Pharmacological targeting of VEGFR signaling with axitinib inhibits Tsc2-null lesion growth in the mouse model of lymphangioleiomyomatosis (LAM)

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ABSTRACT

Pulmonary lymphangioleiomyomatosis (LAM), a rare progressive lung disease associated with mutations of the Tuberous Sclerosis Complex (Tsc2) tumor suppressor gene, manifests by neoplastic growth of LAM cells, induction of cystic lung destruction and respiratory failure. LAM severity correlates with up-regulation in serum of the prolymphangiogenic vascular endothelial growth factor D (VEGF-D) that distinguishes LAM from other cystic diseases. The goals of our study was to determine whether Tsc2-deficiency up-regulates VEGF-D and whether axitinib, the FDA-approved small molecule inhibitor of VEGFR signaling, will reduce Tsc2-null lung lesion growth in a mouse model of LAM. Our data demonstrate up-regulation of VEGF-D in the serum and lung lining in mice with Tsc2-null lesions. Progressive growth of Tsc2-null lesions induces recruitment and activation of inflammatory cells and increased nitric oxide production. Recruited cells isolated from the lung lining of mice with Tsc2-null lesions demonstrate up-regulated expression of pro-vasculogenic Vegfa, pro-lymphangiogenic Figf and pro-inflammatory Nos2, Il6, Ccl2 genes. Importantly, axitinib is an effective inhibitor of Tsc2-null lesion growth and inflammatory cell recruitment which correlates with reduced VEGF-D levels in serum and lung lining. Our data demonstrate that pharmacological inhibition of VEGFR signaling with axitinib inhibits Tsc2-null lesion growth, attenuates recruitment and activation of inflammatory cells and reduces VEGF-D levels systemically and in the lung lining. Our study suggests a potential therapeutic benefit of inhibition of VEGFR signaling for treatment of LAM.
INTRODUCTION

Pulmonary lymphangioleiomyomatosis (LAM) is a rare progressive lung disease affecting predominantly women of childbearing age which manifests by neoplastic growth of atypical smooth muscle-like LAM cells in the pulmonary interstitial space leading to cystic lung destruction and spontaneous pneumothoraces (20, 24). Mutations of the Tuberous Sclerosis Complex 1 (TSC1) and TSC2 tumor suppressor genes induce abnormal growth of LAM cells in the lung (12, 20). Importantly, LAM severity correlates with up-regulated serum levels of pro-lymphangiogenic VEGF-D and the degree of lymphatic vessel density within the lung (40, 46). Clinical data obtained from the MILES trial demonstrated that VEGF-D level in serum could serve as a novel biomarker for evaluating LAM severity, response to treatment and to prospectively distinguish LAM from other cystic lung diseases (34, 40, 46) such as pulmonary Langerhans cell histiocytosis, emphysema, Sjögren syndrome, and Birt-Hogg-Dubé syndrome (10, 47).

Correlation of VEGF-D levels with the severity of LAM measured by LAM CT grade, the abundance of chylous effusions and lymphatic involvement, has been considered as a prognostic tool for disease progression (46). Despite these findings, little is known about the mechanism by which growth of TSC2-null LAM lesions induces VEGF-D up-regulation and abnormal lymphangiogenesis, or whether VEGF-D signaling and lymphangiogenesis can be targeted therapeutically in LAM.

Lymphangiogenic VEGF-D promotes the formation of tumor lymphatic vessels and facilitates metastatic spread of cancer cells by regulating prostaglandin synthesis (27, 43). VEGF-D is a secreted glycoprotein that, in the form of a homodimer, binds and activates receptor protein tyrosine kinases VEGF receptor 2 (VEGFR2) and VEGFR3.
which are highly expressed by lymphatic endothelium in humans, and to VEGFR3 in
mice (1, 43). Histopathological studies in LAM show marked VEGFR3 staining
surrounding LAM lesions and abundant lymphatic channels within LAM lesions detected
by lymphatic vessel endothelial hyaluronan receptor 1 (LYVE-1), VEGFR3 and
podoplanin expression (9, 10, 23, 31). This evidence suggests that up-regulation of
VEGF-D might recruit lymphatic endothelial cells forming lymphatic channels, which
might induce lung remodeling in LAM disease (20).

Previously we demonstrated up-regulation of VEGF-D in a mouse model of LAM
(13). Tsc2-null lung lesions develop throughout the lung and display marked up-
regulation of lymphatic channels similar to those observed in the human LAM (13). The
goal of this study is to determine whether inhibiting VEGF-D represents a viable
pharmacological approach for preventing Tsc2-null lesion growth. Several therapeutic
approaches have been developed to target VEGF-D and VEGFR2/3 signaling including
small molecule inhibitors and neutralizing antibodies for VEGF-D and VEGFR2/3
receptors, some of which successfully completed clinical trials and received FDA
approval (43). In our study we used axitinib, the FDA-approved selective small-
molecule inhibitor (35), to dissect the role of VEGFR signaling in preventing Tsc2-null
lesions growth using a mouse model of LAM.
MATERIALS AND METHODS

Cell Lines

Tsc2-deficient MKOC kidney epithelial cells, derived from renal tubular cystadenoma of Tsc2+/- mice created on C57BL/6 background, were generously provided by Dr. Okio Hino from the Cancer Institute, Tokio, Japan (13, 29). To enhance the invasive characteristics of MKOC cells, 5x10^6 cells were injected subcutaneously into flanks of NCRNU female athymic nude mice (Taconic) (13). After tumors reached 1.5 cm in diameter, mice were sacrificed, the tumors were removed, enzymatically digested and maintained in Dulbecco’s modified Eagle’s medium (DMEM) with 10% fetal bovine serum (FBS) for 2 days. MKOC cells derived from these tumors were named TMKOC, as we described previously (13). Tsc2-expressing kidney tubular epithelial M-1 cells dissected from C57BL/6 mice were purchased from the American Type Culture Collection (ATCC, CRL-2038) and were used as a control. MKOC, TMKOC and M-1 cells were maintained in DMEM supplemented with 10% FBS and further characterized by immunoblot analysis to confirm Tsc2 loss and mTORC1 activation in Tsc2-null cells.

Animals

All animal procedures were performed according to a protocol approved by the University of Pennsylvania Animal Care and Use Committee. Briefly, 8-week-old, athymic nude female mice (NCRNU, Taconic) were injected with TMKOC cells (10^6/100 μL of PBS) into the tail vein as previously described (4, 13). Starting 3 days post-injection, mice received daily intraperitoneal (i.p.) injections of either diluent or 25 mg of axitinib (AG-013736; Tocris Bioscience, Bristol, UK; Batch # 1A/133671) per kg of body weight. Each experimental group included a minimum of 10 mice per condition.
**BAL Fluid Analyses**

Mice were sacrificed 3 weeks post-TMKOC cells injection, lungs were lavaged with PBS to a total volume of 5 ml. Cells isolated from BAL were collected, counted (Beckman-Coulter, Inc., Miami, FL), differentiated and the remaining cells, not used for differential analysis, were frozen and further analyzed by quantitative RT-PCR as previously described (4, 14). Aliquots of the cell-free BAL fluid were stored at -80°C and further analyzed for cytokine levels by Multiplex (Aushon, Billerica, MA) and NO metabolites by chemical reduction and chemiluminescence using the Ionics/Sievers Nitric Oxide Analyzer 280 (NOA 280; Ionics Instruments, Boulder, CO) as previously described (3, 4). Following lavage, lungs were fixed in formalin for H&E staining and immunohistochemical (IHC) analysis.

**Western blotting**

Total proteins from MKOC, TMKOC and M-1 were extracted through 15-minute incubation with Nonidet P-40 lysis buffer and resolved on 4-12% Bis-Tris SDS polyacrylamide gel, (Life Technologies, NP0335). Phosphorylation of ribosomal protein S6 (2217), pS-6 (4856), tuberin (TSC2, 4308) and tubulin (2125) antibodies were obtained from Cell Signaling Technology (Danvers, MA). Equal volume of BAL fluids from axitinib treated or untreated mice were analyzed with VEGF-D antibodies (R&D Systems, Inc., AF469) or SP-D antibodies provided by Michael F Beers (2-4).

**IHC analysis**

IHC analysis of lungs to detect lymphatic vessels were performed by immunostaining with LYVE-1 antibody (Fitzgerald Industries Int. Acton, MA; 70R-LR005) and with 4′,6-
Diamidino-2-phenylindole (DAPI) staining as described (11, 14, 15, Gonchariova 2012). Lesions from a minimum of five animals per each treatment condition were analyzed using the Nikon Eclipse TE2000-E microscope equipped with an Evolution QEi digital video camera.

**qRT-PCR**

M-1, MKOC, TMKOC cell lines and cells recovered from lung lining of treated and untreated mice were analyzed for mRNA expression levels by qRT PCR. The reactions were carried out with SYBR® Green PCR master mix on an ABI Prism 7300 sequence detection system (Applied Biosystems) Obtained Ct values were normalized to β-actin signals and further analyzed using the ΔΔCt method as previously described(4, 14, 28).

**VEGF-D ELISA**

Serum VEGF-D levels were analyzed by commercially available enzyme linked immunoassay (ELISA) kit according to the manufacturer’s protocol (R&D Systems, catalog # DY469) with analysis on a Molecular Devices plate reader. Data were represented as means ± SEM from triplicate measurements and shown as pg of VEGF-D per ml of sample.

**Data Analysis**

Data are shown as mean ± standard error of the mean (SEM). For direct comparisons between two groups, significance was assessed by Student’s t-test. In experiments with multiple treatment groups significance tests were made within the confines of a significant two-way ANOVA and using Bonferroni correction for multiple comparisons.
Assessment was performed by means of GraphPad Prism version 4.00 (GraphPad Software, San Diego, CA). Values of $p < 0.05$ were considered significant.
RESULTS

Tsc2 deficiency up-regulates VEGF-D protein and mRNA expression

To determine whether Tsc2 deficiency up-regulates VEGF-D levels, we used Tsc2-null mouse cells (MKOC and TMKOC) (13) derived from renal tubular cystadenoma of Tsc2 heterozygous mice as described in Methods (29). Since these Tsc2-null cells originated from the renal tubular epithelium, we used Tsc2-expressing renal tubular epithelial M-1 cells as a control. As seen in Figure 1A, marked up-regulation of VEGF-D in Tsc2-null but not in Tsc2-expressing M-1 cells was detected by immunoblot analyses using anti-mouse VEGF-D antibody, which has been previously used to detect VEGF-D levels in mouse cells (5). Tsc2 deficiency in MKOC and TMKOC cells was confirmed by immunoblot analysis as shown in Figure 1A. The constitutive activation of the mTORC1 signaling pathway, a hallmark of Tsc2 loss (12, 16), in Tsc2-deficient cells, was demonstrated by phosphorylation of ribosomal protein S6 (Fig. 1A). Thus, the cells, named TMKOC, utilized in the LAM mouse model were validated by demonstrating the Tsc2 loss, mTORC1 activation and up-regulation of VEGF-D.

To investigate whether Tsc2 deficiency affects VEGF-D expression, Tsc2-null and Tsc2-expressing cells were analyzed by qRT-PCR for specific VEGF family genes. As seen in Figure 1B, the Figf gene expressing VEGF-D is markedly increased in TMKOC cells as compared to M-1 cells. As additional controls we examined mRNA levels of Vegfa, a well-known angiogenic growth factor which expression is regulated by Tsc2 (6, 7). Figure 1B shows marked up-regulation of Vegfa in correspondence with high Figf levels in TMKOC cells (Fig. 1B). In contrast, the Vegfc gene, that is required for lymphangiogenesis during development (26), shows undetectable levels in TMKOC
cells (Fig. 1B). Interestingly, TMKOC cells exhibited increased expression of the inflammatory gene Nos2.

Collectively, these data demonstrate that TMKOC cells utilized in the mouse LAM model are characterized by Tsc2 loss, mTORC1 activation and up-regulation of the VEGF-D protein level and gene expression.

**Axitinib treatment inhibits Tsc2-null lung lesion growth and abnormal lymphangiogenesis**

To investigate whether inhibition of the VEGF receptor signaling will affect Tsc2-null lung lesion growth we used a mouse model of LAM (4, 13) utilizing Tsc2-null cells described above. In accordance with our previously published data, multiple Tsc2-null lesions of various sizes developed in the lungs 21 days post-injections of Tsc2-null TMKOC cells, with total tumor burden of approximately 30% of the lung as it was detected by morphometric analysis of H&E staining of lung tissue sections (Fig. 2A).

Axitinib treatment markedly suppressed tumor development and density of lesion size and it significantly reduced total tumor burden to 20% of the lung tissue sections (32.0±2.9% versus 20.2±2.4% of lesions per untreated or axitinib treated lung, n=7 per group, p<0.005), (Fig. 2B).

Progressive growth of Tsc2-null lesions induces lymphangiogenesis in mouse model of LAM (13). IHC analysis revealed marked up-regulation of lymphatic channels within Tsc2-null lesions (Fig. 2C) detected by staining with antibody for LYVE-1, a marker of lymphatic vessels. Lymphatic endothelial cells forming new lymphatic vessels express VEGFR3 receptor, which is known to be activated by VEGF-D (5). Importantly,
as seen in Figure 2C, axitinib treatment markedly inhibited sprouting of lymphatic channels in \textit{Tsc2}-null lesions.

Collectively, these data demonstrate that pharmacological inhibition of VEGFR signaling in lung with axitinib has a potential beneficial effect in decreasing \textit{Tsc2}-null lesion growth and lymphatic sprouting within these lesions.

**Axitinib attenuates VEGF-D up-regulation in serum and lung lining fluid**

In order to investigate whether VEGF-D levels is increased with progressive \textit{Tsc2}-null lesion growth and whether this increase is affected by axitinib, serum and BAL fluid from mice untreated or treated with axitinib were analyzed for VEGF-D level. Figure 3 demonstrates that mice with \textit{Tsc2}-null lesions exhibit a significant systemic increase of VEGF-D levels in serum and BAL fluid as determined by ELISA (Fig. 3A) and Western blot (Fig. 3B), respectively. As seen in Figure 3, treatment with axitinib significantly reduces \textit{Tsc2}-null dependent VEGF-D levels in serum and BAL fluid. Moreover, this down-regulation of VEGF-D corresponded with reduction of \textit{Tsc2}-null lesions per lung (Fig. 2B) and inhibition of lymphangiogenesis (Fig. 2C).

**Axitinib attenuates cytokine up-regulation in BAL**

Progressive growth of \textit{Tsc2}-null lesions correlate with released cytokines and chemokines into the lung (4, 13). To determine whether axitinib treatment will attenuate up-regulated cytokine release in the lung, BAL fluids from untreated and axitinib treated mice were analyzed for relevant cytokines. As shown in Table 1, axitinib treatment significantly attenuates up-regulated levels of VEGF-A, MCP-1 and TGF-1\(\beta\) but not IL-6, KC and MMP9, suggesting a modulatory role of VEGFR signaling on their expression.

**Axitinib reduces recruitment of leukocytes into the lung lining**
Previously, we have shown that Tsc2-null lesion growth promotes recruitment of immature myeloid cells to the lungs (4). To determine whether axitinib treatment affects the recruitment of leukocytes to the lung with Tsc2-null lesions, cells were isolated from the lining of the lungs with and without axitinib treatment, counted and differentiated according to cell morphology. Table 2 shows that axitinib treatment significantly attenuated total number of cells recruited to the lung lining. Among all cell populations, there were significant reductions of multinucleated and progenitor cell populations (Table 2). Interestingly, the total number of macrophages was not significantly affected by axitinib (Table 2).

**Axitinib inhibits pro-vasculogenic, pro-lymphangiogenic, and inflammatory gene expression in recruited BAL cells**

Progressive growth of Tsc2-null lesions promotes inflammatory cells recruitment (Tables 1 and 2) which correlates with increased expression of VEGF-D level in serum and lung lining (Fig. 3). To determine whether cells recruited to the lung with Tsc2-null lesions express Figf, we performed qRT-PCR analysis of BAL cell pellets. As seen in the Figure 4, cells recruited to the lung lining expressed increased mRNA levels of Figf and Vegfa but not Vegfc. There was also marked up-regulation of pro-inflammatory genes Hif1a, Il6 and Ccl2. Importantly, treatment with axitinib significantly attenuates up-regulation of Figf, Vegfa and pro-inflammatory genes expression in BAL cells.

**Axitinib abrogates NOS2-mediated nitric oxide production**

We previously demonstrated that inflammatory cells recruited to the lung with Tsc2-null lesions expressed increased level of NO metabolites (4). In accordance with our previous observation, recruited leukocytes to the lung with Tsc2-null lesions exhibited...
marked expression of $\text{Nos2}$ level (Fig. 5A). Treatment with axitinib significantly attenuates up-regulation of this pro-inflammatory gene (Fig. 5A). In order to investigate whether increased NO metabolites production observed in the BAL fluid of lung with $\text{Tsc2}$-null lesions was $\text{Nos2}$-dependent, BAL fluids from untreated and axitinib treated mice with $\text{Tsc2}$-null lesions were examined for total NO content. Figure 5B shows that axitinib treatment also significantly decreases production of NO in the lung lining fluid.

**Axitinib attenuates S-nitrosylation of SP-D**

Similar to other cystic mouse models, our mouse model of LAM is characterized by increased $\text{Nos2}$ function and SP-D modification (4). We have recently shown that the increase in SNO-SP-D, a marker of pulmonary inflammation, was associated with increased $\text{Nos2}$ activity and NO production (4). In order to investigate whether axitinib treatment attenuates S-nitrosylation of SP-D level, lung lining fluids from untreated and axitinib treated mice with $\text{Tsc2}$-null lesions were analyzed for total SP-D and SNO-SP-D. Figure 6 shows that axitinib treatment attenuates SNO-SP-D level. The observed decreased level of SNO-SP-D was in agreement with decreased NO production within the lung lining fluid.
DISCUSSION

LAM is a devastating lung disease that primarily affects women of childbearing age. Both the genetic and spontaneous forms are caused by loss of TSC1 or TSC2 gene function in the smooth muscle-like LAM cells in the lung. When LAM cells overgrow, they form cysts that progressively enlarge and destroy the surrounding normal lung tissue, obstruct airways and blood vessels which eventually leads to respiratory failure. In LAM, the lymphatic system intensively expands, which result in loss of basement membrane integrity and spreading of LAM cells to other organs. While the origin of LAM cells is not well understood, one can observe that LAM manifests as a type of benign tumor (20, 30). Dr. Henske and colleges identified the same TSC2 mutations in kidney and lung lesions of LAM patients suggesting that kidney angiomyolipoma and lung LAM cells have common origin (8). This hypothesis is confirmed by the observation that Tsc2 null kidney epithelial cells (29) can be used to generate an animal model of LAM (4, 13). In this mouse LAM model, progressive growth of Tsc2-null lesions correlated with mTORC1 activation detected by pS6, and SM α-actin expression, abnormal lymphangiogenesis detected by LYVE-1 and up-regulation of VEGF-D within lesions, similar to human LAM cells (13).

In the present study, using the same mouse LAM model we demonstrated that mice with Tsc2-null lesions exhibit increased levels of VEGF-D in both the serum and lung lining. This parallels the human disease in which VEGF-D level in serum has been reported as a novel diagnostic biomarker for evaluating disease severity in LAM (40) and to prospectively distinguish LAM from other cystic diseases (32). From a mechanistic perspective it is of interest that, within animal models of cancer, VEGF-D...
promotes tumor angiogenesis, lymphangiogenesis and remodeling of collecting lymphatic vessels that facilitate solid tumor growth and/or metastatic spread to regional lymph nodes and distant organs (41, 42). Taken together, these observations provide a link between the metastatic nature of LAM and the lung tissue destruction, and identify VEGF-D signaling as a potential therapeutic target within this unique pathology.

Secreted VEGF-D exerts its signaling function via the activation of the cell surface receptor VEGFR-3 (25, 1). This receptor is a tyrosine kinase, whose activation within the lymphatic endothelium leads to growth of lymphatic vessels (42, 44). Within cancer models, disruption of VEGF-D binding to VEGFR-3, using either a soluble form of VEGFR-3 (19, 33), neutralizing antibodies to VEGF-D (42) or VEGFR-3 (21, 37), has been shown to reduce tumor lymphangiogenesis and lymphatic metastasis. Previously, direct inhibition of VEGFR-3 signaling has not been considered as an approach to limit LAM. Here, we have used axitinib, also known as AG013736, a small molecule tyrosine kinase inhibitor to target VEGFR-3 signaling. Axitinib targets angiogenesis and shows activity against all three VEGF receptors, including VEGFR-1, 2, and 3 (22). It has been shown to inhibit the growth of breast cancer in xenograft models (45) and has been used successfully in trials with various tumor types, including renal cell carcinoma (38).

The present study demonstrates that pharmacological targeting of VEGFR signaling with axitinib attenuates Tsc2-null lung lesion growth. In addition, it reduces the increased levels of VEGF-D seen in the serum and BAL fluid in this mouse model of LAM. Interestingly, the leukocytes recruited to the lung lining are a significant source of VEGF-D (figure 4). Axitinib only partially reduced the increased cellularity in the lining of lungs with Tsc2-null lesions, whereas up-regulation of Vegfa and Figf gene
expression in BAL cells was markedly reduced by axitinib. Leukocytes recruited to the lung lining produced are in an activated state, as there is up-regulation of pro-inflammatory genes such as Nos2, Ccl2 and Il6 in BAL cells from lung with Tsc2-null lesions. As with Figf, the increased expression of these genes in BAL cells was reduced by axitinib, suggesting that the activated state may be critical to VEGF-D expression.

Importantly, VEGF-D expression is increased in Tsc2-deficient cells used in LAM mouse model (Figure 1). These data suggest that Tsc2-null lung lesions express VEGF-D, which might contribute to inflammatory cell recruitment, which further increases VEGF-D levels in serum. Further study determining the specific contribution of Tsc2-null lesions and inflammatory cells in abnormal lymphangiogenesis in LAM are warranted.

It has been shown that axitinib dose-dependently reduced the phosphorylation of Akt, endothelial NOS (eNOS), and extracellular signal-regulated kinase 1/2 (ERK1/2), key downstream signaling molecules of VEGF (22). This suggests that the reduction in Akt, eNOS, and ERK1/2 phosphorylation may be due to antagonism of upstream VEGFRs by axitinib. Axitinib-dependent inhibition of NOS2 expression demonstrates a role of VEGF signaling in modulating its expression. Because axitinib affects activity not only VEGFR2 and VEGFR3 but also PDGFR, cKit, and BRC-ABL (36), further study will determine specific role of these receptors in up-regulation (18) of NOS2, VEGF-A, and VEGF-D.

The data presented here demonstrate that, in a mouse model of LAM, axitinib is an effective inhibitor of Tsc2-null lesion growth. However, several clinical trial reports
have demonstrated that tyrosine kinase inhibitors, such as axitinib, have been shown to cause frequent cardiovascular adverse events during treatment of renal cell carcinoma (17, 39), and thus caution in axitinib use for the treatment of LAM is warranted. In the mouse model of LAM, growth of Tsc2-null lesions involves recruitment and activation of inflammatory cells; these processes are also inhibited by axitinib. Reduction in lesion growth and cell recruitment by axitinib leads to reduced VEGF-D expression. Collectively, our study demonstrates proof-of-concept of the pharmacological inhibition of VEGF signaling to induce beneficial effects on lung inflammation and Tsc2-null lung lesions seen in LAM.
FIGURE LEGENDS

Figure 1. Tsc2 deficiency up-regulates VEGF-D protein and mRNA expression

(A) Representative micrographs of 3 independent immunoblot analysis of Tsc2-positive (M-1) and Tsc2-null (MKOC and TMKOC) cells with indicated antibody. Equal amount of total protein of M-1, MKOC and TMKOC were subjected to SDS-PAGE under reduced conditions followed by immunoblotting with antibody against tuberin (TSC2), VEGF-D, pS6, S6 and tubulin. (B) M-1 or TMKOC cells were analyzed for gene expression by qRT-PCR as described in Methods. Obtained Ct values were normalized to β-actin signals and further analyzed using the relative quantization (ΔΔCt) method. Data are expressed as fold change (means ± SEM, n=2 in each group, 3 independent experiments), *p<0.05 versus Tsc2-expressing cells (M-1) by Student’s t-test.

Figure 2. Axitinib treatment inhibits Tsc2-null lung lesion growth and abnormal lymphangiogenesis

(A) Representative micrographs of H&E staining of lung sections from control mice and mice with Tsc2-null lesions treated with diluent or axitinib. (B) Statistical analysis of the percentage of lesions per lung treated with diluent (n=9) or axitinib (n=6) was performed as described (13). Values are shown as means ± SEM, # p <0.05 by Student’s t-test. (C) Lung tissue of mice with Tsc2-null lesions treated with diluent or axitinib were analyzed for lymphatic vessels by immunohistochemical analysis with specific anti-LYVE-1 antibodies (red). DAPI staining was performed to detect nuclei (blue).
Representative images were taken using a Nikon Eclipse TE-2000E microscope (n = 5 per group, magnification: X 20).

**Figure 3. Axitinib attenuates VEGF-D up-regulation in serum and lung lining fluid**

Serum and BAL fluid was collected from control mice or mice with Tsc2-null lesions treated with diluent or axitinib and analyzed for VEGF-D level. **(A)** Serum samples were analyzed for VEGF-D level by ELISA kit. Data were represented as means ± SEM from triplicate measurements and shown as pg of VEGF-D per ml of sample. *p < 0.05 versus control/diluent group; # p<0.05 versus Tsc2-null/diluent group by two-way ANOVA and Bonferroni correction for multiple comparisons, n=10 per each group. **(B)** Upper panel: Representative micrographs of BAL VEGF-D protein content. BAL samples containing equal volume were subjected to SDS-PAGE under reduced conditions followed by immunoblotting with anti-VEGF-D antibody. **Lower panel:** densitometric quantification of VEGF-D content. Mean values of all the samples from Tsc2-null lung treated with diluent or axitinib were calculated and presented as a percentage of Tsc2-null lungs treated with diluent. Values are shown as means ± SEM, n=10 per group. *p <0.05 versus control/diluent group; # p<0.05 versus Tsc2-null/diluent group by t-test, n=10 per each group.

**Table 1. Axitinib attenuates cytokine up-regulation in BAL**

BAL fluid was collected at day 21 post TMKOC cells injection and analyzed for cytokine level by the Aushon Searchlight Protein Array multiplex ELISA. Data are expressed as mean values ± SEM, *p <0.05 versus control/diluent group; # p<0.05 versus Tsc2...
null/diluent group by two-way ANOVA and Bonferroni correction for multiple comparisons, n=10 per each group.

Table 2. Axitinib reduces recruitment of leukocytes into the lung lining

Total cells were collected from lung lavage of control mice or mice with Tsc2-null lesions treated with diluent or axitinib at day 21 post TMKOC cells injection. Total count was determined by Coulter counting, differential cell counts were done using Diff-Quick staining of cytospin slides. Cells were identified as macrophages, eosinophils, multinucleated or progenitors by standard morphology. The data are expressed as cell numbers X 10,000. Values are shown as means ± SEM. Comparisons between groups were made by two-way ANOVA and Bonferroni correction for multiple comparisons, *p<0.05 versus control/diluent group; # p<0.05 versus Tsc2-null/diluent group, n=10 per each group.

Figure 4. Recruited cells expressed increased mRNA levels that attenuated by axitinib

RNA was extracted from BAL cells isolated from lungs of control mice and mice with Tsc2-null lesions treated with diluent or axitinib. Expression of gene markers were quantified by RT-qPCR as described in Methods. Ct values obtained were normalized to β-actin signals and further analyzed using the relative quantization (ΔΔCt) method. Data are expressed as fold change (means ± SEM, n=3-5 per each group). Comparisons between groups were made by two-way ANOVA and Bonferroni correction for multiple comparisons. *p<0.05 versus control/diluent group and # p<0.05 versus Tsc2-null/diluent group.
Figure 5. Axitinib abrogates NOS2-mediated nitric oxide production

(A) Cells recovered from BAL fluid of control mice and mice with Tsc2-null lesions treated with diluent or axitinib were analyzed for Nos2 mRNA expression by quantitative RT PCR and normalized to β-actin. Data are expressed as fold change (means ± SEM, n=3-5 per each group); *p <0.05 versus control/diluent group, # p<0.05 versus Tsc2-null/diluent group by two-way ANOVA and Bonferroni correction for multiple comparisons. (B) BAL fluid was collected at 21 day post TMKOC cells injection and analyzed for NO metabolites by chemical reduction as described in Methods. Values are shown as means ± SEM. *p <0.05 versus control/diluent; # p<0.05 versus Tsc2-null/diluent group by two-way ANOVA and Bonferroni correction for multiple comparisons, (control/diluent, n=5; control/axitinib, n=5; Tcs2-null/diluent, n=15; Tsc2/axinitib, n=10).

Figure 6. Axitinib attenuates S-nitrosylation of SP-D

BAL fluid was collected at 21 day post TMKOC cells injection and samples containing equal volume were subjected to SDS-PAGE under reduced conditions followed by immunoblotting with anti-SP-D antibody as described in the Methods. Upper panel: Representative micrographs of total SP-D protein content. Lower panel: Representative micrographs of SNO-SP-D content by Biotin-Switch assay.
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Figure 1

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B

Gene expression/β-actin

- Vegfa
- Vegfc
- Figf
- Nos2

* indicates statistical significance compared to M-1.
Figure 2

B

% of lesions per lung

![Graph showing % of lesions per lung between Diluent and Axitinib](image)

- **Diluent**
- **Axitinib**

Tsc2-null Lesions

C

Diluent

Axitinib
Figure 3

A

B

Figure 4

- mRNA expression/β-actin, (fold of Control/diluent)

Markers:
- *: Significant difference from Control/Diluent
- #: Significant difference from Control/Axitinib
- Tsc2-null/Diluent
- Tsc2-null/Axitinib

Genes analyzed:
- Vegfa
- Vegfc
- Figf
- Hif1a
- Il6
- Ccl2
Figure 5

A

B

$\frac{\text{Nos2/β-actin mRNA induction}}{(\text{fold of Control/diluent})}$

$\text{BAL nitrogen oxides, pmoles}$

**Control**
**Tsc2-null**

* Diluent
# Axitinib
Figure 6

<table>
<thead>
<tr>
<th>Control</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Diluent</td>
<td>Axitinib</td>
<td>Diluent</td>
</tr>
</tbody>
</table>

- Reduced SP-D
- SNO-SP-D
Table 1

<table>
<thead>
<tr>
<th></th>
<th>IL-6 pg/mL</th>
<th>KC pg/mL</th>
<th>MCP-1 pg/mL</th>
<th>VEGF-A pg/mL</th>
<th>TGF-1β pg/mL</th>
<th>MMP3 pg/mL</th>
<th>MMP9 pg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control/Diluent</td>
<td>11.7±1.8</td>
<td>10.7±2.7</td>
<td>12.6±2.3</td>
<td>0.6±0.1</td>
<td>254.3±94.6</td>
<td>356.9±96.4</td>
<td>11.3±0.8</td>
</tr>
<tr>
<td>Control/Axitinib</td>
<td>12.6±5.2</td>
<td>13.5±1.4</td>
<td>11.4±3.4</td>
<td>0.5±0.1</td>
<td>251.5±105.6</td>
<td>396.6±91.0</td>
<td>13.4±6.5</td>
</tr>
<tr>
<td>Tsc2-null/Diluent</td>
<td>66.3±12.5*</td>
<td>29.6±4.2*</td>
<td>523.2±56.9*</td>
<td>20.5±2.6*</td>
<td>3,075±375*</td>
<td>23,982±1,731*</td>
<td>631.7±88.2*</td>
</tr>
<tr>
<td>Tsc2-null/Axitinib</td>
<td>54.9±16.6</td>
<td>21.2±3.2</td>
<td>267.7±48.1#</td>
<td>10.3±3.1#</td>
<td>1,867±283#</td>
<td>17,391±4,570</td>
<td>507.2±127.2</td>
</tr>
</tbody>
</table>

* p<0.05 versus Control/Diluent group
# p<0.05 versus Tsc2-null/Diluent group
<table>
<thead>
<tr>
<th></th>
<th>Total Cells (x10^5)</th>
<th>Macrophages (x 10^5)</th>
<th>Multinucleated (x 10^5)</th>
<th>Eosinophil (x 10^5)</th>
<th>Progenitors (x 10^5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control/Diluent</td>
<td>2.35±0.15</td>
<td>2.35±0.15</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Control/Axitinib</td>
<td>2.2±0.48</td>
<td>2.2±0.48</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Tsc2-null/Diluent</td>
<td>18.86±1.09*</td>
<td>11.6±0.74*</td>
<td>4.67±0.60*</td>
<td>0.50±0.30</td>
<td>2.34±0.31*</td>
</tr>
<tr>
<td>Tsc2-null/Axitinib</td>
<td>14.13±1.28#</td>
<td>9.95±0.78</td>
<td>2.44±0.42#</td>
<td>0.35±0.12</td>
<td>1.44±0.33#</td>
</tr>
</tbody>
</table>

* p<0.05 versus Control/Diluent group
# p<0.05 versus Tsc2-null/Diluent group