Imatinib Disrupts Lymphoma Angiogenesis by Targeting Vascular Pericytes

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None
KEY POINTS

- Blockade of PDGFRβ impairs lymphoma growth by depleting vascular mural cells.
- Pericytes may represent a novel, anti-angiogenic target for lymphoma therapy.

ABSTRACT

Pericytes and vascular smooth muscle cells (VSMC), which are recruited to developing blood vessels by platelet-derived growth factor BB, support endothelial cell survival and vascular stability. Here, we report that imatinib, a tyrosine kinase inhibitor of PDGFRβ, impaired growth of lymphoma in both human xenograft and murine allograft models. Lymphoma cells themselves neither expressed PDGFRβ nor were growth-inhibited by imatinib. Tumor growth inhibition was associated with decreased microvascular density and increased vascular leakage. **In vivo**, imatinib induced apoptosis of tumor-associated PDGFRβ⁺ pericytes and loss of perivascular integrity. **In vitro**, imatinib inhibited PDGFRβ⁺ VSMC proliferation and PDGF-BB signaling, while siRNA knockdown of PDGFRβ in pericytes protected them against imatinib-mediated growth inhibition. FACS analysis of tumor tissue revealed depletion of pericytes, endothelial cells, and their progenitors following imatinib treatment. Compared to imatinib, treatment with an anti-PDGFRβ monoclonal antibody partially inhibited lymphoma growth. Lastly, microarray analysis (GEO database accession number: GSE30752) of PDGFRβ⁺ VSMC following imatinib treatment showed down-regulation of genes implicated in vascular cell proliferation, survival, and assembly, including those representing multiple pathways downstream of PDGFRβ. Taken together, these data indicate that PDGFRβ⁺ pericytes may represent a novel, non-endothelial, anti-angiogenic target for lymphoma therapy.
INTRODUCTION

Despite the fact that tumor cell-directed, multi-modality treatment with chemotherapy, radiation, and biologic agents can induce remission in many subtypes of non-Hodgkin’s lymphoma (NHL), a significant proportion of patients continue to succumb to incurable disease \(^1\)-\(^6\). Recent studies have shown that stromal and vascular cell genetic signatures within the tumor microenvironment can predict disease behavior and clinical outcome in NHL subtypes \(^7\),\(^8\). These findings highlight the importance of tumor stromal cells in the pathogenesis and potential therapy of lymphoma.

The tumor microenvironment supports the initiation and progression of cancerous growth, in part by building and sustaining the tumor’s vascular network \(^9\)-\(^11\). Emerging data on the pro-angiogenic properties of lymphoma cells and the mechanisms of vascular assembly suggest that angiogenesis is highly relevant to the biology and therapy of NHL \(^12\). Drawing parallels from the extensive literature on solid malignancies, anti-angiogenic lymphoma therapy has focused largely on vascular endothelial growth factor (VEGF), which can drive proliferation of both tumor and endothelial cells \(^12\),\(^13\). However, small phase II clinical studies with the anti-VEGF monoclonal antibody bevacizumab have so far shown equivocal efficacy in aggressive NHL \(^14\),\(^15\), suggesting that non-VEGF angiogenic pathways may be highly relevant.

Platelet-derived growth factor-type BB (PDGF-BB) directs the recruitment of PDGFR-expressing pericytes and their progenitors to neovessels, thereby promoting vascular maturation and stabilization \(^16\),\(^17\). Genetic ablation of either PDGF-BB or PDGFR\(\beta\) in developing mouse embryos leads to lethal microvascular leakage and hemorrhage \(^18\)-\(^20\). PDGF may also modulate the expression of other stromal angiogenic factors, such as basic fibroblast growth factor and VEGF \(^21\),\(^22\). Pharmacologic intervention with receptor tyrosine kinase inhibitors that target
PDGFRβ, such as imatinib mesylate or sunitinib malate, have shown efficacy in solid tumor models 22-27, partly by reducing pericyte density and attenuating angiogenesis. To date, however, specific targeting of PDGFRβ has not been extensively evaluated in lymphoid malignancies.

We previously characterized vascular assembly in human NHL subtypes 28, and hypothesized that blood vessel stability depends upon pericyte integrity. Here, we postulate that agents that selectively target pericytes will selectively disrupt tumor vascular integrity and attenuate lymphoma growth. To test this hypothesis, we have treated both human diffuse large B-cell lymphoma in SCID mice and murine EL4 lymphoma in wild type mice with either a pharmacologic PDGFRβ inhibitor, imatinib mesylate, or a PDGFRβ-specific monoclonal antibody. Our data indicate that both agents compromise tumor vascular integrity, mainly by targeting vascular mural cells, thereby attenuating lymphoma growth.
MATERIALS AND METHODS

Cell Lines and Reagents
All culture media and reagents, with the exception of fetal bovine serum (FBS) (Hyclone, Logan, UT) and pericyte culture medium (ScienCell, Carlsbad, CA), were purchased from Mediatech Inc. (Manassas, VA). The human DLBCL cell line OCI-Ly7 was grown in 90% Iscove’s Modified Dulbecco’s medium (IMDM) and 10% FBS with penicillin/streptomycin (P/S), while DLBCL cell lines Karpas422 and Farage were grown in 90% RPMI 1640 and 10% FBS with P/S, L-glutamine and HEPES. The murine lymphoma cell line EL4 was grown in DMEM containing 10% horse serum with P/S. The rat vascular smooth muscle cell line A10 (ATCC) was grown in DMEM containing 10% FBS with P/S. The primary murine vascular smooth muscle cells (see Supplemental Material and Methods) were grown in DMEM containing 10% FBS with P/S, while the primary human brain pericytes were purchased from ScienCell and grown in its proprietary pericyte culture medium. All cell cultures were maintained at 37°C in a humidified incubator containing 5% CO2.

Cell Growth Inhibition Assays
PDGFRβ+ VSMCs and DLBCL cell lines were grown at concentrations sufficient to keep untreated cells in exponential growth over the drug exposure period. Cell viability was measured using a fluorometric resazurin reduction method (CellTiter-Blue, Promega, Madison, Wisconsin) and trypan blue dye-exclusion. Unless stated otherwise, the experiments were carried out in triplicate. The CompuSyn software package (Biosoft, Cambridge, UK) was used to plot dose-effect curves and determine the drug concentration that inhibits the growth of cells by 50% compared to control (GI50).
Mouse Lymphoma Models

All animal procedures were approved by the Institutional Animal Care and Use Committee of Weill Cornell Medical College. For human lymphoma xenograft experiments, 6-8-week-old SCID (NCI, Bethesda, MD) mice were injected subcutaneously with low-passage 1 x 10^7 human Farage, OCI-Ly7 or Karpas422 cells. Tumor size was measured with electronic digital calipers (Fisher Scientific, Waltham, MA), and tumor volume (mm^3) was calculated as length x width^2 x .52. When tumor volumes reached 75 to 100 mm^3, the mice were randomized to receive intraperitoneal (IP) injections of the following compounds for 2-3 weeks: imatinib (LC Laboratories, Woburn, MA) dissolved in PBS at 45 mg/kg twice daily, humanized anti-PDGFRβ antibody 2C5 (provided under a material transfer agreement with ImClone Systems, New York, NY) at 40 mg/kg three times weekly, PBS control, or human IgG control (Jackson ImmunoResarch Lab, West Grove, PA). For murine lymphoma allograft experiments, 6-8-week-old C57BL/6 mice (Jackson Laboratory, Bar Harbor, ME) were injected subcutaneously with low-passage 1.5 x 10^6 EL4 cells. When tumors reached 75 to 100 mm^3, the mice were randomized to receive either imatinib or PBS by IP injections for 2 weeks. In some animals, tumor microvasculature was visualized by staining endothelial cells with Alexa fluor-647-conjugated isolectin GS-IB4 (Invitrogen) injected via lateral tail vein 20 minutes prior to sacrifice. All mice were euthanized by CO2 inhalation at the time of study completion.

Immunohistochemistry

For frozen specimens, 8-10-μm cryosections were fixed in ice-cold acetone/methanol (1:1 v:v), and then subjected to standard color- or fluorescence-based staining procedures as described 28. For confocal microscopy, either 50-75- or 10-μm cryosections were incubated with rat anti-mouse CD31 mAb (clone MEC13.3; BD Pharmingen, San Diego, CA), PDGFRβ mAb (clone
APB5; eBioscience, San Diego, CA), phospho-PDGFR-β (sc-12908, Santa Cruz Biotechnology, Santa Cruz, CA), NG2 (Chemicon, Temecula, CA), CD68 (AbD Serotec, Raleigh, NC), Cy3-conjugated anti-α-SMA mAb (clone 1A4; Sigma-Aldrich, St. Louis, MO), or rabbit anti-cleaved caspase 3 mAb (clone 5A1E; Cell Signaling Technology, Danvers, MA) in various combinations overnight at 4°C, and then developed with Alexa Fluor 488-conjugated goat anti-rabbit or Alexa Fluor 555-conjugated goat anti-rat (Invitrogen). Apoptosis was detected on frozen tissue sections by TUNEL assay (Trevigen, Gaithersburg, MA).

**Image Analysis**

Stained sections were scanned and analyzed under a Zeiss Eclipse 80i microscope using Qimaging Retiga EX camera. A Zeiss LSM510 laser scanning confocal microscope was used to assess co-expression of cellular markers in the same cell. Immunofluorescence signals were captured by an argon laser emitting at 488 nm and a helium-neon laser emitting at 546 nm, and collected using proprietary Zeiss software. To quantify staining markers, an average of 5-10 representative fields were photographed at 100X and 200X magnification, and at least 2 sections at different depths were analyzed for each tumor sample. The CD31⁺, NG2⁺, and α-SMA⁺ area densities, as well as the TUNEL⁺ apoptosis index, were defined as the percent of pixels covered by each individual staining marker, and were scored using NIH ImageJ as previously reported 28.

**FACS Analysis**

Mononuclear cells were prepared from blood, bone marrow and tumor tissues. Tumor tissues were minced, digested with an enzyme cocktail containing 0.5% collagenase I (Worthington, 37°C, 1h), and filtered through a 30-μm strainer to isolate single-cell suspensions. For each flow reaction, $0.2 - 1.0 \times 10^6$ mononuclear cells were pre-treated with Fc block (CD16/CD32, BD Pharmingen, San Diego, CA), and then labeled with the following antibodies: anti-human CD45-
FITC, anti-mouse CD45-PerCP, anti-mouse VEGFR2-PE, anti-mouse CD31-APC, anti-mouse CD117-APC (all from BD Pharmingen), and anti-mouse CD140b-PE (eBioscience, San Diego, CA). Controls included unstained samples, samples stained with isotype-specific antibodies, and BD CompBeads. Analyses were performed in a Cytomics FC500 flow cytometer (Beckman Coulter, Brea, CA), and data analyzed using FlowJo software (Treestar, Ashland, Oregon).

**Immunoblotting**

After a period of serum deprivation, PDGFRβ⁺ primary murine vascular smooth muscle cells, primary human brain pericytes, or A10 cells were pretreated with imatinib (LC Laboratories) for 90 min and then stimulated with 50 ng/ml of recombinant PDGF-BB (Peprotech, Rocky Hill, NJ and R&D Systems, Minneapolis, MN) for 10 min. Cell lysates were then subjected to SDS-polyacrylamide gel electrophoresis and immunoblotting with antibodies against PDGFRβ (Cell Applications, San Diego, CA), phospho-PDGFRβ (Santa Cruz Biotechnology), PDGFRα (Santa Cruz Biotechnology), phospho-PDGFRα (Abcam, Cambridge, MA), c-Kit (Santa Cruz Biotechnology), phospho-c-Kit (Abcam, Cambridge, MA), c-Abl (Santa Cruz Biotechnology), phospho-c-Abl (Cell Signaling Technology, Boston, MA), ERK1/2, phospho-ERK1/2, Akt, and phospho-Akt (all from Cell Signaling Technology). Immunoblots were developed using the ECL kit (PerkinElmer, Boston, MA).

**PDGFRβ small interfering RNA knockdown**

Primary human brain vascular pericytes (HBVP) and human DLBCL cell lines (Karpas422 and Farage) were transfected with either a siRNA targeting PDGFRβ (s10241, Invitrogen Life Technologies, Grand Island, NY) or a non-targeting siRNA (control) using Lipofectamine (Promega) or the Amaxa Nucleofector kit (Lonza, Basel, Switzerland), respectively.
Transfection efficiency, assessed by co-transfection of a GFP-containing plasmid, was 80-95% for HBVP, and 55-65% for DLBCL cells. Viable cells were enumerated by hemocytometer at 24, 48 and 72 h after transfection using trypan blue dye exclusion. PDGFRβ mRNA levels were determined using the ΔΔCT RT-qPCR method employing forward (GTTGGGCGAAGGTTACAAAA) and reverse (GGGTGTCACCTCTCAGAAA) primers, and RPL13A as an internal control, and normalized to baseline expression. All experiments were repeated at least once with triplicate samples.

**Biostatistical Analysis**

All data were considered continuous and summarized using means and standard deviations. Differences between and among experimental groups were assessed using t-test and analysis of variance (ANOVA), respectively. Non-parametric methods such as the Wilcoxon rank sum test and Kruskal – Wallis test were used when the underlying assumptions for t-test and ANOVA were violated. All p-values are two-sided with significance evaluated at the 0.05 alpha level.
RESULTS

Imatinib impaired growth of experimental lymphomas

Using an established xenograft model, human Farage, OCI-Ly7, and Karpas422 diffuse large B-cell lymphoma (DLBCL) cell lines were implanted in SCID mice, which were then treated twice daily with IP injections of either imatinib mesylate at 45 mg/kg or vehicle PBS for 2-3 weeks. Imatinib doses of up to 100 mg/kg twice daily (PO or IP) in mice result in mean peak and trough plasma levels of 4.6–6 μM and 1–1.5 μM, respectively, which are similar to those reported for humans receiving a mid-range dose of 400 mg once daily.

Compared to PBS, treatment with imatinib significantly impaired tumor growth, based on tumor volume (p=0.00015, 0.0022, and 0.022, respectively), and tumor weight (p=0.018, 0.0074, and 0.034, respectively) at the time of harvest (Figure 1A-F). Histological analysis revealed an increased fraction of TUNEL-positive, apoptotic cells in the imatinib treated lymphomas, in addition to central necrosis, which was present in all xenografts regardless of treatment (Supplemental Figure 1).

Imatinib disrupted tumor-associated microvasculature

To determine whether apoptosis was associated with impaired angiogenesis, Farage tumors were stained with both anti-cleaved-caspase 3, a marker for apoptosis, and anti-CD31 (PECAM), an endothelial marker. In addition, isolectin GS-IB4 was infused systemically to assess tumor vessel patency (Figure 1G-L). Compared to PBS treatment, administration of imatinib was strongly associated with loss of microvascular density and patency (Figure 1J-L). In imatinib-treated samples, furthermore, differential interference contrast confocal microscopy revealed
patchy areas of isoelectin leakage associated with fragmented structures suggestive of disrupted microvessels (Figure 1K).

Anti-PDGFRβ staining of Farage tumors showed that PDGFRβ+ pericytes formed tubular structures surrounding blood vessels in PBS-treated tumors, but were less organized and distinct in imatinib-treated tumors, and nearly absent within areas of tumor cell death (Figure 1M-N). Confocal analysis revealed the presence of anti-cleaved caspase 3 staining within remaining PDGFRβ+ pericytes (Figure 1O), suggesting that the dropout of pericytes in imatinib-treated samples was associated with the activation of an apoptotic pathway. Endothelial apoptosis and disintegration was evident in areas of disrupted pericyte network (Figure 1P). These data suggested that imatinib treatment led to microvascular disruption and apoptotic cell death of PDGFRβ+ pericytes.

To determine whether pericyte loss was a general consequence of imatinib treatment of experimental lymphomas, we examined Karpas422 and OCI-Ly7 tumors. Anti-α-SMA and NG2 staining revealed shorter, narrower, and more flow-impaired microvessels surrounded by apoptotic pericytes (Figure 2A-C). Loss of pericytes was associated with an overall reduction in tumor vascularity (Figure 2D-F). In contrast, the vasculature of organs not involved with tumor, including lung, heart, liver and kidney, in imatinib-treated mice showed normal histology and no increase in apoptosis (not shown).

To determine whether immune deficits in the SCID mouse contributed to tumor susceptibility to imatinib, we studied a second model of lymphomagenesis. In the immunologically intact EL4 lymphoma-bearing C57Bl/6 mouse, imatinib treatment was associated with both impaired tumor growth, based on assessment of mass (Figure 3A; p=0.036), and decreased pericyte coverage
with reduced vascularization, based on immunohistologic staining using three separate mural cell markers and an endothelial cell marker CD31 (Figure 3B-C; p=0.0007 for NG2, p=0.0052 for PDGFRβ, p<0.0001 for α-SMA, and p<0.0001 for CD31). Thus, the effect of imatinib on tumor vascularity is not a reflection of immunologic deficiency.

**Imatinib reduced lymphoma-associated vascular cells**

To quantify more precisely the impact of imatinib on tumor vasculature, fluorescence activated cell sorting (FACS) was carried out using single cell suspensions isolated from experimental lymphomas. In untreated human and murine experimental lymphomas, non-tumor stromal cells represented up to 15% of the total cell population. In EL4 tumors propagated in wild type mice, pericytes, defined as CD45-CD11b-CD31-PDGFRβ+, comprised 2.61 ± 0.25% of the total, while endothelial cells, defined as CD45-CD11b-CD31+, represented 0.42 ± 0.07%, and CD45+CD11b+ inflammatory cells, 8.66 ± 2.05% (Supplementary Figure 2). Of note, less than 5% of total endothelial cells, and less than 1% of total inflammatory cells, expressed PDGFRβ.

Upon imatinib treatment, populations of mature VEGFR2+CD31+ endothelial cells, mature PDGFRβ+ vascular stromal cells, and c-Kit+ endothelial and pericyte cell progenitors all declined in both murine (Figure 4A-B) and human lymphomas (Supplemental Figure 3), whereas myeloid cells in both tumor types remained stable. In blood and bone marrow, on the other hand, these cell populations were not affected (not shown). Thus, recruitment, retention, and maturation of tumor endothelial and pericyte progenitors may require a microenvironment conditioned by intact PDGFRβ+ stromal cells.
Imatinib targeted tumor vasculature by inhibition of PDGFRβ signaling in mural cells.

Primary murine VSMCs, unlike human DLBCL or murine EL4 cells, expressed PDGFRβ in a uniformly robust fashion in both FACS and immunoblot assays (Figure 5A-B), and were highly sensitive to imatinib (Figure 5C, GI₅₀ of 3.9 μM). In contrast, primary microvascular endothelial cells, less than 1% of which expressed PDGFRβ, were resistant to imatinib (Figure 5C, GI₅₀ of 52 μM). Similarly, Farage, Karpas422, OCI-Ly7, and EL4 lymphoma cell lines all failed to express PDGFRβ (Figure 5A-B) and displayed GI₅₀ values (18-296 μM) that far exceeded therapeutic plasma levels of imatinib reported in mice (Figure 5C). The variable responses to imatinib in lymphoma cell lines may reflect the effect of imatinib on cellular survival pathways unrelated to PDGFRβ.

Next, we explored whether the sensitivity of vascular mural cells to imatinib was PDGFRβ-dependent. First, we noted that PDGFRβ⁺ VSMC adopted a narrow or rounded appearance, and became cleaved-caspase 3-positive following imatinib treatment (Supplemental Figure 4). Second, PDGFRβ-specific siRNA reduced expression of PDGFRβ mRNA in human brain vascular pericytes (HBVP) by 72% within 72 hours, and rendered them resistant to imatinib-mediated cell death (Figure 5D-E). Karpas422 lymphoma cells, on the other hand, remained insensitive to imatinib after transfection with PDGFRβ siRNA (Figure 5E). Third, while knockdown of PDGFRβ had no effect on the growth of Karpas422 and Farage human lymphoma cell lines, it specifically impaired growth of cultured human brain vascular pericytes (Figure 5F). Finally, we noted that NG2-positive pericyte-like cells expressed anti-phospho-PDGFRβ reactive material in PBS-treated tumors, but not in imatinib-treated tumors (Figure 5G). These data suggest that the therapeutic effect of imatinib in experimental lymphoma...
derives mainly from its effect on pericytes, rather than endothelial cells or lymphoma cells themselves.

**Imatinib regulated multiple signaling pathways downstream of PDGF-BB in vascular mural cells**

We employed several approaches to examine the mechanism by which imatinib impaired PDGFRβ signaling in vascular mural cells. In imatinib-treated murine VSMCs, transcriptional profiling identified 36 and 276 genes displaying higher and lower expression, respectively (**Supplemental Table 2**). Upon Ingenuity Pathway Analysis, the regulated genes clustered into 11 networks, predominant among which were several related to PDGFRβ signaling and activation of downstream mediators, including PI3K/AKT and MAPK/ERK1/2 (**Supplemental Table 3**) \(^{37}\). In addition, we noted dramatic down-regulation of VEGF and TGF-β pathways essential for endothelial cell proliferation, survival, and vascular integrity (**Supplemental Figure 5A,B**) \(^{38}\). Validity of these findings was confirmed for selected genes by real time PCR conducted on cultured primary murine vascular smooth muscles cells harvested 24 and 48 h after imatinib treatment and on FACS-sorted, CD45-PDGFRβ⁺ lymphoma-associated stromal cells 72 h after imatinib treatment (**Supplemental Figure 5C, panels 1 and 2**, respectively). Reduction in smooth muscle cell production of VEGF protein, and smooth muscle and endothelial cell production of TGFβ were confirmed by ELISA (**Figure 6A-B**). Together, these data indicate that imatinib induced decreases in expression of both PDGF pathway and non-PDGF pathway genes that govern vascular integrity.
To define the PDGFRβ-related signaling pathways impacted by imatinib in vascular mural cells, we conducted a series of phospho-immunoblot analyses (Fig. 6C). At baseline, rat embryonic vascular smooth muscle cells (A10)\textsuperscript{39}, primary murine vascular smooth muscle cells (MVSMC), and primary human brain vascular pericytes (HBVP) all expressed PDGFRβ, PDGFRα, c-Kit, c-Abl, AKT and MAPK. In a dose-dependent fashion, imatinib decreased PDGF-BB-mediated phosphorylation of cell surface receptors PDGFRβ, PDGFRα, and c-Kit, as well as intracellular signaling mediators, AKT and MAPK, while total expression of these proteins remained unchanged. Phosphorylation of c-Abl, on the other hand, was not detected in response to PDGF-BB, and, therefore, not impacted by imatinib in any of the mural cell types tested. These data reveal that multiple downstream signaling pathways, active in PDGFRβ\textsuperscript{+} mural cells, could be impacted by imatinib.

**Antibody blockade of PDGFRβ disrupted lymphoma vascular integrity in vivo.**

Finally, we asked whether selective blockade of PDGFRβ signaling could attenuate lymphoma growth in vivo. SCID mice bearing Farage and Karpas422 xenografts were treated with the monoclonal antibody 2C5, which blocks both human and murine PDGFRβ signaling. Compared to controls treated with either PBS or human IgG, 2C5 significantly inhibited Farage tumor growth, as assessed by tumor volume (p=0.0045) and weight (p=0.01) (Figure 7A-B). Immunohistochemical analysis revealed that 2C5 depleted pericytes to 6% of the control level, whereas imatinib reduced pericytes to 26% of control (Figure 7C). Microvascular density, as assessed by CD31 staining, decreased with 2C5 treatment compared to control (p=0.04), although the degree of vascular attenuation with 2C5 treatment was less pronounced compared to imatinib (p=0.065). Interestingly, while 2C5 did not significantly inhibit the growth of
Karpas422 lymphoma, it did induce a significant reduction of pericyte and vascularization (p=0.005), which mirrored the effects seen in Farage tumors (not shown).

Because hematopoietic cells have been reported to contribute to tumor angiogenesis, we next identified perivascular CD68+ hematopoietic myeloid cells, which intermingled with NG2+ pericytes, in Farage (Figure 7D) and Karpas 422 tumors (not shown) by immunostaining. Following imatinib treatment, CD68+ cells became diffusely scattered as vascular organization was disrupted (Figure 7D2). In contrast, tumors treated with 2C5 retained abundant perivascular CD68+ cells (Figure 7D3). These data suggest that both pericytes and myeloid cells may contribute to vascular stability, and that, when PDGFRβ+ pericytes are selectively depleted, CD68+ hematopoietic cells might serve as surrogate mural cells.
DISCUSSION

Our study demonstrates for the first time that blockade of PDGFRβ impairs lymphoma growth by depleting vascular mural cells, disrupting tumor neovascular integrity, and reducing endothelial cell survival. Treatment with either a semi-selective receptor tyrosine kinase inhibitor, imatinib mesylate, or a highly selective, PDGFRβ-specific monoclonal antibody impaired lymphoma growth dramatically without inducing systemic toxicity. Perivascular PDGFRβ+ pericyte-like stromal cells appear to be the major treatment target, providing proof of concept for future anti-lymphoma therapy that might combine both anti-tumor and anti-stromal cell strategies.

Here, imatinib mesylate impaired the growth of lymphoma in both human xenograft and murine allograft models. Inhibition of lymphoma growth correlated with loss of vascular integrity and perivascular apoptosis within tumor tissue. These data suggest that the tumor vasculature is a major target for imatinib. Indeed, treatment with imatinib had a direct anti-proliferative effect on primary murine PDGFRβ+ vascular smooth muscle cells, but not endothelial cells, in vitro, and inhibited PDGFRβ activation in lymphoma microvascular pericytes in vivo. Because the lymphoma cell lines tested neither expressed PDGFRβ nor responded with significant growth inhibition when challenged with imatinib in vitro, it is unlikely that these cells represented its primary target in vivo. Similarly, imatinib had no effect on the survival of stromal inflammatory cells in vivo. On the other hand, both VSMC and pericytes expressed PDGFRβ and exhibited high sensitivity to imatinib. Moreover, knockdown of PDGFRβ expression in vascular pericytes protected them from imatinib-mediated growth inhibition, identifying PDGFRβ as a primary target in these cells.
Analysis of gene expression before and after imatinib provided insight into the potential mechanism of the drug’s action in vascular mural cells. A majority of the 312 differentially expressed genes (88%) were down-regulated, and reflected overall anti-proliferative and pro-apoptotic effects. The top scored networks included genes regulated directly by PDGF-BB and PDGFRβ, suggesting that disruption of PDGFRβ signaling was the primary action of imatinib. In addition, perturbation of proteolytic balance, manifested by increased expression of metalloproteinases, such as MMP3, MMP12 and MMP13, and decreased expression of proteinase inhibitors, such as TIMP1 and PAI-1, may have further destabilized the vascular integrity due to heightened turnover of extracellular matrix 40,41. In evaluating other potential effects of imatinib in VSMC or pericytes, we observed inhibition of PDGF-BB-dependent phosphorylation of PDGFRβ, PDGFRα, and c-Kit, possibly extending its usefulness to a broader range of stromal targets.

In addition to directly interfering with pericyte assembly and survival, imatinib appeared to deplete endothelial cells within the tumor stroma via an indirect pathway. Primary mature microvascular endothelial cells, the majority of which did not express PDGFRβ, were resistant to growth inhibition in the presence of therapeutic concentrations of imatinib. By down-regulating the expression and/or secretion of pericyte-derived proangiogenic growth factors such as VEGF and TGF-β, imatinib might, in an indirect manner, also induce secondary endothelial apoptosis and dropout. Sequential loss of tumor vessel pericytes, followed by endothelial cell dropout, in response to inhibition of PDGF-BB and PDGFRβ has been reported in solid tumor models as well 24,25,42. Because imatinib reduced vascular cell populations only in the tumor compartment, and not in the bone marrow or blood, we conclude that its therapeutic effect reflected
suppression of local recruitment, proliferation and differentiation of progenitors, through blockade of PDGFRβ and, possibly, c-Kit signaling.

Selective PDGFRβ blockade with the monoclonal antibody 2C5 had a highly significant, and more focused anti-angiogenic effect on lymphoma xenografts. Compared to imatinib, treatment with 2C5 was associated with statistically equivalent suppression of Farage tumor growth, and even more dramatic suppression of pericyte numbers. Interestingly, 2C5 had a less dramatic effect on angiogenesis attenuation in Karpas422 lymphoma, and a modest effect on tumor growth inhibition. The potential divergence of anti-angiogenic and anti-tumor effects might reflect varying angiogenic adaptations by individual lymphomas. For example, the differential recruitment of perivascular myeloid CD68+ cells observed upon 2C5, but not imatinib, treatment suggests that a PDGFRβ-independent, but imatinib-sensitive, pathway may partially compensate for the profound pericyte loss caused by PDGFRβ blockade in some tumors. By targeting a broader range of stromal targets, including PDGFRα+ activated fibroblasts and c-fms+ myelomonocytic cells37,43, imatinib may potentially deliver more effective anti-vascular and anti-tumor effect by antagonizing multiple angiogenesis-dependent and angiogenesis-independent pathways43,44. In addition, individual lymphomas may possess distinct resistance mechanisms; inhibition of PDGFRβ targeting may have minimal impact on tumors that overexpress VEGF45, as high levels of VEGF may sustain endothelial proliferation and survival in the absence of pericytes. Finally, pericyte dysfunction and loss have been reported to lead to unexpectedly aggressive growth of some tumors with local invasion and distant metastasis both in a mouse model and in human patients46,47.
In conclusion, this study provides evidence that the pericyte, through its dependence on PDGFRβ signaling, represents a high-value, anti-angiogenesis therapeutic target in experimental mouse lymphoma. This work also alludes to the possible role of additional imatinib-sensitive stromal cell types in sustaining the tumor vasculature and growth. Finally, our data suggest that a multifocal attack, aimed at multiple stromal cell types within the tumor microenvironment, could enhance the therapeutic efficacy of treatment strategies directed toward lymphoma cells alone, thus achieving a more complete and durable response.
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AUTHORSHIP CONTRIBUTIONS AND DISCLOSURE OF CONFLICTS OF INTEREST

**Jia Ruan** wrote the manuscript, designed the studies, coordinated the logistics, and performed the experiments and data analysis on the mouse xenograft studies and immunohistochemical staining.

**Min Luo, Chunjie Wang, Lei Fan, Shao Ning Yang and Mariano Cardenas** performed experiments.

**Humin Geng** performed microarray data analysis.

**John P. Leonard** and **Ari Melnick** reviewed the manuscript and coordinated logistics.

**Katherine A. Hajjar and Leandro Cerchietti** contributed critically to the experimental design, coordination of logistics, interpretation of the data, and writing of the manuscript.

The authors declare they have no conflicts of interest related to this study.
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FIGURE LEGENDS

Figure 1. Imatinib impaired growth of human lymphoma xenografts by disruption of tumor-associated microvasculature.  A-C: Tumor growth curves based on tumor volumes (mm$^3$), comparing imatinib mesylate treatment (red) vs. PBS control (black).  D-F: Tumor weight (g) at the time of tissue harvest, comparing imatinib (red) vs. PBS (black).  G-I: Microvessels delineated by CD31 stain (red) in PBS- (G) and imatinib-treated (H) tumors, and microvessel density normalized to PBS control (I).  J-L: Confocal DIC capture of functional vascular flow measured by isolectin (red), and normalized to PBS control.  M-P: Confocal analysis of pericyte marker staining in tumors.  Blue marks functional vascular flow measured by isolectin, red outlines PDGFR$\beta^+$ pericytes, while green shows cleaved caspase 3 in apoptotic cells.  M: PBS-treated tumor. N: Imatinib-treated tumor, which displays microvascular disintegration and flow leakage (white arrows), and patchy areas of tumor apoptosis/necrosis.  O: White arrows indicate apoptotic, yet structurally intact, PDGFR$\beta^+$ cells in close proximity to functional flow.  P: White arrows mark apoptosis of isolectin$^+$ endothelial cells, and white asterisk (*) indicates leakage of infused intravascular isolectin. Scale bar = 50 $\mu$m.

Figure 2. Imatinib treatment of human DLBCL tumors was associated with pericytic dropout and disruption of microvasculature.  A1-4: Analysis of $\alpha$-SMA$^+$ pericytes and CD31$^+$ vessels in Karpas422 (A1,3) and OCI-Ly7 (A2,4) xenografts.  B1-4: Analysis of NG2$^+$ pericytes and CD31$^+$ vessels in Karpas422 (B1,3) and OCI-Ly7 (B2,4) xenografts.  C: Confocal analysis of pericytes (red) in relation to intraluminal blood flow (isolectin, blue) in OCI-Ly7 tumors.  C1-2: Two z-stack images focused at different depths within the same tissue; white arrows indicate disrupted microvessels with scanty PDGFR$\beta^+$ staining and abundant cleaved-caspase 3$^+$, apoptotic nuclei; white asterisks (*) indicate microvessels with relatively intact blood flow.
flow and perivascular PDGFRβ+ staining. C3-4: White arrows indicate apoptotic α-SMA+ pericytes surrounding regions of functional blood flow. D: Pericyte coverage was quantified as α-SMA+ area (red), and normalized to the PBS control. E: Pericyte coverage was quantified as NG2+ area (green), and normalized to the PBS control. F: Microvessel density was quantified as CD31+ staining area (blue), and normalized to the PBS control. “*” denotes p<0.05, compared with control. Scale bar = 50 μm.

Figure 3. Imatinib treatment of murine EL4 tumors impaired growth, decreased pericyte coverage, and reduced vascularization. A: Tumor weight (g) at the time of tissue harvest, comparing imatinib (red) vs. PBS (black). B: Analysis of pericytes with NG2 (1,2), PDGFRβ (3,4), α-SMA (5,6) staining, and CD31+ vessels in EL4 tumors, in response to either imatinib or PBS. C: Microvessel density was quantified as CD31+ staining density, and normalized to the PBS control. Pericyte coverage was quantified as NG2+, PDGFRβ+ or α-SMA+ area, and normalized to the PBS control. “*” denotes p<0.05. Scale bar = 50 μm.

Figure 4. FACS analysis of lymphoma vascular cell populations. A: Quad-gate analysis of the EL4 tumor-associated cells. Murine EPC (mCD45−VEGFR2+CD117+), mature EC (mCD45−VEGFR2+CD31+), murine PPC (mCD45−CD140b+CD117+), and mature PC (mCD45−CD140b+ NG2+) are shown. Blue-dotted squares outline individual quad-gates for EPC, PPC, EC and PC. B: Quantification of stromal cell populations in EL4 tumors treated with imatinib vs. PBS. “*” denotes p<0.05, compared with control.

Figure 5. Imatinib inhibited PDGFRβ signaling in vascular mural cells. A: FACS analysis of PDGFRβ expression in VSMC and lymphoma cells. B: Immunoblot analysis of PDGFRβ protein expression in stromal and lymphoma cells. C: Imatinib-mediated growth inhibition in
VSMC, endothelial cells, and lymphoma cells.  

**D:** Relative abundance of PDGFRβ mRNA at 24 h, 48 h, and 72 h following transfection of siRNA #1 targeting PDGFRβ in human brain vascular pericytes (HBVP), and expressed as the percentage normalized to expression level at 24 h post transfection.  

**E:** Viable cell counts at 48 h after treatment with imatinib (20 μM for HBVP, 50 μM for Karpas422), following transfection with siRNA for 24 h in HBVP and Karpas422 cells. All cell counts were normalized to values at 24h post siRNA transfection.  

**F:** Viable cell counts at 24 h, 48 h, and 72 h following transfection of siRNA non-targeting (NT) or PDGFRβ-directed siRNA #1 in HBVP, Karpas422, and Farage DLBCL cell lines, and normalized to the values at 24 h post transfection. Data are representative of triplicate experiments in C-F.  

**G:** Immunostaining for phospho-PDGFRβ in NG2+ pericytes in Karpas422 tumors treated with either PBS (1) or imatinib (2). White arrow indicates expression of phospho-PDGFRβ in NG2+ pericytes. PDGFRβ signaling was quantified as phospho-PDGFRβ+ area, and normalized to PBS control (3). “*” denotes p<0.05, compared with control. Scale bar = 50 μm.

**Figure 6. Imatinib blocked angiogenic pathways in vascular mural cells.**  

**A:** Effect of imatinib on VEGF secretion from VSMC at 3h, 24h and 48h post treatment.  

**B:** Effect of imatinib on TGF-β secretion from VSMC (1) and cardiac microvascular endothelial cells (CMEC) (2) at 48h post treatment.  

**C:** Effect of imatinib on PDGF-BB induced phosphorylation of PDGFRα, PDGFRβ, c-Kit, c-Abl, AKT, and ERK1/2 by immunoblot of vascular smooth muscle cells and pericytes in culture.

**Figure 7. Anti-PDGFRβ antibody 2C5 inhibited growth of Farage xenografts in SCID mice.**  

**A:** Tumor growth curves based on tumor volumes (mm^3) for PBS or hIgG control (black squares), imatinib (red circles) and 2C5 (blue triangles) treatments.  

**B:** Tumor weight (g) at the
time of tissue harvest, comparing 2C5 (blue) vs. imatinib (red) vs. control (black). Inset photo depicts gross morphology of the resected tumor xenografts treated with control (1), imatinib (2) and 2C5 (3).  C: Staining of endothelial cells (CD31, red) and pericytes (NG2, green) in Farage xenografts treated with control (1), imatinib (2) and 2C5 (3). NG2⁺ pericytic density (C4) and CD31⁺ endothelial density (C5) were normalized to their respective controls.  D: Confocal analysis of pericytes in Farage tumors treated with control (1), imatinib (2) and 2C5 (3). Blue marks functional vascular flow by isolectin staining; red outlines NG2⁺ pericytes; and green stains CD68⁺ myelomonocytic cells. White arrows indicate perivascular CD68⁺ cells in close association with microvessels. “*” denotes p<0.05, compared with control. Scale bar = 50 μm.
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Imatinib disrupts lymphoma angiogenesis by targeting vascular pericytes

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