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ORIGINAL ARTICLE

α2,6-Sialylation promotes immune escape in hepatocarcinoma cells by regulating T cell functions and CD147/MMP signaling

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Abstract

Altered glycosylation is a common feature of cancer cells and plays an important role in tumor progression. β-Galactoside α2-6-sialyltransferase 1 (ST6Gal-I) is the critical sialyltransferase responsible for the addition of α2-6-sialic acid to the terminal N-glycans on the cell surface. However, the functions and mechanism of ST6Gal-I in tumor immune escape remain poorly understood. Here, we found that ST6Gal-I overexpression promoted hepatocarcinoma cell proliferation, migration, and immune escape by increasing the levels of CD147, MMP9, MMP2, and MMP7. When $CD8^+$ T cells were co-cultured with cell lines expressing different levels of ST6Gal-I, we found that ST6Gal-I upregulation inhibited the T cell proliferation and increased the secretion of IL-10 and TGF-β1, while secretion of IFN-γ and TNF-α was diminished. In a syngeneic tumor transplant model, ST6Gal-I upregulated Hca-P. In addition, Hepa1-6 cells formed significantly larger tumors and suppressed intratumoral penetration by $CD8^+$ T cells. In combination, these results suggest that ST6Gal-I promotes the immune escape of hepatocarcinoma cells in the tumor microenvironment and highlight the importance of assessing ST6Gal-I status for immunotherapies.

Keywords ST6Gal-I · Immune escape · T cell · HCC · CD147

Introduction

Hepatocellular carcinoma (HCC) is a major cause of mortality, being the fifth most common cancer worldwide and the second leading cause of cancer-related deaths [8]. Although surgical resection and liver transplantation are potentially curative treatments [16], the success of these approaches is limited by the stage of the tumor and local ablative therapy. With the high intrinsic and acquired drug resistance potential of HCC, chemotherapeutic choices are also limited [2, 27]. In terms of molecular-targeted therapy [30], sorafenib is a multiple kinase inhibitor with tumor suppression potential in HCC as indicated by the increased survival data from two phase III trials [4, 13]. Unfortunately, these studies also showed that HCCs rapidly became sorafenib-resistant with a short time to progression. Therefore, new therapeutic targets for HCC are urgently required.

According to the model of cancer immunoediting, the occurrence of the tumor can be divided into three dynamic stages: the elimination, equilibrium and escape phase [5, 21]. The ability to escape immune surveillance is regarded as one of the typical characteristics of tumors [10, 28]. In addition, T lymphocytes play a crucial role in controlling tumor growth and the progression of HCC [18, 20]. Altered glycosylation is a common feature of cancer cells and plays an important role in tumor progression [6]. Upregulated expression of α2,6-sialylation catalyzed by β-galactoside α2,6 sialyltransferase-1 (ST6Gal-I) has been observed in many types of the cancers, including breast, ovarian, cervix, and liver carcinomas [6, 7, 23]. In addition, ST6Gal-I is critical

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for aspects of tumor malignancy including metastasis and invasion both in vitro and in vivo [15, 22]. A study revealed that ST6Gal-I mediates the tumor-associated sialylation that stimulates tumor cell escape from Fas-mediated apoptosis [24]. This indicates that sialic acids play an important role in the immune escape of tumors. However, the mechanisms of immune evasion induced by ST6Gal-I mediated α2,6-sialylation in HCC remain poorly understood.

CD147 is a highly glycosylated immunoglobulin superfamily transmembrane protein with three potential N-linked glycosylation sites. And it is best known as stimulating matrix metalloproteinase (MMP) synthesis in tumor and neighboring stromal cells, promoting tumor growth and lymphatic metastasis [14, 26].

In this study, we investigated the roles of ST6Gal-I in HCC cells and the possible molecular mechanisms involved in the regulation of tumor immunity. The results showed that ST6Gal-I overexpression promoted the escape of HepG2 cells from immune surveillance in vitro and enhanced tumor-mediated suppression of intratumoral $CD8^+$ T cell function in HCC in vivo. In addition, ST6Gal-I upregulation resulted in activation of the CD147/MMP signaling pathway in HCC. The results of this study indicate an important function of ST6Gal-I in tumor immune escape and a novel mechanism by which anti-tumor immunity is modulated in HCC.

Materials and methods

Cell lines and animals Human and mouse hepatoma cell lines (HepG2 and Hepa1-6, respectively) were purchased from the Cell Bank of the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China) and maintained in DMEM (Gibco, Thermo Scientific, Waltham) and supplemented with 10% fetal bovine serum (FBS; Gibco). Human $CD8^+$ T cells were identified and sorted from human blood by flow cytometry. All healthy donors were confirmed to have given written informed consent to a tissue and blood procurement study allowing ex vivo experimentation, which is approved by The First Affiliated Hospital of Dalian Medical University. Human T cells and the mouse hepatoma cell line Hca-P were cultured in RPMI 1640 (Gibco) supplemented with 10% FBS (Sciencell), respectively. Female C57BL/6J mice (aged 6–8 weeks) were housed under specific pathogen-free conditions and provided with a standard rodent laboratory diet obtained from Dalian Medical University. All experiments were carried out in accordance with guidelines for the use of laboratory animals and were approved by the Institutional Animal Care and Use Committee of Dalian Medical University.

Stable transfectants The expression vector containing pcDNA3.1/ST6Gal-I (human) sequences and pcDNA3.1/ST6Gal-I (mouse) sequences was constructed and transfection performed according to previously described methods [3, 31]. HepG2 cells were transfected with the recombinant pcDNA3.1/ST6Gal-I vector, while Hca-P and Hepa1-6 cells were similarly transfected with pcDNA3.1/ST6Gal-I. Stable clones were confirmed by real-time PCR, Western blot, *Sambucus nigra* agglutinin (SNA) lectin blot, and flow cytometric analyses.

Cell survival analysis assays Cell Counting Kit-8 (Dojindo Laboratories) was used to assess cell proliferation. HCC cells and derivative cells were seeded in 96-well plates (2×10^3 cells/well) and cultured for 12–48 h. Following incubation for 1 h at 37 °C with CCK-8 solution, the absorbance in each well was measured at 450 nM using a microplate reader.

Cell migration assay Following resuspension in serum-free medium, hepatocarcinoma cells or derivative cells (3×10^4 cells/well) were seeded in the upper chamber of Transwell chambers (Corning Costar, 8 μm), while medium supplemented with 10% serum was added into the lower chamber. After incubation for 18 h at 37 °C in 5% CO_2 humidified incubator, hepatocarcinoma cells on the upper side were removed. Then, migrated cells were fixed with formaldehyde and stained with crystal violet. The cell numbers in five separate high-power fields were counted, and the mean number of invaded cells per field was calculated.

T cell-tumor cell co-culture assay Human $CD8^+$ T cells were sorted using a FACSAria™ III cell sorter (FACSDiva, BD) and stimulated with the Streptamer® CD3/CD28 kit (IBA). T cells (2×10^5 cells/well) were cultured for 48 h in the upper chambers of a Transwell plate (Corning Costar; 0.4-μm membrane pore size) and co-cultured with HepG2 cells (1×10^5 cells/well) in the lower chamber. The cells were then dissociated for the evaluation of apoptosis and proliferation, while the supernatant was collected for Enzyme-linked immunosorbent assay (ELISA). Human blood was obtained from the First Affiliated Hospital of Dalian Medical University.

ELISA assay Cell culture supernatants were harvested to analyze the concentrations of human IL-10, TGF-β1, IFN-γ and TNF-α using enzyme-linked immunosorbent assay kits (Abcam) according to the manufacturer's protocols.

Flow cytometric analysis of apoptosis HepG2 cell apoptosis was quantitated by flow cytometry using an annexin V-FITC/PI apoptosis detection kit (Dojindo Laboratories). The cells were co-cultured with $CD8^+$ T cells for 48 h, harvested, and washed twice with cold PBS. Following resuspension in 1 × annexin V Binding Solution, 1×10^5 cells were suspended in

buffer. Cells were incubated with PI and annexin V-FITC at room temperature in the dark for 15 min and then analyzed by flow cytometry (BD Biosciences).

Western blot analysis Total protein concentrations were measured using the BCA kit (Beyotime). Proteins (30 μg) were resolved by SDS-PAGE and then transferred to polyvinylidene fluoride (PVDF) membranes (Pall Corporation). Specific antibodies for the detection of ST6Gal-I (Abcam, 1:500), CD147, Fas, MMP2, MMP9 (Proteintech, 1;600, 1;600, 1:500, 1:600), Bcl-2, Bax, Bad (Sangon, 1:500), MMP7, GAPDH (Bioworld, 1:500, 1:5000), and integrin-α3 (Santa Cruz, 1:300) were used as the primary detection antibodies. Anti-mouse or rabbit horseradish peroxidase-linked antibodies (ZSGB-BIO, 1:8000) was used as secondary detection antibodies. Immunoreactive proteins were detected using an electrochemiluminescence (ECL) kit (Advansta) and quantitated by densitometry using Gel-Pro software.

Real-time PCR Total RNA was extracted from the aorta or spleen tissues using the RNAiso Reagent Plus (Takara Bio Inc., Shiga, Japan), and complementary DNA (cDNA) was synthesized using a PrimeScript RT reagent Kit (TaKaRa) according to the manufacturers' recommendations. Table 1 shows the primer sequences and expected sizes of the PCR products. Relative changes in mRNA expression were normalized against GAPDH and calculated using the $2^{-\triangle\triangle CT}$ method.

Lectin blot and affinity analyses Cell lysates containing 30 μg protein were resolved by 10% SDS-PAGE. Proteins in one gel were visualized by Coomassie Brilliant Blue (CBB) staining, and those of the second gel were transferred to a PVDF membrane. Identification of proteins was performed with 2 μg/ml biotinylated *S*NA lectin (Vector Laboratories), a lectin that binds specifically to α2-6-linked sialic acids. Immunoreactive proteins were detected using an ECL kit (Advansta) and quantitated by densitometry using Gel-Pro software.

Cell lysate proteins (> 500 μg) were incubated overnight with 50 μl of immobilized SNA lectin with rotation at 4 °C. The α2,6 sialylated proteins complexed with SNA lectin were collected by brief centrifugation and washed. Sialylated proteins were released from the complexes by boiling in SDS-PAGE sample buffer. The proteins were then resolved by SDS-PAGE and immunoblotted to detect CD147, Fas, and integrin-α3.

In vivo animal experiment and immunohistochemistry Hca-P, Hca-P/mock, Hca-P/St, Hepa1-6, Hepa1-6/mock, and Hepa1-6/St cells (2×10^7 cells/mouse) were injected subcutaneously in the inguen of C57BL/6J mice (8 mice/cell line). After 18 days, tumors were isolated from the mice, weighed, fixed in formalin, and stained with hematoxylin and eosin. Immunohistochemical staining of formalin-fixed and paraffin-embedded tissues was performed according to standard protocols using specific antibodies for the detection of CD4 and CD8 (Proteintech, 1:100) as reported previously [29]. CD4-positive and CD8-positive cells were quantified and analyzed with the Image-Pro Plus 6.0 software.

Table 1 Primers sequences for real-time PCR

Human *GAPDH*	Forward: 5′-TCCAAAATCAAGTGGGGCGA-3′
	Reverse: 5′-AAATGAGCCCCAGCCTT CTC-3′
Mouse *GAPDH*	Forward: 5′-TGGCCTTCCGTGTTCCTAC-3′
	Reverse: 5′-GAGTTGCTGTTGAAGTCGCA-3′
Human *ST6Gal-I*	Forward: 5′-ATCGTAAGCTGCACCCCAAT-3′
	Reverse: 5′-ATGATACCAAGCATCCCAGAGG-3′
Mouse *ST6Gal-I*	Forward: 5′-ATGCGGTCCTGAGGTTTAATGG-3′
	Reverse: 5′-CGCTTTTCTGTGGTGACTAACTGAG-3′
Mouse *IFN-γ*	Forward: 5′-TGAACGCTACA CACTGCATCTTGG-3′
	Reverse: 5′-CGACTCCTTTTCCGCTTCCTGAG-3′
Mouse *TNF-α*	Forward: 5′-GCCTCTTCTCATTCCTGCTTG-3′
	Reverse: 5′-CTGA TGAGAGGGAGGCCATT-3′
Mouse *TGF-β1*	Forward: 5′-TGACGTCACTGGAGTTG TACGG-3′
	Reverse: 5′-GGTTCATGTCATGGATGGTGC-3′
Mouse *IL-10*	Forward: 5′-GATGCCTTCAGCAGAGTGAA-3′
	Reverse: 5′-GCAACCCAGGTAAC CCTTAAA-3′

GAPDH, glyceraldehyde 3-phosphate dehydrogenase; *ST6Gal-I*, beta-galactoside alpha-2,6-sialyltransferase 1; *IFN-γ*, interferon-γ; *TNF-α*, tumor necrosis factor-α; *TGF-β1*, transforming growth factorβ1; *IL-10*, interleukin-10

Statistical analysis Quantitative data were presented as mean ± SD of three independent experiments. Data were analyzed by non-paired *t* test, chi-square test, and one-way ANOVA using Prism software (GraphPad 5.0). $P < 0.05$ was considered to indicate statistical significance.

Results

ST6Gal-I overexpression promotes the proliferation and migration of hepatocellular carcinoma HepG2 cells in vitro To explore the possible functions of ST6Gal-I in hepatocellular carcinoma cells, we used an overexpression plasmid vector pcDNA3.1/ST6Gal-I to upregulate the expression of ST6Gal-I in HepG2 cells and then established two monoclonal overexpression cell lines (HepG2/ST1 and HepG2/ST2). As shown in Fig. 1 A–D, ST6Gal-I expression at the mRNA, protein, and glycan levels was significantly increased after transfection with the pcDNA3.1/ST6Gal-I vector. CCK-8 and Transwell migration assays showed that the ST6Gal-I overexpression increased the survival and migration rates of HepG2 cells (Fig. 1E and F). In combination, these data indicate a functional role of ST6Gal-I in promoting cell proliferation and migration in HepG2 cells.

ST6Gal-I overexpression reduces T cell proliferation and HepG2 cell apoptosis in co-cultures To explore the role of ST6Gal-I expression in anti-tumor immunity responses of T cells, human $CD8^+$ T cells were co-cultured with tumor cell lines expressing different levels of ST6Gal-I. As shown in Fig. 2 A, human blood T cells were isolated and sorted by flow cytometry. Compared with the negative control, $CD8^+$ T cell survival was much lower after co-culture for 24 h or 48 h with

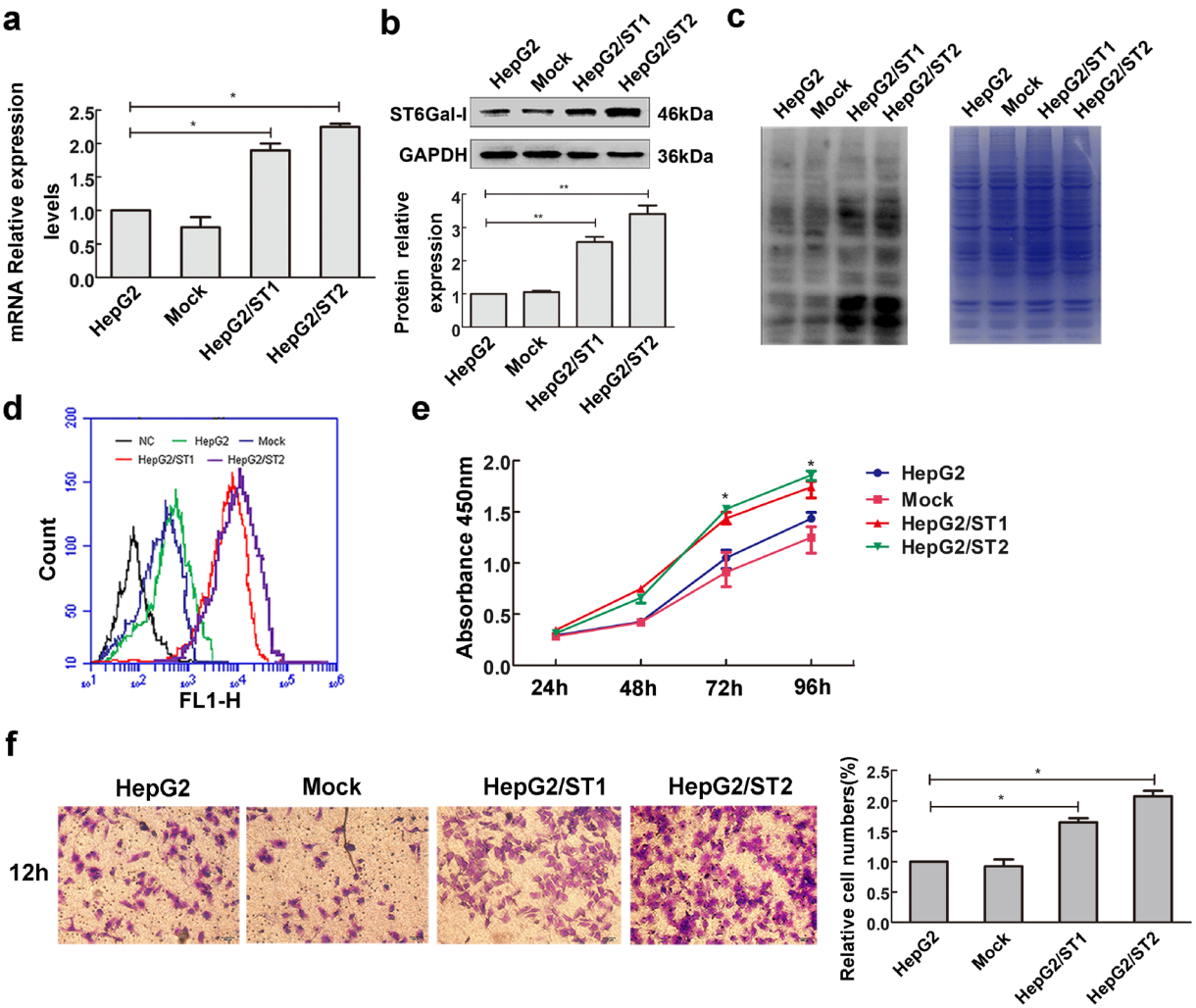

Fig. 1 Upregulation of ST6Gal-I promotes tumor cell proliferation and migration in vitro. Overexpression of ST6Gal-I in HepG2 cells were confirmed by real-time PCR (A), Western-blot assays (B), SNA lectin blot (C), and Flow cytometry analysis (D). (E) Cell proliferation ability measured by a Cell Counting Kit-8 assay. (F) Transwell migration assay was used to determine the migration ability of HepG2 and HepG2/ST cells. Results are the mean of three independent experiments ± SD (*$P < 0.05$, **$P < 0.01$). Mock, pcDNA3.1 vector transfected stable clones; HepG2/ST1 or HepG2/ST2, pcDNA3.1/ST6Gal-I vector transfected stable clones

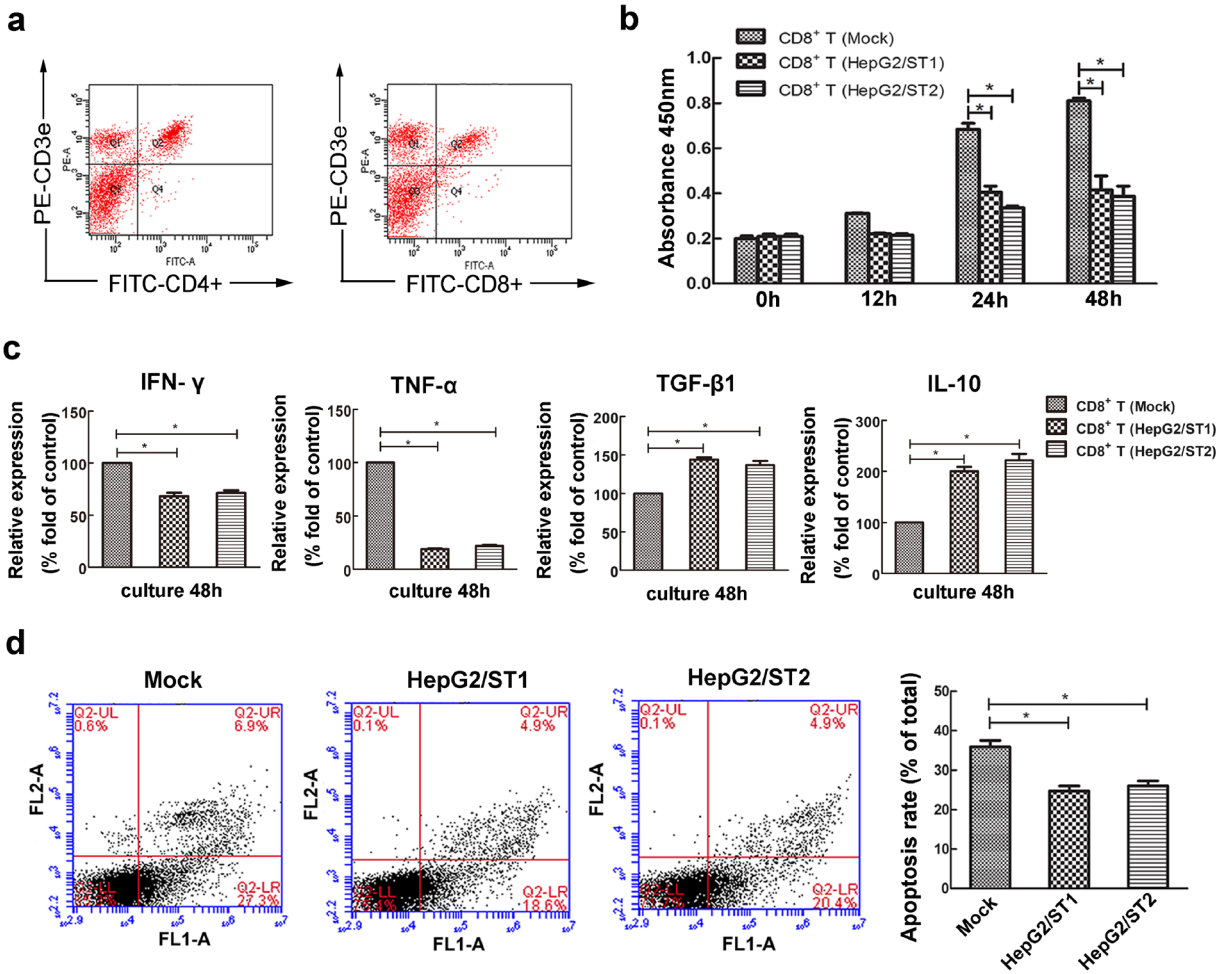

Fig. 2 The immune evasion was strengthened in tumor cells after ST6Gal-I over-expression. (A) Human blood T cells were isolated and sorted by flow cytometry. (B) $CD8^+$ T cells co-cultured with different groups of tumor cells were tested with CCK-8 assay. (C) ELISA examined the levels of IFN-γ, TNF-α, TGF-β1 and IL-10 in co-culture system. (D) Flow cytometry analysis measured the apoptosis of tumor cells in co-culture system. Results are the mean of three independent experiments ± SD (*$P < 0.05$)

cells overexpressing ST6Gal-I (Fig. 2B). Furthermore, $CD8^+$ T cells co-cultured with ST6Gal-I upregulation cells secreted lower levels of the immunoactivating cytokines TNF-α and IFN-γ and higher levels of the immunosuppressive cytokines TGF-β1 and IL-10 compared with the levels detected in the control groups (Fig. 2C). In addition, flow cytometric analysis of annexin V-FITC/PI–stained cells showed that the apoptosis rates of tumor cells overexpressing ST6Gal-I decreased significantly after Transwell co-culture (Fig. 2D). In summary, these findings indicate that ST6Gal-I expression in HCC cells is associated with evasion from immune surveillance.

ST6Gal-I overexpression activates the CD147/MMPs signaling pathway in HepG2 cells Lectin affinity analysis was used to explore the possible target proteins of ST6Gal-I. HepG2 or HepG2/ST1 cell lysates were subjected to SNA lectin pull-down prior to Western blot analysis with specific antibodies for the detection of CD147, Fas, and integrin-α3. As shown in Fig. 3 A, ST6Gal-I upregulation increased the level of α2-6-sialylation on CD147, but not on integrin-α3 and Fas. In addition, the sialylation levels of CD147 both increased when cultured alone or in co-culture with T cells (Fig. 3B).

Next, we further investigated the molecular mechanisms involved in ST6Gal-I-induced tumor escape from T cell killing. The results showed that CD147, MMP2, MMP7, MMP9, and Bcl-2 levels were significantly increased in ST6Gal-I overexpression cells when cultured alone or in co-culture with T cells. In addition, the expression of CD147 and downstream molecules were increased in the co-culture system. Furthermore, Bax and Bad expressions were decreased in ST6Gal-I overexpression cells relative to the levels detected in the control groups (Fig. 3C and D). In combination, these findings suggest that ST6Gal-I upregulation activates the CD147/MMPs signaling pathway in HepG2 cells.

ST6Gal-I overexpression promotes the proliferation and migration of murine hepatoma carcinoma Hepa1-6 and Hca-P cells in vitro Next, we established two murine monoclonal

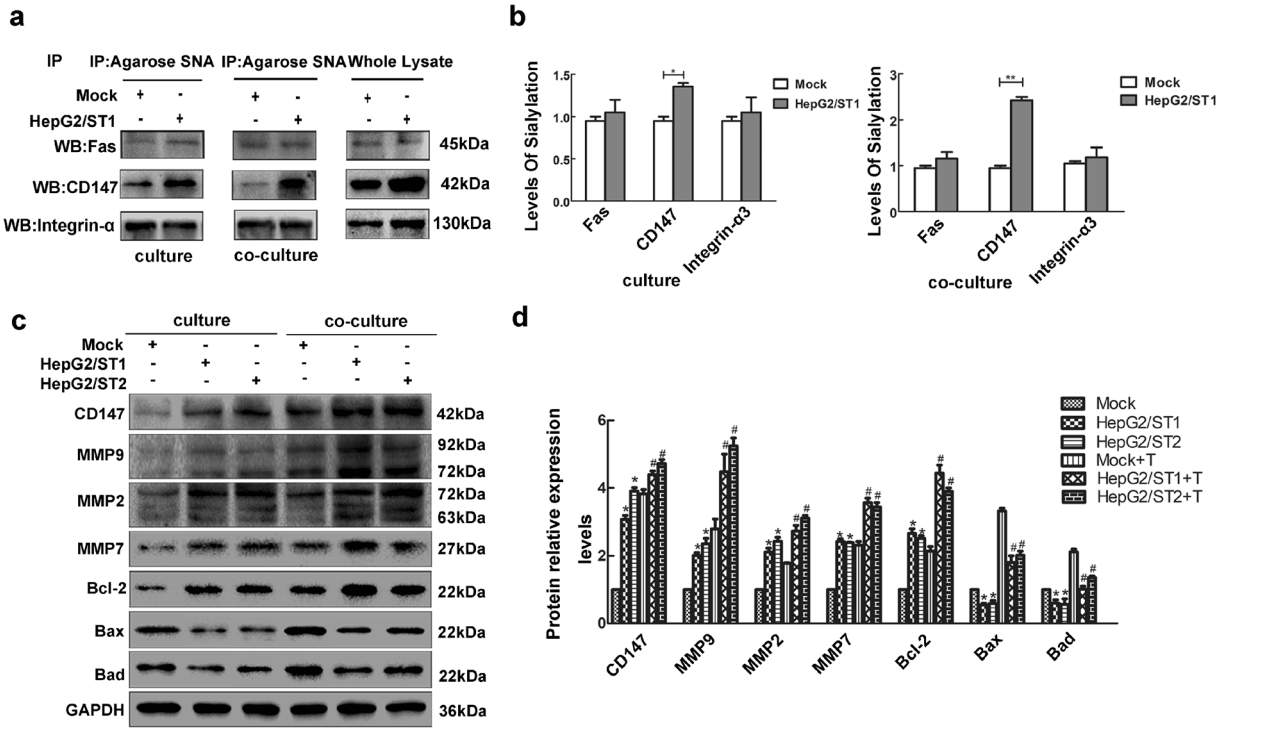

Fig. 3 Overexpression of ST6Gal-I promotes the CD147/MMPs signaling pathway in HepG2 cells. (A) HepG2 or HepG2/ST1 cell lysates were pulled down with SNA lectin and then immunoblotted (WB) with anti-CD147, Fas, and integrin-α3. (C) Whole lysis protein of HepG2 or HepG2/ST1 cell was immunoblotted with anti-CD147, MMP2, MMP7, MMP9, and Bcl-2 family. (B and D) Quantification of protein levels was performed by densitometry. Results represent the mean ± SD of the expression levels from three independent experiments and GAPDH was used as an internal control. (*$P < 0.05$, **$P < 0.01$, compared with the control groups)

ST6Gal-I overexpression cell lines (Hepa1-6/St and Hca-P/St). As shown in Fig. 4 A–F, ST6Gal-I expression at the mRNA, protein, and glycan levels was significantly increased after transfection with a pcDNA3.1/ST6Gal-I vector. Similarly, ST6Gal-I overexpression increased the survival and migration rates of Hepa1-6 and Hca-P cells (Fig. 4G and H). Together, these data indicate a functional role of ST6Gal-I in promoting cell proliferation and migration in murine HCC cells.

ST6Gal-I upregulation increases tumorigenicity of tumor cells in vivo and suppresses intratumoral penetration of $CD8^+$ T lymphocytes in HCC We further characterized the oncogenic function of ST6Gal-I in vivo using a mouse model. On days 18 and 30 after inoculation of ST6Gal-I overexpression cells (Hca-P/St cells and Hepa1-6/St cells), the tumor sizes were significantly increased compared to the that in the control group (Fig. 5Aa–Ac and Ba–Bc, respectively). IHC analysis showed that ST6Gal-I overexpression significantly decreased intratumoral penetration by $CD8^+$ T lymphocytes in both models compared with that observed in control grafts, while there was no significant difference in the fraction of $CD4^+$ T cells in the Hca-P/St tumor grafts (Fig. 5C and D).

To determine the ability of ST6Gal-I overexpression cells to activate tumor-infiltrating T lymphocytes, we evaluated biomarkers of activation in T cells from tumor grafts. The results showed that the expression levels of TNF-α and IFN-γ were markedly decreased in tumor grafts in the Hca-P/St and Hepa1-6/St groups compared to those in the control groups (Fig. 5E and F), while the levels of TGF-β1 and IL-10 were increased. In summary, ST6Gal-I upregulation enhanced the tumorigenicity of tumor cells and suppressed intratumoral penetration of $CD8^+$ T lymphocytes. Furthermore, ST6Gal-I upregulation reduced the expression levels of TNF-α and IFN-γ and increased the levels of TGF-β1 and IL-10 in vivo.

Discussion

Our study showed that ST6Gal-I overexpression protects hepatocarcinoma HepG2 cells from T cell–specific lethal effects. We also provided evidence that the mechanism of immune evasion from tolerogenic to immunogenic in ST6Gal-I overexpression cells is mediated via the CD147/MMP signaling pathway. Furthermore, ST6Gal-I upregulation in mouse hepatoma cells reduced the numbers of $CD8^+$ T cells infiltrating the tumor, suppressed the intra-tumoral levels of IFN-γ and TNF-α, and together promoted tumor growth. Therefore, ST6Gal-I may play a positive role in HCC immune escape.

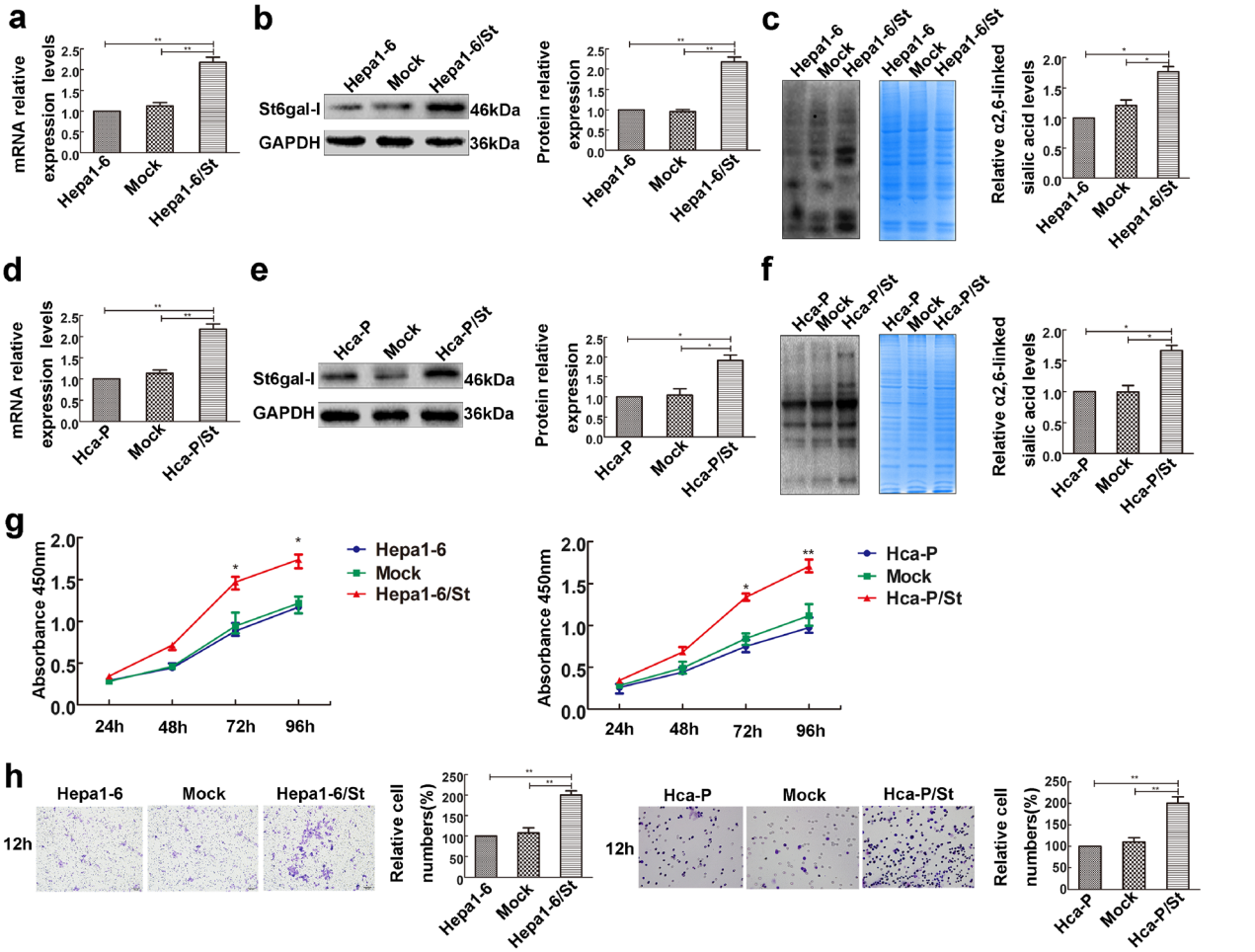

Fig. 4 Upregulation of ST6Gal-I promotes tumor cell proliferation and migration in murine hepatoma carcinoma cells. Overexpression of ST6Gal-I in Hca-P and Hepa1-6 cells were confirmed by real-time PCR (A and D), Western-blot assays (B and E), and SNA lectin blot (C and F). (G) Cell proliferation ability measured by a Cell Counting Kit-8 assay. (H) Transwell migration assay was used to determine the migration ability of cells. Results are the mean of three independent experiments ± SD (*$P < 0.05$, **$P < 0.01$). Hepa1-6/St or Hca-P/St, pcDNA3.1/ST6Gal-I vector transfected stable clones

Although many reports have proposed that aberrant sialylation is correlated with the invasion and metastasis of tumor cells (e.g., cervix and breast carcinomas), its role in cancer is controversial [1, 22, 25]. A study showed that α2,6-sialic acid overexpression in tumors enhanced their migration ability as well as their invasiveness by promoting interactions with matrix proteins [17]. Similarly, α2,6-sialic acid overexpression facilitates the progression of prostate cancer [29]. In this study, we found that increasing sialic acid levels on HepG2 cells surface enhanced tumor immune escape.

CD147, which is a highly glycosylated immunoglobulin superfamily transmembrane protein with three potential N-linked glycosylation sites [26], is enriched on the surface of many malignant tumor cells. Similarly, CD147 contributes to tumor progression and metastasis and functions to induce MMP production [11, 12]. This might explain why increasing levels of CD147 and MMP2/7/9 were observed in the ST6Gal-I overexpression cells. In addition, Bcl-2 family proteins regulate the activation of caspases and the mitochondrial apoptosis pathway [9]. In accordance with this, we found that the pro-apoptotic protein (Bad and Bax) expression was decreased, while anti-apoptotic protein (Bcl-2) expression was increased in ST6Gal-I overexpression cells co-cultured with T cells.

Recently, it has been reported that HCC cells overexpress amphiregulin and promote Treg cell activity, leading to suppression of $CD8^+$ T cell–mediated anti-tumor responses [32]. Furthermore, tumor sialylation promotes murine B16 melanoma cellular immune evasion by interfering with the T effector/Treg balance regulated by innate immune cells [19]. However, the specific molecular mechanisms of cellular immune evasion were not clarified, although the role of T cells in immune evasion is beyond question. Since $CD8^+$ T cells are crucial for inhibiting tumor growth [20], we first analyzed the role of HCC-derived ST6Gal-I in $CD8^+$ T cell regulation. Our results

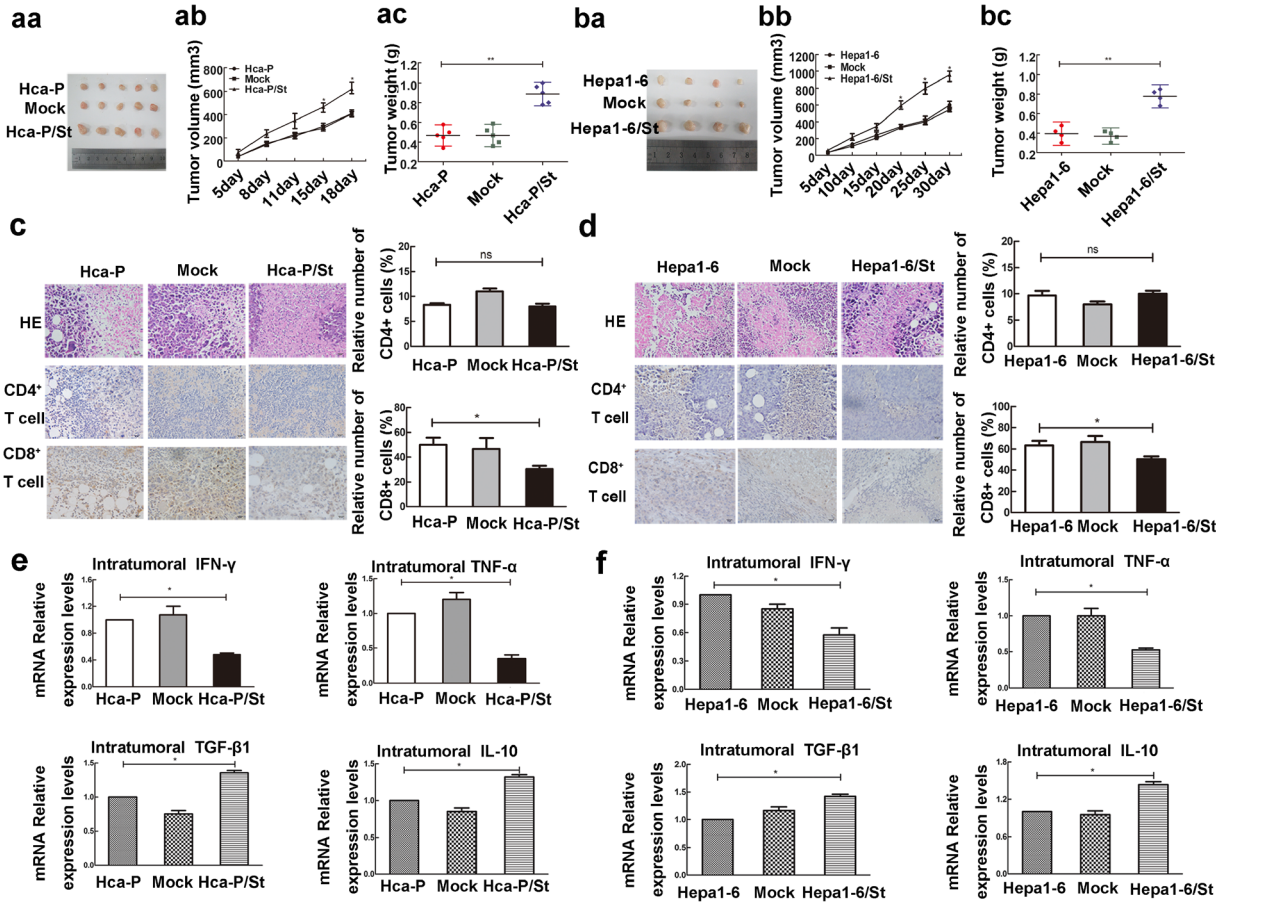

Fig. 5 Overexpression of ST6Gal-I in tumor cells promotes the growth of mouse tumor xenografts, while inhibits anti-tumor immune responses in HCC. (Aa) Images of the Hca-P tumors formed on the inguen of mice at the 18th day after cell inoculations. (Ab and Ac) Average tumor weights and sizes were measured in different groups. (*$P < 0.05$, **$P < 0.01$). (Ba–c) Forced expression of ST6Gal-I in Hepa1-6 cells also facilitated tumor growth as described in (Aa–c). (C and D) Intratumoral penetration of $CD8^+$ and $CD4^+$ T lymphocytes in each group were measured by immunohistochemical staining. The relative rate of $CD8^+$ and $CD4^+$ T cell infiltration were shown in histogram. Photos of inguinal tumor were taken at the magnifications of × 400 (*$P < 0.05$, **$P < 0.01$). (E and F) Expression of IFN-γ, TNF-α, TGF-β1 and IL-10 in intratumoral T cells were analyzed using real-time PCR

indicated that ST6Gal-I overexpression in tumor cells decreased the levels of the immunoactivating cytokines TNF-α and IFN-γ and increased the levels of immunosuppressive cytokines TGF-β1 and IL-10 in a co-culture system. In addition, ST6Gal-I overexpression suppressed intratumoral penetration by $CD8^+$ T cells in Hca-P/St or Hepa1-6/St tumors (Fig. 4), indicating a shift from T cell immunity toward T cell tolerance after tumor implantation.

To our knowledge, although the mechanisms are still not fully understood, we are the first to report the role of ST6Gal-I in modulating immunity in HCC. Moreover, our studies also expose the possible molecular mechanisms involved in tumor immune escape. Hence, our study provides new information indicating that regulating sialic acids on tumors might have therapeutic implications in HCC immunotherapy. This might form the basis of new strategies for immunotherapeutic and molecular-targeted treatments.

Author contributions Wang L. conceived and designed the study. Wang L., Li S., Yu X., Han Y., Wu Y., Wang S., and Chen X. performed the experiments. Wang L. and Li S. wrote the paper. Zhang J. and Wang S. reviewed and edited the manuscript. All authors read and approved the manuscript.

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Compliance with ethical standards

The study protocol conformed to the principles of the Declaration of Helsinki and was approved by the Ethics Committee of The First Affiliated Hospital of Dalian Medical University, Dalian City, P.R. China

Conflict of interest The authors declare that they have no conflicts of interest.

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