High expression of major histocompatibility complex class I overcomes cancer immunotherapy resistance due to IFNγ signaling pathway defects

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Synopsis:

Resistance to immune checkpoint inhibitors can be caused IFNγ signaling pathway defects. The authors show that such resistance can be primarily caused by reduced MHC-I expression and that this can be overcome by NF-κB–targeted therapies.

Abstract

IFNγ signaling pathway defects are well-known mechanisms of resistance to immune checkpoint inhibitors. However, conflicting data have been reported, and the detailed mechanisms remain unclear. In this study, we have demonstrated that resistance to immune checkpoint inhibitors owing to IFNγ signaling pathway defects may be primarily caused by reduced MHC class I (MHC-I) expression rather than by the loss of inhibitory effects on cellular proliferation or decreased chemokine production. In particular, we found that chemokines that recruit effector T cells were mainly produced by immune cells rather than cancer cells in the tumor microenvironment of a mouse model, with defects in IFNγ signaling pathways. Furthermore, we found a response to immune checkpoint inhibitors in a patient with JAK-negative head and neck squamous cell carcinoma whose human leukocyte antigen (HLA)-I expression level was maintained. In addition, CRISPR screening to identify molecules associated with elevated MHC-I expression independent of IFNγ signaling pathways demonstrated that guanine nucleotide-binding protein subunit gamma 4 (GNG4) maintained MHC-I expression via the NF-κB signaling pathway. Our results indicate that patients with IFNγ signaling pathway defects are not always resistant to immune checkpoint inhibitors and highlight the importance of MHC-I expression among the pathways and the possibility of NF-κB–targeted therapies to overcome such resistance.

Introduction

Monoclonal antibodies (mAbs) specific for programmed death-1 (PD-1)/programmed death-ligand 1 (PD-L1) are immune checkpoint inhibitors (ICIs) that have been proven effective against various cancers including melanoma (1,2), lung cancer (3,4), and head and neck squamous cell carcinoma (HNSCC) (5,6). ICIs exhibit efficacy by reactivating suppressed effector T cells in the tumor microenvironment (TME) (7,8). However, the effectiveness of ICIs is limited, and many patients are resistant to ICIs (9,10). The mechanisms of resistance to ICIs involve various factors related to T-cell activation (9,10). For example, the loss of major histocompatibility complex class I (MHC-I) suppresses T-cell activation owing to the loss of antigen presentation (11-13). Reduced chemokine levels induce non-inflammatory TMEs, leading to ICI resistance (14). Various inhibitory immune checkpoint molecules and immunosuppressive cells suppress T-cell effector functions in the TME (15-18).

IFNγ exerts its antitumor effects by increasing MHC-I expression (19), boosting antigen processing (20,21), inhibiting cellular proliferation (22), inducing apoptosis (23), and enhancing chemokine production (24,25). Therefore, IFNγ signaling pathway defects such as *JAK1/2* mutations can cause ICI resistance via reduced MHC-I expression, loss of inhibitory effects on cellular proliferation, or decreased chemokine production (26,27).

However, some patients have responded to ICIs despite impaired IFNγ signaling pathways (28,29), and the mechanisms underlying this remain unclear.

Here, we found loss of *JAK* copy number in a cancer cell line established from a super-responder to anti–PD-1. Thus, we created *JAK*-deleted cancer cell lines and evaluated antitumor immunity, including PD-1 blockade–mediated immunity, against these cell lines both *in vitro* and *in vivo*. We determined that several tumors responded to PD-1 blockade despite *JAK* deletion but others did not respond as previously reported (26,27). To clarify this difference, we elucidated the detailed mechanisms of resistance due to IFNγ signaling pathway defects using these models. In addition, based on the detailed mechanisms, we performed CRISPR screening to search for potential therapeutic molecules to overcome such resistance.

Materials and Methods

Patients and samples

We used autologous cancer cell lines and cultured tumor-infiltrating lymphocytes (TILs) established from three patients with melanoma who underwent surgical resection and received ICI treatment at Yamanashi University Hospital (Yamanashi, Japan) from 2017 to 2019 (**Supplementary Table S1**) (30). All patients provided written informed

consent after receiving an explanation of the study. Samples collected from the patients were immediately treated with enzymes, including collagenase (Sigma-Aldrich, St.Louis, MO, #C4-28-100MG), hyaluronidase (Sigma-Aldrich, #H6254-500MG), and deoxyribonuclease (Sigma-Aldrich, #D5025-15KU), at room temperature and separated by density gradient filtration. We used the digested tumors after filtration and separation according to the density gradient.

In addition, formalin-fixed paraffin-embedded (FFPE) tissues from 48 patients with HNSCC who received anti–PD-1 monotherapy or combination therapy with 5-fluorouracil and cisplatin or carboplatin at Chiba University Hospital (Chiba, Japan) between 2014 and 2022 were used for immunohistochemistry analysis (**Supplementary Table S2**). Clinical information was obtained from the patients' medical records.

The clinical protocols for this study were approved by the appropriate institutional review boards and ethics committees of Chiba University Hospital (1027), Chiba Cancer Center (M04-002), and Yamanashi University Hospital (1795). This study was conducted in accordance with the principles of the Declaration of Helsinki.

In vitro expansion of human melanoma TILs

TILs were cultured and expanded as previously described (12,30,31). Briefly, melanoma tumor digests were initiated in RPMI1640 (Thermo Fisher Scientific, Waltham, Massachusetts, #11875093) supplemented with 10% human AB serum (MP Biomedicals, Irvine, California, # 2931949), penicillin–streptomycin (Thermo Fisher Scientific, #14150122) and recombinant human IL-2 (rhIL-2; 6000 IU/mL, PeproTech, Cranbury, New Jersey, #200-02) in a humidified 37°C incubator with 5% CO_2. Half of the medium was aspirated from the wells and replaced with fresh complete medium and rhIL-2 every 2–3 days.

Cell lines and reagents

To establish cancer cell lines, 1×10^7 digested tumor cells were cultured in RPMI1640, containing 10% FBS (Cytiva, Tokyo, Japan, #SH30396), penicillin–streptomycin, and amphotericin B (Thermo Fisher Scientific, #15290018), as previously described (12). The cells were repeatedly passaged until they reached approximately 80%–90% confluence. Cancer cell lines were used when fibroblasts were free and proliferating beyond the 10th passage. The MEL01 cell line was established from a melanoma patient who acquired resistance to ICI treatment due to the loss of MHC-I

expression after an initial response to PD-1 blockade; MEL02 and MEL03 cell lines were established from melanoma super-responders to PD-1 blockade before therapy initiation (**Supplementary Table S1**) (30). Because we established these cell lines, no authentication was performed.

The EMT6 (mouse breast cancer, #CRL-2755), CT26 (mouse colon cancer, #CRL-2638), B16F10 (mouse melanoma, #CRL-6475), 293T (human epithelial-like cell, #CRL-3216) cell lines were purchased from The American Type Culture Collection (Manassas, Virginia). MC-38 (mouse colon cancer, #ENH204-FP) cells were obtained from Kerafast Inc. (Boston, Massachusetts). EMT6, MC-38, B16F10, and 293T cells were maintained in high-glucose Dulbecco's modified Eagle's medium (FUJIFILM Wako Pure Chemical Corporation, #043-30085) supplemented with 10% FBS. CT26 cells were maintained in RPMI-1640 medium (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan, #189-02025) supplemented with 10% FBS. All cancer cells were used after mycoplasma testing with a PCR Mycoplasma Detection Kit (TaKaRa, Shiga, Japan, #CY232) according to the manufacturer's instructions. Cancer cells were used within 10 passages. Human and mouse IFNγ were purchased from PeproTech (#AF-300-02 and #315-05, respectively). IMD-0354 was purchased from MedChemExpress (Monmouth Junction, New Jersey, #HY-10172).

Copy number assay

Genomic DNA of MEL02, MEL03, and normal lymphocytes derived from the MEL02 patient were extracted using QIAamp DNA Mini Kit (Qiagen, Hilden, Germany #51304). The number of copies of the *JAK1* and *JAK2* genes in the human melanoma cell lines was determined by quantitative real-time PCR using the Applied Biosystems 7500 Real-Time PCR System (Thermo Fisher Scientific) with 10 ng of genomic DNA. For each sample, ΔCt for *JAK1* or *JAK2* versus *LINE-1* used as an internal control was calculated as ΔCt = Ct (*JAK1* or *JAK2*) – Ct (*LINE-1*). ΔΔCt for MEL02 or MEL3 versus normal lymphocytes from the MEL02 patient was calculated as ΔΔCt = ΔCt (MEL02 or MEL03) – ΔCt (normal lymphocytes from the MEL02 patient). The copy number was calculated by doubling $2^{-\Delta\Delta Ct}$, considering the normal lymphocytes as diploid. The experiments were performed in triplicate. The primers used in this study are listed in **Supplementary Table S3**.

Human *JAK2* deletion, mouse *Jak1* or *Jak2* deletion, and mouse *B2m* deletion

Human *JAK2*-deleted MEL02 and MEL03; mouse *Jak1*-deleted EMT6, CT26, MC-38, and B16F10; mouse *Jak2*-deleted EMT6 and MC-38; and mouse *B2m*-deleted EMT6 were generated using CRISPR/CRISPR associated protein 9 (Cas9) technology. The targeting guide RNA (gRNA) sequences used to edit the genomic locus are summarized in **Supplementary Table S4**. The gRNA and TrueCut Cas9 protein v2 (Thermo Fisher Scientific, #A36498) were transfected into cells using Lipofectamine™ CRISPRMAX (Thermo Fisher Scientific, #CMAX00008). Expression of JAK1/2 and MHC-I was evaluated using Western blotting and flow cytometry, respectively.

Mouse *H2-K1* or *Cd274* overexpression

pLV[Exp]-Puro-EF1A>mH2-K1 (VectorBuilder, Chicago, Illinois) or pGIPz-mPD-L1 (Addgene, plasmid #121488; http://n2t.net/addgene:121488; RRID:Addgene_121488), a gift from Mien-Chie Hung (The University of Texas MD Anderson Cancer Center, Houston, Texas) (32) were transfected into packaging 293T cells with pMD2.G (Addgene, plasmid #12259; http://n2t.net/addgene:12259; RRID:Addgene_12259), pRSV-Rev (Addgene, plasmid #12253; http://n2t.net/addgene:12253; RRID: Addgene_12253), and pMDLg/pRRE (Addgene, plasmid #12251; http://n2t.net/addgene:12251; RRID:Addgene_12251), which were gifts

from Didier Trono (EPFL, Lausanne, Switzerland) (33), using Lipofectamine™ LTX reagent (Thermo Fisher Scientific, #15338100). Viral supernatants were collected 2 days after transfection, and viral particles were transduced into *Jak1*-deleted EMT6 or MC-38 cells with 10 μg/mL of polybrene (Nacalai Tesque, Kyoto, Japan, #12996-81) for 48 hours.

Flow cytometry analyses

Flow cytometry was performed as previously described (31). Briefly, the autologous cancer cells (MEL02 and MEL03) and mouse cancer cells (EMT6, CT26, MC-38, and B16F10) were washed with PBS containing 2% FBS and stained with surface antibodies. The samples were analyzed using BD FACSVerse™ and FlowJo™ software version 10.8.0 (BD Biosciences, Franklin Lakes, New Jersey). The antibodies were diluted for staining according to the manufacturer's instructions. Dead cell staining was performed using the Fixable Viability Dye eFluor 780 (Thermo Fisher Scientific, #65-0865-14) or 7-AAD (BD Biosciences, #559925). The antibodies used are summarized in **Supplementary Table S5**. Gating strategy is shown in **Supplementary Figure S1**. The cells were treated for 24 and 72 hours before analyses when using IFNγ (1,000 IU/mL) and IMD-0354 (1 μM), respectively.

Western blotting

Cell lysates (20 μg protein) from the autologous cancer cells (MEL02 and MEL03) and mouse cancer cells (EMT6, CT26, MC-38, and B16F10) were separated by SDS-PAGE and blotted onto polyvinylidene fluoride membranes (Merck Millipore, Burlington, Massachusetts, #IPVH00010). The membranes were blocked and then incubated with primary antibodies. The membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies after washing. Finally, the bands were detected by enhanced chemiluminescence (Bio-Rad, Hercules, California, #170-5060) and confirmed using a LAS4000 system (Cytiva). The experiments were performed in triplicate, and ImageJ software version 1.53k (Wayne Rasband, NIH, Bethesda, MD) was used for quantification. When we analyzed p-STAT1/3 and LMP7/TAP1, we used IFNγ for 30 minutes or 24 hours before analyses, respectively. Nuclear fractions were isolated using hypotonic buffer. The antibodies used are summarized in **Supplementary Table S6**.

Functional IFNγ release assay

TILs (10^5 cells/well) were added to the autologous cancer cells (MEL02 and MEL03) (10^5 cells/well) and cocultured for 24 hours. The supernatant was used to

evaluate IFNγ levels by ELISA (Thermo Fisher Scientific, #88-7316-22). Anti-MHC-I mAb (W6/32, Thermo Fisher Scientific, #16-9983-85) was added at a concentration of 10 μg/mL as a negative control. The experiments were performed in triplicate.

Killing assay

Killing assays were performed as described previously (12,30). Briefly, autologous cancer cells (MEL02 and MEL03) (target cells [T]) labeled with calcein-AM (Thermo Fisher Scientific, #C1430) were cocultured with paired TILs from the same patients (effector cells [E]) at the indicated E/T ratios and then centrifuged to ensure contact between the cell populations. After 3 hours of incubation, fluorescence was measured at 490 nm using an ARVO X3 Multilabel Reader (PerkinElmer, Waltham, Massachusetts). MEL01 with loss of MHC-I expression was used as a negative control (12). The experiments were performed in triplicate.

Cellular proliferation assay

Cellular proliferation was evaluated using a Cell Counting Kit-8 assay (Dojindo Laboratories, Kumamoto, Japan, #343-07623), according to the manufacturer's instructions. Briefly, EMT6, CT26, and MC-38 cells were seeded in 96-well plates at 1 ×

10^3, 2×10^3, and 1×10^3 cells per well, respectively. The cells were then treated with IFNγ (1,000 IU/mL) for 48 hours. After adding the Cell Counting Kit-8 reagent, the cells were incubated for a further 3 hours at 37°C. The absorbance of each well was measured at 450 nm using an ARVO X3 Multilabel Reader. The experiments were performed in triplicate.

In vivo animal models

Female C57BL/6J and BALB/c mice (6–8 weeks old) were purchased from SLC (Shizuoka, Japan). C57BL/6J- Prkdc<scid>/Rbrc mice (B6 SCID; RBRC01346) were provided by RIKEN BRC (Tsukuba, Japan) through the National BioResource Project of the Japan Ministry of Education, Culture, Sports, Science and Technology/Agency for Medical Research and Development. Mouse cancer cells (EMT6, CT26, and MC-38) (1×10^6) were injected subcutaneously, and the tumor volume was monitored twice weekly with calipers. The mean values of the long and short diameters were used to generate tumor growth curves. Mice were grouped when the tumor volume reached approximately 100 mm^3, and anti-PD-1 (200 μg/mouse, RMP1-14, Biolegend, San Diego, California, #114111) or control mAb (200 μg/mouse, RTK2758, Biolegend, #400502) was administered intraperitoneally three times every 3 days thereafter. *In vivo* experiments

were performed at least twice. Tumors were harvested 7 days after treatment initiation and evaluated for chemokine production and $CD8^+$ T-cell infiltration. All mice were maintained under specific pathogen-free conditions at the animal facility of the Institute of Biophysics. Mouse experiments were approved by the Animal Committee for Animal Experimentation of the Chiba Cancer Center (22:6). All experiments met the US Public Health Service Policy on the Humane Care and Use of Laboratory Animals.

Immunohistochemistry

FFPE tissue sections (3 μm) were dried, deparaffinized with xylene, and rehydrated. Antigen retrieval was performed in an autoclave at 121°C for 5 min. Endogenous peroxidase inhibition was assessed by MtOH containing 0.3% hydrogen peroxide. Individual slides were incubated overnight at 4°C with the primary antibody after blocking using 3% bovine serum albumin (Sigma-Aldrich, #A9647), then incubated with HRP-conjugated secondary antibodies and colored with 3,3-diaminobenzidine (Vector Laboratories, Burlingame, California, #SK-4105). Counterstaining was performed using Mayer's hematoxylin. Intratumoral $CD8^+$ T cells were counted in five randomly selected areas (0.25 mm^2/field) using BZ-X710 digital microscope (Keyence, Osaka, Japan). The average of the five fields of view was used. The antibodies are

summarized in **Supplementary Table S6**.

Cell sorting

The *Jak1*-deleted EMT6 tumors from tumor-bearing mice were digested using gentlMACS™ Dissociator (Miltenyi Biotec, Bergisch Gladbach, Germany, #130-093-235), subsequently the tumor digests were washed with PBS containing 2% FBS and stained with a specific antibody to CD45 to distinguish immune cells **(Supplementary Table S5)**. Dead cell staining was performed using the 7-AAD. The samples were sorted according to CD45 expression using BD FACSMelody™ (BD Biosciences).

Quantitative real-time reverse transcription-PCR (qRT-PCR)

Total RNA of mouse cancer cell lines (EMT6 and MC-38) was extracted using the RNeasy® Plus Mini Kit (Qiagen, #74134) and 100 ng of total RNA was reverse-transcribed into cDNA using Prime-Script RT Master Mix (TaKaRa, #RR036A). Real-time PCR was performed using PowerUp SYBR Green Master Mix (Thermo Fisher Scientific, #A25780) according to the manufacturer's instructions. For each sample, ΔCt for *Cxcl9* or *Cxcl10* versus *Gapdh* used as an internal control was calculated as ΔCt = Ct (*Cxcl9* or *Cxcl10*) – Ct (*Gapdh*). Expression data were calculated as $2^{-\Delta Ct}$. To evaluate

chemokine expression, cancer cells were treated with IFNγ (1000 IU/mL) for 24 hours prior to RNA extraction. The experiments were performed in triplicate. The primers are listed in **Supplementary Table S7**.

CRISPR screening

CRISPR screening was performed using the Mouse Brie CRISPR knockout pooled library (Addgene, Watertown, Massachusetts, #73633-LV), a gift from David Root and John Doench (Broad Institute of MIT and Harvard, Cambridge, Massachusetts) (34), according to the manufacturer's instructions. Mouse cancer cells (EMT6 and CT26) were transfected with lentiCas9-Blast (Addgene, plasmid #52962; http://n2t.net/addgene:52962; RRID: Addgene_52962), a gift from Feng Zhang (Broad Institute of MIT and Harvard, Cambridge, Massachusetts) (35), and subjected to selection using blasticidin (10 μg/mL, InvivoGen, San Diego, California, #ant-bl). Lentiviral prep was used to infect EMT6 and CT26 cells transfected with Cas9 (9.6×10^7) with an efficiency of approximately 30%. After selection with puromycin (10 μg/mL, InvivoGen, #ant-pr), the targeted cell populations were sorted according to MHC-I expression using a FACSMelody™. Genomic DNA was extracted using a Genomic-tips-100/g (Qiagen, #10243), subjected to PCR according to the manufacturer's instructions, and sequenced

using a NovaSeq 6000 system (Illumina, San Diego, California). We used the Model-based Analysis of Genome-wide CRISPR-Cas9 Knockout (MAGeCK) method to assemble single-guide (sg)RNAs and rank genes from the screening data and focused on genes with a false discovery rate (FDR) of ≤0.25.

siRNA transfection

Two different sequences of siRNA targeting mouse *Crkl*, *Proser2*, *Il7*, and *Gng4*, and negative control siRNA were purchased from Integrated DNA Technologies, Inc. (Tokyo, Japan). Cancer cells (EMT6, CT26, MC-38, and B16F10) were transfected with *Crkl*, *Proser2*, *IL7*, *Gng4*, and negative control siRNA using Lipofectamine™ RNAiMAX reagent (Thermo Fisher Scientific, #13778-030) according to the manufacturer's protocol. Cancer cells were collected 48 hours after transfection, and gene expression was confirmed by qRT-PCR. In addition, we analyzed MHC-I expression in cancer cells using flow cytometry. The experiments were performed in triplicate. The siRNA sequences are listed in **Supplementary Table S8**.

Establishment of *Gng4*-knockdown CT26 (CT26/sh-*Gng4*)

CT26/sh-*Gng4* cells were generated by lentiviral transduction. Briefly, pLV(short hairpin [sh]RNA)-Puro-(*Gng4*-1), pLV(shRNA)-Puro-(*Gng4*-2), or pLV(shRNA)-EGFP-Control vectors (VectorBuilder) were transfected into packaging 293T cells with pMD2.G, pRSV-Rev, and pMDLg/pRRE, using Lipofectamine™ LTX reagent (Thermo Fisher Scientific, #15338100). Viral supernatants were collected 2 days after transfection, and viral particles were transduced into CT26 cells with 10 μg/mL of polybrene for 48 hours. The cells were selected with puromycin (10 μg/mL) for three days. The same sequences were used for shRNA and siRNA (see **Supplementary Table S8**).

Statistical analyses

GraphPad Prism V.9.3.1 (GraphPad Software, San Diego, Chicago) or R V.4.1.3 (R Foundation for Statistical Computing, Vienna, Austria) was used for statistical analyses. The relationships of continuous variables between and among groups were compared using t-tests and one-way ANOVA, respectively. The relationships between tumor volume curves were compared using two-way ANOVA. For multiple testing, Bonferroni corrections were employed. *P*-values <0.05 were considered statistically significant.

Data Availability Statement

The data presented in this study are available within the article and its supplementary data files. Derived data are available from the corresponding author upon request.

Results

TILs are cytotoxic against MHC-I-expressing cancer cells despite loss of *JAK*

Previously, we established several pairs of TILs and autologous cancer cell lines from three melanoma patients (**Supplementary Table S1**) (30). Although one cell line (MEL02) from a super-responder to anti–PD-1 had copy number loss of *JAK2* (**Supplementary Fig. S2A**), treatment with IFNγ increased PD-L1 expression, a representative IFNγ–related molecule (**Fig. 1A**) (36-38). In contrast, MHC-I expression, another representative IFNγ–related molecule, was high without IFNγ treatment and comparable even with treatment. The changes in PD-L1 and MHC-I expression levels in another cell line (MEL03) without copy number loss of *JAK1* or *JAK2* from a second super-responder were similar to those of MEL02 (**Fig. 1A** and **Supplementary Fig. S2A**).

To elucidate the role of JAK in these high MHC-I–expressing cell lines, we deleted *JAK2* in MEL02 and MEL03 using the CRISPR/Cas9 system (**Supplementary Fig. S2B**). Similar to parental cell lines, both *JAK2*-deleted cell lines had high MHC-I

expression regardless of IFNγ stimulation, whereas PD-L1 expression did not increase (**Fig. 1B**). When paired TILs were cocultured with autologous cancer cells, IFNγ was released, and cytotoxicity was observed in both parental and *JAK2*-deleted MEL02 and MEL03 cells (**Fig. 1C** and **1D**). These findings suggest that IFNγ signaling pathway defects in cancer cells do not always induce ICI resistance.

Jak-deleted mouse tumors with high MHC-I expression respond to PD-1 blockade

To validate the *in-vitro* data, we searched for mouse cell lines with high MHC-I expression regardless of IFNγ stimulation. While MHC-I expression in the MC-38 and B16F10 cell lines increased after IFNγ treatment, that in the EMT6 and CT26 cell lines was high even without treatment (**Fig. 2A**). In addition, MHC-I expression in the EMT6 and CT26 cell lines was comparable before and after treatment, similar to what was seen with MEL02 and MEL03 (**Fig. 2A**). Next, we deleted *Jak1* in these cell lines using the CRISPR/Cas9 system (**Fig. 2B** and **Supplementary Fig. S2C**). MHC-I expression in *Jak1*-deleted MC-38 and B16F10 cell lines did not increase after IFNγ treatment, and the inhibitory effect of IFNγ on cellular proliferation was abrogated in all *Jak1*-deleted cells (**Fig. 2B** and **2C**). In contrast, high MHC-I expression was maintained in the *Jak1*-deleted EMT6 and CT26 cell lines, and was comparable before and after treatment (**Fig. 2B**).

In addition, we created *Jak2*-deleted EMT6 and MC-38 cell lines (**Supplementary Fig. S2D**). Similar to *Jak1* deletion, MHC-I expression in the *Jak2*-deleted MC-38 cell line did not increase after IFNγ treatment and high MHC-I expression was maintained in the *Jak2*-deleted EMT6 cell line, which was comparable before and after treatment (**Supplementary Fig. S2E**). We also analyzed STAT1/3, which are components of IFNγ downstream pathways related to MHC-I expression. Although IFNγ treatment increased STAT1/3 phosphorylation in the controls, it did not have the same effect in *Jak1*- or *Jak2*-deleted cells (**Supplementary Fig. S2F**), suggesting that both *Jak1* and *Jak2* deletion impair IFNγ signaling pathways but do not always impair MHC-I elevation. Because IFNγ signaling pathways also trigger proteasome subunits and TAP expression, which may alter the peptide repertoire (20,21), we analyzed LMP7 and TAP1 expression. Although IFNγ treatment increased LMP7 and TAP1 expression in the controls, it had no effect in the *Jak*-deleted cells, with expression maintained at baseline levels (**Supplementary Fig. S2G**). These findings indicate that IFNγ–triggered expression of proteasome subunits and TAP are impaired by *Jak* deletion, but the baseline expressions are maintained.

In vivo, Jak1 deletion did not induce resistance to PD-1 blockade in EMT6 and CT26 tumors, which had high MHC-I expression regardless of *Jak1* status (**Fig. 3A** and

Supplementary Fig. S3A), whereas the deletion of *Jak1* resulted in PD-1 blockade resistance in MC-38 tumors, as previously reported (**Fig. 3B and Supplementary Fig. S3A**) (26,27). In contrast, efficacy of PD-1 blockade against *Jak1*-deleted EMT6 tumors was not observed in severe combined immunodeficiency (SCID) mice (**Supplementary Fig. S3B**). $CD8^+$ T cells were scarcely observed in *Jak1*-deleted MC-38 tumors, although were considerably observed in *Jak1*-deleted EMT6 tumors after PD-1 blockade (**Fig. 3C** and **3D**). In addition, we created *Cd274*-overexpressing *Jak1*-deleted EMT6 and MC-38 cell lines (**Supplementary Fig. S3C**). *Cd274* overexpression did not affect the efficacy of PD-1 blockade against *Jak1*-deleted tumors (**Supplementary Fig. S3D**). These findings indicate that PD-1 blockade therapies exhibit efficacy against MHC-I–expressing tumors despite defects in IFNγ signaling pathways.

Immune cells rather than cancer cells play important roles in $CD8^+$ effector T-cell recruitment

Although IFNγ signaling pathways promote chemokine production, which is involved in $CD8^+$ effector T-cell recruitment, our *in vivo* data showed that the TME of *Jak*-deleted tumors was highly infiltrated with $CD8^+$ T cells when high MHC-I expression was maintained. Therefore, we analyzed expression of chemokines involved in $CD8^+$

effector T-cell recruitment, such as CXCL9 and CXCL10 (14,39). *In vitro*, both *Cxcl9* and *Cxcl10* expression increased after IFNγ treatment in EMT6 and MC-38 parental cell lines, although neither increased after IFNγ treatment in *Jak1* or *Jak2*-deleted cell lines, as previously reported (**Fig. 4A, B**, and **Supplementary Fig. S2H**) (40). Next, we analyzed chemokine expression in tumor tissues *in vivo* and found that both *Cxcl9* and *Cxcl10* expression increased after PD-1 blockade in high MHC-I–expressing *Jak1*-deleted EMT6 tumors, whereas this increase was not observed in *Jak1*-deleted MC-38 tumors with low MHC-I expression **(Fig. 4C** and **4D**). Considering the possibility of chemokine production from other immune cells as well as cancer cells in the TME, $CD45^+$ immune cells and $CD45^-$ cells including cancer cells were sorted from the TME; chemokine expression was analyzed in each population (**Supplementary Fig. S3E**). *Cxcl9* and *Cxcl10* were highly expressed in $CD45^+$ immune cells, although not in other $CD45^-$ cells (**Fig. 4E**). Altogether, these findings suggest that chemokines involved in $CD8^+$ effector T-cell recruitment are primarily produced by immune cells in the TME of high MHC-I–expressing tumors with IFNγ signaling pathway defects.

Loss of MHC-I expression contributes to resistance to PD-1 blockade induced by IFNγ signaling pathway defects

To confirm the importance of MHC-I expression, we created a *B2m*-deleted EMT6 cell line using the CRISPR/Cas9 system. This cell line did not express MHC-I regardless of IFNγ signaling pathway status (**Fig. 5A**). Since the IFNγ signaling pathways were maintained, the inhibitory effect on cellular proliferation and stimulatory effect on chemokine production by IFNγ were preserved *in vitro* (**Fig. 5B** and **C**). In contrast, PD-1 blockade–mediated antitumor efficacy was completely canceled by *B2m* deletion in EMT6 cells (**Fig. 5D**). Chemokine expression did not increase after PD-1 blockade in *B2m*-deleted EMT6 tumor tissues (**Fig. 5E**), and $CD8^+$ T cells were scarcely observed in the TME, even after PD-1 blockade (**Fig. 5F**). In addition, we created an *H2-K1*-overexpressing *Jak1*-deleted MC-38 cell line (**Supplementary Fig. S3F**). *H2-K1*-overexpressing *Jak1*-deleted MC-38 tumors responded to PD-1 blockade (**Supplementary Fig. S3G**). These findings suggest that PD-1–blockade therapies are effective for MHC-I–expressing tumors regardless of IFNγ signaling pathway status, indicating that reduced MHC-I expression is the most critical contributor to ICI resistance because of IFNγ signaling pathway defects.

A patient with JAK-negative HNSCC responded to PD-1 blockade therapy

To validate our experimental findings, we analyzed 48 patients with HNSCC who

received PD-1–blockade therapy using immunohistochemistry for JAK1/2. The clinical data for the 48 patients are summarized in **Supplementary Table S2**. We observed two JAK-negative cases (**Supplementary Fig. S4** and **Supplementary Table S9**). While one was resistant to anti–PD-1, the other was sensitive (**Supplementary Fig. S4** and **Table S9**). Consistent with our mouse experiments, the resistant case showed little $CD8^+$ T-cell infiltration without MHC-I expression, whereas the sensitive case showed high $CD8^+$ T-cell infiltration with high MHC-I expression (**Supplementary Fig. S4**).

GNG4 regulates MHC-I expression independently of IFNγ signaling

Finally, to search for molecules that upregulate MHC-I expression independent of IFNγ signaling pathways, we conducted CRISPR screening using EMT6 and CT26 cell lines, which have high MHC-I expression in the absence of IFNγ treatment. We used a CRISPR knockout pooled library of over 76,000 guides, targeting approximately 19,000 genes. After infection, we sorted the cells with low and high MHC-I expression from the infected bulk cells and sequenced them. The number of reads was counted after reading the guide sequences. We used the MAGeCK score to identify the group of genes required for MHC-I expression and selected genes with FDRs less than 0.25. After excluding genes known to be required for MHC-I expression, such as *Tap1*, *Tap2*, and *B2m*, we focused

on several genes with higher expression in EMT6 or CT26 cells. As a result, we identified four candidates regulators of MHC-I expression independent of IFNγ signaling pathways: *Crkl, Il7, Proser2,* and *Gng4* (**Supplementary Fig. S5** and **S6**). Next, we tested whether siRNA knockdown of these four genes reduced MHC-I expression. We observed reduced MHC-I expression in *Gng4*-knockdown CT26 cells, which had high *Gng4* expression at baseline, but not in the other cell lines, which had low *Gng4* expression to begin with (**Supplementary Fig. S6** and **S7**). Next, we created *Gng4*-knockdown CT26 cells using an shRNA lentivirus. As observed with siRNA, MHC-I expression was reduced in *Gng4*-knockdown CT26 cells (CT26/sh-*Gng4*) (**Fig. 6A**).

Knockdown of *Gng4* resulted in reduced MHC-I expression, leading to resistance to PD-1 blockade

GNG4 is reportedly involved in the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt) signaling pathway (41,42), and Western blotting showed that Akt phosphorylation was reduced by *Gng4*-knockdown (**Fig. 6B)**. The NF-κB signaling pathway is downstream of the PI3K/Akt signaling pathway (43-45), and reportedly elevates MHC-I expression (46-50). Consistent with this, NF-κB inhibitor α (IκBα) phosphorylation, p65 phosphorylation, p50, and p52 were also reduced by *Gng4*-

knockdown (**Fig. 6B**). In addition, nuclear expression levels of p65, p50 and p52 also decreased (**Fig. 6C**). Next, we treated the cell line with IMD-0354, an inhibitor of the NF-κB signaling pathway, and this resulted in reduced MHC-I expression in parental CT26 and CT26/sh-Control cells, although MHC-I expression in CT26/sh-*Gng4* cells was comparable with and without IMD-0354 treatment (**Fig. 6D**). Moreover, *in vivo*, *Gng4*-knockdown induced resistance to PD-1 blockade in CT26 tumors (**Fig. 6E**). Immunohistochemistry for NF-κB (p65, p50, and p52) in the HNSCC cases indicated that the responder and non-responder had strongly and weakly positive tumors, respectively (**Fig. 6F**), suggesting that the NF-κB signaling pathways contribute to high MHC-I expression independent of IFNγ signaling pathways.

Discussion

Although ICIs are effective against various types of cancer, a considerable number of cases remain resistant. Various mechanisms of resistance to ICIs have been reported, the most well-known being IFNγ signaling pathway defects, such as those associated with *JAK1/2* mutations (26,27). However, some patients reportedly respond to ICIs even when IFNγ signaling pathways are no longer functional (28,29), and the detailed mechanisms remain unclear. Here, we found that not all patients with IFNγ signaling pathway defects were resistant to ICIs and that ICIs were effective if MHC-I expression was maintained

regardless of IFNγ signaling pathway defects in mouse models and clinical samples. In particular, immune cells in the TME recruited $CD8^+$ effector T cells via chemokines even if cancer cells could not because of IFNγ signaling pathway defects. In addition, CRISPR screening showed that GNG4 compensated for MHC-I expression via NF-κB signaling pathways, even when IFNγ signaling pathways were impaired. These findings indicate the importance of MHC-I expression in ICI resistance due to defects in IFNγ signaling pathways and the possibility of NF-κB–targeted therapies to overcome this resistance.

Since IFNγ plays important roles in antitumor immunity via increasing MHC expression and antigen processing, mediating direct anti-proliferative and apoptotic effects in cancer cells, and enhancing chemokine production, patients with IFNγ pathway defects often become resistant to ICIs (9,26,27). Consistent with this, we found that *JAK*-deleted cells lost IFNγ–mediated effects including anti-proliferative effects and chemokine production *in vitro*. However, some *JAK*-deleted cancer cells had high MHC-I expression, independent of IFNγ signaling pathways, and paired TILs recognized and killed cancer cells. In animal models, *Jak*-deleted tumors with low MHC-I expression were resistant to ICIs, as previously reported (9,26,27). In contrast, those with high MHC-I expression independent of IFNγ signaling pathways did not become resistant. The TMEs of these tumors showed high $CD8^+$ T-cell infiltration even though chemokine production

was not elevated by IFNγ in the same *Jak*-deleted cell lines *in vitro*. However, our *in vivo* experiments clearly showed that *Jak*-deleted tumors with high MHC-I expression have high expression of chemokines. In fact, we found that tumor-infiltrating immune cells but not cancer cells mainly produced chemokines in *Jak1*-deleted tumors with high MHC-I expression *in vivo*, and chemokines were elevated by IFNγ in *B2m*-deleted EMT6 cancer cells with intact *Jak in vitro* but not elevated *in vivo*. From these findings, we conclude that if MHC-I is highly expressed, $CD8^+$ T cells recognize cancer cells, leading to further $CD8^+$ T-cell infiltration as a result of chemokines produced by immune cells in the TME, including $CD8^+$ T cells themselves, independent of the IFNγ signaling pathways in cancer cells. Consistent with this, MHC-I overexpression in *Jak*-deleted resistant tumors with low MHC-I expression overcame resistance to PD-1 blockade. In contrast, MHC-I^- tumors owing to *B2m* deletion became resistant to ICIs despite maintained IFNγ signaling pathways in cancer cells. A previous study reported similar findings of acquired resistance to ICIs in lung cancer (13), i.e., impaired MHC-I expression conferred acquired resistance regardless of IFNγ signaling pathways (13). From these data, elevated MHC-I expression is the most important factor related to IFNγ signaling pathways for antitumor immunity, including antitumor immunity mediated by PD-1 blockade. Thus, defects in IFNγ signaling pathways do not always induce ICI resistance, especially when high MHC-I

expression is maintained. Indeed, we identified an ICI responder whose HNSCC was JAK-negative but had high MHC-I expression.

Besides MHC-I expression, IFNγ elevates PD-L1 expression, which is a well-known predictive biomarker for ICIs (51) and *Jak* deletion abrogated PD-L1 elevation by IFNγ in our *in vitro* experiments. To elucidate the role of PD-L1, we created *Cd274*-overexpressing *Jak1*-deleted cell lines. However, *Cd274* overexpression did not affect the sensitivities to PD-1 blockade, supporting the importance of MHC-I expression. In addition, IFNγ also triggers proteasome subunits and TAP expression, which may alter the peptide repertoire (neoantigen or self-antigen processing) (20,21). In our experiments, *Jak* deletion impaired IFNγ–triggered LMP7 and TAP1 expression, but baseline expression was maintained, and *Jak*-deleted tumors with high MHC-I expression responded to PD-1 blockade. In addition, MHC-I overexpression overcame resistance in *Jak*-deleted MC-38 tumors. From these findings, the response in our tested cell line appeared to be unrelated to these IFNγ–mediated processes if the baseline expression was maintained, whereas these processes might be related to resistance in other cell lines that we did not test. To elucidate the detailed roles, further research is warranted.

To overcome resistance from IFNγ signaling pathway defects, we performed CRISPR screening to search for molecules involved in MHC-I expression independent of

IFNγ signaling pathways. We found that GNG4 is involved in MHC-I expression in CT26 cells. Previous studies have shown that GNG4 is involved in PI3K/Akt and NF-κB signaling pathways (41-45). In addition, NF-κB signaling pathways are reportedly related to MHC-I expression (46-50). Our experiments consistently demonstrated that MHC-I expression was reduced by *Gng4*-knockdown via the NF-κB signaling pathway, and *Gng4*-knockdown induced resistance to PD-1 blockade in the CT26 model. In contrast, other cell lines that we tested had low *Gng4* expression, although MHC-I expression was comparable, suggesting that genetic/epigenetic status might have an effect. In a previous report, CRISPR screening using low MHC-I–expressing cell lines identified TRAF3, an NF-κB signaling pathway repressor, as a factor that downregulates MHC-I expression (49). TRAF3 inhibition by the administration of a SMAC mimetic was proven to increase the efficacy of ICIs by increasing MHC-I expression through NF-κB signaling pathway activation (49), which supports our results.

In our cohort, a patient with JAK-negative HNSCC and anti–PD-1 resistance showed low MHC-I expression and limited $CD8^+$ T-cell infiltration. Conversely, in a responder with JAK-negative HNSCC, high MHC-I expression was observed along with high $CD8^+$ T-cell infiltration. Furthermore, the responder was strongly positive for NF-κB, whereas the non-responder was weakly positive for NF-κB, suggesting that the NF-

κB signaling pathways compensate for MHC-I expression despite IFNγ signaling pathway defects. As our cohort was very small, further analyses should be performed to validate these findings.

Although we demonstrated the importance of MHC-I expression in antitumor immunity even when cancer cells have defects in IFNγ signaling pathways, some studies have shown a response to PD-1–blockade therapies despite the loss of MHC-I expression (52-56). Among them, some studies emphasized MHC class II (MHC-II) expression in cancer cells as important for the efficacy of PD-1 blockade (53-56). Specifically, high MHC-II expression and activated IFNγ signaling pathways in melanoma samples appeared to be correlated (54), and we previously demonstrated an important role of cytotoxic $CD4^+$ T cells in antitumor immunity against MHC-II^+ tumors even with the loss of MHC-I (55). Thus, further research focusing on PD-1 blockade–mediated efficacy, IFNγ signaling pathways, and MHC-I/II expression is warranted.

In summary, we established that not all patients with IFNγ signaling pathway defects are resistant to ICIs, and ICIs can be effective even if IFNγ signaling pathways are lost but MHC-I expression was maintained. CRISPR screening showed that the NF-κB signaling pathway can compensate for MHC-I expression even when IFNγ signaling pathways are impaired. These findings indicate the importance of MHC-I expression in

ICI resistance due to defects in IFNγ signaling pathways and suggest the possibility that NF-κB–targeted therapies could be used to overcome this resistance.

Authors' Contributions

K. Kawase: Data curation, Formal analysis, Investigation, Methodology, Writing - original draft, Writing - review & editing. **S. Kawashima:** Formal analysis, Investigation, Methodology, Writing - review & editing. **J. Nagasaki:** Formal analysis, Investigation, Methodology, Writing - review & editing. **T. Inozume:** Funding acquisition, Methodology, Resources, Writing - review & editing. **E. Tanji:** Investigation, Writing - review & editing. **M. Kawazu:** Funding acquisition, Investigation, Methodology, Supervision, Writing - review & editing. **T. Hanazawa:** Funding acquisition, Resources, Supervision, Writing - review & editing. **Y. Togashi:** Conceptualization, Data curation, Funding acquisition, Methodology, Project administration, Writing - Original draft, Writing - review & editing.

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Figure Legends

Figure 1. IFNγ production and killing activities against parental or *JAK2*-deleted cancer cells with high MHC-I expression

(A) and **(B)** Representative flow cytometry staining of MHC-I and PD-L1 expression in parental **(A)** or *JAK2*-deleted **(B)** cancer cell lines established from two patients with melanoma (MEL02 and MEL03). The cells were treated with or without IFNγ for 24 hours and subsequently analyzed with flow cytometry. **(C**) IFNγ release assay. The MEL02 and MEL03 cancer cell lines were cocultured with paired TILs for 24 hours. Supernatants were analyzed with ELISA for IFNγ. Anti–MHC-I was used as a negative control. **(D**) Killing assay. Calcein-AM-labeled MEL02 and MEL03 cancer cell lines (target cells; T) were cocultured with paired TILs (effector cells; E) at the indicated E:T ratios and then centrifuged. After 3 hours of incubation, fluorescence was measured. The MEL01 cancer cell line with loss of MHC-I expression was used as a negative control. All *in vitro* experiments were performed in triplicate. One-way ANOVA tests with Bonferroni corrections were used in **(C),** and two-way ANOVA tests with Bonferroni corrections were used in **(D)** for statistical analyses; data are presented as means and standard error of the means. ***, $P < 0.001$; ****, $P < 0.0001$; ns, not significant.

Figure 2. MHC-I expression and proliferation in various parental or *Jak1*-deleted mouse cancer cells

(A) and **(B)** Representative flow cytometry staining of MHC-I and PD-L1 expression in parental **(A)** or *Jak1*-deleted **(B)** mouse cancer cell lines (EMT6, CT26, MC-38, and B16F10). The cells were treated with or without IFNγ for 24 hours and subsequently analyzed with flow cytometry. **(C**) Cellular proliferation. EMT6, CT26, and MC-38 cells were seeded in 96-well plates and treated with or without IFNγ for 48 hours. After adding Cell Counting Kit-8 reagent, cellular proliferation was evaluated with absorbance. All *in vitro* experiments were performed in triplicate, and paired t-tests were used in **(C)** for statistical analyses; data are presented as means and standard error of the means. *, $P < 0.05$; **, $P < 0.01$; ns, not significant.

Figure 3. *In vivo* efficacy of PD-1 blockade in various parental or *Jak1*-deleted mouse tumors

(A) Parental or *Jak1*-deleted EMT6 (left) and CT26 (right) tumor growth with or without anti–PD-1 treatment in wild-type Balb/c mice. Cancer cells (1×10^6) were injected subcutaneously, and tumor volumes were monitored twice weekly. Mice were grouped

when the tumor volume reached approximately 100 mm^3 (day 0), and anti–PD-1 or control mAb was administered intraperitoneally on days 0, 3, and 6 (n = 5 per group). **(B)** Parental or *Jak1*-deleted MC-38 tumor growth with or without anti–PD-1 treatment in wild-type C57BL/6 mice. *In vivo* experiments were performed as described in **(A). (C)** and **(D)** $CD8^+$ T-cell infiltration was assessed by immunohistochemistry. *In vivo* experiments were performed as described in **(A)**, and tumors were harvested 7 days after treatment initiation and evaluated for $CD8^+$ T-cell infiltration. Five fields (0.25 mm^2) were randomly selected, and $CD8^+$ T cells were counted on each slide. The average of the five fields was used for statistical analyses. Representative staining (left) and summaries (right) are shown for **(C)** EMT6 and **(D)** MC-38. All *in vivo* experiments were performed in duplicate, with similar results achieved. Two-way ANOVA tests were used in **(A)** and **(B)**, and unpaired t-tests were used in **(C)** and **(D)** for statistical analyses; data are presented as means and standard error of the means. Scale bar, 100 μm; **, $P < 0.01$; ****, $P < 0.0001$; ns, not significant.

Figure 4. *Cxcl9* and *Cxcl10* expression *in vitro* and *in vivo*

(A) and **(B)** *Cxcl9* and *Cxcl10* gene expression in parental or *Jak1*-deleted EMT6 **(A)** and MC-38 cells **(B)** *in vitro*. Cancer cells were treated with or without IFNγ for 24 hours, and gene expression was evaluated with qRT-PCR. *Gapdh* was used as an internal control. **(C)** and **(D)** *Cxcl9* and *Cxcl10* gene expression in parental or *Jak1*-deleted EMT6 **(C)** and MC-38 **(D)** tumors *in vivo*. Cancer cells (1×10^6) were injected subcutaneously, and tumor volume was monitored twice weekly. Mice were grouped when the tumor volume reached approximately 100 mm^3, and anti–PD-1 or control mAb was administered intraperitoneally three times every 3 days thereafter. Tumors were harvested 7 days after treatment initiation for evaluation. Gene expression was evaluated with qRT-PCR. *Gapdh* was used as an internal control. **(E)** *Cxcl9* and *Cxcl10* gene expression in *Jak1*-deleted EMT6 tumors treated with anti–PD-1 *in vivo*. *In vivo* experiments were performed as described in **(C)** and **(D)**. $CD45^+$ cells and $CD45^-$ cells were sorted and analyzed using qRT-PCR. *Gapdh* was used as an internal control.

All *in vitro* experiments were performed in triplicate, and paired t-tests were used in **(A)** and **(B)** for statistical analyses. All *in vivo* experiments were performed in duplicate with similar results achieved. Unpaired t-tests were used in **(C)** and **(D)**, and paired t-tests were used in **(E)** for statistical analyses; data are presented as means and standard error of the means. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns, not significant.

Figure 5. *In vivo* efficacy of PD-1 blockade in *B2m*-deleted EMT6 tumors

(A) Representative flow cytometry staining of MHC-I and PD-L1 expression in *B2m*-deleted EMT6 cells treated with or without IFNγ. The *B2m*-deleted EMT6 cells were treated with or without IFNγ for 24 hours and subsequently analyzed with flow cytometry. **(B)** Cellular proliferation of *B2m*-deleted EMT6 cells. The *B2m*-deleted EMT6 cells were seeded in 96-well plates and treated with or without IFNγ for 48 hours. After adding Cell Counting Kit-8 reagent, cellular proliferation was evaluated with absorbance. **(C)** *Cxcl9* and *Cxcl10* expression in *B2m*-deleted EMT6 cells *in vitro*. The *B2m*-deleted EMT6 cells were treated with or without IFNγ for 24 hours, and gene expression was evaluated using qRT-PCR. *Gapdh* was used as an internal control. **(D)** *B2m*-deleted EMT6 tumor growth with or without treatment with anti–PD-1 in wild-type mice. Cancer cells (1×10^6) were injected subcutaneously, and tumor volume was monitored twice weekly. Mice were grouped when the tumor volume reached approximately 100 mm^3 (day 0), and anti–PD-1 or control mAb was administered intraperitoneally on days 0, 3, and 6 (n = 5 per group). **(E)** *Cxcl9* and *Cxcl10* expression in *B2m*-deleted EMT6 tumors *in vivo*. *In vivo* experiments were performed as described in **(D)**. Tumors were harvested 7 days after treatment initiation for evaluation. Gene expression was analyzed using qRT-PCR. *Gapdh* was used as an internal control. **(F)** $CD8^+$ T-cell infiltration in *B2m*-deleted EMT6 tumors. *In vivo* experiments were performed as described **(D)**, and tumors were harvested 7 days after treatment initiation and evaluated for $CD8^+$ T-cell infiltration. Five fields (0.25 mm^2) were randomly selected, and $CD8^+$ T cells were counted on each slide. The average of the five fields was used for statistical analyses. Representative staining (left) and summaries (right) are shown. All *in vitro* experiments were performed in triplicate, and paired t-tests were used in **(B)** and **(C)** for statistical analyses. All *in vivo* experiments were performed in duplicate, with similar results achieved. A two-way ANOVA test was used in **(D)**, and unpaired t-tests were used in **(E)** and **(F)** for statistical analyses; data are presented as means and standard error of the means. Scale bar, 100 μm; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$; ns, not significant.

Figure 6. MHC-I expression and *in vivo* efficacy of PD-1 blockade in *Gng4*-knockdown CT26 tumors

(A) Representative flow cytometry staining of MHC-I expression in CT26, CT26/sh-Control, and CT26/sh-*Gng4* cells. The CT26/sh-Control, CT26/sh-*Gng4*-1, and CT26/sh-*Gng4*-2 cells were generated using lentivirus and analyzed using flow cytometry. **(B)** Western blotting representative images of whole cell lysates and their quantification. Cell lysates were subjected to immunoblot analysis with antibodies to phosphorylated (p) or total forms of Akt, IκBα, p65, p50, p52, or β-actin (loading control). The bands of p-Akt,

p-IκBα, p-p65, p50, and p52 were quantified using ImageJ software. We calculated the fold change to β-actin and parental CT26 (below each band). **(C)** Western blotting representative images of nuclear fractions and their quantification. Nuclear fractions were subjected to immunoblot analysis with antibodies to p65, p50, p52, or TBP (loading control). The bands of p65, p50, and p52 were quantified using ImageJ software. We calculated the fold change to TBP and parental CT26 (below each band). (**D**) Representative flow cytometry staining of MHC-I expression in CT26, CT26/sh-Control, CT26/sh-*Gng4*-1, and CT26/sh-*Gng4*-2 cells treated with or without IMD-0354. The cells were treated with or without IMD-0354 for 72 hours and subsequently analyzed using flow cytometry. **(E**) CT26/sh-Control and CT26/sh-*Gng4*-1 tumor growth with or without anti–PD-1 treatment in wild-type mice. Cancer cells (1×10^6) were injected subcutaneously, and tumor volume was monitored twice weekly. Mice were grouped when the tumor volume reached approximately 100 mm^3 (day 0), and anti–PD-1 or control mAb was administered intraperitoneally on days 0, 3, and 6 (n = 5 per group). **(F**) Immunohistochemistry for p65, p50, and p52 in HNSCC cases. Sections of FFPE tissue (3 μm) were obtained from HNSCC patients who received PD-1–blockade therapies and analyzed for immunohistochemistry. Representative staining of the responder and non-responder (left, p65; middle, p50; right, p52) with JAK-negative HNSCC are shown. All *in vitro* experiments were performed in triplicate, and all *in vivo* experiments were performed in duplicate, with similar results achieved. A two-way ANOVA test was used in **(E)** for statistical analyses; data are presented as means and standard error of the means. Scale bar, 100 μm; ****, $P < 0.0001$; ns, not significant.