Mitochondrial C1QBP Is Essential for T Cell Antitumor Function by Maintaining Mitochondrial Plasticity and Metabolic Fitness

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Abstract

The metabolic stress present in tumors microenvironment (TME) attenuates T cells anti-tumor capacity, whereas the immune function of T cells is intrinsically controlled by their mitochondrial plasticity including mitochondrial dynamics, metabolism and biogenesis. Previous studies have reported that the complement C1q binding protein (C1QBP), a mitochondrial protein, is responsible for maintenance of the mitochondrial integrity and fitness in dendritic cells and tumor cells. However, its role in T cells, especially in T cells mediated anti-tumor immunity, remains unclear. Here we show that C1QBP is required to maintain T cell anti-tumor immunity by regulating mitochondrial biogenesis, dynamics and metabolic fitness even when only one allele of *C1qbp* is deleted, without affecting T cells development and homeostasis. Further analysis of C1QBP in the chimeric antigen receptor (CAR) T cell therapy against B16 melanoma model confirmed the cell intrinsic role of C1QBP in regulating T cells antitumor function. Mechanistically, we found C1QBP regulated mitochondrial plasticity and metabolic fitness through mitochondrial biogenesis via the AMP-activated protein kinase (AMPK)/peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1 α) signaling pathway, as well as mitochondrial morphology via the phosphorylation of mitochondrial dynamics protein dynamin-related protein 1 (Drp1). In summary, our study provides a novel mitochondrial target to reprogram the immune metabolism of T cells and improve their immunotherapeutic potential against tumors.

Introduction

Mitochondria as the highly plastic organelles are critical to modulate T cells development, determine their fate and impact their antitumor immune function(1–4). Mitochondrial plasticity is normally correlated with mitochondrial dynamics, metabolism and biogenesis(2, 4–7). The amenable mitochondrial plasticity endows the tumor-infiltrating lymphocytes (TILs) with the persistent and durable antitumor function, while the impaired mitochondrial plasticity leads to TILs metabolic insufficiency, exhaustion and hypofunction(6, 8, 9). However, the causal relationship between T cells mitochondrial plasticity and their anti-tumor immune function in response to the metabolic stress imposed by tumor microenvironment (TME) still remains poorly understood.

The complement C1q binding protein (C1QBP) predominantly located mitochondrial matrix is tightly linked to cellular bioenergetics and mitochondrial function in tumor cells. Actually, C1QBP is implicated in metabolic reprogramming through regulation of mitochondrial morphology and metabolism, which is further connected to tumor transformation and metastasis(10–14). However, the metabolic adaptation is required not only for tumor malignant progression but also for tumor-specific T cells to perform their anti-tumor immune response(15–17). Given that C1QBP is indispensable for mitochondrial structure and function, exploring the role of C1QBP in regulation of T cells anti-tumor immune capacity through mitochondrial plasticity will be of interest.

Mitochondria always undergo constant cycles of fission and fusion, which is characterized by the different morphologies(18–22). For example, in response to nutrient restriction, mitochondria exhibit the

elongated and fused morphology, which is correlated to the increased ATP requirement and the enhanced mitochondrial function. On the contrary, facing nutrient excess or cellular damage, mitochondria always prefer to the discrete and fragmented morphology to resist the relentless microenvironment. Mitochondrial dynamics is important for the maintenance of mitochondrial plasticity in cellular energy supply and survival especially under the persistently metabolic perturbations.

On the other side, the *C1qbp* gene is reported to be involved in regulation of mitochondrial oxidative phosphorylation (OXPHOS). *C1qbp*-deficient mouse embryonic fibroblasts (MEFs) show the impaired OXPHOS and ATP production, which results in the retarded embryonic development(23). However, whether C1QBP manipulates T cells metabolic fitness through regulation of OXPHOS and glycolysis still remains unclear. Here, we interrogated the effect of *C1qbp* knocking down on T cells mitochondrial metabolism, spare respiratory capacity (SRC) as well as ATP production.

Notably, mitochondrial biogenesis not only affects mitochondrial plasticity but also regulates T cells anti-tumor immune function. TILs always demonstrate the persistent loss of mitochondrial biogenesis in both human patients and mouse models (24, 25), while the proper maintenance of mitochondrial biogenesis is important to recover T cells anti-tumor response. C1QBP is closely associated with the formation of mitochondrial ribosome and the improvement of mitochondrial protein translation(23, 26). *C1qbp*-knockout MEFs exhibit the severe dysfunction of mitochondrial respiratory chain due to the impairment of mitochondrial protein synthesis. In this study, we further explored whether *C1qbp* knocking down would impact T cells mitochondrial biogenesis and the underlying mechanism.

Besides, in order to confirm whether *C1qbp* knocking down would impact the related anti-tumor immune function of chimeric antigen receptor (CAR) T cell, we evaluated the potential role of C1QBP in the CAR T cell immunotherapy. We found that *C1qbp* knocking down actually attenuated CAR T cell immune function in vitro and in vivo. In this regard, targeting mitochondrial plasticity might provide a novel strategy to enhance T cells adaptation to TME and reinvigorate the dysfunctional TILs, thereby promoting their antitumor immunotherapeutic efficacy.

Materials And Methods

Mice

C1qbp heterozygous mice were donated by Dr. Yanping Zhang from the department of Radiation Oncology and Lineberger Comprehensive Cancer Center, University of North Carolina, USA. *C1qbp* heterozygous mice were generated as previously described (27). All mice were housed and bred under specific pathogen-free (SPF) animal facility of the Experimental Animal Center in Xuzhou Medical University. All experimental animal procedures were performed in compliance with the institutional ethical requirements and approved by the Committee of Xuzhou Medical University for the Use and Care of Animals.

Immunoblot analysis

Lysates of T cells were prepared in lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 0.2% Triton X-100, 0.5% NP-40, and protease inhibitor cocktail). Samples were resolved by SDS/PAGE (12% gel) and transferred to nitrocellulose membranes. The transferred membranes were blocked with 5% (w/v) non-fat dried skimmed milk powder in PBST and immunoblotted using the following commercially available primary antibodies. Membranes were then washed with PBST, incubated with the appropriate HRP (horseradish peroxidase)-conjugated secondary antibodies in 5% (w/v) non-fat dried skimmed milk powder/PBST and visualized using enhanced chemiluminescence reagents (Proteintech) according to the manufacturer's instructions.

Seahorse extracellular flux analysis

For analysis of the metabolic phenotype of *C1qbp*^{+/+} and *C1qbp*^{+/-} T cells, oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were measured using the Seahorse XF-96 metabolic extracellular flux analyzer (Seahorse Bioscience). FACS-sorted T cells were planted in a 24-well plate coated with anti-CD3 ϵ /CD28 antibodies and cultured with RPMI-1640 medium for 48 hours. These activated T cells were collected and spun on poly-D-lysine coated seahorse 96 well plates (2×10^5 cells per well) and preincubated at 37°C for a minimum of 45 min in the absence of CO₂. Cellular OCR (pmol/min) was measured under basal conditions and following treatment with 1 μ M oligomycin, 0.5 μ M trifluoromethoxy carbonyl cyanide phenylhydrazine (FCCP), and 1 μ M rotenone (all from Sigma-Aldrich). Real-time analysis of extracellular oxygen and pH was conducted in a standard humidified incubator with 5% CO₂. Mitochondrial function parameters of basal respiratory capacity (difference in OCR before treatment of mitochondrial inhibitors and after rotenone treatment), maximal respiratory capacity (difference in OCR following treatment of FCCP and rotenone), proton leak (difference in OCR following treatment of oligomycin and rotenone), oxidative ATP turnover (difference in OCR following oligomycin treatment and before any treatment) and spare respiratory capacity (difference in OCR following treatment of FCCP and before any treatment) were determined. At the same time, ECAR (mpH/min) was recorded under basal conditions and following the addition of 10mM glucose, 1 μ M oligomycin, and 50 mM 2-deoxyglucose (2-DG). ECAR (difference in maximum rate measurement before oligomycin and last rate measurement before glucose) represented the cellular glycolysis. The related quantification was analyzed in duplicate for at least three independent experiments.

qRT-PCR analysis of mRNA

Total RNA was isolated from T cells by TRIzol reagent (Invitrogen) following manufacturer's instruction, and then reverse-transcribed into cDNA with HiScript II Q RT SuperMix (Vazyme Biotech Co.). Quantitative real-time PCR was performed on LightCycler 96 real-time PCR system (Roche life Science) with AceQ qPCR SYBR Green Master Mix kit (Vazyme Biotech Co.). The relative mRNA expression of indicated genes

was calculated based on the expression of β -actin. Ct values for each analyzed gene were normalized to β -actin and adjusted for amplification efficiency.

Primers to detect β -actin: (FW) 5'-GGGCATCCTGACCCTCAAG-3',

(RV) 5'-TCCATGTCGTCCCAGTTGGT-3',

PGC-1 α : (FW) 5'-GGATATACTTTACGCAGGTCGA-3',

(RV) 5'-CGTCTGAGTTGGTATCTAGGTC-3',

Complex I(ND-1): (FW) 5'-CTAATCGCCATAGCCTTCCTAA-3',

(RV) 5'-GTTGTTAAAGGGCGTATTGGTT-3',

Complex II(SDHC): (FW) 5'-TTCTGGAAGAAGAACACGAGTT-3',

(RV) 5'-GGGAAGACAAGCACAACTTAG-3',

Complex III(UQCRC1): (FW) 5'-CGCACAGACTTGACTGACTACCTC-3',

(RV) 5'-AGGCACGGCATCTTCTTCATACAC-3',

Complex IV(COXIV): (FW) 5'-ATGTTCTTCATTGGCTTCACTG-3',

(RV) 5'-AGTCACGATCGAAAGTATGAGG-3'.

Construction of targeted huB7-H3 CAR T cells

In order to prepare the targeted huB7-H3 CAR T cells, we firstly constructed the retroviruses containing huB7-H3 CAR. Briefly, the chimeric antigen receptor was pieced together using portions of the humanized B7-H3(8H9 clone3.1) single chain variable fragment and the published portions of the murine CD28 & CD3 ζ sequences(28). This chimeric antigen construct was then cloned into the mouse stem cell virus (MSCV) murine retroviral vector. Then the targeted huB7-H3 CAR retroviruses were prepared by co-transfecting B7-H3.CAR MSCV vector and pCL-Eco helper vector into 293T cells. The supernatant containing the CAR retrovirus targeting huB7-H3 was collected 48 hours later and filtered with a 0.22 μ m filter. On the other side, murine T cells were isolated by Pan T magnetic Microbeads (Miltenyi Biotec) from splenocytes obtained from *C1qbp*^{+/+} and *C1qbp*^{+/-} mice and then stimulated on plates coated with the anti-CD3 ϵ /CD28 antibodies for 24 hours. Activated murine T cells were transduced with the above retroviral supernatants using retronectin-coated plates (Takara Bio Inc., Shiga, Japan). The transfection of murine T cells was performed according to the previous protocols(29). After removal from the retronectin-coated plates, T cells were expanded in complete RPMI-1640 medium, 10% FBS, 4mM L-glutamine, 1% penicillin/streptomycin, 75 μ M β -mercaptoethanol, 2 ng/mL IL-2, 10 ng/mL IL-7 and 5

ng/mL IL-15. After 48 hours, the CAR T cells targeting huB7-H3 were collected for the next in vivo and in vitro experiments.

Murine melanoma B16 cells expressing huB7-H3 antigen

To establish the murine melanoma B16 cells stably expressing huB7-H3 antigen (huB7-H3-B16 cells), melanoma B16 cells were transduced with the lentiviral particles carrying the open reading frame (ORF) of 4Ig-B7-H3 (GenBank: NM_001024736), firefly luciferase (Luc) and the green fluorescent protein (GFP). 48 hours later, the positive huB7-H3-B16 cells expressing high levels of huB7-H3 and GFP were detected (Supplementary Figure 7B) and sorted by using FACS Aria III Cell Sorter (BD biosciences).

ELISA

The CAR T cells targeting huB7-H3 were co-cultured with huB7-H3 B16 tumor cells (2×10^5 cells/well) in 24-well plates at different effector to target ratios (E:T=1:2 or 1:1) without the addition of exogenous cytokines. After 18 hours, the supernatant was collected and the cytokines such as IFN- γ and TNF- α were measured in duplicate by specific ELISA kits (Proteintech) following manufacturer's instructions.

Melanoma B16 subcutaneous xenograft mouse model

The murine melanoma B16 cells (2×10^5) expressing firefly luciferase (Luc) were injected subcutaneously into the flanks of two-month-old female *C1qbp*^{+/-} and *C1qbp*^{+/+} mice on Day 0. To monitor tumor growth, tumor volumes (width $\times$ length $\times$ width/2; cm³) from C1QBP^{+/-} and C1QBP^{+/+} mice were measured with the vernier caliper during tumor progression. On Day 24, the bioluminescence images of *C1qbp*^{+/-} and *C1qbp*^{+/+} mice bearing B16 tumors were recorded through the Night OWL II LB 983 In Vivo Imaging System (Berthold Technology, German). On Day 25, these mice were humanely euthanized by CO₂ asphyxiation. The corresponding tumor tissues were collected for the next experiment or fixed in formalin for immunohistochemistry.

Melanoma huB7-H3-B16 subcutaneous xenograft mouse model

The murine melanoma huB7-H3-B16 cells (2×10^5) expressing human B7-H3 antigen and firefly luciferase (Luc) were injected subcutaneously into one flank of the recipient C57BL/6 mice on Day 0. On Day 15, cyclophosphamide (200mg/kg) was injected intraperitoneally for the depletion of endogenous lymphocytes from these recipient mice. One day later, the non-transduced T cells (NT), *C1qbp*^{+/-} and *C1qbp*^{+/+} CAR T cells (2×10^6) targeting huB7-H3 were transferred intravenously into these recipient mice bearing huB7-H3-B16 tumors. During this process, the tumor progression of recipient mice

bearing melanoma huB7-H3-B16 f was measured by the vernier caliper. On Day 15& 22, the bioluminescence images of these recipient mice before and after adoptive transfer of the NT or CAT-T cells were recorded through the Night OWL II LB 983 In Vivo Imaging System. During this process, the tumor volumes of these recipient mice were measured with the vernier caliper. On Day 25, these recipient mice were humanely euthanized by CO₂ asphyxiation. The corresponding tumor tissues were collected for the next experiment or fixed in formalin for immunohistochemistry.

Melanoma huB7-H3-B16 metastasis mouse model

The murine melanoma huB7-H3-B16 cells (3×10^5) expressing huB7-H3 antigen and firefly luciferase (Luc) were injected intravenously into the recipient C57BL/6 mice on Day 0. On Day 11, cyclophosphamide (200mg/kg) was injected intraperitoneally. One day later, the NT, *C1qbp*^{+/+} and *C1qbp*^{+/-} CAR T cells (2×10^6) targeting huB7-H3 antigen were transferred intravenously into the recipient mice bearing huB7-H3-B16 tumor cells. On Day 14&20, the bioluminescence images of these recipient mice adoptively transferred by NT, *C1qbp*^{+/+} and *C1qbp*^{+/-} CAT-T cells were recorded through the Night OWL II LB 983 In Vivo Imaging System. On Day 20, these mice were humanely euthanized by CO₂ asphyxiation. The corresponding tumor tissues were collected for the next experiment or fixed in formalin for immunohistochemistry.

Statistical analysis

Data were analyzed using Graph Pad Prism 6.0 software (La Jolla, CA). Statistical comparisons were evaluated using the Student's t-test. A *p*-value < 0.05 was considered statistically significant. All results are presented as the means $\pm$ standard deviation.

Results

Compromised antitumor immune response in *C1qbp*^{+/-} mouse

Given that C1QBP homozygous deletion resulted in early embryonic lethality(23), we wanted to investigate whether just one allele of *C1qbp* disruption would impact T cells development and homeostasis. First, we observed that C1QBP protein level of *C1qbp*^{+/-} mouse was significantly downregulated in a variety of organs including brain, lung, liver, colon and kidney when compared to that of *C1qbp*^{+/+} control mouse (S. Fig. 1A). Moreover, we isolated the T cells to detect C1QBP protein level and found that *C1qbp*^{+/-} T cells compared to *C1qbp*^{+/+} T cells also exhibited a significant decrease of C1QBP expression in response to the stimulation of anti-CD3/CD28 antibodies, as shown in S. Fig. 1B. At the same time, we found that the relative percentages of CD4 and CD8 positive T cells in spleen, lymph

nodes and blood were comparable between *C1qbp*^{+/-} mice and *C1qbp*^{+/+} littermates. Moreover, the *C1qbp*^{+/-} and *C1qbp*^{+/+} mice had the similar compartment of effector memory (CD44^{hi}CD62L^{lo}) and naïve (CD44^{lo}CD62L^{hi}) T cells (S. Fig. 2). These above data suggest that one allele of *C1qbp* deletion is unable to alter T cells development and homeostasis without tumor invasion.

However, in order to further evaluate whether C1QBP regulates anti-tumor immunity, we injected subcutaneously murine melanoma B16 cells to the *C1qbp*^{+/+} and *C1qbp*^{+/-} mice and then observed their tumor progression. As shown in Fig. 1A, B&C, *C1qbp*^{+/-} mice exhibited the more striking tumor progression than the corresponding *C1qbp*^{+/+} littermates. The uncontrolled tumor progression in *C1qbp*^{+/-} mice was further supported by increased tumor proliferation (Ki67 expression) and decreased apoptosis (Puma expression), respectively (Fig. 1D). Besides, we further investigated that the proportional percentages of central memory (CD44^{hi}CD62L^{hi}) and effector memory (CD44^{hi}CD62L^{lo}) T cells in tumor and spleen. As shown in Fig. 1E, *C1qbp* knocking down moderately impacted the relative ratio of CD44^{hi}CD62L^{hi} central memory to CD44^{hi}CD62L^{lo} effector memory T cells in TME. At the same time, it exhibited the similar proportional percentages of CD44^{hi}CD62L^{hi} central memory and CD44^{hi}CD62L^{lo} effector memory T cells in spleen. Together, these data suggest that one allele deletion of *C1qbp* aggravates tumor malignant progression and decreases the proportion of CD44^{hi}CD62L^{hi} central memory T cells in TME.

***C1qbp* knocking down dampens T cells antitumor response**

To investigate the cellular mechanism of how C1QBP regulates anti-tumor immunity, we noticed that there was a drastic decrease of T cells in tumor tissue, which is consistent with the relative low expression level of C1QBP in T cells. As shown in Fig. 2A, the percentage of CD4⁺ T cells in *C1qbp*^{+/-} mice TME is about 1.87% comparing to 5.71% in *C1qbp*^{+/+} mice. Similarly, the percentage of CD8⁺ T cells in *C1qbp*^{+/-} mice TME is about 1.67% while that in *C1qbp*^{+/+} mice is about 7.08%. Moreover, the immunohistochemistry staining also confirmed that *C1qbp* knocking down decreased CD4⁺ and CD8⁺ T cells in TME (Fig. 2B). Further analysis demonstrated that there were more dysfunctional and exhausted T cells in tumors of *C1qbp*^{+/-} mice (Fig. 2C). Our results show that *C1qbp*^{+/-} TILs exhibit the higher expression of the inhibitory receptors like PD-1, Tim-3 and LAG-3 than the corresponding *C1qbp*^{+/+} T cells. At the same time, we also detected the expression of PD-1 and Tim-3 in CD8⁺ T cells from the spleen of *C1qbp*^{+/+} and *C1qbp*^{+/-} (S. Fig. 3). Unlike TILs, peripheral T cells expressed low and similar expression of PD-1 and Tim-3, which means the impact of C1QBP on T cell exhaustion mainly restricted to TME, where T cells have more metabolic stress in the relentless microenvironment. Therefore, the sufficient expression of C1QBP is important for prevention of TILs exhaustion. Together, our results reveal that *C1qbp* knocking down dampens T cells anti-tumor response through the decrease of T cells percentage and the increase of these TILs exhaustion in TME.

***C1qbp* knocking down impairs T cells mitochondrial metabolism**

As C1QBP has been reported to regulate cellular bioenergetics and mitochondrial function in tumor cells and dendritic cells(23, 30, 31), we hypothesized that C1QBP also plays an essential role in T cell mitochondrial metabolism, which in turn regulates their anti-tumor function. By measuring O_2 consumption rates (OCR) and extracellular acidification rates (ECAR) after the stimulation of anti-CD3/CD28 antibodies for two days, we found *C1qbp*^{+/-} T cells had lower OCR (the basal respiration and the maximal respiration) but higher ECAR (glycolytic capacity) than the corresponding *C1qbp*^{+/+} T cells, indicating that *C1qbp* knocking down suppressed mitochondria-mediated OXPHOS rather than glycolysis occurring in cytoplasm (Fig. 3). In addition, we found that *C1qbp* knocking down induced T cells with more proton leak and less ATP production, which implied that C1QBP probably sustained the integrity of mitochondrial membrane structure to prefer the catabolic pathways rather than the anabolic pathways.

Spare respiratory capacity (SRC) as an important mitochondrial marker stands for the extra mitochondrial capacity available to produce energy especially under the conditions of metabolic stress and nutrient depletion(32-34). In other words, enhancement of SRC probably endows T cells with the long-term survival and persistent immune function. SRC was measured as OXPHOS activity above basal after treatment of the uncoupler carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP). Here, we found that *C1qbp*^{+/-} T cells exhibited the relatively lower SRC level when compared to the corresponding *C1qbp*^{+/+} T cells, suggesting that *C1qbp* knocking down attenuated T cells extra mitochondrial capacity and impaired T cells adaptation to the challenging microenvironments.

***C1qbp* knocking down impacts T cells mitochondrial morphology and dynamics**

To investigate how C1QBP insufficiency impairs T cells mitochondrial metabolism, we first examined the mitochondrial morphology of T cell in response to T cell receptor (TCR) stimulation. We found that *C1qbp*^{+/-} T cells exhibited the predominantly punctate mitochondria after activation of anti-CD3/CD28 antibodies especially for 3 days while *C1qbp*^{+/+} T cells possessed the more elongated and fused mitochondrial phenotype, as shown in Fig. 4A. Mitochondria are the highly dynamic organelles engaged in the repeated cycles of fission and fusion, which are controlled by a series of regulation proteins such as dynamin-related protein 1 (Drp-1), optic atrophy-1 (OPA1) and mitofusion1/2(Mfn1/2)(35-38). Specifically, mitochondrial fission is regulated by the GTPase Drp1, which is translocated from cytoplasm to the outer mitochondrial membrane (OMM) upon phosphorylation to split mitochondria(35, 36). On the other side, mitochondrial fusion is controlled by three dynamin family GTPases including Mfn1/2 on the OMM and OPA1 on the inner mitochondrial membrane (IMM)(38-40). In order to explore the potential mechanism of C1QBP regulation of T cells mitochondrial morphology, we further detected the protein level of mitochondrial fission protein like Drp1 and fusion proteins including OPA1 and Mfn2 in *C1qbp*^{+/-}

and *C1qbp*^{+/-} T cells. As shown in Fig. 4B, we found that *C1qbp* knocking down obviously enhanced T cells mitochondrial fission protein Drp1 expression and its phosphorylation on Ser616 responsible for the scission of mitochondrial membrane. At the same time, C1QBP insufficiency also induced the decrease of mitochondrial fusion proteins OPA1 and Mfn2 expression. Together, these data suggest that *C1qbp* knocking down prefers to T cells mitochondrial fission through the increase of Drp1 expression and its phosphorylation on Ser616 as well as the decrease of OPA1 and Mfn2 protein levels.

C1QBP is required to maintain T cells mitochondrial biogenesis through PGC1 α signaling pathway

Given that the regulation of mitochondrial dynamics was always connected with the alteration of mitochondrial biogenesis (24), we further explored whether *C1qbp* knocking down would impact T cells mitochondrial proteins synthesis. Peroxisome proliferator-activated receptor γ coactivator 1 α (PGC-1 α) has been proposed to play a central role in regulation of mitochondrial biogenesis and function(?). Here, we investigated whether C1QBP insufficiency would impact T cells mitochondrial biogenesis through regulation of PGC1 α expression. As shown in Fig. 5A&B, *C1qbp* knocking down dramatically diminished the transcription and translation of PGC1 α . On the other hand, AMP-activated protein kinase(AMPK) as a conserved Ser/Thr kinase activated by the cellular AMP/ATP ratio is involved with the bioenergetic switches from anabolic process to catabolic pathways for ATP production(41). Moreover, AMPK activation enhances PGC1 α expression to promote the several mitochondrial proteins transcription and translation(42-44). In this study, we also detected and compared the protein expression and phosphorylation of AMPK between *C1qbp*^{+/-} and *C1qbp*^{+/+} T cells (Fig. 5A). Our data showed that AMPK protein level gradually increased accompanying with the stimulation of anti-CD3/CD28 antibodies, but there was no detectable expression difference between *C1qbp*^{+/-} and *C1qbp*^{+/+} T cells. However, *C1qbp*^{+/-} T cells exhibited the relatively lower phosphorylation of AMPK on Thr172 when compared to *C1qbp*^{+/+} T cells, suggesting that *C1qbp* knocking down-mediated decrease of PGC1 α is at least partially attributed to the reduced phosphorylation of AMPK.

Whether PGC1 α signaling pathway regulates T cells mitochondrial proteins synthesis still remains unclear. Next, we detected the transcription and translation of mitochondrial structure proteins such as electron transport chain (ETC) complexes I, II, III and IV between *C1qbp*^{+/-} and *C1qbp*^{+/+} T cells in response to the TCR-induced stimulation. Our data showed that *C1qbp* knocking down exhibited the impaired transcription and translation of these mitochondrial proteins including ETC complexes I, III and IV. In order to further confirm that C1QBP regulated the mitochondrial protein synthesis through PGC1 α signaling pathway, we treated *C1qbp*^{+/+} T cells with PGC1 α siRNA to observe whether blocking PGC1 α would repress the expression of these mitochondrial structure proteins. These results showed that PGC1 α siRNA still could dampen the expression level of mitochondrial structure proteins such as ETC complex I, III and IV although C1QBP was sufficient, as shown in S. Fig. 4. In other words, PGC1 α plays a pivotal role in C1QBP-mediated mitochondrial biogenesis in response to TCR stimulation. Together, C1QBP

insufficiency impairs T cells mitochondrial biogenesis and mitochondrial protein synthesis through AMPK/PGC1 α signaling pathway.

***C1qbp* knocking down attenuates CAR T cells immunotherapeutic efficacy**

In order to answer the question whether C1QBP would directly impact T cells anti-tumor immune function, we designed CAR T cells with the different amount of C1QBP to clarify its specific role in T cells-related immunotherapy. B7-H3 as one member of B7 immune co-stimulatory and co-inhibitory family possesses two isoforms in humans, 2Ig-B7-H3 and 4Ig-B7-H3. B7-H3 protein has the limited expression in human normal tissues including liver, breast, colon et al., whereas it exhibits the abnormally high expression in the corresponding human malignant tissues(45-47). Moreover, CAR T cells targeting huB7-H3 as a novel CAR T therapeutic target was recently reported to exhibit the potent antitumor effects on hematologic and solid tumors such as glioblastoma(48-50). Here, we constructed the *C1qbp*^{+/-} and *C1qbp*^{+/+} CAR T cells targeting huB7-H3 to detect the effect of C1QBP on CAR T cells antitumor efficacy. Firstly, we transfected the melanoma B16 cells with the lentivirus expressing huB7-H3 antigen to assess the anti-tumor immune capacity of these CAR T cells with the different amount of C1QBP. Firstly, we monitored the CAR T cells killing of huB7-H3-B16 cells through RTCA assay and found that *C1qbp*^{+/-} CAR T cells exhibited relatively weaker tumor suppression at different effector to target ratios (E:T) such as 1:2, 1:1 and 2:1 than the corresponding *C1qbp*^{+/+} CAR T cells, as shown in Fig. 6A. Besides, huB7-H3-B16 cells co-cultured with the CAR T cells targeting huB7-H3 also exhibited the similar trend. As shown in Fig. 6B, *C1qbp*^{+/+} CAR T cells repressed tumor cells to 18.7%, 15.5% and 4.36% at different effector to target ratios. However, under the same condition, *C1qbp*^{+/-} CAR T cells just shrunk tumor cells to 38.8%, 21.5% and 10.4%. In order to further evaluate the CAR T cytotoxicity, we detected the TNF- α , IFN- γ release using ELISA kits. Our data demonstrated that *C1qbp*^{+/-} CAR T exhibited the less TNF- α , IFN- γ release at different effector to target ratios (E:T=1:2 or 1:1) when compared with *C1qbp*^{+/+} CAR T cells, as shown in Fig. 6C, which means *C1qbp* insufficiency actually impaired the related CAR T cells cytotoxicity. Together, these results suggest that *C1qbp* knocking down represses the anti-tumor immune function of CAR T cells in vitro.

At the same time, we further evaluated the exact role of C1QBP in the immunotherapeutic efficacy of CAR T cells. Firstly, we injected huB7-H3-B16 cells into recipient C57BL/6 mice and then adoptively transferred *C1qbp*^{+/+} and *C1qbp*^{+/-} CAR T cells to these mice bearing huB7-H3-B16 tumors. Next, we measured tumors volumes and found that the mice accepted with CAR T cells treatment exhibited the significant tumor suppression than the mice transferred with the control non-transduced T cells (NT), suggesting that these CAR T cells could target huB7-H3 antigen and exert their corresponding anti-tumor immune function. Importantly, *C1qbp*^{+/-} CAR T group exhibited relatively weaker tumor regression than *C1qbp*^{+/+} CAR T group in B16 subcutaneous xenograft mouse model and metastasis mouse model, as shown in Fig. 7B-D & Fig. 8B-C. Moreover, our results confirmed that the more Ki67 and the less CD4⁺ & CD8⁺ T cells were detected in tumor tissues from *C1qbp*^{+/-} CAR T cells group, as shown in Fig. 7E & Fig. 8D. At the

same time, *C1qbp*^{+/+} CAR T cell also exhibited higher proportion than the corresponding *C1qbp*^{+/-} CAR T group in TME (Fig. 7F & Fig. 8E). Notably, following clearance of tumor antigens, T cells convert to two distinct subsets of memory: central memory T cells (CD62L^{hi}) and effector memory T cells (CD62L^{lo}). In our study, *C1qbp*^{+/-} CAR T group possessed the significantly lower ratio of CD62L^{hi} to CD62L^{lo} than *C1qbp*^{+/+} CAR T group (Fig. 7G& Fig. 8F), suggesting that *C1qbp* knocking down prevents CAR T cells to possess more central memory phenotype. At the same time, we also detected tumor-specific CAR T cells exhausted phenotype. As shown in S. Fig. 5A, *C1qbp*^{+/+} CAR T cells exhibited the relatively lower PD-1 inhibitory receptors expression, while *C1qbp*^{+/-} CAR T cells tended to the higher PD-1 expression level. However, CAR T cells from spleen in recipient mice possessed the similar expression of PD-1 no matter the amount of C1QBP (S. Fig. 5B). These data demonstrate that *C1qbp* knocking down aggravates the exhausted phenotype of tumor infiltrating CAR T cells but not peripheral CAR T cells. Together, *C1qbp* knocking down attenuates CAR T cells percentage and central memory phenotype as well as exacerbates their exhaustion in TME, which thus dampens the corresponding immunotherapeutic efficacy.

Discussion

Mitochondrial dynamics and metabolic adaptation are crucial to T cells survival, proliferation and effector function especially under the relentless TME. Mitochondrial fusion endows the competitive advantage to T cells through equipping them for the long-term survival and the ability to rapidly recall upon antigen challenge(2). Our data showed that mitochondrial protein C1QBP as a critical regulator was involved with the modulation of T cells mitochondrial morphology. *C1qbp*^{+/-} T cells exhibited more punctate and fragmented phenotype in mitochondria while *C1qbp*^{+/+} T cells possessed more tubular and fused mitochondria in response to the prolonged stimulation of anti-CD3/CD28 antibodies. Moreover, we found that *C1qbp* knocking down enhanced mitochondrial fission protein Drp1 and its phosphorylation on Ser616 but repressed mitochondrial fusion proteins OPA1 and Mfn2 in response to anti-CD3/CD28 induced TCR activation. These data suggest that *C1qbp* knocking down renders T cells prone to mitochondrial fission rather than mitochondrial fusion, which always reduces cellular adaptation to their challenging microenvironment.

Actually, emerging evidence demonstrated that the intricate balance of mitochondrial morphology between fission and fusion impacted on T cells metabolism and immune function (51–53). Pearce et al. found that fused mitochondria had the tight cristae that yielded more efficient ETC activity and favored OXPHOS as well as FAO, while fragmented mitochondria possessed the loose cristae with less efficient ETC activity and thus preferred to aerobic glycolysis(2). Besides, inhibition of mitochondrial OXPHOS resulted in suppression of T cells proliferation and upregulation of their exhaustion(4). Some previous studies reported that C1QBP as an indispensable regulator maintained mitochondrial OXPHOS because *C1qbp* deficient MEFs performed the dysfunctional OXPHOS(11, 31). Consistently, in our study, *C1qbp*^{+/-} T cells exhibited more fragmented mitochondria and lower OXPHOS as well as less ATP production, which possibly rendered TILs with the decreased metabolic fitness. Moreover, the enhanced mitochondrial SRC was also thought to be an important property for cells with the long-term survival and

durable function(34, 54). Actually, long-lived memory T cells always possess the relatively stronger SRC than short-lived effector T cells. In this study, *C1qbp*^{+/-} T cells exhibited the relatively weaker SRC than the corresponding *C1qbp*^{+/+} T cells, which suggested *C1qbp* knocking down rendered T cells with the less extra mitochondrial capacity to meet bioenergetic demands. Besides, *C1qbp* knocking down repressed the relative ratio of CD44^{hi}CD62L^{hi} central memory to CD44^{hi}CD62L^{lo} effector memory T cells. Similarly, *C1qbp*^{+/-} CAR T cells possessed the lower proportion of central memory phenotype when compared to *C1qbp*^{+/+} CAR T cells. Consequently, whether C1QBP associated with the high SRC may potentiate T cells with the persistent and durable antitumor immune function remains to be elucidated.

Given that the tumor specific T cells always exhibited the decreased mitochondrial biogenesis, the metabolic reprogramming through enhancing expression of PGC1 α was reported to be related to the reinvigoration of mitochondrial function and the promotion of T cells anti-tumor response(24, 55). In our study, *C1qbp* knocking down repressed AMPK phosphorylation and PGC1 α protein expression, which thus inhibited ETC complexes I, III and IV mRNA level and their corresponding protein translation. Moreover, PGC1 α siRNA reversed the C1QBP-mediated mitochondrial biogenesis in response to the TCR stimulation. Therefore, these results highlight that C1QBP is involved in the regulation of mitochondrial biogenesis through AMPK/PGC1 α signaling pathway. We further detected the exact role of C1QBP in the anti-tumor immune function of T cells. Considering that TILs with a progressive loss of PGC1 α lacked mitochondrial biogenesis while the enhancement of PGC1 α contributed to their superior metabolic plasticity and effector function(24), we presume that C1QBP-mediated PGC1 α improvement possibly triggers T cell metabolic reprogramming and mitochondrial biogenesis, which subsequently reinvigorates their mitochondrial plasticity to resist T cells exhaustion and hypofunction in TME.

Taken together, as shown in Fig. 8G, *C1qbp* knocking down induced T cells with the more mitochondria fission, the lower mitochondrial OXPHOS and the weaker mitochondrial biogenesis, which further dampened mitochondrial plasticity and metabolic fitness to impact TILs amount, exhaustion as well as memory phenotype. In this regard, the appropriate improvement of mitochondrial function through C1QBP might endow T cells and CAR T cells with the robust and persistent anti-tumor immune function, which probably provides the novel strategy to promote their anti-tumor immunotherapeutic efficacy.

Declarations

Conflict of interest

The authors declare no conflict of interests.

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Author contributions statement

Conception and design: Hui Tian, Zengli Guo and JunNian Zheng; Development of methodology: Hui Tian, Gang Wang, Qiping Wang and Jingyuan Song; Acquisition of data and critical reagents, animals and facility support: Huizhong Li, Guan Jiang, Jingyuan Song and Lin Fang; Analysis and interpretation of data (statistical analysis, biostatistics computational analysis): Hui Tian, Dafei Chai, Gang Wang and Qiping Wang; Writing, review, and/or revision of the manuscript: Hui Tian, Gang Wang, Qiping Wang and Zengli Guo; Administrative, technical or material support (reporting or organizing data, constructing databases): Huizhong Li, Lin Fang and Meng Wang; Study supervision: Zengli Guo.

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Figures

Figure 1

C1qbp knocking down affects the tumor progression. The melanoma B16 cells (2×10^5) expressing firefly luciferase (Luc) were injected subcutaneously into the flanks of *C1qbp*^{+/+} and *C1qbp*^{+/-} mice on Day 0 (5 mice/group). **(A)** The melanoma B16 in vivo bioluminescence image on Day 24 and **(B)** in vitro image on Day 25 were collected from the *C1qbp*^{+/+} and *C1qbp*^{+/-} mice, respectively. **(C)** The volume and weight of melanoma B16 tumor were calculated and recorded. The bar graphs showed the tumor progression of *C1qbp*^{+/+} and *C1qbp*^{+/-} mice bearing B16 tumors. **(D)** Ki67 (proliferation marker) and Puma (pro-apoptotic marker) in tumor tissues from *C1qbp*^{+/+} and *C1qbp*^{+/-} mice were assessed by immunohistochemistry. Scale bars, 50μm (200 ×), 20μm (400 ×). **(E)** The proportions of CD44^{hi}CD62L^{hi} central memory and CD44^{hi}CD62L^{lo} effector memory T cells in TILs and spleen were detected by flow cytometry. The bar graphs show the mean ± S.D. of three independent experiments (n= 3); *P < 0.05, **P < 0.01.

Figure 2

C1qbp knocking down impacts T cells tumor infiltration and exhaustion. **(A)** The relative percentages of CD4⁺&CD8⁺ T cells from B16 tumor tissues of *C1qbp*^{+/+} and *C1qbp*^{+/-} mice were assessed by flow cytometry. **(B)** The CD4⁺, CD8⁺ and CD3⁺ T cells infiltrating tumor tissues were detected by immunohistochemistry. Scale bars, 50μm (200 ×), 20μm (400 ×). **(C)** T cells exhaustion markers (PD-1, Tim-3, LAG-3) expressed in CD4⁺ and CD8⁺ TILs were analyzed by flow cytometry. The bar graphs show the mean ± S.D. of three independent experiments (n = 3); *P < 0.05, **P < 0.01.

Figure 3

C1qbp knocking down impairs T cells mitochondrial OXPHOS. *C1qbp*^{+/+} and *C1qbp*^{+/-} T cells were stimulated with anti-CD3/CD28 antibodies for 48 hours. **(A)** The cellular OCR (pmol/min) was measured under basal conditions and following treatment with 1 μM oligomycin, 0.5 μM trifluoromethoxy carbonyl cyanide phenylhydrazine (FCCP), and 1μM rotenone through the Seahorse Extracellular Flux Analyzer. Basal respiratory capacity, maximal respiratory capacity, proton leak, ATP turnover and spare respiratory capacity represent the different parameters of mitochondrial OXPHOS. Up, representative curve; Down, quantification of three independent experiments. **(B)** The cellular ECAR (mpH/min) of *C1qbp*^{+/+} and *C1qbp*^{+/-} T cells was measured under basal conditions and following treated with 10 mM glucose, 1 μM

oligomycin and 50 mM 2-DG through the Seahorse Extracellular Flux Analyzer. Left panel, representative curve; Right panel, quantification of three independent experiments. The bar graphs show the mean $\pm$ S.D. *P < 0.05, **P < 0.01.

Figure 4

C1qbp knocking down alters T Cells mitochondrial morphologies. **(A)** T cells isolated from *C1qbp*^{+/+} and *C1qbp*^{+/-} mice were stimulated with anti-CD3/CD28 antibodies for the different time points. Mitochondrial morphologies of *C1qbp*^{+/+} and *C1qbp*^{+/-} T cells were detected by confocal microscopy. Scale bar, 5 μ m. **(B)** The relative protein levels of OPA1, MFN2, Drp1, C1QBP and the phosphorylation of Drp1 at Ser616 compared to β -actin were analyzed by immunoblotting. The bar graphs show the mean $\pm$ S.D. of three independent experiments (n= 3); *P < 0.05, **P < 0.01.

Figure 5

C1qbp knocking down suppresses T cells mitochondrial biogenesis through AMPK/ PGC1 α signaling pathway. **(A)** The *C1qbp*^{+/+} and *C1qbp*^{+/-} T cells were activated with anti-CD3/CD28 antibodies for the indicated time points. The relative protein levels of AMPK, PGC1 α , Complex I (ND1), Complex II (SDHC), Complex III (UQCRC1), Complex IV (COXIV) and phosphorylation of AMPK at Thr172 compared to β -actin were detected by immunoblotting. **(B)** The relative mRNA levels of PGC1 α , Complex I, II, III and IV compared to β -actin were analyzed by qRT-PCR assays. The bar graphs show the mean $\pm$ S.D. of three independent experiments (n= 3); *P < 0.05, ** P < 0.01.

Figure 6

C1qbp knocking down decreases the anti-tumor immune function of CAR T cells in vitro. **(A)** The huB7-H3-B16 cells (1×10^4) planted on 16-well micro-E-plate for 24 hours, and then added the non-transduced T cells (E:T=1:1), the *C1qbp*^{+/+} and *C1qbp*^{+/-} CAR T cells targeting huB7-H3 antigen (E:T=1:2, 1:1 and 2:1) to co-culture for additional 72 hours. The survival of huB7-H3-B16 cells were monitored using the real time RTCA assay. **(B)** The huB7-H3-B16 cells (2×10^5) planted on 24-well plate for 24 hours, and then added *C1qbp*^{+/+} and *C1qbp*^{+/-} CAR T cells to co-cultured for additional 6 hours (E:T=1:2, 1:1 and 2:1). The

percentages of residual huB7-H3-B16 tumor cells (GFP⁺) and CAR T cells (PE-Cy7-CD3⁺) were estimated by flow cytometry. **(C)** The *C1qbp*^{+/+} and *C1qbp*^{+/-} CAR T cells targeting huB7-H3 were co-cultured with huB7-H3 B16 tumor cells (2×10⁵ cells/well) in 24-well plates at different effector to target ratios (E:T=1:2 or 1:1) without the addition of exogenous cytokines. After 18 hours, the supernatant was collected and the cytokines IFN-γ and TNF-α were measured in duplicate using specific ELISA kits. The bar graphs show the mean ± S.D. of three independent experiments (n= 3); *P < 0.05, ** P < 0.01.

Figure 7

C1qbp knocking down attenuates CAR T cells immunotherapeutic efficacy in huB7-H3- B16 subcutaneous xenograft mouse model. **(A)** Schematic representation of the procedure about the recipient mice accepted the subcutaneous injection of huB7-H3-B16 tumor (Day 0) and the adoptive transfer of non-transduced control T cells (NT), *C1qbp*^{+/+} and *C1qbp*^{+/-} CAR T cells (Day 16) (4 mice/group). **(B)** Representative bioluminescence images of the recipient mice of above three groups before (Day 15) and after (Day 22) inoculation of NT, *C1qbp*^{+/+} and *C1qbp*^{+/-} CAR T cells. **(C)** Representative images of the huB7-H3-B16 tumor from recipient mice of above three groups on Day 25. **(D)** The volumes of the huB7-H3-B16 tumor from recipient mice of above three groups were calculated and recorded. The bar graph showed their respective tumor progression. **(E)** The anti-CD4, CD8 and Ki67 antibodies were used for immunohistochemistry staining of tumor tissues from recipient mice of above three groups. Scale bars, 50μm (200 ×), 20μm (400 ×). **(F)** The relative percentage of TILs in the huB7-H3-B16 tumor from the recipient mice of above three groups were assessed by flow cytometry. **(G)** The proportions of CD62L^{hi} and CD62L^{lo} in CD8⁺ T cells from the recipient mice adoptively transferred of *C1qbp*^{+/+} and *C1qbp*^{+/-} CAR T cells were detected by flow cytometry. The bar graph from three independent experiments (n = 3); *P < 0.05, **P < 0.01.

Figure 8

C1qbp knocking down dampens CAR T cells immunotherapeutic efficacy in huB7-H3-B16 metastasis mouse model. **(A)** Schematic representation of the procedure about the recipient mice accepted the intravenous injection of huB7-H3-B16 tumor (Day 0) and the adoptive transfer of non-transduced control T cells (NT), *C1qbp*^{+/+} and *C1qbp*^{+/-} CAR T cells (Day 12) (4 mice/group). **(B)** Representative bioluminescence images of the recipient mice of above three groups adoptively transferred of NT, *C1qbp*^{+/+} and *C1qbp*^{+/-} CAR T cells on Day 14&20. **(C)** Representative lung tissue images of the recipient

mice of above three groups. **(D)** The anti-CD4, CD8 and Ki67 antibodies were used for immunohistochemistry staining of the lung tissues with huB7-H3-B16 tumor from the recipient mice of above three groups. Scale bars, 50µm (200 ×), 20µm (400 ×). **(E)** The relative percentage of TILs in tumor tissues from above three groups were assessed by flow cytometry. **(F)** The proportions of CD62L^{hi} and CD62L^{lo} in CD8⁺ T cells from the recipient mice adoptively transferred of *C1qbp*^{+/+} and *C1qbp*^{+/-} CAR T cells were detected by flow cytometry. **(G)** Schematic model of the impact of *C1qbp* knocking down on T cells anti-tumor immune function. The bar graph from three independent experiments (n = 3); *P < 0.05, **P < 0.01.

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