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Lactate inhibits ATP6V0d2 expression in tumor-associated macrophages to promote HIF-2α-mediated tumor progression

Na Liu1#, Jing Luo1#, Dong Kuang2,3#, Sanpeng Xu2,3, Yaqi Duan2,3, Yu Xia1, Zhengping Wei1, Xiuxiu Xie1, Bingjiao Yin1, Fang Chen1, Shunqun Luo4, Huicheng Liu1, Jing Wang1, Kan Jiang5, Feili Gong1, Zhao-hui Tang6, Xiang Cheng7, Huabin Li8, Zhuoya Li1, Arian Laurence9, Guoping Wang2,3*, Xiang-Ping Yang1*

1. Department of Immunology, School of Basic Medicine, Tongji Medical College, Huazhong University of Science and Technology (HUST), Wuhan, China, 430030
2. Institute of Pathology, Tongji Hospital, HUST, Wuhan, China, 430030
3. Department of Pathology, School of Basic Medicine, Tongji Medical College, HUST, Wuhan, China, 430030
4. The Center for Biomedical Research, Tongji Hospital, Tongji Medical College, HUST, Wuhan, China, 430030
5. Biodata Mining and Discovery Section, NIAMS, NIH, Bethesda, 20892, MD, USA
6. Department of Surgery, Tongji Hospital, HUST, Wuhan, 430030, China
7. Laboratory of Cardiovascular Immunology, Institute of Cardiology, Union Hospital, Tongji Medical College, HUST, Wuhan, China, 430030
8. Department of Otolaryngology, Head and Neck Surgery, Affiliated Eye-Ear-Nose and Throat Hospital, Fudan University, Shanghai, China
9. Institute of Cellular Medicine, Newcastle University, Newcastle, UK

#Those authors contributed equally to this work.
Correspondence: wanggp@hust.edu.cn (W.G), yangxp@hust.edu.cn (X.P.Y)

Correspondence:
Dr. Xiang-Ping Yang
Department of Immunology
School of Basic Medicine
Tongji Medical College, Huazhong University of Science and Technology
Wuhan, China, 430030
Tel: +86-27-83692600
Fax: +86-27-83692608
Email: yangxp@hust.edu.cn

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Abstract

Macrophages perform key functions in tissue homeostasis that are influenced by the local tissue environment. Within the tumor microenvironment tumor associated macrophages can be altered to acquire properties that enhance tumor growth. Here, we found lactate, a metabolite found in high concentration within the anaerobic tumor environment, activated mTORC1 that subsequently suppressed TFEB-mediated expression of a macrophage-specific vacuolar ATPase subunit ATP6V0d2. Atp6v0d2−/− mice were more susceptible to tumor growth with enhanced HIF-2α-mediated VEGF production in macrophages that display a more protumoral phenotype. We found that ATP6V0d2 targeted HIF-2α but not HIF-1α for lysosome-mediated degradation. Blockade of HIF-2α transcriptional activity reversed the susceptibility of Atp6v0d2−/− mice to tumor development. Furthermore, in a cohort of patients with lung adenocarcinoma, expression of ATP6V0d2 and HIF-2α was positively and negatively correlated with survival respectively, suggesting a critical role of the macrophage lactate-ATP6V0d2-HIF-2α axis in maintaining tumor growth in human patients. Together, our results highlight the ability of tumor cells to modify the function of tumor-infiltrating macrophages to optimize the microenvironment for tumor growth.
Introduction

Macrophages are key regulators of tissue homeostasis and perform tissue-specific functions, which may be influenced by the local tissue environment (1, 2). The tumor microenvironment (TME) is generated by a pathological tissue structure that contains carcinoma cells, mesenchymal cells and infiltrating immune cells including tumor associated macrophages (TAMs) (3, 4). All these components interact with each other to maintain or disrupt the viability of the tumor. TAMs can be abundant within tumors and are characterized by the expression of Arginase 1, Vegf and other markers associated with immune cell inhibition or tumor cell growth (5, 6). The TME is able to alter the phenotype of invading macrophages through a complex array of mechanisms that include cytokines and metabolic intermediates (7).

TAMs can drive tumor expansion through the promotion of tumor vascularization via the production of VEGF (8). Key to this is the expression of the two transcription factors, HIF-1α and HIF-2α within TAMs that mediate VEGF production (9, 10). In an oxygen rich environment, expression of these two proteins is limited by oxygen-dependent proteasome-mediated degradation (11). The TME is typically hypoxic as the highly metabolically active cells outstrip their blood supply. Even in the presence of an adequate oxygen supply, tumor cells remain highly glycolytic, generating a significant amount of lactate that facilitates tumor growth via HIF-dependent and -independent pathways (12, 13). Both hypoxia and lactate are able to enhance macrophage expression of Vegf and Arginase 1 (13). Despite much work in the field, the full array of molecular circuits that exist within the TME to prime
macrophages into a pro-tumorigenic phenotype remains incomplete.

Cancer cells typically have disordered autophagy that include disruption of their lysosomal machinery resulting in the accumulation of damaged organelles and misfolded, potentially toxic proteins (14). The vacuolar ATPases (V-ATPases) are large protein complexes that consist of a peripheral V1 domain that hydrolyzes ATP and an integral V0 domain that transports hydrogen ions, acidifying intracellular compartments including lysosomes, which is required for the completion of autophagy (15). Dysregulation of either the ATPase activity or expression of V-ATPase subunits has been linked with a variety of cancers (16). Within cancer cells, V-ATPases have been reported to play crucial functions in Wnt, Notch, and mTOR signaling that can contribute to cancer cell survival as well as invasion, migration, and metastasis in the highly acidic tumor microenvironment (17). By contrast, the function of V-ATPases in the accessory cells of the TME has yet to be fully explored.

Here we found that tumor cell derived lactate actively downregulates Atp6v0d2 expression in TAMs via mTOR-dependent inhibition of the transcription factor regulator of lysosomal proteins, TFEB. We found that subcutaneously transplanted tumor cells grew more rapidly in Atp6v0d2−/− mice due to enhanced expression of protumoral factors in the TAMs including VEGF production. Expression of these factors are enhanced by HIF-2α. We found that ATP6V0d2 mediates lysosomal degradation of HIF-2α in macrophages, limiting its expression. Inhibition of HIF-2α reversed the phenotype observed in the Atp6v0d2−/− mice. Furthermore, ATP6V0d2 expression is positively correlated with survival of a cohort of lung adenocarcinoma
patients. Together, we identified a novel mechanism by which tumor cells actively downregulate the expression of macrophage lysosomal gene $Atp6v0d2$, which results in enhanced tumor vascularization and growth via the maintenance of HIF-2α.
Results

Tumor-derived lactate suppresses ATP6V0d2 expression in macrophages

Vacuolar ATPases are critical for lysosome acidification and function. To investigate the potential regulation of tumor cells on macrophage lysosome function, we co-cultured bone-marrow derived macrophages (BMDMs) with Lewis lung carcinoma (LLC)-derived tumor conditioned medium (TCM) for 6h and measured the expression of all V-ATPase subunits. While LLC-derived TCM enhanced the expression of multiple V-ATPase subunits, the expression of *Atp6v0d2* was significantly reduced in the presence of TCM ([Figure S1A and 1A](#)). We cultured the macrophages with different ratios of LLC-TCM:complete medium and found that at a ratio of 1:3 or above, TCM strongly suppressed the expression of *Atp6v0d2* ([Figure 1B](#)), despite the pH and glucose concentrations were comparable to normal complete medium (Data not shown). We repeated this experiment with murine melanoma cell line B16-F10-derived TCM and also found inhibition of *Atp6v0d2* expression by B16-F10 TCM, despite the inhibitory effect was irrespective to the ratios of TCM to fresh medium ([Figure S1B-C](#)), which might due to the differential presence of some bioactive agents in the two tumor derived media. Furthermore, we found that exogenously added common cytokines presently within tumors such as IL-1β, IL-6, IL-10 and TGF-β had no effect on *Atp6v0d2* expression ([Figure S1D-G](#)). In line with this, neutralizing antibodies against IL-1β, IL-6, IL-10 and TGF-β did not reverse the TCM-mediated inhibition of *Atp6v0d2* expression ([Figure S1H](#)). In addition, boiled TCM (b-TCM) derived from LLC cells, as well as from B16-F10 cells, still inhibited
the *Atp6v0d2* in macrophages (*Figure 1C and S1I*), suggesting that the active agent in the TCM was heat-resistant and likely to be a low molecular weight metabolite.

Lactic acid is a well-known product of tumor cell glycolysis. We found the lactate concentration was around 34.5 mM in LLC-TCM and 45.2 mM in B16-F10-TCM, compared to 1.3 mM in fresh complete medium (*Figure 1D and S1J*). To test the effect of lactate on *Atp6v0d2* expression, we incubated macrophages with media containing varying concentrations of lactate and found that lactate inhibited *Atp6v0d2* expression in a dose-dependent manner over a range of 2 mM to 50 mM (*Figure 1E*).

To confirm that the inhibition of *Atp6v0d2* was due to the presence of lactate in the TCM, we used various concentrations of monocarboxylic acid transport inhibitor 2-Cyano-3-(4-hydroxyphenyl)-2-propenoic acid (CHC) in macrophages to block lactate transport. Addition of CHC (5 mM) almost completely reversed the inhibition of *Atp6v0d2* expression by LLC and partially reversed the inhibition by B16-F10 TCM (*Figure 1F and S1K*).

*Atp6v0d2* is a target gene of TFEB, a master regulator of lysosomal biogenesis, which can be phosphorylated by mTORC1 and held inactive in the cytoplasm (18, 19). Next we asked whether lactate inhibited *Atp6v0d2* through activation of mTORC1. LLC-TCM and boiled LLC-TCM, as well as lactate, induced ribosomal protein S6 phosphorylation, a surrogate marker of mTORC1 activation (*Figure 1G-H*). This was associated with significantly reduced nuclear localization of TFEB upon lactate or TCM treatment (*Figure 1I*). Using ChIP-qPCR, we found that TFEB directly bound to the *Atp6v0d2* promoter and the binding of TFEB was significantly reduced by the
addition of lactate or TCM (Figure 1J). Finally, addition of the mTOR inhibitor Torin reversed the down-regulation of Atp6v0d2 expression by lactate or TCM (Figure 1K-L).

**Atp6v0d2−/− mice were more susceptible to tumor deposit growth**

To investigate the potential role of macrophage ATP6V0d2 in tumor growth, we injected LLC cells subcutaneously into wild type and Atp6v0d2−/− mice and monitored the tumor size daily. Deletion of Atp6v0d2 led to significantly increased tumor size (Figure 2A). The tumor weight was significantly increased in the Atp6v0d2−/− mice, compared with WT control at day 14 (Figure 2B-C), suggesting that macrophage ATP6V0d2 suppresses tumor growth. We repeated the experiment with B16 melanoma cells and obtained similar results (Figure 2D-F).

ATP6V0d2 is expressed in BMDMs and TAMs but not in CD4+, CD8+ T cells and tumor cells (Figure 2G and S2A-B), indicating the increased tumor growth seen in Atp6v0d2−/− mice was due to altered macrophage function. Deletion of Atp6v0d2 did not alter the number of tumor-infiltrating CD4+ and CD8+ T cells (Figure S2C-E) or the presence of CD4+FOXP3+, CD4+IFN-γ+ and CD8+IFN-γ+ within LLC-tumors (Figure S2F-H).

To confirm that the effect of Atp6v0d2 deletion on tumor cell growth was intrinsic to TAM function, we co-injected LLC cells with either wild type BMDMs or Atp6v0d2−/− BMDMs into Atp6v0d2−/− mice and monitored tumor growth. Compared to the groups without or with WT BMDMs, tumor growth was significantly enhanced in
the presence of $\text{Atp6v0d2}^{-/-}$ BMDMs (Figure 2H-J). We obtained similar data with co-injection of B16-F10 cells with WT or $\text{Atp6v0d2}^{-/-}$ BMDMs (Figure S3A-C).

To address this discrepancy, we investigated the functions of ATP6V0d2 in TAMs. WT mice were irradiated and transplanted with bone marrows either from WT mice or $\text{Atp6v0d2}^{-/-}$ mice, followed with xenograft of LLC tumor cells. Consistently, mice transplanted with $\text{Atp6v0d2}$-deficient bone marrows had enhanced tumor growth, and macrophages from that group displayed a more protumoral phenotype (Figure S3D-F), compared with animals transplanted with wild type bone marrow.

Together, these data demonstrate that macrophage-expressed ATP6V0d2 inhibits tumor growth in vivo.

$\text{Atp6v0d2}^{-/-}$ mice were more susceptible to lung metastasis

To investigate whether ATP6V0d2 plays a role in tumor metastasis, we inoculated age- and sex-matched WT and $\text{Atp6v0d2}^{-/-}$ mice with LLC cells intravenously and examined their lungs for metastases. $\text{Atp6v0d2}^{-/-}$ mice exhibited significantly more tumor nodules (Figure 3A-B). In the lungs from $\text{Atp6v0d2}^{-/-}$ mice, the percentages of protumoral CD11b⁺F4/80⁺CD206⁺ were significantly higher, compared with those from WT mice (Figure 3C-D). Furthermore, the metastasized tumor cells (gated on CD45) isolated from the lungs of $\text{Atp6v0d2}^{-/-}$ mice expressed higher amounts of Ki-67 (Figure 3E-F). In addition, the tumor cells from the lungs of $\text{Atp6v0d2}^{-/-}$ mice were less apoptotic (Figure 3G-I).

Together, these data demonstrate that ATP6V0d2 suppresses tumor metastasis and
tumor cell survival in a lung metastasis model.

**Deletion of Atp6v0d2 results in enhanced macrophage polarization into a protumoral phenotype in tumor**

TAMs display a specialized protumoral phenotype. To investigate the contribution of ATP6V0d2 in regulation of macrophage polarization, we quantified the proportion of protumoral TAMs within the LLC-induced tumors. Deletion of Atp6v0d2 resulted in an increased percentage of CD206⁺CD11C⁺ protumoral cells within the F4/80⁺ macrophage compartment but had no effect on the percentage of CD206⁻CD11C⁺ pro-inflammatory macrophages (Figure 4A-B).

The expression of protumoral macrophage associated factors such as Mrc1, Arg1 and Fizz1 within the tumor tissues were significantly higher in Atp6v0d2⁻/⁻ tumor bearing mice compared with tumors in control animals, but pro-inflammatory-associated Il1b and Il6 expression was similar (Figure 4C). Next, we compared the expression levels of macrophage associated genes in the isolated TAMs between wild type and Atp6v0d2⁻/⁻ mice. TAMs isolated from Atp6v0d2⁻/⁻ tumor bearing mice showed elevated expression of protumoral-related genes including Mrc1, Arg1 and Fizz1, and comparable expression of the pro-inflammatory-related genes Il1b and Il6 (Figure 4D).

To test if ATP6V0d2 is directly involved in macrophage polarization by TCM, we incubated WT or Atp6v0d2⁻/⁻ BMDMs with LLC TCM. After 36h, LLC TCM induced significantly higher expression of Arg1 and Fizz1 in Atp6v0d2⁻/⁻ BMDMs, compared
with wild type BMDMs (Figure 4E-F). By contrast, the absence of ATP6V0d2 resulted in reduced expression of Il-1b but not Il-6 in macrophages upon treatment of LLC TCM, compared with wild type BMDMs (Figure 4G-H). We repeated the same experiments with B16-F10-derived TCM and found similar results (Figure S4A-D). Taken together, these data suggest that macrophage-intrinsic ATP6V0d2 suppresses expression of protumoral factors by TAMs.

**Deletion of Atp6V0d2 results in enhanced VEGF production and abnormal vascularization**

We next turned to identify the cause of accelerated tumor growth in Atp6v0d2-/− mice. Fixed tissue sections of tumors were investigated by immunofluorescence. LLC tumors from Atp6v0d2-/− mice had increased vascularization and reduced mature vessel generation as measured by CD31 and α-SMA staining respectively (Figure 5A-C), together with enhanced hemorrhages (Figure 5D-E). These features led us to measure tumor VEGF, which was significantly enhanced within the tumors that grew in the Atp6v0d2-/− mice (Figure 5F). The transcripts of Vegf, a main driver for angiogenesis, were enhanced in tumors and isolated TAMs in the Atp6v0d2-/− mouse group (Figure 5G-H). In vitro derived wild type or Atp6v0d2-/− macrophages expressed little Vegf. The addition of LLC- and B16-F10-TCM led to enhanced Vegf expression after 6 h that was significantly greater in the Atp6v0d2-/− BMDMs, compared to WT controls (Figure 5I-J). We found similar results in B16-F10-induced tumors that grew in Atp6v0d2-/− mice compared with wild type controls (Figure
Together, these data demonstrate that tumor-generated lactate inhibits ATP6V0d2 expression and drives VEGF production in TAM, resulting in enhanced tumor vascularization.

**ATP6V0d2 promotes HIF-2α, but not HIF-1α, lysosomal degradation**

Next we turned to how ATP6V0d2 would regulate VEGF. HIF-1α and HIF-2α are two major transcriptional factors that induce VEGF during tumor development (10, 20, 21). *Hif1a* and *Hif2a* mRNA expression in the LLC-derived tumors or TAMs was comparable between WT control and *Atp6v0d2−/−* mice (Figure S6A-D). Next, we detected HIF-2α protein expression in LLC-induced tumors with immunofluorescence and found that HIF-2α expression was increased in the *Atp6v0d2*-deficient TAMs, suggesting post-translational regulation of HIF-2α by ATP6V0d2 (Figure 6A-B). In addition, lactate induced a rapid increase of in HIF-2α expression (Figure S6E). Next we measured HIF-2α protein levels in a time course in WT or *Atp6v0d2−/−* macrophages after incubation with TCM. TCM induced HIF-2α accumulation while suppressed ATP6V0d2 expression in WT macrophages; the expression levels of HIF-2α were higher after 2h, 4h, and 6h TCM stimulation in the absence of ATP6V0d2 (Figure 6C). By contrast, deletion of ATP6V0d2 did not increase HIF-1α protein concentration upon TCM stimulation (Figure 6C). Retroviral forced expression of ATP6V0d2 inhibited the HIF-2α protein expression, as well as p62 and LC3, after LLC-TCM stimulation (Figure 6D). Hypoxia stimulation of 4h induced
significant accumulation of HIF-1α, as well as HIF-2α. However, deletion of ATP6V0d2 resulted in enhanced HIF-2α but not HIF-1α expression, indicating that the specific regulation of HIF-2α by ATP6V0d2 is oxygen-independent (Figure 6E).

Deletion of ATP6V0d2 enhanced HIF-2α stability (Figure S6F-G). Next, we treated WT or Atp6v0d2-/- BMDMs with proteasome inhibitor MG132 over a time course. We found that treatment of MG132 led to an accumulation of HIF-2α at 2h and persisted until 6h in the WT cells, indicating a proteasome-mediated degradation of HIF-2α (Figure 6F, left panel). However, HIF-2α protein concentration remained significantly higher in Atp6v0d2-deficient cells, compared with WT control in the presence of MG132, suggesting a second proteasome-independent regulation of HIF-2α degradation by ATP6V0d2 (Figure 6F, right panel). Next, we tested if ATP6V0d2 regulates HIF-2α lysosomal degradation. Macrophages incubated in the presence of Bafilomycin A resulted in enhanced HIF-2α in wild type cells but not in Atp6v0d2-/- cells and the expressions of ATP6V0d2 and p62 were comparable after Bafilomycin treatment (Figure 6G), indicating ATP6V0d2 mediates HIF-2α degradation through its promotion of lysosomal function. Incubation of macrophages with MG132 led to enhanced accumulation of HIF-1α, and it seems there was a synergistic effect on HIF-1α stability by MG132 and lysosome inhibitor Bafilomycin (Figure S6H, left panel). However, deletion of ATP6V0d2 did not further stabilize HIF-1α (Figure S6H, right panel).

Next we asked if ATP6V0d2 affected the localization of HIF-2α within lysosomes. Treatment of macrophages with MG132 to block the proteasome degradation pathway
resulted in enhanced co-localization of HIF-2α and LysoTracker in wild type cells whereas the co-localization of HIF-2α and LysoTracker was significantly reduced in the Atp6v0d2−/− cells (Figure 6H-I).

Taken together, these data suggest that ATP6V0d2 promotes lysosomal HIF-2α degradation, but does not alter HIF-1α.

Blockade of HIF-2α activity reversed the enhanced tumorigenesis in Atp6v0d2−/− mice

To test the significance of HIF-2α in mediating the effects seen in Atp6v0d2+/− macrophages, wild type and Atp6v0d2−/− mice were injected with LLC tumor cells. At day 10, when the injected LLC tumor cells grew to a size of 100-200 mm³, mice were treated with the HIF-2α inhibitor PT2385 (5 mg/kg) or PBS control twice daily. At day 14, the mice were sacrificed. The addition of PT2385 reversed the difference in tumor growth seen between wild type and Atp6v0d2−/− mice (Figure 7A-B). At days 14, we found a significant increase in serum VEGF concentration in the Atp6v0d2−/− mice that was reversed in animals that received four days of PT2385 (Figure 7C). We detected enhanced abnormal vascularization in the tumors of Atp6v0d2−/− mice by immuno-florescent staining with anti-CD31 and α-SMA that was reversed by the addition of PT2385 (Figure 7D-F). Furthermore, the expression of enhanced HIF-2α target genes including Vegf1, Glut1, Ccnd1, and Pai1 in tumors isolated from Atp6v0d2−/− mice was again reversed by PT2385 (Figure S7A-D). We obtained similar results for HIF-2α target genes in TCM-stimulated wild type and Atp6v0d2−/− macrophages (Figure S7E-H). We next measured expression of macrophage genes
associated with a protumoral phenotype, including *Mrc1*, *Arg1*, *Fizz1* and *Ym1* and found their expression enhanced in the tumors isolated from *Atp6v0d2*-deficient mice compared to wild type controls; inhibition of HIF-2α transcriptional activity by PT2385 inhibited expression of these genes eliminating the differences between wild type and *Atp6v0d2*-deficient mice (Figure 7G-J). To confirm that these differences could be explained by differences in expression within macrophages, we incubated WT or *Atp6v0d2*-/BMDMs in TCM with or without PT2385 and measured protumoral-related gene expression. We saw little gene expression in unstimulated cells. By contrast, TCM induced expression of *Arg1*, *Fizz1* and *Ym1* in wild type BMDMs that was significantly enhanced in *Atp6v0d2*-/BMDMs (Figure 7K-M). Finally, that addition of PT2385 resulted in a significant reduction in the expression of *Arg1*, *Fizz1* and *Ym1* in both WT and *Atp6v0d2*-/BMDMs and eliminated the differences between the two groups (Figure 7K-M). Taken together, these data demonstrate that the enhanced HIF-2α contributed to the enhanced vascularization, protumoral macrophage polarization and tumorigenesis seen in the absence of ATP6V0d2.

**Macrophage ATP6V0d2 expression is inversely correlated with both the presence of CD163* TAMS and survival in human lung adenocarcinoma patients**

To investigate the significance of the ATP6V0d2-mediated HIF-2α lysosomal degradation in human cancers, we measured the expression of ATP6V0d2 and HIF-2α in lung adenocarcinoma tissues (n=35) in patients that have received surgical resection of their lung tumors. While few CD68* macrophages were present in normal
lung tissue adjacent to the tumors, there was a significant number of macrophages within tumor tissues (Figure S8A); both ATP6V0d2 and HIF-2α expression was restricted to the CD68⁺ macrophages, but not in other leucocytes within lung adenocarcinoma tissue (Figure S8B and 8A), despite that HIF-2α was reported in a very small population of NSCLC cancers (22). In contrast, HIF-1α was expressed in tumor cells, as well as infiltrated immune cells (Figure S8C), indicating the unfounded HIF-2α in our hands is not due to sample preparation. We divided the lung adenocarcinoma patients into two groups on the basis of macrophage ATP6V0d2 expression and found that there were more CD163⁺ protumoral-like macrophages and enhanced HIF-2α in the ATP6V0d2 low expression group (Figure 8B). Next we turned to patient survival using Kaplan-Meir survival analysis in patients after undergoing surgical resection of their lung tumors. We found that the survival time for patients with high ATP6V0d2 expression was significantly extended compared with patients that had low ATP6V0d2 expression (Figure 8C). The proportion of patients expressing ATP6V0d2 in the survival group was higher than that in the deceased group (83% versus 45%) (Figure 8D). Although there was no difference of numbers of CD68⁺ within tumors between ATP6V0d2 high expression and ATP6V0d2 low expression patients, the numbers of CD163⁺ protumoral-like macrophages were significantly higher in the ATP6V0d2 low expression group (Figure 8E and S8D). Consistent with this, when the patients were divided into two groups on the basis of HIF-2α expression, the patients with higher expression levels of HIF-2α had a significantly shorter survival time (Figure 8F). Taken together, these data demonstrate
that the ATP6V0d2-mediated HIF-2α lysosomal degradation in macrophages is linked with the survival of human lung adenocarcinoma patients.

**Discussion**

The reciprocal regulation between tumor cells and tumor-infiltrating immune cells shapes the immune status of the tumor microenvironment and may determine the outcome of cancer progression. Here, we demonstrate that tumor cell derived lactate suppressed macrophage ATP6V0d2 expression via activation of mTORC1 that lead to reduced lysosomal degradation of HIF-2α. Subsequently, macrophages produced more VEGF and were polarized into a protumoral phenotype by lactate. We found that tumors grew faster in *Atp6v0d2*-deficient mice compared with control animals. In tumor deposits from patients, the expression of ATP6V0d2 inversely correlated with HIF-2α expression in TAMs and post-operative survival of lung cancer patients.

Macrophages are influenced by the local environment as sentinels of the immune system and acquire additional activities tailored for their tissue specific functions (23). mTOR signaling senses and integrates nutrition, growth factors and energy status to coordinate cellular function. Lactate has been shown to activate PI3K/Akt in endothelial cells through an unknown mechanism and mTOR in tumor cells via glutamine metabolism (24, 25). It is unclear if lactate also activates mTOR via glutamine metabolism in macrophages. The V-type ATPase is critical for lysosomal acidification. In addition, some subunits have been linked with autophagosomal:lysosomal fusion (26) and have been shown to be regulated by TFEB, the master regulator of lysosomal biogenesis (19). We found that TFEB bound to and
regulated the expression of *Atp6v0d2*, and the activation of TFEB was inhibited by lactate in an mTORC1-dependent manner, demonstrating the importance of the TME on macrophage signaling and function.

In contrast with ubiquitous expression of HIF-1α, HIF-2α expression is restricted and plays a significant role in regulating macrophage function in inflammation and cancer (27, 28). Although HIF-1α is required for *Vegf* induction in macrophages, our data demonstrated that dysregulated HIF-2α expression enhanced *Vegf* expression and facilitated tumor growth in both mouse and human, indicating both HIF members contribute to shape the macrophages within TME form them to acquire the maximal protumoral activities. Despite the enhanced HIF-2α expression in steady *Atp6v0d2* −/− macrophages (Figure 6C), the VEGF production was similar to that in steady WT cells (Figure 5I-J), indicating HIF-2α-mediated VEGF production may need other factors, which are activated during TCM stimulation or tumor progression in vivo. Our data, together with previous report (13), suggest that lactate engages two parallel pathways to regulate ATP6V0d2-independent HIF-1α and ATP6V0d2-dependent HIF-2α expression to enhance protumoral activity of macrophages. In contrast to hypoxia-dependent enhancement of HIF-1α protein expression by lactate (Data not shown), lactate enhances HIF-2α independent of oxygen.

The stability of both HIF-1α and HIF-2α expression is classically regulated by the proteasome (29, 30). Yet recent studies have demonstrated that these two molecules are also subject to lysosome-mediated degradation in cancer cells (31-33). Our work would suggest that distinct lysosomal regulation of these two related transcriptional
factors exists. Considering the restricted expression of ATP6V0d2, it is highly likely that distinct types of cells may employ different machineries for lysosomal degradation of HIF-2α. Rapamycin promoted HIF-2α degradation in WT macrophages but not in Atp6v0d2−/− macrophages (Data not shown), suggesting that ATP6V0d2 promotes HIF-2α degradation via autophagy a process that is dependent lysosomal degradation. Currently, it remains unclear how ATP6V0d2 selectively regulates HIF-2α stability.

Despite that it has been reported that HIF-2α is expressed in a small proportion of human lung adenocarcinomas (34), we found both ATP6V0d2 and HIF-2α were exclusively expressed in macrophages and that their expression is inversely correlated with each other, consistent with our mouse data. In contrast, HIF-1α is expressed in both tumor cells and tumor infiltrating immune cells, which is consistent with the idea of differential expression between HIF-1α and HIF-2α (22). The expression level of ATP6V0d2 and HIF-2α in TAMs correlated positively and negatively with patient survival respectively, highlighting the significance of the regulation of HIF-2α by ATP6V0d2 in human cancers. This could not be explained by differences in the intratumoral inflammatory infiltrate as we found similar numbers of TAMs inside human lung cancers with different outcomes. This suggests that the function rather than the number of TAMs is critical for prognosis, consistent with previous studies (35, 36).

In summary, our findings identified ATP6V0d2 as a key factor that mediates the ability of lactate enriched TME to induce protumoral activities in TAM via the
maintenance of hypoxia-independent regulation of HIF-2α. This scenario provides molecular and cellular insights into the macrophage acquisition of protumoral activities within tumors and expands the number of factors that mediate the effect of the TME.
Methods

Mice

*Atp6v0d2*-/- mice were generated using TALEN (transcription activator-like effector nuclease) technology. A TALEN binding pair was chosen from *Atp6v0d2* CDS in the first exon. The genomic recognition sequences of TALEN left and right arms are CGAGGATGCAAAGCCAGCC (L) and GCACCTAGGTGACATA (R), spaced by 16 bp and anchored by a preceding T base at the -1 position to meet the optimal criteria for natural TAL proteins. TALEN vectors of left and right arms -TALEN-*Atp6v0d2*-L and TALEN-*Atp6v0d2*-R- were obtained by one-step ligation using the Fast TALETM TALEN Assembly Kit (SIDANSAI Biotechnology, Shanghai, China) according to the manufacturer’s instructions. mRNA (400 ng/μl, 10 pl) was injected into cytoplasm of 180 one-cell embryos. After incubation for 24 hr, the selected two-cell embryos transferred into the oviduct of 7 pseudopregnant C57BL/6 mice. We confirmed the genotype of F0 mice by DNA sequencing. C57BL/6 WT mice were purchased from Huafukang (Beijing). All the mice were bred and housed in a specific-pathogen-free facility at the Tongji Medical College, HUST.

Xenograft mouse tumor models

Male C57BL/6 WT or *Atp6v0d2*-/- mice were injected s.c. with either 5×10^5 LLC cells or 2×10^5 B16-F10 cells. Tumor growth was measured every 2 to 3 days after tumor inoculation using digital calipers. Mice were sacrificed 14 days after inoculation and tumors were excised and weighted. For macrophage adoptive transfer experiment,
Atp6v0d2\(^{-/-}\) mice were injected s.c. with 5×10\(^5\) LLC cells alone or 5×10\(^5\) LLC cells plus either 2×10\(^5\) WT BMDMS or Atp6v0d2\(^{-/-}\) BMDMs, or injected s.c. with 5×10\(^5\) B16-F10 cells alone or 2×10\(^5\) B16-F10 cells plus either 2×10\(^5\) WT BMDMs or Atp6v0d2\(^{-/-}\) BMDMs. Tumor growth and weight were determined as described above.

**Generation of chimeric bone marrow mice**

Bone marrow chimera were generated as following. Briefly, 8-12 weeks CD45.2 WT C57BL/6 male mice were sub-lethally irradiated with 7 Gy of whole-body irradiation. Bone marrows were harvested from donor CD45.2 WT and Atp6v0d2\(^{-/-}\) mice or CD45.1 WT mice to monitor the chimeric efficiency. Two hours after irradiation, mice were injected i.v. with 8×10\(^6\) cells from donor BM. After 4 weeks of transplantation, mice were s.c. challenged with 5×10\(^5\) LLC cells. Percentage of chimerism was determined by staining periphery blood cells with anti-CD45.1-PE (clone A20; BD Bioscience) and anti-CD45.2-APC (clone 104; Biolegend) antibodies for analysis by flow cytometry.

For the inhibition of HIF-2α in a mouse model, a total of 5×10\(^5\) LLC cells in a volume of 100 µl of PBS were inoculated s.c. into 6-7 weeks of age C57BL/6 WT or Atp6v0d2\(^{-/-}\) male mice. PT2385 was formulated with 10% absolute ethanol, 30% PEG400, 60% water containing 0.5% methylcellulose and 0.5% Tween 80 according to previous report (37). Once tumor size reached 100-200 mm\(^3\) at day 10, WT and Atp6v0d2\(^{-/-}\) mice were treated with PBS or PT2385 (5mg/kg) twice daily by oral gavage for 4 consecutively days. Tumor sizes were measured and recorded daily. On day 14, mice were sacrificed and tumor tissues were collected for experiments.
Cell culture and collection of tumor conditioned medium (TCM)

Primary bone marrow derived macrophages (BMDMs) were generated by culturing mouse bone marrow cells in the presence of 50 ng/ml M-CSF conditional medium for 7 days. BMDMs were seeded in 12-well plates. For the neutralization experiment, BMDMs were stimulated with LLC-derived TCM in the presence of 10 μg/ml different neutralizing antibodies: anti-IL-1β (clone: B122; BioX cell), anti-IL-6 (clone: MP5-20F3; eBioscience), anti-IL-10 (clone: JES5-2A5; eBioscience) and anti-TGF-β (clone: 19D8; Biolegend). Lewis lung carcinoma (LLC) cells and B16-F10 melanoma cells were obtained from ATCC. 1x10^6 cells were cultured in 10 cm plates with DMEM medium supplemented with 10% heat-inactivated FCS, 100 U/ml penicillin G, and 100 μg/ml streptomycin for 5 days. To collect tumor conditional medium, cultured medium were centrifuged at 10,000 rpm for 5 mins followed by filtration with 0.2 μm filter.

Determination of lactate concentration in TCM

The lactate concentration was measured using a Lactate Assay Kit (Bioassay systems, catalogue number, ECLC-100). The mean values and s.e.m. of the lactate concentration were calculated according to the manufacturer’s instructions.

Quantitative RT-PCR

Total RNA were extracted from tissues or cell lines with TRizol reagent (Invitrogen). cDNA was synthesized from 1 μg RNA using the Reverse Transcription Kit (Toyobo, Japan) following the manufacturer’s protocol. All quantitative RT-PCR was performed by the SYBR green method on Bio-Rad. The quantification of the results was
performed by the comparative Ct ($2^{-\Delta\Delta Ct}$) method. The Ct value for each sample was normalized by the value for Actin or Gapdh gene. Primers sequences for the genes of Atp6v0d2, Atp6v0a2, Atp6v0c, Atp6v0d1, Atp6v1a, Atp6v1c1, Atp6v1d, Atp6v1e1, Atp6v1g1, Atp6v13, Vegf, Glut1, Pai1, Ccnd1, Il1b, Il6, Hif2a, Hif1a, Arg1, Ym1, Fizz1, Mrc1 and Actb are provided in Supplementary Table 1.

**Chromatin-immunoprecipitation assay**

Mouse BMDM/Raw 264.7 cells were treated with TCM or Lactate for 6h, followed by cross-linking for 8 mins with 1% (vol/vol) formaldehyde. Cells were collected and lysed by sonication. Cell lysates were immunoprecipitated with anti-TFEB (ab2636, abcam). After washing and elution, crosslinks were reversed for 4h at 65°C. The eluted DNA was purified, and analyzed by qPCR with custom-designed primers. Forward: AAACTGGGCATGACCTTG, Reverse: GCAGCTCAGAAGGCACA using a Bio-Rad SYBR Green intercalating fluorophore system. The Ct value for each sample was normalized to corresponding input value.

**Isolation of Tumor Infiltration macrophage**

On day 15, mice were sacrificed and tumor tissues were gently minced and excised into small pieces. Afterwards, tumor tissues were disrupted with 1 × PBS containing 200ng/ml collagenase IV (Cat: c17104019, Invitrogen), 250 ng/ml hyaluronidase (Cat: H1115000, Sigma), 40 ng/ml DNase I (Cat: 10104159001, Roche) at 37°C, 100 rpm shaker. Cell suspension were filtered and red blood cells were lysed. Tumor infiltrating lymphocyte were separated by gradient centrifugation with 40%/80% percoll (GE). Collected cells were stained and analyzed by flow cytometry.
Flow cytometry

Single-cell suspensions of tumors were generated following the manufacturer’s protocol. After Fc blockade with anti-CD16/CD32 (clone 93), cells were stained with following antibodies: CD4-FITC (clone RM4-5), CD8-PE (clone 536.7), CD11c-PE (clone N418), F4/80-PerCPcy5.5 (clone BM8), CD206-PE/cy7 (clone C068C2) and CD11b-FITC (clone M1/70) (all from Biolegend). For intracellular staining, cells were stimulated with 50 ng/ml PMA (Sigma-Aldrich) and 500 ng/ml ionomycin (Sigma-Aldrich) in the presence of 5 μg/ml Golgiplug (BD) in complete RPMI 1640 medium for 4 h at 37°C. Cells were fixed, permeabilized and stained with following antibodies: anti-Foxp3-APC(clone FJK-16s; ebioscience), anti-IL-1β-APC (clone NJTEN3; ebioscience) and anti-IFN-γ-BV421 (clone XMG1.2; Biolegend). Samples were collected on a BD Verse Flow cytometer, and data were analyzed using FlowJo software.

ELISA quantitation of VEGF

Every 0.5mg tumor tissues were homogenated with 500µl ice-cold PBS, then supernatant were collected after centrifuge, and stored at -80°C until further use. VEGF-A in the tumor tissue was measured with a ELISA Kit from BioLegend (DKW12-2734-048) according to the manufacturer's instructions.

Immunofluorescence

BMDMs were seeded at 3×10⁴ cells per well in glass slides, and rested overnight for proper attachment. Then the cells were stimulated with MG132 (20μM) for 4h, and then treated with 1:10000 PBS-diluted LysoTracker Red DND-99 dye (DMSO
solution, Life Technologies) for 15 mins. After the treatment, cells were washed twice with sterile PBS and fixed with 4% paraformaldehyde (PFA), permeabilized with 0.05% Triton X-100 and blocked in 5% BSA. Anti-HIF2α (NB100-132, Novus) was incubated overnight in -4°C. Secondary fluorescent antibodies FITC, Cy3 from Life Technology™ or Jackson Laboratories) were added for 1h and DAPI was used for nuclear counterstaining. The human tumor tissues were collected during surgery and fixed in formalin immediately, followed by standard paraffin-embedding and immunohistochemistry (22). For human tissue, immunofluorescence staining was performed overnight with anti-CD3 (GB13014, Servicebio), CD19 (GB11061, Servicebio), CD68 (ab125212, abcam), CD31 (GB13063, Servicebio), α-SMA (GB13044, Servicebio), F4/80 (GB11027, Servicebio), ATP6V0d2 (ab194557, abcam). Secondary fluorescent antibodies (FITC, Cy3 from Life Technology™ or Jackson Laboratories) were added for 1 hr and DAPI was used for nuclear counterstaining. Samples were imaged through a SP5 confocal (Leica) 24h after mounting.

**Immunoblotting**

Immunoblotting was performed according to standard method. Primary antibodies included the following: phospho-PS6 (4851, Cell signaling), Lamin B1 (13435, Cell signaling), TFEB (ab2636, abcam), GAPDH (5174, Cell signaling), HIF-2α (NB100-122, Novus), HIF-1α (14179S, Cell signaling), LC3B (#12741, Cell signaling), p62 (PM045, MBL), anti-ATP6V0d2 (SAB2103221, Sigma), β-actin (4970S, Cell signaling). Followed by appropriate secondary HRP-conjugated antibodies, then developed with ECL (GE healthcare).
**Immunohistochemistry**

Paired cancer and adjacent non-cancer tissue samples were obtained with informed consent from Tongji Hospital of HUST. The specimens were isolated at the time of surgery, formalin-fixed and paraffin-embedded, and stained with hematoxylin and eosin, standard immunohistochemistry was performed with antibodies against ATP6V0d2 (ab194557, abcam), CD163 (ZM-0428, ZSGB-BIO), CD68 (66231-2-Ig, Proteintech), HIF-2α (NB100-132, Novus Biologicals), then examined by two blinded pathologists. The immunoreactive score (IRS) gives a range of 0-8 as a product of multiplication between positive cells proportion score (0-4) and staining intensity score (0-3). The IRS, 0, negative; 1, mild; 2, moderate; 3, strongly positive to measure the expression of ATP6V0d2. The IRS, 0-1, negative; 2-3, mild; 4-6, moderate, 7-8, strongly positive to measure the expression of HIF-2α. The diagnosis of lung cancer or normal lung was confirmed based on histological findings by two independent pathologists. The immunohistochemistry data were evaluated by two blinded pathologists.

**Statistical analysis**

Data were graphed using GraphPad Prism software. Statistical significance was determined either by unpaired 2-tailed Student’s test, one-way ANOVA followed by Turkey’s post-hoc test or two-way ANOVA. A *P* value of less than 0.05 was considered significant.

**Study approval**
All mice studies were approved and used in accordance with institutional guidelines (Tongji Medical College, HUST). Human specimens used in this study have been approved by the Ethics Committee of Tongji Hospital, Huazhong University of Science and Technology and signed informed consent was obtained from all patients’ family (TJ-IRB-20180516, Tongji Hospital, HUST, Wuhan).
AUTHOR CONTRIBUTIONS

Conceptualization, NL, JL, AL, and XPY; Investigation, NL, LJ, DK, SX, YD, YX, ZW, XX, FC, SL, HL, JW, Formal Analysis, BY, KJ, FG, ZT, XC, ZL, GW and XPY.; Resources, HL, GW; Funding Acquisition and Supervision, FG, and XPY; Visualization and writing, NL, LJ, AL, and XPY.

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Address correspondence to: Guoping Wang, Institute of Pathology, Tongji Hospital, HUST, 1095 Jiefang Road, Wuhan, 430030, China. Phone: 86.27.83662856; Email: wanggp@hust.edu.cn and Xiang-Ping Yang, Department of Immunology, Tongji Medical College, Huazhong University of Science and Technology, 13 Hangkong Road, Wuhan, 430030, China. Phone: 86.27.83692600; Email: yangxp@hust.edu.cn
References:


Figure 1

A-F, except D) Atp6v0d2 mRNA expression was determined by q-PCR. (A and B) LLC-tumor conditioned medium (TCM) was collected after 5 days culture of LLC cells to reach 100% confluence. BMDMs stimulated with LLC-TCM (A) or a concentration gradient of LLC-TCM (B) for 6h. (C) BMDMs were stimulated with medium, LLC-TCM, or 100°C 5mins boiled LLC-TCM (b-TCM) for 6h. (D) Lactate concentration in LLC-TCM was determined. (E) BMDMs were stimulated with a concentration gradient of lactate for 6h. (F) BMDMs stimulated with lactate, LLC alone, or with the addition of different doses of CHC (a-cyano-4-hydroxycinnamate), a monocarboxylate channel transporter inhibitor. (G and H) Representative histograms (G) and bar chart (H) show MFI for pS6 expression on macrophages that were starved with EBSS for 2h, followed by replacement with fresh medium (control), fresh medium with lactate, b-TCM, or TCM for 1h. (I) BMDMs were starved with EBSS for 2h, followed by replacement with fresh medium in the presence of lactate (40mM) or complete TCM for the indicated times. The amounts of nuclear-cytoplasmic fractionation of TFEB and pS6 were determined by immunoblot and amounts of TFEB in the nuclei were quantified. (J) RAW264.7 cells were stimulated with lactate (40mM) or TCM for 4h and the binding of TFEB in the Atp6v0d2 promoter was determined by chromatin immunoprecipitation. (K and L) BMDMs were stimulated with lactate (K) or TCM (L) for 6h in the presence or absence of 15min pretreatment of Torin (1μM). Atp6v0d2 mRNA expression was determined by q-PCR. Data are representative of three independent experiments (A-I). Data were assessed by unpaired Student’s t test (A), one-way ANOVA with Turkey’s test (B-L), except D, G) and represented as mean±SEM. *p < 0.05, **p < 0.01, ***p < 0.001.
Figure 2. Atp6v0d2<sup>−/−</sup> mice are more susceptible to tumorigenesis. (A-C) Comparison of LLC tumor growth in WT and Atp6v0d2<sup>−/−</sup> mice (n=7) after implantation, tumor growth rate (A), tumor weight (B), and representative images of excised tumors (C). (D and F) Tumor growth of B16-F10 in WT and Atp6v0d2<sup>−/−</sup> mice (n=5) after implantation, tumor growth rate (D), tumor weight (E), and representative images of excised tumors (F). (G) Immunoblot analysis of ATP6V0D2 expression in macrophages, CD4<sup>+</sup> T cells, CD8<sup>+</sup> T cells, fibroblasts, TAMs, B16-F1, B16-F10, and LLC cells. (H-K) Atp6v0d2<sup>−/−</sup> mice were challenged s.c. with 5x10<sup>4</sup> LLC cells alone, or together with 2x10<sup>5</sup> either WT BMDMs or Atp6v0d2<sup>−/−</sup> BMDMs (n=5). Tumor size was measured every 2 to 3 days (H). Tumor mass was determined at day 15 after inoculation (I). Representative images of tumor were presented (J). All experiments except H-J were repeated two times. Data were assessed by unpaired Student’s t test (A, B, D, E) or one-way ANOVA with Turkey’s test (H, I) and represented as mean±SEM. *p < 0.05, **p < 0.01, ***p < 0.001. Scale bars: 5 mm (C, F, and J).
Figure 3. Atp6v0d2-deficient mice were more susceptible to lung metastasis. (A-I) WT and Atp6v0d2−/− mice were injected i.v. with 8×10^6 LLC cells. On day 15 post-injection, mice were sacrificed. (A-B) Images (A) and quantification analysis of lung metastasis of WT (n=5) and Atp6v0d2−/− mice (n=6). (C-D) Flow cytometric analysis of CD11b^+ F4/80^+ CD206^+ TAMs in lung tissues (C) and comparison of fractions of F4/80^+ CD206^+ TAMs between WT and Atp6v0d2−/− mice (D). (E-F) Ki67 expression by tumor cells in the lung tissues isolated from WT and Atp6v0d2−/− mice. (G-I) Flow cytometric analysis of tumor cells apoptosis in the lung tissues from WT and Atp6v0d2−/− mice (G), then quantification of apoptotic cells (H) and live cells (I). Data are assessed by unpaired Student’s t test and presented as mean±SEM. *p < 0.05, **p < 0.01, ***p < 0.001.
Figure 4. Deletion of Atp6v0d2 leads to enhanced protumoral polarization of macrophages. (A-D) WT and Atp6v0d2−/− mice were injected s.c. with 5×10⁵ LLC cells. On day 15 post-inoculation, mice were sacrificed. (A and B) Representative flow cytometric plot of tumor infiltrating gated F4/80+cells showing CD11C+CD206− (proinflammatory) and CD11C−CD206+ (protumor) TAM fractions in LLC tumor xenograft model (A) and comparisons of fractions of CD11C+CD206−, CD11C−CD206+ in TAMs between WT and Atp6v0d2−/− mice (B) (n=5). (C) The expression of Mr1, Arg1, Fizz1, Il1b and Il6 in WT and KO tumor tissues was determined by q-PCR (n=5). (D) TAMs from tumor-bearing WT and Atp6v0d2−/− mice were isolated with CD11b magnetic beads. The expression of Mr1, Arg1, Fizz1, Il1b and Il6 was determined by q-PCR (n=5). (E and F) WT and Atp6v0d2−/− BMDMs were stimulated with medium, or LLC-TCM for 36h. The expression of Arg1 (E) and Fizz1 (F) was determined by q-PCR. (G and H) WT and Atp6v0d2−/− BMDMs were stimulated with medium, or LLC-TCM for 6h. The expression of Il1b (G) and Il6 (H) was determined by q-PCR. Data are representative of two (A-D) or three (E-H) independent experiments. Data were assessed by unpaired Student’s t test and represented as meansSEM, *p < 0.05, **p < 0.01, ***p < 0.001.
Figure 5. Atp6v0d2-deficiency results in enhanced tumor angiogenesis. (A-I) WT and Atp6v0d2−/− mice were injected s.c. with 5×10^5 LLC cells. On day 15 post-inoculation, mice were sacrificed (n=5). (A-C) Double immunostaining for CD31 (red) and α-SMA (green) vessels in tumor tissues from WT and Atp6v0d2−/− mice (A). Quantifications of the percentage CD31+ vessels (B) and α-SMA+CD31+ vessels (G). (D and E) H&E staining on tumor tissues in WT and Atp6v0d2−/− mice. Histogram depicts the percentage of hemorrhagic area versus the whole tumor area (E). (F) Comparison of tumor tissue VEGF levels at day 15 in WT and Atp6v0d2−/− mice. (G and H) The expression of Vegf in tumor tissues (G) or isolated TAMs (H) from WT and Atp6v0d2−/− mice was determined by q-PCR. (I and J) Expression analysis of Vegf by q-PCR in WT and Atp6v0d2−/− BMDMs that were stimulated with LLC-TCM (I) or B16-F10-TCM (J) for 6h. Data are representative of three independent experiments. Data were assessed by Student’s t test and represented as mean±SEM, *p<0.05, **p<0.01, ***p<0.001. Scale bar: 100μm.
Figure 6

(A-B) Whole-mount immunofluorescence staining of DAPI (blue), F4/80 (red) and HIF-2α (green) in tumor tissues of WT and Atp6v0d2−/− mice (A). Percentage of HIF-2α/F4/80+ among F4/80+ were quantified manually (B).

(C-D) Immunoblotting of HIF-2α, HIF-1α, P62, ATP6V0d2 and LC3 in WT and Atp6v0d2−/− BMDMs that were stimulated with hypoxia (1% O2) for the indicated times. (G) WT and Atp6v0d2−/− BMDMs were untreated, or treated with Bafilomycin A (100nM) for 6h. The expression of HIF-2α, P62, ATP6V0d2 and LC3 was determined by immunoblotting. (H and I) WT and Atp6v0d2−/− BMDMs were untreated or treated with MG132 (20μM) for 4h. Cells were stained with anti-HIF-2α and lysotracker (H). Co-localizations of HIF-2α and lysosome among cytoplasmic HIF-2α were quantified (I). Data were assessed by Student’s t test and representative of two (A and B) or three (C-I) independent experiments. mean±SEM, *p < 0.05, ***p < 0.001. Scale bar: 100μm.
Figure 7. Suppression of HIF-2α activity reverses the enhanced tumorigenesis in Atp6v0d2−/− (n=5) mice. (A-J) WT and Atp6v0d2−/− mice were injected s.c. with 5×10⁶ LLC cells. Once the tumor volumes reached 100-200 mm³ around day 10, mice were gavaged with vehicle or HIF-2α inhibitor PT2385 (5mg/kg) twice daily for 4 consecutive days. Growth curve (A) and tumor weight (B) were plotted. (C) Comparison of tumor tissue VEGF levels. (D-F) Immunostaining of CD31 and α-SMA in tumor tissues. (G-J) q-PCR analysis of Mrcl (G), Argl (H), Fizz1 (I), Ym1 (J) in the tumor tissues. (K-M) WT and Atp6v0d2−/− BMMs were stimulated with LLC-TCM for 24h with or without PT2385 (20nM) and PT2385 was added 18h prior to the TCM stimulation. Expression of Argl (K), Fizz1 (L) and Ym1 (M) was determined by q-PCR. Data were assessed by two-way ANOVA with Turkey’s test and presented as mean±SEM. *p < 0.05, **p < 0.01, ***p < 0.001. Scale bar: 100μm.
Figure 8. ATP6V0d2 is linked with survival for human lung adenocarcinoma patients. (A) Immunofluorescence staining of human primary lung adenocarcinoma tissues with DAPI (blue), anti-CD68 (red), and anti-HIF-2α (green). Scale bar, 200µm. (B) A representative image of H&E staining and the IHC analysis of ATP6V0d2, CD163, and HIF-2α in the lung sections of adjacent non-tumorous lungs or tumor tissues isolated from two lung adenocarcinoma patients. Scale bars, 50µm. (C) Kaplan-Meier plot of overall survival of patients with lung cancer stratified by high IRS (>3) or low IRS (<3) of ATP6V0d2 expression levels. (D) The proportion of ATP6V0d2 expression in survival and deceased patients was calculated and evaluated by the χ² test with Graphpad prism software. A P value less than 0.05 was considered statistically significant. (E) Quantification of numbers of CD163+ TAMs in 5 areas of each patients were determined by IPP image software in two groups of lung adenocarcinoma patients with high ATP6V0d2 expression (n=20, IRS>3) and low ATP6V0d2 expression (n=11, IRS<3). (F) Kaplan-Meier plot of overall survival of patients with lung cancer stratified by high IRS (>3) or low IRS (<3) of HIF-2α expression levels.