Original Research

Dysfunction of Cytotoxic T Lymphocyte Induced by Hepatoma Cells through the Gln-GLS2-Endoplasmic Reticulum Stress Pathway

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Abstract

Background: Metabolic activities of tumor cells lead to a depletion of nutrients within the tumor microenvironment, which results in the dysfunction of infiltrating T cells. Here, we explored how glutamine (gln) metabolism, which is essential for biosynthesis and cellular function, can affect the functions of cytotoxic T lymphocytes (CTLs). **Methods**: Activated CTLs were co-cultured with hepatoma cells. Western blot was used to analyze changes of proteins and ELISA was used to analyze changes of effector. RNA-sequencing was used to detect differentially expressed genes in CTLs. The status of the endoplasmic reticulum (ER) was investigated using transmission electron microscopy experiments. **Results**: Co-culturing CTLs and hepatoma cells revealed that CTLL-2 cells in the co-culture group expressed high levels of PD-1 (Programmed cell death protein 1), TIM-3 (T cell immunoglobulin and mucin domain-containing protein-3), GRP78 (Glucose regulated protein 78), and P-PERK (phosphorylated protein kinase RNA-activated-like endoplasmic reticulum kinase) and secreted low levels of Granzyme B and perforin. Additionally, the substructure of the ER was severely damaged. When CTLs were treated with an inhibitor of ER stress, their functions were restored. Next, complete medium without Gln was used to culture cells, causing CTLs to display dysfunction and ER stress. WB results revealed decreased expression levels of GLS2 and SLC1A5 (Solute carrier family 1 member 5) in CTLs in the co-culture group. Subsequently, glutaminase (GLS) inhibitors were added to the cultures. As expected, CTLs treated with a GLS2 inhibitor had increased protein content of PD-1 and TIM-3, decreased secretion of Granzyme B and perforin, and an enhanced ER stress response. **Conclusions**: In summary, CTLs are functionally downregulated induced by hepatoma cells through the Gln-GLS2-ERS pathway.

Keywords: CTLs; dysfunction; glutamine; GLS2; endoplasmic reticulum stress

1. Introduction

Cytotoxic T lymphocytes (CTLs) are important components of the tumor microenvironment (TME). These immune cells mainly secrete effector molecules such as perforin and granzyme [1] to kill target cells or induce target cell apoptosis through the Fas/FasL pathway [2]. However, the TME can hamper the antitumor immunity of CTLs, including through hypoxia, low nutrition, immunosuppressive cytokines, and immunosuppressive cells [3]. Among these, nutritional deficiency has been shown to suppress CTL-mediated antitumor responses, such as glucose deficiency [4]. However, the specific metabolic mechanisms that regulate the function of CTLs remain to be explored.

The endoplasmic reticulum (ER) is the main site of protein synthesis and folding, maintaining Ca^{2+} homeostasis and lipid metabolism in cells [5]. Imbalances in unfolded and misfolded proteins are detected by ER stress sensors such as protein kinase R (PKR)-like ER kinase (PERK) [6]. Transient stress responses can relieve the burden of ER folding or misfolding proteins to restore proteostasis [7]. However, excessive or prolonged ER stress can induce cell death. The ER stress response is very commonly observed in tumors [8]. The relationship between the ER stress response and CTL functions remains to be investigated.

Any factor that causes an increased secretory load of proteins and the presence of mutant proteins under pathological conditions can induce the ER stress response. The main factors include undernutrition [9], hypoxia [10], viral infection [11], and accumulation of reactive oxygen species [12]. The deprivation of nutrients such as amino acids [13] and glucose [11,14] and the study of ER function have gradually attracted attention in the field. Glutamine (Gln) is the most abundant free amino acid in serum and is an essential nutrient for cells [15]. In tumor cells, Gln deficiency can induce ER stress [16]. Whether Gln can regulate the ER processes of tumor-infiltrating CTLs is still unclear.

In this study, we therefore investigated if Gln can regulate CTL functions through the ER stress pathway to clarify the relationship between the Gln metabolism pathway and CTL dysfunction, as well as to explore new metabolic pathways.

2. Materials and Methods

2.1 Cell Culture and Activation

Hepa1-6 cells (American Type Culture Collection (ATCC)) and Huh-7 cells (ATCC) were cultured in buffered

Dulbecco's modified eagle medium (DMEM, C11995500, Gibco, China). CTLL-2 cells (359785, BeNa Culture Collection (BNCC), China) and CD8$^+$ T cells were cultured in vitro using RPMI-1640 medium (C11875500, Gibco, China). All media were supplemented with 10% fetal bovine serum (FBS; Biological Industries, USA) and 1% penicillin/streptomycin (P1400, Solarbio, China). CTLL-2 cells were activated with 10 μg/mL anti-CD3 antibody (kx10-3A; Beijing KEXIN Biological, China) and 2.5 μg/mL anti-CD28 antibody (kx10-28A; Beijing KEXIN Biological, China) in medium containing 100 U/mL IL-2 (PeproTech, Rocky Hill, NJ, USA) for 2 days. All cell types were maintained at 37 °C in an atmosphere of 5% CO$_2$.

2.2 Reagents and Antibodies

4-Phenylbutyric acid (4-PBA) (P21005) was obtained from Sigma-Aldrich (Germany). Glutaminase C-IN-1/Compound 968 (HY-12682) and Telaglenastat/CB-839 (HY-12248) were purchased from MedChemExpress (MCE) (USA). DMSO (D8371) was obtained from Solarbio (China).

The primary antibodies included anti-Glutaminase (M01272-3, Boster Biological Technology, 1:1000, China), anti-GLS2 (A05334-1, Boster Biological Technology, 1:800, China), SLC1A5 (AF6610, Affinity Biosciences, 1:1000, USA), GRP78 (AF5366, Affinity Biosciences, 1:1000, USA), Phospho-PERK/Thr982 (DF7576, Affinity Biosciences, 1:1000, USA), PERK (ab229912, abcam, 1:1000, UK), PD-1 (ab214421, abcam, 1:1000, UK), TIM-3/D3M9R (83882, Cell Signaling Technology, 1:1000, USA), and anti-GAPDH (BM1623, Boster Biological Technology, 1:5000, China). The Goat anti-rabbit IgG secondary antibody (BA1054, 1:10,000) was obtained from Boster Biological Technology (China).

2.3 Co-Culture

Co-cultures were performed in 6-well plates coated with poly-2-hydroxyethyl methacrylate (poly-Hema) (TCS011006, JET, China). In 6-well plates, 1.5×10^6 CTLs (CTLL-2 and CD8$^+$ T cells) were seeded per well as monocultures, and 1.5×10^6 CTLs and 1×10^6 hepatoma cells (Hepa 1–6 and Huh-7 cells) per well were seeded for co-cultures. These plates were incubated for 2 days at 37 °C in a humidified 5% CO$_2$ atmosphere. Results of co-culture were compared with those of the control group (activated CTLs) and negative control (NC) group (respective monoculture).

2.4 Cell Sorting and RNA Sequencing of CD8$^+$ T cells

Normal human peripheral blood was obtained from healthy volunteer donors with informed consent. Peripheral blood mononuclear cells (PBMCs) were obtained from these samples by centrifugation ($800\times$g, 20 min). Cell sorting was performed using a MofloXDP sorting flow cytometer (Beckman Coulter, Brea, CA, USA).

Total RNA was extracted from CD8$^+$ T cells, and total RNA-Seq libraries were obtained by a three-step method: (1) RNA library construction and on-board sequencing, sequencing data were aligned to the reference genome of the project species to obtain comprehensive transcript information and perform gene expression quantification; (2) the transcriptome sequencing project was completed on the Illumina sequencing platform (San Diego, CA, USA), the IlluminaPE library (approximately 300 bp) was constructed for sequencing; and (3) the obtained sequencing data were analyzed by bioinformatics after performing quality control.

2.5 ELISA Assay

Cell culture supernatants were collected from each group and were then centrifuged for 20 min at $1000\times$g at 2–8 °C. The concentrations of Granzyme B (ELMO003, Boxbio, China) and Perforin (E-EL-M0890c, Elabscience, China) in the supernatants were determined using ELISA kits according to the manufacturer's instructions.

2.6 Glutamine Measurement

The Gln content (EGLN003, BioAssay Systems, USA) in the supernatant (ELx800^{TM}, Kodak, USA) was determined colorimetrically, measured at 565 nm.

2.7 Transmission Electron Microscopy (TEM)

Cells in each group were digested to prepare 1×10^6 cells/mL suspensions and centrifuged. After discarding the supernatant, the cells were fixed with 3% glutaraldehyde fixative for 4 hours and 1% osmic acid for 2 hours. After dehydration with a graded ethanol series and acetone, the samples were immersed in embedding medium for 1 hour. The cells were embedded in epoxy resin. Resin blocks were sectioned using an ultramicrotome (Leica UC-6 ultramicrotome, Germany) for double staining. Images were collected using TEM (H-7650, Hitachi, Tokyo, Japan) followed by photography (Gatan Image Analysis System, California, USA). The ultrastructural changes of the ER in each group of cells were observed by TEM.

2.8 Western Blot

Cells were harvested and lysed with protein sample loading buffer ($1\times$). Lysates were incubated in boiling water for 10 mins. Total proteins were separated on a polyacrylamide gel, electrotransferred to polyvinylidene difluoride (PVDF) membranes, and blocked for 1 hour at 37 °C with 5% skim milk in $1\times$ TBST (T1082, Solarbio, China). The membranes were then incubated with the primary antibodies at 4 °C overnight. After being washed three times in $1\times$ TBST, the membranes were incubated with secondary antibody for 2 hours at room temperature and washed again. Proteins were detected using the ChemiDoc MP Imaging System (Bio-Rad, California, USA) and form pictures. Pictures were analyzed using Image Lab (Bio-Rad, USA) and
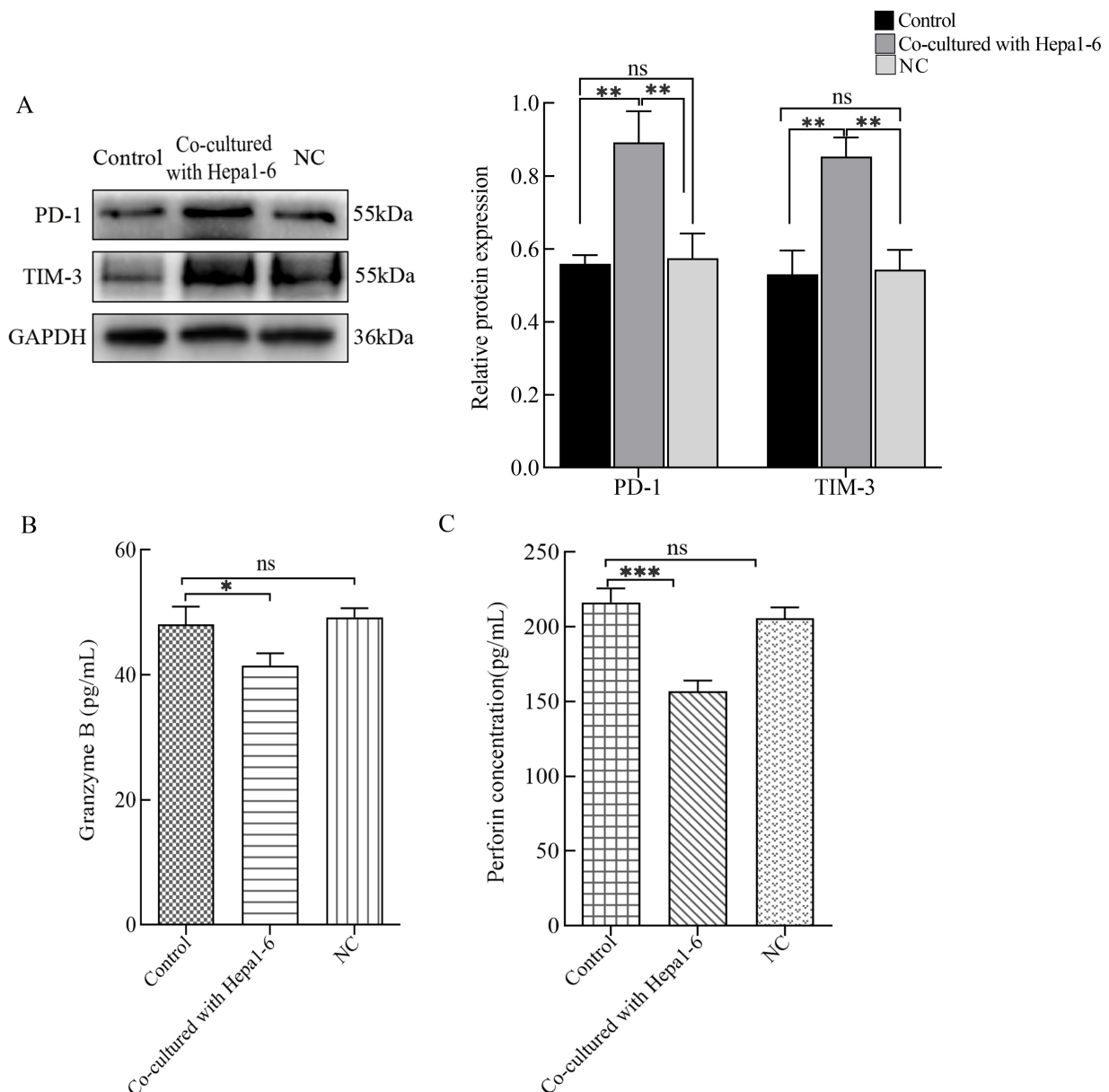
Fig. 1. Detection of cytotoxic T lymphocyte (CTL) functions. Hepatoma cells and CTLs were co-cultured. (A) Western blots were used to detect the protein expression levels of PD-1 and TIM-3. The levels of (B) Granzyme B and (C) perforin in the cell culture supernatants were assessed by enzyme-linked immunosorbent assays (ELISAs). *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

Image J (National Institutes of Health, USA) software.

2.9 Statistical Analysis

The data was represented as the Mean $\pm$ Standard Deviation (SD) in this study. All analyses were performed with SPSS 21.0 software (IBM Corp., Armonk, NY, USA) and GraphPad Prism 8.0.2 (GraphPad Software, Inc., San Diego, CA, USA). Analyses between two groups were performed using a two-tailed Student's *t* test. One-way analysis of variance (ANOVA) was used for multiple group comparisons, and an LSD-*t* test was used for pairwise comparisons between multiple groups. Statistical significance was defined as $p < 0.05$.

3. Results

3.1 CTLs Exhibit Dysfunction Induced by Hepatoma Cells

We previously found that a large number of CTLs can infiltrate liver cancer tissues. To clarify whether hepatoma cells can regulate the function of CTLs, we co-cultured CTLL-2 (CTLs) cells with Hepa1-6 (hepatoma) cells. Compared with cells in other groups, CTLL-2 cells co-cultured with Hepa1-6 cells expressed high levels of PD-1 and TIM-3 and secreted low levels of Granzyme B and perforin (Fig. 1). High expression of immune checkpoint-related proteins and low secretion of effector molecules suggested that Hepa1-6 cells can induce a functional downregulation of CTLL-2 cells.

3.2 ER Stress in CTLs Induced by Hepatoma Cells

To investigate the functional changes of CTLs, we performed RNA-Seq on CD8$^+$ T cells co-cultured with Huh-7 cells. We observed an upregulation of genes involved in ER-related reactions, such as ER to Golgi transport vesicles, and amino acid stimulation responses, such as the cellular response to amino acids (Fig. 2A). The expression of genes involved in cellular metabolism, such as "the regulation of the lipid metabolic process", and "cellular secretion", such as "positive regulation of secretion by cells", was downregulated (Fig. 2B). These results indicate that CD8$^+$ T cells undergo alterations associated with cell metabolism, ER-related reactions, and cell secretion after being co-cultured with hepatoma cells.

Next, we determined if hepatoma cells have any regulatory effects on the ER of CTLL-2 cells. The status of the ER in different groups was analyzed using western blots by detecting the expression of ER stress-related proteins. In contrast to the other groups, GRP78 and P-PERK protein expression levels were significantly upregulated in CTLL-2 cells co-cultured with Hepa1-6 cells (Fig. 2C). Secondly, the substructure of the ER was observed using TEM. The substructure of the ER in the control and NC groups were arranged in order and continuously. The substructure of the ER in the co-culture group was severely damaged, showing gross expansion and degranulation (Fig. 2D). These results show that hepatoma cells can induce CTLs to undergo ER stress.

3.3 ER Stress Regulates the Function of CTLs Induced by Hepatoma Cells

Based on the role of ER stress in cellular function, we investigated whether ER stress was involved in the hepatoma cell-induced dysfunction of CTLL-2 cells. 4-PBA, an inhibitor of ER stress, was added to the co-culture system. The results showed that after 4-PBA intervention, the expression levels of PD-1 and TIM-3 were downregulated (Fig. 2E), while the secretion of Granzyme B (Fig. 2F) and perforin (Fig. 2G) were increased. Taken together, these data suggest that ER stress is involved in the hepatoma cell-mediated inhibition of CTL functions.

3.4 Gln Deprivation Induced ERS and Dysfunction of CTLs

The RNA-Seq results (Fig. 2A) showed that genes related to the amino acid stimulation response were upregulated in CTLs after co-culture with hepatoma cells (Fig. 2A), while Gln is essential for cell metabolic and cellular proliferation. Therefore, we hypothesized that Gln is involved in the process by which hepatoma cells inhibit CTL functions. To examine this, we measured the Gln concentration in each group. The data indicate that cells in the co-culture group consumed more Gln (Fig. 3A). Next, we cultured CTLL-2 cells using Gln-free medium. The results show that CTLs in the group lacking Gln had increased ex-pression levels of PD-1 and TIM-3 (Fig. 3B) and decreased secretion of Granzyme B and perforin (Fig. 3C,D). These data demonstrate that CTL functions are impaired following Gln deprivation. Finally, we examined the state of the ER. When cultured without Gln, there were elevated protein expression levels of GRP78 and P-PERK (Fig. 3E), as well as swollen ER (Fig. 3F). This suggests that ER stress has occurred. However, when 4-PBA was added to the medium when Gln was deprived, the expression levels of GRP78 and P-PERK were not significantly different from those of the control group. Additionally, no significant ER swelling was observed with these conditions. Overall, hepatoma cells compete with CTLs for Gln availability. Gln deficiency inhibited the function of CTLs and induced ER stress.

3.5 Dysfunction of CTLs Induced by Hepatoma Cells Through Gln-GLS2-ERS Pathway

To investigate how Gln is utilized by CTLs, we detected the expression of glutaminase and glutamine transporters (SLC1A5) in cells. The expression levels of GLS2 and SLC1A5 proteins were significantly reduced in CTLL-2 cells co-cultured with Hepa1-6 cells (Fig. 4A). Next, CTLs deprived of Gln also showed low GLS2 and SLC1A5 expression levels (Fig. 4B). Additionally, there was no significant difference in GLS1 expression. Unlike CTLs, GLS1 protein expression levels were significantly reduced in Hepa1-6 cells in the absence of Gln (Fig. 4B). Thus, we speculated that CTLs mainly use GLS2 to utilize Gln. GLS inhibitors (CB-839 and Compound 968) were added to the medium to further investigate the role of glutaminase. From the western blot results, we set the effective concentration of CB-839 to 4 μM (Fig. 5A) and the concentration of Compound 968 to 40 μM (Fig. 5B). CB-839 treatment had no effect on PD-1, TIM-3, GRP78, and P-PERK expression levels and ER stress activation in CTLs compared with controls. However, CTLs treated with Compound 968 had increased protein levels of PD-1 and TIM-3, as well as decreased secretion of Granzyme B and perforin and an enhanced ER stress response (Fig. 5C–G). Overall, these results indicate that GLS2 deficiency inhibits the function of CTLs and induces an ER stress response.

4. Discussion

Tumor-infiltrating CTLs exhibit loss of antitumor immunity due to altered organelle function and abnormal protein expression caused by long-term antigen exposure, tumor microenvironment inhibition [3], and regulation of signaling molecules (e.g., MYD88) [17]. Dysfunctional CTLs highly express inhibitory receptors, such as PD-1, TIM-3, CTLA-4, and 2B4 [18]. Additionally, decreased expression of effector molecules such as Granzyme B and perforin is another important feature of dysfunctional CTLs. Under conditions of chronic viral infection or cancer, immune checkpoint molecules are highly induced on activated
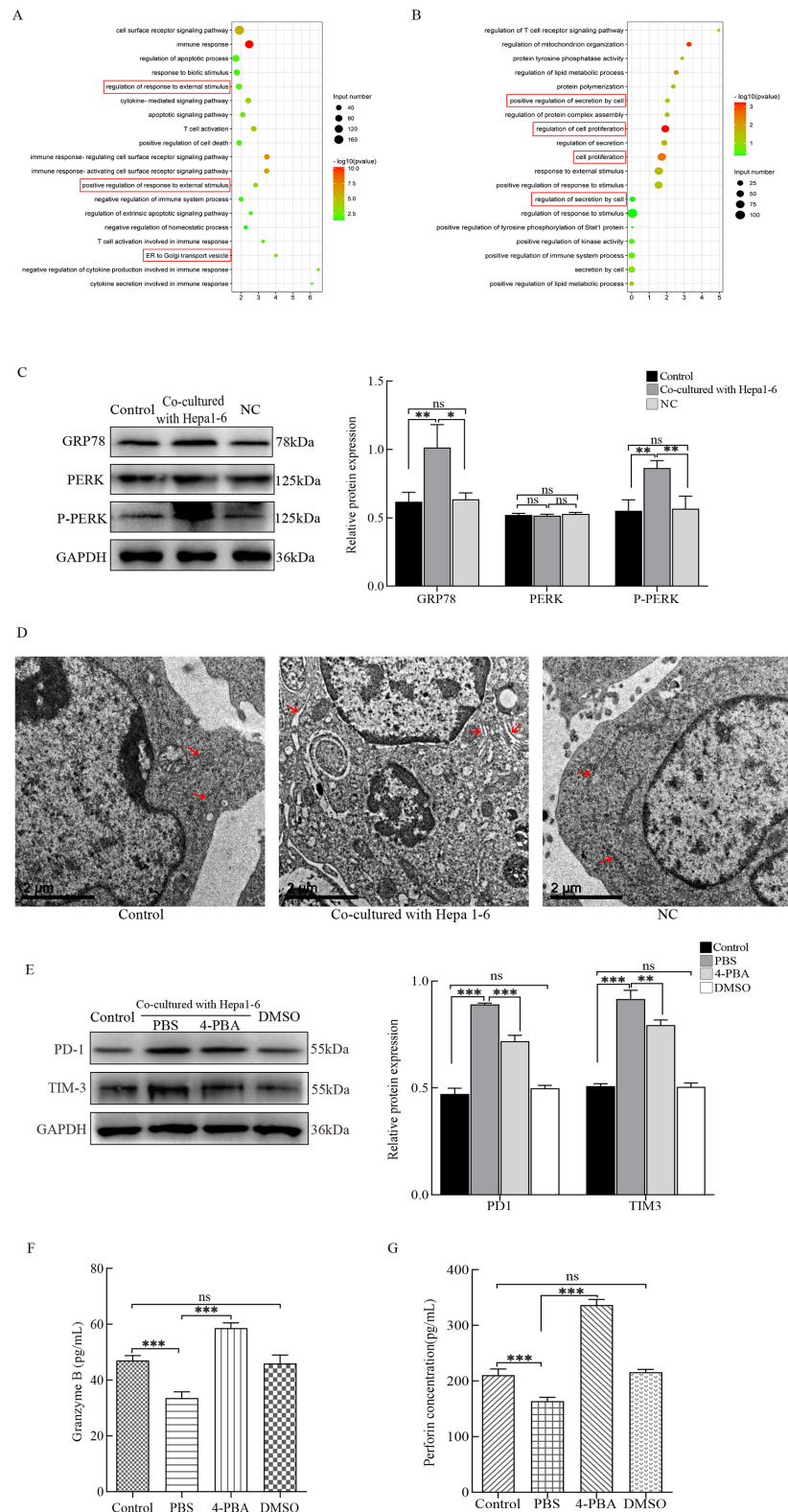
Fig. 2. Detection of endoplasmic reticulum (ER) stress and its effects on cytotoxic T lymphocyte (CTL) functions. RNA-seq was used to detect differentially expressed genes in hepatocarcinoma-infiltrating CTLs. (A) Gene ontology (GO) Enrichment Plot of upregulated genes. (B) GO Enrichment Plot of downregulated genes. (C) The protein expression levels of GRP78, PERK, and P-PERK in CTLs were detected by western blots. (D) Morphological changes of the ER were observed by transmission electron microscopy. (E) Protein levels of PD-1 and TIM-3 in CTLs cultured with 4-PBA were analyzed using western blots. The secreted levels of (F) Granzyme B and (G) perforin in CTLs cultured with 4-PBA were assessed by enzyme-linked immunosorbent assays (ELISAs). *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

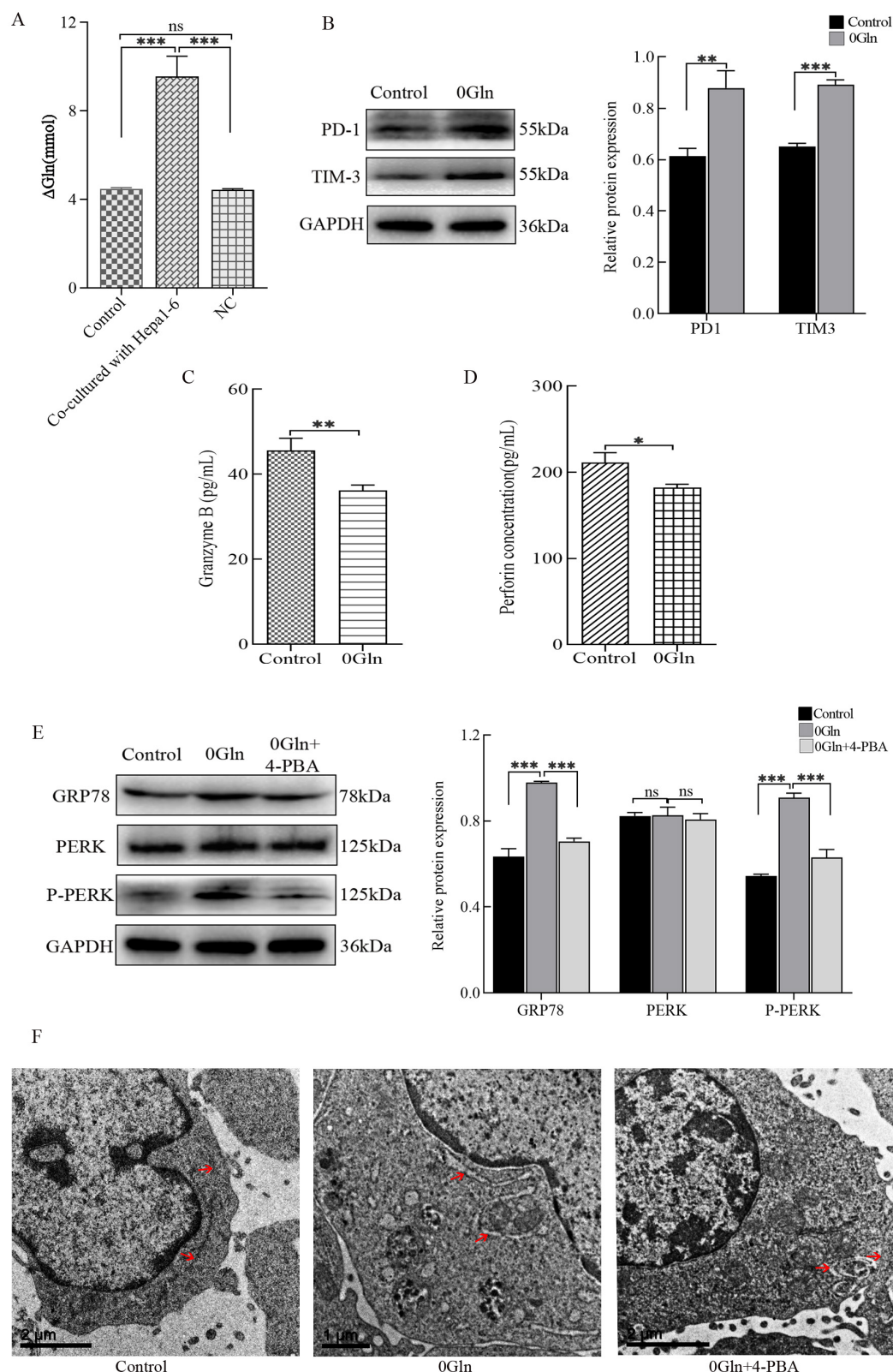**Fig. 3. Effect of glutamine (Gln) deprivation on cytotoxic T lymphocytes (CTLs) co-cultured with hepatoma cells.** (A) The Gln content in the supernatant of each culture group was detected by colorimetry. Activated CTLs were cultured in Gln-deprived RPMI 1640 medium, and (B) PD-1 and TIM-3 protein expression was detected by western blots. (C) Granzyme B and (D) perforin secretion was detected by enzyme-linked immunosorbent assays (ELISAs). (E) 4-PBA was added to Gln-deprived RPMI 1640 medium, and western blots were used to assess GRP78, PERK, and P-PERK protein expression levels. (F) Transmission electron microcopy was used to observe the morphology of the ER in these cells. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.
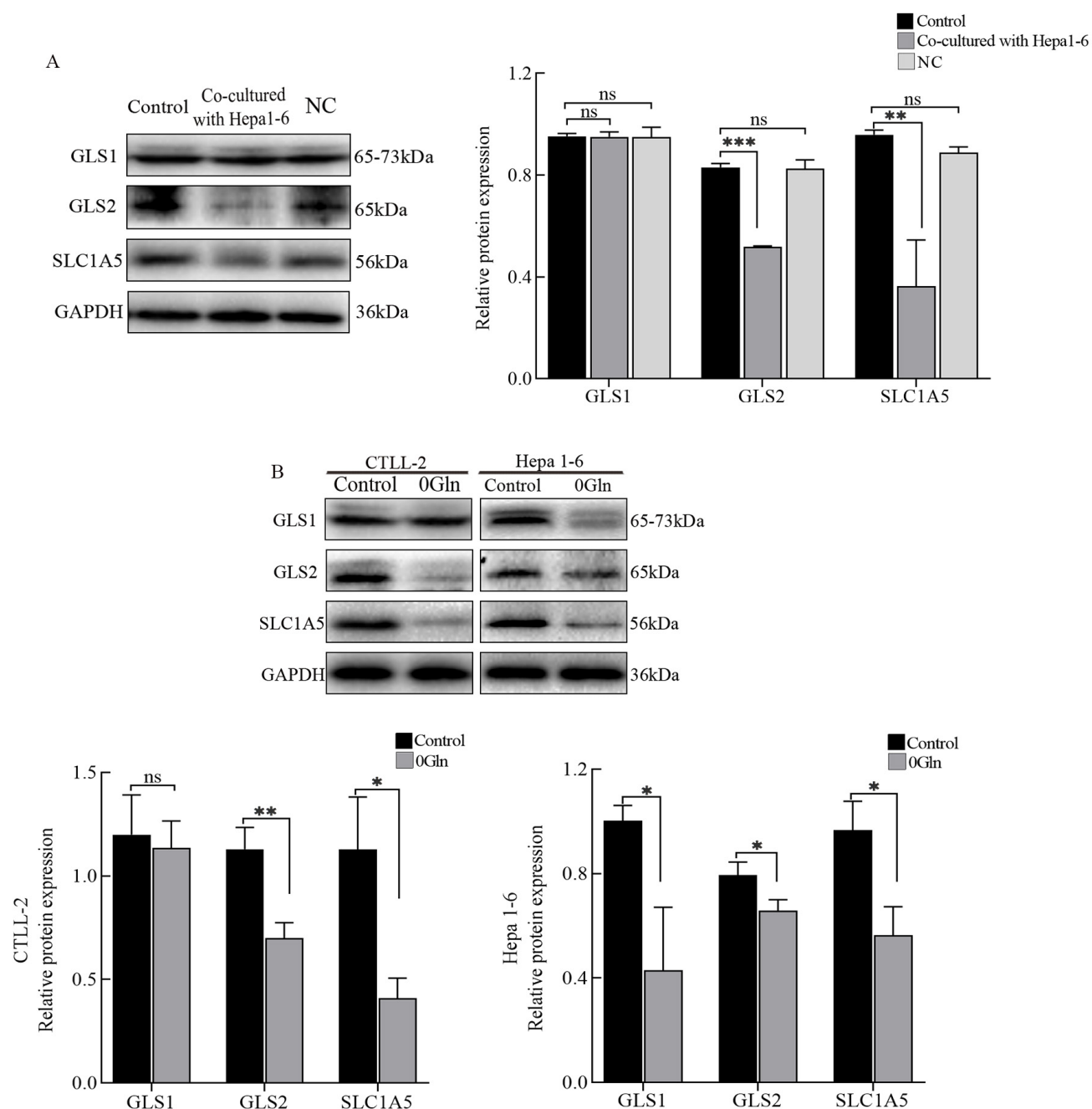
Fig. 4. Glutamine (Gln) utilization in cytotoxic T lymphocytes (CTLs) and hepatoma cells. (A) Western blots were used to detect the protein expression levels of GLS1, GLS2, and SLC1A5 in the different groups. (B) After Gln deprivation, protein expression levels of GLS1, GLS2, and SLC1A5 in both CTLs and hepatoma cells were assessed by western blots. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

T cells to keep the immune response in check [19]. Our experiments here showed that stimulation with hepatoma cells increased the expression levels of PD-1 and TIM-3 in CTLs, while decreasing the secretion of Granzyme B and perforin. These results indicate that an inhibition of CTL functions is induced by hepatoma cells.

The regulation of cellular functions is closely related to organelle functions, such as those of the ER [20] and mitochondria [21]. Reactive metabolic byproducts induced by the TME, like 4-hydroxynonenal (4-HNE), promote the

development of ER stress responses in infiltrating tumor-associated dendritic cells (tDCs) [22]. Our RNA-Seq results indicated that there is an upregulation of genes involved in ER-related reactions. This suggested that the ER of CTLs is altered upon stimulation by hepatoma cells. Further experiments demonstrated that CTLs expressed high levels of GRP78 and PERK, and their ER was swollen after hepatoma cell stimulation. These findings suggest that the ER stress response is activated and ER morphology is altered in CTLs under hepatoma cell induction. Inhibition
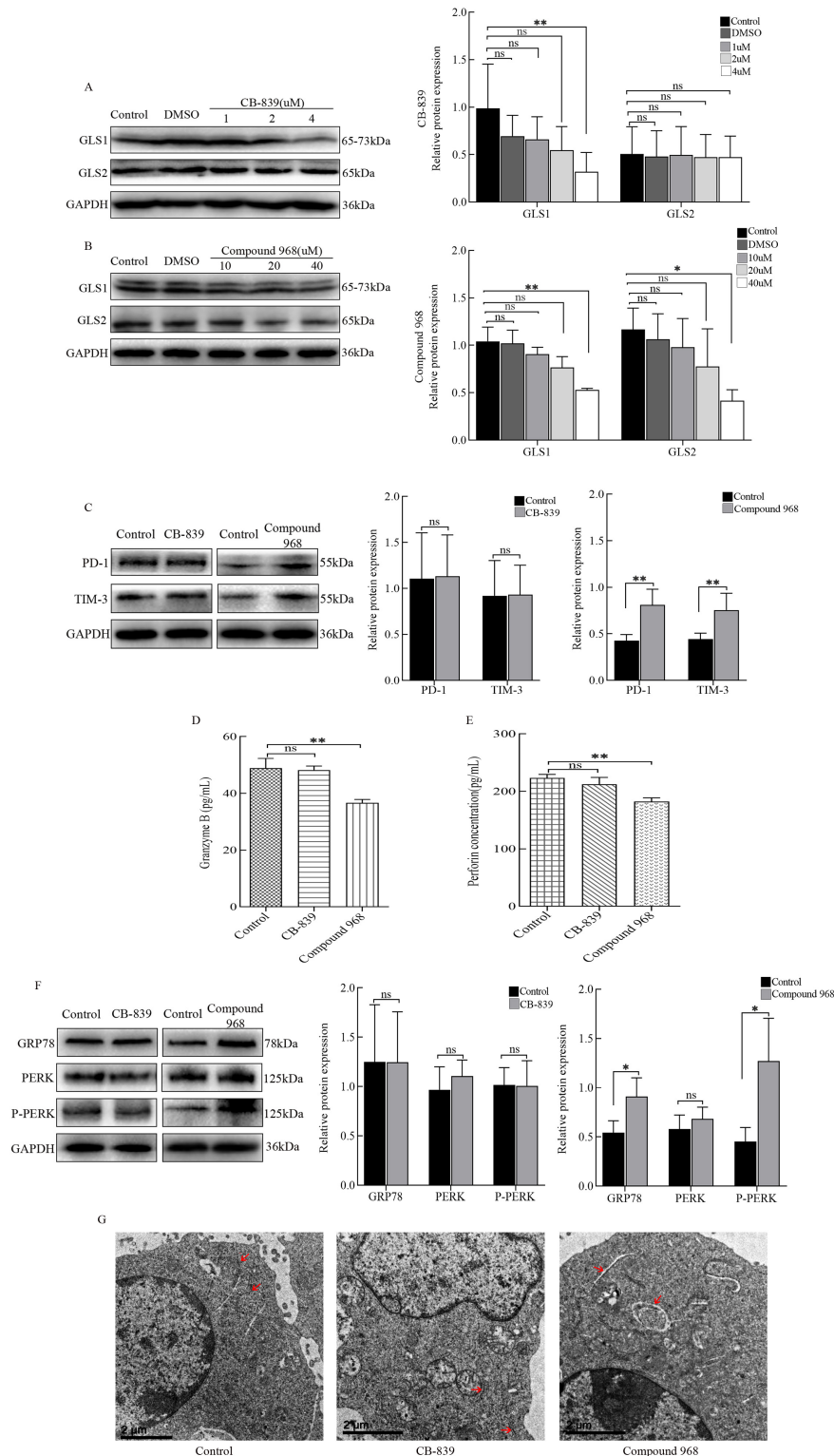
Fig. 5. Regulation of cytotoxic T lymphocytes (CTLs) by various glutaminases. (A) CTLs were cultured using RPMI 1640 medium containing different concentrations of CB-839, and the protein expression levels of GRP78, PERK, and P-PERK were detected by western blots. (B) CTLs were cultured using RPMI 1640 containing different concentrations of Compound 968, and the protein levels of GRP78, PERK, and P-PERK were detected by western blots. CTLs were cultured using RPMI 1640 containing 4 μM of CB-839 or 40 μM of Compound 968. (C) The protein expression levels of PD-1 and TIM-3 were detected by western blots. Secretion of (D) Granzyme B and (E) perforin were measured by enzyme-linked immunosorbent assays (ELISAs). (F) Protein expression of GRP78, PERK, and P-PERK was assessed by western blots. (G) Transmission electron microscopy was performed to observe the morphology of the ER in these cells. *$p < 0.05$; **$p < 0.01$; $^{ns}p > 0.05$.

of the ER stress signaling pathway results in the development of memory T cells responding to acute infection [23]. Next, we explored the relationship between ER stress and cellular function in CTLs. As mentioned earlier, 4-PBA is an ER stress inhibitor. Our data using 4-PBA showed that after ER stress was inhibited, the expression levels of PD-1 and TIM-3 in CTLs were reduced. Additionally, the secretion of Granzyme B and perforin was increased, suggesting the recovery of CTL functions. These data show that inhibition of the ER stress response can upregulate the effector functions of CTLs.

Normally, homeostasis of the ER internal environment is a fundamental condition for maintaining proper ER function. However, hypoxia [12], low pH [4], nutrient deficiency [11], or hyperalimentation (e.g., high glucose) [24], and ER dysfunction can cause ER stress to occur. Multiple stressors enriched in the TME can provoke maladaptive ER stress responses to control the effector function of infiltrating T cells [14]. Because of the high metabolic demand and unlimited proliferative ability of tumor cells, the nutrient availability within the microenvironment is changed, making it difficult for the infiltrating immune cells to obtain the key nutrients required for protein folding [25]. Our RNA-Seq results showed that genes associated with amino acid stimulation responses and the ER in CTLs induced by hepatoma cells were regulated, such as "the regulation of the lipid metabolic process", "cellular secretion" and "positive regulation of secretion by cells". Gln deprivation had a negative effect on the M2 macrophage activation program, reducing the committed cell population by approximately 50% [26]. In ovarian cancer, T cell mitochondrial dysfunction can be caused by inhibition of X-box binding protein 1 (XBP1)-mediated Gln influx [27]. These results shown in the literature suggest a close link between Gln metabolism and organelle function. In the present study, hepatoma cells consumed more Gln when co-cultured. With Gln deprivation, the ER of CTLs became swollen and expression levels of GRP78 and PERK were elevated. This suggested that CTLs underwent the ER stress response. Simultaneously, expression of PD-1 and TIM-3 increased and the secretion of Granzyme B and perforin decreased, indicating that the functions of CTLs were inhibited. These results suggest that Gln deprivation can downregulate CTL functions and the induction of the ER stress response.

Gln is transported into cells through one of many transporter proteins, such as the solute carrier family 1 neutral amino acid transporter member 5 (SLC1A5; also known as ASCT2) [28], and is then available for biosynthesis by glutaminase. Glutaminase, which exists in multiple tissue-specific versions, is encoded by two genes in mammals, kidney-type glutaminase (GLS1) and liver-type glutaminase (GLS2) [29]. GLS1 is more widely expressed in normal tissues and thought to be highly expressed in many cancers, such as liver cancer [30]. However, the data in this study showed that GLS2 was highly expressed in CTLs in-

duced by hepatoma cells. When Gln deprived, CTLs had low GLS2 expression, whereas the change in GLS1 levels was not significant. The CTL functions and the ER status were not affected by the GLS1 inhibitor CB-839. Upon addition of the glutaminase inhibitor compound 968, the protein levels of PD-1 and TIM-3 in CTLs increased, and the secretion of Granzyme B and perforin decreased. GRP78 and P-PERK expression levels also increased, and the ER became swollen. These results indicate that when GLS2 is inhibited, CTLs undergo the ER stress response and further downregulate cellular functions. Overall, GLS2 has an important role in the Gln utilization process in CTLs, which is different from the use of GLS1 by hepatoma cells.

5. Conclusions

In summary, CTLs are functionally downregulated induced by hepatoma cells through the Gln-GLS2-ERS pathway.

Author Contributions

JW conceived the idea and designed the research study. WW and MG performed the research. ZB and WB provided help and advice on data analysis. WW, WC and YS analyzed the data. WW wrote the manuscript. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript.

Ethics Approval and Consent to Participate

The study was approved by the Tangshan Maternal and Child Health Care Hospital affiliated to North China University of Science and Technology (No. 2022-064-01).

Conflict of Interest

The authors declare no conflicts of interest.

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