A Macrophage Dominant PI-3K Isoform Controls Hypoxia Induced HIF1α & HIF2α Stability and Tumor Growth, Angiogenesis and Metastasis

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Running Title: PTEN/PI-3 kinase regulates HIFα-VEGF axis and metastasis in macrophages

Abbreviations. BMDM, Bone marrow derived macrophages; MCSF, Macrophage colony stimulating factor, PI-3K, phosphoinositide 3-kinase; PTEN, phosphatase and tensin homolog

Key words: Hypoxia, HIFα, proteasome, PTEN, PI-3 kinase, macrophages

Abstract
Tumor growth, progression and response to the hypoxic tumor microenvironment (TME) involve the action of hypoxia inducible transcription factors, HIF1 and HIF2. HIF is a heterodimeric transcription factor containing an inducible HIFα subunit and a constitutively expressed HIFβ subunit. The signaling pathways operational in macrophages regulating hypoxia induced HIFα stabilization remain the subject of intense investigation. Here it was discovered that the PTEN/PI-3K/AKT signaling axis controls hypoxia induced HIF1α (HIF1A) and HIF2α (EPAS1) stability in macrophages. Using genetic mouse models and pan-PI-3 kinase as well as isoform specific inhibitors, inhibition of the PI-3K/AKT pathway blocked the accumulation of HIFα protein and its primary transcriptional target VEGF in response to hypoxia. Moreover, blocking the PI-3K/AKT signaling axis promoted the hypoxic degradation of HIFα via the 26S proteasome. Mechanistically, a macrophage dominant PI-3K isoform (p110γ) directed tumor growth, angiogenesis, metastasis and the HIFα/VEGF axis. Moreover, a pan-PI-3K inhibitor (SF1126) blocked tumor-induced angiogenesis and inhibited VEGF and other proangiogenic factors secreted by macrophages. These data define a novel molecular mechanism by which PTEN/PI-3K/AKT regulates the proteasome dependent stability of HIFα under hypoxic conditions, a signaling pathway in macrophages that controls tumor-induced angiogenesis and metastasis.

Implications: This study indicates that PI-3K inhibitors are excellent candidates for the treatment of cancers where macrophages promote tumor progression.

Introduction

Hypoxia, is considered a hallmark of tumor progression in that it induces neoangiogenesis, the recruitment of bone marrow derived cells and degradation of extracellular matrix (ECM) resulting in activation of proliferation, invasion and metastasis of cancer cells (1). Hypoxic regions of tumors are characterized by an increased accumulation of macrophages which contributes to tumor angiogenesis and tumor progression (2). Hypoxic macrophages are known to respond to hypoxia by up-regulating hypoxia-inducible transcription factors (HIFs) consisting of distinct, hypoxia-responsive (HIF1α and HIF2α)
subunits and identical, constitutively expressed (HIF1β and HIF2β) subunits (3, 4). In the presence of oxygen, the α subunits are hydroxylated by oxygen-sensitive enzymes, prolyl hydroxylases, which targets them for degradation by the VHL dependent ubiquitin-proteasomal pathway (5, 6). Under hypoxic conditions, HIFα subunits are stabilized, translocate to the nucleus and, together with their partner factor, basic-helix-loop-helix/PAS protein Arnt, bind to the promoters of genes that mediate glycolysis and neovascularization/angiogenesis (7, 8).

There are conflicting views of the relative contribution of each HIF to the regulation of hypoxic gene expression in macrophages. Some studies suggest that the main form of HIF up-regulated by tumor-associated macrophages (TAMs) is HIF2 (9, 10), and overexpression of HIF2α in normoxic human macrophages up-regulates various proangiogenic genes (11). However, human macrophages also markedly up-regulate HIF1α when exposed to hypoxia in vitro and in tumors (12), and HIF1α deficient murine macrophages express lower levels of such HIF-regulated genes such as VEGF and the glucose receptor GLUT1 in hypoxia than their wild-type counterparts. Interestingly, the exact contribution of HIF1α and HIF2α to the regulation of hypoxic gene expression appears to vary between different cell types (7).

The molecular mechanisms controlling HIFα accumulation in macrophages are not completely elucidated. In the tumor cells, a large number of signaling pathways including Rac-GTP, Src, Fak, phosphoinositide 3-kinase (PI-3K), etc have been linked to the control of HIF1α and VEGF (13, 14). Several groups have implicated the role of PTEN/PI-3K signaling in the regulation of HIF1α (15-17) in tumor cells. However, the exact contribution of the PI-3K pathway to HIFα regulation remains a subject of considerable interest and controversy in both the tumor and stromal compartments (18, 19).

PI-3 kinases (PI-3K) are a family of enzymes that phosphorylate phosphatidylinositol (PI) lipids at the 3’ position (20). The phosphatase and tensin homolog deleted on chromosome 10 (PTEN) is a dual specificity phosphatase that antagonizes PI-3K’s enzymatic activity by dephosphorylating phosphatidylinositol (3,4,5)-trisphosphate to generate phosphatidylinositol (4,5)-biphosphate. PI-
3K/PTEN has been shown to play a prominent role in regulating a number of cellular responses, including growth, survival, and migration (21, 22). PI-3K is known to be associated with different phases of macrophage function (23). The PI-3K/Akt signaling axis is crucial for migration and diapedesis of immune effector cells, such as neutrophils and monocytes, to the site of inflammation and infection (24-26). The role of myeloid specific PTEN in controlling lung infection and inflammation during murine pneumococcal pneumonia and neutropenia associated pneumonia is also well-studied (27, 28).

However, the contribution of PTEN/PI-3K/AKT pathway in enhancing HIFα mediated gene expression in macrophages has not been previously reported. In the present study, using myeloid specific deletion of PTEN and various pan and isoform specific PI-3K inhibitors, we show that PTEN/PI-3K signaling controls hypoxia induced HIF1α and HIF2α stabilization in macrophages. Moreover, using p110γ/-/- mice and pan PI-3K inhibitor SF1126 we demonstrate that PI-3K signaling promotes tumor growth and metastasis in tumor models by increasing the expression and potential secretion of proangiogenic factors by tumor associated macrophages.

Materials and Methods

Animal studies

All procedures involving animals were approved by the University of California San Diego Animal Care Committee, which serves to ensure that all federal guidelines concerning animal experimentation are met. PI-3K γ (p110γ/-/-) mice were obtained from Dr. Joseph Penninger, Institute of Molecular Biotechnology, Vienna, Austria. Floxed PTEN mice and lysozyme M (LysM) Cre recombinase transgenic mice were purchased from Jackson laboratories. LysM cre mice were mated with PTENβ/β mice to delete PTEN from myeloid cell compartment. Depending on the presence of LysM Cre recombinase, double-floxed PTENβ/β mice are referred to as PTENMC-KO or PTENMC-Wt mice, respectively. Macrophages from PTENβ/β mice were used as wild-type controls.

Antibodies and Reagents
MCSF was from Gibco Life technologies. PI-103, BKM 120 and PF4691502 were purchased from Selleck chemicals. SF2523 and SF1126 were obtained from SignalRx Pharmaceuticals (29, 30). Primary or fluorescent antibodies against CD31 (clone MEC13.3), CD11b (clone M1/70) was from BD Biosciences, F4/80 (clone BM8) was from eBiosciences. Alexa Flour 488 was from Invitrogen life Technologies. Bouin’s solution, DAPI, hyaluronidase type V and Dnase I was from Sigma.

**Isolation of bone marrow-derived macrophages, hypoxia and in vitro inhibitor experiments**

Bone marrow-derived macrophages (BMDM) were isolated as described previously and cultured under MCSF (75 ng/ml) + 20% FBS conditions (31). On day 7, most of the adherent cells are macrophages as confirmed by FACS analysis (>90% Mac1 and F4/80 positive cells). In order to study the effect of different inhibitors *in vitro*, macrophages (7 days in culture) were treated with or without pan or isoform specific PI-3 kinase inhibitors followed by hypoxia (1% O₂) for 4 hrs. For hypoxia experiments, macrophages were placed in a modulator incubator chamber (Billups-Rothenberg) that was flushed with a gas mixture consisting of 1% O₂, 5% CO₂, with balance N₂, sealed, and incubated at 37°C. Whole cell lysates were prepared in RIPA buffer (Cell Signaling Technologies) containing complete protease inhibitor cocktail. For HIFα immunoblots, nuclear extracts were prepared using Dignam protocol (32).

**Plasmid and Si-RNA Transfections**

BMDMs were transfected using an AMAXA Mouse Macrophage Nucleofection Kit (Lonza) with 5µg of HA-PTEN or myr-AKT plasmid or 100 nM of siRNA for PI3Kα (Mm_pik3ca_1), PI3Kβ (Mm_pik3cb_2), PI3kγ (Mm_pik3cg_1), PI3Kδ (Mm_pik3cd_1), Akt1/2 siRNA or non-silencing siRNA (Ctrl_AllStars-1) purchased from Qiagen or Akt1 /2 siRNA and control siRNA purchased from Santa Cruz Biotechnology. After transfection, cells were cultured for 36 h. Each siRNA was tested individually for efficient knockdown of protein expression.

**Quantification of gene expression**
Total RNA was isolated from BMDMs using RNAeasy kit (Qiagen, Hilden, Germany). cDNA was prepared from 1 µg RNA sample using iscript cDNA synthesis kit (Bio-Rad, Hercules, CA). cDNA (2 µL) was amplified by RT-PCR reactions with 1× SYBR green supermix (Bio-Rad, Hercules, CA) in 96-well plates on an CFX96 Real time system (Bio-Rad, Hercules, CA), using different primers for mouse gene. Sequence of primers used were reported before (31). Relative expression levels were normalized to GAPDH expression according to the formula \( < 2^{(Ct\,\text{gene\,of\,interest}-Ct\,\text{Gapdh})} > \). Values are multiplied by 100 for presentation purposes.

**Immunoblotting**

Whole cell extract (WCE) or nuclear extracts of BMDMs were prepared and protein was quantitated with Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA) using BSA as a standard. Equal protein from protein lysates were resolved by SDS-PAGE, followed by immunoblotting and probing with primary antibodies against p110\(\alpha\) (Cell Signaling Technologies), p110\(\beta\) (Santa Cruz), p110\(\gamma\) (Cell Signaling Technologies) and p110\(\delta\) (Santa Cruz), pAKT (Cell Signaling), AKT (Cell Signaling), HIF1\(\alpha\) & HIF2\(\alpha\) (Novus Biological) and \(\beta\)-actin (Santa Cruz).

**In vivo tumor experiments**

Lewis lung carcinoma (LLC) and B16 F10 melanoma obtained from the American Type Culture Collection (ATCC) were cultured as described previously (31). LLC cells \((1 \times 10^5)\) were injected subcutaneously into syngenic 4-6 week old mice. Tumor dimensions were recorded regularly and tumors were harvested 25 days post injection. Tumor volume was measured using the following formula: \(\text{Volume} = 0.5 \times \text{length} \times (\text{width})^2\). For experimental metastasis, B16 F10 melanoma cells \((5 \times 10^5)\) were injected intravenously and lungs were harvested after 15 days as described before (31). For drug treatment studies, WT mice implanted subcutaneously with \(1 \times 10^5\) LLC cells were treated with vehicle or 50 mg/kg SF1126 subcutaneously three times a week for 28 days, until tumors were harvested. For B16
F10 melanoma, two doses of 50 mg/kg of SF1126 were given before injecting 5 x 10^5 B16F10 melanoma cells, followed by daily treatment with the drug until lungs were harvested on day 15. For survival studies, three doses of 50 mg/kg of SF1126 were given before injecting 3 x 10^5 B16F10 melanoma cells, followed by daily treatment until mice died. Tumor volumes and weights were measured and the tumors were used for IF, IHC and sorting of macrophages using FACS. For FACS analysis, LLC tumors were excised, minced and digested to single cell population as described (31). CD11b and F4/80 positive cells sorted by FACS were used for RNA isolation and real time PCR studies.

Results

PTEN regulates expression of HIFα protein and HIFα mRNA targets in macrophages

The tumor suppressor PTEN is an antagonist of PI-3K signaling and functions by removing the phosphate at the D3 position of phosphatidylinositol trisphosphate and phosphatidylinositol bisphosphate. In order to validate the role of PTEN in hypoxia induced HIFα stabilization we generated conditional PTEN knockout mice in which PTEN expression was controlled by LysM, a myeloid cell-specific promoter (20). Depending on the presence of LysM Cre recombinase, double-floxed PTEN^β/β^ mice are referred to as PTEN^{MC-KO} or PTEN^{MC-Wt} mice, respectively. Deletion of PTEN was confirmed in primary macrophages (Fig. 1A), and resulting downstream effects of constitutively active PI-3K were reflected by greatly elevated baseline levels of phospho-Akt in PTEN^{MC-KO} macrophages (Fig. 1A). Myeloid specific deletion of PTEN induced high level of HIF1α & HIF2α stabilization in PTEN^{MC-KO} mice as compared to PTEN^{MC-Wt} (Fig. 1B). These results were also supported by a greater degree of HIFα stabilization in BMDMs isolated from PTEN^{+/−} mice (Fig. 1C). Our next point of focus was to investigate the role of PTEN in the transcriptional activation of the HIFα target gene VEGF. Loss of PTEN in myeloid cell compartment increased VEGF mRNA levels in PTEN^{MC-KO} mice (Fig. 1D). In order to further validate the role of PTEN in stabilization of HIFα, PTEN was exogenously expressed in PTEN^{MC-ko} mice. Results in Fig. 1E showed that expression of PTEN blocked the accumulation of HIFα in PTEN^{MC-ko} mice. Taken
together, these results suggest a role for PTEN in the regulation of the HIFα-VEGF axis under hypoxia in macrophages (Fig. 1).

**Inhibition of PI-3K/AKT pathway blocks the hypoxic induction of HIF1α & HIF2α and its transcriptional target VEGF in macrophages**

The observation that PTEN exerts control over HIF1α and HIF2α protein expression and transcriptional activity (Fig. 1) led us to investigate the effects of different clinically relevant PI-3K inhibitors (SF1126, PF4691502, PI-103 and BKM120) on the hypoxic induction of HIFα in macrophages. SF1126, a pan PI-3K inhibitor, is a vascular RGDS/integrin targeted prodrug derivative of LY294002 and is entering phase II clinical trials (29). The other inhibitors employed are in early phase clinical trials include: PI-103, a dual PI-3K /mTOR inhibitor; PF4691502, a pan PI-3K inhibitor; BKM120, a pan PI-3K inhibitor (33-35). The results shown in Fig. 2A clearly demonstrate that SF1126, PI-103, PF4691502 and BKM120 potently inhibit the hypoxic induction of HIF1α & HIF2α in BMDMs. Furthermore, these pan PI-3K inhibitors also blocked the transcriptional activation of HIFα target gene, VEGF (Fig. 2B). In addition to pan PI-3K inhibitors in clinical trial, we used SF2523, a novel pan PI-3K inhibitor developed in collaboration with SignalRx Pharmaceuticals (30) to see the effect of this PI-3K inhibitor on HIFα stabilization. SF2523 blocked accumulation of HIF1α & 2α in dose-dependent manner (Fig. 2C), which correlates with the decreased VEGF mRNA in BMDMs treated with SF2523 (Fig. 2D). To determine the role of AKT in hypoxic HIFα accumulation, experiments were performed where AKT is either knocked down or an activated membrane targeted AKT (myrAKT) is exogenously expressed in BMDMs. Fig. 2E & F showed that knockdown of AKT using siRNA blocked accumulation of HIFα while exogenous expression of myrAKT increased HIFα levels.

**Specific p110δ and p110 γ isoform of PI-3K control HIFα-VEGF axis in macrophages**

We next focused on the specific isoforms of PI-3K involved in regulating HIFα-VEGF axis in macrophages. Recently, published reports (26) and our results provide evidence that p110γ is the
predominant isoform expressed in the myeloid cells and macrophages (Supplementary Fig. S1A). In order to determine the specificity of the PI-3K signaling in HIFα accumulation isoform specific PI-3K inhibitors viz., GDC-0941 (p110α specific inhibitor) (36), TGX-221 (p110β specific inhibitor) (37), Cal101 (p110δ specific inhibitor) (38) and AS605240 (p110γ specific inhibitor) (39) were used. Interestingly, we found that AS605240 (a p110γ isoform inhibitor) showed the most marked inhibition of HIFα accumulation and decreased the transcriptional activation of VEGF in BMDMs, while the effect of inhibitors of α, β and δ isoform didn’t show a marked effect on HIFα accumulation (Fig. 3A & B). However, inhibitors of α, β and δ isoform significantly blocked VEGF mRNA expression. Although isoform specific inhibitors of PI-3K display some level of selectivity, the kinase inhibitory profiles and IC50 potencies against different p110 isoforms is not absolute making conclusions difficult. To confirm a specific role for different PI-3 kinase catalytic isoforms in the regulation of HIF1α accumulation, we therefore knocked down the expression of p110 isoforms in BMDMs by using si-RNA against these isoforms. As shown in Fig. 3C & D, targeting of specific p110γ isoform lead to decreased hypoxia induced HIFα stabilization and reduced expression of VEGF mRNA, whereas there is no effect on HIFα protein and VEGF mRNA levels on targeting α and β isoforms. Interestingly, knockdown of p110δ isoform also blocked accumulation of HIFα protein as well as VEGF gene expression (Fig. 3C & D). Taken together, these results support that HIFα stability is mainly contributed by p110γ isoform and partially by p110δ isoform of PI-3K. To validate our results that p110γ is the major isoform involved in regulating HIFα stability, we used p110γ/-/- mice. The BMDMs isolated from these mice show decreased HIFα accumulation and VEGF secretion (Fig. 3E & F). However, p110γ/-/- BMDMs treated with AS605240 (p110γ specific inhibitor) didn’t show any further reduction in VEGF levels (Fig. 3F), further validating our results that p110γ is the major isoform involved in HIFα accumulation and VEGF secretion.

**Blocking PI-3K signaling axis induces the hypoxic degradation of HIFα via the 26S proteasome pathway**
The above results (Fig. 1-3) clearly establish a key role for the PTEN-PI-3K signaling pathway in hypoxia induced HIFα accumulation in macrophages. In order to explore the mechanism by which PTEN-PI-3K pathway affects HIFα levels in macrophages, we utilized the proteasome inhibitor, MG132 to ask whether this process was related to proteasome dependent degradation of HIF. BMDMs from PTEN\textsuperscript{MC-WT} and PTEN\textsuperscript{MC-KO} mice were treated with the proteasome inhibitor, MG132, prior to exposure to hypoxia. Our data supports the hypothesis that PTEN affects HIFα under hypoxic conditions by inducing the hypoxic degradation of HIFα as this effect can be reversed by pretreatment with the proteasome inhibitor, MG132 (Fig. 4A). To further confirm that the disappearance of HIFα is a proteasome dependent event and that PI-3K activation is required for the hypoxic stability of HIFα we treated macrophages with a panel of PI-3K inhibitors. The decrease in HIFα accumulation observed in BMDMs treated with pan PI-3K inhibitors, p110γ isoform specific PI-3K inhibitor, AS605240 and novel pan PI-3K inhibitor SF2523 was reversed by treatment with MG132 (Fig. 4B, C & D). The degradation of HIFα by VHL under normoxic conditions is well known, however its degradation under hypoxia is poorly understood. \textbf{Importantly}, our results provide direct evidence that HIFα is degraded under hypoxic conditions in a PTEN and PI-3K dependent manner in macrophages.

\textbf{PI-3Kγ signaling in macrophages promotes tumor growth and metastasis}

It is well-documented that macrophages exposed to hypoxia accumulate both HIF1α and HIF2α, and overexpression of HIF2α in tumor-associated macrophages (TAMs) is specifically correlated with high-grade human tumors and poor prognosis (9). On the same note, Doedens et al. has reported that macrophage expression of HIF1α suppresses T cell function and promotes tumor growth (40). Based on the results observed in the present study showing the important role of PI-3K signaling axis in controlling both HIF1α & HIF2α accumulation in macrophages, we hypothesize that PI-3K signaling should promote tumor growth by increasing secretion of proangiogenic factors in TAMs. To validate this hypothesis, syngeneic Lewis lung carcinoma (LLC) was injected subcutaneously into WT and p110γ\textsuperscript{-/-} mice. Tumor
growth and angiogenesis were drastically reduced in p110γ/-/- mice (Fig. 5A & B). Immunofluorescence CD31 staining showed that the microvessel density was significantly reduced in tumors propagated in p110γ/-/- mice (Fig. 5B). Based on previously published report that p110γ is the isoform predominantly expressed in myeloid cell compartment (26) and our observation that p110γ isoform is the major contributor of hypoxia-induced both HIF1α and HIF2α accumulation in macrophages (Fig. 3C-F), we hypothesized that macrophages infiltrating into the tumors implanted in p110γ/-/- mice would be defective in secretion of proangiogenic factors that promote tumor growth. To validate this hypothesis, macrophages were FACS sorted (on the basis of CD11b and F4/80 staining) from LLC tumors implanted in WT and p110γ/-/- mice and evaluated for expression of genes required for tumor growth and progression. For this, a panel of TAM associated genes VEGF, MMP9, uPA, Cox2, Arginase, MMR, TGFβ, IL1, TNFα were used to examine the gene expression profile of macrophages present in the tumor microenvironment of WT and p110γ/-/- mice. The expression levels of Cox 2, uPA, MMP9, MMR, arginase and VEGF are significantly higher in macrophages sorted from LLC tumors of WT as compared to p110γ/-/- mice (Fig. 5C). In contrast, the macrophages sorted from tumors implanted in p110γ/-/- animals showed higher levels of IL1 and TNFα (Fig. 5C). Consistent with this, existing literature suggests that tumor associated macrophages secrete several proteins including MMP-9 (41), uPA (42) and VEGF (43), which promotes tumor growth, angiogenesis and metastasis. IL1 and TNF α are pro inflammatory cytokines which are reported to be expressed at a lower level in tumor associated M2 macrophages and are known to promote tumor suppression (44). These results suggest that macrophages isolated from tumors implanted in p110γ/-/- mice express higher levels of proinflammatory cytokines seen in M1 macrophages which promote tumor suppression, while macrophages isolated from WT mice shows higher expression of genes which promotes tumor growth and progression. Tumor growth and angiogenesis are prerequisites for metastasis hence, we examined if p110γ/-/- mice display any defect in metastasis. In order to explore the role of p110γ in experimental lung metastasis, B16F10 melanoma cells were injected into mouse tail veins of WT and p110γ/-/- animals. Two weeks later, we observed massive numbers of
metastatic foci in the lungs of WT mice compared with a marked reduction in number of nodules in p110\(\gamma\)-/- mice (Fig. 5D). A microscopic analysis of lung tissue sections confirmed the results from gross specimens and demonstrated larger numbers of metastatic foci in the lungs of WT mice (Fig. 5D). These observations led us to examine if the BMDMs prepared from p110\(\gamma\)-/- mice in vitro display any defect in expression of genes promoting tumor growth and progression. We found that macrophages isolated from LLC tumors growing in p110\(\gamma\)-/- mice were defective in the expression of genes previously implicated in the promotion of tumor growth (M2 markers) (Fig. 5E) including VEGF described in Fig. 3F.

**SF1126, pan PI-3 kinase inhibitor blocks tumor growth and metastasis and reduces the expression of proangiogenic factors by TAMs.**

Considering the role of PTEN and PI-3K signaling in the macrophage dependent promotion of tumor growth and tumor-induced angiogenesis, we examined the effects of a pan PI-3K inhibitor, SF1126, in the LLC model for its effects on tumor growth, angiogenesis and metastasis in vivo (Fig. 6). SF1126 has completed phase I clinical trial in solid tumors and B cell malignancies and is headed for a Phase II trial in 2014 (29). The administration of 50 mg/kg dose of SF1126, three times a week significantly blocked tumor growth in WT animals implanted with LLC (Fig. 6A). Hence, we determined if SF1126 exerts an effect on the neovascularization of these tumors. Immunofluorescence CD31 staining showed that the microvessel density was significantly reduced in the mice treated with SF1126 (Fig. 6B). Since TAMs are known to have a marked influence on the regulation of tumor-induced angiogenesis (45), we propose that SF1126 blocks angiogenesis in the tumors by blocking the proangiogenic factors secreted by TAMs within the tumor microenvironment. The supporting evidence for this notion comes from the real-time PCR data obtained from macrophages (FACS sorted on basis of CD11b and F4/80 staining) isolated from the LLC tumors implanted into WT mice and treated with or without SF1126 (Fig. 6C). Interestingly, the macrophages sorted from the mice treated with SF1126 inhibitor showed reduced expression of VEGF, MMP9, arginase and Cox-2 (Fig. 6C). The results in Fig. 6C, suggest that
macrophages isolated from SF1126 treated tumors display a reduced expression of proangiogenic factors promoting metastasis. So, we next explored if administration of SF1126 can block tumor metastasis. We observed a marked increase in metastatic foci in the lungs of WT mice compared to the 60% reduction in metastatic nodules seen in SF1126 treated mice injected with B16F10 melanoma (p < 0.01; Figure 6D &E). Our results demonstrating that SF1126 blocked metastasis lead us to explore the effect of SF1126 on survival in the B16 experimental metastasis model. The data in Fig. 6F demonstrates that SF1126 treatment prolongs the survival of mice by average 15 days, a result which directly correlates with the inhibition of experimental metastasis in this model and validating the efficacy of this drug either alone or in combination with other drugs in the treatment of cancer. Taken together, we conclude that SF1126 blocks tumor growth and metastasis potentially by blocking HIF1α and HIF2α and the downstream expression and secretion of proangiogenic factors by tumor infiltrating M2 macrophages. Based on these results, we propose a hypothetical model in which E3 ligase degrades HIF1α under hypoxic conditions in the cytoplasm in a proteasome dependent manner. The model would predict that the stimulation of PI-3K and AKT via upstream signals will serve to promote translocation of E3 ligase into the nucleus and promote the stabilization of hypoxic HIF1α. The pathway will bring hypoxic HIF1α under the control of the PTEN-PI-3K/AKT signaling axis to regulate an E3 ligase to control hypoxic HIF1α levels and angiogenesis and tumor metastasis in vivo (Fig. 7).

Discussion

It is well-established that TAMs accumulate in hypoxic regions of tumors and that hypoxia triggers a pro-angiogenic program in these cells stimulating the production of angiogenic factors and matrix regulatory proteins such as VEGF and matrix metalloproteinases (MMPs), respectively (46, 47). Therefore, macrophages recruited in situ represent an indirect pathway of amplification of angiogenesis, in concert with angiogenic molecules directly produced by tumor cells. Thus, we speculate that targeting
the common pathway promoting angiogenesis in macrophages as well as tumor cell compartment will likely provide more effective therapy in the treatment of cancer. The PI-3K pathway is known to play a critical role in tumor cell survival, angiogenesis, invasion, apoptosis and cellular metabolism (15, 22) and is considered as the most frequently activated pathway in cancer. Herein, we provide direct evidence that PI-3K pathway controls tumor growth and angiogenesis and regulates the HIFα-VEGF axis in macrophages. Moreover, SF1126, a pan PI-3K inhibitor entering phase II clinical trials, blocks macrophage mediated tumor growth, angiogenesis and metastasis, suggesting efficacy of SF1126 and other PI-3K inhibitors in the treatment of cancer in which macrophages are important in tumor progression.

Recent studies have clearly demonstrated that HIF1α and HIF2α are stabilized in hypoxic macrophages in vitro (12). Talks et al. demonstrated that HIF2α protein accumulates at high levels in TAMs detected in various human cancers (9). Recent reports suggest the important role of HIF2α in macrophage functions in mouse models of tumor inflammation (48). In the present study, using genetic mouse models, siRNA approach and clinically relevant PI-3K inhibitors, we provide evidence that PTEN/PI-3K signaling controls both HIF1α and HIF2α accumulation in macrophages. The role of PTEN-PI-3K signaling in promoting HIFα stabilization in tumor cells is well documented. However, there are no reports examining the role of this important signaling axis in HIFα stabilization in macrophages. Our studies clearly demonstrate that the loss of PTEN in the myeloid cell compartment promotes hypoxic HIFα stabilization in BMDMs (Fig. 1). Our studies provide convincing evidence that the expression of PTEN and inhibition of PI-3K/AKT signaling induces the hypoxic degradation of HIF1α & HIF2α in a proteasome dependent manner. While much is known about the transcriptional and post-translational regulation of HIFα, there is very limited evidence that HIFα can be degraded under conditions of hypoxia by the 26S proteasome (49). Liu et al. recently described oxygen-independent degradation of HIF1α by a novel HIF1α-interacting protein, receptor for activated C-kinase 1 (RACK1) (50). They suggested that similar to the E3 ligase pVHL, RACK1 increased poly-ubiquitination of HIF1α and was unable to
mediate degradation of HIF1α in the presence of proteasomal inhibitor MG132. In the same context, recent study suggests that SHARP promotes proteasomal degradation of HIF1α independent of pVHL, hypoxia and ubiquitination machinery (51). Recently published work from our laboratory performed in glioma tumor models has demonstrated that PTEN and PI-3K inhibitors control the hypoxic degradation of HIF1α and that the E3 ligase, MDM2, is required for hypoxic degradation of HIF1 α (52) The E3 ligase and other signaling pathways controlling the degradation of HIF1α & HIF2α under hypoxic conditions in macrophages remains an active area of investigation and will be a major focus of our future research efforts.

Moreover, we observed that p110γ, the most dominant isoform of PI-3K expressed in macrophages, controls HIF1α and HIF2α accumulation in macrophages. Interestingly, our data provide evidence that p110δ isoform contributes significantly to HIFα stabilization and VEGF secretion in BMDMs (Fig. 3C & D). More importantly, we demonstrate that the expression of p110γ in macrophages promotes tumor growth and metastasis potentially by controlling the expression of proangiogenic and tumor growth promoting factors by TAMs (Fig. 5C & E). A recent report by Schmid et al has shown that p110γ promotes tumor inflammation by controlling the trafficking of myeloid cells into tumor (26). Our work extends these findings and suggests that the expression of p110γ in macrophages controls the hypoxia driven HIFα-VEGF axis which plays a crucial role in neovascularization and tumor growth. Moreover, our studies provide evidence that administration of SF1126, blocks macrophage mediated tumor growth and angiogenesis. SF1126 has completed phase I clinical trial in B cell malignancies and solid tumors with excellent safety profile (29). It is currently slated for a Phase II trial in PIK3CA mutated human cancers. Herein, we demonstrate that SF1126 which potently blocks tumor proliferation and survival (29, 53), exerts a marked effect on the tumor stromal macrophage compartment as it blocks tumor growth, angiogenesis and metastasis in vivo. In conclusion, the major findings of this paper are: 1) we identified an important role of PTEN-PI-3K/AKT signaling axis in HIF1α and HIF2α stabilization in macrophages 2) PTEN and several clinically relevant PI-3K inhibitors exert their control over HIFα-
VEGF axis by inducing the hypoxic degradation of HIFα subunit in proteasome dependent manner 3) Expression of p110γ in macrophages promotes tumor growth and metastasis 4) SF1126 potently blocked macrophage-mediated tumor angiogenesis and metastasis. These studies suggest SF1126 and other PI-3K inhibitors are excellent candidates for the treatment of cancers where macrophages promote tumor progression.

Acknowledgements

This research effort was funded by CA94233 and HL091385 from NIH to DLD and grants from ALSF (Springboard Grant) and Hyundai Hope on Wheels Foundation, Hope Grant. D.L. Durden discloses a financial conflict of interest regarding the SF1126 compound under study in this manuscript. This relationship has been reviewed by the UCSD committee on conflict of interest.

References


Legends to Figures:

**Figure 1** Deletion of PTEN in myeloid cells increase hypoxic HIF1α and HIF2α accumulation. A, Western blot analysis showing deletion of PTEN in myeloid cells. Cell lysates from BMDMs isolated from PTENMC-KO or PTENMC-Wo mice were subjected to Western blot analysis for PTEN, pAKT and AKT. β-actin was used as loading control. (B & C), BMDMs isolated from PTENMC-WT and PTENMC-KO (B) and PTEN +/- and PTEN+/- (C) mice were incubated under hypoxic (1% O₂) conditions for 4 hrs followed by preparation of nuclear extracts (for HIF1α & HIF2α) and Western blot analysis. Each lane in A, B & C represent lysate prepared from individual mice. Mouse ID numbers are provided for each genotype. D, mRNA was isolated from PTENMC-WT and PTENMC-KO BMDMs incubated under hypoxia (1% O₂) for 24 hrs. VEGF mRNA expression was measured by real time PCR. Data represent mean ± SEM (n = 3 or 4; P < 0.001; pair wise two-sided Student's t test). (E) BMDMs isolated from PTENMC-KO mice were transfected with 5 µg of HA-PTEN, using AMAXA mouse macrophage Nucleofaction kit. After 36 hrs of transfection, cells were exposed to normoxia (21% O₂) or hypoxia (1% O₂) for 4 hrs followed by preparation of either nuclear extracts (for HIF α blots) or WCE (PTEN blot) and Western blot analysis. Experiments were repeated 4-5 times with 2-3 mice in each group.

**Figure 2** PI-3K/AKT signaling axis control HIFα-VEGF axis. A & B, BMDMs from WT animals were treated with different concentrations of PI-3K inhibitors (500 nM PF 4691502, PI 103, BKM 120 & 25
μM SF1126) followed by hypoxia for 4 hrs for Western blots or 24 hrs for Real Time PCR studies. These macrophages were either used for lysate preparation (nuclear extracts for HIFα or WCE for pAKT and AKT) and Western blot analysis (A) or RNA and real time PCR for VEGF (B). C & D, WT BMDMs were treated with different conc. of SF2523 (10, 20 and 50 μM) followed by hypoxia for 4 hrs for Western blots (C) or 24 hrs for RNA isolation and real time PCR for VEGF (D). E & F, BMDMs from WT animals were transfected with either with 5μg of Myr-AKT plasmid or 100 nM control siRNA or 100 nM siRNA against AKT1/2 using Amaxa Mouse Macrophage Nucleofaction kit (Lonza). 36 hrs after transfection, hypoxia was given to these transfected BMDMs for 4 hrs followed by cell lysate preparation and Western blot analysis. Graphs in B & D represent mean ± SEM (n = 3-4). Statistical significance is assessed by two sample t-test where *denotes P<0.05, ** denotes P<0.01 and *** denotes P<0.001. Experiments were repeated 3-4 times with similar results.

Figure 3 Specific p110γ and p110δ isoform of PI3K control HIFα-VEGF axis in macrophages A & B, BMDM from WT animals were treated with 100nM of GDC0941 or TGX221 or Cal101 or AS605240 followed by hypoxia for 4 hrs for Western blot analysis or 24 hrs for RNA isolation and Real time PCR analysis. C & D, BMDMs from WT animals were transfected with either 100 nM control siRNA or siRNA against p110α or β or δ or γ isoform using Amaxa Mouse Macrophage Nucleofaction kit (Lonza). 36 hrs after transfection, hypoxia was given to these si-RNA transfected BMDMs for 4 hrs for Western blot analysis (C) or 24 hrs for RNA isolation and Real Time PCR analysis (D). E, Western blot analysis of HIF1α & HIF2α from WT and p110γ-/- BMDMs kept in hypoxic (1% O2) conditions for 4 hrs followed by preparation of nuclear extracts. Each lane in figure represents lysate prepared from an individual mouse. F, Relative VEGF expression in WT, p110 γ-/- BMDMs treated with or without 100nM AS605240 and kept in hypoxia (1% O2) for 24 hrs, followed by RNA isolation and real time PCR studies. Graphs in B, D & F represent mean ± SEM (n = 4-5) for three independent experiments. Statistical significance is assessed by two sample t-test where *denotes P<0.05, ** denotes P<0.01 and *** denotes P<0.001.
Figure 4. Hypoxic degradation of HIF1α & HIF2α by PTEN-PI-3K/AKT pathway occurs via the 26S proteosome. A, Western blot analysis of HIF1α & HIF 2α from BMDMs from PTENMCWT and PTENMC-KO treated with 10 µM MG132 and kept in hypoxic conditions for 4 hrs. B-D, WT BMDMs were treated with the proteosome inhibitor 10 µM MG132 for 5 min before being pulsed with the pan-PI-3K inhibitors, 25 µM SF1126 & 500 nM PF4691502 (B), 100 nM AS605240 (C) and 10 µM SF2523 (D) followed by normoxia (21% O2) or hypoxia (1% O2) for 4 hrs. Nuclear extracts were prepared for HIFα Western blots as described before. Experiments were repeated 3-4 times with similar results.

Figure 5. Expression of p110γ isoform in macrophages promotes tumor growth and angiogenesis. A, Tumor growth of LLC cells inoculated subcutaneously in WT and p110γ-/- mice (n=6-8). B, Left panel shows representative immunofluorescent staining of tumor vasculature by CD31 (green) and counterstain by DAPI (blue) on frozen tumor sections of LLC tumors implanted subcutaneously in WT and p110γ-/- mice. Right panel shows reduced microvascular density (MVD) in tumors isolated from p110γ-/- animals as compared to WT animals. MVD was determined by counting the number of microvessels per high-power field (HPF) in the section with an antibody reactive to CD31. Microvessels were counted blindly in 5-10 randomly chosen fields and data is representative of three independent experiments with 4-5 mice. * P<0.05 vs. WT. C, Relative expression levels of specific genes required for tumor growth in macrophages sorted from LLC tumors implanted into WT and p110γ-/- animals. D, Left panel shows representative photograph of pulmonary metastatic foci produced 15 days after intravenous injection of B16F10 cells in WT and p110γ-/- mice. Right panel shows number of experimental pulmonary B16F10 metastases in lungs counted under dissecting microscope. (E) Expression of specific tumor promoting genes in the BMDMs isolated from WT and p110γ-/- mice and cultured in MCSF in vitro. Graphs present mean ±SEM of 7 mice for A, B and D and 3-4 mice for C. *P <0.05, **P <0.01 and ***P <0.001 vs. WT, as determined using two sample t-test.
Figure 6. SF1126, a pan PI-3kinase inhibitor blocks tumor growth, angiogenesis, metastasis and reduced the expression of proangiogenic factors by TAMs. A, Tumor volume of LLC inoculated subcutaneously in WT mice treated with or without 50 mg/kg SF1126 three times a week. Treatment was started 10 days after inoculation of LLC cells and was continued until tumors were harvested (n=6-8). B, MVD was scored by manual counting of CD31 positive vessels in subcutaneous LLC tumor sections was reduced in tumors isolated from WT animals treated with SF1126. Right panel shows reduced microvascular density in tumors isolated from untreated or SF1126 treated animals as confirmed by quantification of CD31 positive area in high power field (HPF) C, Relative expression levels of specific genes required for tumor growth in macrophages sorted from LLC tumors implanted into WT mice and treated with 50 mg/kg of SF1126, three times a week until tumors were harvested. D, Left panel shows representative photograph of pulmonary metastatic foci produced in WT animals injected with B16F10 melanoma and treated with daily dose of 50 mg/kg SF1126 for 15 days. Right panel shows number of experimental pulmonary B16F10 metastases in lungs. E, Figure shows representative lung H&E sections of B16 metastases from WT mice treated with or without 50 mg/kg SF1126. Arrows point to metastatic foci. F, Kaplan Meir survival curve for B16 metastasized mice treated with or without 50 mg/kg SF1126 daily. Data are representative of 2-3 independent experiments. Graphs present mean ±SEM of 8-10 mice for A, B, D, E and F and 3-4 mice for C. *P <0.05 and **P <0.01 vs. WT, as determined using two sample t-test.

Figure 7. Mechanism by which the PTEN-PI-3K-AKT signaling axis exerts control over HIF α stability, tumor growth and metastasis by secretion of proangiogenic factors by macrophages. PI-3K inhibitors block the phosphorylation of an E3 ligase resulting in cytoplasmic localization of E3 ligase and degradation of HIF1α; this could have therapeutic implications for HIF1α-VEGF axis in PI-3K inhibitor cancer therapeutics.
Fig. 1

A

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Mouse ID

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AKT     
β actin

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Mouse ID

HIF1α    
HIF2α    
β actin

C

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Mouse ID

HIF1α    
HIF2α    
PTEN

D

E

- +    - +    HA-PTEN
Normoxia Hypoxia

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***
Fig. 5

A

Tumor Volume (mm³)

0
2500
500
14
17
21
25
11

Days

WT

p110γ−/−

B

CD 31 Staining

WT

p110γ−/−

C

Relative Gene Expression

0
5
10
15
20
25
30
35
IL-1
TNFα
Ccr2
Lrp
Mmp-9
Aggrecan
Vegf

D

WT

p110γ−/−

E

No. of Metastatic Nodules

0
25
50
75
WT

p110γ−/−
Fig. 6

A

B

C

D

E

F

**Tumor Volume (mm³)**

- **Vehicle**
- **SF1126 Treated**

**Time of Treatment (Days)**

0 3 6 9 12 15 18

- 0
- 500
- 1000
- 1500
- 2000
- 2500
- 3000
- 3500

**Relative Gene Expression**

- **IL-6**
- **MMP-2**
- **IL-1β**
- **TNF-α**
- **Arginase**
- **VEGF**

**Vehicle**

**SF1126 Treated**

**Percent survival**

0 5 10 15 20 25 30 35 40

- **Vehicle**
- **SF1126 Treated**

* indicates statistical significance compared to vehicle.