**LETTER**

**ATP6V0d2 mediates leucine-induced mTORC1 activation and polarization of macrophages**

**Dear Editor,**

mTORC1, as a center regulatory hub of metabolism, senses the cellular energy status, nutrition and extracellular stimuli and regulates cell growth, differentiation and functions of immune cells (Powell et al., 2012). Lysosomal localization of key signal components is critical for mTORC1 activation: mTORC1 activation requires co-localization of activated Rheb and mTORC1 to the lysosome membrane (Buerger et al., 2006). Signals including growth factors, cellular stresses and energy levels act on the disruption the formation of tuberous sclerosis complex (TSC) complex, comprised of TSC1, TSC2 and TBC1D7, which leads to the translocation and activation of Rheb on the lysosome membrane (Dibble et al., 2012). In response to nutrient levels, specifically the availability of amino acids and glucose (Efeyan et al., 2013), mTORC1 is recruited to the lysosomal surface by Rag GTPases that are heterodimers of RagA or RagC bound to RagB or RagD. Multiple protein complexes have been implicated in regulation of mTORC1 upon nutrient sensing including Ragulator, GATOR1, GATOR2, KICSTOR and vacuolar ATPases (Wolfson et al., 2017). Vacular ATPases are large multiple-protein complexes that acidify the lysosome and may mediate additional functions independent of their proton pump activity (Nishi and Forgac, 2002).

Although a number of amino acid sensors have been identified (Chantranupong et al., 2016), the regulation of mTORC1 activation by amino acids remains largely elusive. Furthermore, the majority of studies examining amino acid-induced mTORC1 signaling were performed in cell lines and little is known about the amino acid- and cell type-specificity of mTORC1 activation.

We recently identified ATP6V0d2 as a macrophage-specific subunit of vacuolar ATPase, whose expression is restricted in macrophages and inhibited by inflammatory stimuli and tumor cell-derived lactate (Liu et al., 2019). ATP6V0d2 inhibits inflammation and bacterial infection by promoting autophagosome and lysosome fusion (Xia et al., 2019). In addition, ATP6V0d2 can mediate HIF-2α degradation, limiting macrophage protumoral activity (Liu et al., 2019). Given the restricted expression of ATP6V0d2 and its localization in lysosome membrane, we speculated that ATP6V0d2 might play a role in amino acid-mediated mTORC1 activation in macrophages.

First, we stimulated amino acid-starved HEK293T cells with increasing amounts of leucine, arginine or glutamine and measured mTORC1 activation. All three amino acids induced mTORC1 activation in a dose-dependent manner, measured by the phosphorylation of ribosome protein S6 and 4-EBP1 (Fig. S1A–C). Next, we used the optimized amino acid concentration (4 mmol/L leucine, 20 mmol/L glutamine, 2 mmol/L arginine) to stimulate bone marrow derived macrophages (BMDMs). In comparison to HEK293T cells, only leucine induced the phosphorylation of S6, 4-EBP1 and p70S6K in macrophages but to a lesser extent (Fig. 1A). Neither glutamine nor arginine induced any detectable mTORC1 activation, indicating amino acid-induced mTORC1 activation is cell-type specific (Fig. 1B and 1C).

To investigate the possible function of ATP6V0d2 in amino acid-induced mTORC1 activation, we compared leucine-induced mTORC1 activation in WT and Atp6v0d2−/− BMDMs. Deletion of ATP6V0d2 resulted in impaired phosphorylation of S6, 4-EBP1, and p70S6K upon leucine treatment in macrophages, despite comparable mTORC1 activation upon complete amino acids stimulation (Fig. 1D), indicating that ATP6V0d2-independent activation of mTORC1 induced by other amino acids may exist. Conversely, overexpression of ATP6V0d2 enhanced leucine-induced mTORC1 activation measured by S6 phosphorylation (Fig. S1D). Immunofluorescence staining showed that absence of ATP6V0d2 reduced the co-localizations of mTOR and LAMP1 (Fig. 1E and 1F), a lysosome marker, indicating that ATP6V0d2 may promote the recruitment of mTOR to the lysosome membrane.

Given the role of mTORC1 in macrophage polarization (Byles et al., 2013), next we compared the macrophage polarization under M1 and M2 conditions between WT and Atp6v0d2−/− BMDMs. The expression of M1-associated genes Tnfa and iNOS was reduced in LPS and IFN-γ polarized Atp6v0d2−/− BMDMs, compared to WT counterparts (Fig. S2A and S2B). This was associated with impaired phosphorylation of S6, 4-EBP1 and p70S6K in Atp6v0d2−/− macrophages upon LPS and IFN-γ stimulation (Fig. S2C). In contrast, the expression of M2-associated genes...
Arginase-1, Fizz-1 and Ym-1 was enhanced in IL-4-polarized Atp6v0d2−/− BMDMs, compared to WT cells (Fig. S2D–F). Furthermore, deletion of ATP6V0d2 significantly suppressed the macrophage polarization into F4/80⁺CD11c⁺ M1 phenotype (Fig. S3A and S3B); in contrast, the polarization into F4/80⁺CD206⁺ M2 phenotype was enhanced (Fig. S3C and S3D). The expression of another M2 marker CD301 upon IL-4 stimulation was also enhanced in the absence of ATP6V0d2 (Fig. S3E and S3F). These data indicate that ATP6V0d2 promotes M1 polarization but suppresses M2 polarization of macrophages in vitro.

To test if ATP6V0d2 plays a role in the regulation of mTORC1 activation and macrophage polarization in vivo, wild-type and Atp6v0d2−/− mice were fasted for 16 h and administrated with leucine for 1 h for detection of mTORC1 activation or twice a day for 48 h for measuring the...
Figure 2. ATP6V0d2 regulates leucine-induced mTORC1 activation and macrophage polarization in vivo. Wild type and Atp6v0d2−/− mice (n = 5) were starved for 16 h, and then gavaged with 200 μL PBS (A) or 200 μL leucine (54.0 g/L in PBS) (B and C). After 1 h, the S6 phosphorylation of splenic macrophages was determined by flow cytometry. Wild type and Atp6v0d2−/− mice were starved for 16 h, and then gavaged with 200 μL leucine (54.0 g/L) twice a day for 48 h. After 6 h the last gavage, the expressions of F4/80 and CD11c (D and E) or CD206 (F and G) of splenocytes were determined by flow cytometry. The expressions of Tnf-a (H), iNOS (I), Arginase-1 (Arg-1) (J), Fizz-1 (K) and Ym-1 (L) of F4/80+ macrophages were determined by RT-PCR. Data shown are representative of three independent experiments for leucine-induced mTORC1 activation. Bars = mean ± SEM, *P < 0.05, **P < 0.01, ***P < 0.005, ****P < 0.0001 (Students’s paired t-test).
macrophage polarization. There was no difference in the phosphorylation of S6 in splenic F4/80+ macrophages between wild-type and Atp6v0d2−/− mice upon starvation (Fig. 2A). However, after administration of leucine, the phosphorylation of S6 in macrophages from Atp6v0d2−/− mice was significantly reduced compared with macrophages from control animals (Fig. 2B and 2C). Consistent with the in vitro data, deletion of ATP6V0d2 significantly reduced splenic F4/80+CD11c+ M1 polarization but enhanced F4/80+CD206+ M2 polarization upon leucine gavage (Fig. 2D–G). The expression levels of M1-associated genes Tnfα and iNOS were reduced in the F4/80+ splenic macrophages from Atp6v0d2−/− mice, compared to WT counterparts (Fig. 2H and 2I). Conversely, the expression levels of M2-associated genes Arginase-1,Fizz-1 and Ym-1 were enhanced, in the F4/80+ splenic macrophages from Atp6v0d2−/− mice (Fig. 2J–L). These data suggest that ATP6V0d2 regulates leucine-induced mTORC1 activation and macrophage polarization in vivo.

Amino acids engage overlapping and specified components for mTORC1 activation (Efeyan et al., 2015). It is not clear why only leucine induces mTORC1 activation in macrophages. Macrophages are critical sentinel for immune responses and tissue homeostasis (Ginhoux and Guilliams, 2016). One possibility is that macrophages lack proteins that are required for sensing arginine and glutamine. We speculate that unresponsiveness to arginine and glutamine may help prevent unwanted activation of macrophages. The current study did not completely elucidate the mechanism by which ATP6V0d2 regulates leucine-induced mTORC1 and this question warrants further investigation. Interestingly, ATP6V0d2 also promotes mTORC1 activation under M1 macrophage polarization. We previously showed that ATP6V0d2 does not regulate lysosome acidification, which is consistent with other studies (Lee et al., 2006). It is highly likely that ATP6V0d2 serves as an adaptor protein that facilitates the interaction between mTORC1 and other critical protein complexes i.e., Rag GTPases or Ragulator. Our previous study implied that macrophage specific ATP6V0d2 might exist in other protein complexes independent of V-ATPase (Xia et al., 2019). Atp6v0d2-deficient BMDMs have reduced M1 differentiation but enhanced M2 differentiation, which is consistent with enhanced presence of M2 macrophages within tumors in Atp6v0d2-deficient mice in xenograft tumor model (Liu et al., 2019). In line with our results, constitutive activation of mTORC1 in the Tsc1−/− BMDMs inhibits M2 polarization but promotes M1 polarization; mice with myeloid specific Tsc1 deficiency spontaneously develop inflammatory disorders (Zhu et al., 2014). These data highlight that the amount of mTORC1 activation might be a critical parameter in the determination of differentiation and functions of immune cells, which is consistent with previous studies (Hukelmann et al., 2016).

In summary, here we identified that ATP6V0d2 mediates leucine-induced mTORC1 activation in macrophages, which further regulates macrophage differentiation. These data demonstrate a cell-specific role of V-ATPase subunit in mediating amino-acid-induced mTORC1 activation.

FOOTNOTES

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REFERENCES

Buergel C, DeVries B, Stambolic V (2006) Localization of Rheb to the endomembrane is critical for its signaling function. Biochem Biophys Res Commun 344:869–880
Byles V, Covarrubias AJ, Ben-Sahra I, Lamming DW, Sabatini DM, Manning BD, Homg T (2013) The TSC-mTOR pathway regulates macrophage polarization. Nat Commun 4:2834
Chantranupong L, Scaria SM, Saxton RA, Gygi MP, Shen K, Wyant GA, Wang T, Harper JW, Gygi SP, Sabatini DM (2016)
CASTOR proteins are arginine sensors for the mTORC1 pathway. Cell 165:153–164
Dibble CC, Elis W, Menon S, Qin W, Kekota J, Asara JM, Finan PM, Kwiatkowski DJ, Murphy LO, Manning BD (2012) TBC1D7 is a third subunit of the TSC1-TSC2 complex upstream of mTORC1. Mol Cell 47:535–546
Efeyan A, Comb WC, Sabatini DM (2015) Nutrient-sensing mechanisms and pathways. Nature 517:302–310
Efeyan A, Zoncu R, Chang S, Gumper I, Snitkin H, Wolfson RL, Kirak O, Sabatini DD, Sabatini DM (2013) Regulation of mTORC1 by the Rag GTPases is necessary for neonatal autophagy and survival. Nature 493:679–683
Ginhoux F, Guilliams M (2016) Tissue-resident macrophage ontogeny and homeostasis. Immunity 44:439–449
Hukelmann JL, Anderson KE, Sinclair LV, Grzes KM, Murillo AB, Hawkins PT, Stephens LR, Lamond AI, Cantrell DA (2016) The cytotoxic T cell proteome and its shaping by the kinase mTOR. Nat Immunol 17:104–112
Lee SH, Rho J, Jeong D, Sul JY, Kim T, Kim N, Kang JS, Miyamoto T, Suda T, Lee SK et al (2006) v-ATPase V0 subunit d2-deficient mice exhibit impaired osteoclast fusion and increased bone formation. Nat Med 12:1403–1409
Liu N, Luo J, Kuang D, Xu S, Duan Y, Xia Y, Wei Z, Xie X, Yin B, Chen F et al (2019) Lactate inhibits ATP6V0d2 expression in tumor-associated macrophages to promote HIF-2alpha-mediated tumor progression. J Clin Invest 129:631–646
Nishi T, Forgac M (2002) The vacuolar (H+)-ATPases—nature’s most versatile proton pumps. Nat Rev Mol Cell Biol 3:94–103
Powell JD, Pollizzi KN, Heikamp EB, Horton MR (2012) Regulation of immune responses by mTOR. Annu Rev Immunol 30:39–68
Wolfson RL, Chantranupong L, Wyant GA, Gu X, Orozco JM, Shen K, Condon KJ, Petri S, Kedir J, Scaria SM et al (2017) KICSTOR recruits GATOR1 to the lysosome and is necessary for nutrients to regulate mTORC1. Nature 543:438–442
Xia Y, Liu N, Xie X, Bi G, Ba H, Li L, Zhang J, Deng X, Yao Y, Tang Z et al (2019) The macrophage-specific V-ATPase subunit ATP6V0D2 restricts inflammasome activation and bacterial infection by facilitating autophagosome-lysosome fusion. Autophagy. https://doi.org/10.1080/15548627.2019.1569916
Zhu L, Yang T, Li L, Hou Y, Hu X, Zhang L, Tian H, Zhao Q, Peng J et al (2014) TSC1 controls macrophage polarization to prevent inflammatory disease. Nat Commun 5:4696

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