Lymphotoxin-α promotes tumor angiogenesis in HNSCC by modulating glycolysis in a PFKFB3-dependent manner

Jie-Gang Yang1, Wei-Ming Wang2, Hou-Fu Xia1, Zi-Li Yu1,3, Hui-Min Li1, Jian-Gang Ren1,3, Gang Chen1,3, Bei-Ke Wang1, Jun Jia1,3, Wei Zhang4,5,6 and Yi-Fang Zhao3

1The State Key Laboratory Breeding Base of Basic Science of Stomatology and Key Laboratory of Oral Biomedicine Ministry of Education, School and Hospital of Stomatology, Wuhan University, Wuhan, China
2Centre of Stomatology, Xiangya Hospital, Central South University, Changsha, China
3Department of Oral and Maxillofacial Surgery, School & Hospital of Stomatology, Wuhan University, Wuhan, China

Tumor angiogenesis is critical for tumor progression as the new blood vessels supply nutrients and facilitate metastasis. Previous studies indicate tumor associated lymphocytes, including B cells and T cells, contribute to tumor angiogenesis and tumor progression. The present study aims to identify the function of Lymphotoxin-α (LT-α), which is secreted by the activated lymphocytes, in the tumor angiogenesis of head and neck squamous cell carcinoma (HNSCC). The coculture system between HNSCC cell line Cal27 and primary lymphocytes revealed that tumor cells promoted the LT-α secretion in the cocultured lymphocytes. In vitro data further demonstrated that LT-α promoted the proliferation, migration and tube formation of human umbilical vein endothelial cells (HUVECs) by enhancing the PFKFB3-mediated glycolytic flux. Genetic and pharmacological inhibition of PFKFB3 suppressed the enhanced proliferation and migration of HUVECs. We further identified that LT-α induced PFKFB3 expression was dependent on the TNFR/NF-κB signaling pathway. In addition, we proved that PFKFB3 blockade decreased the density of CD31 positive blood vessels in HNSCC xenografts. Finally, the results from the human HNSCC tissue array revealed that the expression of LT-α in HNSCC samples positively correlated with microvessel density, lymphocytes infiltration and endothelial PFKFB3 expression. In conclusion, infiltrated lymphocyte secreted LT-α enhances the glycolysis of ECs in a PFKFB3-dependent manner through the classical NF-κB pathway and promotes the proliferation and migration of ECs, which may contribute to the aberrant angiogenesis in HNSCCs. Our study suggests that PFKFB3 blockade is a promising therapeutic approach for HNSCCs by targeting tumor angiogenesis.

Introduction
Head and neck squamous cell carcinoma (HNSCC) is the sixth most susceptible malignant tumor worldwide with a global incidence of approximately 600 thousand cases per year.1 Despite the improvement of treatments in the past few decades, most HNSCC patients are still suffering from the long-term prognosis. The 5-year overall survival rate of HNSCC patients remains low, at around 40–50%.2 The main treatment options are surgery, chemotherapy and radiation therapy, but the clinical outcomes are challenged by the high recurrence rates and frequent metastasis.3 Angiogenesis, the formation of new blood vessels, is the process involving the migration, proliferation and differentiation of endothelial cells (ECs).4 Angiogenesis plays a critical role in tumor progression as blood vessels support the tumor growth by supplying nutrients and oxygen, and facilitate the metastasis of tumor cells to distant organs.5 Lymphangiogenesis, the formation of new lymphatic vessels, is also thought to be crucial for cancer cells to metastasize to the regional lymph...
nodes in many cancers, including HNSCC. Therefore, targeting angiogenesis and/or lymphangiogenesis is considered promising for cancer therapy, and many inhibitors have already been approved by the US Food and Drug Administration (FDA). Although some research indicated that the infiltration of lymphocytes in tumors facilitates the anti-tumor immune response and therefore benefits the patients’ survival, increasing studies suggest that the infiltrated lymphocytes also promote tumor progression. A recent study has demonstrated that the infiltrated B cells in melanoma, termed tumor-associated B (TAB) cells, were important for the melanoma cells to acquire drug resistance against targeted therapy. The IGF-I secreted by TAB cells was able to activate the PI3K/AKT pathway in melanoma cells. Additionally, the tumor infiltrated B cells were also capable of activating IKK-α and STAT3 signaling in cancer cells in order to enhance its therapy-resistance and promote the tumor progression in a prostate carcinogenesis model. Accumulating evidence has proved that increased regulatory T cells positively correlated with poor prognosis in a great number of tumors. Studies have reported that the infiltrated lymphocytes contributed to the enhanced angiogenesis in cancers. Local synthesis of angiogenic factors by infiltrated T cells was regarded deleterious because of increased capillary ingrowth. The infiltrated B cells in tumors also involved tumor angiogenesis in murine melanoma models by activating the STAT3 signaling pathway in melanoma cells.

Lymphotoxin alpha (LT-α), a member of the tumor necrosis factor (TNF) superfamily, is one of the predominant chronic pro-inflammatory cytokines secreted by lymphocytes. It is reported that LT-α was able to promote angiogenesis at low concentrations by inducing endothelial cell sprouting and tube formation. Moreover, a pervious study reported the impaired lymphatic vessels formation in the LT-α−/− mice, revealing the importance of LT-α during lymphangiogenesis. And our previous research also suggested that LT-α might promote lymphatic vessel formation in lymphatic malformation. LT-α is a strong chemokine for the recruitment of immune cells, including B cells, CD4 T cells and dendritic cells. These infiltrated immune cells further secrete pro-angiogenic factors and enhance tumor angiogenesis and lymphangiogenesis. Activation of the main LT-α receptor, TNFR-1, can stimulate new lymphatic and blood vessel formation under various pathological conditions. Additionally, LT-α functions as an autocrine cytokine and triggers the production of IL-6, contributing to angiogenic response in ECs in cutaneous T cell lymphoma. Lymphotoxin is also proven to increase angiogenesis by releasing angiogenic chemokine CXCL2 in fibrosarcoma.

In the present study, we proved that increased LT-α, which could be secreted by the infiltrated lymphocytes in HNSCCs, promoted the proliferation, migration and tube formation of ECs by enhancing PFKFB3-mediated glycolysis flux. Furthermore, for the first time, we proved that LT-α up-regulated PFKFB3 expression through the classical NF-κB signaling pathway in a TNFR-dependent manner. Genetic (PFKFB3 shRNA) and pharmacological (PFK15) inhibition of PFKFB3 were able to deteriorate glycolysis and angiogenic activities of HUVECs in vitro. By establishing HNSCC xenograft mice models, we proved that PFK15 significantly decreased CD31 positive vessels in tumors. The results from human HNSCC tissue assays revealed that LT-α expression was positively related to micro-vessel density (MVD), lymphocyte infiltration and endothelial PFKFB3 expression. Our research suggests a therapeutic strategy targeting PFKFB3 for suppressing tumor angiogenesis.

**Materials and Methods**

**Cell culture**

HNSCC cell line Cal27 was purchased from ATCC and cultured in DMEM containing 10% fetal bovine serum (FBS). Primary human B cells and T cells were purified from peripheral blood of healthy donors using Dynabeads™ Untouched™ human B cells or T cells Kit (Invitrogen) according to the manufacturer’s instruction. Primary human umbilical vein endothelial cells (HUVECs) were isolated from human umbilical cord veins as described and cultured in endothelial cell medium (ECM, Lonza, CA). Protocols for sampling primary human cells were approved by the review board of the ethics committee of Hospital of Stomatology, Wuhan University. Informed consent was obtained from healthy donors and parents of the newborn children from whom umbilical cords were obtained. For coculture, T cells or B cells and Cal27 cells were cocultured in RPMI1640 containing 10% (v/v) FBS, 20 mM L-glutamin, 50 μM β-Mercaptoethanol.

**Bioinformatics analysis**

Tumor Immune Estimation Resource (TIMER) web server (http://cistrome.shinyapps.io/timer/) was utilized to detect
the expression of LT-α in various cancer types. To study the differential mRNA expression of LT-α between tumor and adjacent normal tissue, "DiffExp module" was performed to show the statistical significance of differential expression using Wilcoxon test. As for visualizing the correlation of the expression of LT-α with immune infiltration levels in HNSCCs, "Gene module" was performed to show the purity-corrected partial Spearman’s correlation.24

Detection of cytokine production
To measure the LT-α production in lymphocytes, flow cytometry and enzyme-linked immunosorbant assay (ELISA) were performed. For flow cytometry, after coculture for 24 h, PerCP-labeled CD20 and AF700-lebeled CD3 antibodies (Biolegend) were used to label B cells and T cells, respectively. After fixation and permeabilization, the cells were stained with PE-labeled LT-α antibody for 1 h at 4°C. All data acquisition was performed on LSR II Flow Cytometry (BD Biosciences) and analyzed with FlowJo (TreeStar). The level of LT-α was calculated as mean fluorescence intensity (MFI). For ELISA, the 100 μL supernatants from the coculture system were added to each well that were pre-coated with LT-α antibody and incubated overnight at 4°C. Biotin LT-α antibody and HRP-conjugated streptavidin (BD Biosciences) were then added and incubated for 1 h. The plates were developed with TMB substrate Kit (Pierce) and read in Tek Reader. The recombinant LT-α (Biolegend) was used to establish the standard curve.

Measurement of glycolysis
The glucose uptake, intracellular 1-lactate level and ATP generation were detected with the Glucose Uptake Cell-Based Assay Kit (Cayman Chemical), 1-lactate Assay Kit (Cayman Chemical) and Firefly Luciferase-Based ATP Assay Kit (Beyotime) according to manufacturers’ instructions. Details were provided in Supporting Information Materials and Methods.

Determination of cell viability, proliferation and cell cycle progression
The cell viability was measured by MTT assay with cells plated in 96-well. The cell proliferation was determined by EdU (5′-ethynyl-2′-deoxyuridine) incorporation assays with cells seeded on cover slides. And the cell cycle was tested by the approved guidelines and regulations. According to previous study,25,26 about 1 × 10^5 viable Cal27 cells re-suspended in 100 μL PBS were subcutaneously injected into 12 mice to establish the HNSCC xenografts. After 2 weeks when subcutaneous tumors were visible, the mice were then randomly divided into two groups, which received either intraperitoneal injection of PFK15 (20 mg/kg; n = 6) or PBS (vehicle, 100 μL; n = 6) three times per week for 2 weeks. The mice were euthanized and sacrificed at the end of the time point (20 days after the first injection of PFK15), and the tumors were harvested for successive histology and molecular analysis according to standard

Transfection and RNA interference
To construct PFKFB3 knockdown HUVECs, HUVECs were plated at a density of 4 × 10^5 / well in a 6-well plate 18 h before transfection. pLV-U6-shPFKFB3-hEF1a lentivirus was purchased from Beijing Syngentech Co., LTD. Stable knockdown was selected with puromycin (2 μg/mL) for 48 h. For RNA interference, HUVECs were cultured in 6-well plates at a density of 50% confluence 18 h prior to transfection. Transfection was performed with Lipofectamine 2000 according to the instructions. The cell lysate was harvested 24 h after transfection of siRNA. TNFR-1 and TNFR-2 siRNAs were purchased from Ribobio Co., Ltd. Knockdown effects were confirmed by real-time PCR and western blot.

Transwell migration and tube formation assays
Transwell migration assays were performed to measure the cell migration. Briefly, 100 μL 5 × 10^4 cells were seeded into the upper chamber, while 800 μL of ECM with indicated cytokines were added to the bottom chambers as chemoattractant. The migrated cells on the lower surface of the filter were fixed and counted. For tube formation, 2 × 10^5 cells were seeded on 24-well plates coated with BD Matrigel™ Matrix and incubated in indicated medium for 24 h at 37°C. The formation of capillary-like structures was observed and captured under a light field microscope (Leica). The total length of tubes was analyzed by ImageJ.

Establishment and PFK15 treatment of squamous cell carcinoma xenograft model
Female BALB/C nude mice (18–20 g; 6–8 weeks of age) were purchased from the Hunan SJA Laboratory Animal Co.Ltd. Animal handling and procedures were approved and overseen by the Ethics Committee for Animal Research, Wuhan University, China. The methods were carried out in accordance with the approved guidelines and regulations. According to previous study,25,26 about 1 × 10^7 viable Cal27 cells re-suspended in 100 μL PBS were subcutaneously injected into 12 mice to establish the HNSCC xenografts. After 2 weeks when subcutaneous tumors were visible, the mice were then randomly divided into two groups, which received either intraperitoneal injection of PFK15 (20 mg/kg; n = 6) or PBS (vehicle, 100 μL; n = 6) three times per week for 2 weeks. The mice were euthanized and sacrificed at the end of the time point (20 days after the first injection of PFK15), and the tumors were harvested for successive histology and molecular analysis according to standard
procedures. The number of CD31 positive vessels indicated the level of angiogenesis in tissues.

**Human HNSCC samples**

Twenty HNSCC specimens and 6 normal mucosa (Supporting Information Tables S1 and S2) were chosen for double-labeling immunofluorescence. One hundred and fourteen pathologically confirmed HNSCC specimens and 59 samples of normal human oral mucosa (Supporting Information Tables S3 and S4) were collected from the Hospital of Stomatology, Wuhan University and made into a HNSCC tissue array with the assistance of Kindstar Global Co. Ltd. All the procedures and protocols were performed in accordance with the National Institutes of Health guidelines regarding the use of human tissues and approved by the review board of the Ethics Committee of Hospital of Stomatology, Wuhan University, China. Written informed consent for this study was obtained from all the participants.

**Tissue immunofluorescence, immunohistochemistry (IHC), scoring system and hierarchical analysis**

The immunofluorescence and IHC were performed in accordance with our previous procedures. Briefly, the tissue sections of formalin-fixed and paraffin-embedded tissues were dewaxed in xylene, rehydrated in ethanol and double-distilled water. Then, the antigen was retrieved through high pressure dewaxed in xylene, rehydrated in ethanol and double-distilled water. Then, the antigen was retrieved through high pressure fixation and paraffin embedding. Aperio ScanScope CS scanner was utilized for scanning sections and quantifying the histoscore of each sample with background subtraction.

**Statistical analysis**

All experiments were performed at least in triplicate. Normality of distribution was determined by D’Agostino-Pearson omnibus normality test and variance between groups was assessed by the F-test. For normally distributed data, significance of mean differences was determined using two-tailed paired or unpaired Student’s t-tests; for groups that differed in variance, unpaired Welch t-test was performed. Non-parametric Mann-Whitney U-tests was applied if normality test failed. For multiple comparisons, one-way ANOVA was applied. As for two independent factors, two-way ANOVA was applied. Correlations were determined by Spearman rank test. All the statistical analyses were performed in GraphPad Prism. Data were presented as mean with standard error (SEM). p-Value of <0.05 was considered statistically significant. See “Supporting Information Materials and Methods” for more information.

**Results**

**Infiltrated lymphocytes in HNSCC secret lymphotxin-α**

With TIMER, a web server for comprehensive analysis of tumor-infiltrating immune cells, the mRNA expression of LT-α was found to be significantly higher in HNSCCs, compared to adjacent normal tissues (Fig. 1a). The purity-corrected partial Spearman’s correlation analysis revealed the positive correlation between LT-α expression and lymphocytes infiltration, including B cells, CD4 and CD8 T cells in HNSCCs (Fig. 1b). A pervious study reported that B cells could be stimulated by tumor cells and then release growth factors to facilitate tumor growth. Here we examined whether lymphocytes cocultured with HNSCC cells could secrete more LT-α. To this end, freshly isolated lymphocytes from the peripheral blood of healthy donors were cocultured with the human HNSCC cell line Cal27. As expected, coculture with Cal27 significantly up-regulated the mRNA expression of LT-α in both B cells and T cells (Fig. 1c). Furthermore, flow cytometric analyses revealed that LT-α positive B cells were increased from 4.41% to 69.5%, and LT-α positive T cells were increased from 38.0% to 74.3% when cocultured with Cal27 cells (Figs. 1d and 1e). The significant increase of LT-α in the culture medium was confirmed by ELISA assays. A threefold increase of LT-α was found in the coculture system containing B cells and Cal27 cells (Fig. 1f).

Taken together, the higher expression of LT-α in HNSCC might be attributed to the stimulated lymphocytes, especially the tumor associated B cells.

**LT-α promotes glycolysis activity via up-regulation of PFKFB3 in HUVECs**

A previous study reported that LT-α was able to promote angiogenesis through production of angiogenic chemokines. Since the enhanced glycolytic flux was essential for the activity of ECs during angiogenesis, we then explored whether LT-α modulated the glucose metabolism of ECs. First of all, the supernatants and lysates of HUVECs treated with LT-α were examined for glucose uptake, lactate production and ATP generation. The results showed that recombinant LT-α (20 ng/mL) significantly increased the 2-NBDG uptake by HUVECs, total lactate level and ATP concentration in cell lysis (Supporting Information Figs. S1a-1c), indicating the enhanced glycolytic flux in HUVECs. In accordance with the increased glycolysis, LT-α promoted cell growth of HUVECs in a time-dependent manner (Supporting Information Fig. S1d).

The expression of glycolytic enzymatic proteins was then examined to explore the possible mechanisms. Western blots analyses revealed that LT-α increased the expression of PFKFB3, an enzyme synthesizing fructose-2,6-bisphosphate, which was able to accelerate glycolysis through stimulating PFK-1. The other glycolic enzymes, including PKM2, HK II, FPKP and LDHA, did not show an increase in LT-α treated HUVECs (Figs. 2a and 2b). The immunofluorescence results verified the increased expression of PFKFB3 in LT-α-treated HUVECs.
Figure 1. Infiltrated lymphocytes in head and neck squamous cell carcinoma (HNSCC) secreted LT-α. (a) Differential expression of LT-α between tumor and adjacent normal tissues from various types of cancer. Data were extracted from the TIMER web server. HNSCCs were highlighted in gray. (b) The purity-corrected partial Spearman’s correlation of LT-α and immune infiltration level in HNSCCs. Human B cells or T cells were cocultured with Cal27 for 48 h before detection. (c) The mRNA expression of LT-α was measured by real-time PCR in B cells or T cells with indicated treatment. (d) Detection of LT-α (PE) expression in B cells (CD20-PerCP) or T cells (CD3-AF700) by flow cytometry. (e) The quantitative data from flow cytometric analysis. (f) Detection of LT-α in coculture supernatant from Cal27 and lymphocytes by ELISA. Cal27 supernatant was set as a negative control. Quantitative data were from 3 independent experiments. Data were presented as mean ± SEM. *, p < 0.05; **, p < 0.01; ***, p < 0.001. [Color figure can be viewed at wileyonlinelibrary.com]
especially in the cytoplasmic part (Fig. 2c). The mRNA expression of PFKFB3 was also increased (Fig. 2d), indicating that PFKFB3 was at least regulated at the transcriptional level by LT-α in HUVECs.

We next asked whether LT-α up-regulated PFKFB3 expression and enhanced glycolysis directly, as previous studies reported that VEGF-A/C increased PFKFB3 expression in endothelial cells.31 ELISA assay results showed that the concentration of VEGF-A and VEGF-C in the culture supernatant of untreated HUVECs was extremely low and barely detectable. LT-α treatment did not stimulate VEGF-A and VEGF-C secretion in HUVECs, especially those treated with LT-α (Fig. 2e–2g). PFKFB3 knockdown also suppressed cell growth in the LT-α treated HUVECs (Fig. 2h). Taken together, our data demonstrated that LT-α was able to directly enhance the glycolysis flux of HUVECs through up-regulation of the glycolytic rate-limiting enzyme PFKFB3, and knockdown of PFKFB3 impaired the glycolytic activity stimulated by LT-α in HUVECs.

**LT-α accelerates cell cycle in HUVECs in a PFKFB3-dependent manner**

PFKFB3 also regulated cell proliferation in a glycolysis-independent manner.32,33 The cell proliferation of HUVECs was measured with EdU incorporation assays. As shown in Figures 3a and 3b, the ratio of EdU positive HUVECs was increased significantly, from 22.55% to 40.08%, after LT-α

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**Figure 2.** LT-α promoted glycolysis activity of human umbilical vein endothelial cells (HUVECs) by up-regulating PFKFB3. HUVECs were transfected with either shPFKFB3 or Vector, and then treated with PBS (Ctrl) or LT-α (100 ng/mL) for 24 h. (a) Immunoblotting was performed to examine the enzymes involved in glycolysis in HUVECs. (b) The quantitative data from immunoblotting. Protein expression was standardized by the expression of the interior reference β-actin. (c) Detection of PFKFB3 (red) expression in HUVECs by immunofluorescence. The nuclei were stained with DAPI (blue). (d) The mRNA expression of PFKFB3 was measured by real-time PCR in HUVECs with indicated treatment. (e) Glucose uptake was examined in HUVECs using 2-NBDG. (f) Total lactate production including intracellular and supernatant lactate was detected in HUVECs. (g) ATP generation in HUVECs was determined. Both lactate production and ATP generation were standardized by indicated number of HUVECs (1 × 10⁷ cells). (h) Cell growth was measured by MTT assays in transfected HUVECs after treatment of LT-α for 24 h, 48 h and 72 h, respectively. Quantitative data were from 3 independent experiments. Data were presented as mean ± SEM. *, p < 0.05; **, p < 0.01; ***, p < 0.001. [Color figure can be viewed at wileyonlinelibrary.com]
Knockdown of PFKFB3 resulted in a dramatic decrease of EdU positive population in the presence of LT-α. The cell cycle phases of HUVECs treated with LT-α was then measured by flow cytometry. The cell population in G1 phase was decreased from 64 ± 2% to 55 ± 1% (Figs. 3c and 3d), and cell population in G2/S phases was increased after LT-α treatment. PFKFB3 knockdown remarkably halted the ECs in G1 phase. Furthermore, the cell cycle related proteins, including cyclins, CDK2, phospho-Rb (p-Rb) and p27, the Kip proteins family protein in the HUVECs were examined. As shown in Figures 3e and 3f, knockdown of PFKFB3 in HUVECs resulted in the increase of the cell cycle inhibitory protein p27 and the decrease of Cyclin D1, Cyclin D3 and CDK2 in the LT-α treated HUVECs. Consistent with a previous study, treatment of LT-α promoted the expression of p-Rb, while PFKFB3 knockdown significantly inhibited the expression of p-Rb. In summary, our results demonstrated that LT-α treatment enhanced the proliferation of HUVECs through acceleration of the cell cycle, which could be abolished by knockdown of PFKFB3.
LT-α promotes migration and tube formation of HUVECs in a PFKFB3-dependent manner

Cell migration and tube formation abilities of ECs are crucial for angiogenesis. Next, we sought to understand the effects of LT-α on cell motility through transwell migration assays. After treatment of LT-α for 24 h, the migrated cells were almost twice as motile as those in vector control (Ctrl) group (Figs. 3g and 3h), indicating that cell motility was enhanced. Meanwhile, PFKFB3 knockdown in HUVECs significantly suppressed the migration of HUVECs (Figs. 3g and 3h). Furthermore, tube formation assays revealed that LT-α strongly promoted the formation of capillary-like structures (Figs. 3i and 3j), which was also significantly impaired by the knockdown of PFKFB3 in HUVECs.

Cellular protrusions, the dynamic cell-matrix contact structures facilitating cell migration and invasion,26 were essential during new blood vessel formation. As the cellular protrusion formation was also regulated by PFKFB3,25,31 we then explored whether LT-α regulated the cellular protrusions in HUVECs. Alexa Fluor 488 labeled phalloidin staining indicated that LT-α stimulation obviously increased the number and the length of filopodia in HUVECs (Figs. 3k–3m). While PFKFB3 knockdown in HUVECs led to fewer and shorter filopodia in the LT-α treated HUVECs. Additionally, by quantifying lamellipodia formation, we found that HUVECs treated with LT-α possessed larger lamellipodia compared to the untreated ones. Moreover, in the PFKFB3-knockdown HUVECs, we only found the smaller lamellipodia even in the cells treated with LT-α (Figs. 3k and 3n). Podosomes are actin-rich, adhesive structures present in monocytic myeloid cells and activated endothelial cells, which are able to degrade extracellular matrix (ECM). By seeding cells on Alexa Fluor 568-labeled gelatin-coated coverslips, the podosomes in HUVECs treated with LT-α were studied. The degradation of Alexa 568-labeled Gelatin by the podosomes could be photographed under a confocal laser scanning microscope and then analyzed. LT-α slightly enhanced the gelatin degradation capability of HUVECs, which was further inhibited by PFKFB3 knockdown; however, due to the limited degradation ability of the ECs, the changes were not significantly different (Supporting Information Figs. S4a and S4b). A recent study pointed out that in ECs, PFKFB3, which binds to actin and generates ATPs for protrusion assembling, could mediate the protrusions formation.31 Subcellular co-localization of PFKFB3 and F-actin, as well as black holes on the gelatin was then detected by immunofluorescence (Supporting Information Figs. S4a and S4c). More F-actin and PFKFB3 positive dots were colocalized with the black holes in HUVECs treated with LT-α, while PFKFB3 knockdown decreased the F-actin positive protrusive structures. Taken together, our above data demonstrated that LT-α could enhance the formation of subcellular protrusions in a PFKFB3-dependent manner and increase the motility of HUVECs.

LT-α up-regulates PFKFB3 through activation of classical NF-κB signaling pathway

To verify whether NF-κB signaling pathway was involved in LT-α-stimulated PFKFB3 expression, western blots were performed. LT-α treatment in HUVECs significantly decreased the expression of IκBα and increased the phosphorylation of NF-κB p65 over fourfold, indicating the activation of the classical NF-κB pathway (Fig. 4a; Supporting Information Figs. S5a and S5b). Knockdown of PFKFB3 showed no effects on the activation of the classical NF-κB pathway in HUVECs (Supporting Information Figs. S5a–S5c). Bay 11–7085, a selective IκBα phosphorylation inhibitor, is widely used for specific inhibition of the classical NF-κB pathway.37 Application of Bay 11–7085 significantly rescued the decreased expression IκBα and suppressed the phosphorylation of p65 as expected (Fig. 4a). Bay 11–7085 also suppressed the expression of PFKFB3 in the LT-α-treated HUVECs (Figs. 4a and 4b), indicating the up-regulation of PFKFB3 was mediated by the classical NF-κB pathway. TNFR-1 and TNFR-2 were regarded as the receptors with equal affinity to LT-α in various cell types.38 Knockdown of either TNFR-1 or TNFR-2 significantly reduced the expression of PFKFB3 as well as the phosphor-p65 in the LT-α-treated HUVECs (Figs. 4c–4e). The results proved that both TNFR-1 and TNFR-2 were essential for the LT-α stimulation in HUVECs, although the effect was more pronounced upon knockdown of TNFR-1. Moreover, inhibition of the classical NF-κB pathway significantly decreased 2-NBDG uptake, total lactate production and ATP generation in LT-α treated HUVECs (Figs. 4f–4h). In summary, the abovementioned results demonstrated that by activating the classical NF-κB signaling pathway, LT-α up-regulated the expression of PFKFB3 and increased glycolytic flux in endothelial cells.

PFK15, a specific PFKFB3 inhibitor, impairs tumor angiogenesis in vitro and in vivo

To explore the potential therapeutic value of PFKFB3 inhibition, PFK15, a highly effective inhibitor against PFKFB3,25,26 was used to treat HUVECs. Glycolytic flux promoted by LT-α was obviously alleviated by PFK15 treatment (Supporting Information Figs. S6a-S6c). Moreover, PFK15 suppressed cell proliferation, migration and tube formation of HUVECs (Figs. 5a–5f). To further verify the therapeutic effects of PFK15 in vivo, we established HNSCC xenografts in nude mice by injecting Cal27 cells subcutaneously. The mice were then treated with PFK15 (20 mg/kg) three times a week for two weeks. PFK15 treatment significantly reduced the tumor volume (Fig. 5g), in accordance with our previous research.25 Our immunohistochemistry results verified that PFK15 administration significantly reduced the CD31 positive vessels in HNSCC xenografts compared to those in the tumors treated with vehicles (Figs. 5h and 5i). In summary, the administration of PFK15 impaired tumor angiogenesis both in vitro and in vivo, indicating the therapeutic potentials of PFK15 as an angiogenesis inhibitor in HNSCC.
High expression of LT-α in HNSCCs correlates with enhanced angiogenesis and increased endothelial PFKFB3 expression

Immunohistochemical analysis of tissue array was performed to verify the expression of endothelial PFKFB3 in HNSCCs. As shown in Figure 6a, PFKFB3 positive blood vessels were easily found in HNSCC samples (n = 114), but not observed in normal mucosa (n = 59). Through immunofluorescence analysis of HNSSC (n = 20) and oral mucosa (n = 6) samples, we analyzed the proportion of PFKFB3 and CD31 double-positive ECs (Supporting Information Figs. S7a and S7b). The results showed that most ECs in HNSSC were PFKFB3 positive. In addition, the infiltration of T cells and B cells was analyzed by the staining of their markers, CD3 and CD20, respectively. The LT-α in stromal cells was also analyzed in the tissue array (Fig. 6a). The histoscores of the stained proteins were analyzed by Aperio QuantiFication System. The results showed that the expression of CD3, CD20 and LT-α was enhanced in HNSSC samples, compared to normal oral mucosa, suggesting that the enhanced infiltration of lymphocytes might be responsible for the increased expression of LT-α in HNSSCs (Fig. 6a–6f). These results were consistent with the TCGA data shown in Figure 1.

The spearman rank correlation analysis showed that the elevated expression of LT-α positively correlated with the infiltration of lymphocytes, the high expression of PFKFB3 in ECs and increased CD31+ microvessels in HNSSCs (Figs. 6g and 6h). The quantitative data of CD3, CD20, LT-α, PFKFB3 and CD31 for each sample was further analyzed by hierarchical cluster (Fig. 6i). Most HNSSCs were clustered together, in which the levels of the infiltrating lymphocytes, the expression of LT-α in stromal cells, endothelial PFKFB3 and CD31+ microvessels were higher than those of OMs clustered in the other group. Additionally, the expression of LT-α positively correlated with angiogenesis biomarkers in HNSSCs according to TCGA data (Supporting Information Fig. S8). Taken together, the results suggested that high expression of LT-α in stromal cells, potentially secreted by infiltrated B cells and T cells, was positively correlated to the increased expression of PFKFB3 in ECs and aberrant angiogenesis. This suggests PFKFB3 a promising target for the HNSSCs therapeutics.

Discussion

Tumor associated stromal cells in the tumor microenvironment (TME) are crucial for tumor angiogenesis by secreting growth factors and inflammatory cytokines to promote ECs survival,
Recently, lymphocytes were also reported to mediate tumor angiogenesis directly and indirectly. Yang et al. reported B cells secreted pro-angiogenic factors in a STAT3-dependent manner to facilitate tumor angiogenesis. In ovarian cancer, CD4^+CD25^+ Tregs secreted VEGF-A and promoted EC proliferation in vitro and in vivo. Lymphocytes, especially the Tregs, also stimulated tumor angiogenesis by recruiting and polarizing macrophages into a M2-like phenotype, which produced pro-angiogenic factors and MMPs to degrade extracellular matrix (ECM). In the present study, we reported a novel mechanism by which lymphocytes promoted tumor angiogenesis. We found in HNSCCs that the infiltrated lymphocytes released LT-α and were able to stimulate ECs and promote tumor angiogenesis by enhancing the PFKFB3-mediated glycolysis.

Figure 5. PFK15 impaired tumor angiogenesis in vitro and in an HNSCC xenograft mouse model. Five micromolar PFK15 was applied to competitively inhibit PFKFB3 during culture. The nude mice bearing xenograft were given 20 mg/mL PFK15 intraperitoneally (i.p) every other day for a fortnight. (a) EdU incorporation assays suggested that PFK15 reduced HUVECs proliferation promoted by LT-α. (b) The quantitative data of the EdU assays. (c) PFK15 impaired the enhanced migration of HUVECs led by LT-α in Transwell experiments. HUVECs were stained with crystal violet. (d) The quantitative data of the Transwell migration assays. (e) PFK15 attenuated increased tube formation ability caused by LT-α in HUVECs seeded on Matrigel. (f) The quantitative data of the tube formation assays. (g) Tumor growth was measured at the end of the experiment (Day 20). (h) Immunohistochemical staining of PFKFB3 and CD31 (PECAM-1) in tumors. The expression of CD31 demonstrated the presence of endothelial cells, which helped to evaluate tumor angiogenesis. Black arrows indicated CD31 positive blood vessels. (i) The number of CD31 positive blood vessels. Quantitative data were from 3 independent experiments. Five random microscope fields were chosen for quantitative data. Data were presented as mean ± SEM. **, p < 0.01; ***, p < 0.001. [Color figure can be viewed at wileyonlinelibrary.com]
Metabolic adaptation of ECs was recognized as a driving force of angiogenesis.\textsuperscript{44} Glycolysis in ECs provided sufficient energy and also intermediates, ensuring indispensable biosynthetic processes for cell growth. By producing fructose-2,6-bisphosphate (F-2,6-BP), a strong allosteric activator of PFK1, PFKFB3 triggered robust glycolysis in ECs.\textsuperscript{31} Loss of PFKFB3 in ECs alleviated vessel formation.\textsuperscript{31} Deletion of endothelial PFKFB3 suppressed neovascular growth in mice oxygen-induced retinopathy models, stressing the role of PFKFB3 in pathological angiogenesis.\textsuperscript{45} Moreover, VEGF-A/C, the most powerful pro-angiogenic growth factors, were able to regulate PFKFB3 at transcriptional and post-transcriptional levels.\textsuperscript{45} Additionally, PFKFB3 was induced by hypoxia as the PFKFB3 gene promoter contained HIF-1 binding sites, which was necessary for the transactivation of neovascular growth in mice oxygen-induced retinopathy models, stressing the role of PFKFB3 in pathological angiogenesis.\textsuperscript{45}
PFKFB3 in response to hypoxia.\textsuperscript{46} Though the practical scenario might be complicated considering the interaction between LT-\(\alpha\) heterotrimer of with LT-\(\beta\) and TNF receptors, we found that the homotrimer complex of LT-\(\alpha\), a common inflammation related cytokine, was able to regulate the mRNA and protein expression of PFKFB3 in ECs. Moreover, our results showed that LT-\(\alpha\)-induced up-regulation of PFKFB3 in ECs did not attribute to VEGF-A/C, which was actually not increased after LT-\(\alpha\) treatment. Instead, LT-\(\alpha\) promoted PFKFB3 expression through activating the NF-\(\kappa\)B signaling pathway in a TNFRI/2-dependent manner. These results were consistent with a previous study,\textsuperscript{37} in which the researchers found the PFKFB3 gene promoter contained the NF-\(\kappa\)B p65 binding sites and activation of NF-\(\kappa\)B signaling pathway could modulate the cellular glucose metabolism by regulating PFKFB3. 

PFKFB3 was also importantly involved in cell cycle progression, as well as cell migration and invasion. The main product of PFKFB3, F-2,6-BP was able to stimulate the phosphorylation of the Cip/Kip protein p27, resulting in ubiquitination and proteasomal degradation of the protein.\textsuperscript{27} p27 was a potent suppressor of the G\(_i\)/S transition and activator of apoptosis; therefore, F-2,6-BP promoted cell cycle and prevent cell apoptosis.\textsuperscript{48} Recent evidence showed that PFKFB3 also directly bound to PFKFB3 in response to hypoxia.\textsuperscript{46} Though the practical scenario

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