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

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Introduction

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 that lactate, a metabolite found in high concentration within the anaerobic tumor environment, activated mTORC1 that subsequently suppressed TFEB-mediated expression of the 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.

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vival as well as invasion, migration, and metastasis in the highly acidic TME (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 down-regulates 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 is 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. Taken together, these results identify what we believe is a novel mechanism by which tumor cells actively downregulate the expression of the 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. V-ATPases are critical for lysosome acidification and function. To investigate the potential regulation of macrophage lysosome function by tumor cells, we cocultured bone marrow–derived macrophages (BMDMs) with Lewis lung carcinoma–derived (LLC-derived) tumor-conditioned medium (TCM) for 6 hours 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 1A and Supplemental Figure 1A; supplemental material available online with this article; https://doi.org/10.1172/JCI23027DS1). 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 being comparable to normal complete medium (data not shown). We repeated this experiment with murine melanoma cell line B16-F10–derived TCM and found that Atp6v0d2 expression by B16-F10 TCM, despite the inhibitory effect being irrespective of the ratios of TCM to fresh medium (Supplemental Figure 1, D–G). 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 being comparable to normal complete medium (data not shown).

We found that exogenously added common cytokines in fresh complete medium (Figure 1D and Supplemental Figure 2, A–C). To confirm that 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 Supplemental Figure 1K).

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 WT 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 2, B and C), suggesting that macrophage ATP6V0d2 suppresses tumor growth.

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Atp6V0d2 is expressed in BMDMs and TAMs but not in CD4+ or CD8+ T cells and tumor cells (Figure 2G, and Supplemental Figure 2, A and B), indicating that 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 (Supplemental Figure 2, C–E) or the presence of CD4+ T cells within LLC tumors (Supplemental Figure 2, F–H).

To address this discrepancy, we investigated the functions of ATP6V0d2 in TAMs. WT mice were irradiated and transplanted with BMDMs from WT mice or Atp6v0d2–/– mice, followed with xenograft of LLC tumor cells. Consistently, mice transplanted
Together, these data demonstrate that macrophage-expressed ATP6V0d2 inhibits tumor growth in vivo.

**Atp6v0d2**−/− mice were more susceptible to lung metastasis. To investigate whether ATP6V0d2 plays a role in tumor metastasis,
Figure 2. *Atp6v0d2−/−* mice are more susceptible to tumorigenesis. (A–C) Comparison of LLC tumor growth in WT and *Atp6v0d2−/−* mice (n = 7) after implantation: tumor growth rate (A), tumor weight (B), and representative images of excised tumors (C). (D–F) Tumor growth of B16-F10 in WT and *Atp6v0d2−/−* 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+ T cells, CD8+ T cells, fibroblasts, TAMs, B16-F1, B16-F10, and LLC cells. (H–K) *Atp6v0d2−/−* mice were challenged s.c. with 5 × 10^5 LLC cells alone, or together with 2 × 10^5 either WT BMDMs or *Atp6v0d2−/−* 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 tumors are presented (J). All experiments except H–J were repeated 2 times. Data were assessed by unpaired Student’s t test (A, B, D, and E) or 1-way ANOVA with Turkey’s post hoc test (H and I) and are represented as mean ± SEM. *P < 0.05; **P < 0.01; ***P < 0.001. NS, not significant. Scale bars: 5 mm (C, F, and J).
we inoculated age- and sex-matched WT and Atp6v0d2<sup>−/−</sup> mice with LLC cells intravenously and examined their lungs for metastases. Atp6v0d2<sup>−/−</sup> mice exhibited significantly more tumor nodules (Figure 3, A and B). In the lungs from Atp6v0d2<sup>−/−</sup> mice, the percentages of protumoral CD11b<sup>+</sup>F4/80<sup>+</sup>CD206<sup>+</sup> TAMs were significantly higher, compared with those from WT mice (Figure 3, C and D). Furthermore, the metastasized tumor cells (gated on CD45<sup>+</sup>) isolated from the lungs of Atp6v0d2<sup>−/−</sup> mice expressed higher amounts of Ki-67 (Figure 3, E and F). In addition, the tumor cells from the lungs of Atp6v0d2<sup>−/−</sup> mice were less apoptotic (Figure 3, G–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 tumors. TAMs display a spe-
cialized 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^+ protumoral-related macrophages (Figure 4, A and B).

The expression of protumoral macrophage–associated factors such as Mrc1, Arg1, and Fizz1 within the tumor sections was significantly higher in ATP6V0d2^−/− tumor-bearing mice compared with tumors in control animals, but proinflammatory-associated Il1b and Il6 expression was similar (Figure 4C). Next, we compared the expression levels of macrophage-associated genes in the isolated TAMs between WT 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 proinflammatory-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 36 hours, LLC TCM induced significantly higher expression of Arg1 and Fizz1 in ATP6V0d2^−/− BMDMs compared with WT BMDMs (Figure 4, E and F). By contrast, the absence of ATP6V0d2 resulted in reduced expression of Il1b but not Il6 in macrophages upon treatment with LLC TCM, compared with WT BMDMs (Figure 4, G and H). We repeated the same experiments with B16-F10–derived TCM and found similar results (Supplemental Figure 4, A–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 identifying 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-smooth muscle actin (α-SMA) staining, respectively (Figure 5, A–C), together with increased hemorrhagic areas (Figure 5, D and 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 5, G and H). In vitro–derived WT or ATP6V0d2^−/− macrophages expressed little VEGF. The addition of LLC and B16-F10 TCM led to enhanced VEGF expression after 6 hours that was significantly greater in the ATP6V0d2^−/− BMDMs, compared with WT controls (Figure 5, I and J). We found similar results in B16-F10–induced tumors that grew in ATP6V0d2^−/− mice compared with WT controls (Supplemental Figure 5, A–G).

Together, these data demonstrate that tumor-generated lactate inhibits ATP6V0d2 expression and drives VEGF production in TAMs, 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 2 major transcription factors that induce VEGF during tumor development (10, 20, 21). Hif1α and Hif2α mRNA expression in the LLC-derived tumors or TAMs was comparable between WT control and ATP6V0d2^−/− mice (Supplemental Figure 6, A–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 posttranslational regulation of HIF-2α by ATP6V0d2 (Figure 6, A and B). In addition, lactate induced a rapid increase in HIF-2α expression (Supplemental Figure 6E). Next we measured HIF-2α protein levels in a time-course experiment in WT or ATP6V0d2^−/− macrophages after incubation with TCM. TCM induced HIF-2α accumulation, while it suppressed ATP6V0d2 expression in WT macrophages; the expression levels of HIF-2α were higher after 2, 4-, and 6-hour 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 6D). 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 for 4 hours 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 (Supplemental Figure 6, F and 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 2 hours and persisted until 6 hours in the WT cells, indicating a proteasome-mediated degradation of HIF-2α (Figure 6F). 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). Next, we tested if ATP6V0d2 regulates HIF-2α lysosomal degradation. Macrophages incubated in the presence of bafilomycin A resulted in enhanced HIF-2α in WT cells but not in ATP6V0d2^−/− cells, and the expression of ATP6V0d2 and p62 was comparable after bafilomycin treatment (Figure 6G), indicating that 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 the lysosome inhibitor bafilomycin (Supplemental Figure 6H). However, deletion of ATP6V0d2 did not further stabilize HIF-1α (Supplemental Figure 6H).

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 colocalization of HIF-2α and LysoTracker in WT cells, whereas the colocalization of HIF-2α and LysoTracker was significantly reduced in the ATP6V0d2^−/− cells (Figure 6, H and 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, WT 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 sac-
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 after inoculation, mice were sacrificed. (A and B) Representative flow cytometric plots of tumor-infiltrating, gated F4/80+ cells showing CD11C+CD206− (proinflammatory) and CD11C CD206+ (protumoral) TAM fractions in LLC tumor xenograft model (A) and comparisons of fractions of CD11C+CD206− and CD11C CD206+ in TAMs between WT and Atp6v0d2−/− mice (B) (n = 5). (C) The expression of Mrcl, Arg1, Fizz1, Il1b, and Il6 in WT and Atp6v0d2−/− (KO) tumor tissues was determined by qRT-PCR (n = 5). (D) TAMs from tumor-bearing WT and Atp6v0d2−/− mice were isolated with CD11b magnetic beads. The expression of Mrcl, Arg1, Fizz1, Il1b, and Il6 was determined by qRT-PCR (n = 5). WT and Atp6v0d2−/− BMDMs were stimulated with medium or LLC TCM for 36 hours. The expression of Arg1 (E) and Fizz1 (F) was determined by qRT-PCR. WT and Atp6v0d2−/− BMDMs were stimulated with medium or LLC TCM for 6 hours. The expression of Il1b (G) and Il6 (H) was determined by qRT-PCR. Data were assessed by unpaired Student’s t test and are represented as mean ± SEM, *P < 0.05, **P < 0.01, ***P < 0.001. NS, not significant.
rificed. The addition of PT2385 reversed the difference in tumor growth seen between WT and Atp6v0d2–/– mice (Figure 7, A and B). At day 14, we found a significant increase in serum VEGF concentration in the Atp6v0d2–/– mice that was reversed in animals that received 4 days of PT2385 (Figure 7C). We detected enhanced abnormal vascularization in the tumors of Atp6v0d2–/– mice by immunofluorescent staining with anti-CD31 and anti-α-SMA that was reversed by the addition of PT2385 (Figure 7, D–F). Furthermore, the enhanced expression of HIF-2α target genes including Vegf1, Glut1, Ccnd1, and Pdil in tumors isolated from Atp6v0d2–/– mice was reversed by PT2385 (Supplemental Figure 7, A–D). We obtained similar results for HIF-2α target genes in TCM-stimulated WT and Atp6v0d2–/– macrophages (Supplemental Figure 7, E–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 with WT controls; inhibition of HIF-2α transcriptional activity by PT2385 inhibited expression of these genes, eliminating the differences between WT and Atp6v0d2-deficient mice (Figure 7, G–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 WT BMDMs that was significantly enhanced in Atp6v0d2–/– BMDMs (Figure 7, K–M). Lastly, the 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 2 groups (Figure 7, K–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 (Supplemental Figure 8A); both ATP6V0d2 and HIF-2α expression was restricted to the CD68+ macrophages, but not in other leucocytes within lung adenocarcinoma tissue (Supplemental Figure 8B and Figure 8A), despite that HIF-2α was reported in a very small population of non–small cell lung carcinoma samples (22). In contrast, HIF-1α was expressed in tumor cells, as well as infiltrated immune cells (Supplemental Figure 8C), indicating that the undetected HIF-2α in our hands is due to sample preparation. We divided the lung adenocarcinoma patients into 2 groups on the basis of macrophage ATP6V0d2 expression and found that there were more CD163+ protumoral-like macrophages and enhanced HIF-2α in the low-ATP6V0d2-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 in numbers of CD68+ cells within tumors between high-ATP6V0d2-expression and low-ATP6V0d2-expression patients, the numbers of CD163+ protumoral-like macrophages were significantly higher in the low-ATP6V0d2-expression group (Figure 8E and Supplemental Figure 8D). Consistent with this, when the patients were divided into 2 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 TME 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 led 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 postoperative 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. V-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 in 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 that both HIF members contribute to shaping the macrophages within the TME for them to acquire maximal protumoral activities. Despite the enhanced HIF-2α expression in steady-state Atp6v0d2–/– macrophages (Figure 6C),
Figure 5. Atp6v0d2 deficiency results in enhanced tumor angiogenesis. (A–I) WT and Atp6v0d2−/− mice were injected s.c. with 5 × 10⁵ LLC cells. On day 15 after 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). Quantification of the percentage CD31+ vessels (B) and α-SMA+CD31+ vessels (C). (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 qRT-PCR. (I and J) Expression analysis of Vegf by qRT-PCR in WT and Atp6v0d2−/− BMDMs that were stimulated with LLC TCM (I) or B16-F10 TCM (J) for 6 hours. Data are representative of 3 independent experiments. Data were assessed by Student’s t test and are represented as mean ± SEM. *P < 0.05; **P < 0.01; ***P < 0.001. Scale bars: 100 μm.
In summary, our findings identified ATP6V0d2 as a key factor that mediates the ability of a lactate-enriched TME to induce protumoral activities in TAM via the maintenance of hypoxia-independent regulation of HIF-2a. 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 genome recognition sequences of TALEN left and right arms are CGAGATGGCAAAAGCGCC (L) and GCACAGGTTGACATA (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 1-step ligation using the FastTALE TALEN Assembly Kit (SIDANSAI Biotechnology) according to the manufacturer’s instructions. mRNA (400 ng/μl, 10 pl) was injected into the cytoplasm of 180 one-cell embryos. After incubation for 24 hours, the selected 2-cell embryos were 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. 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 × 10⁶ LLC cells or 2 × 10⁶ 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 weighed. For macrophage adoptive transfer experiments, Atp6v0d2–/– mice were injected s.c. with 5 × 10⁶ LLC cells alone or 5 × 10⁶ LLC cells plus either 2 × 10⁶ WT BMDMs or Atp6v0d2–/– BMDMs, or injected s.c. with 5 × 10⁶ B16-F10 cells alone or 5 × 10⁶ B16-F10 cells plus either 2 × 10⁶ WT BMDMs or Atp6v0d2–/– BMDMs. Tumor growth and weight were determined as described above.

Generation of BM-chimeric mice. BM chimeras were generated as follows. Briefly, 8- to 12-week-old CD45.2 WT C57BL/6 male mice were sublethally irradiated with 7 Gy of whole-body irradiation. BMs 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 × 10⁶ cells from donor BM. After 4 weeks of transplantation, mice were s.c. challenged with 5 × 10⁶ LLC cells. Percentage of chimerism was determined by staining peripheral blood cells with anti-CD45.1-PE (clone A20; BD Biosciences) and anti-CD45.2-APC (clone 104; Biolegend) antibodies for analysis by flow cytometry.

For the inhibition of HIF-2a in a mouse model, a total of 5 × 10⁵ LLC cells in a volume of 100 μl of PBS were inoculated s.c. into 6- to 7-week-old C57BL/6 WT or Atp6v0d2–/– male mice. PT2385 was formulated with 10% absolute ethanol, 30% PEG400, 60% water con-
Figure 7. Suppression of HIF-2α activity reverses the enhanced tumorigenesis in Atp6v0d2−/− mice. (A–F) WT and Atp6v0d2−/− mice (n = 5) were injected s.c. with 5 × 10⁵ LLC cells. Once the tumor volumes reached 100–200 mm³ at around day 10, mice were gavaged with vehicle or HIF-2α inhibitor PT2385 (5 mg/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 (D) and quantification of CD31 area percentage (E), CD31-alpha-SMA vessels as a percentage of CD31 vessels (F) in the tumor areas. (G–J) qRT-PCR analysis of M2 marker genes Mct1 (G), Arg1 (H), Fizz1 (I), Ym1 (J) in the tumor tissues. (K–M) WT and Atp6v0d2−/− BMDMs were stimulated with LLC TCm for 24 hours with or without PT2385 (20 μM) and PT2385 was added 18 hours prior to the TCm stimulation. Expression of Arg1 (K), Fizz1 (L), and Ym1 (M) was determined by qRT-PCR. Data were assessed by 1-way ANOVA with Dunnett’s test and are presented as mean ± SEM. *P < 0.05; **P < 0.01; ***P < 0.001. Scale bars: 100 μm.

GACCTTG; reverse, GCAGCTCAGAGGCACA) using a Bio-Rad SYBR Green intercalating fluorophore system. The Ct value for each sample was normalized to the corresponding input value.

Isolation of TAMs. On day 15, mice were sacrificed and tumor tissues were gently minced and excised into small pieces. Afterwards, tumor tissues were disrupted with 10% PBS containing 200 ng/ml collagenase IV (catalog c17104019, Invitrogen), 250 ng/ml hyaluronidase (catalog H115000, MilliporeSigma), 40 ng/ml DNase I (catalog 10104159001, Roche) at 37°C on a shaker at 100 rpm. Cell suspensions were filtered and red blood cells were lysed. Tumor-infiltrating lymphocytes 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 the following antibodies: CD4-FITC (clone RM4-5), CD8-PE (clone 5367), CD11c-PE (clone N418), F4/80-PerCP/Cy5.5 (clone BM8), CD206-PE/cy7 (clone C068C2), and CD11b-FITC (clone M1/70) (all from Biolegend). For intracellular staining, cells were fixed and stained with 50 ng/ml MPA (MilliporeSigma) and 500 ng/ml ionomycin (MilliporeSigma) in the presence of 5 μl GolgiPlug (BD) in complete RPMI 1640 medium for 4 hours at 37°C. Cells were fixed, permeabilized, and stained with the following antibodies: Foxp3-APC (clone FJK-16s; eBioscience), IL-17-APC (clone JT-17; eBioscience), and TGF-β (clone 11D11; Biolegend). Samples were collected on a BD FACSVerse flow cytometer, and data were analyzed using FlowJo software (Tree Star).

ELISA quantitation of VEGF. Tumor tissues (0.5 g) were homogenized with 500 μl ice-cold PBS, and then supernatants were collected after centrifugation, and stored at −80°C until further use. VEGF-A in the tumor tissue was measured with an 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 incubated overnight for proper attachment. Then the cells were stimulated with MG132 (20 μM) for 4 hours, and then treated with 1:10,000 PBS-diluted LysoTracker Red DND-99 dye (DMSO solution, Life Technologies) for 15 minutes. After the treatment, cells were washed twice with sterile PBS and fixed with 4% paraformaldehyde, permeabilized with 0.05% Triton X-100, and blocked in 5% BSA. Anti-HIF-2α (NB100-132, Novus) was incubated with the cells overnight at 4°C. Secondary fluorescent antibodies labeled with FITC and Cy3 (from Life Technologies or Jackson Laboratories) were added for 1 hour 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 antibodies against CD3 (GB13014, Servicebio), CD19 (GB11061, Servicebio), CD68 (ab125212, Abcam), CD31 (GB13063, Servicebio), α-SMA (GB13044, Servicebio), F4/80 (GB11027, Servicebio), and ATP6V0d2 (ab194557, Abcam). Secondary fluorescent antibodies (FITC, Cy3 from Life Technologies or Jackson Laboratories) were added for 1 hour and DAPI was used for nuclear counterstaining. Samples were imaged through an SP5 confocal microscope (Leica) 24 hours after mounting.

Immunoblotting. Immunoblotting was performed according to standard methods. Primary antibodies included the following: phospho-PS6 (4851, Cell Signaling Technology), lamin B1 (13435, Cell Signal-
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 bars: 200 μm. (B) A representative image of H&E staining and immunohistochemical analysis of ATP6V0d2, CD163, and HIF-2α in the lung sections of adjacent nontumorous lungs or tumor tissues isolated from 2 lung adenocarcinoma patients. Scale bars: 50 μm. (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 surviving 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 2 groups of lung adenocarcinoma patients with high ATP6V0d2 expression (n = 20, IRS > 3) and low ATP6V0d2 expression (n = 11, IRS < 3). Data are the mean ± SEM. (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. ***P < 0.001 by Student’s t test.

Author contributions
NL, JL, AL, and XPY conceptualized the study. NL, JL, DK, SX, YD, YX, ZW, XX, FC, SL, H. Liu, and JW carried out the investigations. BY, KJ, FG, ZT, XC, ZL, GW, and XPY conducted formal analyses of the data. H. Li and GW provided resources. FG and XPY acquired funding and supervised the study. NL, JL, AL, and XPY prepared the data and figures and wrote the manuscript.

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