TIGIT Can Exert Immunosuppressive Effects on CD8+ T Cells by the CD155/TIGIT Signaling Pathway for Hepatocellular Carcinoma In Vitro

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Summary: The efficacy of adoptive cellular immunotherapy against cancer cells is limited due to the presence of immunosuppressive cells within the solid tumor microenvironment. The upregulation of certain coinhibitory receptors may lead to exhaustion of the immune effector cells. T-cell immunoglobulin and immunoreceptor tyrosine-based inhibitory motif domain (TIGIT) is an immune inhibitory receptor expressed by regulatory T cells and activated T cells and natural killer cells. The aim of this study was to determine the immunosuppressive effects of CD155/TIGIT signaling on CD8+ T cells of adoptive cellular immunotherapy in hepatocellular carcinoma (HCC). Our studies found that CD155 was overexpressed in HCC, and CD155+ HCC cells upregulated TIGIT on CD8+ T cells, which decreased the secretion of interferon-γ, tumor necrosis factor-α, and interleukin-17A and increased that of interleukin-10 from the effector cells. However, TIGIT blockade or CD155-knockdown reversed the inhibitory effect of HCC cells on CD8+ T-cell effector function. These results indicate that TIGIT can exert an immunosuppressive effect on CD8 T cells by modulating cytokine production through CD155, and is a promising target to optimize adoptive cellular immunotherapy against HCC.

Key Words: TIGIT, HCC, CD8+ T cell, immunosuppressive effect, immunotherapy

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Primary liver cancer is the sixth most commonly diagnosed malignancy, and the second most common cause of cancer-related deaths worldwide. In fact, the number of deaths recorded among liver cancer patients (745,500) every year is almost equal to that of the newly diagnosed cases (782,500).1 Hepatocellular carcinoma (HCC) is a prevalent primary liver cancer with poor long-term prognosis and high frequency of recurrence, despite therapeutic options such as surgery, transarterial chemoembolization, radiofrequency ablation, etc.2,3 Thus, it is imperative to develop novel strategies to improve survival of HCC patients, especially those in the advanced stages.

In recent years, plenty of studies have shown that the clinical benefits of immune-based therapies for HCC and other malignant diseases are emerging. Efforts to mobilize the body’s immune system to treat cancer, collectively known as cancer immunotherapy, have garnered the most attention so far, on the basis of new mechanisms and technology development in this area.4 Cancer immunotherapy is based on the activation and recruitment of antitumor immune cells.5–7 Adoptive cellular immunotherapy, a type of adjuvant therapy, induces a durable response against tumor cells,8,9 through cytokine-induced killer (CIK) cells, tumor-infiltrating lymphocytes (TILs), expanding activated autologous lymphocytes, central memory T cells, and chimeric antigen receptor T cells, or natural killer (NK) cells. However, its therapeutic efficacy is limited within the solid tumor microenvironment10 due to the upregulation of immune coinhibitory receptors, such as cytotoxic T-lymphocyte–associated protein 4 (CTLA-4), lymphocyte activation gene-3, T-cell immunoglobulin-3, cytokine-inducible T-cell costimulator (ICOS), programmed cell death 1 (PD-1), and T-cell immunoglobulin-3, ligand for TIGIT that is also expressed on monocytes, dendritic cells (DCs), fibroblasts, endothelial cells, epithelial cells, platelets, and activated T and B cells.11–17 CD112 (nectin-2, also known as PRR2 or PVRL2), on the other hand, is a low-affinity ligand for TIGIT that is also expressed on monocytes, DCs, endothelial cells, hematopoietic cells, and activated T and B cells.18–20 Both CD155 and CD112 also bind to the costimulatory counterparts to TIGIT, such as CD226 (DNAM-1) or CD96, which act in concert with lymphocyte function-associated antigen 1 to activate T-cell responses.21,22 It is interesting to note that, CD155 and CD112 are also expressed by multiple solid tumors, including colorectal carcinoma,22 melanoma,23,24 ovarian cancer,25 etc.
Studies indicate that TIGIT exerts its immunosuppressive effects by enhancing interleukin (IL)-10 production by DCs via CD155, which impedes CD4+ T-cell proliferation and function. In addition, TIGIT also inhibits CD4+ T cells by recruiting SHP phosphatases that suppress cytokine production and proliferation, and competes with CD226 for CD155. The TIGIT locus is demethylated in Tregs and may potentially bind to FOXP3. In fact, TIGIT+ Tregs are highly active and secrete fibrinogen-like protein 2, which selectively inhibits Th1 and Th17 responses. Recent gene expression analyses have detected TIGIT in the CD8+ TILs in several solid tumors, including lung, colon, breast, uterine, and renal cancers. Furthermore, elevated TIGIT expression in these TILs correlates with CD8 and PD-1 levels, and their immune function. However, the significance of TIGIT+ TILs in adoptive cellular immunotherapy is not completely clear. We found that TIGIT was upregulated in the HCC-targeting CD8+ T cells and exerted an immunosuppressive effect by binding to CD155.

MATERIALS AND METHODS

Cell Lines and Culture
The human HCC cell lines SNU423, Hep3B, and Bel-7402 were obtained from China Type Culture Collection (Shanghai, China). SNU423 and Bel-7402 cells were cultured in RPMI-1640 medium (Gibco) supplemented with 10% fetal bovine serum and 100 U/mL penicillin/streptomycin at 37°C under 5% CO2. The Hep3B cells were cultured in complete MEM (Gibco) under the same conditions.

Isolation of Peripheral Blood Mononuclear Cells (PBMCs)
Blood samples were collected from HCC patients and healthy volunteers. The PBMCs were isolated by Ficoll-Hypaque density gradient centrifugation within 2 hours of sample collection. The immune cells were depleted by positive selection using EasySep Human CD45 Depletion Kit (STEMCELL Technologies Inc., Vancouver, BC, Canada).

Immunohistochemistry (IHC) and Immunofluorescence (IF)
Formalin-fixed, paraffin-embedded HCC and paratumor tissues were dehydrated, dewaxed, and rehydrated. For IHC, endogenous peroxidase activity was visualized using a Hitachi laser scanning microscope. To expand the T cells, the cell density was adjusted to 1×106 cells/mL with the addition of fresh medium every 2–3 days. For long-term expansion, the cells were harvested and resuspended every 7–10 days in fresh medium and restimulated with ImmunoCult Human CD3/CD28 T-Cell Activator, in addition to adjusting the cell density every 2–3 days, as described. The CD8+ T cells were obtained by immunomagnetic positive selection using EasySep human CD8+ T-cell Isolation Kit (STEMCELL Technologies Inc.), and purity was verified to be >95%.

Flow Cytometry
Primary or cultured CD8+ T cells were stained with FITC-CD8, PE/cy7-TIGIT, and PE-CD25 antibodies, and the HCC cell lines with PE-CD155 antibody (Biolegend, San Diego, CA), as per standard protocols. The stained samples were analyzed by fluorescence-activated cell sorting and the data analyzed using FlowJo software. The gating strategies used in this study are shown in Figure S3 (Supplemental Digital Content 1, http://links.lww.com/JIT/A560). Isotype controls were used to set the gate.

Western Blotting
Total proteins were extracted from the cultured cells and analyzed by Western blotting with antibodies against TIGIT, β-actin, CD226, interferon (IFN)-γ, AKT, p-AKT, ERK, p-ERK, and p-1x8ζ (CST, Danvers, MA), as per standard protocols. When necessary, the gray value of the band is obtained by the Image J software; the gray value of the target protein is divided by the gray value of the internal reference protein, and then normalized for comparison.

Cytokine Measurement
The cytokines released by the CD8+ T cells were measured using the LEGENDplex Multianalyze Flow Assay Kit (Biolegend) according to the manufacturer’s instructions.

Cell Coculture
The activated CD8+ T cells were sorted and cocultured with HCC cell lines either in regular culture plates or in transwells (Fig. S1, Supplemental Digital Content 1, http://links.lww.com/JIT/A560). In the transwell coculture format, the HCC cells were seeded in the bottom chambers and the effector CD8+ T cells in the lower chamber of the inserts. The functional anti-human TIGIT antibody (clone MBSA43, mouse IgG1; eBioscience, San Diego, CA) and recombinant human CD155/Fc protein (Sino Biological Inc., Beijing, China) were added to the culture medium, as appropriate. The cells were incubated for 2 days at 37°C under 5% CO2, harvested, and analyzed by Western blotting or flow cytometry. The culture supernatants were also collected and evaluated for cytokine levels, as described.

CD155 Knockout and Lentivirus Transduction
CRISPR/Cas9 was used for CD155 knockout. The sgRNA’s sequence (sgRNA-1 5′-AATCGGGCATGGCC GAGCCA-3′) was cloned into pLV vectors. HEK293T cells were cotransfected with pLV vector, pSPAX2, and pMD2. G plasmids, and the lentiviruses were precipitated using PEG8000-NaCl. The HCC cells were transduced with the lentivirus and selected 48 hours later using 3 µg/mL puromycin (MCE). Single-cell-derived clones were isolated.

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FIGURE 1. A, Representative immunohistochemical images showing in situ CD155 expression in hepatocellular carcinoma sections from 10 patients. B, Representative immunofluorescence images showing CD155 expression in SNU423, Hep3B, and Bel-7402 cells. C, Flow cytometry plots showing CD155 expression in SNU423, Hep3B, and Bel-7402 cells. D, The 2-dimensional visualization of CD8 T-cell clusters of 5 patients by t-SNE. Each dot corresponds to a single cell. The depth of each dot color indicates the level of T-cell immunoglobulin and immunoreceptor tyrosine-based inhibitory motif domain (TIGIT) expression. E, The violin plots showing the level of TIGIT expression in 5 CD8 T-cell clusters of all and individual patients. F, Representative immunofluorescence images showing the TIGIT+ CD8+ T cells (white arrows) in hepatocellular carcinoma sections.
through flow cytometric sorting, and CD155-knockdown (KD) was confirmed.

**Statistical Analysis**

A standard 2-tailed Student t test was used for comparing groups, and \(P\)-value < 0.05 was considered statistically significant.

**RESULTS**

**CD155 Was Overexpressed in HCC Tissues**

The in situ expression of CD155 was significantly higher in the HCC tissues compared with the paired para-tumor tissues by IHC (Fig. 1A) and in HCC cell lines by IF (Fig. 1B). Consistent with this, the CD155 positivity rates were 99.5%, 99.5%, and 99.1%, respectively, in the SNU423, Hep3B, and Bel-7402 cell lines (Fig. 1C).

**TIGIT Levels Were Higher in Activated CD8+ T Cells**

Analysis of previously published single-cell sequencing GEO data of HCC immune cells\(^{32}\) revealed variations in TIGIT expression levels on CD8+ T cells from 5 HCC patients (Fig. 1D). Furthermore, the CD8+ TILs showed higher TIGIT expression compared with the CD8+ T cells in peripheral blood and adjacent tissues (Fig. 1E), and most TIGIT+ CD8+ TILs were distributed in the fourth C4_CD8-LAYN cluster that was predominantly composed of TILs expressing high levels of exhaustion markers such as CTLA-4, PDCD1, and HAVCR2. Consistent with these findings, the TILs in the HCC tissues of our cohort coexpressed CD8 and TIGIT (Fig. 1F). In addition, the percentage of TIGIT+ CD8+ T cells in the PBMCs was also significantly elevated following activation (Fig. 2A), as well as upon coculturing with the SNU423, Bel-7402, and Hep3B cells (Figs. 2B–D). Taken together, the CD155hi HCC cells upregulated TIGIT on CD8+ T cells.

**HCC Cells Inhibited CD8+ T-Cell Effector Function Via CD155/TIGIT Signaling**

To determine whether the CD155/TIGIT signaling pathway inhibited CD8+ T-cell effector function, we treated them with recombinant human CD155. The cells cultured with CD155 secreted significantly less amount of IFN-\(\gamma\), tumor necrosis factor (TNF)-\(\alpha\), and IL-17A, and higher levels of IL-10 compared with the unstimulated CD8+ T cells (Fig. 2D). The gray value of the target protein is divided by the gray value of the internal reference protein, and then normalized for comparison. PBMC indicates peripheral blood mononuclear cell; TIGIT, T-cell immunoglobulin and immunoreceptor tyrosine-based inhibitory motif domain.
cells. Furthermore, blocking TIGIT reversed the secreted cytokine profile of T cells (Fig. 3A), indicating that the CD155/TIGIT axis relays inhibitory signals to these effector cells. Similarly, the CD8+ T cells cocultured with the CD155hi Bel-7402 cells (Figs. 1B, C) also released significantly lower amounts of IFN-γ, TNF-α, IL-17A, perforin, granzyme B, and granulysin, and higher levels of IL-10 compared with the control cells (Fig. 3B). Antibody-mediated neutralization of TIGIT increased the production of effector cytokines and cytotoxic factors and decreased that of IL-10 in the cocultured CD8+ T cells (Fig. 3B). To further validate the immunosuppressive function of CD155, we generated a stable CD155-KD Bel-7402 cell line (Bel-7402-CD155 RNAi) using siRNA (Fig. 3C). CD8+ T cells cocultured with CD155hi Bel-7402 cells secreted lower levels of IFN-γ, TNF-α, IL-17A, perforin, and granulysin, and higher IL-10 compared with the control group with Bel-7402-CD155 RNAi (Fig. 3D), which was neutralized by blocking TIGIT (Fig. 3D). Taken together, CD155-expressing HCC cells inhibited CD8+ T-cell effector function through the CD155/TIGIT pathway.

Mechanism of CD155/TIGIT Signaling Pathway

Studies have implicated phosphoinositide 3-kinase (PI3K), mitogen-activated protein kinase (MAPK) and nuclear factor-κB (NF-κB) signaling pathways in TIGIT/CD155-mediated immunosuppressive effects on NK cells. To this end, we analyzed the expression levels of related proteins in the CD8+ T cells cocultured with SNU423 and Bel-7402 cells. Blocking TIGIT during the coculture of T cells and wild-type HCC cells significantly increased the ratio of p-AKT/AKT and p-ERK/ERK and decreased p-IκBα levels (Fig. 4A). To this end, we analyzed the expression levels of related proteins in the CD8+ T cells cocultured with SNU423 and Bel-7402 cells. Blocking TIGIT during the coculture of T cells and wild-type HCC cells significantly increased the ratio of p-AKT/AKT and p-ERK/ERK and decreased p-IκBα levels (Fig. 4A). To this end, we analyzed the expression levels of related proteins in the CD8+ T cells cocultured with SNU423 and Bel-7402 cells. Blocking TIGIT during the coculture of T cells and wild-type HCC cells significantly increased the ratio of p-AKT/AKT and p-ERK/ERK and decreased p-IκBα levels (Fig. 4A). To this end, we analyzed the expression levels of related proteins in the CD8+ T cells cocultured with SNU423 and Bel-7402 cells. Blocking TIGIT during the coculture of T cells and wild-type HCC cells significantly increased the ratio of p-AKT/AKT and p-ERK/ERK and decreased p-IκBα levels (Fig. 4A).
blocking TIGIT (Fig. 4D). Taken together, the TIGIT/CD155 interaction suppresses effector functions of CD8⁺ T cells by inhibiting multiple signaling pathways.

**DISCUSSION**

Adoptive cellular immunotherapy using NK cells, CIK cells or TILs, expanding activated autologous lymphocytes, central memory T cells, and chimeric antigen receptor T cells has gained considerable attention in recent years as a strategy against solid tumors. However, these cells function suboptimally within the tumor microenvironment on account of various immune inhibitory receptors such as PD-1, CTLA-1, T-cell immunoglobulin-3, and lymphocyte activation gene-3 that impair antitumor effects.

TIGIT is a newly identified inhibitory receptor that is expressed on effector CD8⁺ T cells, the major effector cell type...
in antitumor immune responses and adaptive cellular immunotherapy. In addition, its ligand CD155 is overexpressed on human colon cancer,22 glioma,34 pancreatic cancer,35 and lung cancer cells.36 Not surprisingly, therefore, the TIGIT/CD155 pathway inhibits the tumor-infiltrating CTK, NK, and CD4+ T cells.37-39 However, CD155 expression in HCC cells has not been elucidated so far, and the role of CD155/TIGIT signaling on the CD8+ T cells infiltrating HCC tumors has also not been clearly defined. We found that CD155 was overexpressed in HCC compared with adjacent liver tissues, as well as in HCC cell lines such as SNU-423, Hep3B, and Bel-7402. In addition, the HCC tissues had a high density of TIGIT and CD8 coexpressing T cells. This is consistent with the findings of Zhang et al32 who reported significantly higher proportion of TIGIT+ CD8+ T cells in the HCC tumors compared with that in paracarcinoma tissues and peripheral blood. In addition, most of these TIGIT+ CD8+ TILs were distributed in the C4_CD8-LAYN cluster, which predominantly consisted of cells expressing high levels of exhaustion markers such as CTLA-4, PDCD1, and HAVCR2. Therefore, the CD155/TIGIT signaling pathway likely plays an important role in immunosuppression in the HCC tumors.

It is interesting to note that, TIGIT levels were significantly upregulated on CD8+ T cells cocultured with HCC cells, indicating that the latter suppresses the tumor-infiltrating immune cells in an exocrine manner. This is consistent with previous studies showing that TIGIT can be rapidly induced by antigenic and other inflammatory stimuli.32,35,36,39 Furthermore, the immunosuppressive effects of HCC cells and recombinant CD155 on CD8+ T cells—measured in terms of the levels of effector cytokines released by the latter—was reversed after blocking TIGIT. Consistent with this, the CD155-KD HCC cells also failed to suppress the effector function of the cocultured CD8+ T cells. Studies indicate that TIGIT binding to CD155 induces phosphorylation at Tyr225 in the ITT-like motif and that it recruits SH2 domain-containing inositol-5-phosphatase 1 (SHIP1) through the cytosolic adaptor growth factor receptor-bound protein 2.40,41 TIGIT-mediated recruitment of SHIP1 inhibits NK cells by blocking the PI3K and MAPK signaling pathways. In addition, the ITT-like motif of phosphorylated TIGIT binds to β-arrestin2 and recruits SHIP1 to limit NF-κB signaling.41 Consistent with this, we found that CD155/TIGIT blocked the PI3K, MAPK, and NF-κB signaling pathways in CD8+ T cells.

To summarize, we have shown, for the first time, that CD155 is overexpressed on HCC cells, and the latter upregulates TIGIT on the CD8+ TILs. TIGIT/CD155 binding then relays inhibitory signals into the effector cells by blocking multiple signaling pathways. Thus, tumors overexpressing CD155 likely escape the host immune response by upregulating TIGIT on the TILs, and inhibiting this blockade may improve the efficacy of adoptive cellular immunotherapy against HCC. In fact, the combination of targeted anticancer therapy and immune checkpoint inhibition has shown better clinical outcomes in HCC.42,43 Furthermore, a recent study reported coexpression of TIGIT and PD-1 in melanoma TILs.44 These data strongly support dual TIGIT and PD-1 blockade to optimize CD8+ T-cell-driven adoptive cellular immunotherapy in HCC, and will need further experimental and clinical validation.

CONFLICTS OF INTEREST/FINANCIAL DISCLOSURES

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