TM4SF5-mediated liver malignancy involves NK cell exhaustion-like phenotypes

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Received: 13 July 2021 / Revised: 8 November 2021 / Accepted: 18 November 2021 / Published online: 18 December 2021
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Abstract
Aberrant extracellular matrix and immune cell alterations within the tumor microenvironment promote the pathological progression of liver carcinogenesis. Although transmembrane 4 L six family member 5 (TM4SF5) is involved in liver fibrosis and cancer, its mechanism avoiding immune surveillance during carcinogenesis remains unknown. We investigated how TM4SF5-mediated signaling caused immune evasion using in vitro primary cells and in vivo liver tissues from genetic or chemically induced mouse models.

TM4SF5-transgenic and diethylnitrosamine (DEN)-induced liver cancer mouse models exhibited fibrotic and cancerous livers, respectively, with enhanced TM4SF5, pY705STAT3, collagen I, and laminin γ2 levels. These TM4SF5-mediated effects were abolished by TM4SF5 inhibitor, 4′-(p-toluenesulfonylamido)-4-hydroxychalcone (TSAHC). TM4SF5-dependent tumorigenesis involved natural killer (NK) cell exhaustion-like phenotypes including the reduction of NK cell number or function, which were blocked with TSAHC treatment. TM4SF5 expression in cancer cells downregulated stimulatory ligands and receptors for NK cell cytotoxicity, including SLAMF6, SLAMF7, MICA/B, and others. TM4SF5 suppression or inhibition reduced STAT3 signaling activity and recovered the receptor levels and NK cell surveillance, leading to reduced fibrotic and cancerous phenotypes, and longer survival. Altogether, these findings suggest that TM4SF5-mediated STAT3 activity for extracellular matrix modulation is involved in the progression of liver disease to HCC and that TM4SF5 appears to suppress NK cells during liver carcinogenesis.

Keywords Immune checkpoint · NK cell immune therapy · Liver cancer · Signal transduction · L6 family member

Introduction
Chronic liver injury progressively causes liver diseases, including nonalcoholic fatty liver disease (NAFLD), via excessive production of the extracellular matrix (ECM), which drives fibrosis and cirrhosis and ultimately results in hepatocellular carcinoma (HCC) [1]. Specifically, the excessive ECM deposition during fibrosis and cirrhosis activates abnormal cell proliferation signaling for HCC development [2].

Nonalcoholic injury-mediated liver inflammation results in the production of diverse cytokines, which can facilitate ECM production for preneoplastic progression and eventual carcinogenesis. In addition to the influence of the inflammatory environment, immune cells in the microenvironment are critically associated with the carcinogenic processes [3]. After their transformation from normal to malignant cells, cancer cells present tumor antigens to T cells, and antigen-presenting cells (APS, including dendritic cells, B cells, and
Unlike cytotoxic CD8+ T-cells, NK cells are activated by apoptosis-inducing ligand (TRAIL) and Fas Ligand (FasL), as well as via death receptor signaling (TNF-related apoptosis-inducing ligand 1 (PD-L1) via T-cell checkpoint blockade in obese cancer patients [5] and non-small cell lung cancer [6]. Despite significant immune surveillance in the liver, HCC can still occur as a result of chronic liver infection or inflammation. Natural killer (NK) cells make up approximately 5% of the liver lymphocyte population and kill tumors via perforin and granzyme B. NK cells can also function to target and prevent liver cancer and metastases [12]. The high frequency of liver-resident NK cells in the healthy liver also suggests an essential role of NK cells in targeting and preventing liver metastasis. Although we currently have a good understanding of the complex and often redundant activation/inactivation pathways of NK cells [13], HCC-specific checkpoints in hepatocytes and NK cells warrant further investigation.

Transmembrane 4 L six family member 5 (TM4SF5) is a potential target for NK cell-related immunotherapy against HCC. TM4SF5 activates non-receptor tyrosine kinase c-SRC via direct physical association [20], which leads to signal transducer and activator of transcription 3 (STAT3) activation [21]. Although TM4SF5 is involved in animal liver fibrosis and xenograft growth, the mechanism by which TM4SF5 is influenced by the immune system, particularly via NK cells, during hepatic carcinogenesis remains unknown.

In this study, the roles of TM4SF5 in the development of precancerous and cancerous phenotypes in genetically engineered or chemically treated animals were explored in regard to TM4SF5-dependent signaling and crosstalk between cancer cells and immune cells. We found that TM4SF5-mediated STAT3 activity caused excessive ECM deposition during hepatic carcinogenesis, which was blocked by TM4SF5 inhibition, using an anti-TM4SF5 small compound. TM4SF5 inhibition or suppression downregulated co-stimulatory activation ligands in hepatocytes, which caused decreased cognate receptor expression on NK cells and eventually led to immune surveillance of TM4SF5-positive tumors. Thus, TM4SF5 is a potential target for NK cell-related immunotherapy against HCC.

Materials and methods

Cells

Human hepatocarcinoma cell lines that lacked TM4SF5 expression (SNU449 and empty-vector control SNU449Cp) or that expressed wild-type (WT) TM4SF5 ([exogenous expression] SNU449T7, [endogenous expression] HepG2, Huh7, and HepG2 cells) were used for in vitro experiments and were previously described [17]. Cells were purchased from either the Korean Cell Bank (Seoul, Korea) or American Type Culture Collection (ATCC, Manassas, VA, USA). Hepatocytes were cultured in Roswell Park Memorial Institute (RPMI) 1640 Medium or Dulbecco’s Modified Eagle Medium (DMEM) (Cytiva, Marlborough, MA, USA) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (GenDEPOT Inc., Barker, TX, USA) at 37 °C in 5% CO2. Cells were stably infected with empty pLJM1-EGFP (Addgene) or TM4SF5-encoding pLJM1-EGFP lentiviral vectors. Human NK92 cells were cultured in α-Minimum Essential Medium (MEM) (Invitrogen, Grand Island, NY, USA) containing 200 U/mL recombinant human interleukin (IL)-2 (PeproTech, Rocky Hill, NJ, USA), 12.5% FBS, and 12.5% fetal horse serum (GenDEPOT Inc.). Stable cells were cultured in RPMI 1640 (WelGene) containing 10% FBS, geneticin (G418; 250 μg/mL), and antibiotics (Invitrogen, Grand Island, NY, USA). Cells were passaged every 3–4 days at ratios specified by the suppliers. Cells were monitored for mycoplasma contamination.
Transfection and infection

The shRNA or cDNA expression plasmids were transfected for 48 h using Lipofectamine RNAiMAX or Lipofectamine 3000 (Thermo Fisher Scientific, Waltham, MA, USA), respectively, according to the manufacturer’s protocols. Alternatively, cells were infected with lentivirus for shRNA or cDNA expression for 24 h. Lentiviruses encoding shRNA against TM4SF5 (5′-CCCAGGACATGTTGATCGGGAAA ATGTGCCCTAGGCAGCATTCTCCTGACGTGT TTGTTG-3′ for sequence #2, 5′-CCGAGCATCAGCTGGATC TTGGTTTGGTGTTTTTG-3′ for sequence #4) were prepared using lentiviral vector pLKO.1 (Addgene). Infected cells were selected with puromycin (2 μg/mL, GenDEPOT).

Mice

Age-matched WT and Tm4sf5-knockout (Tm4sf5−/−) C57BL/6 male mice as well as WT and TM4SF5-transgenic (TgTM4SF5) FVB/N male mice were used for in vivo experiments. TgTM4SF5 FVB/N mice with a CMV promoter on the Tg construct for expression throughout the entire mouse were generated by breeding WT FVB/N and TgTM4SF5 C57BL/6 mice [22] for 10 generations. The FVB/N strain was selected because it is susceptible to spontaneous development of lung cancer, relatively than cancer of any other organs, including the liver. Furthermore, the ratio of liver tumors is lower in FVB/N mice than in other strains [23]. Tm4sf5−/− C57BL/6 mice were generated via embryo injection and transfer to normal, healthy female C57BL/6 recipient mice (Macrogen, Seoul, Korea), as described previously [24]. Briefly, the exon 1 of C57BL/6N-Tac Tm4sf5 gene by Cas9 proteins single guide RNAs (50 μg, 5′-GAG GTTGCCGTCGCCAGGTGG-3′ and 5′-GCTAGGGTG GCCGTCCGCCAGG-3′ was targeted, and its deletion was proved by genotyping using primers for mouse-Tm4sf5 (forward 5′-CCAAGGCTCCACCTGTA-3′, reverse 5′-GCTCCAGCTTCCCCACCAT-3′ for KO exon1). We further regularly backcrossed the KO mice with WT mice. F1 heterozygous littersmates (Tm4sf5+/−) were bred to generate homozygous mice (Tm4sf5−/−). The littersmates of WT, Tm4sf5−/− C57BL/6, WT or TgTM4SF5 FVB/N mice (isolated from a variety of different C57BL/6 or FVB/N litters, respectively) were used in experiments after randomized assignment following genotyping.

Chemically induced animal models

Four-week-old mice (BALB/c) were purchased from Orient Co. Ltd (Seungnam, Korea). The mice were housed in a specific pathogen-free room with controlled temperature and humidity. Five-week-old mice (n ≥ 5) were injected intraperitoneally with or without CCl4 (Sigma-Aldrich, St. Louis, MO, USA; 1 mg/kg body weight, 1 time in the beginning of the experiment), or diethylnitrosamine (DEN, Sigma-Aldrich; 50 mg/kg body weight, one time/week) in 40% olive oil for 16 or 27 weeks, respectively. For 4′-(p-toluenesulfonylamido)-4-hydroxychalcone (TSAHC) [25] administration [50 mg/kg in 40% dimethylsulfoxide (DMSO)], mice were injected intraperitoneally for 16 weeks (every 2 or 3 days per week). TSAHC injection was started either at week 0 (DEN + TSAHC1 group) or week 10 (DEN + TSAHC2 group) for overall 27-week-treatment for DEN-treated liver cancer model. Alternatively, age-matched (2-week-old) WT and Tm4sf5−/− mice (n ≥ 5) were injected intraperitoneally with or without DEN (25 mg/kg body weight) at day 0. TSAHC was also injected intraperitoneally twice a week (5 mg/kg body weight). After 45 weeks, the mice were sacrificed for analyses. The mice were euthanized with ether, and the liver and spleen tissues were resected. One piece of liver tissue was immediately frozen in liquid N2, whereas a second piece was embedded in paraffin or alternatively used for primary hepatocyte preparation. Plasma samples were also collected for plasma parameter analyses.

Western blot analysis

Sub-confluent cells in normal culture media were transfected or infected with control or specific siRNA, shRNA vectors, or virus encoding the indicated molecules for 48 or 24 h, respectively, in the presence of vehicle or TSAHC [26] treatment. Animal liver tissues were harvested for whole-cell or tissue extraction using a modified RIPA buffer, as previously described [17, 21]. The primary antibodies (generally at 1:1000 dilution) used included antibodies against laminins, pY397FAK, MICA/B (Abcam, Cambridge, UK), p-ERKs, ERKs (Cell Signaling Technology, Danvers, MA, USA), pY705STAT3 (Millipore, Billerica, MA, USA), β-actin, α-tubulin, SLAMF7 (CS1), MICA/B, STAT3, pY577FAK (Santa Cruz Biotechnology, Santa Cruz, CA, USA), FAK (BD Transduction Laboratories, Bedford, MA, USA), collagen I (Acris Antibodies, Herford, Germany), and TM4SF5 [17]. The TM4SF5 C-ter (epitope region of RKKQDTPH197) antibody was custom designed (Pro-Sci, Poway, CA, USA).

Natural killer cell cytotoxicity assay

Evaluation of NK cell cytotoxic activity was performed using a lactate dehydrogenase (LDH) cytotoxicity assay kit (Cytotoxicity Detection Kit Plus, Roche). Human HCC cell lines (Huh7 or HepG2 cells with endogenous TM4SF5 expression; target cancer cells) were seeded (0.5 × 106 cells/well; 50 μL) into a 96-well plate in triplicate with assay medium (RPMI 1640 with 1% FBS). Human NK92 cells
pre-treated with IL-2 (effector cells) were adjusted to a concentration of 1 × 10^6 cells/mL in assay medium, and then two-fold dilutions of the NK cells were prepared (1 × 10^6, 0.5 × 10^6, 0.25 × 10^6, and 0.125 × 10^6 cells/mL). NK cell suspension was pipetted into the wells of the 96-well plate in triplicate at effector (E) to target (T) cell ratios (E:T) of 1:2.5, 1:5, and 10:1 to see the TM4SF5-dependent effects. Cells were incubated in the 96-well plate for 4 h at 37 °C in 5% CO_2. LDH released from the dead target cells was measured using an ELISA reader at 492–690 nm. Percentage (%) of cytotoxicity was calculated as follows: [LDH (effector-target cell mix) − LDH (effector cell)] − LDH (target cell low control) × 100. After co-culturing, non-adherent NK cells were collected and centrifuged at 150×g for 5 min to remove the target cancer cell debris. Quantitative RT-PCR was then performed to analyze the ligand or receptor mRNA levels of the hepatocytes or NK92 cells, respectively.

**Murine splenic and intrahepatic immune cell analysis**

Mouse spleens and livers were dissected, and cell suspensions were generated by mechanical disruption through 70- or 100-μm nylon mesh filters, respectively. Immune cells were isolated from the spleens and suspended in RPMI 1640 (with 2% FBS). The immune cell suspension was then incubated with Fc Block (BD Bioscience, Cat. No: 554657) and centrifuged at 480×g for 8 min, and the supernatant was removed. The cell pellets were resuspended in ammonium-chloride-potassium (ACK) buffer (RBC lysis buffer, Thermo Fisher Scientific, Cat. No: A1049201) and centrifuged at 480×g for 8 min, and the supernatant was removed. The cell pellets were washed twice using FACS staining buffer (BD Bioscience, Cat. No: 554657) and centrifuged at 480×g for 8 min. The cell pellet suspensions with the staining buffer were then incubated with Fc Block (BD Bioscience, Cat. No: 564219) at 4 °C for 15 min to block the lymphocyte FcγII/III receptors. Erythroid cells were lysed with red blood cell (RBC) lysis buffer (Invitrogen). To assess IFN-γ, perforin 1 (PRF1), and granzyme B (GZMB) expression, lymphocytes were incubated with 5 ng/mL phorbol 12-myristate 13-acetate (PMA, Sigma), 500 ng/mL ionomycin (Sigma), and 10 ng/mL monensin (Sigma), respectively, for 4 h at 37 °C in 5% CO_2. Lymphocyte FcγII/III receptors were blocked with Fc Block (BD Bioscience), and surface antigens were stained with conjugated and biotinylated monoclonal antibodies (mAbs). Splenic or intrahepatic immune cells were then fixed and permeabilized using Cytotox/ Cytoperp (BD Bioscience), and intracellular antigens were detected using conjugated and biotinylated mAbs at 4 °C for 30 min. Phycocerythrin (PE)-conjugated anti-human PRF1 allophycocyanin (APC)-conjugated anti-mouse GZMB, PE-conjugated anti-mouse PRF1 fluorescein isothiocyanate isothiocyanate (FITC)-conjugated anti-mouse CD45, PE-conjugated anti-mouse CD4, APC/Cy7-conjugated anti-mouse CD3, FITC-conjugated anti-mouse CD8a (BioLegends), BV421-conjugated anti-mouse CD8a, and BV421-conjugated anti-mouse NK1.1 antibodies (BD Bioscience) were used for staining. Flow cytometry analysis was performed on a FACS LSR Fortessa X-20 (BD) and analyzed with FlowJo version 10.6.1. Graphic data were presented the mean ± standard error of the mean (SEM).

**Immunohistochemistry and tissue staining**

Liver tissues from human liver cancer patients, Tg*TM4SF5* FVB/N mice, or C57BL/6 (WT or Tm4sf5−/− KO) mice treated with or without DEN and in the absence or presence of TSAHCl were processed for immunohistochemistry. Liver sections were fixed with 3.7% formaldehyde and embedded in paraffin. The fixed liver sections were deparaffinized and rehydrated. Antigen retrieval was performed with heat-induced epitope retrieval (HIER) using sodium citrate buffer (pH 6.0). Quenching and blocking were performed using 3% H_2O_2 in distilled water and 1% normal goat serum in phosphate-buffered saline (PBS). Antigens were stained using the avidin–biotin complex (ABC) method (VECTASTAIN Elite ABC HRP Kit, Vector) and were detected with 3,3′-diaminobenzidine (DAB) stain (Vector). Antibodies against TM4SF5 [17], collagen I (Acris Antibodies), pY705STAT3 (Cell Signaling Technology), normal rabbit or mouse IgG, α-SMA (Sigma-Aldrich), α-fetoprotein (AFP), CD34, Ki67, laminins (Abcam), α-L-fucosidase [FUCA B; (AFU)], and laminin γ2 (Santa Cruz Biotechnology) were used for immunostaining. Ten random images per slide were saved using a digital slide scanner (MoticEasyScan, Motic, British Columbia, Canada). The tissues were also processed with Masson’s trichrome as well as hematoxylin and eosin stains, as previously described [27].

**Polymerase chain reaction**

Total RNA from animal liver tissues or cells was isolated using TRIzol reagent (Invitrogen), and total RNA isolated from the samples was treated with ReverTra Ace qPCR RT
Master Mix (TOYOBO) with gDNA Remover to generate complementary DNA (cDNA). The cDNA was subjected to RT-PCR using the Dream Taq Green PCR Master Mix (Thermo Scientific, San Jose, CA, USA). Quantitative RT-PCR was performed with LaboPass EvaGreen Q Master (Cosmo Genetech, Seoul, Korea) and performed with the CFX Connect Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). The mRNA levels were normalized against 18S ribosomal RNA, using the ddCq method. The CFX Maestro Software (Sunnyvale, CA, USA) was used to analyze the data. Primers were purchased from Cosmo Genetech (Seoul, Korea). The primer sequences are shown in Table 1.

### Analysis of public RNA expression data

The mRNA expression levels of some genes were searched in the public Gene Expression Omnibus (GEO, https://www.ncbi.nlm.nih.gov/geo/) database (accession numbers GSE6764, GSE14520, and GSE76427). The mRNA expression levels were extracted using the built-in function, ‘Analyze with GEO2R’. The specific identification numbers for TM4SF5 and SOCS1 were 8003939 and 7999423 from GSE6764, respectively, and TM4SF5 and LAMC2 were ILMN 2167808 and ILMN 1653824 from GSE76427, respectively. Public identification numbers of GSE14520 were for TM4SF5 (ID No. 4507538), SLAMF7 (12711663), MICA (4557750), ULBP1 (13376825), and ULBP2 (13376823). The available mRNA expression levels for the indicated groups were further analyzed for comparison. The

| Table 1 | The primer sequences for RT-PCR or q-PCR in the current study |
|----------|---------------------------------------------------------------|
| **Gene name** | **Sequence** |
| Human | |
| TM4SF5 | CTTGCTCAACCGCACTCTAT | ATCCCAACAGTACTATCTCCCA |
| hSLAMF6 | GTCGAAAATCCACGTGACTAA | GTAAGAGCTGGTGCTCTTCATC |
| hSLAMF7 | TTGGTCTCGAGAACGTAATG | CAGGGCCACTCGGATTTAAT |
| hMIC | CTTTGGCTATGAACGTCACA | CCCTCTGAGACCTGGTCTCA |
| hULBP1 | TTTCCCTAAGGCCAATCTCTG | AGGAACCTCCAAAGTCTCTT |
| hULBP2 | CATTACCTCCTAATGGGAGAGTCT | TGGTGCCTGAGACATGCG |
| hULBP3 | CTAATGCAACGAAGAAGAG | TATGCTTTGGGTTGAGCTAA |
| hULBP5 | GACTGCGCTGATCTAATTACT | GGCTGCGCGTTATATTTG |
| hCD111 (Nectin-1) | ATCCGCTTGGTGTTGTGTT | TGGTCTTTGCTGCTGTAGT |
| hCD112 (Nectin-2) | TTCCTGGATCTAGAGGAGTAG | CCTTGCCCTGTTAGAATCA |
| hCD113 (Nectin-3) | TTCCCTGGATCTAGAGGAGTAG | CCTTGCCCTGTTAGAATCA |
| hCD155 (PVR) | AGGTATCATCTCCTGGCTATGA | CCATGTCGCTGCTCATAA |
| hCDM1 | GCTTCTGCTGTTGTCTCTTT | CTCGATCTAGTCTGATTTT |
| hKlrk1 (NK2D) | CAGCAAAAGAGAGCAGGATTTA | GTATGAGTTGGTGAGAGAATG |
| hPRF1 | CCCAGTGACACACACAGG | TGGTCCGAGGTGTAG |
| hGZMB | CTCTCGTATACGAGACAGCCT | CCGCTCTGCTTCTCTGATATG |
| hCD107a | CTTTCAGAGCTGCCGATCGAG | CATGTTCTTAGGGCCACCTTT |
Mann–Whitney U test was applied for statistical analysis using GraphPad Prism 7.0. A box-and-whisker plot was drawn using GraphPad Prism 7.0.

**Study approval**

Human liver tissues were collected after obtaining informed consent from the liver cancer patients at the Seoul National University under institutional review board (IRB)-approved protocols (Seoul, Korea). All animal procedures were performed in accordance with the Seoul
Three isolated experiments were performed to determine statistical significance. * and ** represent \( p < 0.05 \) and \( p \leq 0.001 \), respectively, and ns indicates no significance, following Student’s \( t \) tests.

**Results**

**TM4SF5-mediated hepatic carcinogenesis**

Because C57BL/6-\( T_g^{TM4SF5} \) mice exhibited nonalcoholic steatohepatitis (NASH)-associated fibrotic livers [24], we determined whether TM4SF5 overexpression in a disease-susceptible mouse strain (i.e., FVB/N [23]) caused an enhanced malignant cancer phenotype. The livers of 1-year-old FVB/N-\( T_g^{TM4SF5} \) mice were analyzed to test this hypothesis. Out of seven mice, we found two with hepatic nodules, suggesting hepatic carcinogenesis. Interestingly, the \( T_g^{TM4SF5} \) mice also exhibited enlarged spleens compared with age-matched wild-type (WT) mice (Fig. 1A).

The FVB/N-\( T_g^{TM4SF5} \) mice showed higher aspartate aminotransferase (AST), alanine aminotransferase (ALT), and low-density lipoprotein (LDL) levels with comparable levels of albumin and serum triglycerides (TGs) compared with age-matched WT mice (Fig. 1B). Furthermore, \( CD34 \) and \( Ki67 \) mRNA levels were higher in the livers of FVB/N-\( T_g^{TM4SF5} \) mice than in age-matched WT mice (Fig. 1C), indicating cell proliferation. Interestingly, liver monocyte chemoattractant protein 1 \([Mcp-1 (chemokine ligand 2, Ccl2)]\) and \( F4/80 \) mRNA levels were also elevated in the \( T_g^{TM4SF5} \) mice compared with age-matched WT mice, indicating immune system involvement (Fig. 1D). Indeed, serum MCP-1 was reported to be positively correlated with \( \alpha \)-fetoprotein (AFP) levels, suggesting it as another tumor marker in HCC [28].

The levels of laminins and \( pY^{705} \) \( \text{STAT3} \) (Fig. 1E), collagen I, \( \alpha \)-smooth muscle actin (SMA), laminins, laminin \( \gamma_2 \), and TM4SF5 expression levels were elevated in FVB/N-\( T_g^{TM4SF5} \) mice compared with age-matched WT mice, in addition to \( CD34 \), \( \alpha \)-fetoprotein (AFP), \( \alpha \)-l-fucosidase [FUCA (AFU)], in addition to \( pY^{705} \) \( \text{STAT3} \) and extracellular matrix factors, including collagen I, laminin \( \gamma_2 \), and laminins. Data shown represent three isolated experiments.

National University Laboratory Animal Maintenance Manual and were approved by the IRB of the Institute of Laboratory Animal Resources Seoul National University (SNU-IACUC) (SNU-140423-11-7, SNU-130911-2-3, SNU-170920-9, SNU-190122-6-3).

**Statistics**

Statistical analyses were performed using Prism Software (GraphPad 7.0, La Jolla, CA, USA). Two-way analysis of variance (ANOVA), ordinary one-way ANOVA, an unpaired, one-tailed Mann–Whitney \( U \) test, or unpaired, two-tailed Student’s \( t \) tests were performed to determine statistical significance. A \( p \) value < 0.05 was considered statistically significant.

**Discussion**

TM4SF5-mediated liver malignancy involves NK cell exhaustion-like phenotypes
Next, we investigated the roles of the immune system in TM4SF5-mediated hepatic carcinogenesis using $Tm4sf5^{-/-}$ and age-matched WT mice treated with or without diethylnitrosamine (DEN) in the absence or presence of TM4SF5 inhibitor $4'-p$-toluenesulfonlamido)-4-hydroxychalcone (TSAHC) treatment. The mice were treated once with DEN and twice a week with TSAHC. After 45 weeks, the livers of the mice were analyzed, as depicted in the scheme (Fig. 3A). The livers of the mice exhibited dramatic differences in nodule formation depending on TM4SF5 expression and function (i.e., activity). Age-matched WT and knockout mice without DEN treatment did not form liver nodules. However, WT mice treated with DEN showed dramatic nodule formations, which was abolished by additional TSAHC treatment (Fig. 3B). Indeed,
**Experiments and Results**

**Tm4sf5−/− mice did not show substantial nodule formation** with being similar in the DEN-treated WT mice with TSAHC treatment (Fig. 3B). Quantitative analysis in terms of the number of tumors larger than 0.1 mm in diameter, tumor load (as the sum of tumor diameter of all tumors with diameters > 1.0 mm), and the maximal tumor size resulted in greater values for DEN-treated WT mice than DEN-treated knockout mice and DEN/TSAHC-treated WT mice, although the change tendencies were statistically significant (p = 0.0470) or insignificant (p = 0.0757–0.1910) (Fig. 3C). Unlike the FVB/N-TgTM4SF5 mice, DEN-treated WT mice did not have significantly larger spleen sizes compared with DEN-treated knockout mice and DEN/TSAHC-treated WT mice (p = 0.4065, Fig. 3D).

Hematoxylin and eosin (H&E) staining and immunohistochemical analyses of the liver tissues showed dramatically different tumor lesions among the mice. More aggressive tumor lesions were shown in the DEN-treated WT mice by positive Ki67 and AFP staining, whereas DEN-treated knockout and DEN/TSAHC-treated WT mice showed fewer tumors with less positive or negative Ki67 and AFP staining (Fig. 3E). Interestingly, we found significant formation of noninfectious granulomatous inflammation (with immune cell infiltrates) near the portal tracks with positive AFP staining in the DEN-treated WT mice, which was abolished by additional TSAHC treatment (Fig. 3F). These observations indicate that TM4SF5 activation may be involved in the inflammation during DEN-induced hepatic carcinogenesis and that inhibition of TM4SF5 by TSAHC may interfere with the DEN-mediated effects.

**Natural killer cells were inhibited by TM4SF5 during diethylnitrosamine-induced hepatic carcinogenesis**

Because the hepatic granuloma-like lesions within the DEN-induced, AFP-positive tumors exhibited immune cell infiltration, we investigated the potential mechanism of the immune cells in carcinogenesis. First, we used immunostaining and FACS to analyze the population of splenic immune cells for CD3+/CD4+ (T-helper cells, Th), CD3+/CD8+ (cytotoxic T cells), and CD3−/NK1.1+ (NK cells) cell markers (Fig. 4A). Splenic CD4+ Th-cell populations were not significantly different between vehicle- and DEN-treated WT or KO mice; however, DEN/TSAHC-treated WT mice showed significantly (p = 0.0331) lower CD4+ Th-cell populations compared with DEN-treated WT mice (Fig. 4B, left). CD8+ T-cell populations were not differential between vehicle-treated WT and KO mice (p = 0.9155), and significantly increased in KO mice upon DEN treatment (p = 0.0362) or in DEN-treated WT mice upon further TSAHC treatment (p = 0.0376) (Fig. 4B, middle). Therefore, CD4+ Th- and CD8+ T-cell populations appeared not to be significantly involved in the immune effects during TM4SF5 expression (e.g., WT vs. KO) but in TM4SF5 function/activity-dependent carcinogenesis (e.g., WT-DEN vs. WT-DEN/TSAHC). However, consistent with the tumor load patterns, DEN-treated WT mice showed a slight but insignificant reduction in the population of NK cells compared with vehicle-treated WT mice (p = 0.1195), and DEN-treated KO mice and DEN/TSAHC-treated WT mice exhibited slight but statistically insignificant recoveries in the splenic NK cell population (p = 0.1510 or 0.1528, respectively), suggesting a possible involvement (though insignificant presumably due to smaller sample numbers) of NK cells in response to TM4SF5 blockade with TSAHC during DEN-induced carcinogenesis (Fig. 4B, right).

We also examined the intrahepatic immune cell populations via immunostaining and FACS for CD3+/CD4+ Th-cells, CD3+/CD8+ T-cells, and CD3−/CD45+/NK1.1+ NK cells (Fig. 4C). Unlike splenic CD4+ Th-cells, hepatic CD4+ Th-cells were reduced in DEN-treated WT mice and were not recovered by TSAHC treatment or Tm4sf5-knockout (Fig. 4D, left). Furthermore, hepatic CD8+ T-cell populations were increased in WT mice upon DEN treatment (p = 0.0076), which were not changed by TSAHC treatment or Tm4sf5-deficiency (Fig. 4D, middle). Additionally, DEN-treated WT mice showed higher populations of NK cells than vehicle-treated WT mice (p = 0.0435), which was similar to the levels of DEN-treated KO mice (p = 0.4295) or DEN/TSAHC-treated WT mice (p = 0.9655, Fig. 4D, right). Therefore, intrahepatic immune cells, including Th, cytotoxic T, and NK cell populations, did not appear to be correlated with the TM4SF5-dependent carcinogenesis in DEN-treated mice. However, splenic NK cell numbers might still have roles in DEN-induced carcinogenesis, depending on TM4SF5 activity, although their effects were not statistically insignificant presumably due to small sample numbers (Fig. 4B).

We therefore analyzed further intrahepatic NK cell activity by immunostaining for perforin, granzyme, and cytokine IFN-γ. Although DEN-treated WT mice exhibited lower levels of IFN-γ and granzyme than vehicle-treated WT mice, their levels were not significantly different by additional TSAHC treatment (p = 0.6920 and p = 0.3897, respectively).
and from those in DEN-treated KO mice \((p > 0.9999\) and \(p = 0.2779\), respectively) (Fig. 4E). Meanwhile, only the perforin levels in WT mice were significantly decreased by DEN treatment \((p < 0.0001)\), which were recovered by additional TSAHC treatment \((p = 0.0453)\), suggesting NK cell activity appeared to be associated with TM4SF5 function/activity-dependent carcinogenesis (Fig. 4E). That is, perforin expression from intrahepatic NK cells appeared to correlate with TM4SF5 function and activity during carcinogenesis, since TM4SF5 inhibition using TSAHC could lead to reduction of NK cells number or function for NK cell exhaustion-like phenotypes.

**TM4SF5 in hepatocytes modulated molecules stimulatory for NK cell surveillance**

We next examined how the NK cell cytotoxicity could depend on the TM4SF5 expression and/or activity in hepatocytes. NK92 (effector) cells were co-cultured with hepatocytes (target cancer cells) at different ratios before measurement of lysis of target cells. Endogenous TM4SF5 in Huh7 cells were suppressed (Fig. 5A), before the co-cultures. Cytotoxicity of NK92 cells following the co-cultures increased when they targeted TM4SF5-suppressed cells, compared to non-suppressed target cells (Fig. 5B). Furthermore, we examined membrane proteins (or ligands) on the target hepatocytes, which could be involved in the regulation of NK cell cytotoxicity. qRT-PCR data from three different target cells, including Huh7, Hep3B, and HepG2, showed increased mRNA levels of co-stimulatory ligands on the target hepatocytes with suppression of endogenous TM4SF5, compared with those of non-suppressed (NS) target hepatocytes (Fig. 5C, red-highlighted). They include SLAMF6 [signaling lymphocytic activation molecule (SLAM) family member 6], SLAMF7 (SLAM family member 7), MICA, (major histocompatibility complex (MHC) I-related chain A), MICB (MHC class I related protein B), CADM1 (Cell adhesion molecule 1), ULBP1/2 (UL16-binding protein), and others.

Next, we investigated the TM4SF5-mediated effects on ligand levels and NK cell cytotoxicity after the co-culturing of NK92 effector cells with TM4SF5-expressing or –suppressed target hepatocytes. Analysis of cognate receptor expression of the NK92 cells after co-culture with TM4SF5-suppressed target cells showed increased mRNA levels of natural killer receptor group 2 member D \((NKG2D)\), stimulatory receptor for MICA/B and ULBP1/3 ligands), SLAMF6, and SLAMF7 (Fig. 5D). Consistently, suppression of TM4SF5 in the target cells resulted in increased NK92 cell cytotoxicity via elevated mRNA levels of PRF1, CD107a, TNF-α, FasL, and TRAIL in the NK92 cells (Fig. 5E). Co-culturing of NK92 cells with TM4SF5-suppressed target HepG2 hepatocytes (another target cell line) also resulted in increased cytotoxicity of the NK92 cells (Fig. 5F). Following co-culture with TM4SF5-suppressed target cells, NK92 cells showed higher ERK1/2 activity (Fig. 5G), which is known to be associated with the upregulation of NK cell stimulatory receptors \([32]\). Consistently, when the target hepatocytes were treated with the TM4SF5 inhibitor, TSAHC, the NK92 cell cytotoxicity increased in a dose-dependent manner (Fig. 5H). Additionally, we found that TM4SF5 expression levels in the target cancer cells showed an inverse relationship with the stimulatory ligand levels for NK cell cytotoxicity (Fig. 5I, left and middle). The levels of stimulatory ligands in TM4SF5-positive hepatocytes were increased or highly maintained after TSAHC treatment, whereas the levels appeared to decrease in TM4SF5-negative cells with TSAHC treatment (Fig. 5I, right).
A  
**Splenic immune cells**

| Group           | n |
|-----------------|---|
| WT+Veh          | 6 |
| KO+Veh          | 6 |
| WT+DEN+Veh      | 7 |
| KO+DEN+Veh      | 7 |
| WT+DEN+TSAHC    | 4 |

FSC-A  | CD3  | CD4  | CD8  | NK1.1

B  

**Intrahepatic immune cells**

| Group           | n |
|-----------------|---|
| Veh             | 5 |
| DEN             | 5 |

FSC-A  | CD3  | CD4  | CD8  | NK1.1

D  

| Group           | n |
|-----------------|---|
| Veh             | 5 |
| DEN             | 5 |

FSC-A  | CD45 | CD8  | NK1.1

E  

| Group           | n |
|-----------------|---|
| Veh             | 5 |
| DEN             | 5 |

FSC-A  | IFNγ+ | PRF1+ | GZMB+
TM4SF5 inhibitor abolished TM4SF5/STAT3-mediated precancerous and cancerous phenotypes

Because we showed that hepatic fibrosis/cirrhosis and cancer involves TM4SF5 (Fig. 2B, C), blockade of TM4SF5 function/activity may be a beneficial strategy for the treatment of TM4SF5-dependent liver diseases. Anti-TM4SF5 compound, TSAHC, was used to block DEN-induced carcinogenesis either at the beginning (DEN + TSAHC1) or during DEN treatment (DEN + TSAHC2). DEN treatment caused cancerous liver nodules, which were abolished by TSAHC treatment (Fig. 6A). Particularly, earlier TSAHC treatment (DEN + TSAHC1) led to increased survival and reduced pY705STAT3 and laminin expression levels, whereas later TSAHC treatment (DEN + TSAHC2) did not reduce laminin expression, indicating that earlier TM4SF5 inhibition might be more effective to block the carcinogenesis (Fig. 6B, C). Consistent with these results, decreased laminin expression was evident in mice with earlier TSAHC treatment, but not in those with later TSAHC treatment, leading to a greater survival rate (Fig. 6B). These observations suggest that laminins play an important role in DEN-mediated hepatic carcinogenesis (Fig. 6C, D). Furthermore, public GEO data (GSE14520) analysis showed that TM4SF5 expression was highly elevated in HCC patients (p < 0.0001), and among samples those showing increased TM4SF5 expression in HCC, SLAMF7 (p < 0.0001), MICA (p = 0.0161), ULBP1 (p < 0.0001), and ULBP2 (p = 0.0926) were oppositely decreased (Fig. 6E). These observations thus suggest that increased TM4SF5 expression in HCC samples over control counterparts could be linked to decreased expressions of stimulatory NK cell ligand/receptors. Taken together, these results suggest that blockade of TM4SF5 function/activity inhibits liver carcinogenesis, possibly by abolishing TM4SF5-mediated STAT3 activity, the expression of ECM factors, and improvement of NK cell cytotoxicity.

Discussion

This study provides evidence that TM4SF5-mediated STAT3 signaling promoted collagen I and laminin expression, which were involved in hepatic fibrosis/cirrhosis and eventual carcinogenesis in TM4SF5-transgenic and chemically induced mouse models. Furthermore, inhibition of TM4SF5 expression or activity and thereby of STAT3 activity abolished ECM production and cancerous phenotypes in the mice livers. DEN-mediated hepatic carcinogenesis was abolished by Tm4sf5 gene knockout or TM4SF5 protein inhibition, resulting in an increased population and thereby cytotoxic activity of NK cells. Meanwhile, during blockade of tumorigenesis via the TM4SF5 inhibition but not via TM4SF5-deficiency, Th and/or T cells might play roles in immune surveillance. Suppression or inhibition of TM4SF5 in target hepatocytes (cancer cells) increased the mRNA levels of stimulatory ligands in hepatocytes and cognate receptors in NK cells and also led to enhanced NK cell surveillance. (Fig. 7). Therefore, TM4SF5 may be targeted using an anti-TM4SF5 reagent, such as a small compound TSAHC [25], or its antibody [19] as a potential NK cell-related immunotherapy for HCC, in addition to immunotherapies against PD-1, PD-L1, or CTLA-1 [12]. Indeed, we have reported by mutation study and molecular modeling that TSAHC binding to TM4SF5 occurs at a region of the long extracellular loop (W124 to E129 of LEL) caused induced fit changes, leading to inhibitions of protein–protein interaction, L-arginine binding, and its downstream signaling activity [26]. TSAHC is quite specific for TM4SF5 at 0.3–5 μM, since TM4SF5-negative hepatocytes were not responsive to its treatment [25, 33, 34].

On the cell surface, there are certain domains in which specific membrane proteins and receptors are enriched to transduce specific intracellular signaling pathways. Such domains include focal adhesions, lipid rafts/caveolae, and tetraspanin-enriched microdomains (TERMs). Similar to focal adhesions with integrins that are engaged with the ECM and lipid rafts/caveolae that are enriched with glycosylphosphatidylinositol-linked proteins, TERMS are enriched with tetraspanins for massive protein–protein complexes that include tetraspanins, integrins, and growth factor receptors [35]. TM4SF5, a member of the transmembrane 4 L six family or L6 four transmembrane superfamilies also forms TM4SF5-enriched microdomains (T₃ERMs) [15]. Thus, TM4SF5 at T₃ERMs may function differentially...
Data shown represent three independent experiments, HepG2
(A, D), Hep3B, and HepG2 cells with endogenous TM4SF5 expression (C) were infected with lentivirus for non-specific sequence (NS) or shTM4SF5 (targeting sequences #2 and #4 of TM4SF5) before expression level analysis by qRT-PCR for TM4SF5 or ligands for natural killer cell (NK) cell stimulation (A, C) or before a NK cell cytotoxicity assay using a co-culture system [at different ratios (E:T) of target (T) Hep3B hepatocytes and effector (E) NK92 cells] for 4 h (B). D-G Huh7 (D, E) or HepG2 (F, G) target (T) cells with or without TM4SF5 suppression (A, F, left) were co-cultured with NK92 effector (E) cells for 4 h at an E:T ratio of 10:1 (D, E) or the indicated ratios (F, G) for NK92 cytotoxicity analysis (F, right), qRT-PCR (D–F, left), or whole-cell extract preparation prior to immunoblot analysis (G). H For NK92 cytotoxicity analysis, Huh7 target cells and NK92 effector cells were co-cultured for 6 h (E:T = 10:1), and 4′-p-toluenesulfonylamido)-1,4-dihydroxychalcone (TSAHC) was administered at the indicated concentrations. Statistical significance is indicated by *, **, ***, or ****, which represent a p-value ≤ 0.05, 0.01, 0.001, or 0.0001, respectively, and ns indicates no significance, but red-highlighted ns indicates 0.05 ≤ p-values ≤ 0.10. p-values were calculated by one-way analysis of variance (ANOVA) or one-tailed and unpaired Mann–Whitney U tests. I, J Whole-cell extracts from hepatocytes with different TM4SF5 expression levels (I) and from hepatocytes transiently transfected with empty vector (EV) or TM4SF5 plasmids with or without TSAHC treatment at the indicated concentrations for 24 h (J) were prepared for immunoblot analysis of the indicated molecules. MICA/B blot from stable control SNU449Cp cells showed multiple bands, although stably TM4SF5-expressing SNU449Tc, or Tc cells and other transiently transfected SNU449 cell lines showed a single band. Data shown represent three independent experiments depending on cellular needs, via changes in binding partners or signaling activities/pathways to transduce diverse intracellular signaling pathways in a spatio-temporal manner [36]. TM4SF5 expression in hepatocytes promotes phosphorylations of FAK [37], c-SRC [20], STAT3 [21, 38], and mTOR/S6K1 [26]. TM4SF5 also binds EGFR [39], integrin α5 [38, 39], CD133 [34], CD151 [40], CD44 [41], and mTOR [26] membrane receptors or proteins. Interaction of TM4SF5 with integrin α5 induces VEGF secretion for hepatic angiogenesis via enhanced proliferation and migration of endothelial cells [38]. In addition, we have observed that TM4SF5 is involved in bidirectional crosstalks between hepatocytes and macrophages during development of NASH and is induced in differentiation/activation of macrophages or T cells (data not shown).

TM4SF5-mediated protein–protein complex formation at the TcERMs may serve as a signaling hub of diverse signaling pathways for gene expression or suppression at the transcriptional level. It may also regulate the expression levels of its binding partners via subcellular trafficking or compartmentalization prior to degradation or stabilization. In lung epithelial cells, TM4SF5 expression leads to suppression of ZEB2, which in turn increases alternative splicing factors for CD44 isoforms (from the standard CD44 form to CD44(α8,10) variant form) during idiopathic pulmonary fibrosis [42]. Furthermore, TM4SF5 expression in hepatocytes leads to the downregulation of E-cadherin mRNA levels, thus promoting the loss of contact inhibition via epithelial–mesenchymal transition [17]. Here, we have shown that TM4SF5 decreased the mRNA levels of NK cell stimulatory receptors, such as NKG2D (receptor of MICA/B and ULBP1/3 ligands), SLAMF6, and SLAMF7, and of hepatocyte cognate ligands, via immunoblot experiments and public GEO data analyses. Therefore, it can be likely that TM4SF5 transcriptionally regulated gene expression and was involved in tumor progression. Here, we also found that ERK1/2 phosphorylation increased in NK cells after co-culturing with TM4SF5-suppressed target hepatocytes (Fig. 5G). Consistently, NK cell activation via the expression/activation of stimulatory receptors including those of the self-specific SLAM family for lymphocyte activation [43] and via lytic granule polarization and secretion [44], requires ERKs activation [32].

In addition, TM4SF5-mediated STAT3 activation appeared involved in excessive ECM production and regulation of stimulatory ligands and receptors levels for NK cell cytotoxicity. It is well known that collagen I expression is important for liver fibrosis [45]. Meanwhile, our present study shows that active STAT3 correlated with laminin and laminin γ2 expression, in addition to collagen I, and also with subsequent hepatic carcinogenesis. Active STAT3 in hepatic inflammatory environment has been shown to induce ECM expressions by binding to their promoter regions [46]. Considerable immunostaining for laminin γ2 is shown in the cytoplasm of hepatic carcinoma cells [47] and laminin γ2 is suggested as a novel serum biomarker for HCC [48]. Indeed, TM4SF5 has been previously reported to be related to liver cancer progression, together with cell markers CD133+, CD44+ (bound to TM4SF5)/CD24−/ALDH+/Bmi+, and protein tyrosine phosphatase receptor type F (PTPRF) [34, 41], in addition to laminin γ2+, AFP+, MCP-1+, CD34+, and FUCA+ (AFU) shown in this study. TM4SF5 interacts with IL6R, leading to STAT3 activation independent of IL6 in hepatic cancer cells [21]. Because c-SRC is upstream of STAT3 [49] and TM4SF5 directly binds and activates e-SRC [20], TM4SF5 may therefore activate STAT3 in a ligand-independent manner. Indeed, IL6 activates STAT3 during HCC growth [50]. However, small in-frame deletions around the gpi130 binding site for IL6 [51] and disruption of negative regulators of STAT3 such as SOCS3 and SHP1/2 [52, 53] are involved in ligand-independent STAT3 activations. Recently we reported that TM4SF5 activates STAT3 for the pathological progression to NASH-associated with fibrosis via hepatic inflammation-concomitant SOCS1/3 down-regulation [24]. Here, a negative correlation was shown between TM4SF5 and SOCS1 expression in HCC patient groups. Therefore, TM4SF5 at TcERMs can promote STAT3 activity during the progression toward fibrotic and cancerous liver phenotypes. The blockade of TM4SF5-dependent
A. PBS, DEN, DEN+TSAHC1, DEN+TSAHC2

C. Laminins, pY705STAT3, STAT3, β-tubulin, p-Erk, TM4SF5

D. IgG Rabbit, IgG Mouse, TM4SF5, pY705STAT3, Collagen I, Laminins, Laminin γ2

E. Roessler Liver2 (GSE14520)

- TM4SF5: p = 0.00155
- SLAMF7: p = 3.779e-12
- MICA: p = 0.01613
- ULBP1: p = 1.739e-07
- ULBP2: p = 0.09264
STAT3 activity and liver carcinogenesis by TSAHC treatment appeared further to be correlated with improved NK cell cytotoxicity. The inhibition of TM4SF5 in (target) hepatocytes appeared to activate NK cell surveillance via different signaling pathways; (1) inactivation of STAT3 activity leading to apoptosis directly and activation/induction of stimulatory ligands in hepatocytes for NK cell surveillance indirectly, and (2) activation of ERKs in NK cells for increased SLAM and NKG2D receptor families for improvement of NK cell cytotoxicity. Consistently, STAT3-blocked (or inhibited) hepatocellular carcinoma cells showed upregulated NKG2D ligands (i.e., MICA/B and ULBPs), and thereby cytotoxicity of NK cells treated with supernatant from STAT3-blocked hepatocytes was augmented with a concomitant elevation of molecules associated with NK cytolysis [54, 55]. Interestingly, we have not found any cytokines, chemokines, growth factors, or extracellular matrix that could induce TM4SF5 in NK92 cells upon their treatment alone or in combinations (i.e., tested with approximately 40–50 different factors, data not shown). Indeed, here TM4SF5 knock-out and inhibition (by TSAHC) led to less significant tumor formations in xenografts. However, NK cell cytotoxicity appeared functional upon TM4SF5 inhibition in vitro cells and in vivo animals but not upon Tm4sf5 gene knock-out. Although Th and T cells were shown to be involved in the TM4SF5 inhibition-mediated blockade of tumorigenesis, those immune cells appeared not to be critical for the DEN-mediated tumorigenesis in WT mice, compared to significantly less tumorigenesis in KO mice. Thus, we may speculate that in vivo complicated system without Tm4sf5 gene and related pathway may adopt other immune system (such as Th and/or T cells) or cytotoxic pathway (no survival/growth due to no TM4SF5-STAT3 activation). Presumably, conditional Tm4sf5 KO may be informative on whether the compensatory mechanisms may be the development of the immune system without TM4SF5 involvement. By the way, TM4SF5 in hepatocytes can still be targeted by an anti-TM4SF5 reagent, including TSAHC [25] and its antibody [19].

Altogether, we provide evidence that TM4SF5 plays an important role in the pathological progression of liver carcinogenesis via TM4SF5-mediated STAT3 signaling for ECM production. Concomitantly, TM4SF5 mediates the downregulation of stimulatory membrane ligands on target hepatocytes that stimulate NK cell cytotoxicity, leading to NK cell immune exhaustion-like phenotypes including reduction of NK cells number or function upon TSAHC treatment. Thus, TM4SF5 (as a molecule to suppress NK surveillance) is a promising target for the treatment of advanced liver diseases.

Fig. 6 Abolishment of diethylnitrosamine-mediated pre-cancerous and cancerous phenotypes by TM4SF5 inhibition. A–D Five-week-old BALB/c mice (n ≥ 5) were treated with diethylnitrosamine (DEN) for 27 weeks with or without intraperitoneal (IP) injection of DMSO as the vehicle control or 4′-(p-toluenesulfonylamido)-4-hydroxychalcone (TSAHC), as explained in the Materials and Methods section. TSAHC was administered either at the beginning of DEN treatment (DEN + TSAHC1) or 10 weeks after DEN treatment (DEN + TSAHC2). Liver tissues were imaged and processed for hematoxylin and eosin staining (A). Survival rates of the mice in each experimental group were graphed (B). Whole-tissue extracts were also prepared and processed for immunoblot analysis (C), or tissues were processed for immunohistochemical analysis of the indicated molecules (D). Immunoglobulins (IgG) from rabbit or mouse were used for the negative control stains without the primary antibodies. p-values were calculated by Gehan–Breslow–Wilcoxon test for *p = 0.0435, p-value > 0.05 was considered statistically insignificant (ns; p = 0.3679). Data shown represent three independent experiments. E Gene expression profiles of liver cirrhosis [normal (Nor, n = 220) vs. HCC (n = 225)] from GSE14520 were analyzed and showed HCC with significantly increased TM4SF5 (p < 0.0001), and among the samples showing the increased TM4SF5 expressing in HCC (n = 143) SLAMF7 (p < 0.0001), MICA (p = 0.0161), and ULBP1 (p < 0.0001) were significantly decreased in HCC, compared with normal counterparts. Meanwhile, ULBP2 was insignificantly decreased (p = 0.0926). p-values were calculated by the unpaired, two-tailed Student’s t tests.
Author contributions HS, JHR, and EMK performed most experiments; HJL helped animal experiments; JWJ, JEK, HSL, EAS, and YP helped with imaging experiments and with reagents; JHL and JHY helped with clinical tissue samples; SK helped with analysis on NK cell cytotoxicity and discussion on manuscript; JWL designed the experiments and wrote the manuscript.

Funding This work was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Science, ICT and Future Planning (NRF-2020R1I1A1A01070020 to EMK, NRF-2018M3A9C8020027 to SK and JWL, 2020R1A2C3008993, and NRF-2021M3A9D3024752 to JWL) for the Tumor Microenvironment GCRC (2011-0030001) to JWL.

Data availability Upon written requests to the corresponding author, the data and materials can be available.

Code availability Not applicable.

Declarations

Conflict of interest The authors declare no potential conflicts of interest.

Ethics approval All animal procedures were performed in accordance with the Seoul National University Laboratory Animal Maintenance Manual and were approved by the IRB of the Institute of Laboratory Animal Resources Seoul National University (SNU-IACUC) (SNU-140423-11-7, SNU-130911-2-3, SNU-170920-9, SNU-190122-6-3).

Consent to participate Not applicable.

Consent for publication All authors have consented for a publication in the CMLS.

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