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B7-H3 suppresses anti-tumor immunity via the CCL2–CCR2–M2 macrophage axis and contributes to ovarian cancer progression

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Running title: B7-H3 immunosuppression via M2 macrophages in ovarian cancer

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Synopsis: A role of B7-H3 in M2 macrophage-mediated immunosuppression in ovarian cancer is demonstrated. The data reveal B7-H3's potential as a therapeutic target to enhance anti-tumor responses in ovarian cancer, highlighting a differential role from other B7 family members.

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Abstract

New approaches beyond PD-1/PD-L1 inhibition are required to target the immunologically diverse tumor microenvironment (TME) in high-grade serous ovarian cancer (HGSOC). In this study, we explored the immunosuppressive effect of B7-H3 (CD276) via the CCL2–CCR2–M2 macrophage axis and its potential as a therapeutic target. Transcriptome analysis revealed that B7-H3 is highly expressed in PD-L1-low, non-immunoreactive HGSOC tumors, and its expression negatively correlated with an IFNγ signature, which reflects the tumor immune-reactivity. In syngeneic mouse models, B7-H3 (*Cd276*) knockout (KO) in tumor cells, but not in stromal cells, suppressed tumor progression, with a reduced number of M2 macrophages and an increased number of IFNγ^{+}CD8^{+} T cells. CCL2 expression was downregulated in the B7-H3 KO tumor cell lines. Inhibition of the CCL2–CCR2 axis partly negated the effects of B7-H3 suppression on M2 macrophage migration and differentiation, and tumor progression. In HGSOC patients, B7-H3 expression positively correlated with CCL2 expression and M2 macrophage abundance, and patients with B7-H3-high tumors had fewer tumoral IFNγ^{+}CD8^{+} T cells and poorer prognosis than patients with B7-H3-low tumors. Thus, B7-H3 expression in tumor cells contributes to CCL2–CCR2–M2 macrophage axis-mediated immunosuppression and tumor progression. These findings provide new insights into the immunological TME and could aid the development of new therapeutic approaches against the unfavorable HGSOC phenotype.

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Introduction

Ovarian cancer is the eighth leading cause of cancer death in women worldwide, with an estimated figure of more than 200,000 deaths per year (1). In addition to conventional surgery and chemotherapy, the introduction of anti-VEGF and PARP inhibitors as therapeutic modalities has remarkably prolonged the duration of progression-free survival, even though progression remains inevitable in the majority of cases (2,3). The efficacy of PD-1/PD-L1 inhibition therapy is limited in ovarian cancer, as evidenced by clinical trials, and immunotherapy has not yet become the standard treatment option (4-6).

Of all the histological types of ovarian cancer, high-grade serous ovarian cancer (HGSOC) remains the most common and the most aggressive (7,8). Although the tumor microenvironment (TME) of HGSOC varies widely, it is well-characterized by four subtypes based on gene expression profiles: differentiated, immunoreactive, mesenchymal, and proliferative subtypes (9,10). The immunoreactive subtype is characterized as tumors with high expression of IFNγ and PD-L1, a representative anti-tumor cytokine and an immunomodulatory molecule upregulated in response to IFNγ, respectively, and patients have a better prognosis (10-12). The mesenchymal type is characterized as epithelial-mesenchymal transition (EMT)-high, immunosuppressive tumors with poor prognosis (10,13,14). The differentiated and proliferative subtypes consist of immune 'desert' tumors (14). PD-1/PD-L1 inhibition therapy is relatively effective in treating PD-L1-high immunoreactive tumors. However, non-immunoreactive phenotypes, which comprise a majority of HGSOC tumors, respond poorly to the treatment (4). To elucidate what contributes to the differences in the TME, and new immunological approaches to treat non-immunoreactive phenotypes other than PD-1/PD-L1 inhibition, are required.

B7-H3 is a transmembrane protein from the B7 family; it was first reported in 2001 (15). Developments in therapies targeting B7-H3, including antibody–drug conjugates, radioimmunotherapy, and chimeric antigen receptor T cells, which focus on its high selective expression in tumor tissues, highlight the potential of B7-H3 as a therapeutic target (16-18). B7-H3 seems to play complex roles in modulating the TME. Not only does it exert its immunomodulatory effects by directly acting on target cells as an immune checkpoint (15,19,20), it is also involved in the intracellular signaling of cancer cells, such as the STAT3 pathway (21-23). Its contribution to immunosuppressive TME, other than as an immune checkpoint molecule, remains to be fully elucidated.

Here, we demonstrate that B7-H3 expression is upregulated in the non-immunoreactive tumors of HGSOC and that B7-H3 contributes to ovarian cancer progression via CCL2–CCR2–M2 macrophage axis-mediated immunosuppression, in both mouse syngeneic ovarian cancer models and HGSOC cases. Our results not only augment the current understanding of the immunological heterogeneity of HGSOC but also provide a foundation for stratification of the immunosuppressive HGSOC phenotype with a B7-H3-high, M2 macrophage-rich TME and facilitate the development of new treatment strategies other than PD-1/PD-L1 inhibition that can specifically benefit patient

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groups with extremely poor prognosis.

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Materials and Methods

Study approval. This study was approved by the Kyoto University Graduate School and Faculty of Medicine Ethics Committee (reference number G531) and conforms to the Declaration of Helsinki. Informed consent was obtained from all participants via an opt-in approach (wherein participants signed a printed informed consent document) or an opt-out approach (wherein participants were informed about the study through the website). All animal studies were approved by the Kyoto University Animal Research Committee.

Human samples. Formalin-fixed, paraffin-embedded (FFPE) primary site tumor specimens (n=62) and fresh primary site tumor samples (n=28) were collected from patients with HGSOC, who underwent primary surgery at the Kyoto University Hospital between 1998 and 2015. Patients without primary site resection surgery or those who received chemotherapy prior to surgery were excluded. Blood samples from HGSOC patients (n=23) and healthy female donors (n=10) were also collected between 2012 and 2020. FFPE samples were stored at room temperature, and fresh tumor samples and blood samples were stored at –80 °C until use.

Bioinformatics analysis. Gene expression data of 30 HGSOC specimens from the Kyoto University Hospital that were partially deposited in the Gene Expression Omnibus (RRID: SCR_005012, accession numbers: GSE39204 and GSE55512) were classified into the four transcriptome subtypes (24), according to the Classification of Ovarian Cancer (CLOVAR) subtype signatures (10). Differentially expressed genes (DEGs) between the immunoreactive (n = 8) and non-immunoreactive (n = 22) subtypes were extracted using the Samroc method as described previously (25). The gene expression profiles from The Cancer Genome Atlas-Ovarian Cancer (TCGA-OV) RNA-sequencing dataset (n=263) from TCGA Data Portal (RRID: SCR_003193, illuminahiseq_rnaseqv2_Level_3_RSEM_genes_normalized_data files obtained on October 19, 2015) was used for comparison. Single-sample Gene Set Enrichment Analysis (ssGSEA) (26), using GenePattern software (RRID:SCR_003201, version 3.5.0), was performed to evaluate the IFNγ signature activity scores using the previously reported gene set (27) using R software (RRID: SCR_001905, version 3.6.1).

Cell culture and transfection. The OV2944-HM-1 (HM-1) mouse ovarian cancer cell line was purchased from RIKEN BioResource Center (RRID: SCR_003250, Cat# RCB1483; RRID: CVCL_E954; Tsukuba, Japan) in January 2003. HM-1 cells were cultured and maintained in minimal essential medium (MEM)-alpha (Cat# 12571063, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% (v/v) heat-inactivated FBS (Cat# S1810, Biowest, San Marcos, TX, USA) and penicillin–streptomycin (100 IU/ml penicillin and 100 µg/ml streptomycin; Cat# 26253-84, Nacalai Tesque, Kyoto, Japan) in an atmosphere of 5% CO_2 at 37 °C. The ID8 mouse ovarian cancer cell line was kindly provided by Dr. Katherine Roby of The University of Kansas Medical Center

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(RRID: CVCL_IU14) in September 2009. Human ovarian cancer cell lines OVCAR3 (RRID: CVCL_0465) and OVCA420 (RRID: CVCL_3935) were kindly provided by Dr. Susan K. Murphy of Duke University in June 2007. Both ID8 and human ovarian cancer cells were cultured and maintained in RPMI1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% FBS and penicillin–streptomycin in an atmosphere of 5% CO_2 at 37 °C. All cell line-based experiments were performed before passage 20. All cell lines were regularly tested for mycoplasma contamination (date of last check: April 12, 2021) using a Mycoplasma PCR Detection Kit (Cat# G238; Applied Biological Materials, Richmond, BC, Canada). Human cell lines (OVCAR3 and OVCA420) were re-authenticated on April 14, 2021, by short tandem repeat analysis.

For CRISPR/Cas9-mediated *Cd276* knockout (B7-H3 KO), target sequences were determined as previously described (16). Three single-guide RNAs (sgRNAs) and a control sgRNA were synthesized by FASMAC (Kanagawa, Japan). A mixture of the RNAs and Guide-it recombinant Cas9 (Cat# 632641, Takara Bio Inc., Shiga, Japan) was transfected into the HM-1 and ID8 cells using the TransIT-X2 Dynamic Delivery System (Cat# MIR6003, Mirus Bio, Madison, WI, USA). The cells were incubated with rabbit anti-CD276 (1:1000 dilution, clone: EPNCIR122, Cat# ab134161; RRID: AB_2687929, Abcam, Cambridge, UK) for 20 min at 4 °C and with Goat Anti-Rabbit IgG H&L (Alexa Fluor 647) (1:2000 dilution, Cat# ab150079; RRID:AB_2722623, Abcam) for 10 min at 4 °C. Alexa Fluor 647-negative cell populations were sorted by flow cytometry using BD FACSAria II Cell Sorter (RRID: SCR_018934, BD Biosciences, Franklin Lakes, NJ, USA). The purity of Alexa Fluor 647-negative cells was >99 %. Single cells were isolated by limiting dilution and cultured again on 96-well plates (Cat# 655180, Greiner Bio-One, Kremsmunster, Austria) for 8 days under the same conditions described in the section above, followed by expansion. Finally, B7-H3 expression deficiency was confirmed using FACS analysis and quantitative PCR (qPCR).

B7-H3-knockdown HGSOC cells, OVCAR3-sh-B7-H3 and OVCA420-sh-B7-H3, and their control cells were generated by lentiviral-mediated transfection with short hairpin RNAs (shRNAs) targeting B7-H3 or a non-silencing control (sh1, Cat# TRCN0000128599; sh2, Cat# TRCN0000436667; sh control, Cat# SHC002; Sigma-Aldrich, St. Louis, MO, USA). The sgRNA and shRNA sequences are listed in Supplementary Table S1.

Mice. Five- to six-week-old female C57BL6 (strain code: 027) and B6C3F1 (C57BL6×C3/He F1, strain code: 031) mice were purchased from CLEA Japan (Tokyo, Japan) and used in immunocompetent mouse experiments. Five- to six-week-old female BALB/c-nu (*Foxn1*^{nu}, strain code: 194) mice were purchased from CLEA Japan and used in immunodeficient mouse experiments. The C57BL6 background *Cd276*$^{-/-}$ (B7-H3 KO) mice were kindly provided by Dr. Brad St. Croix of the National Cancer Institute. Animals were maintained under specific pathogen-free conditions.

Tumor models. A total of 1×10^6 HM-1-B7H3 KO cells or control cells were inoculated intradermally into the shaved abdominal lesion of B6C3F1 and nude mice. A total of 5×10^5 HM-1-B7H3 KO or

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control cells were injected into the abdominal cavity of B6C3F1 or nude mice (intraperitoneal model). Similarly, 5.0×10^6 ID8 B7-H3 KO or control cells were injected into the abdominal cavity of C57BL6 or nude mice (intraperitoneal model). Intradermal tumor size was measured using calipers and calculated as follows: volume = length × width × height × π/6. In the intraperitoneal models, tumor growth was evaluated based on changes in body weight, which reflect the amount of ascites and omental tumor weight. Survival analysis was also performed in these models. Intradermal tumor size and body weight were measured once or twice a week. Intradermal tumors of HM-1-B7H3 KO or control cells at days 10, 12, and 25 were used for RNA-sequencing, flow cytometry, immunohistochemistry, and ELISA, respectively. Intraperitoneal tumors of ID8-B7H3 KO or control cells at days 63 and 67 were used for ELISA and flow cytometry, respectively. For B7-H3 KO mouse studies, only *Cd276*$^{+/+}$ and *Cd276*$^{-/-}$ littermates derived from *Cd276* heterozygous intercrosses were used for comparison. The CCR2 antagonist (RS504393: 2 mg/kg body weight; Cat# 17330, Cayman Chemical, Ann Arbor, MI, USA) treatment was initiated a day after tumor cell inoculation and was administered intraperitoneally daily. Mice were euthanized before becoming moribund.

Cell treatments. OVCAR3 and OVCA420 human ovarian cancer cells (1×10^5) were cultured in 6-well plates (Cat# 657160, Greiner Bio-One) as described in the "Cell culture and transfection" section above and treated with 20 ng/mL recombinant IFNγ (Cat# 570202, BioLegend, San Diego, CA, USA) (28) or 0.1 % bovine serum albumin (BSA; Cat# 7284, Sigma-Aldrich, St. Louis, MO, USA) as a control the following day. Cells were analyzed for the expression of B7-H3 (*CD276*), PD-L1 (*CD274*), and PD-L2 (*PDCD1LG2*) using qPCR and flow cytometry (as described below) after treatment for 24 and 48 hours, respectively.
For STAT3 inhibition, 1×10^5 cells were treated with the STAT3 inhibitor C188-9 (Cat# S8605, Selleck Chemicals, Houston, TX, USA) for the mouse cell lines HM-1 and ID8 (29) or with Stattic (Cat# ab120952, Abcam, Cambridge, UK) for the human cell lines OVCAR3 and OVCA420 (30) at 1.25 µM and 5 µM for 24 h, respectively. *Ccl2* and *CCL2* expression levels were analyzed using qPCR as described below.

Tumor cell proliferation assay. HM-1-B7H3 KO cells, ID8-B7-H3 KO cells, or their respective control cells were seeded in 96-well plates at 1×10^3 cells/well (Greiner Bio-One) and incubated for 3 days in an atmosphere of 5% CO_2 at 37 °C. The number of viable cells in each well was examined every 24 h by absorbance at 450 nm using Cell Count Reagent SF (Cat# 07553-44, Nacalai Tesque) and iMark microplate reader (Cat# 168-1130J, Bio-Rad, Hercules, CA, USA). Absorbance values at 72h were compared using Microsoft Excel (RRID: SCR_016137, Redmond, WA, USA).

Macrophage and monocyte selection. For tumor-derived macrophage selection, single cells from the tumors were prepared as described in the "Flow cytometry" section below. F4/80-positive macrophages were positively selected using anti-Mouse F4/80 PE antibody (clone: BM8, Cat#

123109; RRID: AB_893498, Biolegend), EasySep Mouse PE positive selection kit II (Cat# 17666, Stemcell Technologies, Vancouver, BC, Canada), and EasySep Magnet (Cat# 18000, Stemcell Technologies). The purity of F4/80-positive cells was >90% as confirmed by flow cytometry. For mouse monocyte selection, single cells from the bone marrow of the femur and tibia of B6C3F1 or C57BL6 non-tumor–bearing mice were collected by mechanical dissociation. For human monocyte selection, peripheral blood mononuclear cells of healthy donors were separated using Leucosep (Cat# 227290, Greiner) and Lymphosep (Cat# 1692254, MP Biomedicals, Santa Ana, CA, USA). Ly6C-positive mouse monocytes and CD14-positive human monocytes were positively selected by EasySep Magnet as described above using anti-Mouse Ly6C PE antibody (clone: HK1.4, Cat# 128007; RRID: AB_1186133, Biolegend) and anti-Human CD14 PE antibody (clone: M5E2, Cat# 301806; RRID: AB_314188, Biolegend). The purity of Ly6C-positive and CD14-positive cells was >90%, respectively, as confirmed by flow cytometry.

RNA extraction and reverse transcription (RT) qPCR. Human (OVCAR3 and OVCA420) and mouse (HM-1 and ID8) cells or macrophages isolated from the tumors of HM-1-B7H3 KO or control cells (as described in the preceding section) were lysed in RLT buffer (Cat# 1015750, Qiagen, Hilden, Germany) containing 1% 2-mercaptoethanol (Cat# 1610710, Bio-Rad). Intradermal tumors of HM-1-B7H3 KO cells or control cells were treated in a bead homogenizer with RLT buffer and centrifuged at 16,000 ×*g* for 1 min at 4 °C. Total RNA was extracted from the lysates using RNeasy Mini Kit (Cat# 74104, Qiagen).

A ReverTra Ace qPCR RT Kit (Cat# FSQ-101, TOYOBO, Osaka, Japan) was used for cDNA synthesis. Gene amplification was performed using PowerUp SYBR Green Master Mix (Cat# A25742, Thermo Fisher Scientific, Waltham, MA, USA) on a StepOne Plus real-time PCR system (Applied Biosystems, Foster City, CA, USA). Fifty nanograms of RNA was used as the template for PCR to detect the relative expression levels of *CD276*, *CD274* (PD-L1), *PTCD1LG2* (PD-L2), and *CCL2* for human cells, and *Cd276*, *Ccl2*, *Arg1*, *Il10*, and *Ifng* for mouse cells; the primer sequences are listed in Supplementary Table S1. Relative expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method. *GAPDH*/*Gapdh* was used for normalization.

Immunostaining and evaluation. FFPE sections of the 62 HGSOC samples and 3–6 mouse tumors of HM-1-B7H3 KO cells or control cells were immunostained as described previously (31). Epitope retrieval was performed at 120 °C for 10 minutes using citrate buffer at pH 6.0 (B7-H3) or Tris-EDTA buffer at pH 9.0 (αSMA, CD206, CD8, and IFNγ). The kits, antibodies and dilutions used are listed in Supplementary Table S2. Tumor cell B7-H3 expression was evaluated as previously described (32) and scored as follows: 0, negative or very weak; 1, weak; 2, moderate, and 3, strong (16). Scores of 0/1 and 2/3 were defined as B7-H3-low and B7-H3-high, respectively. Tumor-infiltrating $CD206^+$ cells (200×) were counted from five fields, and the average number of cells was calculated. For the evaluation of $CD8^+$ and $IFN\gamma^+CD8^+$ T cells, the number of cells in the representative $CD8^+$ cell-rich

field was counted at 200× magnification. The sensitivity and specificity of the IFNγ antibody was confirmed using appendix (Cat# CS802695, OriGene, Rockville, MD, USA) and tonsil (kindly provided by Kyoto University Department of Otolaryngology, Head and Neck Surgery) tissue as positive controls (Supplementary Figure S1a-b). Slides were analyzed using a BZ 9000 fluorescence microscope (RRID: SCR_015486, Keyence, Osaka, Japan).

Flow cytometry. IFNγ- or BSA-treated OVCAR3 and OVCA420 cells, HM-1-B7H3 KO cells and ID8-B7-H3 KO cells or their respective control cells, and OVCAR3-sh-B7-H3 and OVCA420-sh-B7-H3 or their respective control cells were collected from the culture plates using trypsin (Cat# T4549, Sigma-Aldrich) and incubated for 20 min at 4 °C with the corresponding antibodies listed in Supplementary Table S2. Tumors of HM-1-B7H3 KO cells, ID8-B7-H3 KO cells, or their control cells were minced into thin pieces and dissociated in 100 µg/mL of DNase I (Cat# 11284932001, Roche, Basel, Switzerland) and 1 mg/mL of collagenase IV(Cat# CLS4, Worthington, Columbus, OH, USA) in RPMI 1640 medium. The tissues were incubated for 1 h at 37 °C with agitation. Single cells were prepared through a 70 µm nylon mesh strainer (Cat# 542070, Greiner Bio-One). FcR blocking for leukocytes was performed with FcR Blocking Reagent (for mouse samples: Cat# 130-092-575, RRID: AB_2892833; for human samples: Cat# 130-059-901, RRID:AB_2892112, Miltenyi Biotec, Bergisch Galdbach, Germany) for 10 min at 4 °C. Tumor-infiltrating immune cells were stained to detect $CD4^+$ T cells ($CD3^+CD4^+CD8^-$), CD8+ T cells ($CD3^+CD4^-CD8^+$), M2 macrophages ($CD45^+F4/80^+CD206^+$), M1 macrophages ($CD45^+F4/80^+CD80^+$ or $CD45^+F4/80^+CD86^+$), and dendritic cells (DCs, $CD45^+CD11c^+$MHC class 2^+) (33) with the antibodies listed in Supplementary Table S2 for 30 min at 4 °C. Gating strategies for each cell subset are shown in Supplementary Figure S2. For IFNγ staining, single cells from the tumor were incubated for 4 h with eBioscience Cell Stimulation Cocktail (Cat# 00-4970-93, Invitrogen, Carlsbad, CA, USA) and for 2 h with Brefeldin A (Cat# B7651, Sigma-Aldrich). BD Cytofix/Cytoperm Fixation/Permeabilization Kit (Cat# 554714, BD Biosciences) was used for intracellular staining. Non-viable cells were stained with 7-amino-actinomycin D (AAD) solution (Cat# 51-68981E, BD Biosciences) or DAPI solution (Cat# D1306, Life Technologies, Carlsbad, CA, USA) and gated out. MACS Quant Analyzer 10 (Miltenyi Biotec) and FlowJo (RRID: SCR_008520, FlowJo LLC, Ashland, OR, USA) were used for analysis.

T-cell proliferation assay. For T-cell selection, single cells from the spleens of B6C3F1 mice bearing tumors of HM-1-B7H3 KO cells or control cells harvested at day 12 were collected by mechanical dissociation. Red blood cells were lysed with Pharm Lyse buffer (Cat# 555899, BD Bioscience). The purity of $CD3^+$ cells was >90% as confirmed by flow cytometry. T cells were negatively selected using mouse Pan-T-cell isolation kit II (Cat# 130-095-130, Miltenyi Biotec), an LS column (Cat# 130-042-401, Miltenyi Biotec), and QuadroMACS Separator (Cat# 130-090-976, Miltenyi Biotec).

T cells were labeled using 10 mmol/L carboxyfluorescein succinimidyl ester (CFSE; Cat#

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600121, Cayman Chemical). T cells (1×10^5) were co-cultured with F4/80$^+$ macrophages (obtained as described in the "Macrophage and monocyte selection" section above) at 2:1, 4:1, and 8:1 ratios with RPMI 1640 medium supplemented with 10% FBS, 2 mM of L-glutamine (Cat# 25030-081, Gibco), and 50 µM of 2-mercaptoethanol for 72 h in an atmosphere of 5% CO_2 at 37 °C. During incubation, T cells were activated with Dynabeads Mouse T-activator CD3/28 (1:100 dilution, Cat# 11453D, Thermo Fisher Scientific). Activated T cells without macrophages were used as a positive control. After 72 hours, T-cell proliferation was examined using flow cytometry according to the decrease of the CFSE fluorescence intensity. MACS Quant Analyzer 10 (Miltenyi Biotec) and FlowJo (FlowJo) were used for analysis. The culture supernatants were collected and used for ELISA.

ELISAs. Mouse IFNγ levels in the cell culture supernatant of T cells co-cultured with macrophages were measured using Mouse IFN-γ Quantikine ELISA Kit (Cat# MIF00, R&D Systems). HM-1-B7H3 KO cells, ID8-B7-H3 KO cells or their control cells, and OVCAR3-sh-B7-H3 and OVCA420-sh-B7-H3 or their control cells were seeded at a density of 1×10^5 with 500 µL of the corresponding medium (see "Cell culture and transfection" section) in 24-well plates (Cat# 662160, Greiner Bio-One). Cell culture supernatants were collected after 24 h with a 0.45 µm Millex filter (Cat# SLHVR33RS, Merck Millipore, Burlington, MA, USA) and used for ELISA. These supernatants were also used for migration and monocyte differentiation assays (described in detail below) as the tumor cell-conditioned medium (TCM). Human and mouse tumor tissues were treated in a bead homogenizer, and sonicated in 1× RIPA buffer (Cat# 89900, Thermo Fisher Scientific) containing a protease inhibitor cocktail (Cat# 03969-21, Nacalai Tesque) and a phosphatase inhibitor cocktail (Cat# 07575-51, Nacalai Tesque), followed by centrifugation at 16,000 ×*g* for 5 min at 4 °C. Human tumor lysates were used for B7-H3 and CCL2 detection at 75 µg and 200 µg as protein amount, respectively, and 20 µg as protein amount of mouse tumor lysates were used for CCL2 detection. Mouse CCL2 protein levels in culture supernatants and tumor lysates were measured using the Mouse CCL2/JE/MCP1 DuoSet kit (Cat# DY479-05, R&D Systems). Human CCL2 protein levels in culture supernatants, tumor lysates, and serum samples were measured using Human CCL2/MCP-1 Quantikine ELISA Kit (Cat# DCP00, R&D Systems). Human B7-H3 protein levels in tumor lysates were measured using Human B7-H3 Quantikine ELISA Kit (Cat# DB7H30, R&D Systems). The absorbance readings at 570 nm were subtracted from the readings taken at 450 nm using an iMark microplate reader (Bio-Rad) to establish standard curves for calculation of the concentration. Microsoft Excel was used for analysis.

Immunoblotting. Nuclear proteins were collected from HM-1-B7H3 KO cells, ID8-B7-H3 KO cells or their control cells, and OVCAR3-sh-B7-H3 and OVCA420-sh-B7-H3 or their control cells using NE-PER nuclear and cytoplasmic extraction reagents (Cat# 78833, Thermo Fisher Scientific). Protein (20 µg) of HM-1-B7H3 KO cells or their control cells, and 10 µg protein of the other cells were loaded onto 8% acrylamide gels. Proteins were subsequently separated using SDS-PAGE and

transferred onto PVDF membranes (Cat# 1620177, Bio-Rad). After blocking for 1 h with Blocking One-P (Cat# 05999-84, Nacalai Tesque), the membranes were then immunoblotted with the antibodies listed in Supplementary Table S2 at the indicated dilutions. The membranes were incubated with the primary antibody overnight at 4 °C and with the secondary antibody for 1 h at room temperature. Bands were visualized using ChemiDoc XRS+ Systems (Bio-Rad). Signals were quantified using Image Lab 2.0 (RRID: SCR_014210, Bio-Rad).

RNA-sequencing. Total RNA from HM-1-B7H3 KO cells, ID8-B7-H3 KO cells or their control cells, or intradermal tumors of HM-1-B7H3 KO cells or their control cells at day 10 of tumor growth were extracted as described in the 'RNA extraction and reverse transcription (RT) qPCR' section and used for RNA-sequencing. Sequencing was performed on an Illumina NovaSeq 6000 platform (RRID: SCR_016387, Illumina) to generate 100 bp paired-end reads. Raw reads were trimmed using Trim Galore (RRID: SCR_011847), and the resulting reads were aligned to the mouse reference genome GRCm38/mm10 using STAR (RRID: SCR_004463). Differential expression analysis was performed using DESeq2 (RRID: SCR_015687) between control (n=3) and B7-H3 KO (n=3) groups of the ID8 and HM-1 cell lines, and HM-1 intradermal tumors (processed as indicated in above sections). After excluding the genes showing low expression and low fold change between groups ("baseMean" > 1000; |log2FoldChange| > 2; $padj < 0.01$), the remaining genes were considered as DEGs and listed in Supplementary Tables S3–S5. RNA-sequence data have been deposited in the Gene Expression Omnibus database, under accession number GSE174137.

***In vitro* M2 macrophage generation.** Mouse and human monocytes (obtained as described in the "Macrophage and monocyte selection" section) were cultured in 6-well plates (Cat# 657160, Greiner Bio-One) at 3×10^6 cells/well and 6×10^5 cells/well, respectively, with RPMI 1640 medium supplemented with 40 ng/mL of M-CSF (mouse: Cat# 576402, human: Cat# 574802, BioLegend, San Diego, CA, USA) and 40 ng/mL of IL6 (mouse: Cat# 216-16, PeproTech, Cranbury, NJ, USA, human: Cat# 570802, BioLegend) for 6–7 days in an atmosphere of 5% CO_2 at 37 °C and used for migration assays. After incubation, >70% of the cultured mouse cells were F4/80-positive and CD206-positive, and >85 % of the cultured human cells were CD206-positive as confirmed by flow cytometry.

Migration assays. Monocytes or the generated M2 macrophages (mouse: 1×10^5 cells, human: 5×10^4 cells, isolated as described in the "Macrophage and monocyte selection" and "*In vitro* M2 macrophage generation" sections, pretreated with 2 µM RS504393 (described in "Tumor models") or DMSO were plated in the upper compartment of 8 µm cell culture insert (Cat# 353097, Corning, Corning, NY, USA), and 500 µL of TCM of HM-1-B7H3 KO cells, ID8-B7-H3 KO cells or their control cells, and OVCA420-sh-B7-H3 or their control cells (generated as described in the "ELISAs" section) was added to the lower compartment. Plates were incubated for 1 hour in an atmosphere of 5% CO_2 at 37 °C, and migrated cells were counted using CountBright Absolute Counting beads (Cat# 36950, Thermo

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Fisher Scientific).

Monocyte differentiation assay. Mouse bone marrow monocytes (isolated and cultured as described in the "Macrophage and monocyte selection" section) were plated in the upper compartment of 0.4 µm cell culture inserts (Cat# 353095, Corning) at a density of at 1×10^5, and 500 µL of TCM of HM-1-B7H3 KO cells, ID8-B7-H3 KO cells or their control cells (generated as described in the "ELISAs" section) supplemented with 20 µg/mL anti-mouse CCL2 (Cat# AB-479-NA, RRID:AB_354366; R&D Systems, Minneapolis, MN, USA) or goat IgG control (Cat# AB-108-C, RRID:AB_354267; R&D Systems) was added to the lower compartment. Plates were incubated for 72 hours in an atmosphere of 5% CO_2 at 37 °C, and F4/80+CD206+ cells were then evaluated using flow cytometry, as described above.

Statistics. At least three *in vitro* experiments and at least two *in vivo* experiments were independently performed. Data from one representative experiment are presented. For mouse experiments, cages of mice were randomly allocated to the experimental groups. Sample sizes were chosen to assure reproducibility of the experiments in accordance with the replacement, reduction, and refinement principles of the animal ethics regulation. Grouped data are shown as the average ± standard error of the mean (SEM) in all figures except non-normally distributed data for clinical samples, which shows the median with 95 % confidence intervals. The investigator was not blinded to the group allocation. *A priori* power analysis was not performed for human samples. Survival curves were constructed using the Kaplan-Meier method. Prognostic factors for progression-free survival (PFS) and overall survival (OS) of HGSOC patients were assessed by univariate and multivariate analysis using the Cox proportional hazards regression model. Statistical analysis was performed using R software and GraphPad Prism 7 (RRID: SCR_002798, GraphPad Software, San Diego, CA, USA), and the appropriate tests are indicated in the figure legends. Significance was set as $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$.

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Results

B7-H3 expression is upregulated in the non-immunoreactive TME of HGSOC

We first identified the genes acting antagonistic to the immunoreactive TME in HGSOC based on our RNA expression array data. *CD276* (B7-H3) was the most upregulated gene in the non-immunoreactive subtype (Figure 1a). Analysis of TCGA-OV revealed that B7-H3 expression was higher in the proliferative and mesenchymal subtypes than in the immunoreactive subtypes (P=0.004 and P<0.001, Figure 1b). Among the T-cell suppressive immune checkpoint molecules of the B7 family, only B7-H3 expression was lower in the immunoreactive subtype and higher in the non-immunoreactive subtype (both P<0.001, Figure 1c).

A clear positive correlation was observed between the IFNγ signature, which is a major characteristic of the immunoreactive tumors and can be used as an index for predicting the therapeutic efficacy of immune checkpoint inhibitor (ICI) treatment (27), and the other B7 family molecules. By contrast, the IFNγ signature negatively correlated with B7-H3 expression (P=0.020, Figure 1d). Based on the response to IFNγ, we performed qPCR or flow cytometry and observed significantly increased expression of PD-L1 and PD-L2 and unchanged or slightly decreased expression of B7-H3 in HGSOC cell lines (Supplementary Figure S3a-b). Moreover, PD-L1 expression negatively correlated with B7-H3 expression (Supplementary Figure S3c). These results indicate that B7-H3 expression is associated with the non-immunoreactive TME and that B7-H3 is involved in immunosuppression, albeit via a different mechanism than that of PD-L1.

B7-H3 mediates murine ovarian cancer growth via immune system regulation

To investigate the function of B7-H3 in tumor cells, we generated three B7-H3 knockout (KO) HM-1 and ID8 mouse ovarian cancer cell lines using CRISPR-Cas9. Both cell lines express B7-H3 and are resistant to PD-1/PD-L1 inhibition therapy (34,35). We confirmed B7-H3 knockout by flow cytometry and immunostaining (Figure 2a-b, Supplementary Figure S4a). B7-H3 KO did not affect cell proliferation *in vitro* (Supplementary Figure S4b). In immunocompetent mice, B7-H3 KO delayed the growth of HM-1 intradermal and intraperitoneal tumors, as well as ID8 intraperitoneal tumors (Figure 2c-d, Supplementary Figure S4c). B7-H3 suppression significantly prolonged the survival of mice with ID8 tumors, a trend that was also observed with HM-1 tumors (Supplementary Figure S5a). By contrast, in immunodeficient mice, tumor growth and survival were not affected by B7-H3 expression in either HM-1 or ID8 cells (Figure 2e-f, Supplementary Figure S5b). These data suggest that B7-H3 is involved in tumor development via immune-related mechanisms. Next, to verify the role of B7-H3 in stromal cells, we injected ID8 cells into *CD276* knockout (B7-H3 KO) mice and observed that B7-H3 expression did not significantly affect tumor development (Supplementary Figure S6). Overall, these results indicate that B7-H3 expression in tumor cells is important for tumor progression.

B7-H3 suppression reduces M2 macrophages and increases T cell-produced IFNγ in the TME

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First, we confirmed a direct T-cell suppressive effect of tumor cell B7-H3 *in vitro* (Supplementary Figure S7a-c), which has been reported in several studies (19, 20). Next, we evaluated tumor-infiltrating immune cells. Although there was no difference in the number of $CD8^+$ T cells, $CD206^+$ M2 macrophages decreased in HM-1 B7-H3 KO tumors (Figure 3a, Supplementary Figure S8a). Flow cytometry showed that $F4/80^+CD206^+$ M2 macrophages decreased in HM-1 B7-H3 KO tumors, whereas M1 macrophages, CD8+ T cells, CD4+ T cells, and DCs were unchanged (Figure 3b). ID8 tumors similarly showed a decrease in M2 macrophages but no change in M1 macrophages (Supplementary Figure S8b). These results suggest that B7-H3 KO tumors are not only associated with a decreased number of M2 macrophages within tumor tissues, but also alters macrophage polarization (i.e., a decrease in the immunosuppressive fraction).

In macrophages from the B7-H3 KO tumors, we observed decreased expression of the M2 markers *Arg1* and *Il10* but unchanged expression of the M1 marker *Ifng* (Figure 3c). T-cell proliferation assays with CD3/CD28-activated T cells co-cultured with tumor-derived macrophages showed that macrophages from the B7-H3 KO tumors had reduced ability to inhibit T-cell proliferation compared with those from control tumors (Figure 3d), and IFNγ production in co-culture supernatants was increased (Figure 3e). We also observed an increased number of $IFN\gamma^+CD8^+$ T cells in B7-H3 KO tumors (Figure 3f). These results suggest that B7-H3 suppression in tumor cells reduces immunosuppression in the TME by not only attenuating direct T cell inhibition, but also reducing the number of M2 macrophages, which may contribute to the increased IFNγ production by $CD8^+$ T cells and enhanced anti-tumor immunity.

B7-H3 suppression downregulates CCL2 production in tumor cells, partly via the STAT3 pathway

Next, we elucidated the mechanism by which B7-H3 suppression in tumor cells reduced M2 macrophage infiltration. Using RNA-sequencing, we found 90, 131, and 67 DEGs in the HM-1 and ID8 cell lines and HM-1 intradermal tumors relative to controls, respectively (Supplementary Tables S3–S5). We identified *Ccl2* as a common downregulated gene in the KO groups (Figure 4a). We also confirmed that changes in expression of several chemokines related to T-cell trafficking were not consistent between the cell lines and the B7-H3 KO tumors (Supplementary Figure S9). A reduction in CCL2 protein in the B7-H3 KO groups was confirmed in both culture supernatants and tumor lysates from HM-1 and ID8 cells (Figure 4b-c). Then, we investigated whether B7-H3 suppression also altered CCL2 in human HGSOC cell lines (Supplementary Figure S10) and confirmed a decrease in CCL2 in the culture supernatants of the shB7-H3 cells (Figure 4d).

To investigate the mechanism by which B7-H3 suppression induced CCL2 downregulation, we examined STAT3 (21-23) and NF-κB (36) pathways, which have been reported as downstream pathways of B7-H3. Western blots showed that phosphorylated STAT3 was decreased in the B7-H3-suppressed cells (Figure 4e-f). In contrast, phosphorylated p65, a major factor in the classical NF-κB pathway, was decreased in ID8 and OVCAR3 cell lines; thus, consistent results were not observed among cell lines (Supplementary Figure S11a-b). Next, we examined whether the decrease

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in phosphorylated STAT3 contributed to CCL2 downregulation. Treatment with STAT3 inhibitors C188-9 for mouse cells (29) and Stattic for human cells (30) led to a decrease in CCL2 expression (Figure 4g-h). These results indicate that B7-H3 suppression downregulates the production of CCL2 partly via the STAT3 pathway.

The CCL2–CCR2–M2 macrophage axis is involved in B7-H3-mediated tumor growth

We confirmed whether the CCL2–CCR2 axis contributed to the effects of B7-H3 suppression on M2 macrophage migration and differentiation both *in vitro* and *in vivo*. Compared with controls, the migration of monocytes and M2 macrophages was reduced in the B7-H3 KO TCM, and pretreatment of target cells with a CCR2 inhibitor (RS504393) partially eliminated this effect (Figure 5a, Supplementary Figure S12a-b). M2 macrophage polarization was significantly reduced in B7-H3 KO TCM, and this effect was partially abrogated by CCL2 antibodies (Figure 5b, Supplementary Figure S12c). These results indicate that B7-H3 suppression in tumor cells reduces monocyte migration and macrophage differentiation into the M2 phenotype *in vitro*, and that the CCL2–CCR2 axis is responsible for these effects.

In the HM-1 model, CCR2 inhibition suppressed tumor growth in controls but did not affect B7-H3 KO tumors (Figure 5c). Flow cytometry showed that CCR2 inhibition reduced the number of M2 macrophages and increased the number of IFNγ$^+$CD8$^+$ T cells in control tumors but had no effect on B7-H3 KO tumors (Figure 5d). The number of total CD8$^+$ T cells was not changed by the treatment in either control or B7-H3 KO tumors. These results indicate that CCL2 downregulation induced by B7-H3 suppression contributes to the reduction of M2 macrophages and the increase in IFNγ$^+$CD8$^+$ T cells *in vivo*, and that the CCL2–CCR2 axis and M2 macrophages partly contribute to B7-H3-mediated tumor progression.

Poor prognosis of B7-H3-high HGSOC with an M2 macrophage-rich, IFNγ$^+$CD8$^+$ T cells-sparse TME

Lastly, we investigated the relationships among B7-H3 and CCL2, M2 macrophages, CD8$^+$ T cells, and IFNγ$^+$CD8$^+$ T cells in HGSOC clinical samples. B7-H3 and CCL2 protein in HGSOC primary tumors positively correlated (P=0.040, Figure 6a). Serum CCL2 was elevated in HGSOC patients compared with those in healthy donors (P<0.001, Supplementary Figure S13a); however, there was no significant correlation between serum CCL2 and tumor B7-H3 (P=0.089, Supplementary Figure S13b).

FFPE specimens from the primary tumor site of HGSOC cases were evaluated for tumor cell B7-H3 and CD206$^+$ cells by immunohistochemistry and CD8$^+$ and IFNγ$^+$CD8$^+$ cells by immunofluorescence (Figure 6b). A positive correlation between B7-H3 expression and the number of CD206$^+$ M2 macrophages was observed (P=0.018, Figure 6c). The number of CD8$^+$ T cells and IFNγ$^+$CD8$^+$ cells positively correlated (P < 0.001, Figure 6d). There was no difference in the number of CD8+ T cells in the B7-H3-high and B7-H3-low groups; however, the number of IFNγ$^+$CD8$^+$ cells was significantly lower in B7-H3-high versus B7-H3-low tumors (P=0.047, Figure 6e). Patient

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characteristics are shown in Supplementary Table S6. Higher B7-H3 expression was a potential poor prognostic factor for both PFS and OS in the univariate analysis, although it was not identified as an independent prognostic factor in the multivariate analysis (Figure 6f, Supplementary Table S7). These results show the immunosuppressive properties of B7-H3 in relation to M2 macrophages and $IFN\gamma^{+}CD8^{+}$ T cells in HGSOC patients.

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Discussion

In this study, we investigated B7-H3 upregulation in the non-immunoreactive subtype of HGSOC and hypothesized that B7-H3 exerted immunosuppressive effects via a mechanism distinct from that of other B7 family members, such as PD-L1, overexpressed in immunoreactive tumors. We showed that B7-H3, which has been mainly reported to directly modulate T-cell function (15,19,20), similar to other immune checkpoint molecules, is involved in indirect T-cell suppression via the CCL2–CCR2–M2 macrophage axis.

Several studies have investigated the relationship between B7-H3 and M2 macrophages. B7-H3 promotes the *in vitro* differentiation of macrophages into the M2 phenotype in co-cultures of HepG2 hepatoma cells and THP-1 macrophages (37) and cultures of PBMC-derived monocytes in colon cancer cell culture supernatants (38); however, the underlying mechanism has not yet been clarified. In an immunocompetent transgenic mouse model of head and neck cancer, anti-B7-H3 treatment reduces the number of immature myeloid cells, including macrophages, in the tumor and activates anti-tumor immunity (32). However, whether the decreased number of macrophages was an effect of anti-B7-H3 on tumor cells or on macrophages expressing B7-H3 was not clarified. To the best of our knowledge, this is the first study to demonstrate that CCL2 downregulation following B7-H3 suppression in tumor cells contributes to the reduction in M2 macrophages and the subsequent improvement in anti-tumor responses both *in vitro* and *in vivo*. Using clinical tumor samples, we also clarified the relationship of B7-H3 with CCL2 and M2 macrophages.

The immunologic and non-immunologic roles of B7-H3 have been previously reported (39). In this study, the difference in tumor growth between the control and B7-H3 KO tumors observed in immunocompetent mice was not observed in immunodeficient mice, suggesting that immunologic effects have a greater influence on tumor growth than non-immunologic effects. B7-H3 has been reported to exert direct immunomodulatory effects mainly on T cells (15,20) and NK cells (40). However, the results of our tumor inoculation experiments in immunodeficient nude mice with normal NK cell function suggest that the effect of B7-H3 on NK cells is not significant.

We confirmed both the direct and indirect immunosuppressive effects of B7-H3 on T cells. The increased number of IFNγ$^+$CD8$^+$ T cells in B7-H3 KO tumors and B7-H3-low HGSOC cases, both of which were not accompanied by changes in the number of CD8$^+$ T cells, suggests that B7-H3 is not involved in the recruitment of T cells; instead, it attenuates T-cell activity at the tumor site either directly or indirectly through M2 macrophages. Several studies have reported T cell-suppressive functions for M2 macrophages (41,42). Although it remains unclear which between the direct and indirect effects contribute more to T-cell suppression, the fact that CCR2 blocking *in vivo* decreased the number of M2 macrophages and increased that of IFNγ$^+$CD8$^+$ T cells and inhibited tumor growth indicates that the M2 macrophage-mediated indirect effects significantly affect tumor growth.

In tumor tissues, B7-H3 is expressed not only in tumor cells but also in other stromal cells (16,20). Although B7-H3 expression in antigen-presenting cells has been reported to regulate T-cell function (15,19), its role in other normal cells in tumor tissues remains to be elucidated. Our results

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showed that B7-H3 expression in stromal cells does not significantly affect tumor growth. This result is similar to that reported by a study on MC38 mouse colon cancer cells, in which no difference in tumor growth was observed in the presence or absence of stromal B7-H3 (16). By contrast, another study on E.G7-OVA mouse lymphoma cells showed delayed tumor growth in the absence of stromal B7-H3 (43). These contrasting results suggest that the effect of stromal B7-H3 expression on tumor growth may vary depending on tumor type.

To date, the B7-H3 receptor remains unknown, and it is unclear how B7-H3 acts among cells or within B7-H3-expressing cells. The spontaneous dimerization of B7-H3 *in vitro* suggests that B7-H3 among neighboring cells may transmit signals to each other (44). IL20RA has been identified as a binding partner of B7-H3 (45). The involvement of B7-H3 in the activation of intracellular signals, such as STAT3 in tumor cells, has also been revealed (21-23). We found decreased nuclear phosphorylated STAT3 following B7-H3 suppression and CCL2 downregulation by STAT3 inhibitors in tumor cells. These findings indicate that B7-H3 partly acts via STAT3–CCL2 intracellular signaling in the cancer cells expressing B7-H3 or among neighboring cancer cells. Further elucidation of the molecular mechanism underlying B7-H3-mediated signaling, including the identification of its receptors, is thus required.

In TCGA-OV, we found that B7-H3 expression negatively correlated with an IFNγ signature and PD-L1 expression. Non-immunoreactive, B7-H3-high, and PD-L1-low HGSOC tumors are not expected to benefit from ICI treatment, and therefore, require a different treatment strategy than that used for immunoreactive tumors. M2 macrophages promote tumor progression in ovarian cancer (46,47), and the abundance of M2 macrophages is an important distinctive characteristic of cancer subtypes with poor prognosis (48,49). We found a positive correlation between B7-H3 expression and the number of M2 macrophages in HGSOC cases. CCR2 inhibition had no therapeutic effect on B7-H3-suppressed tumors with sparse M2 macrophages, whereas in B7-H3-expressing tumors with abundant M2 macrophages, it exerted a mild therapeutic effect, suggesting that M2 macrophages are potential therapeutic targets in patients with B7-H3-high, M2 macrophage-rich tumors. Ongoing clinical trials targeting macrophages are expected to be effective in such tumor subtypes (50). Our findings also confirm those of a previous study which stated that anti-B7-H3 treatment reduces immature myeloid cells, including macrophages (32), suggesting that B7-H3 suppression could be effective in reducing the number of M2 macrophages. In addition to its previously reported clinical applications, including as a direct inhibitor of T cells and a pan-tumor antigen with high selectivity, B7-H3 may be a promising therapeutic target in cancers with a M2 macrophage-mediated immunosuppressive TME.

There are several limitations to this study. We did not identify the upstream regulators of B7-H3 expression, and hence the fundamental causes that shape the differential immunological TME. B7-H3 upregulation in tumor cells was not observed by the addition of any cytokines, including IFNγ in our *in vitro* experiments. The relationship between B7-H3 expression and genetic factors, such as tumor mutation burden (TMB), remains unknown. Verhaak et al. report that the

differentiated type has lower TMB and aneuploidy than other subtypes, whereas the proliferative type has fewer germline *BRCA* 1/2 mutations than other subtypes (10). Desbois et al. found no differences in TMB or *BRCA* mutations, homologous recombination deficiency (HRD) status, or microsatellite instability (MSI) status among the molecular subtypes of HGSOC (14). Therefore, it remains unclear when and how B7-H3 expression and the immunoreactive TME are determined. The identification of the definitive drivers is warranted in future studies.

In conclusion, we revealed the involvement of B7-H3 in the CCL2–CCR2–M2 macrophage axis and in the immunosuppressive TME in HGSOC. Our findings further the understanding of the immunological TME and demonstrate the promising potential of B7-H3 as a therapeutic target for the B7-H3-high, M2 macrophage-rich, unfavorable phenotype of HGSOC.

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Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

Figure legends

Figure 1. *CD276* (B7-H3) expression is upregulated in the non-immunoreactive tumor microenvironment of HGSOC. a, Microarray analysis of HGSOC clinical samples from Kyoto University (n=30). The immunoreactive (n=8) and non-immunoreactive (n=22) subtypes were compared using the Samroc method. The magenta dot represents *CD276* (B7-H3). **b,** *CD276* expression among the molecular subtypes of 263 HGSOC samples from TCGA-OV. Data are presented as the mean±SEM; $^{**}P<0.01$, $^{***}P<0.001$, N.S.: not significant by one-way ANOVA with Tukey's multiple comparison test. **c,** Gene expression comparison of T cell-suppressive B7 family molecules in the immunoreactive and non-immunoreactive subtypes of TCGA-OV. Data are presented as the mean±SEM after Z-score normalization. $^{***}P<0.001$ by one-way ANOVA. **d,** Pearson's correlation analysis of the IFNγ signature activity scores and expression of T cell-suppressive B7 family molecules in TCGA-OV. Values after Z-score normalization are plotted. $^{*}P<0.05$, $^{***}P<0.001$. (Green and blue dots represent the immunoreactive and non-immunoreactive subtypes, respectively, in **b-d**.

Figure 2. B7-H3 contributes to tumor progression via an immune-related mechanism in murine ovarian cancer models. a, Flow cytometry of B7-H3 knockout (KO) HM-1 and ID8 and their respective controls. Blue, pink, and gray histograms represent B7-H3 KO cells, B7-H3 control cells, and the isotype control, respectively. **b,** Representative B7-H3 IHC (top) and immunofluorescence (bottom) images of controls and HM-1 B7-H3 KO tumors for the indicated markers. αSMA-positive fibroblasts represented by black and white arrowheads. Scale bar, 50 µm. Positive cells were stained brown in IHC, and red (αSMA), green (B7-H3), and blue (DAPI) in immunofluorescence images. **c,** Intradermal tumor volume (left, n=6) and omental tumor weight at day 10 (right, n=6) of immunocompetent mice injected with HM-1 B7-H3 KO or control cells. **d,** Changes in body weight (top, n=5–6) and omental tumor weight at day 52 (bottom, n=6) of immunocompetent mice intraperitoneally injected with ID8 B7-H3 KO or control cells. **e,** Intradermal tumor volume (left, n=5) and omental tumor weight at day 10 (right, n=6) of immunodeficient mice injected with HM-1 B7-H3 KO or control cells. **f,** Changes in body weight (top, n=6) and omental tumor weight at day 52 (bottom, n=6) of immunodeficient mice intraperitoneally injected with ID8 B7-H3 KO or control cells. **c-f,** Data are presented as the mean±SEM; $^{***}P< 0.001$, N.S.: not significant, unpaired t-test.

Figure 3. B7-H3 suppression in tumor cells decreases the number of intratumoral M2 macrophage and increases IFNγ production of CD8^{+} T cells. a, IHC of CD206 in the B7-H3 KO intradermal HM-1 tumors and the controls (n=3). CD206^{+} cells were stained brown. Scale bar, 50 µm. **b,** Flow cytometry of mice from **a**. Density plots (upper left) show the M2 macrophages at day 12. Each dot represents live CD45^{+} cells, and the boxed area represents F4/80^{+}CD206^{+} M2 macrophages. The percentage of positive cells relative to the total cells is plotted (n=6). **c,** Quantitative PCR for the indicated genes in F4/80^{+} macrophages (Mφ) isolated from the HM-1 intradermal tumors (n=5). **d,** T-cell proliferation in

presence of HM-1 tumor-derived macrophages. Upper histograms show the percentage of proliferating T cells co-cultured (4:1) with macrophages from control or B7-H3 KO tumors. Lower bar graph shows the percentage of proliferating T cells co-cultured with various ratios of macrophages (n=5). **e,** IFNγ levels in the supernatants of co-cultures in **d** (n=5). **f,** Flow cytometry of IFNγ$^+$CD8$^+$ T cells from HM-1 tumors. Each dot represents live CD45$^+$CD3$^+$ cells, and the boxed area represents IFNγ$^+$CD8$^+$ T cells The percentage of IFNγ$^+$CD8$^+$ T cells relative to total CD8$^+$ T cells is plotted (n=6). **a-f,** Data are presented as the mean±SEM; *P<0.05, **P<0.01, ***P<0.001, N.S.: not significant, unpaired t-test.

Figure 4. B7-H3 suppression downregulates CCL2 production, in part, via the STAT3 pathway in ovarian cancer cells. a, Differential gene expression based on RNA-sequencing between controls (n=3) and B7-H3 KO (n=3) groups in HM-1 and ID8 cells and HM-1 intradermal tumors. **b, c,** CCL2 protein in the (**b**) culture supernatants and (**c**) tumors derived from HM-1 B7-H3 KO (top), ID8 B7-H3 KO (bottom), and their respective controls (n=5).
d, CCL2 protein in the culture supernatants of the OVCAR3 shB7-H3 (top), OVCA420 shB7-H3 (bottom), and their respective controls (n=5). **e, f,** Representative Western blot of nuclear phosphorylated-STAT3 (p-STAT3) in (**e**) HM-1 and ID8 and (**f**) OVCAR3 and OVCA420 cells. Lower bar graphs show the signal intensity of p-STAT3 relative to the TBP control in HM-1 and ID8 (both n=10) and OVCAR3 and OVCA420 cells (both n=7). **g, h,** Quantitative PCR for expression of (**g**) *Ccl2* in HM-1 and ID8 cells and (**h**) *CCL2* in OVCAR3 and OVCA420 cells treated with 1.25 and 5 μM of the STAT3 inhibitors (**g**) C188-9 and (**h**) Stattic, respectively. DMSO was used as the control (n=3). Data are presented as the mean±SEM; *P<0.05, **P<0.01, ***P<0.001, unpaired t-test in **b, c, e, f** and one-way ANOVA with Tukey's multiple comparisons test in **d**, **g**, **h**.

Figure 5. The CCL2–CCR2–M2 macrophage axis contributes to B7-H3-mediated tumor progression. a, Chemotaxis of mouse monocytes (left) and generated M2 macrophages (right) in response to HM-1 control or B7-H3 KO tumor cell-conditioned medium (TCM). Monocytes or M2 macrophages were pretreated with 2 μM RS504393 (CCR2 antagonist) or DMSO before plating. MEM-Alpha was used as the negative control (n=3). **b,** Differentiation of mouse monocytes into M2 macrophages in response to HM-1 control or B7-H3 KO TCM supplemented with 20 μg/mL anti-CCL2 or control IgG. MEM-Alpha was used as the negative control (n=3). **c,** Tumor growth in mice intradermally injected with HM-1 B7-H3 KO cells or control cells and treated with RS504393 (2 mg/kg body weight) or DMSO daily following tumor inoculation. **d,** Flow cytometric analysis of the treated tumors in **c** (n=6). Data are presented as the mean ± SEM (*P < 0.05, **P < 0.01, ***P < 0.001, N.S.: not significant, one-way ANOVA with Tukey's multiple comparisons test in **a**, **b**, and unpaired t-test in **c, d**).

Figure 6. B7-H3 expression associates with an M2 macrophage-rich, IFNγ$^+$CD8 T cell-poor TME and a poor prognosis in HGSOC patients. a, Correlation between CCL2 expression and B7-H3 protein in

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primary HGSOC tumors (*n*=28) analyzed by Spearman's correlation analysis. **b,** Representative images of HGSOC samples showing B7-H3 expression and CD206$^+$, CD8$^+$, and IFNγ$^+$CD8$^+$ cells. Cells were stained with hematoxylin and eosin or immunostained with the corresponding antibodies. Scale bar, 100 μm. Positive cells were stained brown in IHC, and red (CD8), green (IFNγ), and blue (DAPI) in immunofluorescence slides. The boxed area represents the zoomed image shown in the upper right corner. **c,** Correlation between the infiltration of CD206$^+$ cells and B7-H3 expression in the corresponding primary tumors of HGSOC (*n*=62) analyzed using Jonckheere-Terpstra test. Data are presented as the median with 95 % confidence intervals. **d,** Correlation between tumor-infiltrating CD8$^+$ and IFNγ$^+$CD8$^+$ T cells (*n*=62) analyzed using Spearman's correlation analysis. Blue and pink dots represent B7-H3-low and -high expression cases, respectively. **e,** Comparisons of the number of tumor-infiltrating CD8$^+$ T cells (left) and IFNγ$^+$CD8$^+$ T cells (right) in B7-H3-low and -high expression groups by immunostaining (*n*=62). Data are presented as the median with 95 % confidence intervals; *P<0.05, N.S.: not significant, Mann-Whitney's U test. **f,** Progression-free survival (left) and overall survival (right) of patients with HGSOC (*n*=62). Patients were classified into a B7-H3-low group (*n*=31) and B7-H3-high group (*n*=31). *P<0.05, log-rank test.

Figure 1

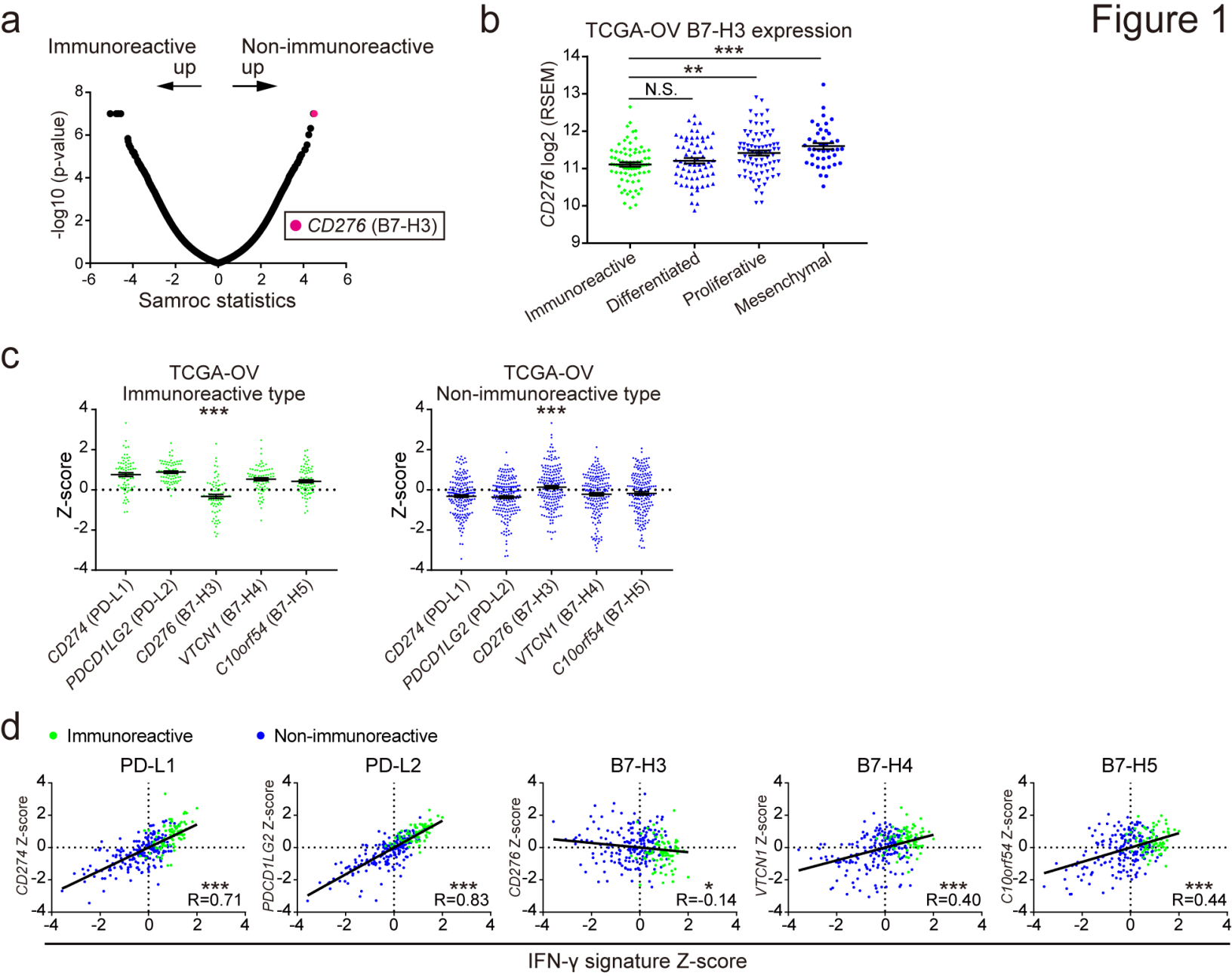
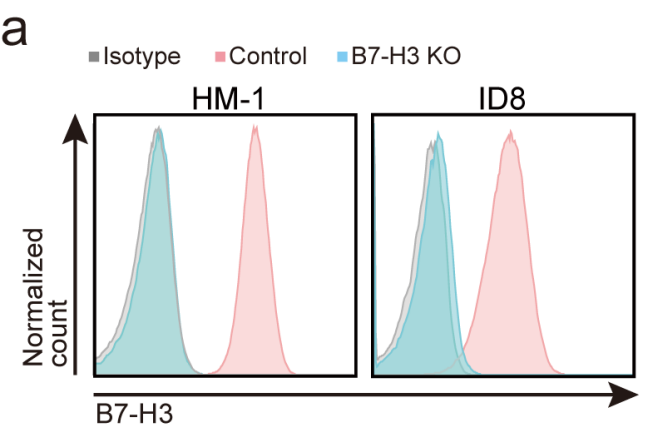
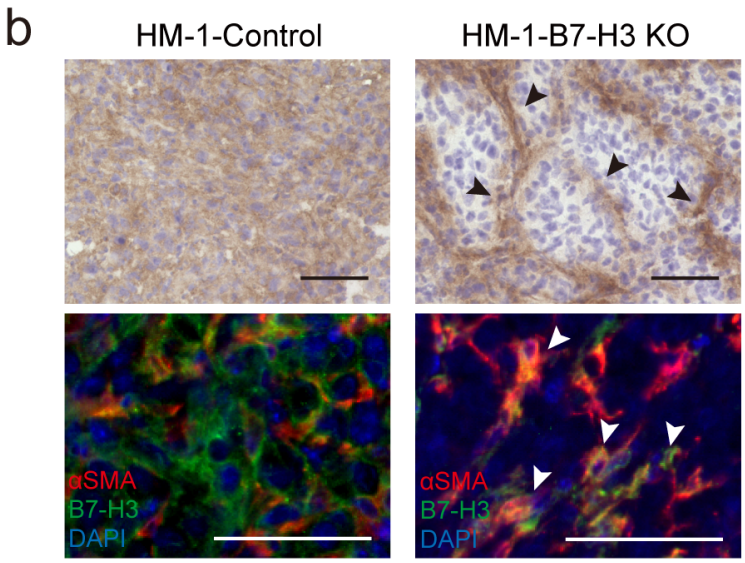
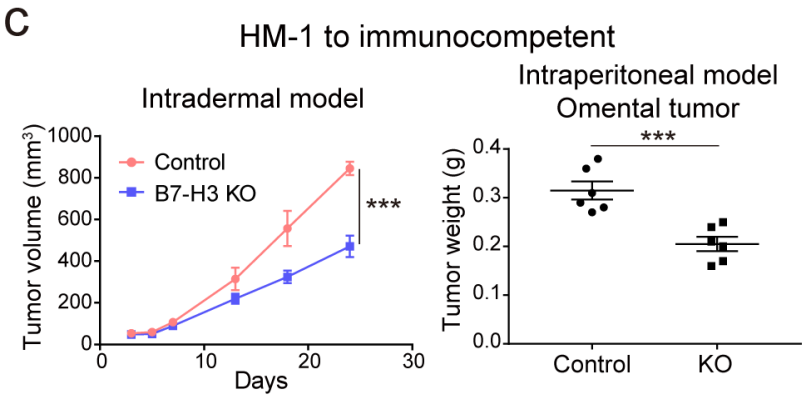
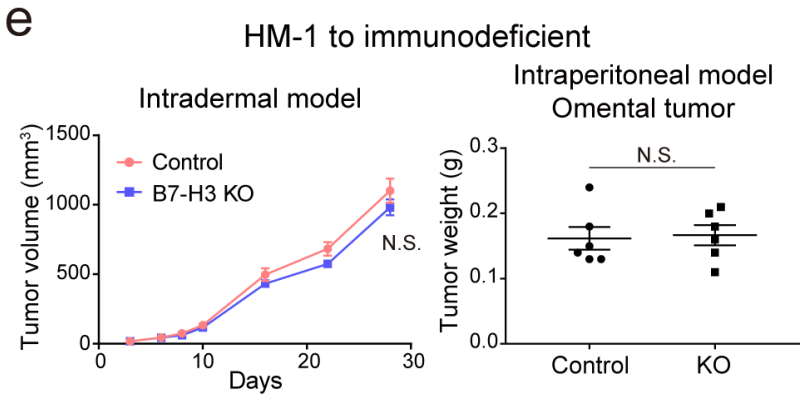


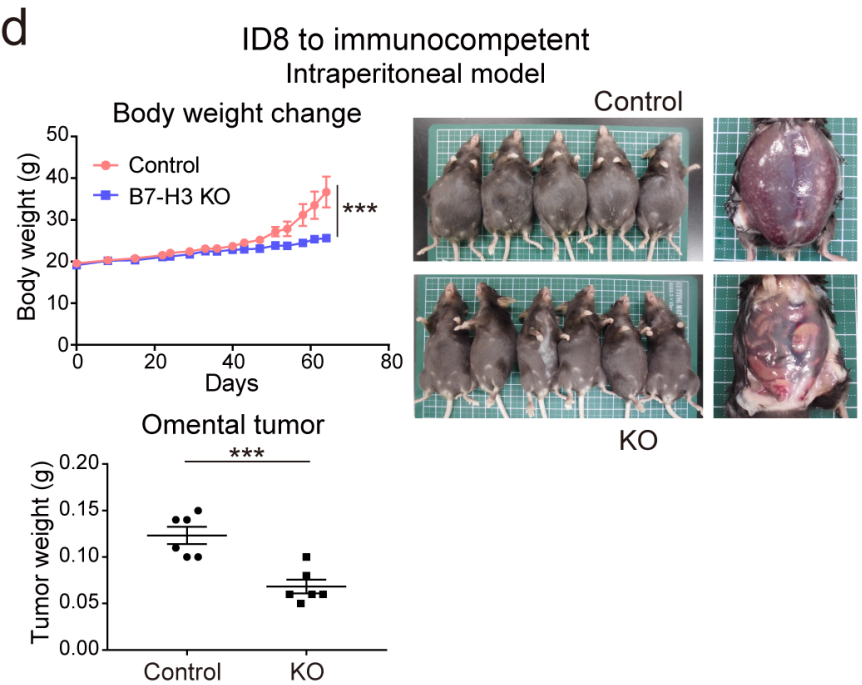
Figure 2

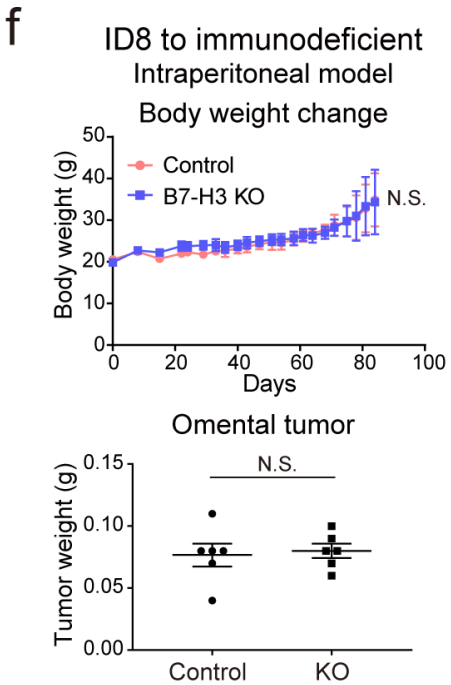












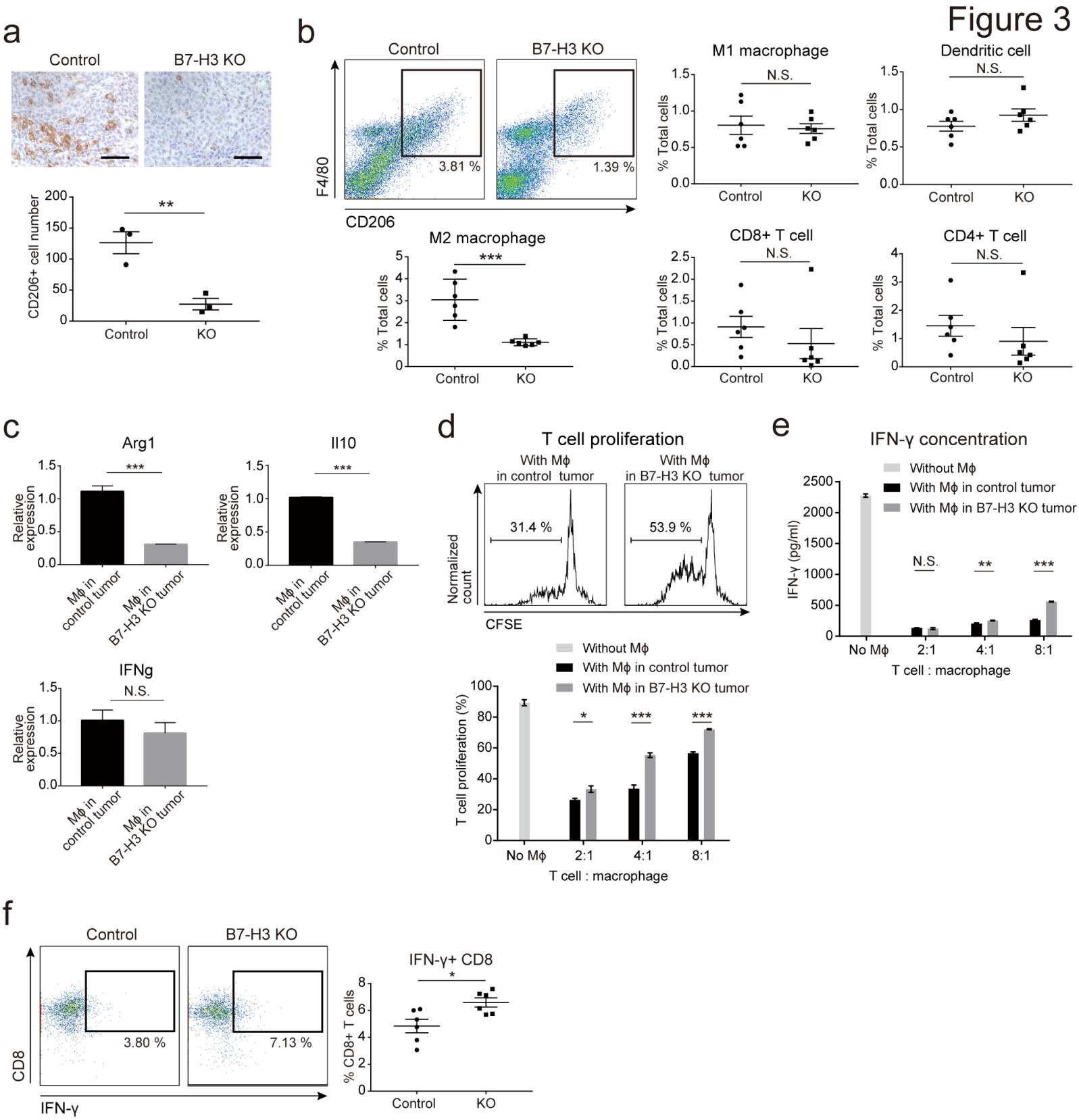


Figure 4

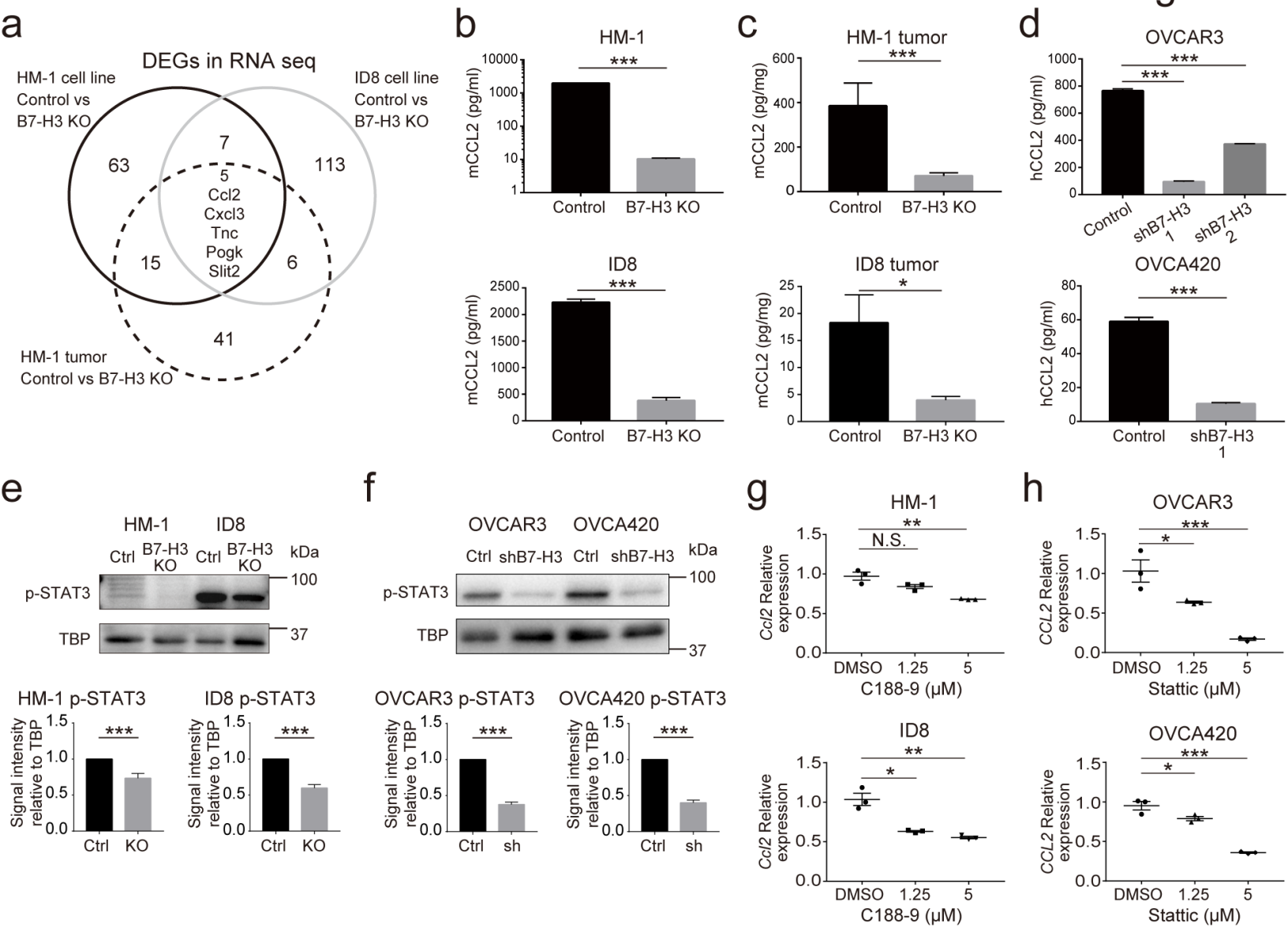
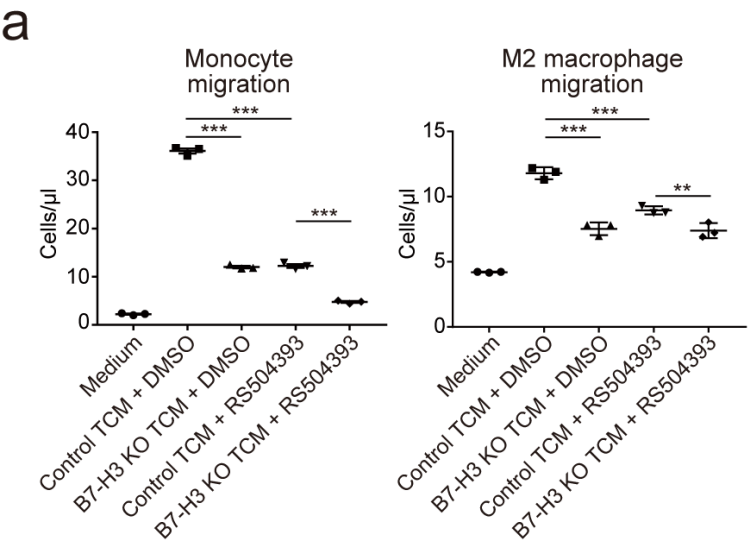
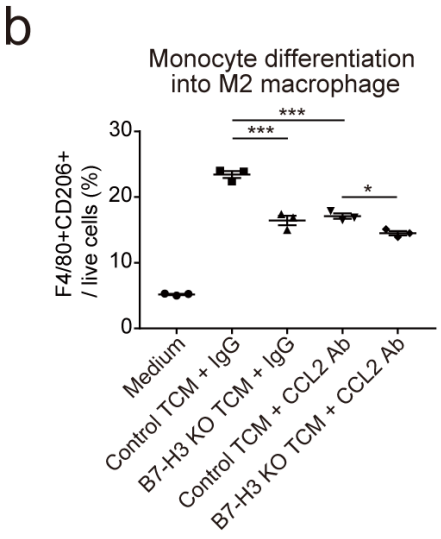
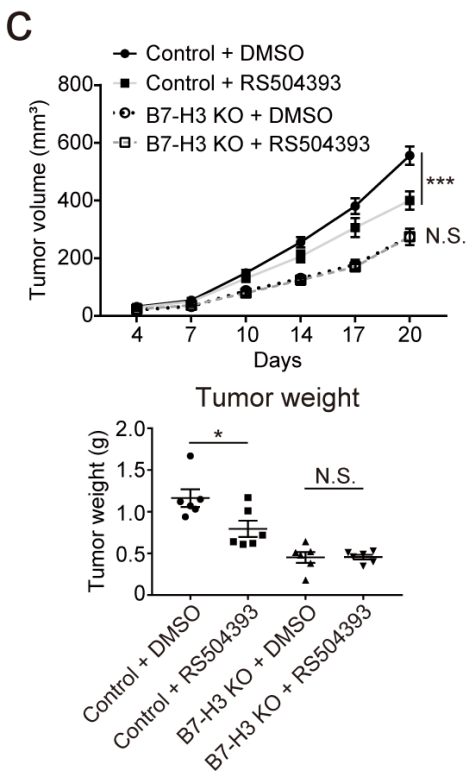


Figure 5







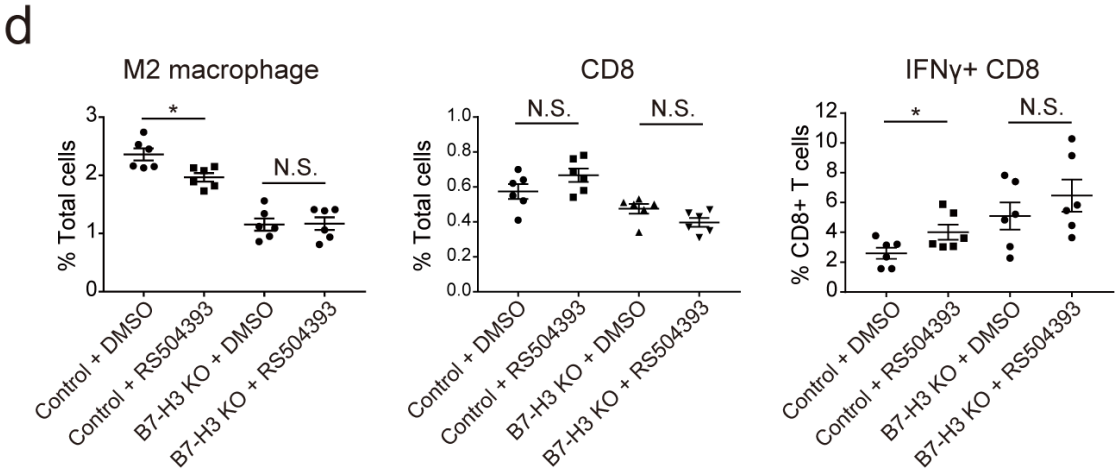
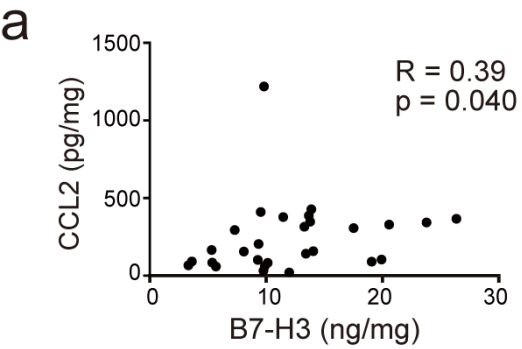
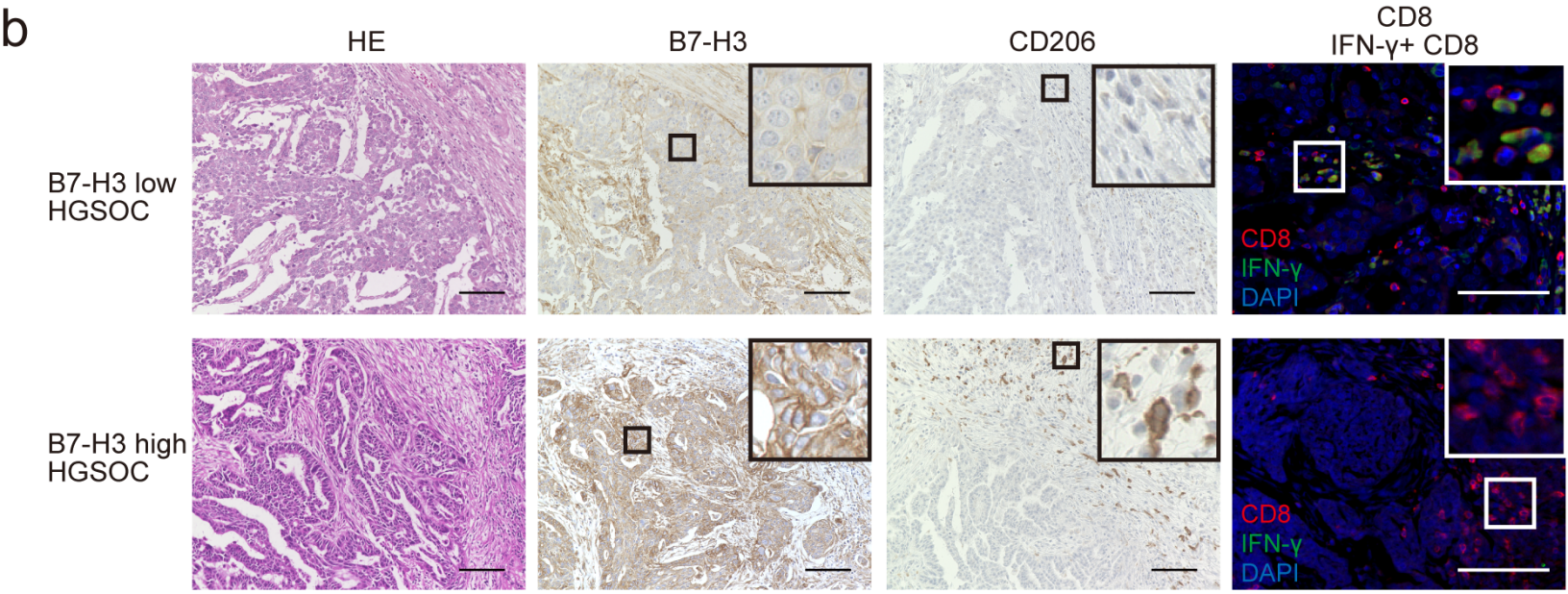
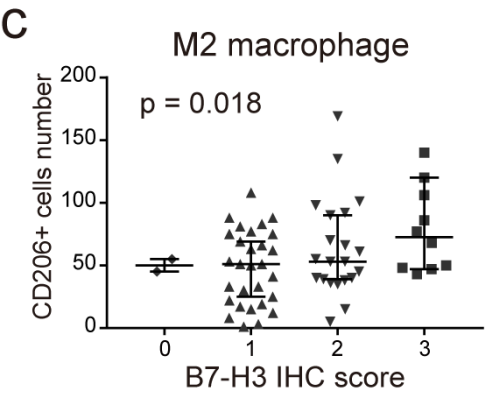
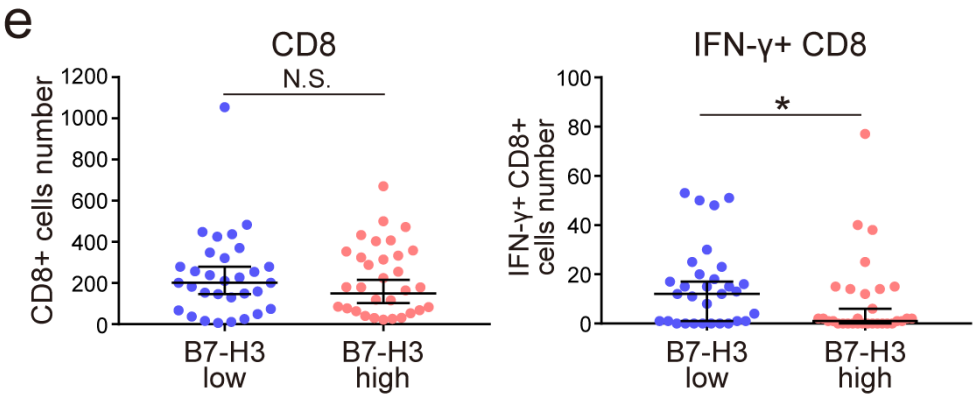


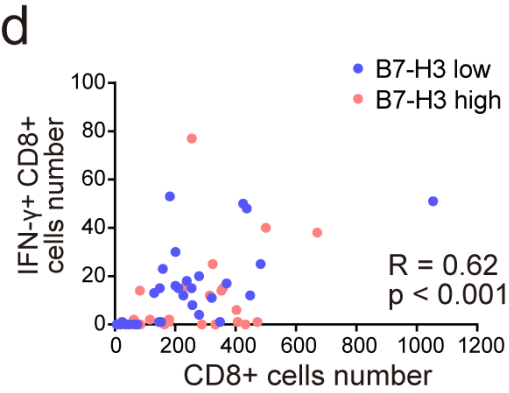
Figure 6

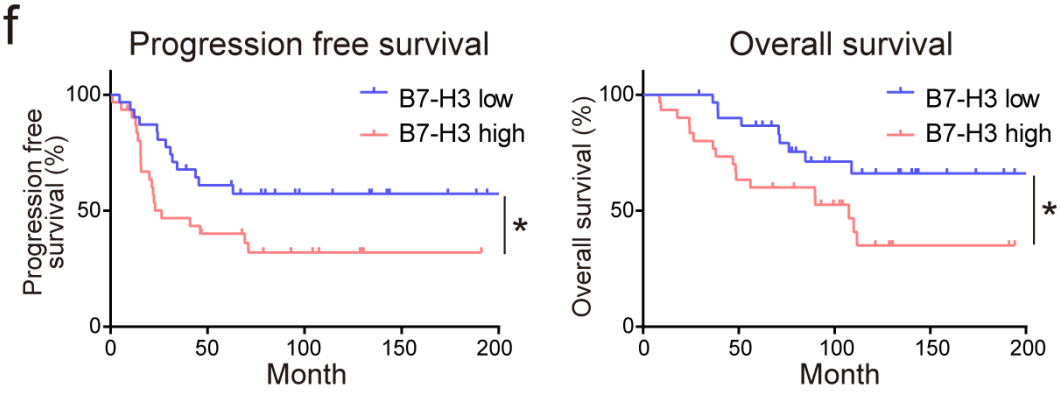












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B7-H3 suppresses anti-tumor immunity via the CCL2-CCR2-M2 macrophage axis and contributes to ovarian cancer progression

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