Published in "Cancer Research doi:0.1158/0008-5472.CAN-10-3684 , 2011"
which should be cited to refer to this work.

Proangiogenic factor PlGF programs CD11b^{+} myelomonocytes in breast cancer during differentiation of their hematopoietic progenitors

Julien Laurent[1**], Eveline Faes-van't Hull[1], Cedric Touvrey[1], Francois Kuonen[1,*], Qiang Lan[1,2,], Girieca Lorusso[1,2], Marie-Agnès Doucey[1], Laura Ciarloni[1,**], Natsuko Imaizumi[1,**], Gian Carlo Alghisi[1], Ernesta Fagiani[3], Khalil Zaman[4], Roger Stupp[4], Masabumi Shibuya[5], Jean François Delaloye[6], Gerhard Christofori[3,7], and Curzio Ruegg[1,2,7,§]

1. Division of Experimental Oncology (DEO), Centre Pluridisciplinaire d'Oncologie (CePO), Centre Hospitalier Universitaire Vaudois (CHUV) and University of Lausanne (UNIL), Faculty of Biology and Medicine, Lausanne, Switzerland

2. Pathology, Department of Medicine, Faculty of Science, University of Fribourg, Fribourg, Switzerland

3. Institute of Biochemistry and Genetics, Department of Biomedicine, University of Basel, Switzerland

4. The Breast Center, Centre Pluridisciplinaire d'Oncologie (CePO), Centre Hospitalier Universitaire Vaudois (CHUV) and University of Lausanne (UNIL), Faculty of Biology and Medicine, Lausanne, Switzerland

5. Tokyo Medical and Dental University, Department of Molecular Oncology, Tokyo, Japan

6. The Breast Center, Department of Obstetrics and Gynecology, Centre Hospitalier Universitaire Vaudois (CHUV) and University of Lausanne (UNIL), Faculty of Biology and Medicine, Lausanne, Switzerland

7. National Center for Competence in Research (NCCR) Molecular Oncology, Swiss Institute of Experimental Cancer Research, Ecole Polytechnique Féderale de Lausanne (ISREC–EPFL-SV), Lausanne, Switzerland

* Current address: Department of Medicine, Centre Hospitalier du Centre du Valais, Hôpital de Martigny, Switzerland

** Current address: Diagnoplex SA, Chemin de Vulliette 4, Lausanne, Switzerland

§ Correspondence to: Curzio Rüegg, Chair of Pathology, Department of Medicine, Faculty of Science, University of Fribourg, 1 Rue Albert Gockel, CH-1700 Fribourg, Switzerland; Phone: +41-26-300-8766; Fax: +41-26-300-9732; E-mail: curzio.ruegg@unifr.ch and Julien Laurent, Division of Experimental Oncology, Avenue Pierre Decker, 4, CH-1011 Lausanne, Switzerland; Phone +41-21-314-0202; Fax: +41-21-314-6839; e-mail: julien.laurent@chuv.ch

Running title: Proangiogenic programming of CD11b$^+$ cells by PlGF

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 (PlGF) is critical in promoting this education. Cultures of human CD34$^+$ progenitors supplemented with conditioned medium from breast cancer cell lines or PlGF, but not from non-tumorigenic 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 progenitors 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 PlGF 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 PlGF 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 PlGF-dependent manner. These findings impact breast cancer biology, detection and treatment.

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). While newly formed tumor vessels originate mostly by sprouting angiogenesis from locally preexisting vessels, increasing 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 upon 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 PlGF was kindly provided by Prof. Kurt Ballmer, Paul Scherrer Institute, Villigen, Switzerland. Human VEGFA, VEGFB, IL8 and IL1β and mouse FGF were purchased from PeproTech EC Ltd (London, UK). Human SCF, Flt-3, IL6 and TPO were purchased from R&D Systems. 4',6-diamidino-2-phenyl-indole, dihydrochloride (DAPI) was purchased from LuBioscience GmbH. Ficoll Histopaque Plus 1.077, were from Sigma-Aldrich Chemistry.

Antibodies

Anti-human antibodies. FITC-conjugated anti-CD11b, anti-CD14, anti-Ki67; PE-conjugated anti-CD14, PerCP-conjugated anti-CD34; APC-conjugated anti-CD11b; 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; 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 Biolegend (San Diego, USA).

Cell lines and primary cells

The human breast cancer cell line MDA MB 231 and human non-transformed breast epithelium-derived cell line MCF10A were purchased from ATCC (HTB-26D and CRL-10317) and cultured in RPMI 1640 medium supplemented with 10% (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, Lausanne (13). Cell conditioned medium (CCM, 2 to 4 days old) was collected from cell lines at near-confluence conditions, filtered at 0.2 µm 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 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) (16).

Umbilical cord blood

CD34+ progenitors cells were isolated using EasySep selection kit (stem cells technologies) from umbilical cord blood obtained from newborn babies of healthy volunteer's pregnant women for whom a caesarotomy was pre-programmed, upon

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 ± 10% filtered CCM from MDA-MB-231, Wnt-1-HMEC, MCF10A, 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 PBMC were isolated using EasySep selection kit for labeled cells (Stemcell Technologies) or using FACS Aria 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. 48 (HUVEC) or 24 (MLEC) hours later 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 ± s.d..

Cytokine measurement

Measurements of human or mouse PlGF were performed in duplicate using the Quantikine ELISA systems (R&D Systems). Results represent means of duplicate determinations ± s.d..

Tumor model

4T1 cells were injected ($5x10^4$ cells in 50 µl PBS/20% Matrigel per injection, respectively) in the fourth right inguinal mammary gland of 4-6 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 = π/6(length x 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 performed 21 days after tumor cell line injection using the VEVO 770 micro Three-dimensional High-Frequency Doppler Ultrasound system (VisualSonics, Toronto, Canada) equipped with a 30MHz 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 3D motor on a rail system above the animal, moved perpendicular to the beam axis, thereby acquiring consecutive two-dimensional images in B mode and power Doppler mode with a slice thickness/interval of 500 µm. A region of interest (ROI) was drawn within every two-dimensional 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 three sections per tumor using Image-J free software (http://rsbweb.nih.gov/ij/). 5-7 tumors per each experimental condition were measured (4T1 wild type, 4T1 non-silenced and 4T1 sh-PlGF).

Corneal angiogenesis assay

Corneal angiogenesis assay was performed based on a modification of the method originally reported (19). CD11b$^+$ cells purified by immunomagnetic beads (Stemcell Technologies) were resuspended at 20 x 10^6 cells/ml in RPMI 1640 medium with 2% FCS. NOD/SCID/IL2 receptor common γ2 chain knockout mice were anesthetized with ketamine and xylazine and 5µl 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 post-injection.

Breast cancer patients and healthy donors

Peripheral blood from healthy donors and cancer patients was obtained according to the declaration of Helsinki and upon 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 (T1-2; N0-1, M0), estrogen receptor 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 (Becton Dickinson).

Human samples processing

Blood was collected in Lithium heparin anti-coagulated Vacutainer tubes (Becton Dickinson). Plasma phase was collected and centrifuged 5 min at 1500xg at room temperature (RT). Plasma was stored at –80°C. PBMC were isolated using Ficoll Histopaque Plus 1.077 (25 minutes centrifugation at 680xg at RT). The PBMC fraction was collected, washed in PBS, counted, aliquoted at 10x10^{6} cells/mL and frozen at -80°C in 90%FCS/10%DMSO medium. 24 hours later, samples are transferred into liquid nitrogen.

Statistical analysis

In all experiments of this study, values are expressed as mean ± 95% confidence intervals. Statistical analyses were performed by paired Student's t test unless indicated otherwise. Differences were considered statistically significant at $p < 0.05$.

RESULTS

Breast cancer cell conditioned medium induces the generation of proangiogenic CD11b$^+$ myelomonocytic in CD34$^+$ hematopoietic progenitors-initiated culture

To test the effect of tumor cells on the generation of proangiogenic CD11b$^+$ myeloid cells, we cultured human CD34$^+$ progenitors in a basal hematopoietic medium (BHM) (16) alone, or in the presence of culture-conditioned medium (CCM) obtained from the tumorigenic metastatic breast cancer-derived cell line MDA-MB-231 or from the normal breast epithelium-derived non-tumorigenic cell line MCF-10A. 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. 1A). The CD34$^-$CD45^{high}CD11b$^+$ subpopulation is not cycling (Ki67^{neg}) compared to CD11b$^-$ populations and has myelomonocytic (non-macrophage) morphology (Supplementary Fig. 1B and 1C). CD11b$^+$ cells isolated from 7 days-old cultures expanded in the presence of MDA-MB-231-derived CCM induced robust sprouting in an *in vitro* endothelial cell-sprouting assay (17), while CD11b$^+$ cells isolated from cultures expanded in basal hematopoietic medium (BHM) alone or BHM supplemented with MCF-10A-CCM, did not (Fig. 1A). In a second experiment, CD11b$^+$ generated from CD34$^+$-initiated cultures exposed to CCM derived from normal primary human mammary epithelial cells (HMEC) did not induce sprouting, while those exposed to CCM derived from the tumorigenic Wnt-1 transformed HMEC line (Wnt-1-HMEC) (13) did (Fig. 1B).

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

Placenta growth factor (PlGF) 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 five for their ability to confer proangiogenic properties to CD11b$^+$ cells: VEGF-A, VEGFB, IL8, IL1β, and PlGF. Only CD11b$^+$ cells derived from CD34$^+$-initiated cultures supplemented with PlGF induced endothelial sprouting (Fig. 2A). This effect was not due to a direct effect of PlGF possibly carried over to the sprouting assay, since PlGF, in contrast to VEGF, did not induce endothelial sprouting even at the highest concentration tested (100 ng/ml) (Supplementary Fig. 2A). PlGF levels were significantly elevated in CCM of Wnt1-HMEC and MDA-MB-231 cell lines compared to MCF-10A and HMEC CCM (Supplementary Fig 2B). 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 PlGF, prevented the generation of CD11b$^+$ cells with sprouting-inducing capacity (Fig. 2B and supplementary Fig. 2C). CD11b$^+$ cells derived from PlGF and MDA-MB-231-CCM stimulated cultures induced a robust angiogenic response *in vivo* in the mouse corneal angiogenesis assay, while CD11b$^+$ cells derived form BHM cultures did not (Fig. 2C). Addition of PlGF a later time points in CD34$^+$-cell-initiated cultures resulted in a progressive decline in the endothelial sprouting activity of generated CD11b$^+$

cells (Supplementary Fig. 3). In CD34$^+$ cell-initiated cultures, Flt-1 was expressed on a minority of CD34$^-$/CD45^{dull}/CD11b- cells (≈15-20%) on CD34$^-$/CD45^{high}/CD11b$^+$ cells (≈10%), while the majority of CD11b$^+$ cells were negative (Supplementary Fig. 4). Differentiated Flt-1$^+$CD11b$^+$ cells issued from CD34$^+$ initiated BHM culture and further cultured in the presence of PlGF did not acquire proangiogenic activity (Fig. 2D).

From these results we conclude that PlGF 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 PlGF in the circulation of breast cancer patients

These results suggest the possibility that in cancer patients PlGF 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 (T1-2, N0-1, 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 individuals, induced endothelial sprouting *in vitro* (Fig. 3A). In the same cohort of patients we observed significantly increased levels of PlGF compared to healthy individuals (Fig. 3B). Exposure of mature CD11b$^+$ myeloid cells isolated from healthy donors to PlGF, however, did not result in proangiogenic programming while they did after one-week culture in presence of MDA-MB-231 CCM (Supplementary Fig. 5A).

Proangiogenic programming of $CD11b^+$ cells by PlGF

Sprouting activity was observed in whole $CD11b^+$ population generated from CD34 progenitors, but not in $Flt\text{-}1^+CD11b^+$ cells (supplementary Fig. 5B), and differentiated $CD11b^+$ cells were insensitive to PlGF-induced proangiogenic programming (Fig. 3C).

These results demonstrate that breast cancer patients have elevated levels of PlGF and proangiogenic $CD11b^+$ cells circulating in their blood, compared to healthy individuals.

Tumor-derived PlGF promotes proangiogenic programming of $CD11b^+$ myelomonocytes and tumor growth

To test whether PlGF induced proangiogenic programming of $CD11b^+$ myelomonocytes *in vivo,* we silenced PlGF expression in the murine mammary carcinoma-derived cell line 4T1 by PlGF shRNA expression (Supplementary Fig. 6A). As control 4T1 cells were transduced with a non-silencing shRNA (21), which did not affect PlGF expression (Supplementary Fig. 6B). Silencing of PlGF significantly lowered plasma PlGF levels in 4T1 tumor-bearing mice (Fig. 4A) and slowed tumor growth compared to wild type 4T1 cells or 4T1 cells transduced with the non-silencing shRNA as measured by tumor volume (Fig. 4B) or weight (Supplementary Fig. 7). The frequency of circulating $CD11b^+$ myelomonocytes in 4T1 tumor-bearing mice was not affected by PlGF silencing (Fig. 2C). However, circulating $CD11b^+$ cells isolated from mice bearing PlGF-silenced tumors did not induce sprouting in an *in vitro* MLEC sprouting assay, compared to $CD11b^+$ cells isolated from control mice bearing wild type or non-silenced tumors (Fig. 4D). Importantly, circulating $CD11b^+$ cells isolated from the blood of tumor-free mice didn't induce endothelial sprouting

(Supplementary Fig. 6C). PlGF silencing profoundly modified the morphology of the tumor vasculature. While in wild type and non-silenced 4T1 tumors the tumor vessels were highly heterogeneous, ranging from larger vessels with well-formed lumen to microvessels without detectable lumen, in PlGF-silenced tumors vessels were uniformly small without detectable lumen (Fig. 5A). Power Doppler analysis revealed a significant reduction of blood perfusion in PlGF-silenced 4T1 tumors compared to wild type or non-silenced tumors (Fig. 5B). These results are consistent with a reduced maturation and functionality of tumor vessels in mice bearing PlGF-silenced tumors.

PlGF silencing did not affect the fraction of F4/80$^+$CD11b$^+$ or F4/80$^-$CD11b$^+$ tumor-recruited cells (Fig. 5C, Supplementary Fig. 8). (21)Real time RT-PCR analysis of selected transcripts encoding for proangiogenic (i.e. *VEGF*, *HBEGF* and *MMP9*) and inflammation-modulatory factors (i.e.*CCL17*, *IL10*, *TNF*) in whole tumor explants, revealed a down regulation of *VEGF*, *HBVEGF*, *MMP9*, *CCL17* and *IL10* and an upregulation of *TNF* expression in PlGF-silenced tumors, compared to control tumors, consistent with decreased angiogenic activity (Fig. 5D).

These data demonstrate that in the 4T1 tumor model, silencing of tumor-derived PlGF reduces proangiogenic activity of circulating CD11b$^+$ myelomonocytic cells, decreases tumor 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 PlGF-programmed CD11b$^+$ myelomonocytic cells.

Proangiogenic programming of CD11b$^+$ cells by PlGF

Next, we searched for factor(s) mediating the endothelial sprouting activity of PlGF-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. 9A). We analyzed the expression of selected candidate genes by real-time RT-PCR in CD34$^+$ progenitors-initiated cultures. *ITGAM* mRNA, the transcript encoding for the CD11b protein, was induced in both BHM and PlGF-supplemented cultures. *MMP9* mRNA was induced 30 to 40-fold by PlGF, while other potentially relevant transcripts (*i.e. VEGFA, TGFB-1*, *Sema4D*, *IF44L*, *ANG1*, or *PROK2*) were not or only marginally affected by PlGF (Fig. 6A). Addition of MMI-270, a synthetic hydroxomate-type MMP inhibitors, previously shown to inhibit MMP9 and to suppress tumor angiogenesis (22), during the sprouting assay fully blocked endothelial sprouting induced by PlGF-programmed CD11b$^+$ cells (Fig. 6B). Sprouting induced by VEGF was unaffected (Supplementary Fig. 9B). The anti-VEGF blocking antibody bevacizumab caused only a minor, statistically non-significant reduction of endothelial cell sprouting induced by PlGF-programmed CD11b$^+$ cells (Fig. 9C). Bevacizumab inhibited VEGF-induced endothelial sprouting, as expected (Supplementary Fig. 9C).

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

DISCUSSION

Different myeloid cell subsets with proangiogenic and tumor-promoting properties have been recently reported, including VEGFR1$^+$CD11b$^+$ cells (8), VEGFR-1$^+$CXCR4$^+$CD11b$^+$ cells (6, 7), Gr1$^+$CD11b$^+$ cells (9), and Tie-2$^+$CD11b$^+$ monocytes (23). While 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 demonstrating that breast cancer can program CD11b$^+$ myeloid cells to acquire proangiogenic activity while differentiating from CD34$^+$ hematopoietic progenitors though a PlGF-dependent mechanism, and that proangiogenic CD11b$^+$ cells circulate in the blood of tumor-bearing mice and cancer patients. While blood-circulating TEM in tumor-bearing mice were reported to stimulate tumor angiogenesis when co-injected together with tumor cells in mice (24), we demonstrate for the first time that circulating CD11b$^+$ monocytes in tumor-bearing mice and breast cancer patients induce endothelial cell sprouting. PlGF only programs myeloid cells while they differentiate from progenitor cells. It is unable to program differentiated CD11b$^+$ cells even if expressing Flt-1, while tumor cell-derived conditioned medium does. These results suggest that further angiogenic programming of CD11b$^+$ cells might occur upon recruitment to the tumor microenvironment.

Two important corollaries stem from these observations. First, endothelial cell sprouting-inducing activity of circulating CD11b$^+$ cells might be 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 pre-metastatic 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 PlGF in promoting human cancer progression, including breast cancer. PlGF expression is increased in breast cancer tissues compared to normal breast and elevated PlGF levels are associated with elevated risk for recurrence, metastasis and reduced survival (28, 29). Elevated levels of PlGF 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 PlGF or antibody-based PlGF inhibition suppressed tumor angiogenesis and tumor growth and enhanced efficacy of anti-VEGF therapy (38-40). The angiogenic activity of PlGF appears indirect and mediated by bone marrow derived myeloid cells recruited to sites of angiogenesis (39, 41). Recently the role of PlGF in promoting tumor-angiogenesis has been challenged by a report (42) in which new anti-PlGF antibodies failed to suppress tumor angiogeneis. By demonstrating that PlGF 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 PlGF and show that MMP inhibition suppresses the proangiogenic activity of programmed CD11b$^+$ cells. MMP9 it 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 a PlGF-mediated angiogenic programming and recruitment of monocytes was not addressed in that study. Thus, our results provide experimental evidence for a functional link between PlGF, 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 PlGF, 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 PlGF/Flt-1 axis in cancer progression.

SUPPLEMENTAL DATA

Supplementary data are available in the online version of the paper at http://cancerres.aacrjournals.org/

ACKNOWLEDGMENTS

The authors thank C. Brisken, Fred R. Miller, K. Hodivala-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, J-C. Stehle for technical assistance, and A. Sarre for Power Doppler analyses.

FUNDING

This work was funded by the Molecular Oncology Program of the National Centre for Competence in Research (NCCR) (CR and GC), a research instrument of the Swiss National Science Foundation; the Swiss National Foundation (MAD, 310030-

120473), Oncosuisse (MAD, 02069-04-2007), a Collaborative Research Grant (CCRP) from Oncosuisse (CR and GC, 01812-12-2005); the Geber Ruef Stiftung (CR); the MEDIC foundation (CR); the TUMIC program of the 7th Frame Program of the European Union (CR and GC).

COMPETING INTERESTS STATEMENT

The authors declare competing financial interests.

REFERENCES

1. Carmeliet P. Angiogenesis in life, disease and medicine. Nature 2005;438:932-6.
2. Kerbel RS. Tumor angiogenesis. N Engl J Med 2008;358:2039-49.
3. De Palma M, Naldini L. Role of haematopoietic cells and endothelial progenitors in tumour angiogenesis. Biochim Biophys Acta 2006;1766:159-66.
4. Murdoch C, Muthana M, Coffelt SB, Lewis CE. The role of myeloid cells in the promotion of tumour angiogenesis. Nat Rev Cancer 2008;8:618-31.
5. Shojaei F, Singh M, Thompson JD, Ferrara N. Role of Bv8 in neutrophil-dependent angiogenesis in a transgenic model of cancer progression. Proc Natl Acad Sci U S A 2008;105:2640-5.
6. Grunewald M, Avraham I, Dor Y, Bachar-Lustig E, Itin A, Jung S, et al. VEGF-induced adult neovascularization: recruitment, retention, and role of accessory cells. Cell 2006;124:175-89.

7. Jin DK, Shido K, Kopp HG, Petit I, Shmelkov SV, Young LM, et al. Cytokine-mediated deployment of SDF-1 induces revascularization through recruitment of CXCR4+ hemangiocytes. Nat Med 2006;12:557-67.

8. Lyden D, Hattori K, Dias S, Costa C, Blaikie P, Butros L, et al. Impaired recruitment of bone-marrow-derived endothelial and hematopoietic precursor cells blocks tumor angiogenesis and growth. Nat Med 2001;7:1194-201.

9. Shojaei F, Wu X, Zhong C, Yu L, Liang XH, Yao J, et al. Bv8 regulates myeloid-cell-dependent tumour angiogenesis. Nature 2007;450:825-31.

10. Venneri MA, De Palma M, Ponzoni M, Pucci F, Scielzo C, Zonari E, et al. Identification of proangiogenic TIE2-expressing monocytes (TEMs) in human peripheral blood and cancer. Blood 2007;109:5276-85.

11. Zacchigna S, Pattarini L, Zentilin L, Moimas S, Carrer A, Sinigaglia M, et al. Bone marrow cells recruited through the neuropilin-1 receptor promote arterial formation at the sites of adult neoangiogenesis in mice. J Clin Invest 2008;118:2062-75.

12. Seandel M, Butler J, Lyden D, Rafii S. A catalytic role for proangiogenic marrow-derived cells in tumor neovascularization. Cancer Cell 2008;13:181-3.

13. Ayyanan A, Civenni G, Ciarloni L, Morel C, Mueller N, Lefort K, et al. Increased Wnt signaling triggers oncogenic conversion of human breast epithelial cells by a Notch-dependent mechanism. Proc Natl Acad Sci U S A 2006;103:3799-804.

14. Heppner GH, Miller FR, Shekhar PM. Nontransgenic models of breast cancer. Breast Cancer Res 2000;2:331-4.

15. Dormond O, Foletti A, Paroz C, Ruegg C. NSAIDs inhibit alpha V beta 3 integrin-mediated and Cdc42/Rac-dependent endothelial-cell spreading, migration and angiogenesis. Nat Med 2001;7:1041-7.

16. Peters R, Leyvraz S, Faes-Van't Hull E, Jaunin P, Gerber S, Rollini P. Long-term ex vivo expansion of human fetal liver primitive haematopoietic progenitor cells in stroma-free cultures. Br J Haematol 2002;119:792-802.

17. Korff T, Augustin HG. Tensional forces in fibrillar extracellular matrices control directional capillary sprouting. J Cell Sci 1999;112 (Pt 19):3249-58.

18. Kuonen F, Touvrey C, Laurent J, Ruegg C. Fc block treatment, dead cells exclusion, and cell aggregates discrimination concur to prevent phenotypical artifacts in the analysis of subpopulations of tumor-infiltrating CD11b(+) myelomonocytic cells. Cytometry A 2010.

19. Gimbrone MA, Jr., Cotran RS, Leapman SB, Folkman J. Tumor growth and neovascularization: an experimental model using the rabbit cornea. J Natl Cancer Inst 1974;52:413-27.

20. Sawano A, Iwai S, Sakurai Y, Ito M, Shitara K, Nakahata T, et al. Flt-1, vascular endothelial growth factor receptor 1, is a novel cell surface marker for the lineage of monocyte-macrophages in humans. Blood 2001;97:785-91.

21. Cullen BR. Enhancing and confirming the specificity of RNAi experiments. Nat Methods 2006;3:677-81.

22. Nakamura ES, Koizumi K, Yamaura T, Saiki I. Anti-tumor angiogenic effect of a matrix metalloproteinase inhibitor MMI270. Anticancer Res 2003;23:411-7.

23. De Palma M, Venneri MA, Galli R, Sergi Sergi L, Politi LS, Sampaolesi M, et al. Tie2 identifies a hematopoietic lineage of proangiogenic monocytes

required for tumor vessel formation and a mesenchymal population of pericyte progenitors. Cancer Cell 2005;8:211-26.

24. De Palma M, Mazzieri R, Politi LS, Pucci F, Zonari E, Sitia G, et al. Tumor-targeted interferon-alpha delivery by Tie2-expressing monocytes inhibits tumor growth and metastasis. Cancer Cell 2008;14:299-311.

25. Sessa C, Guibal A, Del Conte G, Ruegg C. Biomarkers of angiogenesis for the development of antiangiogenic therapies in oncology: tools or decorations? Nat Clin Pract Oncol 2008;5:378-91.

26. Kaplan RN, Rafii S, Lyden D. Preparing the "soil": the premetastatic niche. Cancer Res 2006;66:11089-93.

27. Gupta GP, Nguyen DX, Chiang AC, Bos PD, Kim JY, Nadal C, et al. Mediators of vascular remodelling co-opted for sequential steps in lung metastasis. Nature 2007;446:765-70.

28. Escudero-Esparza A, Martin TA, Douglas-Jones A, Mansel RE, Jiang WG. PGF isoforms, PLGF-1 and PGF-2 and the PGF receptor, neuropilin, in human breast cancer: prognostic significance. Oncol Rep 2010;23:537-44.

29. Parr C, Watkins G, Boulton M, Cai J, Jiang WG. Placenta growth factor is over-expressed and has prognostic value in human breast cancer. Eur J Cancer 2005;41:2819-27.

30. Pompeo E, Albonici L, Doldo E, Orlandi A, Manzari V, Modesti A, et al. Placenta growth factor expression has prognostic value in malignant pleural mesothelioma. Ann Thorac Surg 2009;88:426-31.

31. Cheng SJ, Lee JJ, Kok SH, Chou CH, Chang HH, Ling Chiang M, et al. Expression of placenta growth factor: An independent factor for prediction of progression and prognosis of oral cancer. Head Neck 2010.

32. Zhang L, Chen J, Ke Y, Mansel RE, Jiang WG. Expression of Placenta growth factor (PlGF) in non-small cell lung cancer (NSCLC) and the clinical and prognostic significance. World J Surg Oncol 2005;3:68.

33. Escudero-Esparza A, Martin TA, Davies ML, Jiang WG. PGF isoforms, PLGF-1 and PGF-2, in colorectal cancer and the prognostic significance. Cancer Genomics Proteomics 2009;6:239-46.

34. Matsumoto K, Suzuki K, Koike H, Hasumi M, Matsui H, Okugi H, et al. Placental growth factor gene expression in human prostate cancer and benign prostate hyperplasia. Anticancer Res 2003;23:3767-73.

35. Nomura M, Yamagishi S, Harada S, Yamashima T, Yamashita J, Yamamoto H. Placenta growth factor (PlGF) mRNA expression in brain tumors. J Neurooncol 1998;40:123-30.

36. Ho MC, Chen CN, Lee H, Hsieh FJ, Shun CT, Chang CL, et al. Placenta growth factor not vascular endothelial growth factor A or C can predict the early recurrence after radical resection of hepatocellular carcinoma. Cancer Lett 2007;250:237-49.

37. Chen CN, Hsieh FJ, Cheng YM, Cheng WF, Su YN, Chang KJ, et al. The significance of placenta growth factor in angiogenesis and clinical outcome of human gastric cancer. Cancer Lett 2004;213:73-82.

38. Carmeliet P, Moons L, Luttun A, Vincenti V, Compernolle V, De Mol M, et al. Synergism between vascular endothelial growth factor and placental growth factor contributes to angiogenesis and plasma extravasation in pathological conditions. Nat Med 2001;7:575-83.

39. Fischer C, Jonckx B, Mazzone M, Zacchigna S, Loges S, Pattarini L, et al. Anti-PlGF inhibits growth of VEGF(R)-inhibitor-resistant tumors without affecting healthy vessels. Cell 2007;131:463-75.

40. Van de Veire S, Stalmans I, Heindryckx F, Oura H, Tijeras-Raballand A, Schmidt T, et al. Further pharmacological and genetic evidence for the efficacy of PlGF inhibition in cancer and eye disease. Cell 2010;141:178-90.

41. Pipp F, Heil M, Issbrucker K, Ziegelhoeffer T, Martin S, van den Heuvel J, et al. VEGFR-1-selective VEGF homologue PlGF is arteriogenic: evidence for a monocyte-mediated mechanism. Circ Res 2003;92:378-85.

42. Bais C, Wu X, Yao J, Yang S, Crawford Y, McCutcheon K, et al. PlGF blockade does not inhibit angiogenesis during primary tumor growth. Cell 2010;141:166-77.

43. Bergers G, Brekken R, McMahon G, Vu TH, Itoh T, Tamaki K, et al. Matrix metalloproteinase-9 triggers the angiogenic switch during carcinogenesis. Nat Cell Biol 2000;2:737-44.

44. Heissig B, Hattori K, Dias S, Friedrich M, Ferris B, Hackett NR, et al. Recruitment of stem and progenitor cells from the bone marrow niche requires MMP-9 mediated release of kit-ligand. Cell 2002;109:625-37.

45. Hiratsuka S, Nakamura K, Iwai S, Murakami M, Itoh T, Kijima H, et al. MMP9 induction by vascular endothelial growth factor receptor-1 is involved in lung-specific metastasis. Cancer Cell 2002;2:289-300.

FIGURE LEGENDS

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 basal hematopoietic medium (BHM) or BHM supplemented with cell culture medium (CCM) conditioned by MCF-10A (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 standard deviations (95% confidence intervals).

Figure 2. PlGF 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 basal hematopoietic medium (BHM) alone or BHM supplemented with recombinant VEGFA, VEGFB, IL1β, IL8 or PlGF. Only PlGF 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 progenitors-initiated cultures stimulated with PlGF 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 PlGF were injected into the mouse cornea. Angiogenic response was evaluated 20 days later. Cells exposed to MDA-MB-231 CCM or PlGF, 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 PlGF and their proangiogenic activity was determined in comparison to CD11b$^+$ cells generated from CD34$^+$ initiated culture in the continuous presence of PlGF. PlGF did not program differentiated CD11b$^+$ cells. CSL: cumulative sprout lengths. Bar graphs represent mean values and standard deviations (95% confidence intervals).

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 non-metastatic breast cancer (BC) patients (n=10). BC-, but not HD-derived, CD11b+ cells promoted endothelial sprouting. *B,* Elevated levels of plasma PlGF was observed in non-metastatic breast cancer (BC) patients compared to healthy donors (HD). *C,* CD11b$^+$ cells isolated from peripheral blood of HD and cultured for 4 additional days in the presence of basal hematopoietic medium (BHM) or BHM-PlGF do not acquire proangiogenic activity (n=3). VEGF: induction of sprouting by addition of recombinant VEGFA during the sprouting assay. CSL: cumulative sprout lengths.

Proangiogenic programming of CD11b$^+$ cells by PlGF

Bar graphs represent mean values and standard deviations (95% confidence intervals).

Figure 4. PlGF silencing abrogates the proangiogenic properties of circulating CD11b$^+$ myelomonocytes in 4T1 tumor bearing mice. *A,* Plasma levels of PlGF in BALB/c mice bearing orthotopic tumors derived from parental (4T1), non-silenced (NS) and PlGF-silenced 4T1 cells (shPlGF). Mice bearing PlGF-silenced 4T1 tumors have significantly reduced plasma PlGF levels. *B,* PlGF silencing inhibits 4T1 tumor growth. The volume of tumors derived from parental (4T1), non-silenced (NS) and PlGF-silenced 4T1 cells (shPlGF) was measured clinically at the indicated days. Representative volumes of 3 tumors measured by ultrasound are illustrated in a 3D reconstitution. *C,* Frequency of CD11b$^+$ cells measured by flow cytometry in the peripheral blood of tumor-bearing BALB/c mice at day 17 days after tumor cell injection. *D,* Tumor-derived PlGF 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 PlGF-silenced 4T1 tumors did not induce endothelial sprouting. 4T1, Wild type; NS, non-silenced 4T1 cells; shPlGF 4T1, PlGF-silenced 4T1 cells.

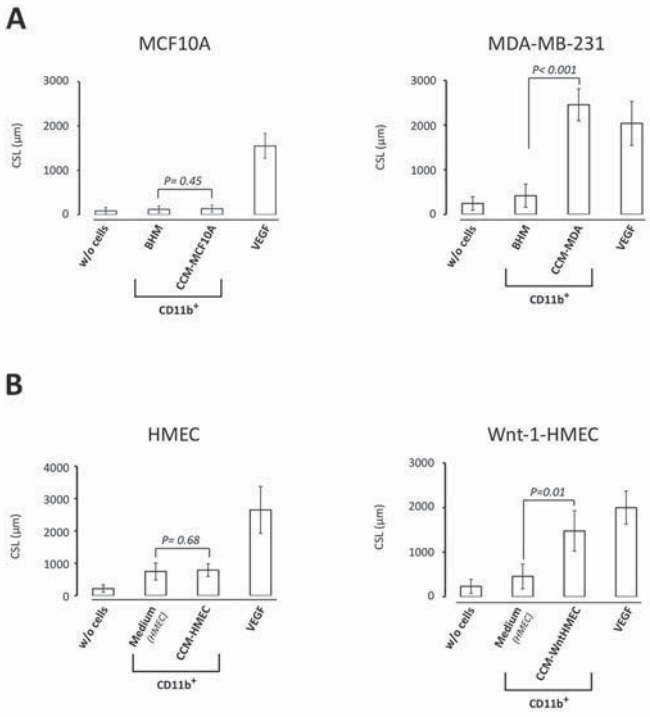
Figure 5. PlGF silencing inhibits 4T1 tumor growth and perfusion and decreases the production of proangiogenic cytokines in the tumor microenvironment. *A,* CD31 and F4/80 immunohistochemistry staining of tumor biopsies from tumor-bearing BALB/c mice 21 days after tumor cell injection. PlGF-silenced tumors lack vessels with

lumen. Endothelial cell surface and vessels with lumen were quantified (lower panels). *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). PlGF 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 (upper panel). Dot plot representation of CD11b$^+$ versus F4/80$^+$ cells in a wild type tumor (lower panel). Infiltrating CD11b$^+$ cells are F4/80$^{+/-}$. *D*, PlGF 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 standard deviations (95% confidence intervals). 4T1, Wild type; NS, non-silenced 4T1; shPlGF 4T1, PlGF-silenced 4T1 cells.

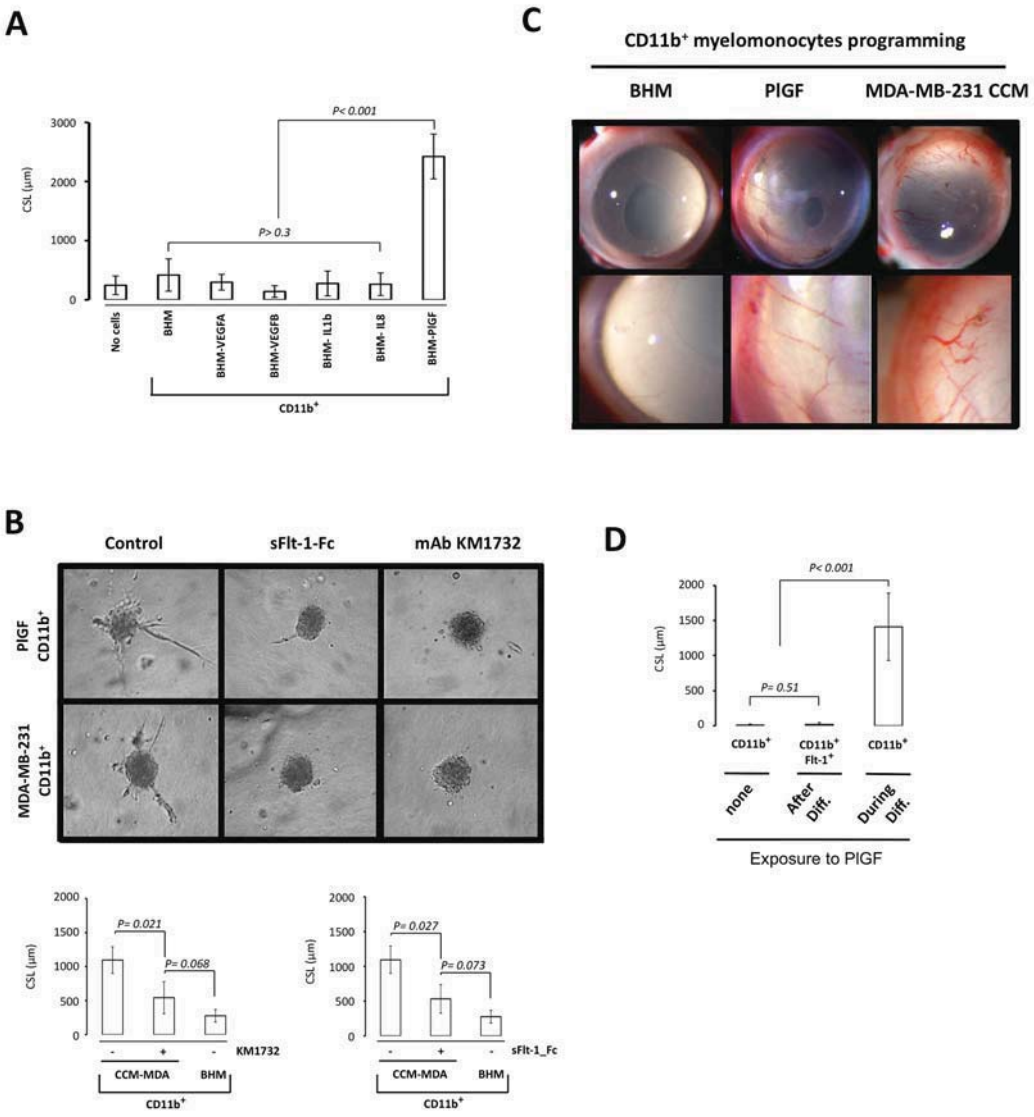
Figure 6. Metalloproteinase activity is required for sprouting activity in PlGF-programmed CD11b$^+$ myelomonocytic cells. *A,* Expression of *ITGAM, TGFB-1, SEMA4D, IF44L, ANG1, MMP9, VEGFA* and *PROK2* mRNA in CD34$^+$ hematopoietic progenitors-initiated cultures was monitored by real time RT-PCR.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

Proangiogenic programming of $CD11b^+$ cells by PlGF

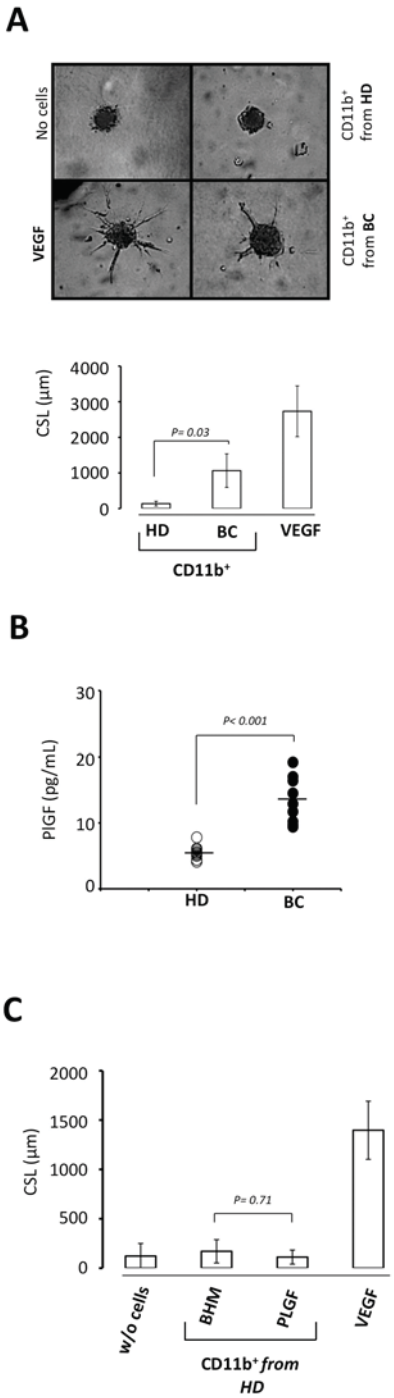
CSL: cumulative sprout lengths. Bar graphs represent mean values and standard deviations (95% confidence intervals).



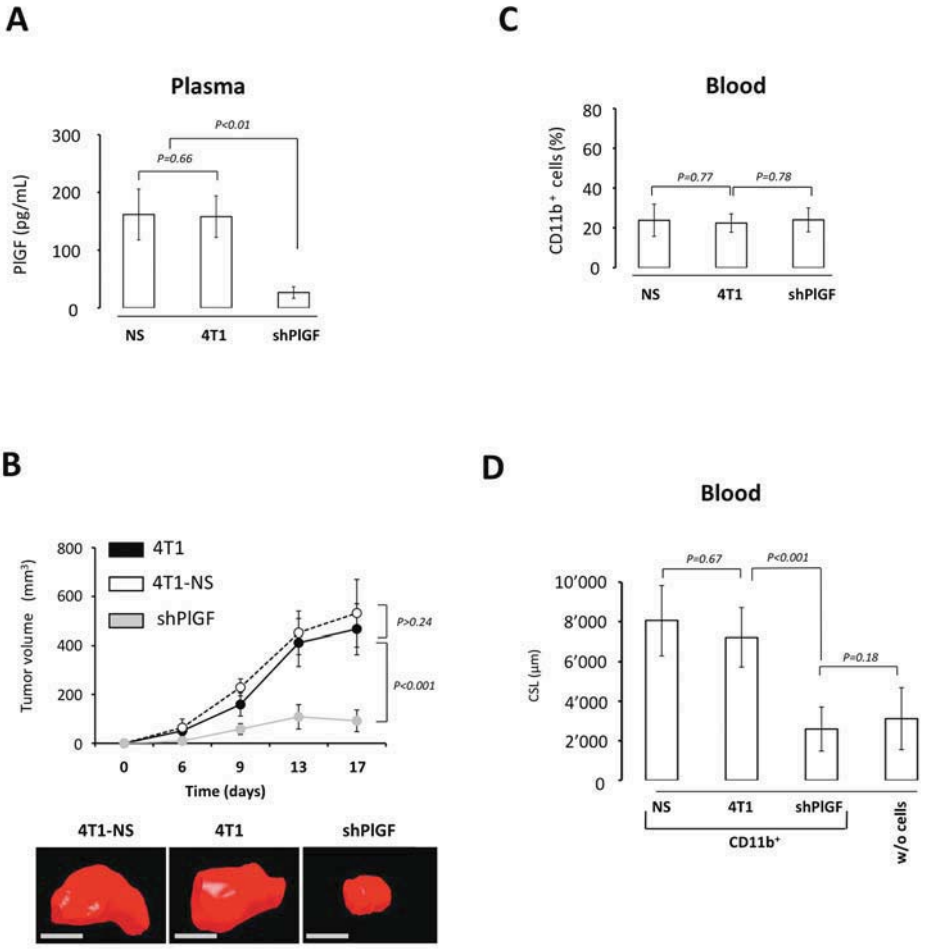
Laurent et al., Figure 1



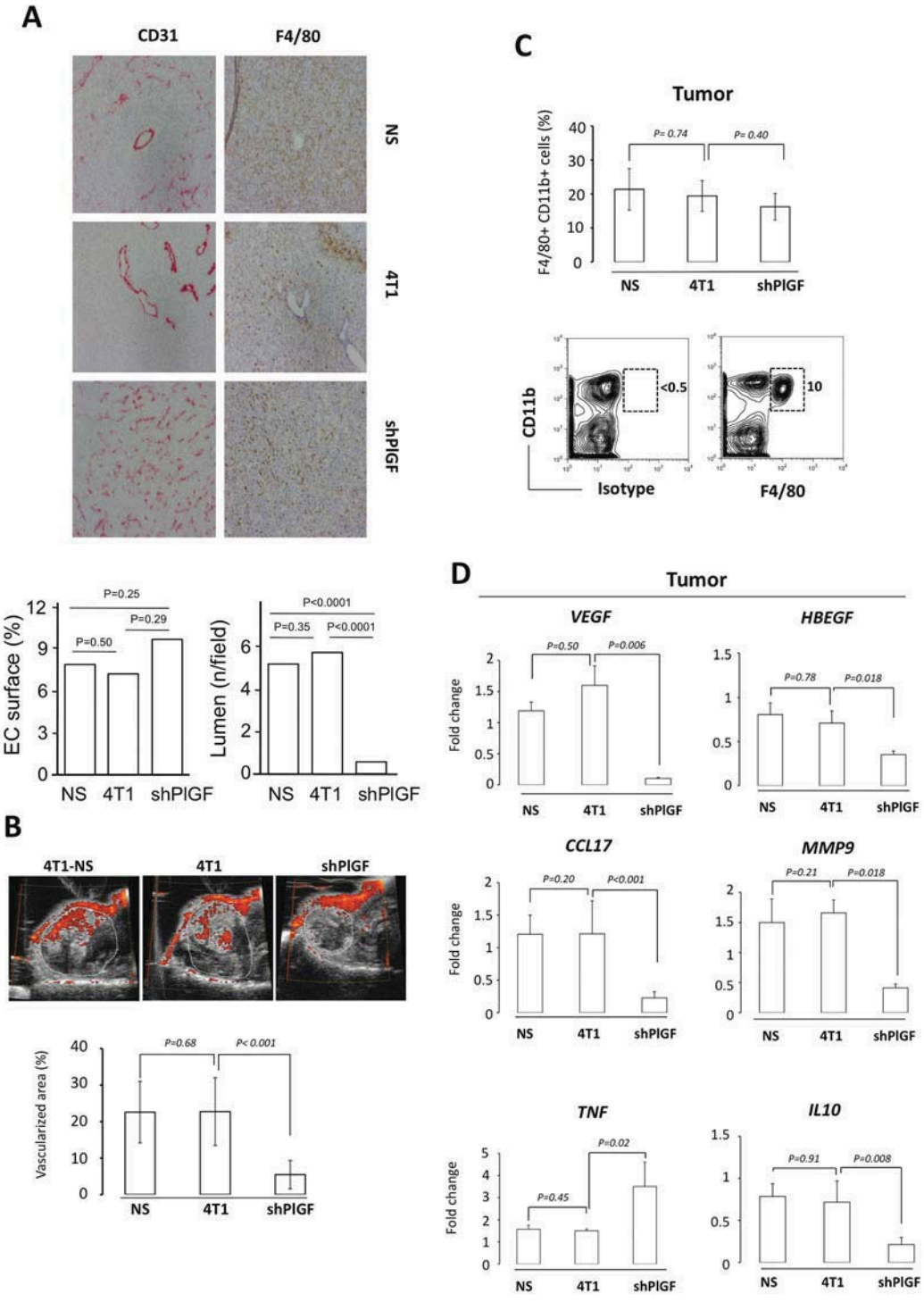
Laurent et al., Figure 2



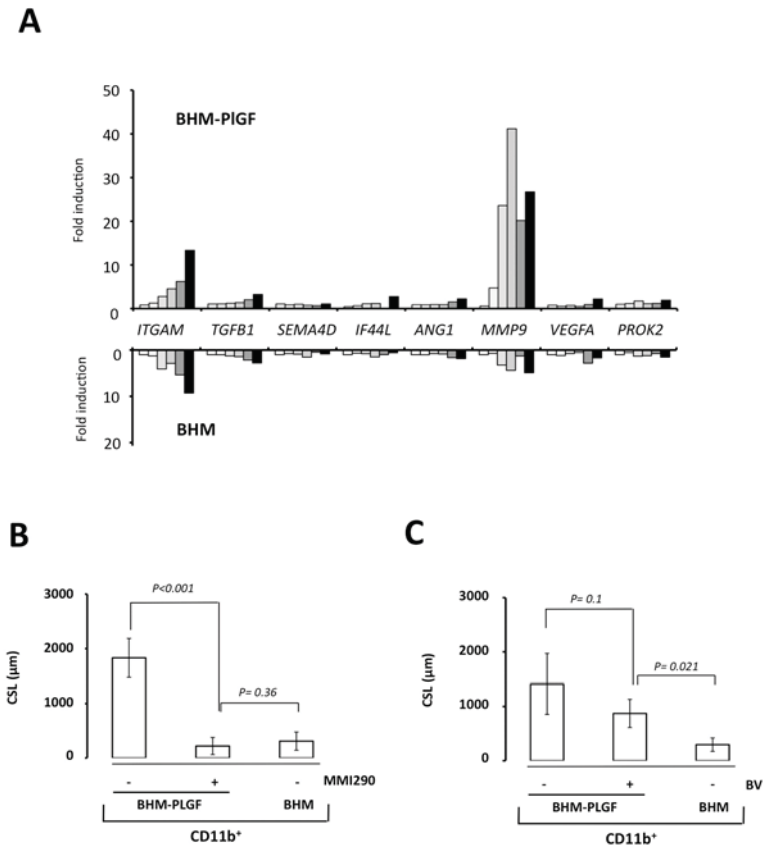
Laurent et al., Figure 3



Laurent et al., Figure 4



Laurent et al., Figure 5



Laurent at al., Figure 6