

Proangiogenic Factor PIGF Programs CD11b⁺ Myelomonocytes in Breast Cancer during Differentiation of Their Hematopoietic Progenitors

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Abstract

Tumor-mobilized bone marrow-derived CD11b⁺ myeloid cells promote tumor angiogenesis, but how and when these cells acquire proangiogenic properties is not fully elucidated. Here, we show that CD11b⁺ myelomonocytic cells develop proangiogenic properties during their differentiation from CD34⁺ hematopoietic progenitors and that placenta growth factor (PIGF) is critical in promoting this education. Cultures of human CD34⁺ progenitors supplemented with conditioned medium from breast cancer cell lines or PIGF, but not from nontumorigenic breast epithelial lines, generate CD11b⁺ cells capable of inducing endothelial cell sprouting *in vitro* and angiogenesis *in vivo*. An anti-Flt-1 mAb or soluble Flt-1 abolished the generation of proangiogenic activity during differentiation from progenitor cells. Moreover, inhibition of metalloproteinase activity, but not VEGF, during the endothelial sprouting assay blocked sprouting induced by these proangiogenic CD11b⁺ myelomonocytes. In a mouse model of breast cancer, circulating CD11b⁺ cells were proangiogenic in the sprouting assays. Silencing of PIGF in tumor cells prevented the generation of proangiogenic activity in circulating CD11b⁺ cells, inhibited tumor blood flow, and slowed tumor growth. Peripheral blood of breast cancer patients at diagnosis, but not of healthy individuals, contained elevated levels of PIGF and circulating proangiogenic CD11b⁺ myelomonocytes. Taken together, our results show that cancer cells can program proangiogenic activity in CD11b⁺ myelomonocytes during differentiation of their progenitor cells in a PIGF-dependent manner. These findings impact breast cancer biology, detection, and treatment. *Cancer Res*; 71(11); 3781–91. ©2011 AACR.

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Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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doi: 10.1158/0008-5472.CAN-10-3684

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Introduction

The formation of new blood vessels within the tumor microenvironment, a process known as tumor angiogenesis, promotes tumor growth and metastatic spreading (1, 2). Although newly formed tumor vessels originate mostly by sprouting angiogenesis from locally preexisting vessels, evidence indicates that tumor-mobilized bone marrow-derived (BMD) cells significantly contribute to tumor angiogenesis. Two mechanisms have been reported. On the one side, tumors mobilize endothelial progenitors capable of differentiating into mature endothelial cells and incorporating into the nascent tumor endothelium (3). On the other side, tumors mobilize myeloid cells, which accumulate in the tumor microenvironment and promote tumor angiogenesis through paracrine mechanisms (4, 5). A number of elegant studies combining cell surface phenotyping, *in vivo* cell tracing, and functional experiments identified multiple angiogenesis-promoting CD11b⁺ BMD cell populations in mice and human (6–11). Little is known, however, on the mechanisms by which tumor-mobilized CD11b⁺ myeloid cells are programmed to acquire proangiogenic properties. One proposed mechanism

is that programming is induced by the tumor on recruitment to the tumor microenvironment (4). Alternatively, programming may occur early on during myeloid cell differentiation from hematopoietic progenitors. In this work, we have addressed this second hypothesis through experimental studies *in vitro* and *in vivo* and correlative analyses in cancer patients.

Materials and Methods

Proteins and chemicals

Human recombinant placenta growth factor (PlGF) was kindly provided by Prof. Kurt Ballmer, Paul Scherrer Institute, Villigen, Switzerland. Human VEGFA, VEGFB, interleukin (IL) 8, and IL1 β and mouse FGF (fibroblast growth factor) were purchased from PeproTech EC Ltd. Human stem cell factor (SCF), Flt-3, IL6, and TPO (thrombopoietin) were purchased from R&D Systems. 4',6-Diamidino-2-phenylindole, dihydrochloride (DAPI) was purchased from LuBioscience GmbH. Ficoll Histopaque Plus 1.077 were from Sigma-Aldrich Chemistry.

Antibodies

Anti-human antibodies. Fluorescein isothiocyanate (FITC)-conjugated anti-CD11b, anti-CD14, anti-Ki67; phycoerythrin (PE)-conjugated anti-CD14, PerCP-conjugated anti-CD34; allophycocyanin (APC)-conjugated anti-CD11b; and Pacific Blue-conjugated anti-CD45 were purchased from BD Pharmingen (Becton Dickinson). PE-conjugated anti-Flt-1, APC-conjugated anti-Flt-1, and IgG1 isotype control were purchased from R&D Systems. The function-blocking anti-human Flt-1 antibody KM1732 was described before (12).

Anti-mouse antibodies. FITC-conjugated anti-CD11b and APC-conjugated anti-CD45 were purchased from BD Pharmingen (Becton Dickinson). Unconjugated anti-F4/80 and anti-CD31, PerCP-conjugated anti-F4/80, and PerCP-conjugated rat anti-IgG2a isotype control were purchased from Biologend.

Cell lines and primary cells

The human breast cancer cell line MDA-MB-231 and human nontransformed breast epithelium-derived cell line MCF10A were purchased from American Type Culture Collection (HTB-26D and CRL-10317) and cultured in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS) and 1% penicillin/streptomycin (P/S; Invitrogen). The primary human mammary epithelial cell (HMEC) line, isolated from normal human breast tissue obtained from reduction mammoplasties and the tumorigenic Wnt-1-transformed HMEC line (Wnt-1-HMEC) were provided by Dr. C. Brisken, ISREC-EPFL (13). Cell-conditioned medium (CCM; 2–4 days old) was collected from cell lines at near-confluence conditions, filtered at 0.2 μ m, and stored at -20° C. The mouse breast cancer cell line 4T1 was provided in 2008 by Dr. Fred R. Miller (Michigan Cancer Foundation, Detroit, MI), who originally generated them (14). We have not tested/authenticated these cell lines after reception in our laboratory. 4T1 cells were cultured in DMEM (Dulbecco's modified Eagle' medium) high glucose

supplemented with 10% FCS and 1% P/S. Human umbilical vein endothelial cells (HUVEC) were prepared and cultured as described previously (15) and used between passage 3 and 5. BALB/c murine lung endothelial cells (MLEC) were generously provided by Dr. K. Hodivala Dilke. CD34⁺ progenitor cells were purified from cord blood using anti-CD34 magnetic beads separation kit (StemCell Technologies Inc.) and cultured in RPMI 1640 medium supplemented with 8% human AB plasma, SCF, Flt-3, IL6, and TPO (basal hematopoietic medium, BHM; ref. 16).

Umbilical cord blood

CD34⁺ progenitors cells were isolated using EasySep selection kit (StemCell Technologies Inc.) from umbilical cord blood obtained from newborn babies of healthy volunteer pregnant women for whom a cesarotomy was preprogrammed, on approval by the ethic committee of the University Hospital of Lausanne (protocol 236/07) and according to the declaration of Helsinki.

In vitro CD34⁺ progenitor differentiation assay

Purified CD34⁺ cord blood hematopoietic progenitor cells were cultured in a 24-well tissue plate in BHM \pm 10% filtered CCM from MDA-MB-231, Wnt-1-HMEC, MCF10A, and HMEC. Cell cultures were harvested at day 7. The morphology of CD11b⁺ cells generated in culture was determined by Wright-Giemsa staining.

Cell sorting

CD11b⁺ cells and/or Flt-1⁺CD11b⁺ cells from CD34⁺-initiated *in vitro* cultures or from peripheral blood mononuclear cells (PBMC) were isolated using EasySep selection kit for labeled cells (StemCell Technologies Inc.) or using FACSAria cell sorting.

In vitro angiogenesis sprouting assay

HUVEC or MLEC cells were trypsinized and cultured during 24 hours in U-bottom 96-well plates to form spheroids (17). Spheroids were collected and transferred to 1% collagen. Cells or cytokines to be tested for angiogenic sprouting activity were added on top of the gel. After 48 (HUVEC) or 24 (MLEC) hours, sprouting was scored by measuring the length of the sprouts using Axiovision Software (Zeiss). Data are presented as mean of cumulated sprout lengths from 10 spheroids \pm SD.

Cytokine measurement

Measurements of human or mouse PlGF were conducted in duplicate using the Quantikine ELISA systems (R&D Systems). Results represent means of duplicate determinations \pm SD.

Tumor model

4T1 cells were injected (5×10^4 cells in 50 μ L PBS/20% Matrigel per injection, respectively) in the fourth right inguinal mammary gland of 4- to 6-week-old BALB/c female mice (Harlan or Charles River Laboratories). Tumor growth was measured twice a week with a caliper and tumor volume was calculated with the equation: volume = $\pi/6$ (length \times width²).

Single-cell suspensions from tumors were obtained as recently reported (18). Mouse experiments were approved by the cantonal veterinary service of Canton Vaud.

Tumor perfusion measurement by power Doppler

Measurements were conducted 21 days after tumor cell line injection using the VEVO 770 Micro Three-dimensional High-Frequency Doppler Ultrasound system (VisualSonics) equipped with a 30 MHz transducer. Mice were anesthetized by inhaling a mixture of isoflurane (1.5%) and oxygen (98.5%). The anesthetized animals were placed on a warmed examination table, and the tumors were covered with ultrasound gel. An ultrasound transducer mounted on a 3-dimensional (3D) motor on a rail system above the animal, moved perpendicular to the beam axis, thereby acquiring consecutive 2-dimensional images in B mode and power Doppler mode with a slice thickness/interval of 500 μ m. A region of interest (ROI) was drawn within every 2D image. Two-dimensional ultrasound images were used to compile a 3D volume using VisualSonic software. Intratumoral perfusion was determined by measuring the Doppler-derived pixels over a constant surface placed over the most perfused area of each of 3 sections per tumor using ImageJ free software (<http://rsbweb.nih.gov/ij/>). Five to 7 tumors per each experimental condition were measured (4T1 wild type, 4T1 nonsilenced, and 4T1 sh-PIGF).

Corneal angiogenesis assay

Corneal angiogenesis assay was conducted on the basis of a modification of the method originally reported (19). CD11b⁺ cells purified by immunomagnetic beads (StemCell Technologies Inc.) were resuspended at 20×10^6 cells/mL in RPMI 1640 medium with 2% FCS. NOD/SCID (severe combined immunodeficient)/IL2 receptor common γ 2 chain knockout mice were anesthetized with ketamine and xylazine and 5 μ L of cell aliquots were injected into the corneas using a 35-gauge NanoFil injection kit (WPI). Eyes' vascularization was monitored with a stereomicroscope and mice were euthanized 20 days postinjection.

Breast cancer patients and healthy donors

Peripheral blood from healthy donors and cancer patients was obtained according to the declaration of Helsinki and on approval by the ethic committee of the University Hospital and University of Lausanne. Breast cancer patients aged from 32 to 75 years (mean: 56.7 ± 13.4) were all previously untreated and presenting at first diagnosis with ductal invasive carcinoma without evidence of distant metastasis (T1 and T2, N0 and N1, M0), estrogen receptor (ER) positive (85%–95%), and scheduled for tumor resection (10 patients). Blood was collected the day before surgery. Healthy volunteers were individuals without history of cancer, chronic disease, or medication other than hormonal contraception. PBMCs were isolated using Ficoll Histopaque Plus 1.077 (Becton Dickinson).

Human samples processing

Blood was collected in Lithium heparin anti-coagulated Vacutainer tubes (Becton Dickinson). Plasma phase was col-

lected and centrifuged 5 minutes at $1,500 \times g$ at room temperature. Plasma was stored at -80°C . PBMC were isolated using Ficoll Histopaque Plus 1.077 (25 minutes centrifugation at $680 \times g$ at room temperature). The PBMC fraction was collected, washed in PBS, counted, aliquoted at 10×10^6 cells/mL, and frozen at -80°C in 90%FCS/10%DMSO (dimethyl sulfoxide) medium. Twenty-four hours later, samples are transferred into liquid nitrogen.

Statistical analysis

In all experiments of this study, values are expressed as mean \pm 95% CI. Statistical analyses were conducted by paired Student's *t* test unless indicated otherwise. Differences were considered statistically significant at $P < 0.05$.

Results

Breast cancer CCM induces the generation of proangiogenic CD11b⁺ myelomonocytic in CD34⁺ hematopoietic progenitor-initiated culture

To test the effect of tumor cells on the generation of proangiogenic CD11b⁺ myeloid cells, we cultured human CD34⁺ progenitors in a BHM (16) alone or in the presence of CCM obtained from the tumorigenic metastatic breast cancer-derived cell line MDA-MB-231 or from the normal breast epithelium-derived nontumorigenic cell line MCF10A. In this model, CD34⁺CD45^{dull} cells progressively disappear to give rise to CD34⁺CD45^{dull} and CD34⁺CD45^{high} populations. CD11b⁺ cells are present within CD34⁺CD45^{high} populations (>70% CD11b⁺) and virtually absent from CD34⁺CD45^{dull} and CD34⁺CD45^{dull} populations (Supplementary Fig. S1A). The CD34⁺CD45^{high}CD11b⁺ subpopulation is not cycling (Ki67^{neg}) compared with CD11b⁻ populations and has myelomonocytic (nonmacrophage) morphology (Supplementary Fig. S1B and C). CD11b⁺ cells isolated from 7-day-old cultures expanded in the presence of MDA-MB-231-derived CCM-induced robust sprouting in an *in vitro* endothelial cell-sprouting assay (17), whereas CD11b⁺ cells isolated from cultures expanded in BHM alone or BHM supplemented with MCF10A-CCM did not (Fig. 1A). In a second experiment, CD11b⁺ generated from CD34⁺-initiated cultures exposed to CCM derived from normal primary HMECs did not induce sprouting, whereas those exposed to CCM derived from the tumorigenic Wnt-1-transformed HMEC line (Wnt-1-HMEC; ref. 13) did (Fig. 1B).

These results show that human breast cancer cell lines confer sprouting-promoting activity to CD11b⁺ myelomonocytic cells generated from CD34⁺ progenitor-initiated cultures.

PIGF programs proangiogenic activity in CD11b⁺ myelomonocytic cells derived from CD34⁺ progenitor-initiated cells cultures

Several tumor-derived cytokines associated with BMD cell mobilization and angiogenesis were expressed in MDA-MB-231 cells (data not shown). Among these, we tested 5 for their ability to confer proangiogenic properties to CD11b⁺ cells: VEGFA, VEGFB, IL8, IL1 β , and PIGF. Only CD11b⁺ cells derived from CD34⁺-initiated cultures supplemented with

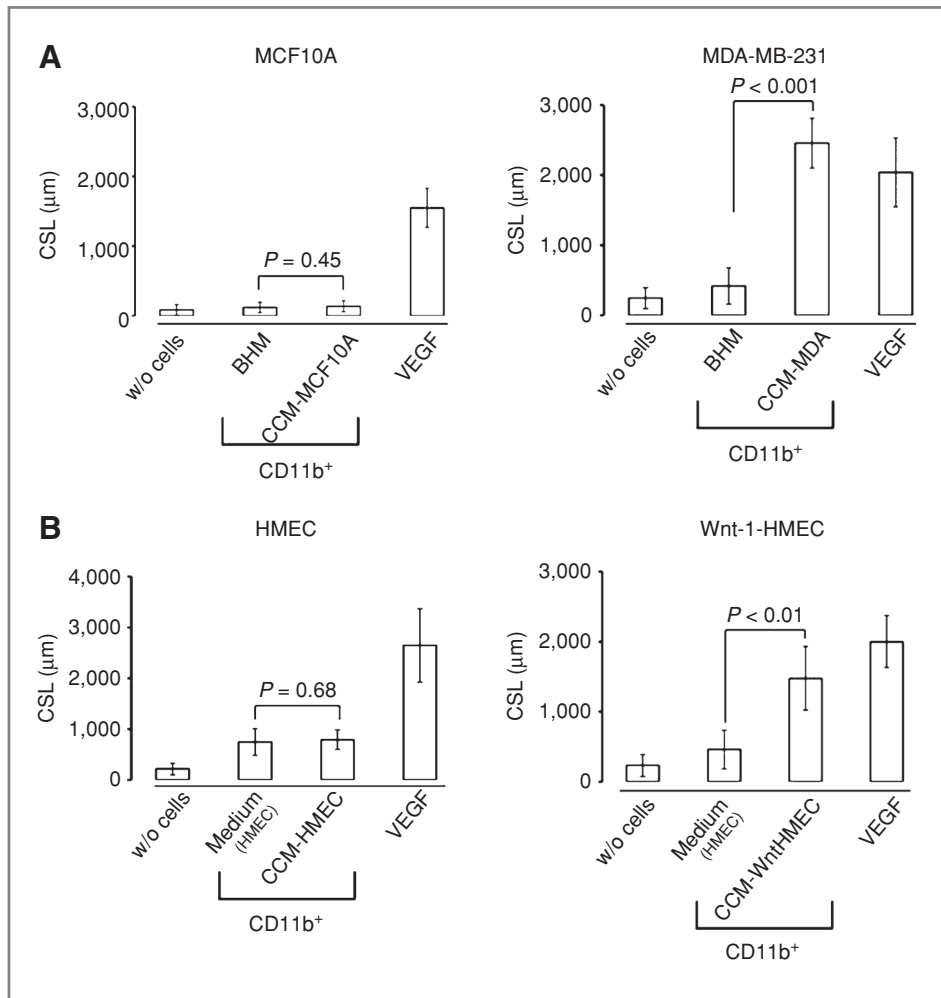


Figure 1. Conditioned medium from breast cancer cell cultures programs proangiogenic activity in CD11b⁺ myelomonocytic cells differentiating from CD34⁺ progenitors. A, endothelial sprouting activity of CD11b⁺ cells isolated from CD34⁺ hematopoietic progenitor-initiated cultures expanded in the presence of BHM or BHM supplemented with cell culture medium (CCM) conditioned by MCF10A (normal breast epithelium derived) or MDA-MB-231 (breast cancer derived) cells. B, endothelial sprouting activity of CD11b⁺ cells isolated from CD34⁺ hematopoietic progenitor-initiated cultures expanded in the presence of BHM or BHM supplemented with CCM derived from HMEC (normal breast epithelium derived) primary cells or Wnt-1-transformed HMEC cell cultures (Wnt-1-HMEC). Only CCM derived from tumor cell lines induced angiogenic programming of CD11b⁺ cells. CSL, cumulative sprout length. Bar graphs represent mean values and SD (95% CI).

PIGF induced endothelial sprouting (Fig. 2A). This effect was not due to a direct effect of PIGF possibly carried over to the sprouting assay, as PIGF, in contrast to VEGF, did not induce endothelial sprouting even at the highest concentration tested (100 ng/mL; Supplementary Fig. S2A). PIGF levels were significantly elevated in CCM of Wnt-1-HMEC and MDA-MB-231 cell lines compared with MCF10A and HMEC CCM (Supplementary Fig S2B). Addition of the function-blocking anti-Flt-1 mAb KM1732 (20) and an Flt-1 trap (sFlt-1-Fc) to CD34⁺ cell-initiated cultures exposed to MDA-MB-231-derived CCM or PIGF prevented the generation of CD11b⁺ cells with sprouting-inducing capacity (Fig. 2B and Supplementary Fig. S2C). CD11b⁺ cells derived from PIGF and MDA-MB-231 CCM-stimulated cultures induced a robust angiogenic response *in vivo* in the mouse corneal angiogenesis assay, whereas CD11b⁺ cells derived from BHM cultures did not (Fig. 2C). Addition of PIGF at later time points in CD34⁺ cell-initiated cultures resulted in a progressive decline in the endothelial sprouting activity of generated CD11b⁺ cells (Supplementary Fig. S3). In CD34⁺ cell-initiated cultures, Flt-1 was expressed on a minority of CD34⁺CD45^{dim}CD11b⁻ cells ($\approx 15\%$ – 20%) on CD34⁺CD45^{high}CD11b⁺ cells ($\approx 10\%$), whereas the majority of

CD11b⁺ cells were negative (Supplementary Fig. S4). Differentiated Flt-1⁺/CD11b⁺ cells issued from CD34⁺ initiated BHM culture and further cultured in the presence of PIGF did not acquire proangiogenic activity (Fig. 2D).

From these results, we conclude that PIGF programs proangiogenic activity in CD11b⁺ myeloid cells during the early stages of differentiation from CD34⁺ hematopoietic progenitors *in vitro*.

Presence of proangiogenic CD11b⁺ myeloid cells and elevated levels of PIGF in the circulation of breast cancer patients

These results suggest the possibility that in cancer patients PIGF may program CD11b⁺ myeloid cells to acquire proangiogenic properties while they are generated during hematopoiesis. To test whether circulating CD11b⁺ are proangiogenic, CD11b⁺ myeloid cells were isolated from PBMC collected from the peripheral blood of first diagnosed ER⁺ breast cancer patients (T1 and T2, N0 and N1, M0) and healthy donors and tested in the endothelial sprouting assay. Indeed, CD11b⁺ cells isolated from the blood of breast cancer patients, but not CD11b⁺ cells isolated from healthy indivi-

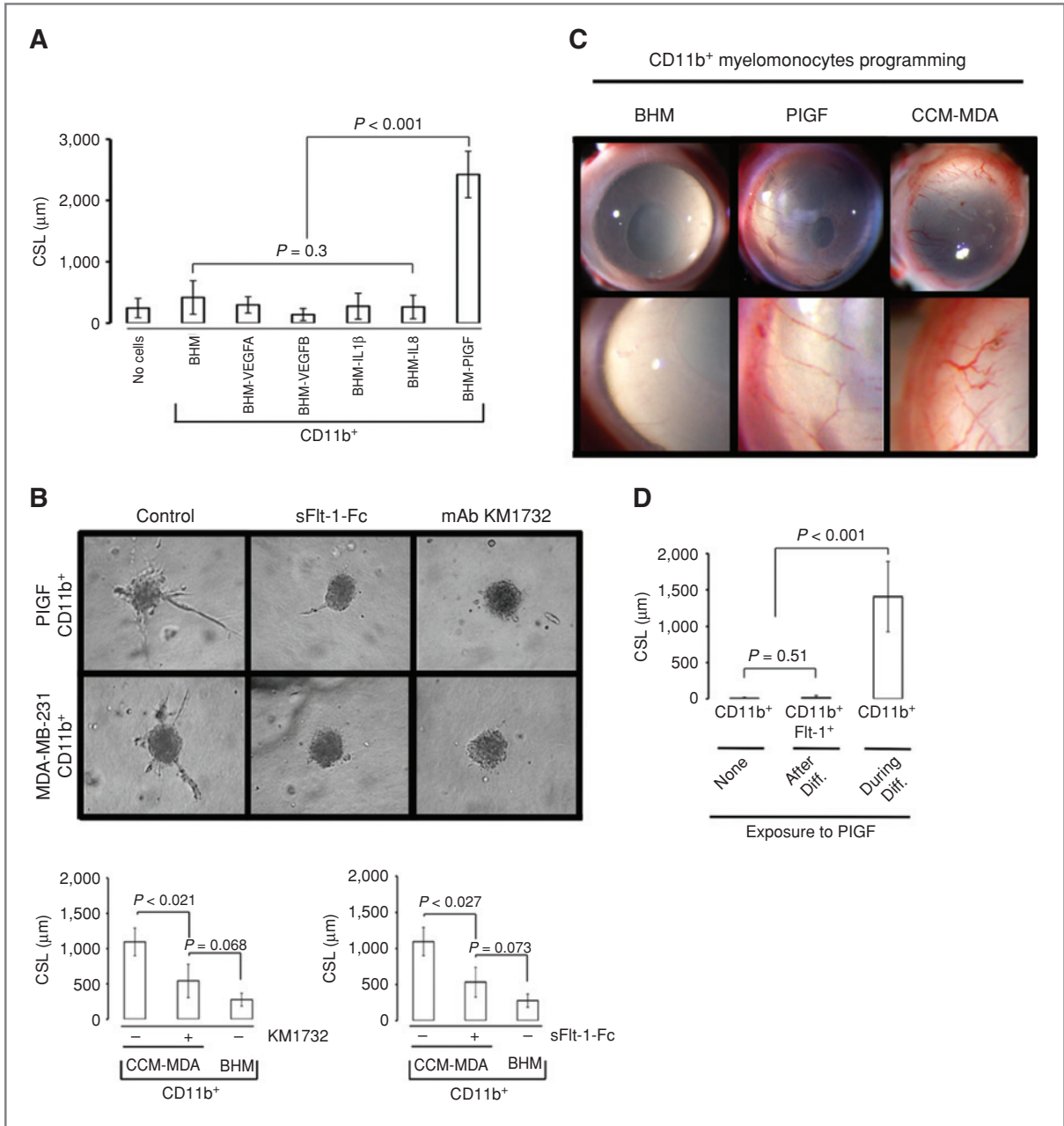


Figure 2. PIGF programs angiogenic activity in CD11b⁺ myelomonocytic cells differentiating from CD34⁺ progenitors. **A**, endothelial sprouting activity of CD11b⁺ cells isolated from CD34⁺ hematopoietic progenitor-initiated cultures in the presence of BHM alone or BHM supplemented with recombinant VEGFA, VEGFB, IL1β, IL8, or PIGF. Only PIGF induced proangiogenic programming. **B**, Flt-1 function-blocking mAb KM1732 and Flt-1 trap (sFlt-1-Fc) inhibited angiogenic programming of CD11b⁺ cells derived from CD34⁺ hematopoietic progenitor-initiated cultures stimulated with PIGF or MDA-MB-231 CCM (CCM-MDA). **C**, *in vivo* corneal angiogenesis assay. CD11b⁺ cells generated *in vitro* from CD34⁺ hematopoietic progenitors in the presence of BHM, MDA-MB-231 CCM, or recombinant PIGF were injected into the mouse cornea. Angiogenic response was evaluated 20 days later. Cells exposed to MDA-MB-231 CCM or PIGF, but not BHM, induced cornea angiogenesis. **D**, angiogenic programming of mature CD11b⁺ cells. CD11b⁺ cells generated from BHM-CD34⁺ cultures were further cultivated for 4 days in the absence or presence of PIGF and their proangiogenic activity was determined in comparison to CD11b⁺ cells generated from CD34⁺ initiated culture in the continuous presence of PIGF. PIGF did not program differentiated (diff.) CD11b⁺ cells. Bar graphs represent mean values and SD (95% CI).

duals, induced endothelial sprouting *in vitro* (Fig. 3A). In the same cohort of patients, we observed significantly increased levels of PIGF compared with healthy individuals (Fig. 3B). Exposure of mature CD11b⁺ myeloid cells isolated from

healthy donors to PIGF, however, did not result in proangiogenic programming, whereas they did after 1-week culture in presence of MDA-MB-231 CCM (Supplementary Fig. S5A). Sprouting activity was observed in whole CD11b⁺ population

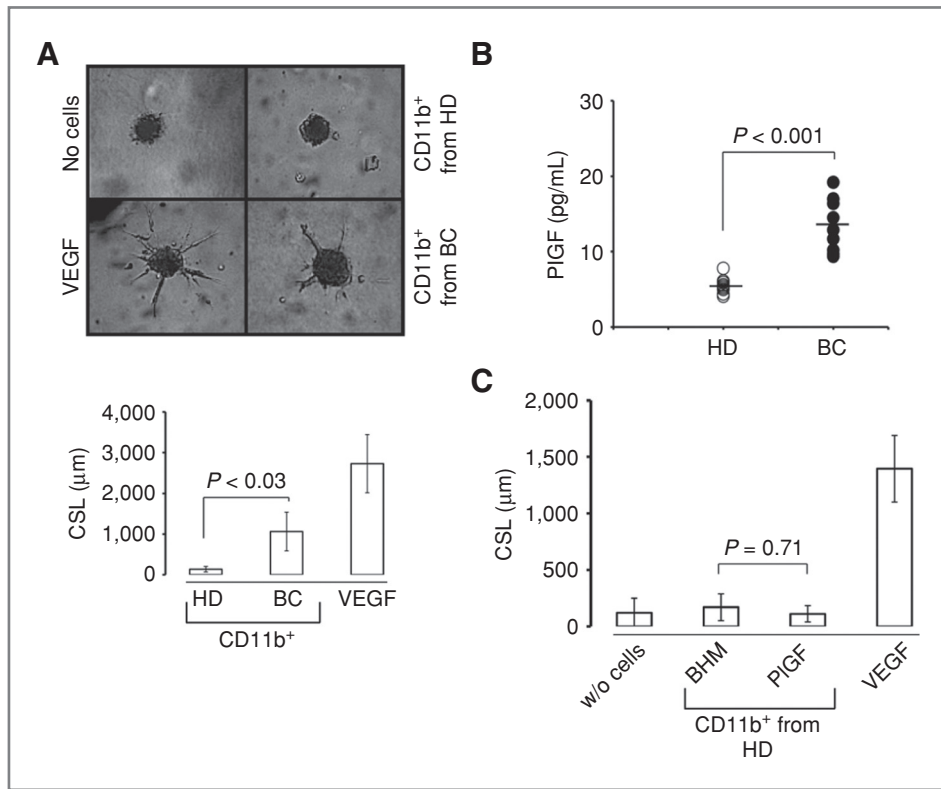


Figure 3. Peripheral blood CD11b⁺ myeloid cells in breast cancer patients are proangiogenic. A, micrographs and quantification of angiogenic sprouting activity of CD11b⁺ cells isolated from the peripheral blood of healthy donor (HD; n = 10) and nonmetastatic breast cancer (BC) patients (n = 10). BC-derived, but not HD-derived, CD11b⁺ cells promoted endothelial sprouting. B, elevated levels of plasma PIGF were observed in nonmetastatic BC patients compared with HD. C, CD11b⁺ cells isolated from peripheral blood of HD and cultured for 4 additional days in the presence of BHM or BHM-PIGF do not acquire proangiogenic activity (n = 3). VEGF, induction of sprouting by addition of recombinant VEGFA during the sprouting assay. Bar graphs represent mean values and SD (95% CI).

generated from CD34 progenitors, but not in Flt-1⁺/CD11b⁺ cells (Supplementary Fig. S5B), and differentiated CD11b⁺ cells were insensitive to PIGF-induced proangiogenic programming (Fig. 3C).

These results show that breast cancer patients have elevated levels of PIGF and proangiogenic CD11b⁺ cells circulating in their blood compared with healthy individuals.

Tumor-derived PIGF promotes proangiogenic programming of CD11b⁺ myelomonocytes and tumor growth

To test whether PIGF induced proangiogenic programming of CD11b⁺ myelomonocytes *in vivo*, we silenced PIGF expression in the murine mammary carcinoma-derived cell line 4T1 by PIGF short hairpin RNA (shRNA) expression (Supplementary Fig. S6A). As control 4T1 cells were transduced with a nonsilencing shRNA (21), which did not affect PIGF expression (Supplementary Fig. S6B). Silencing of PIGF significantly lowered plasma PIGF levels in 4T1 tumor-bearing mice (Fig. 4A) and slowed tumor growth compared with wild-type 4T1 cells or 4T1 cells transduced with the nonsilencing shRNA as measured by tumor volume (Fig. 4B) or weight (Supplementary Fig. S7). The frequency of circulating CD11b⁺ myelomonocytes in 4T1 tumor-bearing mice was not affected by PIGF silencing (Fig. 2C). However, circulating CD11b⁺ cells isolated from mice-bearing PIGF-silenced tumors did not induce sprouting in an *in vitro* MLEC sprouting assay, compared with CD11b⁺ cells isolated from control mice bearing wild-type or nonsilenced tumors (Fig. 4D). Importantly, cir-

culating CD11b⁺ cells isolated from the blood of tumor-free mice did not induce endothelial sprouting (Supplementary Fig. S6C). PIGF silencing profoundly modified the morphology of the tumor vasculature. While in wild-type and nonsilenced 4T1 tumors, the tumor vessels were highly heterogeneous, ranging from larger vessels with well-formed lumen to microvessels without detectable lumen, in PIGF-silenced tumors vessels were uniformly small without detectable lumen (Fig. 5A). Power Doppler analysis revealed a significant reduction of blood perfusion in PIGF-silenced 4T1 tumors compared with wild-type or nonsilenced tumors (Fig. 5B). These results are consistent with a reduced maturation and functionality of tumor vessels in mice-bearing PIGF-silenced tumors.

PIGF silencing did not affect the fraction of F4/80⁺CD11b⁺ or F4/80⁺CD11b⁺ tumor-recruited cells (Fig. 5C and Supplementary Fig. S8; ref. 21). Real-time reverse transcriptase PCR (RT-PCR) analysis of selected transcripts encoding for proangiogenic [i.e., *VEGF*, *HBEGF* (heparin-binding epidermal growth factor), and *MMP9* (matrix metalloproteinase)] and inflammation-modulatory factors (i.e., *CCL17*, *IL10*, and *TNF*) in whole tumor explants, revealed a downregulation of *VEGF*, *HBVEGF*, *MMP9*, *CCL17*, and *IL10* and an upregulation of *TNF* expression in PIGF-silenced tumors, compared with control tumors, consistent with decreased angiogenic activity (Fig. 5D).

These data show that in the 4T1 tumor model, silencing of tumor-derived PIGF reduces proangiogenic activity of circulating CD11b⁺ myelomonocytic cells, decreases tumor

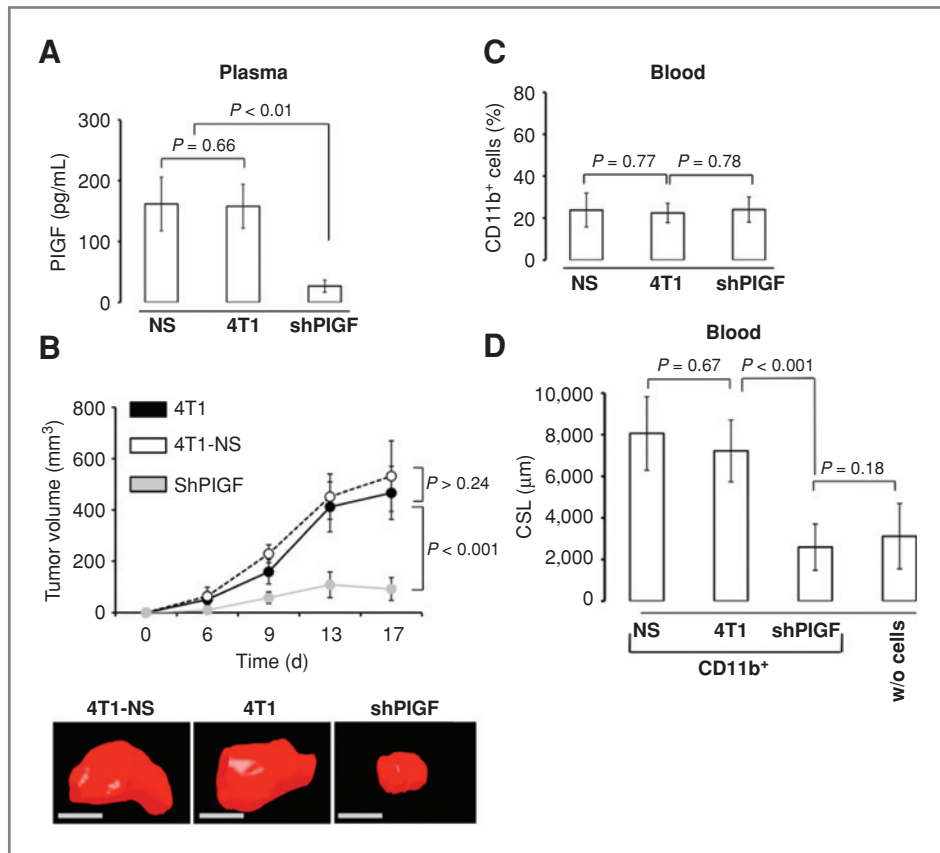


Figure 4. PIGF silencing abrogates the proangiogenic properties of circulating CD11b⁺ myelomonocytes in 4T1 tumor-bearing mice. **A**, plasma levels of PIGF in BALB/c mice bearing orthotopic tumors derived from parental (4T1), nonsilenced (NS), and PIGF-silenced 4T1 cells (shPIGF). Mice bearing PIGF-silenced 4T1 tumors have significantly reduced plasma PIGF levels. **B**, PIGF silencing inhibits 4T1 tumor growth. The volume of tumors derived from 4T1, NS, and shPIGF was measured clinically at the indicated days. Representative volumes of 3 tumors measured by ultrasound are illustrated in a 3D reconstruction. **C**, frequency of CD11b⁺ cells measured by flow cytometry in the peripheral blood of tumor-bearing BALB/c mice at 17 days after tumor cell injection. **D**, tumor-derived PIGF programs proangiogenic activity in CD11b⁺ cells *in vivo*. CD11b⁺ cells isolated from peripheral blood of tumor-bearing BALB/c mice 17 days after tumor cell injection were tested in the sprouting assay. CD11b⁺ cells derived from mice bearing PIGF-silenced 4T1 tumors did not induce endothelial sprouting. 4T1, wild type.

growth, decreases the maturation of the tumor vasculature and perfusion, and impinges on the production of proangiogenic cytokines in the tumor microenvironment.

Metalloproteinase activity is required for sprouting induced by PIGF-programmed CD11b⁺ myelomonocytic cells

Next, we searched for factor(s) mediating the endothelial sprouting activity of PIGF-programmed CD11b⁺ cells. Endothelial sprouting was still induced when CD11b⁺ cells and endothelial spheroids were physically separated using a Transwell system, thus implying the involvement of soluble factors (Supplementary Fig. S9A). We analyzed the expression of selected candidate genes by real-time RT-PCR in CD34⁺ progenitor-initiated cultures. *ITGAM* mRNA, the transcript encoding for the CD11b protein, was induced in both BHM- and PIGF-supplemented cultures. *MMP9* mRNA was induced 30- to 40-fold by PIGF, whereas other potentially relevant transcripts (i.e., *VEGFA*, *TGFB-1*, *SEMA4D*, *IF44L*, *ANG1*, or *PROK2*) were either unaffected or only marginally affected by

PIGF (Fig. 6A). Addition of MMI270, a synthetic hydroxamate-type MMP inhibitors, previously shown to inhibit MMP9 and to suppress tumor angiogenesis (22), during the sprouting assay fully blocked endothelial sprouting induced by PIGF-programmed CD11b⁺ cells (Fig. 6B). Sprouting induced by VEGF was unaffected (Supplementary Fig. S9B). The anti-VEGF blocking antibody bevacizumab caused only a minor, statistically nonsignificant reduction of endothelial cell sprouting induced by PIGF-programmed CD11b⁺ cells (Supplementary Fig. S9C). Bevacizumab inhibited VEGF-induced endothelial sprouting, as expected (Supplementary Fig. 9C).

From these results, we conclude that PIGF induces MMP9 expression during programming of CD11b⁺ cells and that metalloproteinase activity is required for sprouting activity of PIGF-programmed CD11b⁺ cells.

Discussion

Different myeloid cell subsets with proangiogenic and tumor-promoting properties have been recently reported

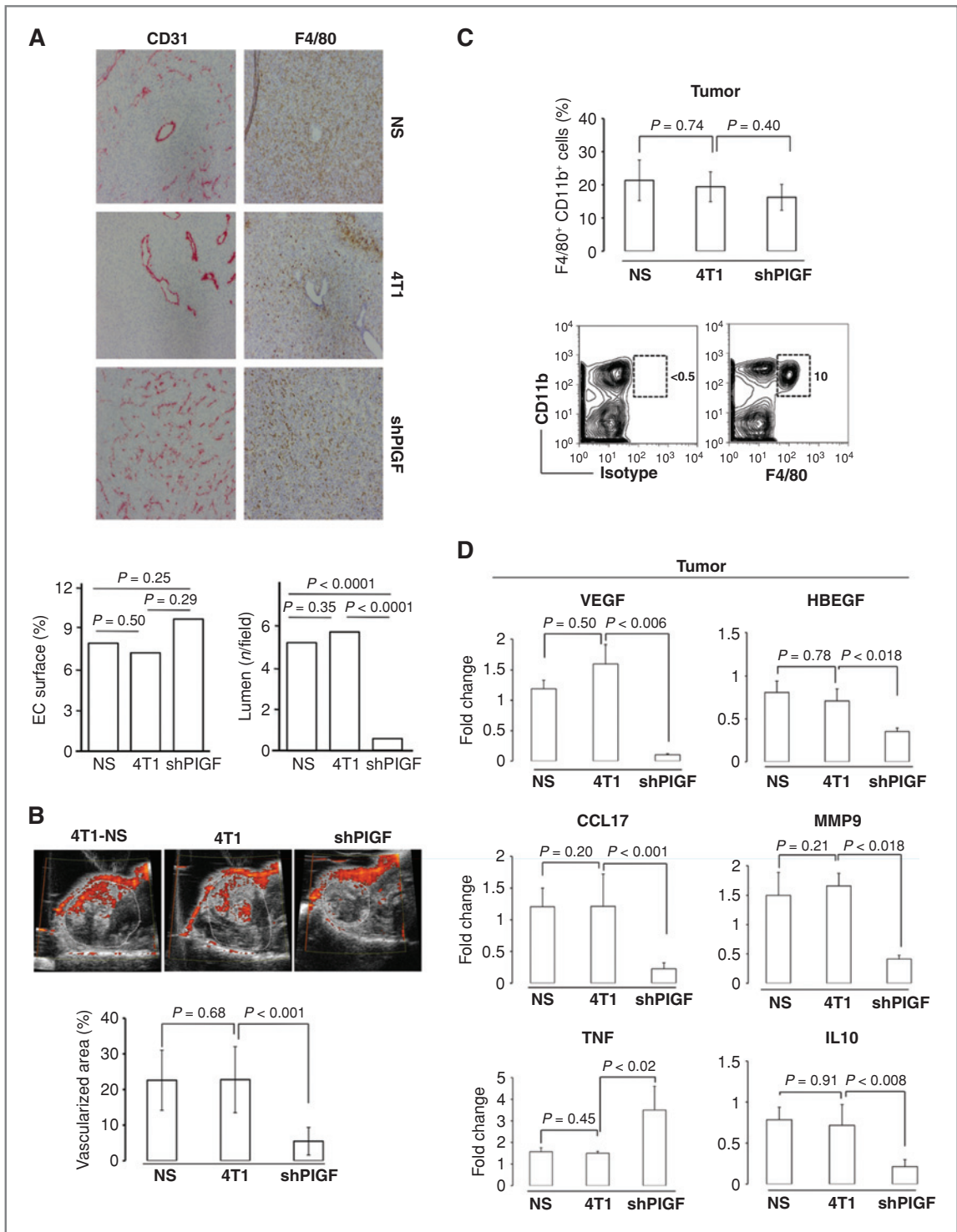
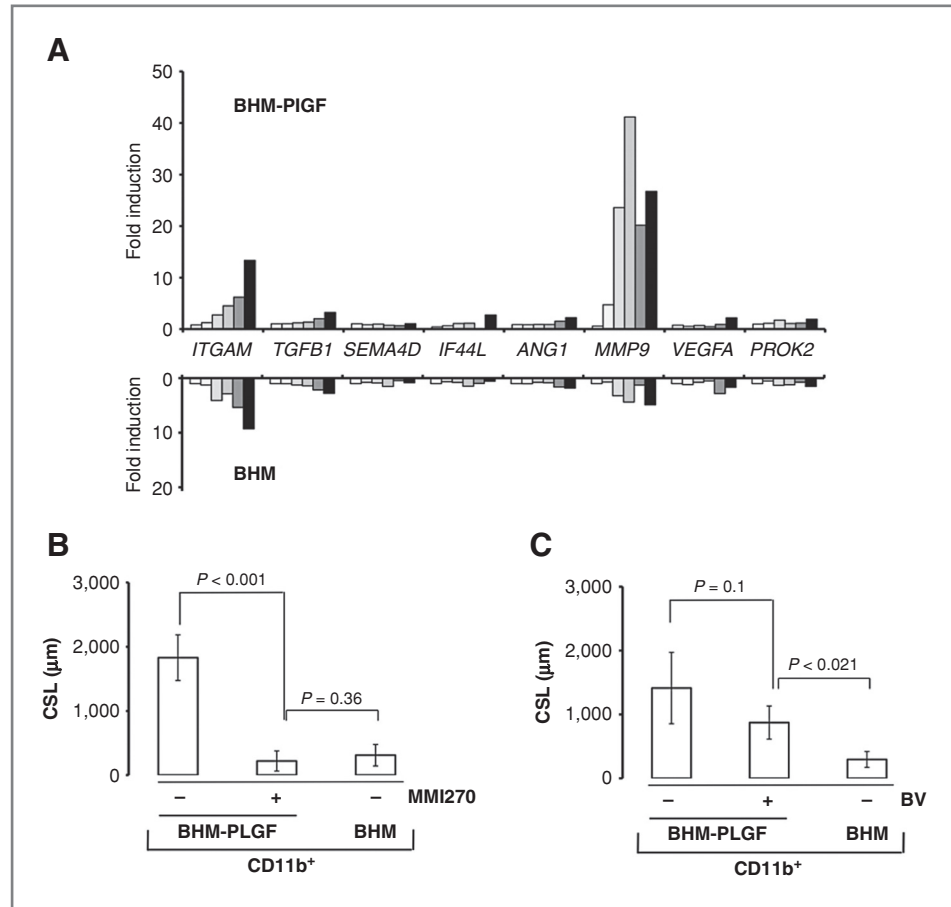


Figure 5. PIGF silencing inhibits 4T1 tumor growth and perfusion and decreases the production of proangiogenic cytokines in the tumor microenvironment. **A**, CD31 and F4/80 immunohistochemistry staining of tumor biopsies from tumor-bearing BALB/c mice 21 days after tumor cell injection. PIGF-silenced tumors lack vessels with lumen. Endothelial cell (EC) surface and vessels with lumen were quantified (bottom). **B**, Power Doppler–based perfusion analysis of orthotopically implanted tumors in BALB/c mice. The images show an ultrasonographic tumor cross-section (black and white) overlaid with the perfusion signal (color). PIGF silencing in 4T1 tumors decreased tumor perfusion. **C**, frequency of F4/80⁺CD11b⁺ cells within CD45⁺ cells recovered from tumors and analyzed by flow cytometry 17 days after tumor injection (top). Dot plot representation of CD11b⁺ versus F4/80⁺ cells in a wild-type tumor (bottom). Infiltrating CD11b⁺ cells are F4/80⁺. **D**, PIGF silencing modulates expression of the angiogenic factors VEGFA, HBEGF, and MMP9 and proinflammatory cytokines CCL17, IL10, and TNF in whole tumors. mRNA was measured by real-time RT-PCR. Bar graphs represent mean values and SD (95% CI).

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Figure 6. Metalloproteinase activity is required for sprouting activity in PIGF-programmed CD11b⁺ myelomonocytic cells. **A**, expression of *ITGAM*, *TGFB-1*, *SEMA4D*, *IF44L*, *ANG1*, *MMP9*, *VEGFA*, and *PROK2* mRNA in CD34⁺ hematopoietic progenitor-initiated cultures was monitored by real-time RT-PCR. PIGF induced MMP9 expression compared with BHM cultures. The time course is coded by gray shading of the bars (days 2 to 7 from left to right). **B**, the MMP inhibitor MMI-270 inhibited endothelial cell sprouting activity induced by PIGF-programmed CD11b⁺ cells. **C**, the anti-VEGFA antibody bevacizumab (BV) added during the sprouting assay did not inhibit endothelial sprouting induced by PIGF-educated CD11b⁺ cells. Bar graphs represent mean values and SD (95% CI).



including VEGFR1⁺CD11b⁺ cells (8), VEGFR1⁺CXCR4⁺CD11b⁺ cells (6, 7), Gr1⁺CD11b⁺ cells (9), and Tie-2⁺CD11b⁺ monocytes (23). Although it is well accepted that CD11b⁺ cells and subsets thereof promote tumor angiogenesis once recruited at tumor sites (12), the question of whether proangiogenic education may already occur during CD11b⁺ myeloid cell generation from progenitors has not been formally addressed. Here, we provide experimental and clinical evidence showing that breast cancer can program CD11b⁺ myeloid cells to acquire proangiogenic activity while differentiating from CD34⁺ hematopoietic progenitors through a PIGF-dependent mechanism, and that proangiogenic CD11b⁺ cells circulate in the blood of tumor-bearing mice and cancer patients. Although blood-circulating TEM (Tie-2-expressing monocytes) in tumor-bearing mice were reported to stimulate tumor angiogenesis when coinjected together with tumor cells in mice (24), we show for the first time that circulating CD11b⁺ monocytes in tumor-bearing mice and breast cancer patients induce endothelial cell sprouting. PIGF programs only myeloid cells while they differentiate from progenitor cells. It is unable to program differentiated CD11b⁺ cells even if expressing Flt-1, whereas tumor cell-derived conditioned medium does. These results suggest that further angiogenic programming of

CD11b⁺ cells might occur on recruitment to the tumor microenvironment.

Two important corollaries stem from these observations. First, endothelial cell sprouting-inducing activity of circulating CD11b⁺ cells might be a potential biomarker of angiogenesis (25). Second, circulating CD11b⁺ monocytes possessing proangiogenic capacity and homing to normal tissues (e.g., lung and bone) might promote metastatic spreading by facilitating initial angiogenesis and thereby contributing to tumor cell extravasation and formation of the premetastatic niche (26, 27). A translational study aimed at testing these hypotheses is currently in progress in our breast cancer clinic.

Cumulating evidence point to a role of PIGF in promoting human cancer progression including breast cancer. PIGF expression is increased in breast cancer tissues compared with normal breast and elevated PIGF levels are associated with elevated risk for recurrence, metastasis, and reduced survival (28, 29). Elevated levels of PIGF also correlate with progression and reduced survival in mesothelioma (30), oral (31), non-small cell lung (32), colon (33), renal (34), brain (35), hepatocellular (36), and gastric (37) cancers. Genetic ablation of PIGF or antibody-based PIGF inhibition suppressed tumor angiogenesis and tumor growth and

enhanced efficacy of anti-VEGF therapy (38–40). The angiogenic activity of PIGF appears indirect and mediated by BMD myeloid cells recruited to sites of angiogenesis (39, 41). Recently, the role of PIGF in promoting tumor angiogenesis has been challenged by a report (42) in which new anti-PIGF antibodies failed to suppress tumor angiogenesis. By showing that PIGF programs proangiogenic activity in differentiating CD11b⁺ cells, we provide further evidence for its role in cancer (38, 41).

Furthermore, we identified MMP9 as being strongly upregulated by PIGF and show that MMP inhibition suppresses the proangiogenic activity of programmed CD11b⁺ cells. MMP9 is thought to promote angiogenesis through the release of matrix-bound VEGF at tumor sites (43) and by mobilizing BMD cells (44). MMP9 is also induced by primary tumors in an Flt-1-dependent manner in endothelial cells and macrophages at premetastatic sites in the lung (45). Whether MMP9 expression in the lungs was due to a PIGF-mediated angiogenic programming and recruitment of monocytes was not addressed in that study. Thus, our results provide experimental evidence for a functional link between PIGF and MMP9 expression during proangiogenic programming of CD11b⁺ cells and requirement of MMP activity in promoting endothelial sprouting.

Taken together, our work unravels a previously unrecognized activity of PIGF, namely the proangiogenic programming of differentiating CD11b⁺ myelomonocytic cells, with relevant implications to breast cancer biology, detection, and

treatment and reinforces published observations on the contribution of the PIGF/Flt-1 axis in cancer progression.

Disclosure of Potential Conflicts of Interest

C. Ruegg: commercial research support, Novartis; cofunder and stock owner, Diagnoplex. The other authors disclosed no potential conflicts of interest.

Acknowledgments

The authors thank C. Brisken, Fred R. Miller, K. Hovalva-Dilke, and K. Ballmer for providing cells and reagents; T. Petrova and O. Hartle for helpful discussion; S. Monnier-Benoit for assistance with real-time PCR; M. Vicari, L. Ponsonnet, and J.-C. Stehle for technical assistance; and A. Sarre for Power Doppler analyses.

Grant Support

This work was funded by the Molecular Oncology Program of the National Centre for Competence in Research (NCCR; C. Ruegg and G. Christofori), a research instrument of the Swiss National Science Foundation; the Swiss National Foundation (M.-A. Doucey; 310030-120473), Oncosuisse (M.-A. Doucey; 02069-04-2007), a Collaborative Research Grant (CCRP) from Oncosuisse (C. Ruegg and G. Christofori; 01812-12-2005); the Geber Ruff Stiftung (C. Ruegg); the MEDIC foundation (C. Ruegg); and the TUMIC program of the 7th Frame Program of the European Union (C. Ruegg and G. Christofori).

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Received October 9, 2010; revised March 15, 2011; accepted April 5, 2011; published OnlineFirst April 20, 2011.

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