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Loss or Inhibition of Stromal-Derived PlGF Prolongs Survival of Mice with Imatinib-Resistant Bcr-Abl1$^+$ Leukemia

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SUMMARY

Imatinib has revolutionized the treatment of Bcr-Abl1$^+$ chronic myeloid leukemia (CML), but, in most patients, some leukemia cells persist despite continued therapy, while others become resistant. Here, we report that PlGF levels are elevated in CML and that PlGF produced by bone marrow stromal cells (BMSCs) aggravates disease severity. CML cells foster a soil for their own growth by inducing BMSCs to upregulate PlGF, which not only stimulates BM angiogenesis, but also promotes CML proliferation and metabolism, in part independently of Bcr-Abl1 signaling. Anti-PlGF treatment prolongs survival of imatinib-sensitive and -resistant CML mice and adds to the anti-CML activity of imatinib. These results may warrant further investigation of the therapeutic potential of PlGF inhibition for (imatinib-resistant) CML.

INTRODUCTION

Chronic myeloid leukemia (CML) is caused by chromosomal translocation t(9;22)(q34;q11) ("Philadelphia chromosome") that gives rise to the Bcr-Abl1 fusion kinase (Druker, 2008; Quintas-Cardama et al., 2009). This leukemogenic tyrosine kinase (TK) promotes survival and proliferation of CML cells (Van Etten, 2004). Because of its pivotal role in CML

Significance

Imatinib has revolutionized the treatment of chronic myeloid leukemia (CML). Yet, failure to eradicate the disease, the emergence of resistance, and the limited efficacy in advanced disease stages warrant alternative approaches. Here, we report that PlGF, upregulated in bone marrow (BM) stromal cells by close contact with leukemia cells, increases leukemia cell proliferation and creates a "fertile soil" for CML. Treatment with an anti-PlGF antibody prolongs survival of CML mice, alone and in combination with imatinib. Anti-PlGF is also effective in an imatinib-resistant model, warranting further exploration of these therapeutic avenues.

pathogenesis, most therapies have focused on targeting Bcr-Abl1.

The Bcr-Abl1 TK inhibitor (TKI) imatinib has revolutionized the treatment of CML (Druker et al., 2006). Nonetheless, a subset of patients fails imatinib therapy because of poor tolerance, loss of response or resistance mostly due to acquired mutations in the Abl1 TK domain (Quintas-Cardama et al., 2009). While imatinib induces molecular remission in most patients, it generally fails to completely eradicate the leukemic stem cell pool. Even though some CML patients can arrest treatment without disease recurrence, most relapse after treatment discontinuation (Mahon et al., 2010) and responses are short-lived in end-stage disease (Druker, 2008). Second-generation TKIs have been developed, but some Bcr-Abl1 mutations convey resistance to these drugs. The T315I mutation is particularly problematic as it is one of the common mechanisms of escape and resistance against second generation TKIs (Druker, 2008).

Microenvironment-targeted treatment has gained increasing attention in hemato-oncology. Targeting the bone marrow (BM) environment may provide alternative opportunities to improve the efficacy of anti-CML treatment for TKI-refractory or intolerant cases (Konopleva et al., 2009; Lane et al., 2009). In line with findings that CML is highly vascularized and angiogenesis contributes to leukemogenesis (Aguayo et al., 2000; Zetterberg et al., 2004), vessel density is a predictor of CML progression and patient survival (Korkolopoulou et al., 2003). However, the relevance of angiogenesis in CML remains poorly characterized.

The angiogenic factor VEGF is upregulated by Bcr-Abl1 in CML cells while its levels and BM angiogenesis are reduced by imatinib (Li et al., 2008). VEGF also promotes leukemic cell growth and survival via autocrine effects. Nonetheless, monotherapy with inhibitors targeting VEGF or angiogenesis-related factors has shown limited success in leukemia (Li et al., 2008; Zahiragic et al., 2007), and clinical effects in CML have not been reported yet.

Placental growth factor (PlGF) is a VEGF homolog that binds to Flt1 (VEGFR1) and coreceptor neuropilin-1 (Npn1) (Fischer et al., 2008). PlGF is a disease-specific cytokine, which is dispensable in health but contributes to malignant, inflammatory and ischemic disorders (Carmeliet et al., 2001; Fischer et al., 2007, 2008; Van de Veire et al., 2010). PlGF blockade with the anti-PlGF monoclonal antibody (mAb) 5D11D4 inhibits solid tumor growth and angiogenesis (Fischer et al., 2007; Van de Veire et al., 2010). PlGF is a multitasking cytokine that not only affects endothelial, tumor, inflammatory, and stromal cells, but also promotes survival of hematopoietic precursors (Hattori et al., 2002). In vitro, PlGF stimulates the growth of acute lymphoblastic leukemic (ALL) and acute myeloid leukemia (AML) cells, while Flt1 is expressed by human CML (Fragoso et al., 2006). However, the in vivo role of PlGF in leukemia has not been validated. Prompted by the suggestive link between PlGF and leukemia, we explored the disease-candidate role of PlGF and the therapeutic potential of pharmacological PlGF inhibition in CML models.

RESULTS

PlGF Is Upregulated in CML

To identify angiogenic targets in CML, we profiled a mouse model of CML-like myeloproliferative disease, induced by transplanting donor BM cells, coexpressing the leukemogenic Bcr-Abl1 oncoprotein and GFP ("CML mice") (Daley et al., 1990). Healthy mice or mice receiving mock-transduced BM were used as "controls." Among a selection of candidates, implicated in CML mouse models or patients (Konopleva et al., 2009; Li et al., 2008), PlGF and IL-6 were upregulated the highest in CML mice (Figures 1A and 1B). As IL-6 has already been implicated in CML and PlGF expression was upregulated the most, we focused on PlGF.

PlGF levels were low in the peripheral blood (PB) and BM plasma in healthy and control mice, but increased in CML mice (Figure 1C; see Figure S1A available online). Quantification of $GFP^{(+)}$ $Bcr\text{-}Abl1^{(+)}$ leukemia cells in the BM confirmed that leukemia burden correlated with PB and BM plasma PlGF levels (Figures 1D and 1E). sFlt1 was also elevated, but PlGF was upregulated more, increasing the PlGF/sFlt1 molar ratio by 7.1 ± 1.6-fold in the PB (Figure S1B) and by 2.7 ± 0.3-fold in the BM plasma in end-stage CML mice ($N = 6–9$; $p < 0.05$). PlGF levels were also upregulated in CML patients. Compared to healthy human subjects, plasma PlGF levels were elevated in CML patients in chronic phase upon initial diagnosis and in blast crisis of treated patients (Figure 1F). PlGF was also detectable in the BM plasma of CML patients (62 ± 12 pg/ml; $N = 7$). The elevated PlGF levels in murine and human CML prompted us to study its role further.

PlGF Is Produced by BM Stromal Cells

Cultured $Bcr\text{-}Abl1^{(+)}$ cell lines of human (BV-173, K562) and murine (32D; BaF/3) origin produced only negligible amounts of PlGF (<9 pg/ml/48 hr/10^6 cells; $N = 3$). Leukemic $CD34^{(+)}$ cells from CML patients and $GFP^{(+)}$ cells from CML mice also produced minimal levels of PlGF mRNA (Figures 2A and 2B). In contrast, high PlGF levels were produced by BM stromal cells (BMSCs) from healthy mice (pg/ml/48 hr/10^6 cells: 4190 ± 204; $N = 3$) and murine BMSC lines (pg/ml/48 hr/10^6 cells: 7280 ± 280 for OP9; 636 ± 11 for S17; $N = 3$). Similar results were obtained when using BMSCs from CML patients (Figure 2A). Analysis of sorted BM cells from CML mice confirmed that PlGF was predominantly expressed in $GFP^{(-)}CD45^{(-)}$ stromal cells and negligibly in non-malignant $GFP^{(-)}CD45^{(+)}$ hematopoietic cells or $GFP^{(+)}$ leukemia cells (Figure 2B). Notably, PlGF levels in $CD45^{(-)}$ BM cells were higher in CML than healthy mice, suggesting that the presence of CML cells induced PlGF production in BMSCs in vivo (Figure 2B).

We then analyzed which $CD45^{(-)}$ BMSC subtype produced PlGF in CML mice. Analysis after FACS sorting showed that only low amounts of PlGF were produced by $CD45^{(-)}CD133^{(+)}$ cells, enriched in nonhematopoietic progenitors, by $CD45^{(-)}CD31^{(+)}$ or $CD45^{(-)}VEGFR3^{(+)}$ cells, enriched in endothelial cells, or by $CD45^{(-)}CD31^{(-)}CD44^{(+)}$ cells (Figure 2C). Additional sorting revealed that BM cells, immunonegative for CD45, CD31, CD133 and CD44 produced the highest amounts of PlGF (Figure 2C). These cells also expressed early osteogenic/fibroblastoid markers, such as collagen type I (Col1A1), Runx-2, alkaline phosphatase (ALP), but not PPARγ (adipogenic lineage) or Sox9 (chondrogenic lineage) (Figure 2D).

PlGF Production by Stromal Cells Is Induced by Leukemia Cells

Leukemia cells crosstalk with stromal cells and regulate each other's function (Ding et al., 2010a; Konopleva et al., 2009;

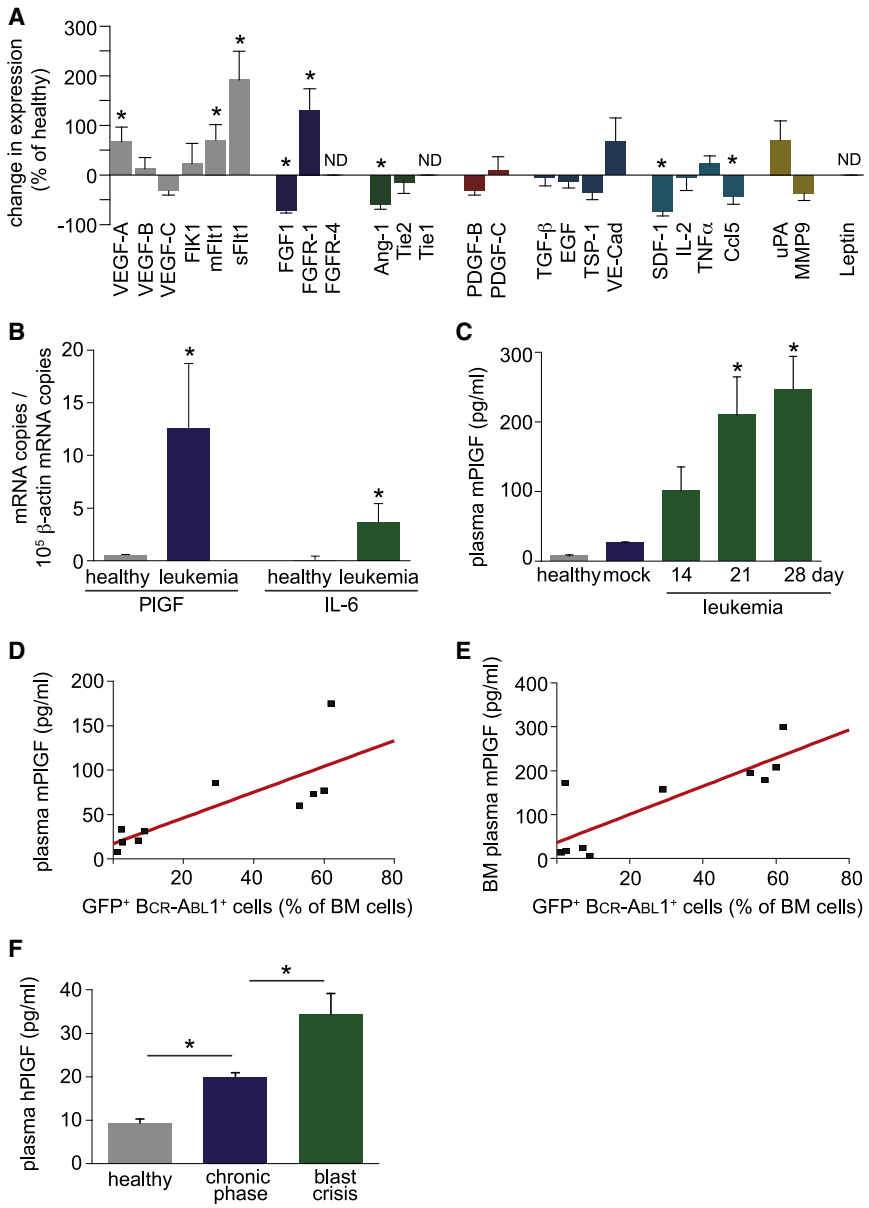

Figure 1. PlGF Levels in Different Stages of CML

(A) RT-PCR analysis of mRNA transcript levels of angiogenic genes in BM from CML mice, expressed as % change of the levels in BM of healthy mice (N = 8; *p < 0.05). ND: not detectable.

(B) RT-PCR analysis of PlGF and IL-6 mRNA levels in BM from healthy and leukemia mice (N = 8; *p < 0.05).

(C) ELISA of plasma PlGF levels in healthy, control mock, or leukemia mice (N = 3–9; *p < 0.05).

(D and E) Correlation of leukemia burden GFP$^{(+)}$ Bcr-Abl1$^{(+)}$ cell fraction) with PlGF protein levels in PB plasma (N = 10; p < 0.01; r^2 = 0.62) (D) or BM plasma (N = 10; p < 0.01; r^2 = 0.68) (E).

(F) ELISA of human PlGF plasma levels in healthy individuals and in CML patients in chronic phase and blast crisis (N = 9–32; *p < 0.05).

Nair et al., 2010). We therefore evaluated if such paracrine interaction regulated PlGF expression and cocultured human leukemia cells on top of murine BMSCs. The use of this system together with a specific ELISA for human or murine PlGF allowed us to examine whether a tumor-stromal communication regulated PlGF expression in the tumor (human) versus host compartment (murine). When cultured alone, CML cells did not produce detectable human PlGF, while BMSCs released murine PlGF (see above). However, in coculture conditions, murine but not human PlGF levels were increased above those in monocultures, indicating that CML cells stimulated BMSCs to upregulate PlGF expression, while the reverse paracrine communication did not affect PlGF production in leukemia cells (Figure 3A). CML and other leukemic cells upregulated PlGF production by BMSCs in a dose-dependent manner (Figure 3B; Figure S2A). Similar results were obtained with S17 stromal cells, but not with osteoclasts or endothelial cells (Figures S2B–S2D). Thus, BMSCs upregulated PlGF when cocultured with CML cells, while PlGF expression was undetectable in leukemia cells.

Role of NF-κB in Production of PlGF

To study how leukemia → stromal cell interactions upregulated PlGF, we first focused on CML cells. The PlGF-inducing activity was not restricted to Bcr-Abl1$^{(+)}$ CML cells alone, since Bcr-Abl1$^{(-)}$ leukemia and other solid tumor cells (expressing distinct oncogenes) also induced PlGF expression by BMSCs (Figures S2E and S2F). Nonetheless, not every tumor cell line upregulated PlGF in BMSCs and some even suppressed PlGF production (Figure S2G). Furthermore, inhibition or silencing of Bcr-Abl1 or some of its downstream signaling molecules (PI3K, RAS, MEK) did not inhibit the ability of Bcr-Abl1$^{(+)}$ CML cells to upregulate PlGF in BMSCs (Figures S2H–S2J).

We then explored how CML cells induced PlGF expression in BMSCs and focused on NF-κB, as this transcription factor upregulates PlGF expression (Cramer et al., 2005). Overactivation of NF-κB in BMSCs leads to a premalignant hematopoietic disorder via a stromal → leukemia crosstalk, but this pathway has not been implicated in leukemia → stromal cell interactions yet. Compared to BMSC monocultures, BMSCs in coculture with CML cells expressed higher levels of a NF-κB responsive luciferase reporter (% of control: 177 ± 2%; N = 3; p < 0.005) as well as of phosphorylated p65, a transcriptionally active NF-κB subunit (Figure 3C). Moreover, BMSCs lacking IKKß, the kinase that activates NF-κB signaling by phosphorylating the NF-κB inhibitors Iκ-Bα and Iκ-Bß, failed to upregulate PlGF when cocultured with CML cells (Figure 3D). In accordance, treatment of BMSC monocultures with pharmacological NF-κB

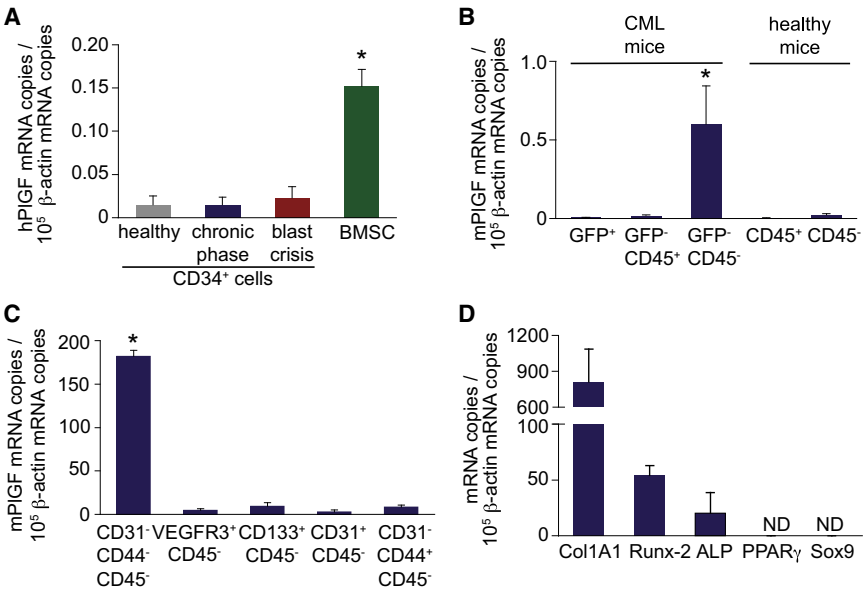

Figure 2. PlGF Production by Bone Marrow Stromal Cells

(A) RT-PCR analysis of human PlGF mRNA levels in $CD34^{(+)}$ cells of healthy donors, in $CD34^{(+)}$ cells of CML patients in chronic phase or blast crisis, and in BMSCs of CML patients (N = 3–7; *$p < 0.005$).

(B) RT-PCR analysis of mouse PlGF mRNA levels in $GFP^{(+)}$ leukemia cells, in $GFP^{(-)}CD45^{(+)}$ hematopoietic cells, and in nonhematopoietic $GFP^{(-)}CD45^{(-)}$ BMSCs from CML mice, and in $CD45^{(+)}$ or $CD45^{(-)}$ cells from healthy mice (N = 3–4; *$p < 0.05$).

(C) RT-PCR analysis of mouse PlGF mRNA levels in subfractions of $GFP^{(-)}CD45^{(-)}$ BMSCs from CML mice (N = 4, *$p < 0.05$).

(D) RT-PCR analysis of mRNA levels of Col1A1, Runx-2, and alkaline phosphatase (ALP) in $CD45^{(-)}CD44^{(-)}CD31^{(-)}CD133^{(-)}$ BMSCs (N = 4). ND, not detectable.

inhibitors lowered baseline PlGF production (indicating active NF-κB signaling in baseline conditions) but, importantly, BMSCs could no longer be stimulated by CML cells to upregulate PlGF expression (Figure 3E). Also, immunostaining of sorted BM cells revealed that phosphorylated p65 was detected in a larger fraction of the PlGF-producing $CD45^{(-)}CD44^{(-)}$ cells in CML than control mice (% of control: 122% ± 6%; N = 5; $p < 0.05$).

The induction of PlGF release by BMSCs required contact with CML cells, suggesting a possible role for integrins. We focused on VCAM-1 and VLA-4 ($\alpha_4\beta_1$ integrin) as NF-κB signaling is enhanced by activation of VCAM-1 with VLA-4 (Zohlnhofer et al., 2000), and expression of VLA-4 on leukemia cells and of VCAM-1 on BMSCs plays a role in residual disease in AML and resistance of ALL cells to chemotherapy (Matsunaga et al., 2003). VLA-4 was expressed by CML cells (mRNA copies/10^5 copies β-actin: 440 ± 20; N = 5), while VCAM-1 was produced by BMSCs (mRNA copies/10^5 copies β-actin: 45 ± 5; N = 5). When supplementing a neutralizing anti-VLA-4 antibody (PS/2) to the coculture, the upregulation of BMSC-derived PlGF by leukemia cells was attenuated (Figure 3F). Similar findings were obtained when using a VCAM-1/Fc, which traps VLA-4 (Figure 3F). Thus, via VLA-4/VCAM-1, leukemia → BMSC interactions induce PlGF production in BMSCs.

Loss or Inhibition of PlGF Prolongs Survival of Leukemia Mice

To explore if PlGF is functionally important for CML, we transplanted BM cells, transduced with Bcr-Abl1, from WT donors in WT recipients (WT→WT) and transplanted Bcr-Abl1$^{(+)}$ $PlGF^{-/-}$ BM cells in $PlGF^{-/-}$ recipients (KO→KO). WT→WT mice developed progressive $GFP^{(+)}$ leukocytosis and abundant $GFP^{(+)}$ cells in the BM, which persisted until death (Figure 4A). Affected mice succumbed to splenomegaly and pulmonary hemorrhage, as reported (Daley et al., 1990). PlGF was a disease-modifier, as CML onset occurred later in KO→KO than WT→WT mice, and KO→KO mice survived longer (Figure 4B). This phenotype was observed in a Balb/c and C57Bl/6 background, though the disease developed more slowly in the C57Bl/6 background (Figure S3A).

We also explored if inhibition of PlGF phenocopied the loss of PlGF and prolonged the survival of CML mice, using the anti-PlGF mAb 5D11D4 (Van de Veire et al., 2010). WT→WT mice were randomized and treatment with 5D11D4 or an isotype-matched IgG control was initiated at 60 hr after transplantation, when CML cells had engrafted the BM (see below). 5D11D4 prolonged the survival of CML mice (Figure 4C) and reduced the leukemia burden, evidenced by lower white blood cell (WBC) counts, spleen weight, $GFP^{(+)}$ leukemia cells in the BM and higher hemoglobin (Hb) levels (Figures S3B–S3E).

At end stage, the BM in IgG-treated CML mice was hypercellular, with densely packed leukemia cells and vessels, but few adipocytes and hematopoietic cells (Figure 4D). In contrast, when analyzed on the same day, the BM of 5D11D4-treated mice contained fewer leukemia cells, but more adipocytes and hematopoietic cells, indicative of less advanced disease (Figure 4E). Moreover, when injecting Bcr-Abl1$^{(+)}$ BaF3 cells, in which PlGF levels in the BM were 2.2-fold higher than in healthy mice (N = 7/8; $p < 0.01$), 5D11D4 prolonged survival of these mice (Figure 4F). Overall, 5D11D4 delayed leukemia in different Bcr-Abl1^{+} CML models and phenocopied the slower disease upon PlGF deficiency.

Blockade of PlGF Does Not Alter Disease Characteristics

PlGF blockade delayed onset and slowed down the course of CML rather than changing the disease characteristics. Indeed, analysis of the retroviral integration sites by Southern blotting of genomic DNA from BM cells of CML mice showed the presence of one to three clonal integration sites per mouse and no differences in the number of clones or proviral content between genotypes or treatment conditions (Figure S3F). These results suggest that the delay in disease onset and the less severe disease upon PlGF-blockage were not due to disease alteration

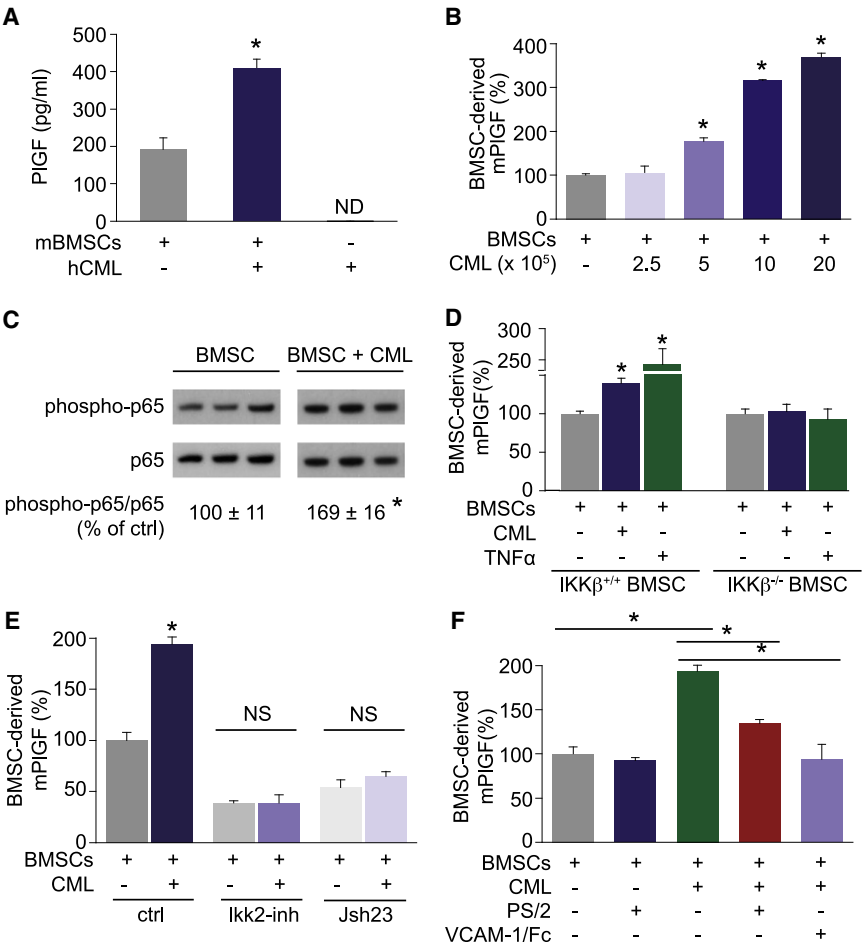

Figure 3. Effect of NF-κB Signaling on Stromal Cell PIGF Expression

(A) ELISA measurement of murine and human PIGF in the culture media of murine BMSCs (mBMSCs), human CML (hCML) (BV-173) cells, or coculture of both (N = 3; *p < 0.05). ND, not detectable.

(B) ELISA measurement of murine PIGF in the culture media of murine BMSCs cultured alone or cocultured together with increasing numbers of human CML (BV-173) cells (N = 3; *p < 0.05).

(C) Immunoblot of phosphorylated p65 (phospho-p65) and total p65 levels in BMSC monocultures and in BMSCs cocultured with CML (K562) cells; quantification of phospho-p65/total p65 is shown below each lane (mean ± SEM; N = 3, *p < 0.05).

(D) ELISA measurement of murine PIGF in the culture media of murine IKKβ$^{+/+}$ or IKKβ$^{-/-}$ BMSCs, cultured alone or together with human (BV-173) CML cells; effect of TNFα on BMSCs monocultures is shown as control (N = 3, *p < 0.05).

(E) ELISA measurement of PIGF in the culture media of murine BMSCs, cultured alone or together with human CML (K562) cells, in the absence or presence of NF-κB pathway inhibition; IKK-2 inhibitor IV (Ikk2-inh), or NF-kB activation inhibitor II (Jsh23) (N = 3, *p < 0.05). NS: not significant.

(F) ELISA measurement of PIGF in the culture media of murine BMSCs, cultured alone or together with human (BV-173) CML cells, in the absence or presence of the VLA-4 inhibitor PS/2 or the VLA-4 trap VCAM-1/Fc (N = 3, *p < 0.05).

with loss of Bcr-Abl1 vector integration or a difference in oligoclonal reconstitution. Furthermore, GFP$^{(+)}$ cells in the BM and PB of all mice of each genotype (WT→WT; KO→KO mice) or treatment condition (control IgG; 5D11D4) were uniformly Gr1$^{(+)}$Mac1$^{(+)}$ myeloid without shift to a B220$^{(+)}$ lymphoid phenotype (Table S1). Also, myeloperoxidase staining of granulocytes did not reveal evidence for a phenotypic shift (Figure S3G). PlGF-blockade did also not induce ectopic growth of solid tumors or CD68$^{(+)}$ histiocytic sarcoma in the skull, brain, kidneys, or other organs (observed in none of the IgG- and 5D11D4-treated CML mice; N = 10; p = NS).

Blockade of PlGF Does Not Affect Homing or Engraftment

We next assessed if the prolonged survival upon PlGF blockage was due to defective homing or engraftment of Bcr-Abl1$^{(+)}$ BM cells. To analyze homing, PKH26 dye-labeled Bcr-Abl1$^{(+)}$ cells were transplanted. FACS analysis at 16 hr after transplantation revealed no difference in the fraction of GFP$^{(+)}$PHK26$^{(+)}$ cells in the BM of WT→WT and KO→KO mice (GFP$^{(+)}$PHK26$^{(+)}$ cells, % of BM cells in WT→WT mice: 100 ± 17 for WT→WT versus 96 ± 7 for KO→KO; N = 5; p = NS), suggesting unimpaired homing. Noteworthy in this respect, in all survival experiments, 5D11D4 was administered from day 3 onward, i.e., after transduced cells had homed to the BM.

To analyze if engraftment was altered in the absence of PlGF, we transplanted Bcr-Abl1$^{(+)}$ cells and counted GFP$^{(+)}$ cells in the PB at weekly intervals. All WT→WT and KO→KO mice maintained GFP$^{(+)}$ cells throughout the study and exhibited a fully penetrant disease until death (Table S2), confirming sustained short-term engraftment of Bcr-Abl1$^{(+)}$ cells upon PlGF blockage. Also, a comparable fraction of GFP$^{(+)}$Lin$^{(-)}$Sca1$^{(+)}$c-Kit$^{(+)}$(LSK) cells was recovered in the BM of WT→WT and KO→KO mice at 18 days post-transplantation (GFP^{+} LSK cells, % of CML cells: 0.09 ± 0.02 for WT→WT versus 0.05 ± 0.02 for KO→KO; N = 3; p = NS). Thus, the prolonged survival of KO→KO mice is not explained by reduced engraftment of leukemia-initiating cells, but rather due to a slowing of the disease.

Loss of Host-Derived PlGF Slows Down CML Disease

To obtain further evidence that PlGF production by stromal rather than leukemia cells regulated Bcr-Abl1$^{(+)}$ leukemic disease, additional cross-over studies were performed. Transplantation of PlGF$^{-/-}$ donor Bcr-Abl1$^{(+)}$ BM cells did not prolong survival of WT recipients (KO→WT) while, conversely, transplantation of WT donor Bcr-Abl1$^{(+)}$ BM cells in PlGF$^{-/-}$ recipients (WT→KO) induced a survival advantage comparable to that seen in KO→KO mice (Figure 4G).

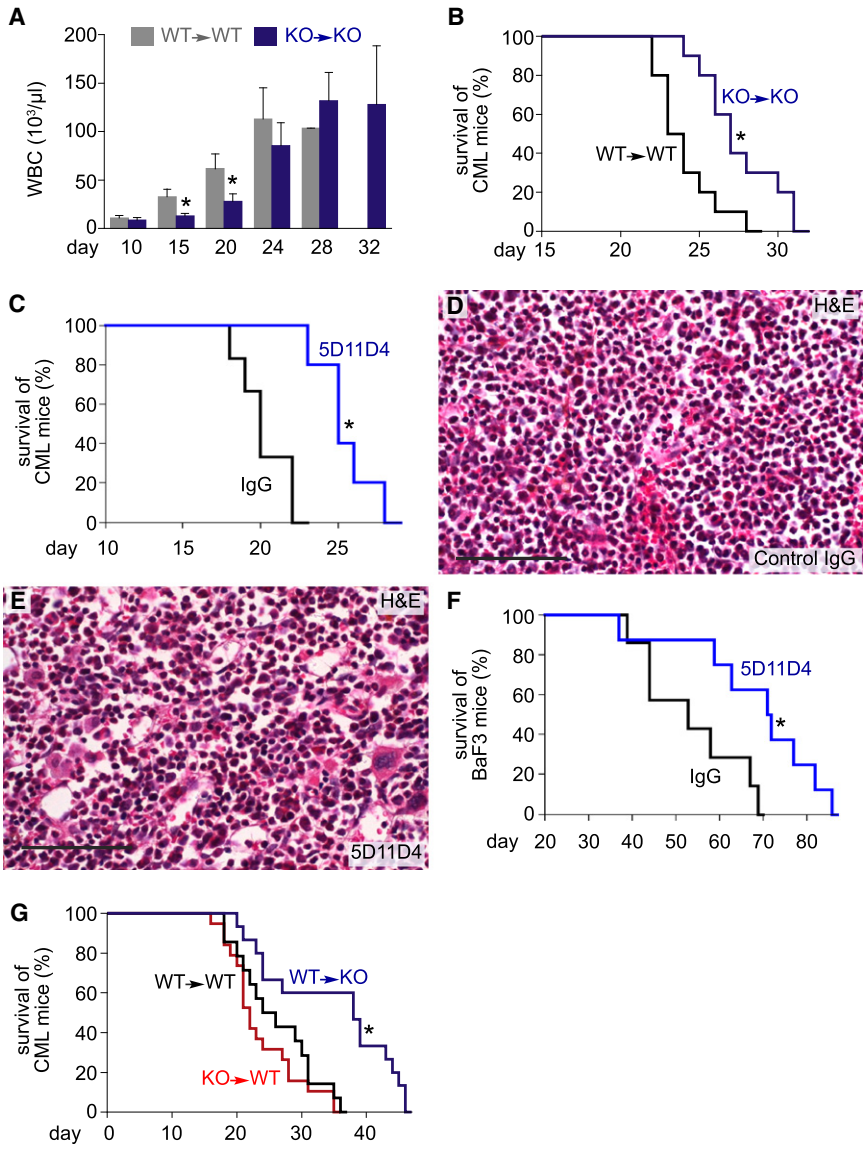

Figure 4. Effects of Loss or Inhibition of PlGF on Survival of Leukemia Mice

(A) Analysis of white blood cell counts (WBC) in PlGF$^{-/-}$ mice receiving Bcr-Abl1$^{(+)}$ BM from PlGF$^{-/-}$ donors (KO→KO) or in WT mice receiving Bcr-Abl1$^{(+)}$ BM from WT donors (WT→WT) (N = 11; *p < 0.05).

(B) Kaplan-Meier survival analysis of KO→KO and WT→WT mice (N = 11; *p < 0.05).

(C) Kaplan-Meier survival analysis of CML mice treated with 5D11D4 or control IgG (N = 10–11; *p < 0.02).

(D and E) H&E staining of BM in IgG-treated (D) or 5D11D4-treated CML mice (E) (bar: 150 μm).

(F) Kaplan-Meier survival analysis of mice with Bcr-Abl1$^{(+)}$ BaF3 leukemia treated with 5D11D4 or IgG (N = 8/9; *p < 0.05).

(G) Kaplan-Meier survival analysis of WT→WT, KO→WT, or WT→KO mice (N = 14–19; *p < 0.05).

Control studies showed that only a negligible fraction of donor-derived BMSCs "contaminated" the transplantation of Bcr-Abl1$^{(+)}$ BM cells. Indeed, after transplantation of Bcr-Abl1$^{(+)}$ BM cells from β-actin:GFP WT donors (expressing GFP in BMSCs and other cells) in WT recipients, only very few GFP$^{(+)}$CD45$^{(-)}$ stromal cells were detected by FACS analysis of recipient BM cells, even at preterminal stages (GFP$^{(+)}$CD45$^{(-)}$ cells, % of BM cells at day 18 and 28: 1.0% ± 0.6% and 2.1% ± 0.7%; N = 5). Similar results were obtained for PlGF$^{-/-}$ recipients (1.3% ± 0.6% and 1.0% ± 0.5%; N = 5). In addition, in WT and PlGF$^{-/-}$ recipients, 99.1% ± 0.2% and 99.7% ± 0.2% of CD45$^{(-)}$CD44$^{(-)}$ cells, the stromal population producing PlGF, were GFP$^{(-)}$ and thus host derived. Moreover, in WT→KO mice, only negligible PlGF protein levels were detected in the BM plasma of recipient mice (3.1% ± 1.9% of the levels found in WT→WT mice; N = 5), while PlGF was undetectable in the PB (N = 5) (Figure S3H). Taken together, it is unlikely that the negligible amount of PlGF, produced by the very few cotransplanted donor stromal cells contributed to the phenotype in a relevant manner. Indeed, if a contamination of PlGF by donor-derived WT stromal cells would be relevant, the WT→KO and WT→WT phenotype should phenocopy each other, which was not the case. Moreover, if the contribution of PlGF$^{-/-}$ donor stromal cells would be relevant, then KO→WT mice should survive longer than WT→WT mice, which was again not the case.

To explore whether PlGF signaling in cells of the BM milieu affected CML, we used the genetic Flt1-TK$^{-/-}$ mouse model, which expresses a "knock-in" Flt1 variant lacking the cytosolic signaling TK domains (Hiratsuka et al., 1998); Flt1-TK$^{-/-}$ mice were only available on a C57Bl/6 background, in which CML develops more slowly (see above). Bcr-Abl1$^{(+)}$ BM cells of WT donors were transplanted into recipient Flt1-TK$^{-/-}$ mice (WT→TK) or control WT mice (WT→WT). Notably, WT→TK mice developed less severe disease than WT→WT mice: on day 59 after transplantation, 63% of WT→WT mice (N = 19) but only 43% of WT→TK mice (N = 21) had succumbed ($p < 0.05$ by Chi- square), while the median survival was 41 days for WT→WT mice versus 75 days for WT→TK mice (N = 19–21; $p < 0.05$). These results indicate that PlGF determined CML disease severity in part through Flt1 signaling in the BM microenvironment.

5D11D4 Reduces Matrix Deposition and Osteolysis

Given that Flt1 signaling in BM stromal cells determined CML, we studied how PlGF-blockage delayed CML by exploring various stromal mechanisms. BM matrix accumulation is an adverse prognostic factor in CML and associated with resistance to imatinib (Buesche et al., 2007; Konopleva et al., 2009). Staining

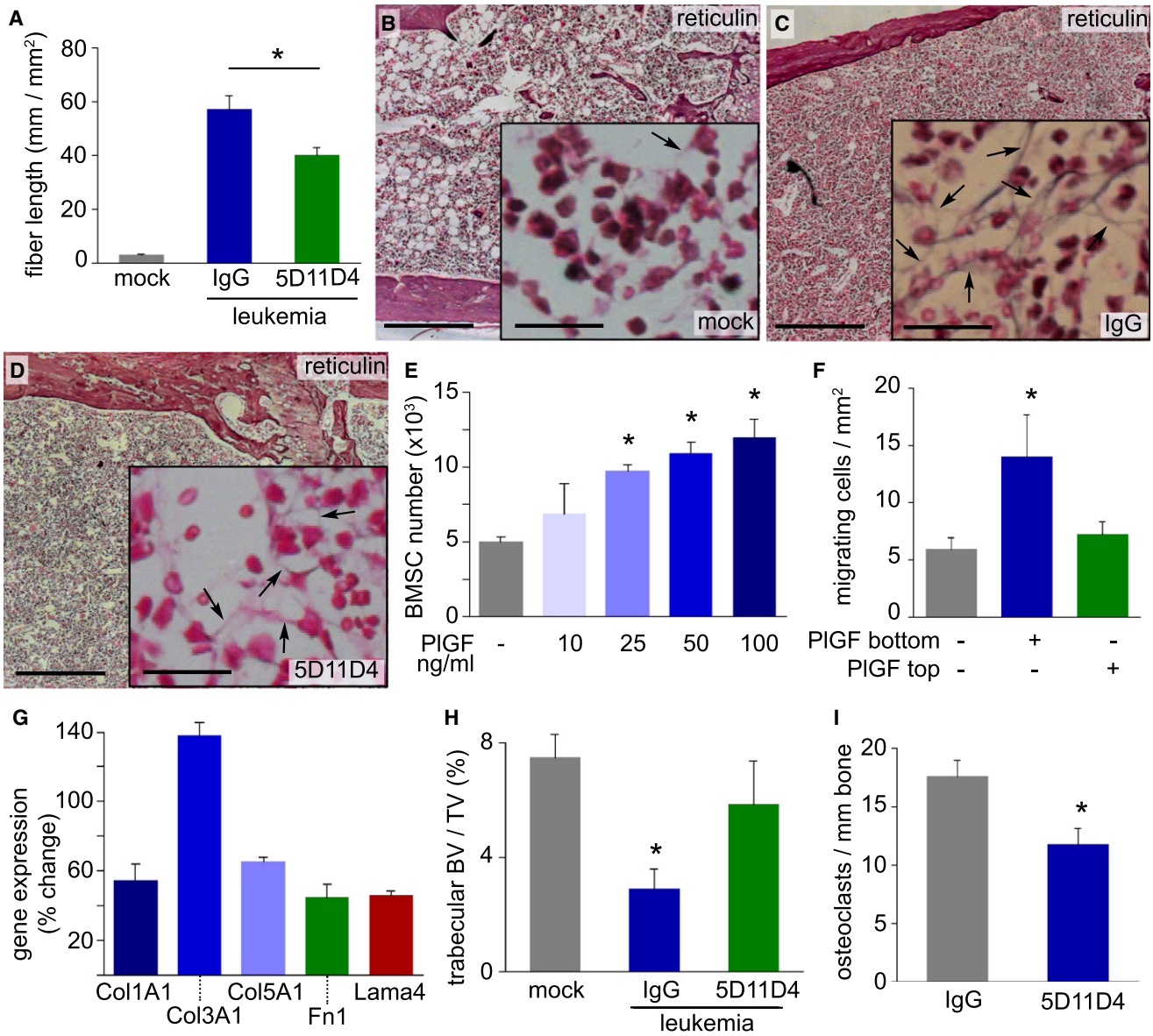

Figure 5. Effect of PlGF on BM matrix deposition in murine CML

(A) Morphometric analysis of reticulin-stained BM matrix accumulation in healthy mock mice, or in CML mice treated with control IgG or 5D11D4 (N = 5-7; *p < 0.05).

(B–D) Representative examples of BM reticulin staining in healthy mock mice (B), and in IgG-treated (C) or 5D11D4-treated CML mice (D) (bar: 400 μm). Insets show higher magnification (bar: 20 μm); arrows denote reticulin fibers.

(E) Analysis of PlGF$^{-/-}$ BMSC cell accumulation in response to increasing concentration of PlGF in vitro (N = 3, *p < 0.05).

(F) Analysis of PlGF$^{-/-}$ BMSC cell migration in the Boyden chamber assay with PlGF supplemented to the bottom or top chambers (N = 3, *p < 0.05).

(G) RT-PCR analysis of mRNA transcript levels of genes encoding matrix components in PlGF$^{-/-}$ BMSCs treated with PlGF, expressed as % change of the levels in PlGF$^{-/-}$ BMSCs treated with control vehicle (N = 3, for all: p < 0.05).

(H) Measurement of trabecular bone volume (BV), as a % of the total bone volume (TV) in healthy mock mice, and in CML mice treated with control IgG or 5D11D4 (N = 5–7; *p < 0.05).

(I) Analysis of osteoclast numbers in the bone of CML mice treated with control IgG or 5D11D4 (N = 5, *p < 0.05).

for reticulin revealed an increase in reticulin deposition at end-stage disease in IgG-treated CML mice as compared to healthy mock mice, while 5D11D4 partially inhibited this deposition (Figures 5A–5D). When staining for Sirius Red (SR) to identify the deposition of reticulin, various types of collagen and other matrix components, only few SR^{+} fibers were detected in the healthy BM; in the BM of IgG-treated CML mice, ~3-fold more SR^{+} fibers were present, while 5D11D4 treatment partially prevented this increase in SR^{+} fiber deposition (Figures S4A–S4D).

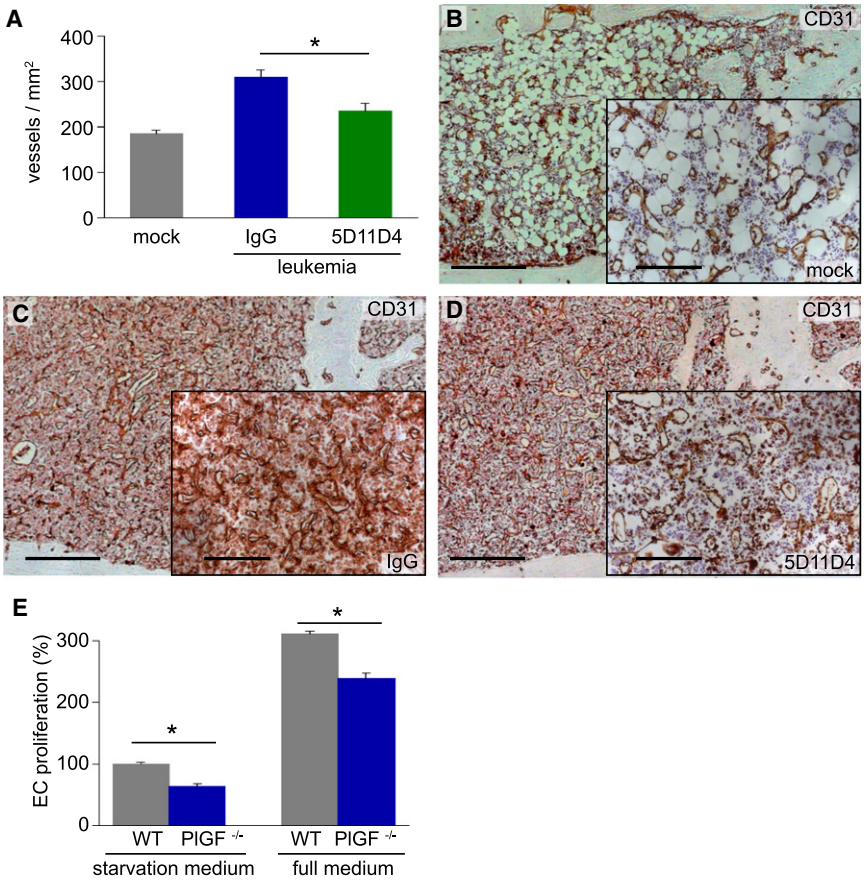

Figure 6. Effect of PlGF on BM Angiogenesis in Murine CML

(A) Microvessel density in CD31 stained bone marrow from healthy mock mice, and from CML mice treated with control IgG or 5D11D4 (N = 5–7; *p < 0.05).

(B–D) Representative images of CD31$^+$ bone marrow sections (bar: 400 μm) from healthy mock mice (B), IgG-treated CML mice (C), and 5D11D4-treated CML mice (D). Insets show higher magnification (bar: 150 μm).

(E) Analysis of the accumulation of WT and PlGF$^{-/-}$ ECs, cultured in conditions of serum starvation or full medium (N = 5, *p < 0.05).

5D11D4 Inhibits Bone Marrow Angiogenesis

We also studied BM vascularization in CML mice at a time when IgG-treated mice had end-stage disease. Morphometry of CD31$^+$ sections revealed an increase in vessel density in IgG-treated CML mice as compared with healthy mice (Figures 6A–6C). 5D11D4 treatment nearly completely blocked this angiogenic burst (Figures 6A and 6D). To assess whether PlGF affected angiogenesis directly, we tested the effect of PlGF on ECs. Since primary mouse BM-derived sinusoidal ECs (BMECs) could not be obtained in sufficient numbers, we isolated pulmonary ECs (pECs) and observed lower proliferation of PlGF$^{-/-}$ than WT pECs, indicating that endogenous PlGF stimulated ECs via an autocrine loop (Figure 6E). Similar differences were seen for migration (% decrease versus control: 36% ± 5%; N = 5; P < 0.05). These findings are consistent with reports that PlGF induces various responses in cultured ECs (Landgren et al., 1998; Van de Veire et al., 2010).

We also tested if PlGF activated BMECs in healthy mice in the absence of leukemia cells and therefore intravenously injected an adenovirus, encoding PlGF (AdPlGF) or a control adenovirus (AdCtrl), as this gene transfer transduces hepatocytes, which then release PlGF in the PB sufficient to stimulate angiogenesis in peripheral organs (Van de Veire et al., 2010). Immunostaining for CD31 after 7 days revealed that PlGF-gene transfer increased the BM microvascular density by 36% ± 13% (N = 5; p = 0.02). In accordance, counting of BrdU$^{(+)}$CD45$^{(-)}$CD31$^{(+)}$ ECs showed that PlGF gene transfer stimulated BMEC proliferation by 32% ± 3% (N = 3; p < 0.05).

We also assessed whether PlGF affected BMSCs, as these cells are known to produce matrix components in the BM stroma. PlGF regulated the growth of these cells in an autocrine manner, since PlGF deficiency reduced BMSC proliferation by 55% ± 4% (N = 6; p < 0.05), while PlGF dose-dependently stimulated growth of PlGF$^{-/-}$ BMSCs (Figure 5E). PlGF also chemoattracted PlGF$^{-/-}$ BMSCs, since PlGF in the lower but not in the upper Boyden chamber stimulated BMSC migration (Figure 5F). Similar mitogenic and chemotactic effects were observed when treating WT BMSCs with PlGF (Figures S4E and S4F). Treatment of PlGF$^{-/-}$ BMSCs with PlGF also upregulated the expression of genes, encoding matrix components in the BM stroma, including collagen type III (Col3A1), the main fiber type in reticulin, and, more modestly, collagen type I (Col1A1), collagen type V (Col5A1), fibronectin (Fn1), and laminin-α4 (Lama4) (Figure 5G). PlGF did, however, not alter the production of the profibrotic factors PDGF-A, PDGF-B, PDGFRα and PDGFRß, TGF-ß, or FGF2 (Figure S4G).

Osteolysis is a rare complication in CML. Compared to untreated mock mice, CML mice treated with IgG showed signs of trabecular bone loss at end-stage disease (Figure 5H; Figures S4H–S4J). 5D11D4 treatment prevented this bone loss in CML (Figure 5H; Figures S4H–S4J) and reduced the number of osteoclasts (Figure 5I). These findings are in line with results that PlGF affects Flt1-expressing osteoclasts (Coenegrachts et al., 2010).

Bcr-Abl1-Independent Signaling of PlGF in CML Cells

We also explored if PlGF affected CML cells. Given that Bcr-Abl1$^+$ cells express Flt1 and Npn1 (mRNA copies/10^5 copies β-actin: for Flt1, 7.2 ± 1.7 for BV-173 and 1.8 ± 0.4 for K562; for Npn1: 7.4 ± 1.4 for BV-173 and 0.45 ± 0.04 for K562; N = 5), we analyzed if PlGF modulated Bcr-Abl1 activation. Immunoblotting for a key phosphorylated tyrosine residue in Abl1 (pY^{421}-Abl1) showed that PlGF did not activate Abl1 in

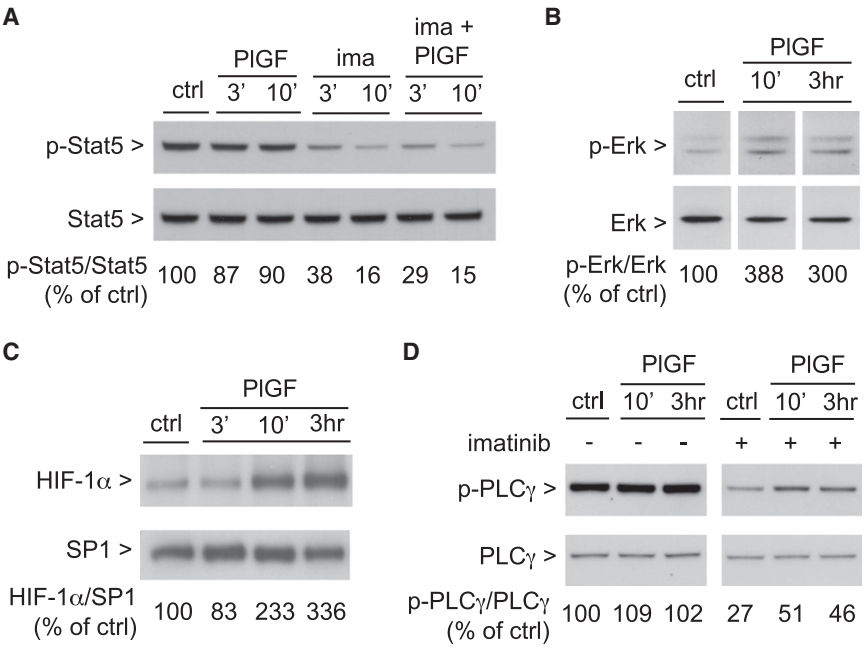

Figure 7. Analysis of Signaling Pathways in CML cells

In each panel, the numbers below the immunoblots refer to the densitometric quantification of the phosphorylated versus total protein levels, % of baseline.

(A) Immunoblot of phosphorylated P-STAT5 and total STAT5 levels in human CML (BV-173) cells, treated with PlGF, imatinib (ima), or a combination of both.

(B) Immunoblot of phosphorylated P-ERK and total ERK levels in human CML (BV-173) cells, treated with PlGF.

(C) Immunoblot of HIF-1α and SP1 (loading control) levels in human CML (BV-173) cells, treated with PlGF.

(D) Immunoblot of phosphorylated p-PLCγ and total PLCγ levels in human CML (BV-173) cells, treated with PlGF, imatinib, or a combination of both.

CML cells (pY^{421}-ABL1/total ABL1: 1.07 ± 0.02 after vehicle versus 0.97 ± 0.10 after PlGF; N = 3; p = NS).

Analysis of signaling targets downstream of BCR-ABL1 revealed high baseline levels of phosphorylated STAT5 (P-STAT5) which PlGF failed to further increase (Figure 7A). To exclude that the high baseline activity of BCR-ABL1 masked an effect of PlGF, we blocked BCR-ABL1 signaling by imatinib and then treated CML cells with PlGF, but also in these conditions, PlGF was ineffective in upregulating P-STAT5 levels (Figure 7A). Similar findings were obtained for CRKL, PI3K, and p38MAPK (Figures S5A–S5C). These findings are consistent with reports that PlGF does not regulate STAT5 (Bartoli et al., 2000; Bellik et al., 2005) or variably affects p38MAPK (Taylor et al., 2010). Phosphorylated AKT (P-AKT) levels were low in baseline conditions and only insignificantly increased by PlGF (P-AKT/total AKT, % of control: 116% ± 39%; N = 3; p = NS).

However, PlGF increased the phosphorylation of ERK1/2 (Figure 7B), a downstream target of both PlGF and BCR-ABL1 (Hazlehurst et al., 2009; Olsson et al., 2006). Another key pathway is the hypoxia-inducible transcription factor HIF-1α, which is stabilized in normoxic conditions by BCR-ABL1 in CML cells and by PlGF in endothelial cells (Mayerhofer et al., 2002; Zhao et al., 2010). Treatment of normoxic BCR-ABL1$^{(+)}$ cells with PlGF also increased HIF-1α protein levels (Figure 7C), consistent with findings that PlGF reduced by 38% ± 12% mRNA levels of the prolyl-hydroxylase PHD2 (a known negative regulator of HIFs); similar changes in PHD2 expression cause biological effects (Mazzone et al., 2009). In accordance, PlGF enhanced the transcriptional HIF-activity in normoxic BCR-ABL1$^{(+)}$ cells, as measured by transfecting CML cells with a HIF-inducible luciferase reporter (luciferase activity, % of control: 139% ± 5%; N = 4; p < 0.05). PlGF also elevated the levels of phosphorylated PLCγ, another common target of BCR-ABL1 and Flt1 (Arroyo et al., 2004), but only after imatinib treatment, likely because its levels were too high in baseline conditions (Figure 7D).

PlGF Stimulates CML Cell Responses

We also assessed if PlGF regulated CML responses in vitro. PlGF dose-dependently stimulated proliferation of BCR-ABL1^{+} cells, as evidenced by measuring ^{3}H-Thymidine incorporation or counting CML cell numbers, an effect mediated via Flt1 that was blocked by 5D11D4 (Figures 8A–8C; Figure S6A). To document that PlGF also induced proliferation of CML cells in vivo, we injected BrdU in CML mice at day 25 and sorted BrdU^{+} leukemia cells from the BM of mice treated with IgG or 5D11D4. This analysis showed that 5D11D4 reduced the number of BrdU^{+} leukemia cells by 38% ± 14% (N = 4; p = 0.06). PlGF also increased CML cell migration by 37% ± 10% in a Boyden chamber assay in vitro (N = 15; p < 0.05). Metabolic measurements further indicated that PlGF stimulated the glycolytic flux (Figure 8D), a known HIF-1α driven process that fuels malignancy (Zhao et al., 2010). PlGF enhanced glycolysis despite inhibition of BCR-ABL1 signaling (Figure 8D), showing that it can affect CML cells independently of BCR-ABL1.

PlGF-Dependent Stromal → Leukemia Crosstalk

Stromal → leukemia interactions affect CML proliferation, survival and drug resistance (Nair et al., 2010). We therefore analyzed if BMSC-derived PlGF regulated BCR-ABL1$^{(+)}$ CML growth. In BMSC/CML cocultures, BMSCs are the only source of (murine) PlGF, which can be selectively blocked by 5D11D4. In this assay, 5D11D4 inhibited CML cell proliferation to levels, observed when these cells were cultured alone, indicating that the mitogenic effect was induced by paracrine PlGF release from BMSCs (Figure 8E). Moreover, when coculturing CML cells with BMSCs, proliferation of CML cells was stimulated more by WT than PlGF$^{-/-}$ BMSCs (Figure 8F). CML cells also stimulated BMSC proliferation by inducing release of PlGF from BMSCs (Figures S6B and S6C).

5D11D4 Prolongs Survival of Imatinib-Treated CML Mice

Given that PlGF affected both the BM stromal environment and CML cells, and PlGF and BCR-ABL1 acted partially in

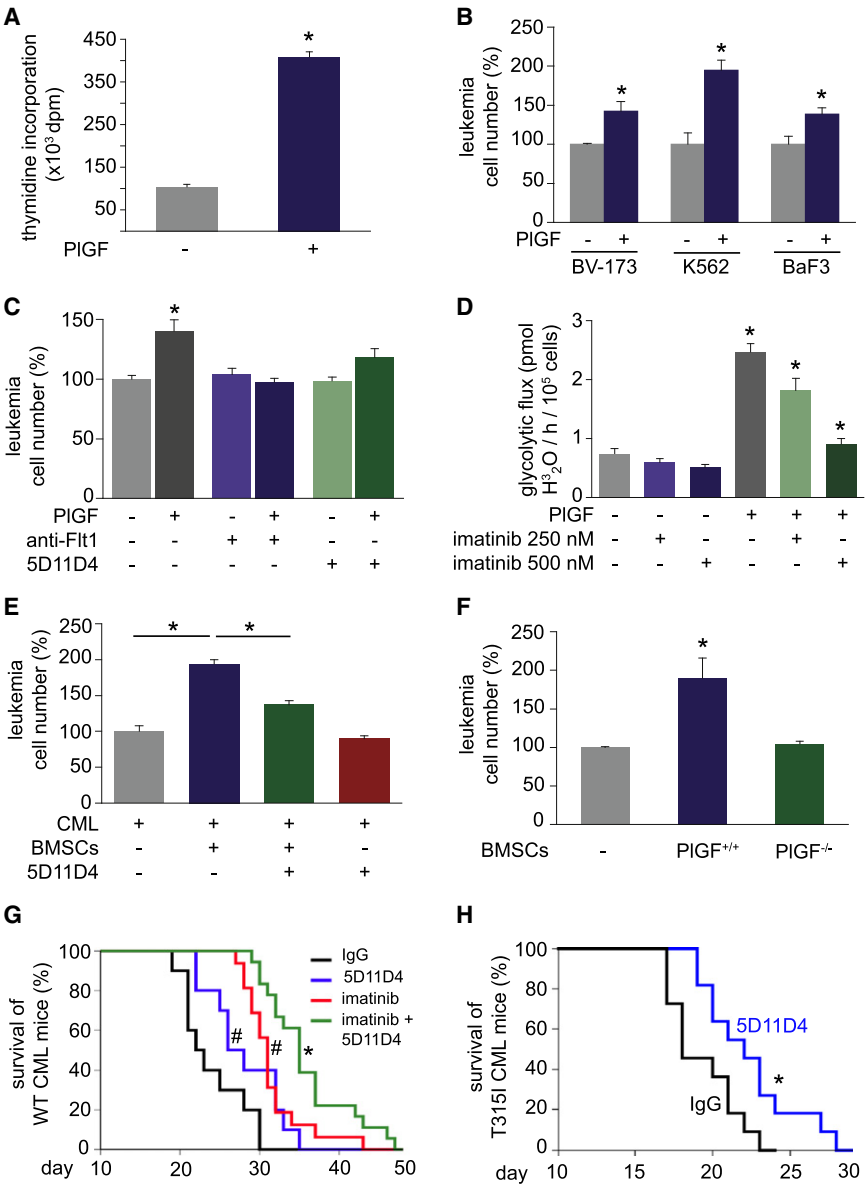

Figure 8. Effects of PlGF on CML Cells In Vitro and In Vivo

(A) ^{3}H-Thymidine incorporation assay of human CML (K562) cells treated with vehicle or PlGF (N = 3; *p < 0.05).

(B) Measurement of the accumulation of Bcr-Abl1$^{(+)}$ leukemia cell lines of murine (BaF3) or human origin (BV-173, K562) upon treatment with control vehicle or PlGF (N = 3; *p < 0.05).

(C) Measurement of the accumulation of CML (BV-173) cells in response to PlGF in the absence or presence of an anti-Flt1 antibody or 5D11D4 (N = 3; *p < 0.05).

(D) Measurement of glycolytic flux in CML (K562) cells in response to PlGF, in the absence or presence of imatinib (N = 3; *p < 0.05 versus control).

(E) Measurement of the accumulation of CML (BV-173) cells, cultured alone or together with BMSCs in the presence or absence of 5D11D4 (N = 3; *p < 0.05).

(F) Measurement of the accumulation of CML (BV-173) cells, cultured alone or together with PlGF$^{+/+}$ or PlGF$^{-/-}$ BMSCs (N = 3; *p < 0.05).

(G) Kaplan-Meier survival curve of CML mice upon monotherapy with 5D11D4 (N = 10; #p < 0.05 versus IgG) or imatinib (N = 10/16; #p < 0.05 versus control; p = NS versus 5D11D4), or upon combination therapy with 511D4 plus imatinib (N = 10-18; *p < 0.05 versus imatinib alone and 5D11D4 alone); imatinib was administered once a day.

(H) Kaplan-Meier survival curve of imatinib-resistant CML mice, carrying the T315I Bcr-Abl1 mutation, treated with control IgG or 5D11D4 (N = 12; *p < 0.05).

a non-overlapping and complementary manner, we explored if combination treatment of CML mice with 5D11D4 and imatinib was superior to each monotherapy alone. Pilot experiments showed that a dose of 100 mg/kg imatinib, given 1×/day (qd) or 2×/day (bid) yielded similar results (Figure S6D). We therefore treated CML mice with 5D11D4, imatinib or a combination of both. Notably, monotherapy with imatinib or 5D11D4 prolonged the survival of CML mice, but the combination therapy prolonged survival of leukemia mice significantly longer than each monotherapy alone, both when imatinib was given once or twice per day (Figure 8G; Figure S6D).

5D11D4 Prolongs Survival in an Imatinib-Resistant CML Mouse Model

These results also prompted us to investigate whether 5D11D4 could prolong the survival of mice developing an imatinib-resistant variant of CML. Therefore, a retrovirus was used, expressing the imatinib-resistant T315I variant of the Bcr-Abl1 oncoprotein, found in CML patients who fail to respond to imatinib and second generation TKIs (Druker, 2008). This virus was used to transduce BM cells (from WT donors) and generate a mouse model of imatinib-resistant CML by transplanting these Bcr-Abl1^{T315I} BM cells into WT recipients. Notably, treatment with 5D11D4 induced a survival advantage in this imatinib-resistant model as well (Figure 8H).

DISCUSSION

This study provides evidence for a role for PlGF in CML. First, PlGF levels in the BM plasma or PB correlate with Bcr-Abl1$^{(+)}$ leukemia load in CML mice and are elevated in CML patients. Second, this cytokine stimulates BM angiogenesis and CML cell growth. Third, PlGF gene deletion or inhibition by an anti-PlGF antibody prolongs survival of CML mice. And fourth, the beneficial effect of anti-PlGF treatment adds to the anti-CML activity of imatinib in sensitive CML mice and prolongs the survival of imatinib-resistant CML mice.

While current anti-CML therapies have been largely "leukemia cell-centered," emerging evidence highlights the importance of the BM stroma for the growth, survival and TKI resistance of leukemia cells (Hazlehurst et al., 2009; Konopleva et al., 2009; Lane et al., 2009; Nair et al., 2010). Leukemia cell ↔ stromal cell interactions are bidirectional, with forward signaling from leukemia to stromal cells ensuring that the BM stroma becomes a nurturing environment for the malignant cells, and reverse signaling from stromal to leukemia cells promoting malignancy and therapy resistance. Here, we show that PlGF is only minimally produced by CML cells. However, leukemia cells instruct BMSCs to produce increasing amounts of PlGF, a multitasking cytokine that not only stimulates proliferation, migration, and metabolism of CML cells, but also promotes angiogenesis in the BM stroma. As such, PlGF drives a self-sustaining, reinforcing vicious cycle, whereby CML cells induce the production of PlGF by BMSCs, which results in increased leukemia load, that in turn stimulates production of PlGF by the stroma, and so on. Hence, by upregulating PlGF, leukemia cells create for themselves a fertile tumor-promoting "soil," rich in vessel supply, that fuels expansive growth. This mechanism can explain why PlGF levels correlate with disease burden in CML mice. The finding that PlGF is only upregulated by BMSCs when CML cells are present in the BM clarifies why this cytokine is critical in pathologic leukemic conditions but not in normal hematopoiesis.

Precisely the fact that PlGF not only affects CML cells but also the BM stroma may explain why PlGF-blockade not only adds to the anti-CML activity of imatinib in the imatinib-sensitive model but also prolongs survival in the imatinib-resistant model. Several mechanisms contribute to the anti-CML effects of PlGF-blockade. First, PlGF acts, at least partially, in parallel to Bcr-Abl1 in Flt1$^+$ CML cells. Activation of CML cells by PlGF did not increase tyrosine phosphorylation of Bcr-Abl1 or its downstream targets Stat5, Crkl, Akt, Pi3K, or p38Mapk, but activated Plcγ, Erk1/2 and HIF-1α, targets shared by Flt1 and Bcr-Abl1. PlGF can thus act independently of Bcr-Abl1 signaling and induce pathways, that only partially overlap with those induced by Bcr-Abl1 (Bellik et al., 2005; Ding et al., 2010b; Hazlehurst et al., 2009; Landgren et al., 1998; Quintas-Cardama et al., 2009). Such molecular interactions could provide a mechanistic framework to explain why PlGF partially overcomes the blockage of Bcr-Abl1 by imatinib or acts in parallel of imatinib-resistant T315I Bcr-Abl1. Identifying leukemia cell-extrinsic mechanisms that contribute to the resistance against Bcr-Abl1–specific TKIs is of great medical interest. So far, only a few examples, other than PlGF, have been documented, including GM-CSF, BMSC-medium, and Src-kinases (Donato et al., 2003; Hazlehurst et al., 2009).

A second mechanism is that PlGF induced key changes in the leukemic BM microenvironment, as evidenced by the prolonged survival of CML mice with defective Flt1 signaling in cells of the BM milieu. Infiltration of the BM with leukemia cells is known to evoke an angiogenic response in hematological malignancies including CML, and microvascular density is an independent prognostic parameter for CML patients' survival (Korkolopoulou et al., 2003). Nonetheless, besides VEGF, angiopoietin-2 and a few other molecules, the molecular basis of the angiogenic switch in CML remains poorly defined (Li et al., 2008; Maffei et al., 2009). PlGF is known to stimulate proliferation, migration and survival of cultured ECs (Landgren et al., 1998; Van de Veire et al., 2010). Moreover, PlGF stimulated BM angiogenesis in healthy mice, indicating that leukemia cells were not absolutely required for its angiogenic activity. Besides direct effects on ECs, PlGF could also act as an "indirect angiogenic factor" via effects on CML or stromal cells, for instance by stimulating the release of additional angiogenic factors. Emerging evidence indicates that the BM milieu and the marrow vasculature are capable of promoting the growth, survival and resistance of leukemic cells by providing vital nutrients, cytokines and cell contact-mediated signals (Konopleva et al., 2009; Lane et al., 2009).

PlGF-blockade partially blocked the deposition of reticulin- and collagen-rich matrix in the BM of CML mice. In CML patients, prominent BM fibrosis is a known adverse prognostic factor and associated with resistance to imatinib (Buesche et al., 2007; Konopleva et al., 2009). While a statistically significant increase in matrix accumulation in the BM stroma of CML mice was observed, it remains to be determined to what extent the reduced BM matrix deposition in PlGF-blocked mice is causally involved in reducing disease severity or only an indicator of the less severe disease stage. Also, the overall relevance of the relatively subtle matrix accumulation in CML mice for human CML remains to be further defined. Moreover, PlGF-blockage reduced osteolysis, a rare complication of CML, by inhibiting osteoclast accumulation. We previously reported that PlGF affects Flt1-expressing osteoclasts (Coenegrachts et al., 2010).

Bidirectional communication between leukemia and stromal cells promotes CML, but only in a few cases have the responsible signals produced by each class of cell types been identified. For instance, leukemia cell-released PDGF-B upregulates VEGF in stromal cells (Ding et al., 2010a), while stromal cell-produced factors (G-CSF, IL-3, or GM-CSF) mediate leukemia cell resistance against TKIs (Nair et al., 2010). In this study, we identified PlGF as a stromal cell-derived signal with activities on both the malignant and host compartments, but also unveiled that leukemia cells upregulate PlGF production in stromal cells by activating NF-κB signaling. Over-activation of this pathway in stromal cells leads to uncontrolled hematopoietic cell growth, but NF-κB signaling has so far not been implicated in leukemia → stroma cell communication. Interestingly, binding of VLA-4 to VCAM-1 activates NF-κB (Zohlnhofer et al., 2000), while NF-κB in turn upregulates VCAM-1 expression (Rajan et al., 2008). This may not only explain why cell contact between CML cells and BMSCs was required for the upregulation of PlGF, but also suggests that this cell contact fuels a positive feedback loop, whereby VCAM-1$^+$ BMSCs reinforce binding of VLA-4$^+$ CML cells and ensure continued PlGF expression. Other CML-derived signals that activate NF-κB signaling could be involved as well. Interesting in this respect are recent findings that cancer-associated fibroblasts (CAFs), a related population of stromal cells in solid tumors, also exhibit a NF-κB gene signature (Erez et al., 2010; Quante et al., 2011). However, the precise resemblance of the BMSC subpopulations, releasing PlGF, producing matrix components or even stimulating angiogenesis in CML on one end with the CAFs in solid tumors on the other end, remains to be further elucidated. The ability of malignant cells to induce PlGF in stromal cells is not restricted to CML alone, but appears to be a more common paradigm, that other Bcr-Abl$^-$ leukemogenic and Bcr-Abl-independent tumors also

use to hijack the stroma for their own benefit. Thus, while applicable for CML per se, the present findings may have more far-reaching implications for tumor biology in general than for CML alone.

Finally, while these preclinical studies suggest other therapeutic opportunities, the translational applicability of PlGF-blockade for CML will require further testing. Compared with the overwhelming therapeutic benefit of imatinib, the therapeutic potential of PlGF-blockade in this CML mouse model is obviously more modest. Nonetheless, our findings may prime further interest in targeting the BM stroma or blocking BM angiogenesis for improved treatment of CML in the future.

EXPERIMENTAL PROCEDURES

More detailed methods are found in the Supplemental Experimental Procedures.

Animals

Wild-type Balb/C or C57BL/6 mice and PlGF$^{-/-}$, Flt1-TK$^{-/-}$ or IKKß$^{l/l}$ mice were used. Adenoviral PlGF or control vector was administered intravenously. Animal procedures were approved by the Institutional Animal Care and Research Advisory Committee (KU Leuven); mouse experiments were performed in accordance with the institutional and national guidelines and regulations.

Patient Samples

Patient samples from healthy controls, untreated patients upon primary diagnosis, and treated CML patients in blast crisis were collected in the university hospitals of Leuven, Mannheim, Hamburg, Liverpool, and Glasgow and analyzed for PlGF protein and Bcr-Abl1 transcripts, in accordance with the declaration of Helsinki and patients' informed consent, and with the approval of the ethical committee of the respective university hospitals.

Cell Culture and In Vitro Functional Assays

Human and murine Bcr-Abl1^{+} or Bcr-Abl1^{-} tumor cell lines (K562, BV-173, Molm13, MV4-11, REH, BaF3, EOL-1, HL-60, PC3, MDA-MB-231, Nalm-6, CRL-8024, CaKi-1, 32D), primary murine ECs, BMSCs or BMSC lines (S17, OP9) were used in BMSC/tumor cell cocultures as detailed in Supplemental Experimental Procedures. Proliferation, migration and luciferase reporter assays are described in the Supplement. siRNA transfection was done by electroporation.

Elisa, Immunoblot , and RT-PCR

Human and murine PlGF, VEGF, Flt1, and sFlt1 were determined in cell culture supernatants, blood plasma and BM by ELISA; immunoblotting of (phospho) protein was with the antibodies listed in Supplemental Experimental Procedures; RNA expression analysis was by Taqman qRT-PCR.

Leukemia Models and Treatments

For the BaF3 model, 1 × 10^{6} Bcr-Abl1^{+} BaF3 cells were injected i.v. into Balb/c recipients. The CML transplantation model using GFP^{+} or GFP^{+}Bcr-Abl1^{+} BMSC was carried out as described (Daley et al., 1990; De Keersmaecker, 2008). Treatment with 5D11D4 (50 mg/kg; 3×/wk) or an unrelated control antibody (1C8) and/or imatinib (100 mg/kg/once or twice per day) was started 2 days after transplantation. Survival of mice was monitored daily; Bcr-Abl1 proviral integration was evaluated by Southern blot.

Flow Cytometry and Sorting

Bcr-Abl1$^{(+)}$ cells in peripheral blood and BM were analyzed as GFP^{+} cells by flow cytometry. FACS or immunomagnetic bead sorting using specific marker antibodies was as in Supplemental Experimental Procedures.

BM Histology, Morphometry, and μCT Analysis

For BM stainings, paraffin sections from decalcified hind limb bones were stained with hematoxylin, TRAP (osteoclasts), eosin (H&E), and Sirius Red and immunostained for reticulin fibers, αSMA, collagen type I, and CD31.

Statistics

Data represent mean ± SEM of representative experiments, unless otherwise stated. Statistical significance was calculated by Student's t test or ANOVA. For correlations, Pearson's r coefficients were calculated, and survival of mice was analyzed by log-rank tests.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, six figures, and two tables and can be found with this article online at doi:10.1016/j.ccr.2011.05.007.

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