CD155/TIGIT Signaling Regulates CD8+ T Cell Metabolism and Promotes Tumor Progression in Human Gastric Cancer

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Abstract

The T cell surface molecule TIGIT is an immune checkpoint molecule that inhibits T cell responses, but its roles in cancer are little understood. In this study, we evaluated the role TIGIT checkpoint plays in the development and progression of gastric cancer (GC). We show that the percentage of CD8 T cells that are TIGIT+ was increased in GC patients compared to healthy individuals. These cells showed functional exhaustion with impaired activation, proliferation, cytokine production and metabolism, all of which were rescued by glucose. In addition, GC tissue and cell lines expressed CD155, which bound TIGIT receptors and inactivated CD8 T cells. In a T cell-GC cell co-culture system, GC cells deprived CD8 T cells of glucose and impaired CD8 T cell effector functions; these effects were neutralized by the additional glucose or by TIGIT blockade. In GC tumor cells, CD155 silencing increased T cell metabolism and IFNγ production, whereas CD155 overexpression inhibited T cell metabolism and IFNγ production; this inhibition was neutralized by TIGIT blockade. Targeting CD155/TIGIT enhanced CD8 T cell reaction and improved survival in tumor bearing mice. Combined targeting of TIGIT and PD-1 further enhanced CD8 T cell activation and improved survival in tumor bearing mice. Our results suggest that GC cells inhibit CD8 T cell metabolism through CD155/TIGIT signaling, which inhibits CD8 T cell effector functions, resulting in hyporesponsive antitumor immunity. These findings support the candidacy of CD155/TIGIT as a potential therapeutic target in gastric cancer.

Key Words: gastric cancer, CD155/TIGIT, CD8 T cells, glucose metabolism
Introduction

Gastric cancer (GC) is one of the most common malignancies worldwide (1). Five-year survival rate of GC is lower than 30% and the current therapeutic methods show little improvement for GC survival (2). Tumor-specific cytotoxic T cells are present in GC tissue, but are unable to contain tumors because of poor immune responses in the tumor microenvironment (3). Reversing this effect would be a potential therapeutic approach to enhance the effectiveness of current treatments. The mechanisms by which GC inhibits antitumor immune responses are poorly understood that targets cannot be identified.

The balance of positive and negative signals is crucial to maintain host immune tolerance and activation (4,5). Immune checkpoints are molecules in the immune system that either turn on a positive (co-stimulatory) signal or turn off a negative (co-inhibitory) signal. Antibody treatments that target immune checkpoints have significantly improved clinical outcomes of solid and hematological malignancies (6,7). Malignant tumors, including GC, escape antitumor immune responses by upregulating co-inhibitory signals, such as PD-1/PD-L1, in the tumor microenvironment (8,9). Phase I clinical trial has provided promising antitumor activity by targeting PD-1/PD-L1 signal (10), warranting further investigation to the immune checkpoints to have better outcomes for GC.

The T cell immunoreceptor with immunoglobulin and ITIM domains (TIGIT) has emerged as an important immune checkpoint in recent years. TIGIT, which belongs to the CD28 family (11), and CD226 share the common ligand CD155; binding of CD155 to TIGIT suppresses T cell activation (12), while binding to CD226 enhances T cell activation (13). TIGIT expression is...
increased in tumor-infiltrating lymphocytes (TILs) and tumor antigen-specific CD8 T cells in melanoma patients (14). Blocking TIGIT enhances CD8 T cell effector functions in tumor-bearing mice (15). In addition, CD155 expression is increased in melanoma cells and the T cell response is inhibited via TIGIT-CD155 interactions (16). However, the mechanisms of CD155/TIGIT-induced immune suppression and subversion in GC remain poorly understood.

Co-stimulatory and co-inhibitory signals interact to activate T cells by regulating metabolic activity (17,18). T cell metabolism is highly dynamic and controls T cell activation, proliferation and differentiation (19,20). Upon initial antigenic stimulation, T cells increase in size and switch their metabolism to glycolysis, which permits proliferation and effector functions (21,22). During T cell clonal expansion, T cells preferentially metabolize glucose to fulfill their increased energy requirements (23). Failure to fulfill the increased bioenergetic demands of cell growth results in deleted or unresponsive T cells (20).

T cell activation, which is critical for the antitumor immune response (24), depends on the AKT/mTOR signaling pathway. AKT promotes glucose metabolism by increasing glucose transporter 1 (Glut1) expression, which facilitates glucose uptake in T cells (17), so mTOR signaling integrates immune signals and metabolic cues in T cells (25). Previous studies have demonstrated that AKT/mTOR signaling and T cell metabolism are decreased in the tumor microenvironment (26), and that limited nutrients in the tumor microenvironment impairs the T cell antitumor immune response (27).
Here, we report that TIGIT\(^+\) T cells were significantly increased in GC patients. TIGIT\(^+\) CD8 T cells underwent metabolic reprogramming and exhibited functional exhaustion. CD155 expressed by GC cells interacted with TIGIT, resulting in the inhibition of glucose uptake and impaired T cell effector functions. CD155/TIGIT pathway blockade enhanced T cell effector functions and suppressed tumor progression. This study provides a potential treatment target for GC.

**Materials and methods**

**Patients**

Peripheral blood and primary tumor tissues were collected from 138 clinically and pathologically verified gastric cancer (GC) patients from the First Affiliated Hospital, Sun Yat-sen University, Guangzhou China; Eighth Affiliated Hospital, Sun Yat-sen University, Shenzhen China; Sun Yat-sen University Cancer Center and Guangdong Provincial Hospital, Guangzhou, China. Infection and autoimmune diseases were excluded. A previous study has shown that TIGIT is upregulated in CMV\(^+\) CD8 T cells. To exclude the contaminated effects in CD8 T cells induced by CMV infection, CMV-infected cases were excluded from the present study. This study was approved by the Institutional Review Board of First Affiliated Hospital, Sun Yat-sen University. Consent was informed and consent forms were obtained from every patient. Patient studies were conducted in accordance with ethical guideline: Declaration of Helsinki. Clinical and pathological characteristics of the included patients are shown in Supplementary Table 1.
Cell isolation

Blood samples were collected from GC patients or age- and sex-matched healthy controls (HCs). Peripheral blood mononuclear cells (PBMCs) were isolated with Ficoll-Hypaque by density gradient centrifugation within 2 h of sample collection. Fresh tumor tissues were obtained from GC patients during surgical tumor resection. Samples were minced and digested with type I collagenase (Sigma, USA) in RPMI 1640. Digested cells were filtered through a nylon mesh (70μm) and washed with phosphate-buffered saline (PBS). CD8\(^+\)TIGIT\(^+\), CD8\(^+\)TIGIT\(^-\), CD4\(^+\)TIGIT\(^+\), or CD4\(^+\)TIGIT\(^-\) cells were sorted using a BD FACS Influx. Total and naïve CD8 T cells were purified from PBMCs by negative selection using the EasySep™ human total or naïve CD8\(^+\) T Cell enrichment kits (STEMCELL Technologies Inc., Vancouver, Canada). Cell purity was checked (>95%, Supplementary Fig. 1 A-C).

Cell culture

Human GC cell lines SGC7901, HGC27, and BGC823 were obtained from the type Culture Collection of Chinese Academy of Sciences (Shanghai, China) (28). Cell lines were authenticated by cell viability analysis, short tandem repeat (STR) profiling, and isoenzyme analysis. Cell lines were screened for mycoplasma contamination. Cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 50 U/mL penicillin, and 50 mg/mL streptomycin in a humidified atmosphere at 37°C with 5% CO\(_2\).
Plasmids, retroviral infection and transfection

CD155 constructs were generated by sub-cloning PCR-amplified full-length human CD155 cDNA into pcDNA3.1. To deplete CD155, siRNA sequences were cloned into GV248 to generate GV248-RNAi(s) targeting CD155. siRNA duplexes were synthesized and purified by RiboBio Inc. (Guangzhou, China). The CD155 siRNA sequences were as follows: sense, 5'-GGUAUCCAUCUCUGGCUAUTT-3'; antisense, 5'-AUAGCCAGAGAUGGAUACCTT-3'. siRNAs transfection was carried out using Lipofectamine 2000 reagent (Invitrogen Co., Carlsbad, CA) according to the manufacturer's instructions. Stable cell lines expressing CD155 or CD155 RNAi(s) were selected via treatment with 0.5 μg/ml puromycin for 10 days beginning 48 h after infection. Following selection, GC cell lysates prepared from the pooled cell populations in sampling buffer were fractionated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS–PAGE) to detect protein levels via western blotting (29).

Flow cytometry

PBMCs isolated from GC patients or HCs were stained with the following antibodies: FITC-conjugated anti-CD4, PE-conjugated anti-CD8, APC-conjugated anti-TIGIT, and FITC-conjugated CD226. Single-cell suspensions from gastric tumor tissues were stained with FITC-conjugated anti-CD4, PE-conjugated anti-CD8, and APC-conjugated anti-TIGIT antibodies. Sorted CD8+TIGIT+ or CD8+TIGIT- cells were stained with PE-Cy7-conjugated CD69, FITC-conjugated anti-PD-1, and V450-conjugated anti-TIM-3 antibodies. Cultured T cells were stained with APC-conjugated p-AKT, PE-CY7-
conjugated p-S6K and Pe-Cy5.5 conjugated p-4EBP1 antibodies. GC tumor cell lines were stained with an APC-conjugated CD155 antibody. A commercial kit from Roche was used to analyze cell apoptosis. Samples were analyzed using a BD FACS ARIA (BD Bioscience).

**T cell function assays**

For T cell activation assays, cells were seeded in 96-well plates and stimulated with anti-CD3/CD28 Dynabeads (αCD3/CD28) for 12 h to measure CD69 expression by flow cytometry. For the proliferation assay, cells were labeled with CFSE and stimulated with αCD3/CD28 at 37°C with 5% CO₂ for 4 days. Cells were collected, and the dilution of intracellular CFSE caused by proliferation was calculated using a flow cytometer (30). For intracellular cytokine stimulation assays, cells were stimulated with 500 ng/ml PMA and 1 μg/ml ionomycin (Sigma–Aldrich) for 5 h at 37°C with 5% CO₂. During the last 2 h, 1 μg/ml Brefeldin A was included. Cells were collected and stained with V450-conjugated anti-IFNγ and PE-Cy7-conjugated anti-TNF-α antibodies.

**Co-culture**

CD8⁺TIGIT⁺ or CD8 T cells were sorted and co-cultured with GC SGC7901 cells in 48-well plates at a ratio of 5:1. Cells were stimulated with αCD3/CD28 in the presence of 5 μg/ml anti-TIGIT blocking antibody (BPS Bioscience, California, USA) or isotype control. Alternatively, cells were treated with 10 mM glucose. T cells were collected to determine the activation, proliferation and cytokine production using the described T cell function assays. The
phosphorylation of AKT, mTOR, S6K and 4E-BP1 in CD8 T cells was measured by western blotting or flow cytometry.

**Glucose consumption assay**

2-Deoxyglucose (2-DG) is a glucose analog taken up by glucose transporters and metabolized to 2-DG-6-phosphate (2-DG6P). 2-DG6P cannot be further metabolized and accumulates in cells. 2-DG6P is oxidized to generate NADPH, which can be measured by an enzymatic recycling amplification reaction. T cells (2 × 10^5/well) were stimulated with αCD3/CD28 for 8 h. Cells were washed with PBS three times and then glucose-starved by plating with 100 μl of Krebs-Ringer-Phosphate-HEPES buffer containing 2% BSA for 40 minutes. Then, cells were stimulated with or without insulin (1 μM) for 20 minutes. A total of 10 μL 10 mM 2-DG was added to the cells for 20 minutes. Glucose levels in the cells were analyzed using a glucose assay kit (Sigma–Aldrich, USA) according to the manufacturer's instructions.

**Lactate production assay**

CD8^+TIGIT^+ or CD8^+TIGIT^- cells (2 × 10^5/well) were stimulated with αCD3/CD28 for 8 h, then cultured with fresh complete medium containing glucose. Lactate concentrations were analyzed in triplicate using a lactate assay kit (Abcam, UK) according to the manufacturer’s instructions.

**Humanized NOG mouse tumor model**

NOD.Cg-Prkdc^{scid}Il2rg^{tm1Sug}JicCrl (NOG) mice (Weitonglihua experimental animal Co., Ltd, Beijing, China) are immunodeficiency that they can receive
human immune cells. In the humanized mice, we can study immune reaction of human cells against tumor and the underlying mechanisms. PMBC were isolated from HCs and 2×10⁷ human PBMCs were injected into the mice peritoneally to reconstitute human immune system. Circulating human T cells were evaluated by flow cytometry. To investigate the antitumor effects by target human T cells, mice were subcutaneously inoculated with 2 × 10⁶ SGC7901 or SGC7901-CD155 RNAi. Mice were treated with an anti-PD-L1 antibody or isotype control. The mice were monitored three times per week for evidence of morbidity and mortality associated with tumor growth and metastasis. In vivo bioluminescence imaging was performed by using the IVIS Imaging System. The Living Image acquisition and analysis software (Caliper Life Sciences) were used together as described before (31).

Statistical analyses

Data are expressed as means ± SEM. Statistical analysis was performed using SPSS version 13.0. Differences were assessed using either the Student’s t-test or one way ANOVA with or without repeated measurements followed by Bonferroni’s multiple comparison post-test, as appropriate. Two-tailed P values < 0.05 were considered statistically significant.

Results

TIGIT+ T cells are associated with immune subversion in patients with gastric cancer.
TIGIT+ T cells expand during malignancy (32). Here, to determine whether TIGIT+ T cells are expanded in GC, we compared TIGIT expression in T cells from GC patients or HCs by flow cytometry. The percentage of CD4+TIGIT+ and CD8+TIGIT+ T cells was increased significantly in GC patients compared with age- and sex-matched HCs (Fig. 1A–C). The percentage of TIGIT+ T cells decreased after surgical removal of the tumor tissue and increased again after tumor recurrence (Fig. 1D–F). Additionally, TIGIT was strongly expressed in tumor-infiltrating lymphocytes (TILs) (Fig. 1G–I). TIGIT+ T cells from PBMCs displayed a memory phenotype. TIGIT was expressed in CD45RA–CD45RO+ memory T cells, but not in CD45RA+CD45RO- naïve T cells (Supplementary Fig. 2A and B). CD226 is the co-stimulatory molecule competing with TIGIT for CD155 and the initiation of CD226 results in T cell activation (33). Compared to HCs, fewer CD226-positive CD8 T cells had been identified in GC patients (Supplementary Fig. 3A and B). CD4 and CD8 T cell compartments were not significantly different in HCs and GC patients (Supplementary Fig. 4A and B), indicating that increased TIGIT expression on T cells is responsible for the higher number of TIGIT-positive T cells in GC patients.

TIGIT is associated with T cell exhaustion. Next, we investigated the effect of increased TIGIT expression on T cell effector functions in patients with GC. CD8+TIGIT+ or CD8+TIGIT- cells were sorted from GC PBMCs by flow cytometry and stimulated with anti-CD3/CD28 Dynabeads (αCD3/CD28). More TIGIT+ T cells were CD69-positive than TIGIT+ T cells (Fig. 1J and K). Additionally, proliferation rate was significantly lower in TIGIT+ CD8 T than TIGIT- CD8 T cells (Fig. 1L and M). Cytokines of IFNγ and TNF-α production...
(Fig. 1N and O) and cell migration (Fig. 1P) were impaired in TIGIT\textsuperscript{+} CD8 T cells. While apoptosis was higher in TIGIT\textsuperscript{+} CD8 T cells (Supplementary Fig. 5A and B). Furthermore, TIGIT\textsuperscript{+} CD4 T cells from GC patients exhibited characteristics functional exhaustion that was similar with CD8 T cells (Supplementary Fig. 6A-F).

In summary, the expansion of TIGIT\textsuperscript{+} T cells was in accordance with immune subversion in GC, implying immune escape in GC through the upregulation of TIGIT.

**Metabolic reprogramming of TIGIT\textsuperscript{+} CD8 T cells in gastric cancer patients.**

Glucose uptake and glycolysis increase rapidly when T cells are activated (20). To explore metabolic changes in TIGIT\textsuperscript{+} CD8 T cells, we evaluated the expression of genes involved in metabolic reprogramming by RT-PCR first. Expression of metabolism-associated genes was significantly reduced in TIGIT\textsuperscript{+} CD8 T cells compared with TIGIT\textsuperscript{−} CD8 T cells (Fig. 2A). Glut1 has been very important for glucose uptake in T cells and hexokinase 1 and hexokinase 2 (HK1/HK2) are the key kinases that initiate the process of glycolysis. The downregulation of Glut1 and HK1/HK2 in TIGIT\textsuperscript{+} T cells was confirmed by flow cytometry and western blotting, respectively (Fig. 2B–D). The AKT/mTOR pathway regulates glycolysis and is important for cell growth and proliferation. Western blot analysis revealed that phosphorylation of AKT and mTOR was significantly downregulated in TIGIT\textsuperscript{+} T cells (Fig. 2E). Furthermore, flow cytometric analysis showed decreased expression of p-S6K
and p-4EBP1, which are downstream targets of mTOR, in TIGIT+ T cells (Fig. 2F–H).

To determine whether these changes were associated with changes in T cell metabolism, we measured glucose uptake and lactate production in TIGIT+ and TIGIT− T cells. Glucose uptake was impaired in TIGIT+ CD8 T cells compared with TIGIT− CD8 T cells (Fig. 2I). In addition, lactate production was significantly lower in TIGIT+ CD8 T cells (Fig. 2J).

Taken together, these findings indicated that TIGIT reduced glucose uptake and inhibited T cell metabolism in GC patients.

**Glucose reverses impaired TIGIT+ T cell metabolism and rescues T cell functional exhaustion**

As glucose is the major cellular fuel that promotes T cell proliferation and survival, we investigated whether the addition of exogenous glucose could reverse TIGIT-associated T cell exhaustion. To do this, TIGIT− CD8 T or TIGIT+ CD8 T cells were sorted from GC PBMCs. Glucose treatment reversed metabolic activities of TIGIT+ CD8 T cells. Glucose uptake and lactate production were increased by glucose. Glucose also increased glucose uptake and lactate production in TIGIT− CD8 T cells (Supplementary Fig. 7A and B). Interestingly, fructose, another hexoses, could not reverse metabolic activities of TIGIT+ CD8 T cells (Fig. 3A and B). Next we were to explore whether glucose can reverse the exhaustion of TIGIT+ CD8 T cells. We found that glut1 expression was increased by glucose treatment, as determined by flow cytometry (Fig. 3C). This increased metabolism in TIGIT+ T cells was accompanied by enhanced T cell effector functions, as the percentage of
CD69-positive T cells and T cell proliferation were increased after glucose treatment (Fig. 3D-G). In addition, TIGIT$^+$ T cell migration and cytokine production were restored by glucose (Fig. 3H-J). Additionally, glucose increased IFNγ production in a dose-dependent manner (Fig. 3K-L).

Taken together, these results indicate that TIGIT may have negative regulatory effects on T cell metabolism, which can be reversed by glucose.

**Gastric cancer cells deprive CD8 T cells of glucose**

To investigate why T cell metabolism is inhibited from the GC, we used the co-culture of CD8 T cells from HC PBMCs and GC SGC7901 cells. As shown in the heat map in Fig. 4A, metabolism-associated gene expression was suppressed in CD8 T cells co-cultured with SGC7901 compared to CD8 T cells without co-cultured with GC cells. Glut1 expression was reduced and CD8$^+$ T cell expression of the downstream molecules, HK2, was also downregulated when compared to CD8 T cells without co-cultured with GC cells (Fig. 4B–D). Glucose uptake by CD8 T cells was significantly inhibited when CD8 T cells were co-cultured with SGC7901 (Fig. 4E). In addition, lactate production in CD8 T cells was much lower in SGC7901-T cell co-cultures (Fig. 4F). Furthermore, we found that the phosphorylation of mTOR and its downstream molecules, S6K and 4E-BP1, was inhibited in T cells when co-cultured with SGC7901 (Fig. 4G–K). Additionally, T cell cytokine production (IL-2, TNF-α and IFNγ) was decreased significantly in SGC7901-T cell co-cultures (Fig. 4L-N). We confirmed these findings in another GC cell line, HGC27 (Supplementary Fig. 8A–D). These data demonstrate that GC
cells could inhibit T cell metabolism in tumor microenvironment to turnover T cell effector functions.

Next we were to investigate whether glucose can affect the effector function of CD8 T cells that are co-cultured with GC cells. CD8 T cells from HC PBMC were co-cultured with SGC7901 with or without the additional glucose. We found that the inhibition T cell function was neutralized by glucose when co-cultured with SGC7901. The percentage of IFNγ-producing T cells increased after supplementation of the co-culture system with glucose (Supplementary Fig. 9A and B). However, TIGIT expression was not affected by glucose supplementation (Supplementary Fig. 9C and D).

These findings suggest that GC cells impair T cell function by depriving them of glucose, and that this impairment can be reversed by the addition of exogenous glucose.

**TIGIT blockade reverses the inhibition of T cell metabolism and cytokine production by gastric cancer cells**

As data shown above that GC cells can inhibit T cell metabolism. We were to investigate how GC cells affect T cell metabolism and effector functions. CD8 T cells were isolated from HC PBMCs and co-cultured with GC cell line of SC7901. TIGIT expression was unregulated in T cells when co-cultured with SGC7901 (Fig. 5A). Circulating tumor cells (CTCs) were enumerated using a NanoVelcro system, as previously described (34). The number of CTCs correlated closely with the percentage of CD8⁺TIGIT⁺ T cells (Fig. 5B), indicating CTCs might contribute to the increased TIGIT⁺ CD8 T cells in the circulation of GC patients.
As we observed that GC cells could induce TIGIT expression in CD8 T cells. And TIGIT was associated with T cell metabolism and T cell exhaustion. We next investigated whether TIGIT blockade could reverse SGC7901-induced changes in T cell metabolism and effector functions. CD8 T cells were sorted from HC PBMCs and co-cultured with GC cell line of SGC7901. TIGIT blocking antibody was used to block TIGIT signal. Blocking TIGIT increased metabolism-associated gene expression in CD8 T cells (Supplementary Fig. 10A). Increased Glut1 expression in CD8 T cells was confirmed at the protein level by flow cytometry (Fig. 5C and D). AKT and mTOR phosphorylation was increased in CD8 T cells after TIGIT blockade (Fig. 5E–G) compared to isotype control. Furthermore, blocking TIGIT increased p-S6K and p-4EBP1 expression by CD8 T cells when co-cultured with SGC7901 (Fig. 5H–J). These metabolic changes were associated with increased glucose uptake and lactate production in CD8 T cells (Fig. 5K and L). Consistent with this, GC cell-mediated inhibition of IFNγ production by CD8 T cells was reversed by blocking TIGIT (Fig. 5M, and N).

These observations demonstrate that GC cells induce TIGIT expression on CD8 T cells, through which GC cells inhibit T cell metabolism and impair T cell effector function.

**Gastric cancer cells inhibit T cell metabolism through CD155/TIGIT signaling**

Melanoma cells suppress T cell responses through CD155-TIGIT interactions (16). To investigate whether CD155 is involved in the inhibition on T cells mediated by GC cells, we tested CD155 expression on GC tissue and GC cell
lines. We found that CD155 expression was detected and significantly increased in GC tissue compared to normal gastric tissue (Fig. 6A and B). And the GC cell lines SGC7901, HGC27, and BGC823 all expressed CD155 (Fig. 6C) as detected by flow cytometry. These data indicate that GC could interact with T cells through CD155-TIGIT and affect T cell functions.

To determine whether CD155 expressed by GC cells regulates T cell metabolism and effector functions, we generated a SGC7901 cell line with stable downregulation of CD155 expression, SGC7901-CD155 RNAi. The downregulation of CD155 was confirmed by flow cytometry (Fig. 6D). CD8 T cells sorted from HC PBMCs were co-cultured with SGC7901-CD155 RNAi or SGC7901-vector. AKT, S6K and 4EBP1 phosphorylation in CD8 T cells was decreased by co-culture with SGC7901-vector cells. Downregulation of CD155 in SGC7901 cells (SGC7901-CD155 RNAi) increased AKT, S6K and 4EBP1 phosphorylation in CD8 T cells (Fig. 6E). Glucose uptake and lactate production in CD8 T cells were both decreased by SGC7901-vector cells, but this effect was reversed when CD155 was downregulated in SGC7901 cells (Fig. 6F and G). In addition, SGC7901-vector cells inhibited IFNγ production in CD8 T cells and this inhibition was reversed by CD155 downregulation in SGC7901 cells (Fig. 6H and I).

We also investigated the effect of CD155 overexpression in SGC7901 cells (SGC7901-CD155) on IFNγ production in T cells. CD8 T cells were co-cultured with SGC7901-vector or SGC7901-CD155 cells. Overexpression of CD155 in SGC7901 was confirmed by flow cytometry (Fig. 6J). SGC7901-vector inhibited IFNγ production compared to T cells stimulated with αCD3/CD28 alone. SGC7901-CD155 cells further decreased IFNγ production.
in CD8 T cells compared to SGC7901-vector cells, which could be neutralized by blocking TIGIT (Fig. 6 K and L).

Taken together, these findings indicated that GC cells inhibit T cell metabolism through CD155/TIGIT signaling pathways.

**TIGIT and PD-1 are co-expressed in CD8 T cells, and combined inhibition of TIGIT and PD-1 signals has synergistic effects.**

Study has shown that exhausted CD8 T cells co-expressed TIGIT and PD-1 and combined blockade these two signals demonstrates stronger effects in T cell activation compared to blocking either. To study whether TIGIT⁺ CD8 T cells from GC co-express PD-1, we analyzed PD-1 expression in CD8⁺TIGIT⁺ and CD8⁺TIGIT⁻ cells by flow cytometry. PD-1 expression was significantly higher in TIGIT⁺ CD8 T cells than in TIGIT⁻ CD8 T cells (Fig. 7A and B). In addition, the expression of another checkpoint molecule TIM-3, was also higher in TIGIT⁺ CD8 T cells (Supplementary Fig. 11A–D). We next investigated whether TIGIT and PD-1 have synergistic effects on T cell activation when co-cultured with GC cells. Blocking TIGIT or PD-1 increased IFNγ production in CD8 T cells that were co-cultured with SGC7901 cells. Blocking both TIGIT and PD-1 further enhanced IFNγ production in CD8 T cells (Fig. 7C and D). These findings indicate that TIGIT and PD-1 act synergistically to induce CD8 T cell exhaustion.

**Targeting CD155/TIGIT suppresses tumor progression in vivo**

To assess the antitumor effects of targeting CD155/TIGIT signaling, we used a humanized mouse tumor model using NOG mice. NOG mice are
immunodeficient mice that can be used for reconstitution of human immune cells in the mice. Mice were first injected with HC PBMCs. NOG mice were then subcutaneously inoculated with SGC7901-vector cells or SGC7901-CD155-RNAi one week later when human immune system is established. Mice were treated with a PD-L1 blocking antibody or isotype control. CD8+ T cell infiltration in the tumor was increased in mice received SGC7901-CD155-RNAi or mice received anti-PD-1 treatment, when compared to mice received SGC7901-vector as measured by IHC (Fig. 7E). Anti-PD-1 treatment combined with SGC7901-CD155-RNAi further enhanced T cell infiltration to the tumor (Fig. 7E). In addition, TCR, IL-2 and IFNγ production in the tumor quantified by RT-PCR further confirmed the increased T cell and enhanced immune response in the mice received SGC7901-CD155-RNAi or anti-PD-1 treatment (Supplementary Fig. 12A-C). Mice received SGC7901-CD155-RNAi showed inhibited tumor progression and improved survival when compared to mice received SGC7901-vector. Also, tumor progression was inhibited by anti-PD-1 treatment and survival was improved by anti-PD-1 treatment. Tumor progression was further inhibited in mice received SGC7901-CD155-RNAi and anti-PD-1 treatment. Survival was further improved in mice received SGC7901-CD155-RNAi and anti-PD-1 treatment (Fig. 7F-H).

Taken together, these results suggest that downregulation of CD155 in GC cells combined with PD-L1 blockade mediated synergistic effects in terms of the inhibition of tumor progression and increased survival compared with the effects downregulation of CD155 in GC cells or PD-L1 blockade alone.
Discussion

Immune surveillance is important for maintaining cellular homeostasis and preventing carcinogenesis (35). Immune escape, which is a defect of the immune system that facilitates carcinogenesis. It is promoted by upregulation of immune checkpoints, such as PD-1, and induce T cell exhaustion (36). CD8 T cells are the major effector cells in antitumor immunity. These cells are exhausted and rendered dysfunctional by immune checkpoints in tumor-bearing hosts (37). In this study, we found that the percentage of CD8+ T cells that are TIGIT+ is dramatically increased in GC patients, and that these cells exhibit functional exhaustion and reduced metabolic activity. We showed that GC cells inhibited glucose uptake and reduced metabolic activity in CD8 T cells and that these effects were reversed by the addition of glucose or blocking CD155/TIGIT. Targeting CD155 pathway suppressed tumor progression and improved survival in tumor bearing mice.

We demonstrated that activation, proliferation, migration, and cytokine production are impaired in TIGIT+ T cells from GC patients. These findings are in agreement with previous studies that have shown that TIGIT deficiency causes T cell hyperproliferation and increased susceptibility to autoimmunity (12,33). Our results suggest that TIGIT+ T cells in GC patients contribute to immune dysfunction, leading to impaired antitumor immunity and accelerated tumor progression. TIGIT may represent a potential therapeutic target to enhance antitumor immunity and control GC progression.

The immune system plays a key role in controlling tumor initiation and progression (38). Activated T cells require adequate energy supplies and changes in cellular metabolism for antitumor immune responses (26,39). In
the present study, we revealed abnormal metabolic reprogramming and lower metabolic activity in TIGIT⁺ CD8 T cells than TIGIT⁻ CD8 T cells. Glucose uptake and lactate production were low in TIGIT⁺ CD8 T cells than TIGIT⁻ CD8 T cells from GC patients. Strikingly, glucose could reverse the hypometabolic profile of TIGIT⁺ T cells. Glucose reversed the metabolic pathway of AKT/mTOR in TIGIT⁺ CD8 T cells. Cytokine production was increased in TIGIT⁺ CD8 T cells along with the reversed metabolism. These observations suggest that TIGIT regulates T cell metabolism and induces T cell dysfunction in GC patients. Harness T cell metabolism may be the potential method in reversing metabolic activity and effector function of TIGIT⁺ CD8 T cells.

T cells from leukemia patients are metabolically impaired (40) and tumor cells outcompete T cells for glucose consumption and induce T cell exhaustion, (26), implying tumor cells may inhibit the metabolism in T cells and damage T cell antitumor effects subsequently. In the present study, we found that CD8 T cells were metabolically impaired when co-cultured with GC cells. GC cells deprived CD8 T cells of glucose and downregulated the AKT/mTOR metabolic pathway in CD8 T cells. These findings suggest that GC cells inhibit AKT/mTOR signaling pathway in CD8 T cell, which resulted in reduced glucose uptake and lactate production. In agreement with our findings, extremely low levels of glucose and high lactate have been reported in gastric tumor tissues (41). Further, we found that GC cells induced TIGIT expression in CD8 T cells. TIGIT blockade activated metabolic pathway in CD8 T cells. The phosphorylation of AKT/mTOR pathway was increased by TIGIT blockade, resulting in increased metabolism and cytokine production in CD8 T
cells. These findings demonstrate that TIGIT signaling in CD8 T cells leads to decreased phosphorylation of AKT/mTOR pathway, resulting in the inhibition of metabolism. PD-1 inhibits mTOR pathway activation and suppresses glycolysis in T cells (42). The immune checkpoints may share some common mechanism in the regulation of T cell function. However, this signaling is different from the previous report that TIGIT signals through ZAP70 and ERK1/2 in NK cells (43). Together, GC cells inhibit AKT/mTOR pathway in CD8 T cells through upregulating TIGIT expression on CD8 T cells.

Next, we demonstrated that CD155, ligand of TIGIT, is overexpressed in GC tumor tissue and cell lines, which was in accordance to the previous report that soluble CD155 is increased in GC serum (44). The expression of CD155 on GC cells downregulated AKT/mTOR pathway and inhibited glucose uptake in CD8 T cells when they were co-cultured together. Downregulation of CD155 in GC cells increased T cell metabolism and cytokine production in the co-culture system. Moreover, mice inoculated with CD155-knockdown GC cells showed enhanced immune responses and improved survival. Conversely, CD155 overexpression in GC cells decreased T cell metabolism and inhibited cytokine production. The effects of CD155 overexpression were reversed by inhibiting TIGIT. Together, GC cells upregulate CD155 expression and inhibit CD8 T cell metabolism through CD155-TIGIT interaction.

Our finding that co-expression of TIGIT and PD-1 promoted cell exhaustion and immune escape in CD8 T cells from GC patients, indicates that combined blockade of these immune checkpoints is a potential therapeutic option for GC. In the T cell-tumor cell co-culture, combined blockade of TIGIT and PD-1
showed stronger in cytokine production. In addition, combined blockade of TIGIT and PD-1 further enhanced immune response in the tumor bear mice and had better tumor control and survival compared to targeting either one. These findings indicate the potential of combined immunotherapies to treat cancer, and this is now receiving more attention from researchers (45,46). Targeting PD-1/PD-L1 signal has improved the clinical outcome in cancer patients dramatically, but treatment responses vary, from 24% in renal-cell cancer and 87% in Non-Hodgkin’s lymphoma (6,47,48). A combination of PD-1 and CTLA-4 blockade has been shown to exert synergistic antitumor effects on B16 melanoma tumors (49). And the combined blockade of TIGIT and PD-1 demonstrates further enhancement of immune activation as reported before (14). Together, TIGIT or TIGIT combined with PD-1 may be the potential therapy for GC.

In conclusion, our findings provide insights into the mechanism by which CD8 T cell metabolism and function is impaired by TIGIT. Mediators that enhance CD8 T cell metabolism and promote maximum antitumor immunity may be novel therapeutic targets for the treatment of gastric cancer.
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Figure Legends

Figure 1. Increased TIGIT-positive T cells disrupt the immune response in gastric cancer patients. (A) TIGIT expression in CD4 and CD8 T cells from PBMCs of gastric cancer (GC) patients and healthy controls (HCs) was analyzed by flow cytometry. (B, C) Percentage of CD4+TIGIT+ or CD8+TIGIT+ cells in PBMCs (Age (y): controls, 66.23 ± 6.627 n = 16; patients, 68.87 ± 6.146 n = 24). (D) The percentages of TIGIT-positive T cells were measured in PBMCs from post-surgery or recurrent GC patients by flow cytometry. Percentages of CD4+TIGIT+ (E) and CD8+TIGIT+ (F) cells in PBMCs (n = 8). (G) Single-cell suspensions were prepared from gastric tumor tissues. Percentages of TIGIT-positive CD4 or CD8 T cells from tumor tissues were measured by flow cytometry. (H, I) Individual percentages of TIGIT+ and TIGIT− T cells from eight independent samples. (J–P) CD8+TIGIT+ and CD8+TIGIT− cells in PBMCs from GC patients were sorted by flow cytometry. Cells were stimulated with anti-CD3/CD28 Dynabeads (αCD3/CD28). (J) CD69 expression was determined by flow cytometry after 12 h of stimulation. Representative flow histograms are shown. (K) CD69-positive rates are summarized from 12 samples. (L) Cells were stained with CFSE, and proliferation rates were measured by flow cytometry. (M) Proliferation of CD8+TIGIT+ versus CD8+TIGIT− cells in 12 independent samples. (N) IFNγ and TNF-α production by CD8 T cells was measured by flow cytometry. Representative flow charts are shown. (O) Percentages of IFNγ- and TNF-α-producing CD8+TIGIT+ or CD8+TIGIT− cells. (P) CD8+TIGIT+ or CD8+TIGIT− cell migration was measured in a Transwell assay and percentage of cells that
migrated to the lower chamber were calculated by flow cytometry. Data were collected from 12 samples. **P < 0.01, ***P < 0.001.

Figure 2. Metabolic reprogramming in TIGIT-positive CD8 T cells from gastric cancer patients. CD8+TIGIT+ and CD8+TIGIT− cells were sorted from PBMCs of GC patients. (A) Cells were stimulated with αCD3/CD28 for 4 h. Heat map shows expression of metabolism-associated gene expression quantified by RT-PCR. (B) CD8+TIGIT+ or CD8+TIGIT− cells were stimulated with αCD3/CD28 for 24 h. Glucose transporter 1 (Glut1) expression in CD8+TIGIT+ and CD8+TIGIT− cells was determined by flow cytometry. Representative histograms are shown. (C) Glut1 MFI is summarized from six independent samples. (D) CD8+TIGIT+ and CD8+TIGIT− cells were stimulated with αCD3/CD28 for 24 h. Hexokinase 1 (HK1) and hexokinase 2 (HK2) expression was measured by western blotting. Representative blots are shown. (E) CD8+TIGIT+ and CD8+TIGIT− cells were stimulated with αCD3/CD28 for 5 min. AKT and mTOR phosphorylation was quantified by western blotting. (F) Cells were stimulated with αCD3/CD28 for 3 min; p-S6K and p-4EBP1 were measured by flow cytometry. Representative histograms are shown. Phosphorylation of S6K (G) and 4EBP1 (H) is summarized. CD8+TIGIT+ or CD8+TIGIT− cells were stimulated with αCD3/CD28. (I) Glucose uptake by T cells was measured indirectly using a 2-deoxyglucose (2-DG)-based assay 2-DG is a glucose analog that is taken up by glucose transporters and metabolized to 2-DG-6-phosphate (2-DG6P). 2-DG6P cannot be further metabolized and accumulates in cells. (J) Lactate
production was measured by a colorimetric assay 4 h after stimulation. (n = 6),
*P < 0.05, **P < 0.01, ***P < 0.001.

Figure 3. Glucose reverses the effects of CD8*TIGIT* on T cell metabolism and rescues T cell exhaustion. CD8*TIGIT* or CD8*TIGIT* T cells were sorted from PBMCs of GC patients. Cells were stimulated with αCD3/CD28 in the presence or absence of 10 mM glucose (Glu) or 10mM fructose. (A, B) Cells were stimulated with αCD3/CD28 in the presence or absence of 10 mM glucose for 24 h. Cells were then collected to measure glucose consumption (A) and lactate production (B) as described above. (C) Glut1 expression in T cells was determined by flow cytometry after stimulation for 24 h. Representative histograms of 6 experiments were presented. (D) CD69 expression was determined by flow cytometry after stimulation for 12 h. (E) The percentages of CD69-positive CD8 T cells. (F) CD8 T cell proliferation was quantified by flow cytometric analysis of CFSE dilution after 4 d of stimulation. Representative histograms of 6 experiments are shown. (G) CD8 T cell proliferation rates are summarized from 6 experiments. (H) CD8 T cell migration was measured using a Transwell system. Transmigrated cells were enumerated by flow cytometry. (I) IFNγ production was measured by flow cytometry after 24 h of stimulation. (J) Percentages of IFNγ-producing CD8 T cells. (K) CD8 T cells were stimulated with αCD3/CD28 for 48 h and treated with different concentrations of glucose. IFNγ production was measured by flow cytometry. Representative flow charts are shown. (L) Percentages of IFNγ-producing CD8 T cells. (n = 6), *P < 0.05, **P < 0.01, ***P < 0.001.
Figure 4. Gastric cancer cells deprive CD8 T cells of glucose. (A–L) Naïve CD8 T cells isolated from HCs were stimulated with αCD3/CD28 and co-cultured with GC cells (SGC7901) at a 5:1 ratio. (A) Glycolytic gene expression levels in CD8 T cells were measured by RT-PCR after 12 h of stimulation. Relative gene expression is shown as a heat map. (B) Glut1 expression in CD8 T cells was determined by flow cytometry after 48 h stimulation. A representative histogram is shown. (C) Summary of Glut1 MFI. (D) HK1 and HK2 expression in CD8 T cells was determined by western blotting. Glucose consumption (E) and lactate production (F) in CD8 T cells were measured as described above after 24 h of co-culture. (G) Phosphorylation of mTOR in CD8 T cells was measured by flow cytometry. (H) p-mTOR (S2448) MFI is summarized. (I) Phosphorylation of SK6 and 4EBP1 was measured by flow cytometry. (J, K) Summary of MFIs (L) CD8 T cells were stimulated with αCD3/CD28 and co-cultured with SGC7901 for 48 h. IFNγ production by CD8 T cells was determined by flow cytometry. (M) Percentages of IFNγ-producing CD8 T cells. (N) CD8 T cell cytokine production in the supernatant was measured by ELISA. Data were analyzed relative to the control group. (n = 6), *P < 0.05, **P < 0.01, ***P < 0.001.

Figure 5. TIGIT blockade neutralizes gastric cancer cell-induced inhibition of T cell metabolism and cytokine production. TIGIT+ CD8 T cells were sorted from HCs by flow cytometry. Cells were stimulated with αCD3/CD28 and co-cultured with SGC7901 at a ratio of 5:1. TIGIT was inhibited by an anti-TIGIT blocking antibody (αTIGIT). An isotype control was used as a control. (A) TIGIT expression in CD8 T cells was measured by flow...
cytometry. Representative histograms are shown. (B) Circulating tumor cells in GC patients were determined by NanoVelcro. Correlation of CTCs and CD8^+TIGIT^+ cells are shown (n = 25). (C) Cells were stimulated with αCD3/CD28 for 2 d, and Glut1 expression was measured by flow cytometry. (D) Summary of Glut1 MFI. (E) AKT and mTOR phosphorylation in CD8 T cells was determined by western blotting. (F, G) Semi-quantification of p-AKT and p-mTOR. (H) S6K and 4EBP1 phosphorylation in CD8 T cells was measured by flow cytometry. The phosphorylation rates of S6K and 4EBP1 are summarized in I and J, respectively. Glucose (glu) consumption (K) and lactate production (L) in CD8 T cells. (M) Total CD8 T cells were isolated from healthy PBMCs. Cells were co-cultured with GC cells at a ratio of 5:1 in the presence of αTIGIT or isotype control. IFNγ production was measured by flow cytometry after 2 d of stimulation with αCD3/CD28. (N) Percentages of IFNγ-producing CD8 T cells. (n = 6), *P < 0.05, **P < 0.01, ***P < 0.001.

**Figure 6. Gastric cancer cells inhibit T cell metabolism through CD155/TIGIT.** (A) CD155 expression in normal gastric tissue or GC tissue was measured by western blotting. Representative blots are shown. (B) Relative expression of CD155. (C) CD155 expression in GC cell lines SGC7901, HGC27, and BGC823 determined by flow cytometry. Representative histograms are shown. (D) CD155 knockdown efficiency was confirmed by flow cytometry. (E–I) CD8 T cells were stimulated with αCD3/CD28 and co-cultured with SGC7901-vector or SGC7901-CD155-RNAi for 48 h. (E) Phosphorylation of AKT, S6K and 4E-BP1 in CD8 T cells was determined by flow cytometry. Representative histograms are shown. Glucose
uptake (F) or lactate production (G) in CD8 T cells. (H) IFNγ production in CD8 T cells measured by flow cytometry. (I) Percentages of IFNγ-producing CD8 T cells. (J) CD155 overexpression was confirmed by flow cytometry. (K) CD8 T cells were co-cultured with SGC7901-CD155 or SGC7901-vector cells. TIGIT was blocked using αTIGIT. IFNγ production in CD8 T cells determined by flow cytometry. Representative flow charts are shown. (L) Percentages of IFNγ-producing CD8 T cells. (n = 6), N: normal gastric tissue; P: GC tumor tissue. *P < 0.05, **P < 0.01, ***P < 0.001.

Figure 7. Combined inhibition of TIGIT and PD-1 signaling has synergistic effects *in vitro* and *in vivo*. (A) PBMCs from gastric cancer patients were stained with anti-human CD8, anti-human TIGIT, and anti-human PD-1 antibodies. Representative flow charts were gated on CD8\(^+\)TIGIT\(^+\) or CD8\(^+\)TIGIT\(^-\) cells. (B) Percentages or MFI of PD-1 in CD8\(^+\)TIGIT\(^+\) or CD8\(^+\)TIGIT\(^-\) cells. (C, D) Total CD8 T cells were isolated from healthy PBMC. Cells were stimulated with αCD3/CD28 and co-cultured with or without SGC7901 for 2d. Anti-TIGIT (αTIGIT), anti-PD-1 (αPD-1), isotype control or a combined of αTIGIT and αPD-1 was included. IFNγ production in CD8 T cells was determined by flow cytometry. Percentages of IFNγ producing CD8 T cells were summarized from 8 samples. (E-H) NOG mice were inoculated subcutaneously with Vector-SGC7901 or SGC7901-CD155-RNAi gastric carcinoma cells. At the same time, mice were reconstituted with human PBMC. When tumor sizes reached approximately 200 mm\(^3\), mice were treated with isotype control or anti-PD-1 antibody (αPD-L1) for 3 weeks. (E) Representative images of showing CD8 T cell infiltration in tumor.
microenvironment as detected by IHC. (F) Mouse survival over time (n=12). (G) Mean (upper panel) or individuals of tumor volume (lower 4 panels) over time (n=8). (H) In vivo bioluminescence images of the tumor bearing mice. \*P < 0.05, \**P < 0.01, \***P < 0.001.
Figure 1

A. CD4 T cells and CD8 T cells with TIGIT.

B. Percentage of CD4+ TIGIT+ cells in HCs and Tumor.

C. Percentage of CD8+ TIGIT+ cells in HCs and Tumor.

D. CD4 T cells and CD8 T cells with TIGIT.

E. Percentage of CD4+ TIGIT+ cells in Recurrence.

F. Percentage of CD8+ TIGIT+ cells in Recurrence.

G. TILs with TIGIT.

H. Percentage of TIGIT+ cells in CD4 T cells.

I. Percentage of TIGIT+ cells in CD8 T cells.

J. TIGIT+/TIGIT- with CD69.

K. Percentage of CD69+ T cells.

L. TIGIT+/TIGIT- with CFSE.

M. Proliferation rates of TIGIT+ and TIGIT-.

N. CD8+ TIGIT+ and CD8+ TIGIT- with IFNγ and TNFα.

O. Percentage of cytokine-producing CD8+ T cells.

P. Percentage of migrated CD8 T cells.
Figure 2

A. TIGIT+ vs. TIGIT-

GLUT1, HK1, HK2, PFK, GAPDH, PGK1, PGM1, PGM2, PKM1, PKM2, PDK1, HIF-1α, c-Myc, PFKFB3

B. Scatter plots showing Glut1 expression.

C. Graph showing Glut1 mean fluorescence intensity (MFI).

D. Western blots for HK1, HK2, and β-actin.

E. Western blots for p-AKT (S473), AKT, p-mTOR(S2448), and β-actin.

F. Flow cytometry for p-S6K and p-4EBP1.

G. Bar graphs for % p-S6K+ T cells and % p-4EBP1+ T cells.

H. Bar graphs for 2-DG6P and lactate production.

I. Bar graphs for 2-DG6P and lactate production.

J. Bar graphs for 2-DG6P and lactate production.

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Figure 4

A

GLUT1
HK1
HK2
PFK
PGK1
PGM1
PGM2
PKM1
PKM2
PDK1
HIF-1α
c-Myc
-3
Log2
+3

B

αCD3/CD28/αCD3/CD28+SGC7901

C

Glut1 MFI

D

HK2
β-actin

E

2DG6P (pmol/l)

F

Lactate production (mM)

G

αCD3/CD28/αCD3/CD28+SGC7901

H

p-mTOR (MFI)

I

αCD3/CD28/αCD3/CD28+SGC7901

J

% p-S6K+ T cells

K

% p4EBP1+ T cells

L

αCD3/CD28
αCD3/CD28+SGC7901

M

% CD8+IFNγ cells

N

Relative concentration

IL-2
TNF-α
IFNγ
CD155/TIGIT Signaling Regulates CD8+ T Cell Metabolism and Promotes Tumor Progression in Human Gastric Cancer

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