggesting that vasculogenesis may be a determinant of leucocyte differentiation. Consequently the Flk-1 antagonist SU5416 which inhibits vasculogenesis of ES cells significantly blunted leucocyte differentiation. Confrontation culture of embryoid bodies with multicellular breast tumour spheroids initiated significant increase of leucocyte cell numbers and invasion of leucocytes into the tumour tissue. In summary our data demonstrate that during ES cell differentiation vasculogenesis precedes leucocyte differentiation, and point towards the direction that leucocyte cell invasion into tumour tissue may initiate the pro-inflammatory microenvironment necessary for tumour vascularization.

Keywords: embryonic stem cells • tumour angiogenesis • haematopoiesis, leucocytes • confrontation culture

Introduction

It is commonly accepted that tumour angiogenesis is a prerequisite for tumour growth. Angiogenesis is stimulated by angiogenic factors released by tumour cells, though other cells, such as tumour-associated macrophages, neutrophils, natural killer (NK) cells and dendritic cells also contribute towards increasing the angiogenic process in cancer [1]. Current opinion holds that native immune cells recruited into tumours in turn stimulate the endothelium and are responsible for an indirect pathway of tumour vascularization [2], which is based on the observation that tumours are infiltrated by different leucocyte cell types. It is now becoming clear that the tumour microenvironment is largely orchestrated by inflammatory cells, which participate in the neoplastic process, fostering proliferation, survival and migration [3]. However, it is not known whether leucocyte cell types could arise from endothelial cells during their invasion into the tumour mass.

Differentiating embryonic stem (ES) cells are increasingly emerging as an important source of haematopoietic progenitors with a potential to be useful for both basic and clinical research applications. During recent years it has been shown that pluripotent ES cells are capable to differentiate to the endothelial cell lineage [4–6] as well as into different haematopoietic lineages including leucocyte subtypes *e.g.* monocytes/macrophages [7], T lymphocytes [8], NK cells [9], neutrophil cells [10] and dendritic cells [11]. It is meanwhile generally accepted that the haematopoietic cell lineage and the endothelial cell lineage derive from a common precursor the haemangioblast [12]. In mice, cell aggregates consisting of mesodermal cells originating from the

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primitive streak form blood islands within the yolk sac. The central cells within the blood islands differentiate towards primitive haematopoetic cells, whereas the peripheral cells differentiate into endothelial cells. Upon fusion of blood islands the first extra-embryonic vascular plexus is formed [13]. This early concept was corroborated by various studies performed on ES cells that demonstrated that early haematopoietic cells and angioblasts express a number of common genes, including Flt1 (vascular endothelial growth factor-R1 [VEGF-R1]), Flk-1 (VEGF-R2), Scl1, Tie1 and Tie2 [14]. Gene targeting studies demonstrated that *Flk-1*$^{-/-}$ embryos do not develop yolk sac blood islands and die around day 9.5 [15]. Cells lacking Flk-1 are unable to reach the correct location to form blood islands, suggesting that Flk-1 is involved in the movement of cells from the posterior primitive streak to the yolk sac and, possibly, to the intra-embryonic sites of early haematopoiesis [15]. Currently it is not well known whether and how cells within the haemangioblast interact and how the differentiation towards specific leucocyte cell types is regulated. In studies on stem cell niches within the bone marrow investigators have shown that haematopoietic stem cells (HSCs), as defined by newly recognized cell-surface markers, reside in perivascular as well as endosteal niches [16] thus suggesting that cell interactions between endothelial cell and HSCs are involved in the control of stem cell self-renewal and differentiation. Although much is known about endothelial ligands and receptors which mediate the docking and extravasation of leucocytes at sites of inflammation the time sequence of differentiation of specific leucocyte cell types in relation to the process of embryonic vascularization is currently not well described. Furthermore, not much is known about the involvement of leucocytes in the process of tumour angiogenesis. In the present study, the ES cell model of vasculogenesis and confrontation cultures of ES cell-derived embryoid bodies and multicellular tumour spheroids are used to investigate the interplay between vasculogenesis and differentiation of functional cell leucocyte cell types. It is demonstrated that endothelial cell differentiation precedes and directs leucocyte differentiation because pharmacological interference with Flk-1 signalling inhibits leucocyte differentiation. Upon confrontation culture with mouse mammary tumour spheroids leucocyte differentiation and/or proliferation is considerably stimulated which points towards an involvement of leucocytes in the process of tumour-induced angiogenesis.

Materials and methods

Materials

Lipopolysaccharide (LPS) from *E. coli* K-235 was provided by Sigma-Aldrich (Taufkirchen, Germany) and used in concentrations of 0.01, 0.1, 1, 10 and 100 ng/ml. Recombinant murine macrophage colony-stimulating factor (M-CSF) was obtained from CellSystems (St. Katharinen, Germany) and used in concentration of 250 ng/ml.

ES cell culture

The ES cell line CGR8 was obtained from the European Collection of Cell Cultures (ECACC). Originally, the germ-line competent cell line CGR8 was established from the inner cell mass of a 3.5-day male pre-implantation mouse embryo (Mus musculus, strain 129). The CGR8 cell line was cultured on gelatine-coated cell culture dishes in Glasgow minimal essential medium (GMEM; Sigma) to keep the cells in stem cell stage for proliferation. Medium was supplemented with 9% heat-inactived (56°C, 30 min.) foetal bovine serum (FBS, Sigma), 2 mM L-glutamine (Biochrom, Berlin, Germany), 45 μM ß-mercaptoethanol (Sigma) and 10^3 U/ml leukaemia inhibitory factor (LIF) (Chemicon, Hampshire, UK) in a humidified environment containing 5% CO_2 at 37°C and passaged every 2–3 days. At day 0 of differentiation, adherent cells were enzymatically dissociated using 0.25% trypsin with 1 mM ethylenediaminetetraacetic acid (EDTA) in Hanks' balanced salt solution (HBSS) (Invitrogen, Karlsruhe, Germany). A total of 1×10^7 cells were seeded in 250-ml siliconized spinner flasks (Integra Biosciences, Fernwald, Germany) containing 125 ml Iscove's medium supplemented with 16% FBS (Sigma), 100 μM ß-mercaptoethanol (Sigma), 2 mM L-glutamine (Biochrom) and 2mM non-essential amino acids (Biochrom). After 24 hrs, 125 ml medium was added to give a final volume of 250 ml. The spinner flask medium was stirred at 22.5 r.p.m. using a stirrer system (Integra Biosciences) and 150 ml cell culture medium were exchanged every day.

Cultivation of multicellular tumour spheroids

The mouse mammary tumour cell line 4T1 was cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) supplemented with 8.7% FBS (Invitrogen), 2 mM L-glutamine, 100 μM ß-mercaptoethanol (Sigma), 2 mM non-essential amino acids (Biochrom), 43 U/ml penicillin and 43 μg/ml streptomycin (both Biochrom). Cell monolayers were dissociated enzymatically with 0.25% trypsin with 1 mM EDTA in HBSS (Invitrogen) and seeded into 250-ml spinner flasks. For the experiments, tumour spheroids with an age of 5–7 days were used.

Generation of confrontation cultures

For the generation of confrontation cultures, multicellular tumour spheroids and embryoid bodies were removed from spinner flasks. To discriminate tumour spheroids grown in confrontation culture from embryoid bodies, tumour spheroids were labelled with the long-term cell tracker dye 5-chloromethylfluorescein diacetate (CMFDA) as described previously [17]. One embryoid body (10 days old) and one tumour spheroid (5–7 days old) were inoculated in a 35 μl drop of mixed culture medium (50% spheroid medium, 50% embryoid body medium) placed onto the lid of a 10-cm Petri dish, the lid was turned around and placed on the Petri dish, which was filled with 10 ml of sterile phosphate buffered saline (PBS). Within 24 hrs, embryoid bodies and tumour spheroids closely attached within the hanging drops and were then plated onto gelatine-coated glass slides in 24-well plates.

Immunohistochemistry

Immunohistochemistry was performed with outgrown embryoid bodies plated on day 4 to gelatine-coated glass slides in 24-well-plates. For

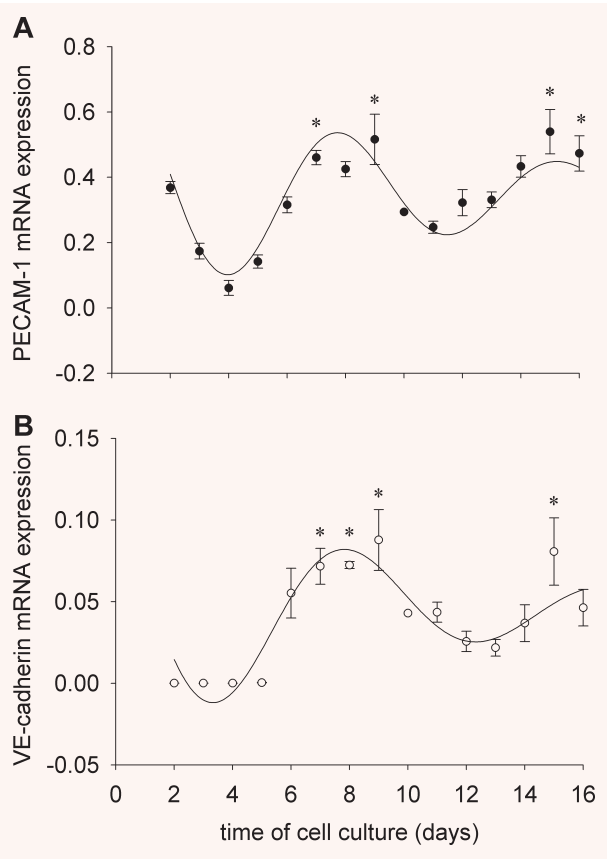

Fig. 1 mRNA expression of PECAM-1 (**A**) and VE-cadherin (**B**) during the differentiation of mouse ES cells of the CGR8 cell line. Note that PECAM-1 and VE-cadherin expression displayed two peaks, suggesting an initial phase of vascular plexus formation followed by vascular remodelling at later stages of differentiation. The expression level of PECAM-1 and VE-cadherin is presented in relation to the expression of the housekeeping gene Polymerase 2a. In each data point $n = 3$ experiments are represented. $^*P < 0.05$, statistically significant as compared to day 4 of cell culture.

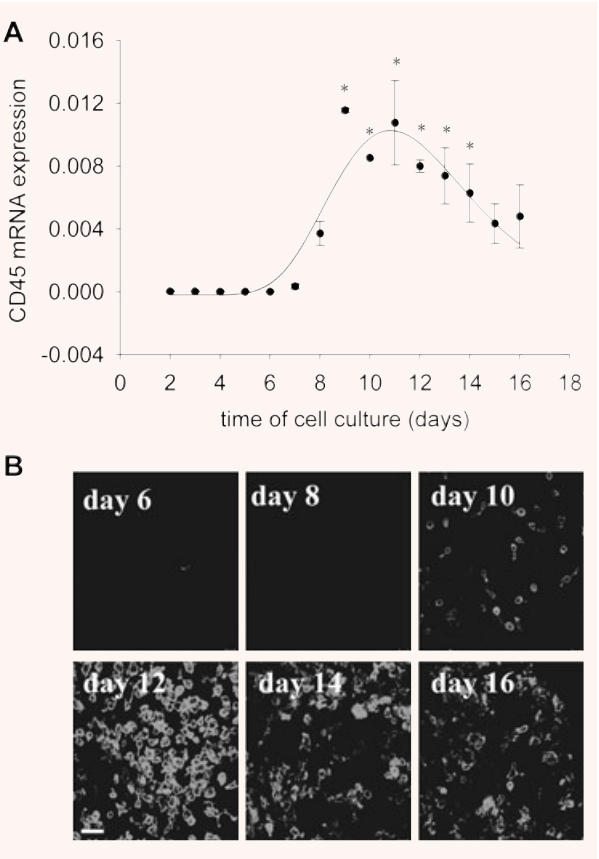

Fig. 2 mRNA expression of the pan leucocyte marker CD45 during the differentiation of CGR8 ES cells (**A**) and appearance of $CD45^+$ cells in plated embryoid bodies (**B**). Note that CD45 mRNA up-regulation was apparent on day 8 of differentiation which was followed by the appearance of $CD45^+$ cells on day 10 of cell culture. The expression level of CD45 is presented in relation to the expression of the housekeeping gene Polymerase 2a. The bar in (**B**) represents 20 μm. In each data point $n = 3$ experiments are represented. $^*P < 0.05$, statistically significant as compared to day 2 of cell culture.

primary antibodies, we used monoclonal antimouse platelet endothelial cell adhesion molecule (PECAM-1; CD31) (Chemicon), monoclonal antimouse CD45 (Chemicon), monoclonal antimouse CD68 (Serotec, Dusseldorf, Germany) and monoclonal antimouse Neutrophil antigen (Serotec). All antibodies were used in 1:100 dilution.

The tissues were fixed in ice-cold methanol/acetone (7:3) for 60 min. at –20°C, and washed with PBS containing 0.01% Triton X-100 (PBST; Sigma). Blocking against unspecific binding was performed for 60 min. with 10% fat-free milk powder (Heirler, Radolfzell, Germany) dissolved in PBST. The tissues were subsequently incubated overnight at 4°C with primary antibodies dissolved in PBST supplemented with 10% milk powder. The tissues were thereafter washed three times with PBST and re-incubated with either a Cy2- or a Cy5-conjugated goat anti-rat IgG (Chemicon) at a dilution of 1:100 in PBST containing 10% milk powder. After washing three times with PBST the tissues were stored in PBST until inspection.

Confocal laser-scanning microscopy

Fluorescence recordings were performed by means of a confocal laser scanning setup (LSM 410, Zeiss, Jena, Germany) connected to an inverted microscope (Axiovert 135, Zeiss). The confocal setup was equipped with a 5 mW helium/neon laser, single excitation 633 nm (excitation of Cy5) and an argon laser, single excitation 488 nm (Cy2 and CMFDA). Emission was recorded using the band-pass filter BP 505–570 IR and long-pass filter sets LP 475 and LP 650, respectively. A 20×, numerical aperture (N.A.) 0.75 and a 25×, N.A. 0.8, water-corrected objective (Neofluar, Zeiss) were used.

Fluorescence recordings were performed by a confocal laser-scanning microscope (LSM 510, Zeiss) connected to an inverted microscope (Axiovert 135, Zeiss). For excitation of the Cy2-fluorochrome or CMFDA,

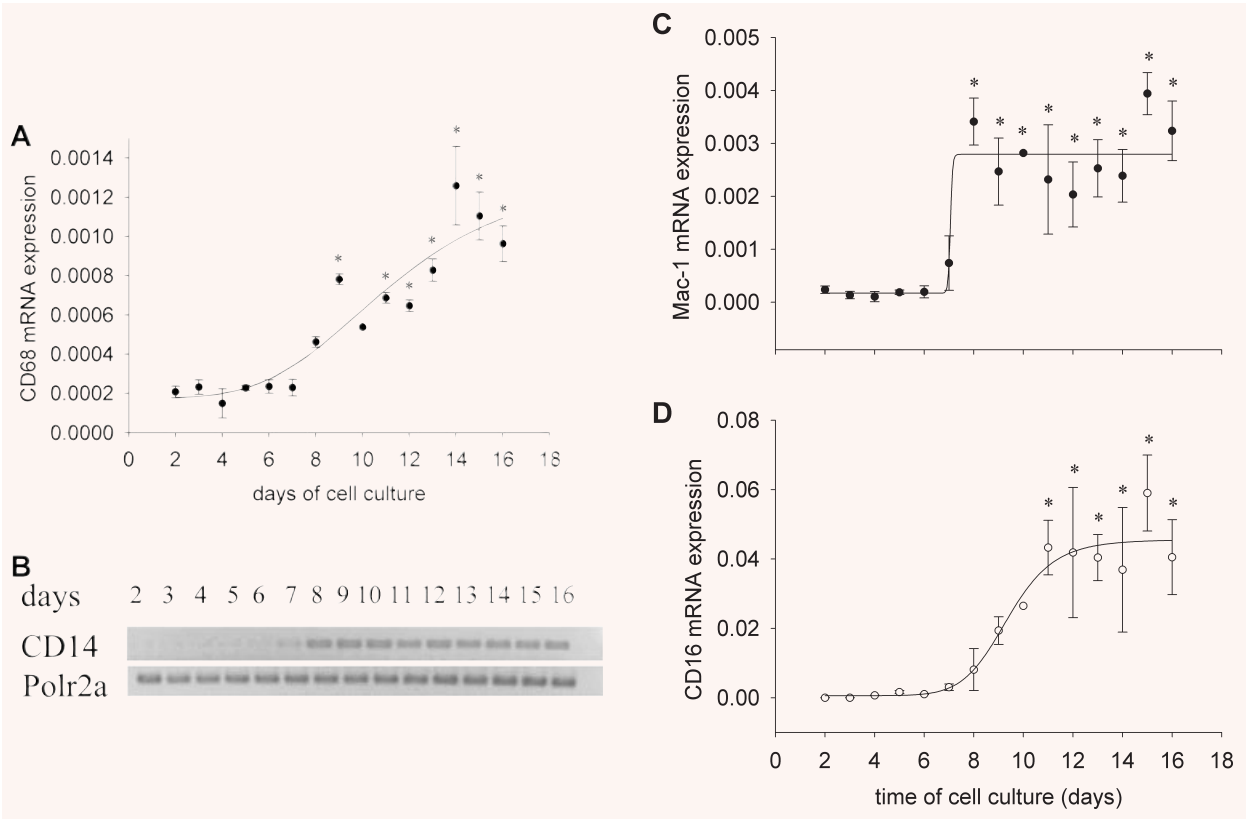

Fig. 3 mRNA expression of the monocyte/macrophage markers CD68 (**A**), CD14 (**B**), the granulocyte/monocyte/NK-cell marker Mac-1 (**C**) and the neutrophil marker CD16 (**D**) during the differentiation of CGR8 ES cells. Note that mRNA expression of the respective genes increased from day 8 of cell culture and remained constantly elevated until day 16. The mRNA expression levels are presented in relation to the expression of the housekeeping gene Polymerase 2a. In each data point $n = 3$ experiments are represented. $^*P < 0.05$, statistically significant as compared to day 2 of cell culture.

the 488-nm band of an argon laser setup and for the excitation of Cy5 fluorochrome, the 633-nm band of a helium/neon laser setup was used. Emission was recorded using a 505–570-nm band-pass or a 650-nm long-pass filter set. Fluorescence values of control samples stained only with secondary antibody were subtracted from samples stained with primary and secondary antibody. The pinhole settings of the confocal setup were adjusted to give a full-width half maximum of 6 μm Fluorescence was determined from the top to a depth of 20 μm of the tissue, composed of five optical sections with an interval of 5 μm in *z*-direction and the fluorescence values in the respective optical section were evaluated by the image analysis software of the confocal setup.

Real-time RT-PCR

Total RNA from CGR8 embryoid bodies was prepared using Trizol (Invitrogen) according to manufacturer's recommendations followed by genomic DNA digestion using DNaseI (Invitrogen). Total RNA concentration was determined by the optical density (260 nm) method. cDNA synthesis was performed with 2 μg RNA in a total volume of 40 μl with SuperScript RTase (Invitrogen).

Primer sequences for quantitative real-time PCR were:
PECAM: forw CAGGTGTGCGAAATGCTCT
rev ATGGGTGCAGTTCCATTTTC
CD45: forw TCACACAAAAGCAGATCGTC
rev GGTTTTAGGGCCATTAGTTTCA
CD68: forw TAAAGAGGGCTTGGGGCATA
rev CTCGGGCTCTGATGTAGGTC
CD16: forw GAGTGATTTCTGACTGGCTGCT
rev TGGAAGAATGAGATCCTGTTCA
Mac-1: forw ACCTCTAATGGTCCCTTGCTG
rev TGGTTGTGTTGATGAAGGTGA
VE-cadherin: forw GTCAGCTATAGGGACCTCTGTCAT
rev TCATTTCCTTTCACGATTTGG
CD14: forw GACCATGGAGCGTGTGCTT
Rev ACCAATCTGGCTTCGGATCT
Polymerase 2a: forw: GACAAAACTGGCTCCTCTGC
rev: GCTTGCCCTCTACATTCTGC

The primer concentration was 10 pM. Amplifications were performed in a Cycler Optical Module (Applied Biosystems 7500, Darmstadt, Germany) using SYBR®-Green (Qiagen). The following programs were used: 95°C for 15 min. hot start step/denaturation

94°C for 30 sec., AT 30 sec., 72°C 30 sec., 40 times
50°C for 10 min.
Annealing temperatures were:
61°C for PECAM, CD68, CD16, Mac-1, CD14
60°C for VE-cadherin
59°C for CD45

Fluorescence increase of SYBR Green was automatically measured after each extension step.

Amplified transcripts were loaded on a 2% agarose gel. CT values were automatically obtained. Relative expression values were obtained by normalizing CT values of the tested genes in comparison with CT values of the housekeeping genes using the ΔΔCT method.

Statistical analysis

Data are given as weighted mean value ± S.E.M., with *n* denoting the number of experiments unless otherwise indicated. In each experiment at least 10 embryoid bodies were analysed. Either one-way ANOVA or Student's t-test for unpaired data was applied as appropriate. A value of $P < 0.05$ was considered significant.

Results

Expression of vascular markers during differentiation of CGR8 ES cells

The present study was undertaken to characterize the time sequence of ES cell-derived vasculogenesis and leucocyte differentiation and to investigate their interdependence. To achieve these aims cell culture time-dependent expression of the endothelial markers PECAM-1 and VE-cadherin was investigated by real time RT-PCR (Fig. 1) and compared to the expression of leucocyte-specific genes (Figs 2 and 3). As previously described [18], PECAM-1 expression was evident in undifferentiated ES cells but was down-regulated until day 4 of cell culture. From day 5 of cell culture PECAM-1 was again up-regulated with two peaks of expression occurring at days 8–9 and day 15–16, indicating an early-phase of vascular plexus formation and later phase of vascular remodelling. In contrast VE-cadherin was only marginally expressed during early stages of differentiation, *i.e.* from day 2 to day 5 of differentiation but followed a similar scheme of expression with two peaks at days 8–9 and day 15 comparably to the expression of PECAM-1.

Expression of CD45, CD14, CD11b, CD68 and Mac-1 during differentiation of CGR8 ES cells

To correlate the expression of endothelial cell markers and leucocyte cell markers mRNA expression of CD45, which is a pan leucocyte marker was investigated (Fig. 2A). In parallel immunohistochemical analysis of $CD45^+$ cells was performed (Fig. 2B).

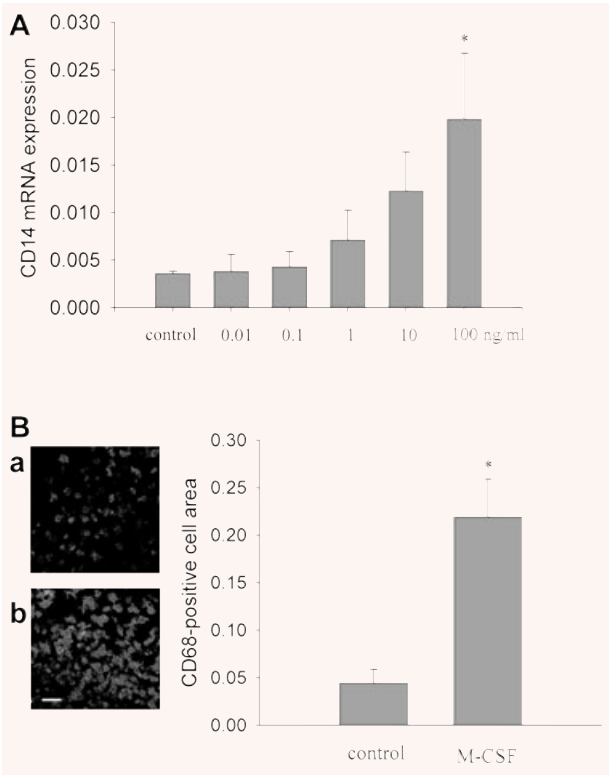

Fig. 4 Stimulation of the expression of the LPS receptor CD14 with LPS (**A**) and increase in $CD68^+$ cell numbers upon incubation with M-CSF (**B**). (**A**) mRNA expression of CD14 was investigated following treatment of 8-day-old embryoid bodies with different concentrations of LPS as indicated. The mRNA expression level of CD14 is presented in relation to the expression of the housekeeping gene Polymerase 2a. (**B**) Treatment with 250 ng/ml M-CSF significantly increased $CD68^+$ cell numbers as evaluated by assessing the size of the $CD68^+$ cell area in outgrown embryoid bodies. The representative images show (a) untreated control, (b) M-CSF-treated sample stained for CD68. The bar represents 20 μm. $*P < 0.05$, statistically significant as compared to the untreated control.

Increased expression of CD45 was evident from day 8 of cell culture with maximum expression between days 9 and 11. During subsequent days down-regulation of CD45 mRNA was observed. Expression of CD45 mRNA was paralleled by the appearance of $CD45^+$ cells in the cell culture, which became apparent on day 10 of cell culture, reached maximum values at day 12 and decreased until day 16 of cell culture. For CD68 (Fig. 3A) and CD14 (Fig. 3B) mRNA expression was observed from day 8 and 7 on, respectively. In contrast to CD45, mRNA expression remained on a constant plateau during the whole time of investigation (until day 16 of cell culture). Comparable results were achieved for the expression of Mac-1 (Fig. 3C) and CD16 (Fig. 3D), which were up-regulated from day 8 of cell culture and were constantly expressed until day 16 of cell culture.

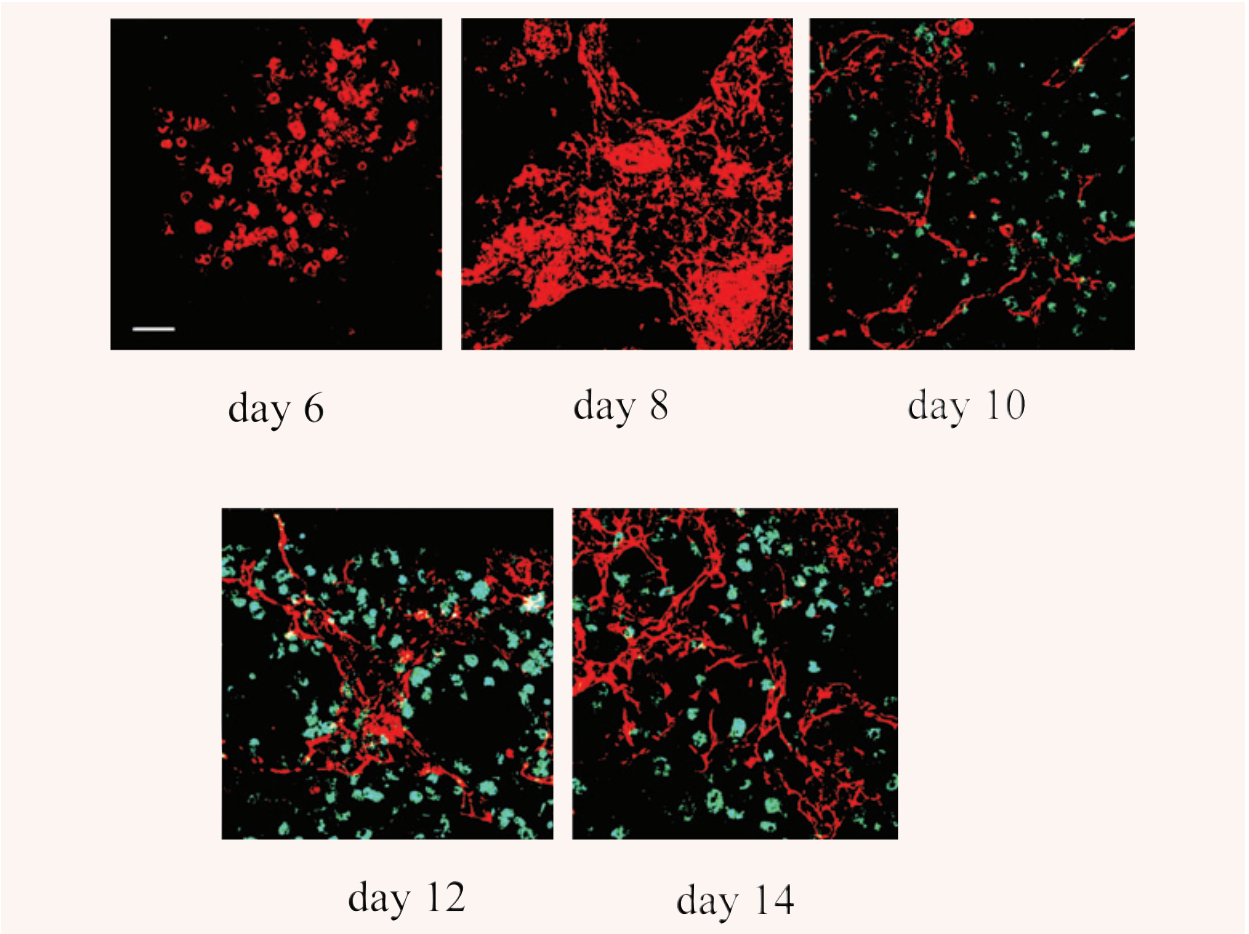

Fig. 5 Time sequence of blood vessel formation *versus* the expression of the monocyte/macrophage marker CD68. Double immunoflourescence images show PECAM-1$^+$ capillaries (red) and CD68$^+$ cells (green). It is apparent that PECAM-1$^+$ cell structures are already visible on day 6 of cell culture, whereas CD68$^+$ cells appear not earlier that on day 10 of differentiation. The bar represents 20 μm.

Induction of CD14 by LPS and stimulation of monocyte/macrophage differentiation by M-CSF

Expression of leucocyte-associated genes does not necessarily mean that functional cells are differentiated. To investigate cell function 8-day-old embryoid bodies were treated with LPS in increasing concentrations. Subsequently mRNA expression of the LPS receptor CD14 was assessed as a read out of leucocyte function (Fig. 4A, $n = 3$). It was evident that CD14 expression increased with the dose of LPS, indicating the occurrence of a physiological immunological inflammation reaction which is typically associated to an up-regulation of the CD14 LPS receptor following stimulation with LPS. To demonstrate cytokine-induced proliferation of macrophages embryoid bodies were treated with 250 ng/ml M-CSF from day 8 to day 10 of cell culture (Fig. 4B, $n = 3$). It was evident that this treatment increased the cell area positive for CD68 expression cells approximately threefold, indicating that the cell system of embryoid bodies responded towards agents that are known to induce macrophage differentiation and proliferation.

Vasculogenesis of ES cells precedes monocyte/macrophage differentiation

The analysis of mRNA expression of leucocyte and endothelial cell markers revealed that endothelial cell markers were expressed about 2 days earlier (day 5–6 of cell culture) as compared to leucocyte cell markers (day 7–8) (see Figs 1–3). To investigate whether this differential increase in gene expression was likewise reflected in the expression of structural proteins, double immunohistochemistry was performed by the use of the endothelial cell

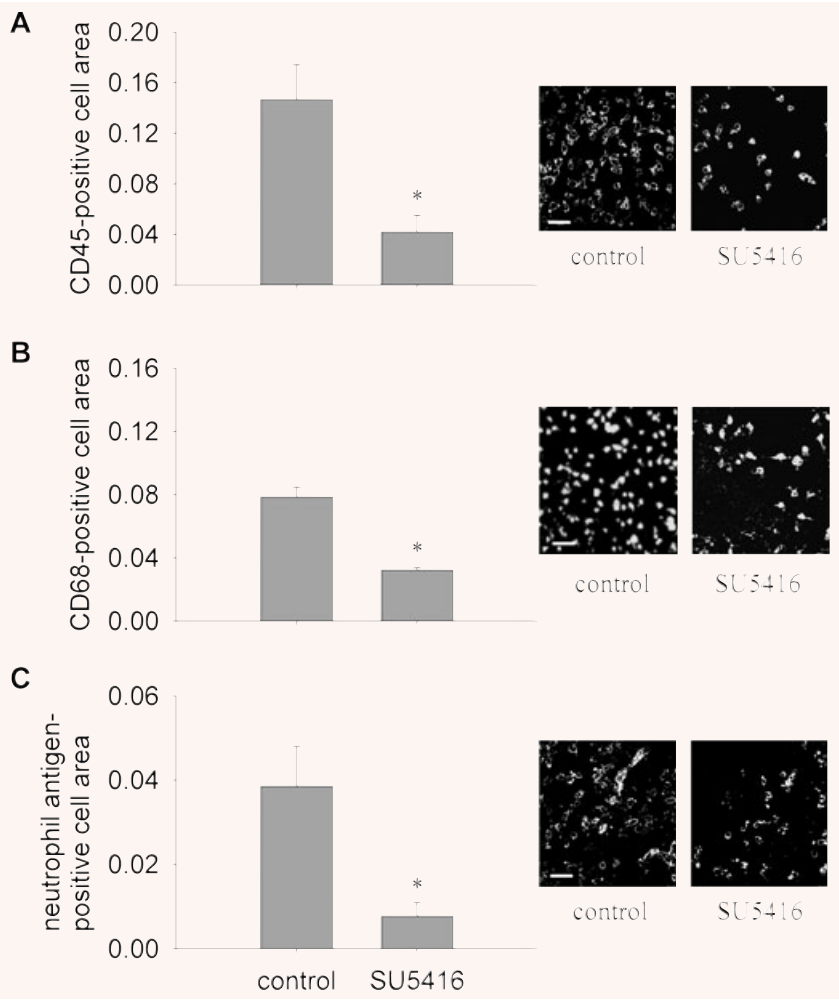

Fig. 6 Effects of the Flk-1 receptor antagonist SU5416 on **(A)** $CD45^+$, (**B**) $CD68^+$ and (**C**) neutrophil antigen-positive cell numbers. Treatment with the anti-angiogenic agent SU5416 significantly down-regulated cell numbers that were positive for CD45, CD68 and neutrophil antigen. Cell numbers were assessed as the cell area positive for the respective antigen in relation to the total cell area of outgrown embryoid bodies. The images show representative immunostainings. The bar represents 20 μm. $*P < 0.05$, statistically significant as compared to the untreated control.

marker PECAM-1 in parallel to the monocyte/macrophage marker CD68 (Fig. 5, $n = 3$). PECAM-1 staining of cells was evident already on day 6 of cell culture with increasing cell areas positive for PECAM-1^+ cells until day 8. Cells positive for CD68 were evident on day 10 of cell culture with increasing cell numbers until day 14, thus clearly indicating that vasculogenesis/angiogenesis preceded the differentiation of leucocytes.

Inhibition of leucocyte differentiation by anti-angiogenic treatment with SU5416

The data of the present study suggest that vasculogenesis/angiogenesis precedes leucocyte differentiation. If vasculogenesis/angiogenesis is a prerequisite for leucocyte differentiation it should be assumed that anti-angiogenic treatment would inhibit leucocyte differentiation. To validate this assumption, 6-day-old embryoid bodies that already displayed PECAM-1^+ cell areas were treated until day 12 of cell culture with SU5416 which inhibits signalling *via* the Flk-1 receptor tyrosine kinase [19]. As shown in Fig. 6A–C ($n = 4$) treatment with SU5416 (4 μM) significantly reduced the cell number of cells positive for CD45 (see Fig. 6A), CD68 (see Fig. 6B) and neutrophil antigen (see Fig. 6C), which strongly suggests that blood vessel development is necessary for proper leucocyte differentiation.

Increase in leucocyte numbers upon confrontation culture of ES cells with tumour tissue

Tumour growth is associated with angiogenesis and inflammation. It is known for several years that blood vessel and macrophage density increase simultaneously with pathological progression during tumour-induced angiogenesis [20]. It is, however, not yet known whether increased numbers of leucocytes within tumour tissues arise from recruitment of these cells to the tumour tissue or whether

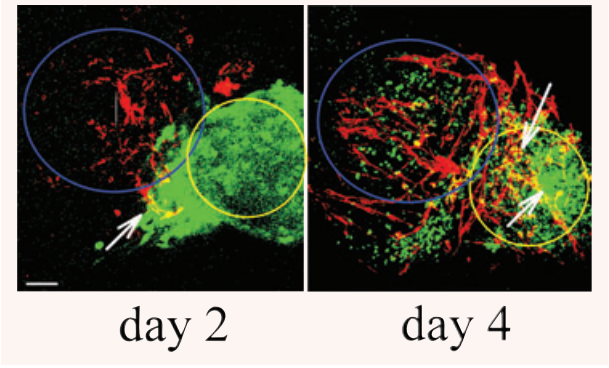

Fig. 7 Tumour angiogenesis upon confrontation culture of embryoid bodies cultivated from 10-day-old CGR8 ES cells and 5–7-day-old 4T1 mouse mammary tumour spheroids. Embryoid bodies derived from CGR8 ES cells (marked by the blue circles) were cultivated in confrontation culture with 4T1 mouse mammary tumour spheroids (marked by the yellow circles). The tumour spheroid was labelled with the long-term cell tracker dye CMFDA to discriminate between embryoid body and tumour tissue. After 2 days (left image) of confrontation culture capillary-like PECAM-1$^+$ cell structures (red) started to invade into the tumour tissue (see arrow). After 4 days of confrontation culture the tumour tissue was traversed with capillary-like structures (see arrows) indicating tumour vascularization. The bar represents 20 μm.

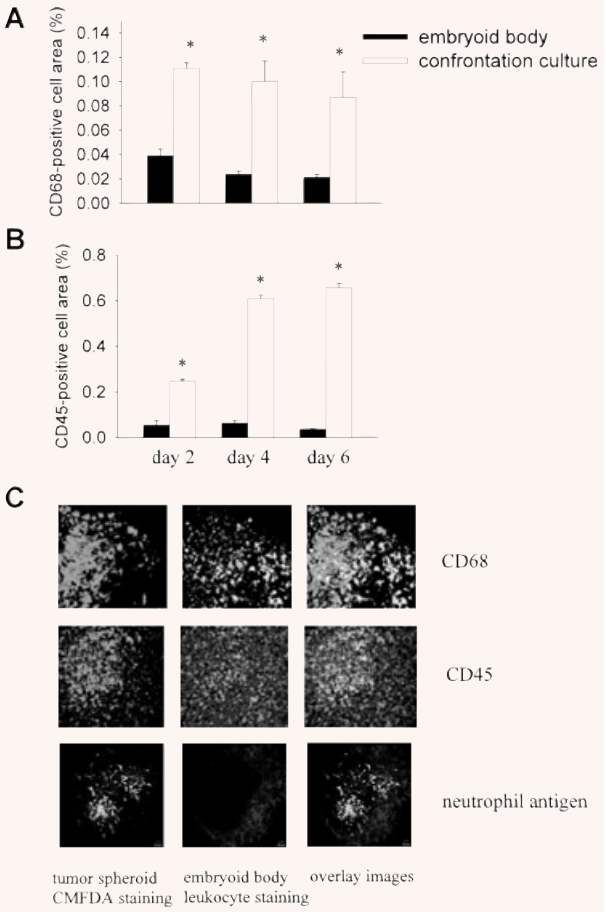

Fig. 8 Stimulation of leucocyte differentiation/proliferation upon confrontation culture of 10-day-old CGR8 ES cell-derived embryoid bodies and 5–7-day-old 4T1 mouse mammary multicellular tumour spheroids. (**A**) CD68$^+$ cell areas, (**B**) CD45$^+$ cell areas on day 2, day 4 and day 6 of confrontation culture as compared to embryoid bodies alone. Cell areas positive for leucocyte markers were assessed by quantification of the area positive for the respective marker in relation to the total cell area of the confrontation culture objects. (**C**) Immunohistochemistry of CD68 (upper row), CD45 (middle) and neutrophil antigen (lower row) in 4-day-old confrontation cultures. The images on the left side show the tumour tissues labelled with the long-term cell tracker dye CMFDA. The middle images show the respective leucocyte markers, and the images on the right side show overlays of the left and middle images. Note that CD68$^+$ and CD45$^+$ cells readily invaded the tumour tissue, whereas cells positive for neutrophil antigen formed a shell around the tumour cells. The bar represents 20 μm.

leucocytes differentiate and proliferate from invading precursor cells. To assess whether a tumour microenvironment would stimulate leucocyte differentiation/proliferation confrontation cultures of embryoid bodies and 4T1 mouse mammary tumour spheroids were generated. Upon 4 days of confrontation culture capillary-like endothelial cell structures invaded the tumour tissue and tumour cells spread into the embryoid body (Fig. 7; $n = 5$). When CD45$^+$, CD68$^+$ and neutrophil antigen-positive cells were assessed in confrontation cultures it became apparent that CD68$^+$ ($n = 4$) and CD45$^+$ ($n = 4$) cells readily invaded into the tumour tissue, whereas neutrophil antigen-positive cells formed a shell around the tumour cells without any penetration ($n = 3$) (see Fig. 8C). Interestingly confrontation culture was accompanied by a significant increase in CD68$^+$ (Fig. 8A, $n = 3$) and CD45$^+$ (Fig. 8B, $n = 3$) cell numbers as compared to the leucocyte cell numbers in embryoid bodies cultured alone, thus indicating that the tumour microenvironment stimulated differentiation and/or proliferation of leucocytes from precursor cells which may belong to the endothelial cell lineage.

Discussion

During embryogenesis haematopoietic cells have been demonstrated to originate from a common precursor which give rise to end othelial progenitors and haematopoietic progenitors, the latter subsequently generating primitive as well as definitive haematopoietic cell lineages [13, 21]. Subsequent studies have refined this concept by demonstrating that during ES cell differentiation specification of Flk-1$^+$ mesoderm into haematopoietic and

endothelial cell lineages occurs in sequential steps with a subpopulation of VE-cadherin-positive endothelial cells that have the capacity of differentiation of multiple definitive haematopoietic progenitors [22, 23]. Comparably it has been demonstrated in human ES cells that haematopoiesis and endothelial maturation occur exclusively from a subset of embryonic endothelium that possesses haemangioblastic properties [24].

In the present study the time sequence of endothelial cell differentiation and differentiation of leucocyte cell types was investigated. It was demonstrated that murine ES cells expressed the endothelial cell markers PECAM-1 and VE-cadherin and formed capillary-like cell structures. With a time delay of approximately 2 days endothelial cell differentiation was followed by the expression of the pan leucocyte marker CD45, the monocyte/macrophage markers CD68, CD14 and Mac-1 and the neutrophil marker CD16. Stimulation of embryoid bodies with LPS resulted in up-regulation of the LPS-receptor CD14. Furthermore, incubation of embryoid bodies with M-CSF increased the number of $CD68^+$ cells suggesting that functional monocytes/macrophages were generated. The time delay between the differentiation of endothelial cells and leucocytes suggested that endothelial cell differentiation may be a prerequisite of leucocyte differentiation. This assumption was tested by incubating 6-day-old embryoid bodies with SU5416 which is an inhibitor of Flk-1 and additionally Flt-1 signalling by inhibiting tyrosine kinase activity [25]. SU5416 has been shown to inhibit endothelial cell migration and angiogenesis [25]. In the embryoid body model SU5416 was shown by us to inhibit vasculogenesis (data not shown). Notably 6-day-old embryoid bodies were already positive for endothelial cell markers whereas cells positive for CD45 were completely absent. Treatment with SU5416 significantly reduced cell areas covered with $CD45^+$, $CD68^+$ and neutrophil antigen-positive cells suggesting that either angiogenesis directly stimulates leucocyte cell differentiation from undifferentiated ES cells or that immature or mature endothelial cells give rise to a leucocyte progeny.

Recently it has been pointed out that a characteristic inflammatory infiltrate is associated with constant tissue remodelling in a growing tumour and with tumour angiogenesis [1]. The origin of these inflammatory cells is not yet defined. Previous studies have demonstrated that bone marrow derived cells are recruited into tumour vasculature in response to angiogenic signals, and some of the cells within the newly forming tumour vessels are HSCs in origin. It seems therefore conceivable that inflammatory leucocyte cell types arise from endothelial progenitor cells within the tumour vasculature, thus establishing the inflammatory microenvironment that may provide the suitable pro-angiogenic cytokine cocktail necessary for proper vascular growth within the tumour tissue. In the present study, we used our previously established confrontation culture system [26] comprising of ES cell-derived embryoid bodies and multicellular 4T1 breast tumour spheroids to demonstrate that within 4 days of confrontation culture capillary-like cell structures were detectable within the tumour tissue. Investigation of leucocyte cell markers within the tissue of confrontation cultures demonstrated that $CD45^+$ and $CD68^+$ cells readily invaded the tumour tissue, which may result in the pro-inflammatory state of increased reactive oxygen species production previously observed in confrontation cultures [27]. In contrast, neutrophil cells positive for neutrophil antigen formed a shell around the embryoid body tissue with no invasion into the tumour. Most notably it was observed that confrontation culture significantly increased tissue areas positive for $CD45^+$ and $CD68^+$ cells, which supports the assumption that during tumour angiogenesis the specific tumour microenvironment either stimulates differentiation of leucocytes presumably from a pre-existing endothelial cell population or promotes a rapid proliferation of these cells. Chronic inflammation that has been attributed to the initiation and progression of cancer as well as to vascular growth and blood vessel formation [28]. If indeed interdependence exists between tumour angiogenesis and leucocyte cell reactions any anti-angiogenic treatment that targets the tumour vasculature should in parallel abolish the inflammatory stage of solid tumours which may be stimulatory for vascular growth.

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