Treatment-induced arteriolar revascularization and miR-126 enhancement in bone marrow niche protect leukemic stem cells in AML

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Abstract

Background: During acute myeloid leukemia (AML) growth, the bone marrow (BM) niche acquires significant vascular changes that can be offset by therapeutic blast cytoreduction. The molecular mechanisms of this vascular plasticity remain to be fully elucidated. Herein, we report on the changes that occur in the vascular compartment of the FLT3-ITD+ AML BM niche pre and post treatment and their impact on leukemic stem cells (LSCs).

Methods: BM vasculature was evaluated in FLT3-ITD+ AML models (MllPTD/WT/Flt3ITD/ITD mouse and patient-derived xenograft) by 3D confocal imaging of long bones, calvarium vascular permeability assays, and flow cytometry analysis. Cytokine levels were measured by Luminex assay and miR-126 levels evaluated by Q-RT-PCR and miRNA staining. Wild-type (wt) and MllPTD/WT/Flt3ITD/ITD mice with endothelial cell (EC) miR-126 knockout or overexpression served as controls. The impact of treatment-induced BM vascular changes on LSC activity was evaluated by secondary transplantation of BM cells after administration of tyrosine kinase inhibitors (TKIs) to MllPTD/WT/Flt3ITD/ITD mice with/without either EC miR-126 KO or co-treatment with tumor necrosis factor alpha (TNFα) or anti-miR-126 miRisten.

Results: In the normal BM niche, CD31+Sca-1high ECs lining arterioles have miR-126 levels higher than CD31+Sca-1low ECs lining sinusoids. We noted that during FLT3-ITD+ AML growth, the BM niche lost arterioles and gained sinusoids. These changes were mediated by TNFα, a cytokine produced by AML blasts, which induced EC miR-126 downregulation and caused depletion of CD31+Sca-1high ECs and gain of CD31+Sca-1low ECs. The impact of miR-126 on LSC activity was evaluated by secondary transplantation of BM cells after administration of tyrosine kinase inhibitors (TKIs) to MllPTD/WT/Flt3ITD/ITD mice with/without either EC miR-126 KO or co-treatment with tumor necrosis factor alpha (TNFα) or anti-miR-126 miRisten.

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miR-126 supply to LSCs. High miR-126 levels safeguarded LSCs, as shown by more severe disease in secondary transplanted mice. Conversely, EC miR-126 deprivation via genetic or pharmacological EC miR-126 knock-down prevented treatment-induced BM miR-126high EC expansion and in turn LSC protection.

**Conclusions:** Treatment-induced CD31+ Sca-1high EC re-vascularization of the leukemic BM niche may represent a LSC extrinsic mechanism of treatment resistance that can be overcome with therapeutic EC miR-126 deprivation.

**Keywords:** Acute myeloid leukemia, BM vascular niche, TNFα, miR-126, Leukemic stem cell, Treatment resistance

**Background**

Acute myeloid leukemia (AML) is a hematopoietic malignancy characterized by somatic mutations occurring in the hematopoietic stem cells (HSCs) and progenitor cells that block hematopoietic differentiation and promote accumulation of leukemic “blasts” in the bone marrow (BM) and/or other extramedullary organs [1]. To date, despite a deep molecular understanding of the pathogenesis, the development of molecular targeting therapeutics and the broadened use of allogeneic HSC transplantation, the overall outcome of AML patients remains poor. Disease refractoriness to initial therapy or post-remission disease relapse [2] are likely due to persistence of treatment-resistant leukemic stem cells (LSCs) [3]. These are primitive leukemic cells capable of unlimited self-renewal and disease initiation [4, 5] and reside in a leukemic BM niche that also comprises several types of non-hematopoietic cells and that preferentially supports homeostasis and competitive growth of LSCs over those of HSCs [6, 7].

Mechanisms of treatment resistance in cancer are multifaceted and often result from the acquisition of genetic mutations that enable malignant cells to escape the therapeutic pressure. Recently, other non-genetic mechanisms of treatment resistance have been also described [8]. While these reported mechanisms have been mainly reported as intrinsic to malignant cells, it is possible that they also include mechanisms that are extrinsic to malignant cells, such as those involving the microenvironment and that protect malignant cells during treatment exposure [7].

Utilizing the FMS-like tyrosine kinase 3 (FLT3) gene internal tandem duplication (ITD) (FLT3-ITD) knock-in mouse and FLT3-ITD+ AML patient-derived xenograft (PDX) models that recapitulate features of human FLT3-ITD+ AML, we report here on previously unrecognized non-genetic, extrinsic mechanisms of treatment resistance in LSCs that involve the vascular compartment of the leukemic BM niche and that are mediated by a TNFα-miR-126 axis in the BM endothelial cells (ECs). FLT3-ITD occurs in approximately 25% of AML patients and the mutated gene encodes a mutant receptor with aberrant, ligand-independent tyrosine kinase (TK) activity that confers growth and survival advantages to leukemic blasts [9]. FLT3-ITD+ AML patients are treated with TK inhibitors (TKIs) in combination with chemotherapy. Although the addition of TKIs to chemotherapy confers a clinical advantage compared to chemotherapy alone, it is not curative in the majority of cases, suggesting treatment resistance arising over time [10].

**Methods**

An extended description of the methods is in the Additional file 1.

**Human samples**

Normal peripheral blood (PB) and BM samples were obtained from healthy donors at the City of Hope National Medical Center (COHNMC). AML samples were obtained from patients from the COHNMC (Additional file 1: Table S1). Mononuclear cells (MNCs) were isolated using Ficoll separation. When necessary, CD34+ cells were then isolated using a positive magnetic bead selection protocol (Miltenyi Biotech, Germany). Sample acquisition was approved by the Institutional Review Boards at the COHNMC, in accordance with an assurance filed with and approved by the Department of Health and Human Services and met all requirements of the Declaration of Helsinki. Healthy donors and AML patients were consented on the IRB # 06229 and IRB# 18067 protocols, respectively.

**Animal studies**

The MllPTD/WT/Flt3ITD/ITD mouse, an AML model in C57Bl/6 (B6) background (CD45.2), was generated and genotyped as previously described [11]. The MllPTD/WT/Flt3ITD/ITD mice (CD45.2, B6) were also bred with CD45.1 B6 mice to produce CD45.1/CD45.2 MllPTD/WT/Flt3ITD/ITD mice as donors for transplant experiments. To obtain conditional miR-126 knock-out (KO) or Spred1 KO in ECs, we bred miR-126fl/fox (CD45.2 B6) [12] and Spred1fl/fox (CD45.2 B6) [13] mice with Tie2cre (CD45.2 B6, from The Jackson Laboratory, 008863) mice and obtained miR-126fl/foxTie2cre+ (EC miR-126 KO, also called miR-126ECΔ/Δ) and Spred1fl/foxTie2cre+ (EC Spred1 KO, also called Spred1ECΔ/Δ, representing a functional EC miR-126 overexpressing model) mice.
miR-126flox/flox/Tie2-cre CD45.1 B6) mice were transplanted into Cre or Cre+ tamoxifen-treated EC miR-126 KO reporter mice [i.e., miR-126 flox/flox/Tie2-cre]. To obtain conditional EC miR-126 KO reporter mice, we also bred Tie2-CreER/TdTomato/Tg(Ly6a-GFP) double fluorescent reporter mice with miR-126 flox/flox mice and obtained inducible EC miR-126 KO reporter mice [i.e., miR-126 flox/flox/Tie2-CreER/TdTomato/Tg(Ly6a-GFP)], miR-126 KO in ECs upon tamoxifen administration.

To evaluate the impact of EC miR-126 on leukemia-induced vascular changes and in turn on LSC burden, BM MNCs from AML (i.e., Mll PTD/WT/Flt3 ITD/ITD, CD45.1/CD45.2 B6) or from normal wild-type (wt, CD45.1 B6) mice were transplanted into Cre+ or Cre-miR-126 flox/flox/Tie2-cre, Spred1 flox/flox/Tie2-cre, and tamoxifen-treated miR-126 flox/flox/Tie2-CreER/TdTomato/Tg(Ly6a-GFP) reporter mice (all CD45.2 B6). To study the vascular changes induced by human FLT3-ITD+ AML blast growth, NOD.Cg-Prkdc scid Il2rgtm1Wjl Tg(CMV-IL3, CSF2, KITLG)1Eav/MloySzJ mice (NSG-GFP and NSGS, from The Jackson Laboratory, 013062) were transplanted with normal cord blood (CB) CD34+ cells or with human FLT3-ITD+ AML blasts (patient-derived xenograft, PDX).

**Immunofluorescent staining and 3D confocal imaging of long bones**

Long bones (femurs and/or tibias) from the mice were processed, sectioned and imaged as described previously [16] with ad-hoc modifications (see Additional file 1 for details).

**Intravital imaging**

Intravital confocal microscopy was used to image the calvarium BM vasculature to study the vascular permeability, as previously described [17] (see Additional file 1 for details).

**Statistical analysis**

Comparison between groups was performed by two-tailed, paired or unpaired Student's t-test, adjusting for multiple comparisons as appropriate. The log-rank test was used to assess significant differences between survival curves. All statistical analyses were performed using Prism version 8.0 software (GraphPad Software). Sample sizes chosen are indicated in the individual figure legends and were not based on formal power calculations to detect prespecified effect sizes but were based on previous experience with similar models. All of the in vitro experiments were performed 3–6 times using biologically independent samples; the in vivo experiments were performed using 6–16 mice in each group. p values <0.05 were considered significant. Results shown represent mean±SEM. *p ≤ 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

**Results**

**Bone marrow vasculature of normal and leukemic mice**

To determine how the vascular compartment of the BM niche adapts to leukemia growth and subsequently to antileukemic treatments, we performed immunofluorescence staining and 3D confocal imaging of vessels in the long bones of normal versus (vs) leukemic mice. To this end, we first analyzed normal mice and identified CD31+α-SMA+Sca-1 high Endomucin (Emcn)– vessels as arteries (Additional file 1: Fig. SIa–c, blue arrow), CD31+α-SMA−Sca-1 low Emcn+ vessels as arterioles (Additional file 1: Fig. SIa–c, yellow arrow), and CD31+α-SMA−Sca-1 low Emcn+ vessels as sinusoids (Additional file 1: Fig. SIa–c, white arrow) [18–21]. In a double fluorescent reporter mouse [i.e., tamoxifen-treated Tie2-CreER/TdTomato/Tg(Ly6a-GFP)], Sca-1 high EC (i.e., tdTomato+GFP high) and Sca-1 low EC (i.e., tdTomato+GFP low) lined vessels were also morphologically consistent respectively with arteries and arterioles (both are tdTomato+GFP high but with different size; Additional file 1: Fig. S1f, blue arrow indicates artery and yellow arrows indicate arteriole) and sinusoids (Additional file 1: Fig. S1f, white arrow). Since Sca-1 high expression appeared to be restricted to arteries and arterioles, we then utilized a simplified CD31+Sca-1 high and CD31+Sca-1 low staining along with morphology examination to mark respectively arterioles and sinusoids in the BM niche [19, 22].

To study changes of the vascular compartment of the BM niche during leukemia growth, we then utilized the Mll PTD/WT/Flt3 ITD/ITD mouse, a model that recapitulates phenotypic, cytogenetic, molecular and pathological features of human FLT3-ITD+ AML, a relatively frequent molecular subset of the disease [11]. Of note, we will refer hereafter to “wt” or “Mll PTD/WT/Flt3 ITD/ITD” to indicate the mouse genotype and to “normal” or “leukemic” to indicate the disease status.

Firstly, we noticed a significant decrease in CD31+Sca-1 high EC lined vessels (i.e., arterioles) in the leukemic Mll PTD/WT/Flt3 ITD/ITD mice compared with normal wt mice (Fig. 1a; Additional file 1: Fig. S2a). These results were corroborated by a flow cytometry...
analysis showing an overall increase in total BM ECs (CD45−Ter119−CD31+) in the leukemic mice (Additional file 1: Fig. S2b, c) but with a lower frequency of CD31+Sca-1high ECs and a higher frequency of CD31+Sca-1low ECs compared with normal wt mice (Fig. 1b; Additional file 1: Fig. S2d). Similar results were also obtained when normal wt mice were engrafted with BM MNCs from congenic leukemic MllITD/WT/Flt3ITD/ITD donors and compared with controls engrafted with BM MNCs from congenic normal wt donors (Fig. 1c, d; Additional file 1: Fig. S2e).

To validate these findings, we then transplanted BM MNCs from leukemic MllITD/WT/Flt3ITD/ITD mice or from normal wt mice into the tamoxifen-induced Tie2-CreER/TdTomato/Tg(Ly6a-GFP) double reporter mice. At 4 weeks after transplantation, we observed reduced Sca-1high EC-lined vessels (i.e., tdTomato+GFPhigh) in the recipients of AML BM MNCs compared with recipients of normal BM MNCs (Fig. 1e; Additional file 1: Fig. S2f). Consistent with these results, flow cytometry showed reduced Sca-1high ECs (i.e., tdTomato+GFPhigh) and increased Sca-1low ECs (i.e., tdTomato+GFPlow) in the double reporter mice engrafted with AML BM MNCs compared with those engrafted with normal BM MNCs (Fig. 1f; Additional file 1: Fig. S2g).

Next, to assess the relevance of these changes to the human disease, we transplanted human primary FLT3-ITD+ AML blasts into NSG-SGM3 (NSGS) mice and generated a patient-derived xenograft (PDX) model. Similar to the murine AML models, the FLT3-ITD+ PDX showed fewer BM CD31+Sca-1high ECs and arterioles as compared with NSGS mice engrafted with normal CB CD34+ cells (Fig. 1g, h; Additional file 1: Fig. S3).

In the BM niche, CD31+Sca-1high ECs reportedly line impermeable vessels such as arteries and arterioles, and CD31+Sca-1low ECs border permeable vessels such as sinusoids [21]. To this end, we imaged the calvarium of normal Sca-1 reporter [i.e., Tg(Ly6a-GFP)] mice with intravital confocal microscopy. Prior to imaging, mice were administered TRITC-dextran (150 kDa, red) intravenously to identify the vasculature [23, 24]. As expected, dextran leakage was visible as diffuse staining preferentially around Sca-1-GFPlow vessels, rather than Sca-1-GFPhigh vessels (Additional file 1: Fig. S4). Consistent with a decrease in CD31+Sca-1high vessels (i.e., arterioles) (Fig. 1a, g), we observed increased vessel permeability in the MllITD/WT/Flt3ITD/ITD AML mouse (TRITC-150 kDa dextran, red) and FLT3-ITD+ PDX (FITC-150 kDa dextran, green) (Additional file 1: Fig. S5a, b). Similar results were obtained when we transplanted BM MNCs from leukemic MllITD/WT/Flt3ITD/ITD mice or from normal wt mice into wt (FITC-150 kDa dextran, green; Additional file 1: Fig. S5c), EC reporter (i.e., tamoxifen-treated Tie2-CreER/TdTomato; FITC-150 kDa dextran, green; Additional file 1: Fig. S5d), or EC/Sca-1 double reporter (i.e., tamoxifen-treated Tie2-CreER/TdTomato/Tg(Ly6a-EGFP); Alex647-150 kDa dextran, blue; Additional file 1: Fig. S5e) recipient mice.

Thus, using different AML models and imaging techniques, flow cytometric analyses and permeability studies of the BM niche, we showed that FLT3-ITD+ AML growth led to a decrease of CD31+Sca-1high vessels (i.e., arterioles) in the BM niche.

**TNFα mediates loss of CD31+Sca-1high vessels in the leukemic BM niche**

To gain insights into the mechanisms leading to CD31+Sca-1high EC and arteriole depletion in the BM niche during leukemia growth, we hypothesized that these effects could be associated with certain secretory...
Fig. 1 (See legend on previous page.)
features of the proliferating leukemic blasts. We therefore measured levels of cytokines and chemokines in the blood and BM of age- and gender-matched leukemic MIIPTD/WT/Flt3TDTIDT and normal wt mice. TNFα was the only cytokine significantly elevated in the BM of leukemic mice (Fig. 2a, left; Additional file 1: Fig. S6a–m). Higher TNFα mRNA levels were observed in myeloid cells and CD45+Lin– progenitors [i.e., Lin–Sca-1–c-Ki67– (L–S–K–) and Lin–Sca-1–c-Ki67+ (LSK)] from the leukemic MIIPTD/WT/Flt3TDTIDT mice compared to the counterparts from normal wt mice (Fig. 2a, right), suggesting an overproduction of this cytokine by the clonal myeloid subpopulations in the leukemic mice. In vitro treatment of BM ECs from normal wt mice with murine recombinant (mr) TNFα (mrTNFα: 1 ng/ml) for 96 h (h) recapitulated the observations in the leukemic mice, with an expansion of ECs (Fig. 2b), a decrease of CD31+Sca-1high EC subfraction and an increase of CD31+Sca-1low EC subfraction (Fig. 2c; Additional file 1: Fig. S7a). Both TNFα receptor type 1 and 2 (TNFαR1 and TNFαR2) were found to be co-expressed on the surface of CD31+Sca-1high ECs (Additional file 1: Fig. S7b) and TNFαR1 and TNFαR2 blocking antibodies (Abs) reversed the mrTNFα effect (Additional file 1: Fig. S7c).

Consistent with these results, normal wt mice given mrTNFα [1 µg/day, intraperitoneal (ip) injection, 3 weeks] [25, 26] showed a significant increase of BM ECs (Fig. 2d), and loss of CD31+Sca-1high ECs and arterioles compared with vehicle-treated controls (Fig. 2e, f). Accordingly, normal wt mice engrafted with BM from leukemic MIIPTD/WT/Flt3TDTIDT donors had higher levels of BM TNFα (Additional file 1: Fig. S7d) and loss of CD31+Sca-1high ECs and arterioles (Fig. 1c, d; Additional file 1: Fig. S2e), compared with mice engrafted with BM from normal wt donors. Similar results were obtained in NSGS mice engrafted with human FLT3-ITD+ AML blasts compared with NSGS mice engrafted with normal CB CD34+ cells (Fig. 1g, h; Additional file 1: Fig. S3, S7e, f). In vivo treatment of MIIPTD/WT/Flt3TDTIDT AML mice with TNFα-neutralizing Ab (anti-TNFα; MP6-XT22, Biolegend; 1 mg/day, ip, 4 times/week for 3 weeks) [27] rescued the loss of CD31+Sca-1high ECs and arterioles (Fig. 2g, h) otherwise observed in the leukemic BM niche. Taken altogether, these results support a TNFα-induced depletion of CD31+Sca-1high vessels in the leukemic BM niche during FLT3-ITD+ AML growth.

**TNFα depletes CD31+Sca-1high ECs via miR-126 downregulation**

The vascular changes induced by TNFα in BM niche were strikingly similar to what we observed in the mice with miR-126 KO in ECs (miR-126flox/floxTie2-cre+) hereafter called miR-126ECA/Δ) obtained by crossing miR-126flox/flox with Tie2-cre mice. This led us to hypothesize that TNFα induces vascular changes during leukemia growth partly via miR-126 downregulation. To this end, it has been reported that in mature ECs, miR-126 contributes to the maintenance of vascular integrity and inhibition of endothelial permeability, which are main features of arterial and arteriolar vessels [12, 28, 29]. In the normal mouse BM niche, we found that ECs expressed at least a log-fold higher level of miR-126 than normal long-term (LT) HSCs (LSK Fit3+CD150+CD48−) and other non-hematopoietic stromal cells, including osteoblasts (OBs; CD45−Ter119−CD31+CD166+Sca-1−) and mesenchymal stromal progenitors (MSPs; CD45−Ter119−CD31−CD166−Sca-1+) [30] (Additional file 1: Fig. S8a, b). Within the ECs, Sca-1high ECs expressed significantly higher levels of miR-126 (Additional file 1: Fig. S8c, d) and lower levels of miR-126 verified targets (i.e., Vcam1 and Spred1) [12, 28, 29] (Additional file 1: Fig. S8e) than Sca-1low ECs. In addition, transcriptomes of Sca-1high and Sca-1low ECs from normal wt mice showed different gene expression profiles (Additional file 1: Fig. S8f), with pathways regulating angiogenesis, cell migration and vasculature development being.

(See figure on next page.)

**Fig. 2** TNFα mediates loss of CD31+Sca-1high vessels in the BM niche of FLT3-ITD+ AML. a Levels of TNFα (pg/ml, left) in the BM and blood analyzed by Lumienx assay (n = 10 mice per group) and levels of TNFα mRNA (right) in the BM CD45+ cell subpopulations analyzed by Q-RT-PCR (n = 4–7 mice for each population) from wt and MIIPTD/WT/Flt3TDTIDT AML mice. b and c Number of ECs from normal wt mice after in vitro exposure to murine recombinant (mr) TNFα (1 ng/ml) or vehicle for 96 h (b) and frequency of Sca-1high EC subfraction after in vitro exposure to mrTNFα (0, 0.2, 1 and 10 ng/ml) for 96 h (c), analyzed by flow cytometry. One of the three independent experiments with similar results is shown. d–f Normal wt mice treated with vehicle or mrTNFα (1 µg/d, ip, 3 weeks) were evaluated by flow cytometry (n = 4 mice per group) for frequencies of BM ECs (d) and Sca-1high and Sca-1low EC subfractions (e, left, representative plots; right, aggregate results) and by bursal CD31 (FITC) and Sca-1 (PE) IF staining (f, left) and quantification (f, right); n = 3 mice per group) of CD31+Sca-1high EC-lined vessels. For f, yellow arrows indicate CD31+Sca-1high EC-lined vessels; white arrows indicate CD31+Sca-1low EC-lined vessels. Scale bars represent a size of 100 µm. Results represent mean ± SEM. Significance values: *p < 0.05, **p < 0.01, ***p < 0.001; ns not significant.
Fig. 2 (See legend on previous page.)
activated in Sca-1<sup>hi</sup> ECs (Additional file 1: Table S2). Q-RT-PCR assays (Additional file 1: Table S3) validated these findings (Additional file 1: Fig. S8g, right panel).

Interestingly, we noticed that miR-126 knock-down (KD) by miRZip anti-miR-126 lentiviral transduction (Fig. 3a, Additional file 1: Fig. S9a) or treatment with a Cpg-miR-126 inhibitor [31] (an anti-miR-126 oligonucleotide hereafter called miRisten; Additional file 1: Fig. S9b, c) depleted the Sca-1<sup>hi</sup> fraction in CD31<sup>+</sup>Sca-1<sup>hi</sup> ECs from normal wt mice. Conversely, miR-126 over-expression (OE) in CD31<sup>+</sup>Sca-1<sup>lo</sup> ECs obtained by transduction with miR-126 precursor lentivirus (Fig. 3b) or treatment with Cpg-miR-126 mimics (Additional file 1: Fig. S9d, e) enriched the Sca-1<sup>lo</sup> fraction. In vivo, mice with EC miR-126 KO (i.e., miR-126<sup>ECΔ/Δ</sup>, Fig. 3c, middle box) [12, 31] had fewer CD31<sup>+</sup>Sca-1<sup>hi</sup> ECs and arterioles than wt reporter mice (i.e., tamoxifen-treated miR-126<sup>wt/wt/Tie2-CreER/TdTomato/Tg(Ly6a-GFP)</sup>) (Additional file 1: Fig. S9f, g). Furthermore, normal wt mice treated in vivo with miRisten (20 mg/kg/day, iv) for 3 weeks showed fewer CD31<sup>+</sup>Sca-1<sup>hi</sup> ECs and vessels (i.e., arterioles) than control mice treated with CpG-scramble (SCR) (Fig. 3h, i). In addition, similar to the leukemic mice, miR-126<sup>ECΔ/Δ</sup> mice had an increase of the BM vessel permeability compared to wt controls (Additional file 1: Fig. S9h).

We previously reported that Spred1, a member of the Sprouty family of proteins and an inhibitor of RAS small GTPases, is both a miR-126 target and a regulator of miR-126 biogenesis [31]. EC Spred1 KO mice (i.e., Spred1<sup>ECΔ/Δ</sup>) [13, 32, 33] therefore express constitutively higher levels of EC miR-126 than Spred1<sup>Tie2-cre+</sup>- and miR-126<sup>ECΔ/Δ</sup> mice (Fig. 3c, right box) and represent a functional model for EC miR-126 OE. Accordingly, Spred1<sup>ECΔ/Δ</sup> mice had more BM CD31<sup>+</sup>Sca-1<sup>hi</sup> ECs and vessels (i.e., arterioles) than wt controls (Fig. 3d–g, right panel).

Taken altogether, these results support a role of miR-126 in determining enrichment of the Sca-1<sup>hi</sup> EC fraction and in turn arteriolar density in the BM niche and a role of TNFα-dependent miR-126 downregulation in the loss of arterioles during leukemia growth.

To this end, we also observed significantly lower BM EC miR-126 levels in the leukemic Mili<sup>TDT/WT</sup>/Flt3<sup>TDT/ITD</sup> mice (Fig. 4a) in addition to fewer BM CD31<sup>+</sup>Sca-1<sup>hi</sup> ECs and vessels (i.e., arterioles; Fig. 1a, b; Additional file 1: Fig. S2a) compared with normal wt controls. Similar changes were observed in normal wt mice engrafted with AML BM MNCs compared with those engrafted with normal BM MNCs (Figs. 1c, d and 4b; Additional file 1: Fig. S2e). Furthermore, murine BM ECs obtained from normal wt mice treated in vitro with mrTNFa (1 ng/ml, 24 h) showed reduced levels of pri/pre- and mature miR-126 (Fig. 4c) and increased levels of miR-126 targets (i.e., Vcam1 and Spred1) (Fig. 4d). Co-treatment with miR-126 mimics rescued the loss of the CD31<sup>+</sup>Sca-1<sup>hi</sup> fraction induced by mrTNFa (Additional file 1: Fig. S10a, b, right panel). TNFαR1 and TNFαR2 blocking Abs also rescued the mrTNFa-induced miR-126 downregulation (Additional file 1: Fig. S10c) and the reduction of the CD31<sup>+</sup>Sca-1<sup>hi</sup> EC fraction (Additional file 1: Fig. S7c).

Consistent with the in vitro results, normal wt mice that were given mrTNFa (1 µg/day, ip injection, 3 weeks) [25, 26] had a significant in vivo reduction of BM EC pri- pre- and mature miR-126 levels (Fig. 4e). As expected, these mice also showed a decrease in CD31<sup>+</sup>Sca-1<sup>hi</sup> ECs and vessels (i.e., arterioles) compared with vehicle-treated controls (Fig. 4f, middle panel; Additional file 1: Fig. S10d, e, middle panel). In vivo co-treatment with miR-126 mimics (20 mg/kg/day, iv, 3 weeks) rescued these changes (Fig. 4f, bottom panel; Additional file 1: Fig. 3a, right box) of Sca-1<sup>hi</sup> EC depletion in the BM niche. a Representative plots (left) and aggregate results (right) of Sca-1<sup>hi</sup> frequency in cultured Sca-1<sup>hi</sup> ECs upon miR-126 knock-down (KD) by GFP-expressing miRZip anti-miR-126 lentiviral transduction, as analyzed by flow cytometry (n = 3). b Representative plots (left) and aggregate results (right) of Sca-1<sup>hi</sup> frequency in cultured Sca-1<sup>lo</sup> ECs upon miR-126 over-expression (OE) by GFP-expressing miR-126 precursor lentiviral transduction, as analyzed by flow cytometry (n = 3). c miR-126 levels in ECs from the BM of wt, miR-126<sup>ECΔ/Δ</sup> (EC miR-126 KO) and Spred1<sup>ECΔ/Δ</sup> (EC miR-126 OE) mice by Q-RT-PCR (n = 4 mice per group) d and e CD31 (FITC) and Sca-1 (PE) IF staining (d) and quantification (e) of CD31<sup>+</sup>Sca-1<sup>hi</sup> EC-lined vessels (i.e., arterioles) in the tibias from wt, miR-126<sup>ECΔ/Δ</sup> and Spred1<sup>ECΔ/Δ</sup> mice. One of the three independent experiments with similar results is shown. Yellow arrows indicate CD31<sup>+</sup>Sca-1<sup>hi</sup> EC-lined vessels; white arrows indicate CD31<sup>+</sup>Sca-1<sup>lo</sup> EC-lined vessels. f and g Representative plots (f) and aggregate results (g) of Sca-1<sup>hi</sup> and Sca-1<sup>lo</sup> subfractions in BM ECs from wt, miR-126<sup>ECΔ/Δ</sup> and Spred1<sup>ECΔ/Δ</sup> mice by flow cytometry analysis (n = 3 mice per group). h CD31 (FITC) and Sca-1 (PE) IF staining (top) and quantification (bottom) of CD31<sup>+</sup>Sca-1<sup>hi</sup> EC-lined vessels (i.e., arterioles) in the tibias from normal wt mice treated with CpG-scramble (SCR) or CpG-miR-126 inhibitor (miRisten, 20 mg/kg/day, iv, for 3 weeks; n = 3 mice per group). Yellow arrows indicate CD31<sup>+</sup>Sca-1<sup>hi</sup> EC-lined vessels; white arrows indicate CD31<sup>+</sup>Sca-1<sup>lo</sup> EC-lined vessels. i Representative plots (left) and aggregate results (right) of Sca-1<sup>hi</sup> and Sca-1<sup>lo</sup> subfractions in BM ECs from normal wt mice treated with SCR or miRisten for 3 weeks (n = 3 mice per group). Results represent mean ± SEM. Significance values: *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001.
Fig. 3 (See legend on previous page.)
miR-126 depletion and loss of CD31 Spred1 ECΔ/Δ; higher expression of miR-126, as occurred in ECs from Fig. S10m, right panel), suggesting that constitutively with wt recipients (Fig. 4g, right panel; Additional file 1: Fig. S10–l). Transplantation of AML BM MNCs was rescued in vivo by blocking TNFα with anti-TNFα Ab (1 mg/day, ip, 4 times/week for 3 weeks) (Additional file 1: Fig. S10g–i). Similar results were observed in NSGS mice engrafted with human FLT3-ITD+ AML blasts compared with those engrafted with normal CB CD34+ cells (Fig. 1g, h; Additional file 1: Fig. S3, S7a and S10j).

Of note, Spred1EC Δ/A mice (EC miR-126 OE, Fig. 3c) treated in vivo with mrTNFα (1 µg/day, ip, 3 weeks) showed no significant difference in vascularization compared with vehicle-treated Spred1EC Δ/A controls (Additional file 1: Fig. S10k–l). Transplantation of AML BM MNCs resulted in a lesser degree of leukemia-induced BM vascular changes in Spred1EC Δ/A recipients compared with wt recipients (Fig. 4g, right panel; Additional file 1: Fig. S10m, right panel), suggesting that constitutively higher expression of miR-126, as occurred in ECs from Spred1EC Δ/A mice, may rescue in vivo TNFα-induced miR-126 depletion and loss of CD31+Sca-1 hi ECs.

While the molecular mechanisms through which TNFα induces endothelial miR-126 downregulation are likely to be multifaceted, we focused on GATA2 since this protein is a verified miR-126 transcription factor [34–36]. We first noticed that Gata2 levels were reduced in BM ECs from AML mice compared with normal mice (Additional file 1: Fig. S11a) and that GATA2 KD by siRNA in human umbilical vein ECs (HUVECs) decreased miR-126 levels (Additional file 1: Fig. S11b, c). We then demonstrated that in vitro, TNFα treatment (1 ng/ml) reduced Gata2 levels in both murine BM ECs and HUVECs (Additional file 1: Fig. S11d–f) as compared with vehicle-treated controls. Using chromatin immunoprecipitation assay, we showed a reduced GATA2 enrichment on the EGFL7/miR-126 promoter [34] in HUVECs exposed to TNFα (Additional file 1: Fig. S11g, h).

Antileukemic treatment restores CD31+Sca-1 hi vessels that safeguard LSCs

Having demonstrated that TNFα secreted by the AML blasts contributes to loss of CD31+Sca-1 hi ECs, we then reasoned that cytoreductive therapy that eliminates TNFα-secreting blasts could reverse the depletion of CD31+Sca-1 hi EC–lined vessels (i.e., arterioles) as observed in the leukemic BM niche. To this end, we transplanted BM MNCs from MiLITD/wt/Flt3 ITD/ITD AML mice (CD45.2) into congenic B6 mice (CD45.1) and generated a cohort of AML mice that developed disease at a similar time. We elected to treat these mice with TKIs rather than chemotherapy to restrict the observed BM niche changes to a direct blast cytoreduction rather than to non-specific chemotherapy cytotoxicity to other non-hematopoietic cells.

Upon confirmation of AML development at 4 weeks after transplantation, we treated these mice with the TKI AC220 [38] to target FLT3-ITD and cytoreduce AML blasts (20 mg/kg/day, oral gavage, Fig. 5a). After 3 weeks treatment, we observed leukemic cytoreduction (Fig. 5b) and decreased BM TNFα levels (Fig. 5c) in AC220-treated AML mice compared with vehicle-treated controls. In AC220-treated mice, we noticed a gain in BM CD31+Sca-1 hi ECs and vessels (i.e., arterioles) (Fig. 5d, e, right panel). Accordingly, normal wt mice engrafted with BM MNCs from MiLITD/wt/Flt3 ITD/ITD AML donors had higher levels of BM TNFα (Additional file 1: Fig. S7d) and reduced levels of BM EC miR-126 (Additional file 1: Fig. S10f), in addition to a decrease in CD31+Sca-1 hi ECs and arterioles (Fig. 1c, d; Additional file 1: Fig. S2e), compared with mice engrafted with BM MNCs from normal wt donors. These changes were rescued in vivo by blocking TNFα with anti-TNFα Ab (1 mg/day, ip, 4 times/week for 3 weeks) (Additional file 1: Fig. S10g–i).
Fig. 4 (See legend on previous page.)
cells (LICs; hereafter called LSCs) as shown by limit subpopulation mostly enriched in leukemia-initiating S12c) compared with vehicle-treated controls. In fact, in secondary (2nd) transplant experiments, recipients of BM MNCs from AC220-treated donors had a higher disease burden (Additional file 1: Fig. S12d) and shorter survival (Fig. 5) than recipients of BM MNCs from vehicle-treated donors, indicating not only persistence but also an expansion of LSCs.

In contrast, AC220-treated mice that also received mrTNFa (1 µg/day, ip, 3 weeks) to prevent the treatment-related TNFa decrease (Fig. 6a) maintained lower levels of BM EC miR-126 (Additional file 1: Fig. S12f) and had fewer CD31+Sca-1high ECs and arterioles (Fig. 6b, c) and less LSCs (i.e., CD45.1/CD45.2 AML LSKs; Fig. 6d; Additional file 1: Fig. S12e) compared with recipients of BM MNCs from AC220-treated donors that received AC220 alone. In 2nd transplant experiments, the recipients of BM MNCs from AC220+mrTNFa-treated donors had a significantly longer survival compared with the recipients of BM MNCs from AC220-treated donors (Fig. 6e, right; median survival: 84 vs. 24 days, p < 0.0001), supporting the hypothesis that prevention of treatment-related CD31+Sca-1high EC enrichment and arteriolar re-vascularization prevents LSC expansion. We confirmed the relevance of these results to human disease using the FLT3-ITD+PDx model (Additional file 1: Fig. S12g–k).

Next, as TNFa acts on the vasculature of the BM niche via miR-126, we reasoned that a direct EC miR-126 deprivation of the BM niche could prevent treatment-induced CD31+Sca-1high EC enrichment, arteriolar re-vascularization and LSC protection. To this end, we transplanted BM MNCs from MiRPTD/WT/Flt3ITD/ITD AML mice into MiR-126ECwt/wt and MiR-126ECΔ/Δ recipient mice and treated them with AC220 (20 mg/kg/day, oral gavage; Fig. 6f). After 3 weeks treatment, we observed reduced CD31+Sca-1high ECs and arterioles (Additional file 1: Fig. S13a–d), a significant reduction of leukemia burden and LSCs, reduced miR-126 levels in AML LSCs (Fig. 6g) likely due to reduced EC supply, and increased survival (Fig. 6h; left) in the AC220-treated MiR-126ECA/Δ primary mice compared with the AC220-treated MiR-126ECA/Δ controls. BM MNCs from AC220-treated MiR-126ECA/Δ or MiR-126ECwt/wt AML donors were then transplanted into 2nd wt recipient mice. Recipients of BM MNCs from the AC220-treated MiR-126ECA/Δ donors had a significantly longer survival than recipients of BM MNCs from the AC220-treated MiR-126ECA/Δ donors (Fig. 6h, right; median survival: 53 days vs. not reached at day 80 post transplantation, p < 0.0001).

Similar results were obtained by pharmacologic miR-126 downregulation with miRisten. A cohort of MiRPTD/WT/Flt3ITD/ITD leukemic mice were generated as described above and then treated with miRisten (20 mg/kg, iv, daily), SCR, miRisten+ AC220 (10 mg/kg, oral gavage, daily), or SCR+ AC220 for 3 weeks, followed by assessment of donor AML cell engraftment in PB, BM and spleen (Additional file 1: Fig. 5).
Fig. 5 (See legend on previous page.)
Fig. S14). We confirmed miR-126 KD in ECs from miRisten-treated mice compared with SCR-treated mice (Additional file 1: Fig. S14b). Mice receiving the combination of miRisten+ AC220 had a significant reduction in the percentage of AML cells in PB, BM and spleen (Additional file 1: Fig. S14c, d), a significant decrease in the frequency of BM AML LSKs (Additional file 1: Fig. S14e) and increased survival (Additional file 1: Fig. S14f) compared to mice treated with SCR+ AC220. Similar to the mrTNFα+ AC220 combination, the miRisten+ AC220 combination also significantly prolonged survival in 2nd transplant experiments (Additional file 1: Fig. S14g) and increased survival (Additional file 1: Fig. S14h) and increased survival (Additional file 1: Fig. S14i). The combination of miRisten+ AC220 had a significant decrease in the frequency of BM AML LSKs, which line mainly non-permeable arterioles, and a gain in CD31+Sca-1low ECs, which line mainly fenestrated, permeable sinusoids. These vascular changes were caused partly by high levels of TNFα produced by the AML blasts, causing downregulation of miR-126 in CD31+Sca-1low ECs, which became depleted while an enrichment in CD31+Sca-1low ECs was observed.

TNFα has been intensively studied for its role in normal and malignant hematopoiesis [41]. Yamashita and Passegue recently reported on the complex role of TNFα in normal and clonal hematopoiesis, showing that while inducing myeloid progenitor apoptosis, TNFα promotes HSC survival [42]. AML blasts express high levels of TNFα [43], and likely hijack TNFα-driven mechanisms of normal hematopoiesis to support leukemia growth [42]. Herein, we show a novel pro-leukemogenic role of TNFα that is extrinsic to AML cells and involves downregulation of miR-126 in the vascular compartment of the BM niche. While TNFα has been implicated in the remodeling of blood vessels and shown to promote angiogenesis during inflammation [44, 45], to our knowledge the...
Fig. 6 (See legend on previous page.)
TNFα-induced switch from Sca-1<sup>high</sup> ECs to Sca-1<sup>low</sup> ECs, and in turn from an arteriole- to a sinusoid-enriched BM niche as observed during leukemia growth has not been previously reported. As these changes were a phenocopy of genetic (EC miR-126 KO) and pharmacologic (i.e., miRisten) EC miR-126 deprivation, we postulated and proved that loss of CD31<sup>+</sup>Sca-1<sup>high</sup> ECs and arterioles were due to TNFα-induced miR-126 downregulation.

MiR-126 is one of the most highly expressed microRNAs in ECs, where it acts as a master regulator of angiogenesis [12, 28, 29]. In developmental vasculogenesis, miR-126 supports differentiation of embryonic stem cells into endothelial precursor cells and mature ECs [46]. In mature ECs, miR-126 contributes to the maintenance of quiescence and vascular integrity and inhibition of endothelial permeability and apoptosis [12, 28, 29]. Furthermore, miR-126 enhances the activity of angiopoietin-1 (Ang-1), a glycoprotein that regulates vessel stabilization, maturation and permeability [47, 48]. Of note, the molecular mechanisms through which TNFα induces endothelial miR-126 downregulation are likely to be multifaceted and remain to be fully elucidated. Herein, we showed that they likely involve GATA2 [34, 35], a miR-126 transcription factor, which is reportedly silenced by TNFα [37]. But how do the TNFα-induced vascular changes in the leukemic BM niche ultimately promote leukemia growth? We previously showed that CD31<sup>+</sup>Sca-1<sup>high</sup> ECs expressed the highest levels of miR-126 in the BM niche and supply miR-126 to maintain LSC quiescence [31]. Thus, by inducing loss of CD31<sup>+</sup>Sca-1<sup>high</sup> ECs, TNFα decreases the endothelial supply of miR-126 to LSCs and enables them to enter the cell cycle and partially differentiate into proliferating “bulk” AML blasts [31, 49].

Of note, these observations may have direct translational and clinical relevance. In fact, under these conditions, therapeutic cytoreduction of AML blasts can cause a drop in the BM levels of TNFα and in turn lead to a post-treatment enrichment of BM CD31<sup>+</sup>Sca-1<sup>high</sup> ECs and arterioles with a consequent increase in the endothelial miR-126 supply to LSCs, which, once enriched in miR-126, are more resistant to therapy [31, 49]. We proved this model in FLT3-ITD+ AML murine and PDX mice treated with TKIs. We showed that after TKI treatment, an increase in CD31<sup>+</sup>Sca-1<sup>high</sup> ECs and arterioles occurred and LSCs persisted as demonstrated using secondary transplant experiments. Of note, LSC persistence could be prevented by blocking the gain in CD31<sup>+</sup>Sca-1<sup>high</sup> ECs and the arteriolar “re-vascularization” of the BM niche with administration of recombinant TNFα or with deprivation of endothelial miR-126 via genetic EC miR-126 KO or pharmacological treatment with the anti-miR-126 miRisten.
(i.e., miRisten), which may represent a novel strategy to overcome non-genetically driven, extrinsic mechanisms of LSC resistance in AML.

Conclusions

The TNFα-miR-126 axis plays a key role in the BM vascular changes induced by antileukemic treatments and mediates non-genetic, extrinsic mechanisms of LSC treatment-resistance that can be overcome with preemptive therapeutic deprivation of EC miR-126.

Abbreviations

AML: Acute myeloid leukemia; BM: Bone marrow; HSC: Hematopoietic stem cell; LSC: Leukemic stem cell; EC: Endothelial cell; TNFα: Tumor necrosis factor alpha; PB: Peripheral blood; MNC: Mononuclear cell; COHNMC: City of Hope National Medical Center; FLT3: FMS-like tyrosine kinase 3; ITD: Internal tandem duplication; TK: Tyrosine kinase; TKI: Tyrosine kinase inhibitor; wt: Wild type; CB: Cord blood; PDX: Patient-derived xenograft; L−S−K−: Lin−Sca-1−c‑Kit−; LSK: Lin−Sca-1+c‑Kit+; LT-HSC: Long-term hematopoietic stem cell; mr: Murine recombiant; Ab: Antibody; OB: Osteoblast; MSP: Mesenchymal stromal progenitor; KO: Knockout; KD: Knock-down; HUVEC: Human umbilical vein endothelial cells; 2nd: Secondary; Ang-1: Angiopoietin-1.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13045-021-01133-y.

Additional file 1. Treatment-induced Arteriolar Revascularization and miR-126 Enhancement in Bone Marrow Niche Protect Leukemic Stem Cells in AML.

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Authors’ contributions

BZ designed and conducted experiments, analyzed data, wrote the manuscript and provided administrative support; LXN and DZ designed and conducted experiments and analyzed data; DF analyzed RNA-sequencing data; HW, DH, JQ, CA, MB, and Y‑LS conducted experiments; AD, DP, ZC, AH, FP, JJ, TT, MC, LL, RCR, NC, and Y‑HK reviewed data and the manuscript; MK designed the CpG‑miR‑126 inhibitor and CpG‑miR‑126 mimics, reviewed data and the manuscript; GM designed experiments, analyzed data, wrote manuscript and provided administrative support. All authors read and approved the final manuscript.

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Availability of data and materials

RNA sequencing data produced in our laboratory and analysed in this study are available at the Gene Expression Omnibus (GEO) repository of the National Center for Biotechnology Information (GSE180104). Supplementary information including Additional file 1: Figs. S1–S15 and Tables S1–S3 are provided with the online version of this paper. All other datasets generated during this study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

Patient sample acquisition was approved by the Institutional Review Boards (IRB) at the COHNMC, in accordance with an assurance filed with and approved by the Department of Health and Human Services and met all requirements of the Declaration of Helsinki. CML patients were consented on the IRB #180867 protocol.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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