LAMTOR1 Phosphorylation of Exo70 Facilitates Tumor-Associated Macrophage Polarization to Restrain CD8⁺ T Cell Function in HCC

Bo Wu

China Medical University

Qian Wang

China Medical University

Bowen Li

China Medical University

Xiaonan Wang

China Medical University

Xiaoni Zhan

China Medical University

Xiang Shi

China Medical University

Gehua Wen

China Medical University

Lingli Wu

China Medical University

Mei Liu

China Medical University

Meixi Jiang (✉ jmx13082206769@163.com)

China Medical University <https://orcid.org/0000-0002-7970-3969>

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Abstract

The tumor microenvironment controls the progression of tissue homeostasis leading to cancer. Accumulation of anti-inflammatory tumor-associated macrophages (TAM) has also been linked to worsening clinical outcomes as well as resistance to treatment in hepatocellular carcinoma (HCC). The current immune landscape for regulation by the presence of TAMs has been studied. It is known that LAMTOR1 undergoes phosphorylation to bind to Exo70 and other exocyst components and is enhancing the secretion of TGF β 1 to facilitate the polarization of TAMs. The tumor-conditioned macrophages (TCM) numbers also correlated with enhanced number of regulatory T cells (Tregs) and decreased CD8⁺T cells in HCC. Mechanistically, TCM enhanced IL-10 production to diminish CD8⁺T cell activities. Our data demonstrate a novel immune therapeutic approach targeting TAMs immune suppression of T cell anti-tumor activities.

Introduction

Hepatocellular carcinoma (HCC) is a common solid cancer in the world and the third leading contributor to cancer-related deaths within Asia [1]. Although there are precise mechanisms in the treatment of cancer, the mortality rate remains high due to the failure of the current anti-tumor therapies in remodel the immune suppressive microenvironment. Therefore, there is an essential need to understand the molecular of immune suppressive microenvironment and recently data suggest that therapeutic strategies targeting the tumor microenvironment have a major role to play in the management of tumors.

LAMTOR1, also called the late endosomal/lysosomal membrane adaptor p18, is a subunit of the Ragulator Complex (composed of LAMTOR1-5) [2]. Notably, the LAMTOR complex senses and transmits amino acid signals and thereby plays an important role in regulating cellular metabolism and growth in response to nutrient supply [3, 4]. Similarly LAMTOR1 is an important intersection of the mTORC1 and MAPK pathways, but the role played in LAMTOR1 during vesicle movement has not been elaborated.

During vesicle secretion, the exocyst mediates a critical step in fusion into the plasma membrane through an octameric complex [5–11]. The exocyst subunit Exo70 interacts with the Arp2/3 complex to enhance its binding to the nucleus-promoting factor WAVE2, thereby promoting lamellipodia formation and cell migration [12–14]. In addition to interacting with the Arp2/3 complex, Exo70 protein shapes homodimers with Sec13 and forms vesicles by inducing membrane bending to form membrane protrusions [15]. Although the role of Exo70 in cellular exocytosis has been verified, we highlighted other cellular processes associated with this interesting protein [16–20].

Hereby that we report a phosphorylation of Exo70 is targeted by LAMTOR1. The phosphorylation at Exo70 by LAMTOR1 suppresses the immune response to HCC. Mechanistically, LAMTOR1 phosphorylation of Exo70 assembly into the exocyst complex, thereby enhance the secretion of TGF β 1. We further showed that tumor-derived LAMTOR1 directly to promote M2 polarization, mediated by the TGF β 1.

Furthermore, we demonstrated that LAMTOR1 regulated M2 polarization of TAMs release IL-10 to suppresses CD8⁺ T cell infiltration. Taken together, these findings identify an important role for M2 polarization of TAMs, a mechanism underlying TAM mediated immunosuppression and this study also provide important potential mechanism to development the immunotherapeutic strategies during treating cancer.

Methods

Cell culture.

Human embryonic kidney cell line 293T, The hepatocellular carcinoma cell lines Huh7, HepG2, SMC7721 and Hepa1-6 were acquired at the American Type Culture Collection (ATCC). All hepatocellular carcinoma cells were grown in DMEM (Corning) with 10% FBS (BI), 1% penicillin, and streptomycin (Gibco) supplementation. Hepatocellular carcinoma cells were grown in a wet incubator at 37°C and 5% CO₂. For starvation condition, Hank's balanced salt solution (HBSS) were used.

IP, GST pull-down and western blot analyses.

Co-immunoprecipitation kit (ab206996, Abcam) was used to prove the intervention between LAMTOR1 and Sec13, Rab11 and Exo70. In vitro GST pull-down assay, recombinant LAMTOR1 protein (2 µg) was cultured with Exo70 (3 µg) or its deletion constructs 1/2/3 in protease inhibitor for 30 min. LAMTOR1 was immunoprecipitated with anti-HA and the deletion constructs of protein was detected with anti-GST antibody. For in vitro phosphorylation assay, Exo70 (M133A) and its mutant variants were purified. The purified Exo70 proteins (without Flag-tag) were released from the glutathion Sepharose beads. Prepared Exo70 or its mutants together with the HA-LAMTOR1 proteins. Samples were then processed for western blotting.

Total proteins were detached on SDS-PAGE and electronically transferred to PVDF membranes, which were incubated at 4°C overnight with the primary antibody and then with BSA blocking.

Immunofluorescence, IHC, and image analysis

HCC tissues staining with antibodies against was performed and scored to determine the protein expression profiles according to previously described standard procedures. The results of staining were scored using the quick (Q) score. All protocols using human specimens were approved by the Institutional Review Board of the China medical university.

Multiplex IHC staining was performed in a sequential order on 3-mm-thick sections from formalin-fixed paraffin-embedded HCC tissues. Antigen retrieval and removal of antibodies from the previous cycle was performed by microwave treatment. A magnification whole-slide scan was acquired followed by selection of regions for multispectral imaging in Phenocart, using the Vectra 3 system (PerkinElmer). Protein signal

intensity was normalized for exposure and spectral unmixing was performed with the in Form Cell Analysis software (PerkinElmer) .

Macrophage isolation and polarization

Monocytes were isolated from PBMCs and cultured for 5 days in MCSF, and polarized toward pro-inflammatory or anti-inflammatory macrophages with LPS (200 ng/mL) or IL-4 (20 ng/mL), respectively.

T-cell function assays

T cells were isolated by CD3 positive selecting microbeads. T-cell proliferation, IFN γ and IL-10 production were assessed following stimulation using CD3/CD28 activation. T-cell function was then evaluated following stimulation with PMA(100 ng/mL) and ionomycin(500 ng/mL) prior to staining.

Flow cytometry analysis

Phenotype and function of tumor cell lines, macrophage and T-cell function were assessed by staining using fluorochrome-conjugated antibodies against CD163, CD68, CD206, CD3, CD4, CD8, Foxp3, IFN γ and Ki67. Fixable live/dead cell dye was used to determine viable cells. All cells were acquired by LSRFortessa and analyzed by FlowJo 10.7.

RNA extraction and real time quantitative qPCR

Total RNA was extracted and RT-qPCR was performed. Primer sequences are available upon request. RNA for sequencing was reverse-transcribed and amplified by PCR.

Elisa and cytokine profiling

Tumor plasmas were generated from dissociated tumors in DMEM medium. After centrifugation, supernatants were collected and concentrated using Protein Concentrator. Cytokine Array (R&D Systems) was used for profiling different cytokines and chemokines using equal amounts (μ g) of tumor lysates.

Mice and in vivo study approval

AAV-sh-Ctrl and AAV-sh-LAMTOR1 Hepa1-6 cells (2.3×10^5 cells per mouse) were injected subcutaneously in seven-week-old C57BL/6 mice. Tumor volumes were measured as previously described. No tumors in the mice exceeded IACUC defined maximum diameters of >2 cm. Sample sizes were determined based on our preliminary experiments and no sample size calculation was done. All animal experiments were approved by the Institutional Ethics Committee of China Medical University.

Statistical analysis

Statistical analyses were performed using R. An unpaired two- or one-tailed t-test was used to determine P-value between indicated groups. Results were represented as mean $\pm$ SEM. A P-value of <0.05 matches for statistical significance.

Results

Exo70 is a direct interacts wit LAMTOR1

To investigate how LAMTOR1 exocytosis,co-expression in cells was carried out(Figure.1a).Candidate LAMTOR1 interacting proteins co-precipitated with Sec13, Rab11 and Exo70.Therefore,we investigated the role of exocytosis regulated by LAMTOR1, we investigated the role of LAMTOR1 protein-protein interaction, which are critically involved in endogenous LAMTOR1-Sec13 and LAMTOR1-Rab11 interaction(Figure.1b,c).We sought to determine whether LAMTOR1 interacts with Exo70 to promote endocytosis,which a subunit of the exocyst complex.Using endogenous proteins in different cell lines by IP assays, we show Exo70 can interact directly with LAMTOR1(Fig.1d), and then with tagged proteins exogenously expressed in cell lines(Fig.1e,f).Co-localization of LAMTOR1 with Exo70 critical for membrane trafficking was observed by immunofluorescence microscopy in HCC patients(Fig.1g).

LAMTOR1 phosphorylate Exo70 at Serine 133

To determine whether Exo70 is due to LAMTOR1 directly interaction, we constructed GST-Exo70, suggesting that LAMTOR1 activity is required for LAMTOR1 interaction(Fig.2a).For more detailed mapping of the region of Exo70, three domains were expressed(Fig.2b).Binding of these constructs to LAMTOR1 was assessed using in vitro GST pull down.Deletion of part of construct 2 or 3 domain abrogated LAMTOR1 binding(Fig.2c).We next investigated whether LAMTOR1 phosphorylates Exo70.Using an in vitro kinase assay, we found that Exo70 is a sub-strate for LAMTOR1 phosphorylation(Fig.2d).To determine whether LAMTOR1 directly phosphorylates Exo70, we performed in vitro assays using both recombinant LAMTOR1 and a purified Exo70(Fig.2e).Together,these data indicating that Exo70(1-140 amino acid) is a alternative phosphorylation sites of LAMTOR1.Through in vitro assays, we found that LAMTOR1 is a substrate for Exo70 phosphorylation, and complete loss of phosphorylation in the S133A mutant resulted in inability to bind to LAMTOR1, suggesting a phosphorylation site leading to exocytosis(Fig.2f). Additionally, using recombinant LAMTOR1 and purified Exo70, suggesting that Ser133 was indeed crucial for LAMTOR1 to phosphorylate Exo70(Fig.2g), suggesting that LAMTOR1 activity is required for Exo70 interaction and directly phosphorylate Exo70 to regulate exocytosis.

Exo70 phosphorylation promotes its interaction with Rab11

How does LAMTOR1 phosphorylation of Exo70 affect exocytosis? Since the assembly of the exocyst complex is regulated from Golgi processing and linked to the plasma membrane via associated vesicle trafficking,we investigated whether phosphorylation of Exo70 by LAMTOR1 regulates the interaction of Sec13 as well as Rab11 to mediate vesicle fusion. Flag-tagged Exo70 or Exo70(S133A) were transduced

in 293T cells, Exo70 was immediately immunoprecipitated with anti-Flag antibodies against Sec13 and Rab11, which have previously been indicated to interact directly in the exocyst complex. To further identify the impact on binding, we performed mutual immunoprecipitation experiments. Flag-tagged Rab11 were co-transfected with Myc-tagged Exo70 or Exo70(S133A), Rab11-Flag was immunoprecipitated with an anti-Flag antibody, and Myc-Exo70 and Sec13 were detected by western blotting. As shown in Figure.3a,b, Exo70 binding to Sec13 and Rab11. The binding of the Exo70(S113A) mutant to Sec13 and Rab11 was reduced.

To simulate the interaction between Exo70 and Rab11 at the endogenous level in cells under stress, we treated performed immunoprecipitation experiments using a monoclonal antibody against endogenous Exo70 treat with HBSS in HCC. The amount of Rab11 that co-immunoprecipitated with Exo70 increased upon HBSS stimulation (Figure 3c). As shown in results, HCC tissues expressed a punctate cytoplasmic distribution of Rab11 that co-localized with LAMTOR1 (Figure.3d) and Exo70 (Figure.3e). Taken together, these data indicate that Exo70 enhances its binding to Sec3 and Rab11.

LAMTOR1 enhances release the TGFB1 by interaction with Exo70

Whether exocyst influenced the cytokine effect of driving immune cells, we used Cytokine Array combined to ELISA assay to test Ctrl vs LAMTOR1 Knockdown. We identified TGFB1 and CCL5 as the major cytokine secreted in Control as compared to LAMTOR1 Knockdown (Fig.4a). These results show that LAMTOR1 expression is associated with a robust anti-inflammatory environment. We next used RNA-seq to analyze differential gene expression in HCC infiltrated by LAMTOR1 positive or negative. Biological pathway analysis performed in "Enrich R" revealed several regulated inflammatory immune response clusters and cytokine activation (Fig.4b,c). Using flow cytometry, we found that the liver cancer cell lines produced TGFB1 and CCL5 (Fig.4d). It is important that cell supernatant of TGFB1 produced by tumor cells correlated with the tumor expression of LAMTOR1 (Fig.4e). In HCC, we found LAMTOR1, EXO70, RAB11A and TGFB1 was a trend toward higher co-expression in the tumor nests (Fig.4f).

Next, we examined whether the effect of immunity activity on Exo70 phosphorylation by LAMTOR1. Stable Hepa1-6 cell lines expressing either Exo70 or the Exo70(S133A) mutant were generated. Treatment with the Exo70(S133A) mutant showed decreased the co-localization TGFB1 with RAB11, Exo70 in mouse HCC (Fig.4g). We also examined whether the secretion of TGFB1 in tumor cells was affected by the Exo70(S133A) mutant. As shown in Figure 4H, knockdown of Exo70 significantly reduced the level of TGFB1, while cells expressing Exo70(S133A) similarly showed reduced levels of TGFB1 secretion. These data suggest that tumor cells secrete TGFB1 via Exo70.

LAMTOR1 promotes TAMs polarization by the exocytosis release of TGFB1

Given the immune-suppressive profile of HCC, we next assessed their capability to suppress immune effector cells. Multiplex immunofluorescent staining and multispectral imaging was to identify CD4⁺T cells, CD8⁺T cells, NK cells, and TAMs. Patients with high infiltration of CD4⁺T cells, CD8⁺T cells, NK cells in adjacent and significantly higher infiltration of TAMs cells in the tumor (Fig.5a). To investigate whether

TGFB1 could be a potential cytokine for the TAMs polarization, we stained tissues expressing with antibodies against CD68 and CD206. We observed that TAMs polarization accumulated on the tumor (Fig. 5b). Likewise, co-staining of CD206 with antibodies targeting TGFB1 showed co-localization of CD206 with TGFB1 (Fig. 5c). LAMTOR1 knockdown cells block the macrophages induction of CD206 expression (Fig. 5d). In in vitro experiments we found that TGFB1 significantly reduced the expression of CD163, and CD86, and simultaneously increased the expression of CD206 (Figure 5e). These data suggest that liver tumor cells produce TGFB1 to drive macrophage polarization through the expression of LAMTOR1.

LAMTOR1 co-culture macrophages inhibit T-cell activation and tumor killing

Next, we used RT-PCR assay to test tumor-conditioned macrophages (TCM) treated with Ctrl and LAMTOR1 knockdown HCC cells (Fig. 6a). And then, Purified T cells from peripheral blood were co-cultured with TCM and assessed for their capacity to produce IFN γ and proliferate. We found that TCM cytokines (IL-10 and IFN γ) suppressed T cell activation. Also, the suppressed T-cell activation correlated with polarization of TCM (Fig. 6b,c). Similarly, proliferation and IFN γ production were suppressed in T cells co-cultured with TCM (Fig. 6d,e). This shows that LAMTOR1 expression can be used as a measure of the suppressive capacity of macrophages. We also found that the T cells that were cultured with TCM were less efficient in killing (Fig. 6f). Thus, LAMTOR1 expressing macrophages actively block T-cell cytotoxicity and inhibit both cytokine production and proliferation.

LAMTOR1 enhanced release of TGFB1, IL-10 in the tumor microenvironment and prevented the infiltration of CD4⁺ and CD8⁺ effector cells

Our results described above strongly argue that therapeutic strategies preventing LAMTOR1 would improve cancer immunotherapies by inducing the infiltration of immune effectors. We first showed that AAV-shLAMTOR1 treatment of HCC cells, under these experimental conditions in vitro, we observe changes in the expression of LAMTOR1, EXOC70, RAB11, TGFB1 and CD206 (Fig. 7a), treatment of tumor bearing mice with AAV-sh-LAMTOR1 significantly increased the infiltration of CD4⁺T and CD8⁺T cells associated with a decrease in the infiltration of TAMs (CD206) cells as well as Treg cells (Fig. 7b,c). Interestingly, AAV combination therapy (AAV-sh-LAMTOR1+anti-TGFB1+anti-IL-10) completely inhibited tumor growth compared to either the combination therapy or control (Fig. 7d), indicating that LAMTOR1 can improve cancer immunotherapy approaches based on immune cytokines blockade and peptide-based vaccination strategies. Our data are in line with several studies highlighted the relevance of targeting LAMTOR1 associated pathways in tumors to enhance immunotherapy efficacy (Fig. 8).

Discussion

Immunotherapy has lately gained attention and has become a well established anticancer therapy to release the brakes on the immune system [20–24]. However, still a minority of all patients with cancer are

expected to respond to the current T-cell–targeted therapies as a result of the immune-suppressive microenvironment [25–27]. Therefore, new targets for immunotherapy are highly desired for combinatory treatments, which could increase the efficacy are being explored [28–30]. TAMs control the tumor microenvironment and shape the anti-tumor responses affecting the clinical response rate in many cancers including HCC [31, 32].

LAMTOR1 expression was recently linked to tumor microenvironment. In this study, we identified the phosphorylation site of LAMTOR1 that directly regulates Exo70, a key component of the exocyst complex critical for the tumor microenvironment, shaping the tumor microenvironment by promoting exocytosis of cytokines. Vesicles mediate the membrane fusion step of SNARE by targeting exocysts. Exo70 interacts directly with PI(4,5)P2 through its C-terminus and is associated with exocyst targeting to the plasma membrane [33]. Our work suggests that the phosphorylation of S133 is likely to lead to conformational changes in Exo70, thus making it easier for other exocyst components to enter the assembly of the complex.

However, the phenotype and function of human LAMTOR1 expressing tumor and their interaction with immune cells in the tumor microenvironment were yet to be defined. In this study, we have visualized the spatial relations between anti-inflammatory TAM subsets and effector T cells in human HCC tumors. Using cytokine generated and tumor-conditioned macrophages (TCM), we demonstrated that tumor expression of the LAMTOR1 is correlated with an immunosuppressive phenotype macrophages. These suppressive were efficient in blocking cytotoxic T cells and enhanced Tregs proliferation and cytokine production [34]. This shows that LAMTOR1 have a similar function in human tumors and that they can be reprogrammed to shift polarization in mouse models.

The host immune system plays a key role within the tumor where different types of immune cells have a contrasting impact on HCC progression, while macrophage infiltration is negatively correlated with survival [35]. We have previously shown in human HCC that LAMTOR1 expression is linked to macrophages, CD4⁺ helper T-cells, and regulatory T-cells [36]. The balance within the immune landscape and interactions with T cells has been highlighted as a resistance mechanism for checkpoint immunotherapies [37]. TAMs as key players in the T cell excluded and suppressive tumor phenotype [38]. The cytokine TGFB1 has been shown to stimulate TAMs anti-inflammatory cytokines IL-10 that promote differentiation of myeloid cells [39, 40]. We show that TGFB1 supports Tregs cells and it has been shown that this cytokine IL-10 influences the suppressive function of these cells. Thus, TGFB1 contributes both to an autocrine and paracrine induction of anti-inflammatory and suppressive environment. Thus, we suggest that AAV-sh-LAMTOR1 treatment can be used in combination with anti-TGFB1 and anti-IL10 to improve effective checkpoint therapies in patients with HCC. Furthermore, anti-LAMTOR1 immunotherapy could support other approaches such as cancer vaccinations in HCC as it increases the immunogenicity by augmenting the antigen presentation in T cells.

Conclusion

To summarize, this study has uncovered an important role of LAMTOR1 in the immune landscape of HCC. Targeting the LAMTOR1 with blockade of TGFB1 and IL-10 are efficient approaches to reprogram suppressive macrophages and to restore T-cell anti-tumor activities. AAV-sh-LAMTOR1 treatment is a promising novel modality for treatment of aggressive cancers with inherent immunosuppressive characteristics.

Abbreviations

TAM tumor-associated macrophages

HCC hepatocellular carcinoma

CO-IP co-immunoprecipitation

IHC immunohistochemistry

TCM tumor-conditioned macrophages

Tregs regulatory T cells

Declarations

Acknowledgements

Not applicable.

Ethics approval and consent to participate

All experiments involving human subjects were performed in accordance with the China Medical University. All experimental protocols were and approved by the Ethics Committee China Medical University. With informed consents from all subjects, paired specimens of HCC and adjacent normal tissues were collected from patients who underwent surgical resection. None of the patients received anticancer therapy before surgery. Our study protocol was approved by the Ethics Committee of China Medical University.

Consent for publication

All authors agree to submit the article for publication.

Availability of data and material

The datasets analyzed during the current study are not publicly available but are available from the corresponding author on reasonable request.

Competing interests

This manuscript has been read and approved by all authors and all declare that they have no competing interests.

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Authors' contributions

BW, ML and MX-J contributed to conception and design of the study. BW, QW, ML and MX-J performed the in vitro experiments with cell lines. BW-L and XN-W provided expertise in mouse models. XN-Z and GH-W performed the flow cytometry analysis, while XS and LI-W carried out the TCGA data set analysis. BW provided expertise in statistical analysis. BW and QW drafted the manuscript. MX-J and ML revised the manuscript critically for important intellectual content.

Contributed equally:

*Meixi Jiang. Department of Neurology, The Fourth Affiliated Hospital, China Medical University, Shenyang 110032, China.

*Mei Liu. Department of internal medicine, The seventh Affiliated Hospital, China Medical University, Shenyang 110032, China.

Contributed equally:

#Bo Wu. Department of General Surgery, The Fourth Affiliated Hospital, China Medical University, Shenyang 110032, China.

#Qian Wang. Department of medical cell biology, School of life science, China medical university, Shenyang 110032, China.

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Figures

Figure 1

Exo70 interacts with LAMTOR1.

A. LAMTOR1 interacts with Sec13 and Rab11 as shown by String Databases.

B. Endogenous LAMTOR1 and Sec13 interaction was shown by IP assay.

C. Endogenous LAMTOR1 and Rab11 interaction was shown by IP assay.

D. The interaction of endogenous Exo70 with LAMTOR1 were examined by IP using anti-Exo70 or anti-IgG antibody in HCC cells.

E. The interaction of targeted Flag-Exo70 and HA-LAMTOR1 was determined by IP assay.

F. Exo70 interaction LAMTOR1 cytoplasmic co-localization. Immunofluorescence staining shows the co-localization of endogenous Exo70 and LAMTOR1 in HCC samples. Scale bar: 20 μ m.

Figure 2

Exo70 is phosphorylated by LAMTOR1 on Ser133.

A. Recombinant proteins shows interaction between HA-LAMTOR1 and GST-Exo70 was shown by vitro GST pull-down binding assay.

B,C.LAMTOR1 and Exo70 was shown by GST pull-down assays.

D.Exo70 is required for interaction with HA-LAMTOR1 transfected in 293T cells.

E.Bacterially purified Exo70 was incubated with purified HA-LAMTOR1 in the in vitro phosphorylation assay.

F. HA-LAMTOR1 was co-transfected with the mutant Flag-Exo70 to immunoprecipitation with anti-Flag antibody.

G.Wild-type and mutant Exo70 by purified LAMTOR1 as shown by vitro phosphorylation assay.

Figure 3

Exo70 phosphorylation promotes its interactions with Rab11 and Sec13

A. Transfected Flag-tagged Exo70 or mutant Exo70(S133A) were treated with HBSS and immunoprecipitation in 293T cells.

B.Co-transfected Flag-tagged Sec13 and Myc-Exo70 or Myc-Exo70(S133A) were treated with HBSS and immunoprecipitation in 293T cells.

C.HCC cells were treated with HBSS to co-immunoprecipitatedas RAB11 with Exo70 using a antibody against the endogenous Exo70.

D.Confocal co-localization analysis of endogenous Rab11(red) and LAMTOR1(green) in human HCC tissues and noncancerous liver tissues.Scale bar, 20μm.

E.Confocal co-localization analysis of endogenous Exo70(red) and LAMTOR1(green) in human HCC tissues and noncancerous liver tissues.Scale bar, 20μm.

B,C.The enrichment plot indicated that LAMTOR1 expression was likely correlated with the cytokine molecules pathway via GSEA analysis

D.Liver cancer cell lines L02, Huh7, HepG2, SMMC7721 and Hepa1-6 were cultured in the presence of protein transport inhibitor for 6 hours and assessed for intracellular CCL5 and TGFB1 by flow cytometry. Pooled data (n=3) are shown as mean SEM.

E.Correlation analyses between HCC cancer cytokines production and tumor expression of LAMTOR1 following are shown in TCGA datasets.

F.Multiplex immunofluorescence staining of paraffin-embedded HCC sections(n=3) for LAMTOR1(green), TGFB1 (red), Exo70 (blue) and RAB11(purple). Scale bar: 200 μ m.

G.Immunofluorescence detects co-localization of endogenous Rab11,Exo70 and TGFB1 in HCC samples. Scale bar: 20 μ m.Stable Hepa1-6 cell lines expressing either Exo70 or the Exo70(S133A) mutant,showed the co-localization TGFB1 with RAB11 and Exo70 in mouse HCC.

H.Elisa analysis of TGFB1 secreted from the cells. The positions of TGFB1 are labeled to the right.

Figure 5

LAMTOR1 derived cytokine TGFBI and tumor-conditioned macrophages have an immune-suppressive phenotype.

A. Multiplex immunofluorescence staining of HCC sections (n=3) for CD4 (green), CD206 (red), CD8 (blue) and NK (purple). Displayed as composite or unmixed image for representative samples defined as either

tumor or adjacent.

B. Confocal co-localization analysis of endogenous CD206 (red) and CD68 (green) in human HCC tissues and noncancerous liver tissues. Scale bar, 200 μ m.

C. Confocal co-localization analysis of endogenous CD206 (red) and TGFB1 (green) in human HCC tissues and noncancerous liver tissues. Scale bar, 20 μ m.

D, E. TCMs (n=3) were blocked with neutralizing antibodies against TGFB1 or isotype control and analyzed for the expression of CD163, CD206 and CD86.

Figure 6

LAMTOR1 expressing TCMs inhibit T-cell function.

A.Heatmap showing differential expression of and IL-10 genes enriched in M2 polarized macrophages(n=6).HCC cell lines(LAMTOR1 konckdown or Control) co-cultured with Tumor associated macrophage indicated macrophages cytokines by RT-PCR.

B. Purified bulk CD3 T cells were co-cultured with IL-4, IL-10 polarized with indicated cytokines for 1–2 days at a 1:1 ratio and evaluated for proliferation Ki67 and IFN γ production.

C. T-cell proliferation and IFN γ production were tested for correlation with TCMs (LAMP1 knockdown or Control+Macrophage). Statistical analyses were performed by simple linear regression test of pooled data (n=40).

D, E. T cells were co-cultured with TCMs (LAMP1 knockdown or Control+ Macrophage) and assessed for proliferation and IFN γ production. Statistical analysis performed using nonparametric Mann–Whitney U tests.

F. T cells were co-cultured with TCMs (LAMP1 knockdown or Control+ Macrophage) and their tumor cell killing capacity was measured. Cell Trace labeled liver cancer cells were added to the T cells and percentage of dead tumor cells was measured by flow cytometry. Pooled data are presented as mean SEM and statistical analyses were performed using paired *t* test.

Figure 7

Targeting of TGFB1 and IL-10 improves the therapeutic benefit combined with LAMTOR1 vaccine-based cancer immunotherapy.

A. Immunohistochemical staining of LAMTOR1, Exo70, RAB11, TGFB1, and CD206 performed on AAV-sh-control and AAV-sh-LAMTOR1 mouse HCC. Scale bars: 200 μ m.

B and C, F4/80 and CD206 (B) and CD4,CD8 and Treg cell (C) were assessed by AAV- sh- LAMTOR1, AAV- sh - LAMTOR1 + aTGFB1 and AAV- sh- LAMTOR1

+aTGFB1+anti-IL-10.

D.C57BL/6 tumor growth curves and tumor weight of mice treated with either isotype (control), combined with AAV-sh-LAMTOR1,AAV-sh-LAMTOR1+anti-TGFB1 and AAV-sh-LAMTOR1+anti-TGFB1+anti-IL-10. Results are reported as the average of 5 mice per group and shown as mean $\pm$ SEM(error bars). Absence of asterisks indicates no significant difference, $p<0.05$ determined by unpaired two-tailed Students *t* test.

Figure 8

LAMTOR1 undergoes phosphorylation to bind to Exo70 and other exocyst components, which is enhancing the secretion of TGFB1 to facilitate the polarization of TAMs. The tumor-conditioned macrophages (TCM) numbers correlated with regulatory T cells (Tregs) and decreased CD8⁺ T cells function by IL-10 in HCC.