Blockade of TIGIT/CD155 Signaling Reverses T-cell Exhaustion and Enhances Antitumor Capability in Head and Neck Squamous Cell Carcinoma

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Abstract

Immunosuppression is common in head and neck squamous cell carcinoma (HNSCC). In previous studies, the TIGIT/CD155 pathway was identified as an immune-checkpoint signaling pathway that contributes to the "exhaustion" state of infiltrating T cells. Here, we sought to explore the clinical significance of TIGIT/CD155 signaling in HNSCC and identify the therapeutic effect of the TIGIT/CD155 pathway in a transgenic mouse model. TIGIT was upregulated in tumor-infiltrating CD8\(^+\) and CD4\(^+\) T cells in both HNSCC patients and mouse models, and was correlated with immune-checkpoint molecules (PD-1, TIM-3, and LAG-3). TIGIT was also expressed on murine regulatory T cells (Treg) and correlated with immune suppression. Using a human HNSCC tissue microarray, we found that CD155 was expressed in tumor and tumor-infiltrating stromal cells, and also indicated poor overall survival. Multispectral IHC indicated that CD155 was coexpressed with CD11b or CD11c in tumor-infiltrating stromal cells. Anti-TIGIT treatment significantly delayed tumor growth in transgenic HNSCC mouse models and enhanced antitumor immune responses by activating CD8\(^+\) T-cell effector function and reducing the population of Tregs. In vitro coculture studies showed that anti-TIGIT treatment significantly abrogated the immunosuppressive capacity of myeloid-derived suppressor cells (MDSC), by decreasing Arg1 transcripts, and Tregs, by reducing TGFβ1 secretion. In vivo depletion studies showed that the therapeutic efficacy of anti-TIGIT mainly relies on CD8\(^+\) T cells and Tregs. Blocking PD-1/PD-L1 signaling increased the expression of TIGIT on Tregs. These results present a translatable method to improve antitumor immune responses by targeting TIGIT/CD155 signaling in HNSCC.

Introduction

Immune therapies are considered low-toxicity, high-affinity, and targeted treatment options that can harness the activity of the host's immune system to prevent tumor escape (1, 2). When used as a monotherapy or in combination with standard therapies, immune therapies have been demonstrated to be an effective therapeutic approach in multiple advanced cancers, including head and neck squamous cell carcinoma (HNSCC; refs. 3–6). HNSCC accounts for 3% to 5% of all cancers (7), with the common features of tumor-mediated immunosuppression and high mutational burden (8). HNSCC has been shown to develop multiple immune-escape mechanisms, attributed to these characteristics (8). Preclinical studies, clinical trials, and our previous work have suggested that immunosuppressive cells contribute to the poor survival of HNSCC patients, whereby immune therapies (such as immune-checkpoint blockades, adoptive cell therapies, and neoantigen-targeting vaccines) can improve clinical outcomes by reversing the immunosuppressive state (9–13). However, the limited efficacy of some immune therapies indicates that new applicable immune checkpoints and therapeutic strategies need to be investigated to overcome the pervasive immune suppression in HNSCC (14).

T-cell immunoglobulin and ITIM domain (TIGIT), which is also known as Vstm3 and VSIG9, is an immunoglobulin superfamily member (15). TIGIT is expressed restrictedly on subsets of activated T cells and natural killer (NK) cells, and interacts with CD155 to induce immunosuppression [16]. However, the expression profile and immunologic effect of TIGIT/CD155 signaling in HNSCC are poorly characterized. Analogous to the B7/CD28/CTLA-4 pathway, which contains both costimulatory and coinhibitory receptors, TIGIT competes with CD226 (also known...
as DNAM-1) to bind CD155 with a high affinity (15).
Multiple groups have shown that the genetic knockout or antibody ablation of TIGIT-enhanced NK cell killing and augmented CD8⁺ T-cell activity against tumors (17–20). In addition, TIGIT⁺ regulatory T cells (Treg) may display a stronger immunosuppressive activity than TIGIT⁻ Tregs (21). It was reported that CD155 was highly expressed in multiple tumor cells and tumor-associated myeloid cells (22–24), and that TIGIT/CD155 signaling may contribute to the potential suppression of conventional NK cells by myeloid-derived suppressor cells (MDSC; ref. 25). Thus, blocking TIGIT/CD155 signaling might provide a promising complement to current immune-checkpoint-based antitumor immunotherapies for clinical intervention.

In this study, we investigated the expression and function of TIGIT⁺ T cells and CD155⁺ myeloid cells in HNSCC patients and mouse models. We observed an elevated number of TIGIT⁺ T cells in HNSCC compared with healthy controls, as well as an increase in CD155-expressing tumor cells and myeloid cells. We also provide evidence that blocking TIGIT/CD155 signaling promoted antitumor immunity, aided in immune homeostasis, and reduced the tumor burden in mouse models by an in vivo TIGIT mAb administration. Our data demonstrate that TIGIT/CD155 signaling is a potential immunotherapeutic target for HNSCC.

Materials and Methods

Mice
Six- to 8-week-old male Tgfbr1/Pten 2cKO mice were used in this study. The time-inducible tissue-specific Tgfbr1/Pten double-knockout mice (K14-CreER<sup>tam</sup>/; Tgfbr1<sup>flox/flox</sup>; Pten<sup>flox/flox</sup>, Tgfbr1/Pten 2cKO) were maintained and genotyped according to previously published protocols (26). All animal studies were carried out in accordance with the NIH guidelines for the use of laboratory animals in a pathogen-free ASBL3 animal center at Wuhan University. All mouse procedures were approved by the Animal Care and Use Committee of Wuhan University (2014LUNSHENZI06 and 2016LUNSHENZI62). The tamoxifen treatment was performed as previously described (27). All the mice had a mixed background of FVBN/CD1/129/C57BL/6.

Human samples
Study cohort 1. A retrospective series of 210 primary HNSCC cases, 35 HNSCC cases with lymph node metastasis, 68 oral

Figure 1. TIGIT is expressed on human TILs. A, Representative flow cytometry contour plots of TIGIT expression on CD4⁺ T cells from healthy donor peripheral blood (HD, n = 10), human HNSCC peripheral blood (n = 16), and matched human HNSCC TILs (n = 12; left). Quantitation of TIGIT expression percentage in total CD4⁺ T cells is shown at right. B, Representative flow cytometry plots of TIGIT expression on CD8⁺ T cells from healthy donor peripheral blood (HD, n = 10), human HNSCC peripheral blood (n = 16), and matched human HNSCC TILs (n = 12; left). Quantitation of TIGIT expression as a percentage of total CD8⁺ T cells is shown at right. C, Representative flow cytometry plots of TIGIT and PD-1 coexpression on CD8⁺ T cells from human HNSCC peripheral blood and TILs (n = 6; left). Quantitation of TIGIT and PD-1 coexpression as a percentage of total CD8⁺ or CD4⁺ T cells is shown at right. Data represent mean ± SD.

www.aacrjournals.org Cancer Immunol Res; 7(10) October 2019 1701

Blockade of TIGIT/CD155 Signaling in HNSCC
epithelial dysplasia (DYS) cases, and 42 normal oral mucosa (MUC) cases was obtained from the Hospital of Stomatology, Wuhan University. All the included HNSCC patients underwent primary surgery (without preoperative adjuvant chemotherapy or radiotherapy) between 2011 and 2016. The specimens were used to generate human HNSCC tissue microarrays. Additionally, 201

Figure 2.
TIGIT is expressed on murine TILs and coordinately with immune checkpoints. **A**, Representative flow cytometry contour plots of TIGIT expression on CD4\(^+\) T cells by WT mice spleen (WT, \(n = 6\)), tumor-bearing mice spleen (TB, \(n = 6\)), and tumor-bearing mice TILs (\(n = 6\); left). Quantitation of TIGIT expression as a percentage of total CD4\(^+\) T cells is shown at right. **B**, Representative flow cytometry contour plots of TIGIT expression on CD8\(^+\) T cells by WT mice spleen (WT, \(n = 6\)), tumor-bearing mice spleen (TB, \(n = 6\)), and tumor-bearing mice TILs (\(n = 6\); left). Quantitation of TIGIT expression as a percentage of total CD8\(^+\) T cells is shown at right. **C**, Representative flow cytometry plots of TIGIT and PD-1 coexpression on CD4\(^+\) or CD8\(^+\) T cells by WT mice spleen (WT, \(n = 6\)), tumor-bearing mice spleen (TB, \(n = 6\)), and tumor-bearing mice TILs (\(n = 6\); left). Quantitation of TIGIT and PD-1 coexpression as a percentage of total CD4\(^+\) or CD8\(^+\) T cells is shown at right. **D**, Quantitation of TIGIT/LAG3 or TIGIT/TIM3 coexpression percentage in total CD4\(^+\) T cells or CD8\(^+\) T cells is shown. Data represent mean \(\pm\) SD with two independent biological duplications.
primary HNSCC cases were included in the survival analysis due to 9 patients lost to follow-up.

Study cohort 2. A prospective series of the whole blood of 16 primary HNSCC cases was obtained from the Hospital of Stomatology, Wuhan University. Additionally, 12 matched fresh surgically resected tumor tissues were obtained to isolate tumor-infiltrating lymphocytes (TIL). All the included HNSCC patients underwent primary surgery (without preoperative adjuvant chemotherapy or radiotherapy) from October 2017 to March 2019. The whole blood of 10 healthy donors was used as a normal control.

Informed consent was obtained from all the patients, and the study was approved by the Institutional Medical Ethics Committee of School and Hospital of Stomatology, Wuhan University (2014LUNSHENZI06 and 2016LUNSHENZI62) and was conducted in agreement with the Helsinki Declaration.

Figure 3.
TIGIT is expressed on murine Tregs and correlated with highly immune suppression. A, Representative flow cytometry contour plots of TIGIT expression on CD25 Foxp3−, CD25medFoxp3−, and CD25hi or medFoxp3+ of WT or tumor-bearing (TB) mice spleen CD4+ T cells (n = 6, respectively). B, Quantitation of TIGIT expression in Tregs. C, Representative suppression assay of WT or tumor-bearing (TB) mice Tregs cocultured with CFSE-labeled CD8+ effector T cells for 72 hours. D, Quantitation of suppression percentage in CD8+ effector T cells. Data represent mean ± SD with two independent biological duplications.
Figure 4.

CD155 is highly expressed on malignant cells and tumor-infiltrating myeloid cells in human and murine HNSCC, and correlated with poor overall survival. 

A, Representative IHC images of CD155 expression on human primary HNSCC and oral mucosa samples in the HNSCC tissue microarrays (scale bar, 50 μm). 

B, Quantitation of CD155 expression score in epithelial cells according to oral mucosa (MUC), dysplasia (DYS), and HNSCC (left). Kaplan–Meier survival curves for overall survival for 201 HNSCC patients according to the presence of a low or high expression of CD155 by median cutoff approach, \( P = 0.0337 \) (right). 

C, Quantitation of CD155 expression score in interstitial cells according to oral mucosa (MUC), dysplasia (DYS), and HNSCC (left). Kaplan–Meier survival curves for overall survival for 201 HNSCC patients according to the presence of a low or high expression of CD155 by median cutoff approach, \( P = 0.0149 \) (right). 

D, Quantitation of CD155 expression score in epithelial cells according to pathologic grade (I, \( n = 53 \); II + III, \( n = 157 \)). (Continued on the following page.)
pathologic diagnosis was made by two independent pathologists of the Department of Oral Pathology, Wuhan University.

IHC

IHC was performed as previously described (28). Briefly, the sections of HNSCC tissue microarray were deparaffinized, rehydrated, and subjected to antigen retrieval by sodium citrate (pH 6.0), followed by blocking endogenous peroxidase. Then, the sections were incubated with CD155 antibody (1:200; Cell Signaling Technology) overnight. On the second day, the sections were incubated with secondary antibodies and stained with ABC kits (Vector). The slides were scanned with Aperio ScanScope CS scanner (Vista) and analyzed by Aperio ScanScope quantification software (Version 9.1). The detailed quantification procedures were performed as before (28).

Multispectral IHC, imaging, and analysis

Multispectral IHC was performed on formalin-fixed paraffin-embedded (FFPE) HNSCC samples with PerkinElmer Tyramide Plus (Opal) reagents according to the Opal serial immunostaining manual. Briefly, paraffin sections were first deparaffinized and rehydrated. After antigen retrieval with AR buffer (pH = 6.0; PerkinElmer), the sections were covered with blocking buffer (PerkinElmer) for 20 minutes at room temperature, and then were incubated with a primary antibody, followed by the horseradish peroxidase–conjugated secondary antibody (PerkinElmer). Sections were washed three times for 2 minutes each in 0.02% Tris-buffered saline– Tween 20 (TBST) followed by signal generation using 100 μL of Opal Fluorophore Working Solution (PerkinElmer) per slide at a dilution of 1:100 in 1 × amplification diluent, incubated at room temperature for 10 minutes as specified by the manual (PerkinElmer). Opal 520 Fluorophore, Opal 540 Fluorophore, Opal 620 Fluorophore, Opal 650 Fluorophore, and Opal 690 Fluorophore (all from PerkinElmer) were applied to each antibody. Multispectral images were acquired with PerkinElmer Vectra platform at × 20 magnification. The following primary antibodies were used in this panel: CD155 (1:200; Cell Signaling Technology), CD11c (1:1,000; Cell Signaling Technology), CD11b (1:400; Cell Signaling Technology), Pan-CK (1:2000; Cell Signaling Technology), PD-L1 (1:1,000; Cell Signaling Technology), and DAPI (PerkinElmer).

In vivo treatments

After 5 consecutive days of tamoxifen administration, all the Tgfb1/Pten 2cKO mice were randomized into a treatment group, which was treated with TIGIT mAbs (10 mg/kg; BE0003-1; Bio X Cell), CD8 (200 μg; BE0004-1; Bio X Cell), CD25 (250 μg; BE0012; Bio X Cell), or Gr-1 (500 μg; BE0075; Bio X Cell) targeting antibodies on the day before tamoxifen administration and on day 4 of tamoxifen administration. On day 5, the blood or lymph nodes were obtained to verify the depletion efficiency by flow cytometry. For the in vivo PD-1 antibody treatment, all Tgfb1/Pten 2cKO mice harboring tumors were randomized into a group treated with PD-1 mAb (10 mg/kg; BE0146; Bio X Cell), and a control group treated with IgG2a isotype control (10 mg/kg; BE0089; Bio X Cell) by intraperitoneal injection three times a week during day 12 to day 40. Mice were euthanized on day 40, and tissues were harvested for flow cytometry and functional analysis.

Peripheral blood mononuclear cell separation

Peripheral blood mononuclear cells (PBMC) were separated from the whole blood samples of patients and healthy donors with Lymphoprep (STEMCELL Technologies) and used for flow cytometry analysis. Briefly, blood was diluted with an equal amount of Dulbecco’s Phosphate-Buffered Saline with 2% fetal bovine serum and was layered on top of Lymphoprep. Then, the sample was centrifuged at 800 × g for 20 minutes at room temperature, and the mononuclear cell layer was retained.

Isolation of TILs

Tumor tissues were harvested and manually minced into small pieces (smaller than 2 mm), digested in RPMI medium containing collagenase D at 1 mg/mL (Roche), hyaluronidase at 0.1 mg/mL (Sigma-Aldrich), and DNases at 0.2 mg/mL (Sigma-Aldrich) for 2 hours at 37°C, and were then filtered with 70-μm cell strainers (Becton & Dickinson). The filtered cells were collected and separated with Lymphoprep (STEMCELL Technologies). Then, the TILs were collected and stored in liquid nitrogen until flow cytometry analysis.

Flow cytometry

The following human antibodies were used for staining: CD45-APC-eFluor 780 (H30), CD3-Alexa Fluor 700 (UCHT1), CD4-FTTC (OKT4), CD8-PE-Cy7 (SK1), TIGIT-PE (MPSA43), purchased from eBioscience, and PD-1-Alexa Fluor 700 (EH12.2H7), purchased from BioLegend. For the TILs, splenocytes and lymph node cells of mice were preincubated with purified anti-mouse CD16/CD32 (eBioscience) before membrane staining. The following mouse antibodies were used for membrane staining: CD3-FTTC (17A2), purchased from BD Biosciences; CD8-PerCP-Cy5.5 (53-6.7), TIGIT-APC (1G9), TIM3-PE (8B.2C12), LAG3-BV421 (C9B7W), Ly6G-PE (1A8), Ly6C-PE-Cy7 (HK1.4), CD11b-FTTC (M1/70), Gr-1-APC (RB6-8C5), purchased from BioLegend; CD4-PE-Cy7.

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Cancer Immunol Res; 7(10) October 2019

Wu et al.

For Treg staining, Mouse Regulatory T-cell Staining Kit #3 (eBioscience) was used. Cells were first stained with CD4-FTC (RM4-5) and CD25-PE (PC61.5) surface marker antibodies, fixed with fixation/permeabilization buffer, and stained with anti-Foxp3-PerCP-Cy5.5 (FJK-16s) in 1× permeabilization buffer.

For intracellular cytokine staining, the TILs were first stimulated with Cell Activation Cocktail with Brefeldin A (BioLegend) in vitro for 6 hours. The cells were collected for CD8-PerCP-Cy5.5 (53-6.7) and CD4-PE-Cy7 (GK1.5) staining, fixed with fixation buffer (BioLegend), and permeabilized with 1× intracellular staining permeabilization wash buffer (BioLegend). Fixed cells were stained with IFNγ-PE (XMG1.2), IL2-PE (JES6-5H4), and TNFα-APC (MIP6-XT22), which were purchased from eBioscience.

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For mouse intracellular cytokine staining, the TILs were first stimulated with Cell Activation Cocktail with Brefeldin A (BioLegend) in vitro for 6 hours. The cells were collected for CD8-PerCP-Cy5.5 (53-6.7) and CD4-PE-Cy7 (GK1.5) staining, fixed with fixation buffer (BioLegend), and permeabilized with 1× intracellular staining permeabilization wash buffer (BioLegend). Fixed cells were stained with IFNγ-PE (XMG1.2), IL2-PE (JES6-5H4), and TNFα-APC (MIP6-XT22), which were purchased from BioLegend.

All samples were analyzed on a CytoFLEX flow cytometer (Beckman Coulter), and data were analyzed using CytoExpert software (Beckman Coulter). Dead cells were excluded based on Fixable Viability Dye-eFluor 506 (eBioscience).

Coculture assays

The sorted Tregs (2 × 10^5/well) from the control group were cultured in RPMI with 10% FBS, 5 mmol/L glutamine, 25 mmol/L HEPES, and 1% antibiotics (Invitrogen). Recombinant GM-CSF (Invitrogen) was added to the media of PMN-MDSCs and M-MDSCs at 10 ng/mL. Then, the TIGIT mAb (10 or 20 μg/mL) or isotype control antibody (10 μg/mL) was administered, respectively. After 48 hours, the supernatants were harvested, and the concentrations of TGFβ1 were detected with an ELISA Kit (Neobioscience) according to the manufacturer's protocol. Briefly, appropriately diluted samples were added to each well with precoated capture antibody. Then, diluted detection antibody and conjugated secondary antibody were added to each well successively. After that, the substrate solution was dispensed to per well. Finally, the absorbance was recorded at 450 nm on a plate reader.

Statistical analysis

GraphPad Prism 7.0 for windows (GraphPad Software, Inc.) was used to conduct statistical analyses. Data analyses were conducted by a two-tailed Student t test for two-group comparisons or by one-way analysis of variance (ANOVA) for multiple-group comparisons. All values are presented as the mean ± SD. P < 0.05 was considered statistically significant. The Kaplan–Meier method followed by the log-rank test was used to analyze the overall survival of patients with HNSCC, and the significance of observed differences was assessed by the log-rank test.

Results

High expression of TIGIT on tumor-infiltrating T cells

TIGIT expression has been shown to be increased on T cells in multiple types of malignant tumors (17, 18). To confirm that TIGIT was expressed in HNSCC, we performed flow cytometry to assess the surface expression of TIGIT within human PBMCs and HNSCC tissues. We found that TIGIT expression on HNSCC patient PBMCs was higher than that on CD8+ T cells from healthy donor PBMCs (Fig. 1A and B). Furthermore, TIGIT was expressed by a large percentage of HNSCC-infiltrating CD4+ and CD8+ T cells, and the expression was significantly higher than those on the matched PBMC (Fig. 1A and B). The coexpression of immune-checkpoint molecules may drive T lymphocyte exhaustion (29, 30). Therefore, we examined the expression of TIGIT with coinhibitory receptor PD-1 in human PBMCs and HNSCC tissues. We observed that TIGIT was coexpressed with PD-1 on CD4+ and CD8+ T cells from human PBMC and TILs. We found that the coexpression of TIGIT and PD-1 on TILs was higher than that on PBMCs (Fig. 1C). Considering these data, we investigated TIGIT expression in a Tgfrb1/Pten 2ckO HNSCC mouse model. Analogously, TIGIT was expressed on 23.96% of CD4+ TILs and 50.15% of CD8+ TILs, which was significantly higher than that on PBMCs (Fig. 1D). The expression of TIGIT was upregulated in the tumors, and the staining was more intense in the tumors than in the stroma.
significantly higher than that in the spleen in WT and in tumor-bearing mice (Fig. 2A and B). Moreover, we found that the coexpression of TIGIT/PD-1, TIGIT/TIM-3, and TIGIT/LAG-3 was upregulated on CD4+ and CD8+ TILs in the mouse model compared with that in the spleen (Fig. 2C and D; Supplementary Fig. S1). These data indicated that TIGIT was highly expressed by HNSCC TILs and correlated with expression of other immune-checkpoint molecules, especially PD-1.

It was reported that TIGIT predominantly regulates the function of Tregs (31). Thus, we then characterized TIGIT expression on Tregs...
Figure 6.
Blocking TIGIT/CD155 signaling increased T-cell resistance to MDSC- and Treg-mediated suppression. A, Representative suppression assay of PMN-MDSCs isolated from the isotype and anti-TIGIT treatment groups cocultured with CFSE-labeled effector T cells for 72 hours (left). Quantitation of suppression assay of the PMN-MDSC and M-MDSC subsets isolated from the isotype and anti-TIGIT treatment groups is shown at right. B, Relative quantification of arginase-1 (Arg1) transcripts in the PMN-MDSC and M-MDSC subsets sorted from the isotype and anti-TIGIT treatment groups. (Continued on the following page.)
in our HNSCC mouse model. We found that CD4⁺CD25⁺Foxp3⁺ Tregs from tumor-bearing mice expressed more TIGIT than that in WT mice (Fig. 3A and B). We observed that the CD25medFoxp3² subset expressed the majority of TIGIT among the CD4⁺ T cells, compared with the CD25⁺Foxp3⁻ subset and CD25medFoxp3⁻ subset (Fig. 3A and B). Suppression assays indicated that Tregs sorted from tumor-bearing mice showed an increased ability to suppress the proliferation of CD8⁺ T cells that were activated by CD3/CD28 antibodies compared with that from WT mice (Fig. 3C and D; Supplementary Fig. S2). These results suggested that TIGIT might act as a negative immune checkpoint to generate an exhausted phenotype in HNSCC.

High expression of CD155 in patients and in a mouse model
TIGIT might transduce negative signals to effector T cells by binding to their inhibitory receptors, such as CD155 (16). Thus, we assessed the expression and functional consequences of CD155 in HNSCC. First, using the TCGA database via GEPIA (32), we found that the CD155 mRNA expression in HNSCC tissues was significantly higher than that in normal tissues (Supplementary Fig. S3A). Survival analysis indicated that HNSCC patients with high CD155 expression demonstrated a worse overall survival than that of patients without CD155 expression (Supplementary Fig. S3B). We detected constitutive expression of CD155 on the epithelial and interstitial cells of human HNSCC tissue (Fig. 4A–C). High CD155 expression on the malignant cells or stromal cells of HNSCC patients was associated with poor survival (Fig. 4B and C). Furthermore, higher expression of CD155 in epithelial cells was correlated with the pathologic grade (Fig. 4D; Supplementary Fig. S4A) and lymph node metastasis (Fig. 4E). However, there was no significant difference in CD155 expression between tumors of different sizes (Supplementary Fig. S4B), and no significance difference in CD155 expression was observed between patient HNSCC tissue and the matched metastatic lymph nodes (Supplementary Fig. S4C). To investigate further, multiplexed IHC analysis showed that CD155 (green) was highly expressed in Pan-CK⁺ (red) HNSCC (Fig. 4F). We also found that CD155 was coexpressed with myeloid cell markers, such as CD11b (yellow) and CD11c (pink), at the invasive front (Fig. 4F). We observed that CD155 and PD-L1 coexpressed on CD11b⁺ myeloid cells (Supplementary Fig. S5). Based on the CD155 expression profiles in patient tissues, we observed that CD155 was similarly overexpressed on CD11b⁺Ly6G⁻Ly6C⁺ PMN-MDSCs and CD11b⁺Ly6G⁻Ly6C⁺ M-MDSCs in the tumor-bearing Tgbri/Pten 2cKO HNSCC mouse model compared with that in the bone marrow and MDSCs of WT mice (Fig. 4G and H; Supplementary Fig. S6A). In our previous work, we verified that these two subsets of MDSCs in tumor-bearing mice had immunosuppressive activity (28). To further confirm this observation, we sorted these cells to detect their capacity to produce arginase-1 (Arg-1). Higher expression of arginase-1 was found in the PMN-MDSCs and M-MDSCs in the tumor-bearing mice, whereas this gene was lowly expressed in the MDSCs of WT mice (Fig. 4G; Supplementary Fig. S6B). These data confirmed the high prevalence of CD155 in human and mouse HNSCC and suggested that CD155 may correlate with an immunosuppressive function in HNSCC. Thus, we hypothesized that blocking TIGIT/CD155 signaling may activate antitumor immunity in HNSCC.

Blocking TIGIT/CD155 signaling inhibits tumor progression
To test our hypothesis, tumor-bearing HNSCC mice were subjected to in vivo TIGIT/CD155 blockade by treatment with a TIGIT mAb. Tgbri/Pten 2cKO mice were administered 5 consecutive days of tamoxifen. When the papilloma had formed, the mice were intraperitoneally injected with a TIGIT mAb (Fig. 5A). The results indicate that blocking TIGIT led to a significant delay in tumor progression compared with that of the control (Fig. 5B). We did not observe significant differences between the anti-TIGIT treatment group and control group in liver and kidney using H&E staining (Supplementary Fig. S7A), indicating that there was no detectable cytotoxicity in our mouse model upon anti-TIGIT treatment, which was consistent with a previous study (33). We also observed a lower frequency of Tregs infiltrating in the peripheral immune organs, local circulation, and the tumor microenvironment (TME) after treatment compared with those in the controls (Fig. 5C). A higher frequency of tumor-infiltrating CD8⁺ T cells expressing IL2/TNF and CD103⁺ T cells expressing IL17 was observed after TIGIT mAb treatment relative to that in the control mice (Fig. 5D). However, treatment with a TIGIT mAb did not reduce tumor-infiltrating MDSC frequencies (Supplementary Fig. S7B). These data suggested that blocking TIGIT/CD155 signaling alleviated CTL exhaustion and delayed tumor growth in HNSCC mouse models.

Blocking TIGIT/CD155 signaling decreases suppression by Tregs and MDSCs
To determine the potential mechanism of the TIGIT/CD155 signaling blockade, we selectively sorted MDSCs and Tregs from Tgbri/Pten 2cKO mice for in vitro investigations. Coculture assays indicated that blocking TIGIT might transduce negative signals to effector T cells for 72 hours (left). Quantitation of suppression assay of Tregs isolated from isotype and anti-TIGIT treatment groups is shown at right. Data represent mean ± SD with two independent biological duplications. D, Representative suppression assay of Tregs isolated from isotype and anti-TIGIT treatment groups cocultured with CFSE-labeled CD8⁺ effector T cells for 72 hours (left). Quantitation of suppression assay of Tregs isolated from isotype and anti-TIGIT treatment groups is shown at right. E, Representative contour plots of annexin V⁻/PI⁺ apoptotic PMN-MDSCs. Isolated MDSCs were cultured with TIGIT mAb or isotype antibody in vitro for 20 hours and stained annexin V and PI to assess apoptotic percentage by flow cytometry (left). Quantitation of annexin V⁻/PI⁺ apoptotic PMN-MDSCs and M-MDSCs from TIGIT mAb or isotype antibody treatment in vitro is shown at right. Data represent mean ± SD with two independent biological duplications. F, Quantitation of TGFβ1 concentrations in the supernatants from TIGIT mAb (10 or 20 μg/mL) or isotype antibody (10 μg/mL) in vitro treated Tregs by ELISA. Data represent mean ± SD with two independent biological duplications.
Then, CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup>Tregs that were isolated from the spleens of TIGIT or isotype antibody–treated mice were cocultured with CD8<sup>+</sup>effector T cells. The results indicated that the anti-TIGIT treatment decreased the suppressive function of Tregs compared with that in the controls (Fig. 6D). The in vitro analysis revealed that the anti-TIGIT treatment did not induce the apoptosis of Tregs (Fig. 6E), but the treatment reduced the secretion of TGFβ in cell supernatants from Tregs compared with that in the controls (Fig. 6F). Overall, these data suggested that blocking TIGIT/CD155 signaling may partially regulate the immunosuppression of Tregs by downregulating TGFβ secretion.

**Anti-TIGIT therapeutic efficacy is mainly dependent on CD8<sup>+</sup>T cells and Tregs**

As the anti-TIGIT treatment may partially influence the function of CD4<sup>+</sup>T cells, CD8<sup>+</sup>T cells, MDSCs, and Tregs, we determined whether the effect of anti-TIGIT was mainly dependent on one of these cell populations. Next, tumor-bearing HNSCC mice were subjected to in vivo depletion of CD4<sup>+</sup>T cells, CD8<sup>+</sup>T cells, MDSCs, or Tregs by depleting antibodies before the blockade of TIGIT antibody. Using depleting antibodies, we found that antitumor effects in tumor-bearing HNSCC mice were abrogated when CD8<sup>+</sup>T cells, but not CD4<sup>+</sup>T cells, were depleted (Fig. 7A). Moreover, we found that when depleting CD25<sup>+</sup>Tregs, there were no differences in the antitumor effects between the anti-CD25 alone and the combination of anti-CD25 and anti-TIGIT. However, the tumor growth was slower with the combination of anti-Gr1 and anti-TIGIT than that with anti-Gr1 alone (Fig. 7B). These results indicated that the therapeutic efficacy by anti-TIGIT mainly relies on CD8<sup>+</sup>T cells and Tregs.

**Blocking PD-1/PD-L1 signaling increases the expression of TIGIT on Tregs**

Our data showed that PD-1 was coexpressed with TIGIT on human and mouse TILs. We therefore examined the expression of TIGIT on CD4<sup>+</sup>T cells, CD8<sup>+</sup>T cells, and Tregs after blocking PD-1/PD-L1 signaling to investigate whether there is a possible rationale to combine the TIGIT and PD-1 treatment in HNSCC. The results showed that blockade of PD-1 increased the expression of TIGIT on Tregs compared with the isotype (Fig. 7C). However, there were no differences on the expression of TIGIT on...
CD8+ T cells between anti-PD-1 treatment group and the control group (Supplementary Fig. S8D). Collectively, these data indicated that blocking PD-1 and TIGIT corporately may elicit better antitumor effects.

Discussion

Cancer immunotherapy with immune-checkpoint blockade has been one of the most successful strategies for cancer therapy (34). Although blocking PD-1, PD-L1, and CTLA-4 has shown to generate antitumor immunity and durable responses, drug resistance still occurs in some patients (14, 35), emphasizing the need for supplementary strategies. TIGIT is an inhibitory checkpoint receptor that has been demonstrated to have immunosuppressive effects on antitumor immunity in several solid tumors and in leukemia (15, 17, 20). The coexpression of TIGIT and other immune checkpoints can lead to an exhausted phenotype in cytotoxic lymphocytes (29). TIGIT+ Tregs are believed to be a distinct Treg subset capable of strong suppression (21). However, direct evidence suggesting a clinical role for TIGIT in HNSCC patients has not been presented. In this study, we found that TIGIT was highly expressed by both human and mouse tumor-infiltrating CD4+ and CD8+ T cells, and was related to several key T-cell checkpoints. In addition, we also demonstrated that TIGIT was more highly expressed on Foxp3+ Tregs than on Foxp3- CD4+ T cells in our HNSCC mouse model, which could be associated with the high amount of suppression on CD8+ T-cell proliferation. CD155 is a ligand of TIGIT that interacts with TIGIT with high affinity. The loss of both host- and tumor-derived CD155 leads to decreased tumor growth and metastasis and increased response to immunotherapy (22). Here, we showed that CD155 was widely expressed in the TME of HNSCC patients, and the overexpression of CD155 in both cancer cells or in tumor-infiltrating stromal cells could predict poor overall survival. The overexpression of CD155 was also associated with pathologic grade and lymph node metastasis, which indicated that the assessment of CD155 expression could be used as an approach to predict the prognostic outcomes of HNSCC patients. These results were consistent with several previous studies in other types of malignant tumors (20, 36, 37). In addition, high CD155 expression on tumor-infiltrating myeloid cells was observed in human and murine HNSCC. In these studies, the blockade of TIGIT/CD155 signaling enhanced the antitumor CTL responses and downregulated the immunosuppressive function of Tregs and MDSCs by decreasing the production of suppressive cytokines.

To our knowledge, this is the first evidence regarding the role of TIGIT/CD155 signaling in HNSCC pathogenesis and immunotherapy.

Current studies have shown that the depletion of CD8+ CTLs or the absence of NK cells might abrogate the therapeutic effects of anti-TIGIT blockade (17, 19). However, the contribution of tumor-infiltrating immunosuppressive cells has not been studied in these investigations. Two previous studies indicated that the expression of PD-L1 on host dendritic cells and macrophages may predict the clinical therapeutic efficacy of PD-L1/PD-1 blockade (38, 39). These data provide evidence that the interaction between CTLs and tumor-associated stromal cells may play an essential role in immune-checkpoint inhibition. In line with our previous work, CD4+CD25+Foxp3+ Tregs, CD11b+Ly6G+Ly6C+ M-MDSCs, and CD11b+Ly6G-Ly6C+ M-MDSCs are the major immune-suppressive cells in the Tgbr1/Pten 2cKO mouse model (28, 40). In this work, a decrease in the suppressive function of Tregs and MDSCs was observed in an HNSCC mouse model by blocking TIGIT, which indicates that CD8+ CTL exhaustion was reversed as a result of the reduction of the immunosuppression of the TME. In pathologic conditions, MDSCs highly express multiple anti-inflammatory cytokines and immunosuppressive factors, inhibiting adaptive immunity and supporting tumor progression (41). In a mouse model, rapid tumor growth and inflammatory infiltrates can result in expansion of the MDSC populations (42). Although the blockade of TIGIT did not induce apoptosis or decrease the MDSCs in this study, the downregulation of ARG1 transcripts was observed in the mouse model. These data indicated that TIGIT mAbs may not directly affect the expansion of MDSCs in the HNSCC TME, but may reduce immunosuppression by inhibiting ARG1 production. The complete mechanism needs to be further investigated. In addition, Tregs are recruited into the TME early and play a prominent role in the regulation of the immune response to tumors via cytokine production or surface molecule interactions (43, 44). Previous studies also revealed that apoptotic Tregs could mediate enhanced immunosuppression via the adenosine pathways (45). In this study, we found that TIGIT mAbs did not directly affect the apoptosis of Tregs in vitro but could downregulate the secretion of typical suppressive cytokine TGFβ1. Paradoxically, we also observed that the blockade of TIGIT/CD155 signaling in vitro could significantly decrease the Treg population in our mouse model. These results may be explained by previous studies, which showed that TIGIT-deficient T cells generate less TGFβ-mediated Treg differentiation (21), but the exact mechanism needs to be further studied.

In summary, TIGIT/CD155 signaling was enhanced in HNSCC patients and in mouse models and was correlated with immunosuppression. Targeting TIGIT/CD155 signaling may be a potential therapeutic strategy for HNSCC treatment.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Acknowledgments

This work was supported by the National Natural Science Foundation of China (NSFC; 81622068, 81472528, 81472529) and the Fundamental Research Funds for the Central Universities (2042017kD017). The authors are grateful to Prof. Ashok B. Kulkarni for kindly proofreading and editing. The
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Received October 11, 2018; revised April 19, 2019; accepted August 1, 2019; published first August 6, 2019.

Published OnlineFirst August 6, 2019; DOI: 10.1158/2326-6066.CIR-18-0725