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Supplemental Information

Interleukin-34 Limits the Therapeutic Effects of Immune Checkpoint Blockade

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Transparent Methods

Cell lines

The ovarian cancer cell line OV2944-HM-1 (HM-1) was purchased from the Japanese Collection of Research Bioresources.The colon cancer cell line CT26 used in this study was kindly provided by Dr. Hidemitsu Kitamura, Hokkaido University. The breast cancer cell line 4T1 was purchased from American Type Culture Collection. HM-1 cell line was maintained in αMEM (Fujifilm Wako Pure Chemical Industries). CT26 and 4T1 cell lines were maintained in RPMI-1640 (Fujifilm Wako Pure Chemical Industries). All culture media were supplemented with 10% fetal bovine serum (Sigma Aldrich), 1% Penicillin/Streptomycin (Nacalai Tesque), and 1% Non-Essential Amino Acid (Nacalai Tesque). Cells were maintained in a 5% CO_2/air environment at 37℃.

Mice and *in vivo* assay

Six to eight-week-old female B6C3F1 and BALB/c mice were purchased from Japan SLC, Inc. The mice were maintained under specific pathogen-free conditions in the animal facility at Hokkaido University. For *in vivo* assay, 2×10^5 tumor cells were inoculated s.c. into the right flank of syngeneic female mice. Antibody treatment (anti-PD-1 (RMP1-14), 250 μg/mouse; CTLA-4 (UC10-4F10), 250 μg/mouse; or IL-34 (C054-35), 200 μg/mouse

was started when tumor size reached 5 mm in diameter. Anti-PD-1 and anti-CTLA-4 antibodies were kindly provided by Dr. Hideo Yagita (Juntendo University). Anti-IL-34 antibody was purchased from BioLegend. Detailed information about antibodies is described in Supplementary Table 1. All animal procedures were approved by the Hokkaido University Animal Care Committee (Approval number: 14-0171).

Generation of *Il34* knockout and *Il34* overexpression cell lines

Il34^{KO} cell line was generated by using IL-34 CRISPR/Cas9 KO Plasmid (m) (Santa Cruz Biotechnology, Inc.). The plasmids were transfected by using *Trans*IT-X2 (Mirus) or Neon® Transfection system (Thermo Fisher Scientific). Cells were selected by GFP expression 48 hours after transfection. For the generation of *Il34* overexpression CT26 cell line, mouse *Il34* coding sequence was cloned into pLenti-EF1a-C-Myc-DDK-IRES-Puro vector (Origene). Lenti-X293T cells were transfected with lentiviral vector and two packaging plasmids pCMV-VSV-G-RSV-Rev and pCAG-HIVgp using *Trans*IT-X2. The complex was added in HEK293T cells and incubated 3 days. After collection of HEK293T medium, CT26 was cultured with 1:1 mixture of HEK293T medium and fresh medium, following selection by puromycin.

Quantitative PCR analysis

Total RNA was extracted using TRIsure reagent (Bioline). cDNA was synthesized using ReverTra Ace® qPCR RT Master Mix (Toyobo). Quantitative PCR was performed on cDNA using KAPA SYBR® FAST qPCR Master Mix (2X) ABI Prism® (Kapa Biosystems) on a StepOnePlus Real-Time PCR System (Thermo Fisher Scientific). The primers are listed in Supplementary Table 2.

Cell viability assay

To assess cell viability, MTT assay was performed using MTT Cell count kit (Nacalai Tesque). Absorbance at a test wavelength of 570 nm and a reference wavelength of 650 nm was measured by using a Multiskan FC (Thermo Fisher Scientific). Cell proliferation was observed up to 4 days.

Enzyme-linked immunosorbent assay (ELISA)

The production of IL-34 in cell lines was measured with ELISA. Culture supernatants were collected at 48 h after seeding the cells at a density of 1×10^6 in 6-well plate. The IL-34 contents was measured with LEGEND MAX Mouse IL-34 ELISA kit with Pre-Coated Plates (Biolegend).

55

56 **Isolation of tumor-infiltrating immune cells from solid tumor**

57 Isolation of tumor-infiltrating immune cells from solid tumors was performed by using BD
58 Horizon™ Dri Tumor & Tissue (Becton, Dickinson, and Company). The recovered tumor-
59 infiltrating cells were used as samples for flow cytometry or RNA extraction.

60

61 **Flow cytometry**

62 Cells were washed and blocked with FcR Blocking Reagent (TONBO biosciences) and
63 stained with 4',6-diamidino-2-phenylindole (DAPI, Cayman Chemical Company) and the
64 antibodies against following molecules; CD3ε, CD4, CD8α, F4/80, CD11b, CD11c, CD45,
65 CD115, IA-IE (MHC class II), iNOS, Arginase 1 and PD-L1 (BioLegend). Data were
66 acquired using BD FACSCanto II, BD FACSAria, or BD FACSCelesta flow cytometer, and
67 analyzed using FlowJo software. Detailed information about antibodies is described in
68 Supplementary Table 1.

69

70 **Next-generation sequencing and data analysis**

71 Total RNA was extracted using PureLinkTM RNA Mini Kit (Life Technologies). Next-
72 generation sequencing was performed at Kazusa DNA Research Institute (illumina HiSeq

2500). The quality and concentration of the RNA was verified with Agilent 2100 Bioanalyzer and Quantus Fluorometer (Promega), respectively. All the samples showed RIN values > 8. Sequencing libraries were prepared using Agilent SureSelect Strand-Specific RNA Library Prep for Illumina according to the manufacturer's instructions. Briefly, poly-A RNA was purified from 300 ng total RNA per sample using oligo dT magnetic beads. The libraries were PCR amplified for 13 cycles and purified with AMPure XP beads. Sequencing of the libraries was conducted on the Illumina HiSeq2500 system performing paired-end 100 bp reads. The reads were mapped to mouse reference genome mm10 with Tophat (v2.1.0), and caluculated FPKM (fragments per kilobase of exon per million reads mapped) value with cufflinks (v2.2.1). The FPKM values were normalized by CD45, and shown as global z-score.

Immunohistochemistry staining

For DAB staining, immunohistochemistry staining was performed on paraffin-embedded tumor tissue sections. PD-L1 was stained using DAB (Dojindo) followed by hematoxylin conterstaining (Fujifilm Wako Pure Chemical Industries). PD-L1 staining was kindly performed by Dr. Yutaka Hatanaka, Research Division of Genome Companion Diagnostics, Hokkaido University Hospital. For multiple immunofluorescent staining, Opal

4-color fluorescent IHC kit (Perkin-Elmer) was used. Tumor sections were objectively judged by two independent researchers at 600× magnification for each section. More than 6 tumor areas in each section were randomly selected for evaluation. FV1000 OLYMPUS software was used for quantification of immunofluorescent staining. Detailed information about antibodies is described in Supplementary Table 1.

PDX model

PDX model was performed at DNA Link, Inc. Firstly, HuNSG mice were generated as previously reported by The Jackson Laboratory (Shultz et al., 2005). In brief, human fetal liver $CD34^+$-purified HSC were purchased from Stem Express and intravenously injected into three-week-old female NSG mice (10^5 cells/mouse), 4h post-140 cGy total body irradiation using the RS-2000 irradiator (Rad Source). The engraftment levels of human $CD45^+$ cells were determined 12 weeks post-HSC transplantation by flow cytometric quantification of peripheral blood. HuNSG mice that had over 25% of human $CD45^+$ cells in the peripheral blood were considered as engrafted and humanized. PDX models were generated using tumor tissues from patients who underwent surgery as the primary treatment strategy for lung cancer at Samsung Medical Center. Twelve weeks post-human HSC transplantation, 30-40 μl finely minced tumors were injected s.c. into the left flank of

HuNSG mice. Treatment was started when the tumor volumes reached 70-120 mm^3. Treatment with anti-human IL-34 (BioLegend; 250 μg per injection, 3 times a week for 4 weeks), anti-human PD-1 (Selleckchem; 10 mg/kg for the first dose, followed by 5 mg/kg dose every 5 days), antibodies combination, or saline was administered intraperitoneally. Vehicle control saline (Sigma Aldrich) was administered 3 times per week until the endpoint. Tumor size was measured by caliper twice a week, and volumes (mm^3) were calculated by (length×$width^2$)/2.

For histological analysis, tumor tissues were fixed with 4% formaldehyde, embedded with paraffin and sections were stained with hematoxylin and eosin.

All animal experiments were performed under the guidelines approved by the Institutional Animal Care and Use Committee of Seoul National University Biomedical Research Institute.

Statistics

Statistical analysis was perfomed with JMP® 14 (SAS Institute Inc.). Significance was determined by Student's *t*-test, Tukey's multiple comparison test, or Steel-Dwass nonparametric multiple comparison test. p-Value was considered statistically significant when < 0.05.

Supplemental Reference

Shultz, L.D., Lyons, B.L., Burzenski, L.M., Gott, B., Chen, X., Chaleff, S., Kotb, M., Gillies, S.D., King, M., Mangada, J., et al. (2005). Human Lymphoid and Myeloid Cell Development in NOD/LtSz- *scid IL2R* γ ^{null} Mice Engrafted with Mobilized Human Hemopoietic Stem Cells. J. Immunol. *174*, 6477–6489.

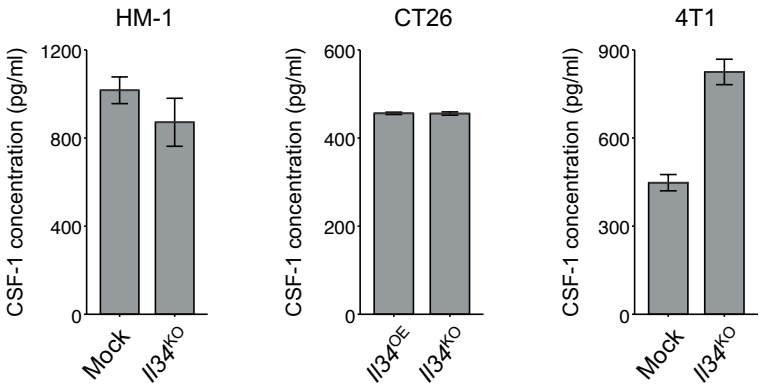

Figure S1 : The expression of CSF-1 from various murine cancer cell lines. Related to Figure 1, 2.

CSF-1 concentration in supernatants of HM-1, CT26 and 4T1 cell lines (n=3/cell line).

Data represent mean ± SEM.

Figure S1

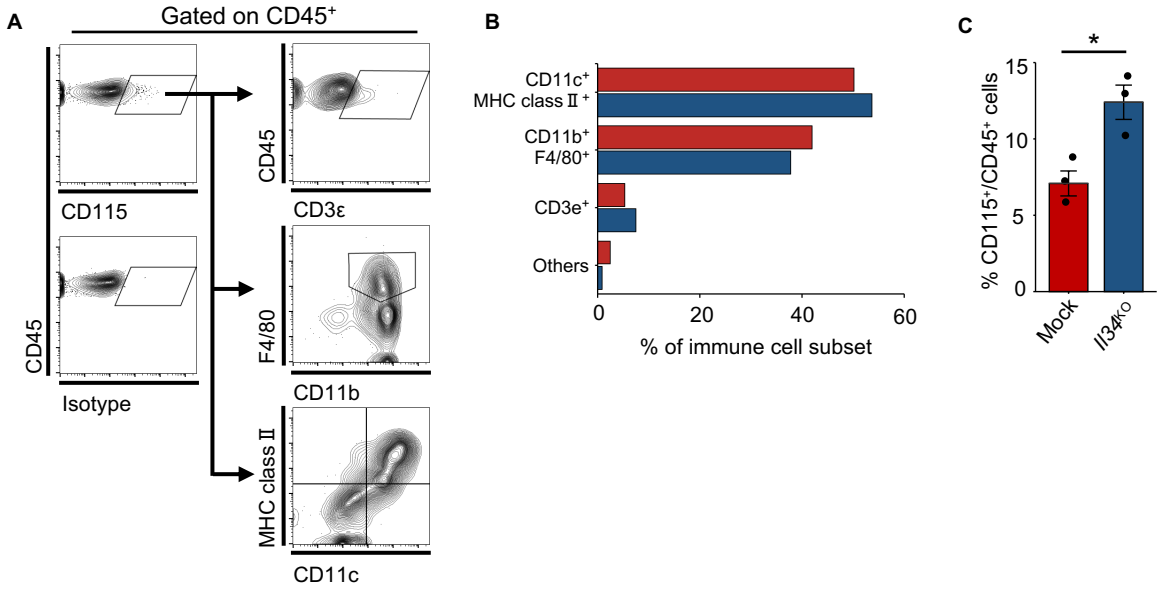

Figure S2 : Identification of the immune cell subset expressing CD115 in mock-HM-1 and *Il34*^{KO}-HM-1 tumors. Related to Figure 1.

(A) Representative flow cytometry profiles showing CD115+ cells within tumor-infiltrating CD45^{+} cells in HM-1 tumor.

(B) Bar graph shows the cell type expressing CD115 within tumor-infiltrating CD45^{+} cells (n=3/group).

(C) Bar graph represent the frequency of CD115^{+} cells within tumor-infiltrating CD45^{+} cells in mock- or *Il34* ^{KO}-HM-1 tumors (n=3/group). Data represent mean ± SEM. *p<0.05; two-tailed Student's t-test.

Figure S2

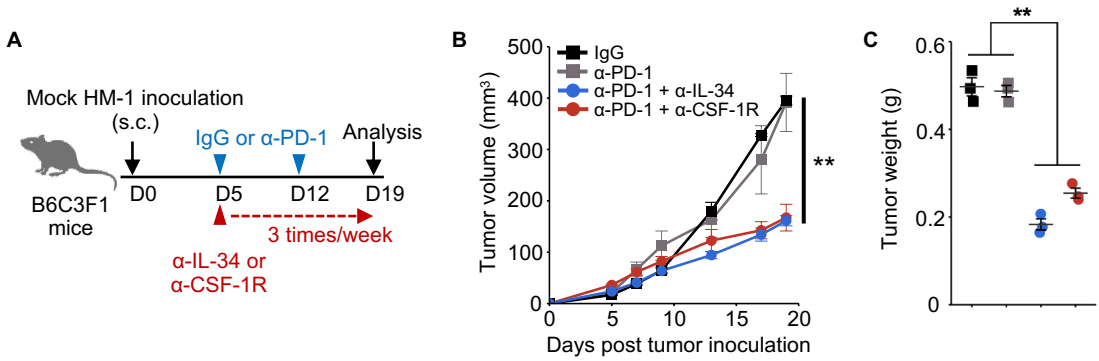

Figure S3 : IL-34 blockade equally enhanced anti-tumor efficacy of α-PD-1 treatment comparing with CSF-1R blockade in HM-1 model. Related to Figure 1.

(A) Schematic of the α-PD-1 mAb treatment in combination with α-IL-34 or α-CSF-1R mAb. The timeline shows the procedure of tumor inoculation and antibody treatment.

(B) Mock HM-1 tumor growth in B6C3F1 mice treated with the indicated antibodies (n=3-4/group).

(C) Tumor weight on day 19 after tumor inoculation (n=3-4/group).

Data represent mean ± SEM. **p<0.01; Tukey's multiple comparison test.

Figure S3

A *Il34*^{KO} vs. *Il34*^{OE} (treated by α-PD-1 mAb)

Canonical Pathway	p-value
T cell receptor signaling pathway	3.68E-10
Graft-versus-host disease	4.47E-10
Antigen processing and presentation	9.52E-10
Systemic lupus erythematosus	3.84E-09
Allograft rejection	5.42E-09
Asthma	3.76E-08
Cell adhesion molecules (CAMs)	3.86E-08
Hematopoietic cell lineage	1.05E-07
Type I diabetes mellitus	2.25E-07
Cytokine-cytokine receptor interaction	2.45E-07

Figure S4 : Clustering the gene expression data on NGS by Gene Ontology analysis. Related to Figure 2.

(A) The list of gene-set clusters enhanced in *Il34*^{KO} CT26 group compared to *Il34*^{OE} CT26 group.

(B) Heatmap shows the differentially of gene expression on selected several gene-set clusters.

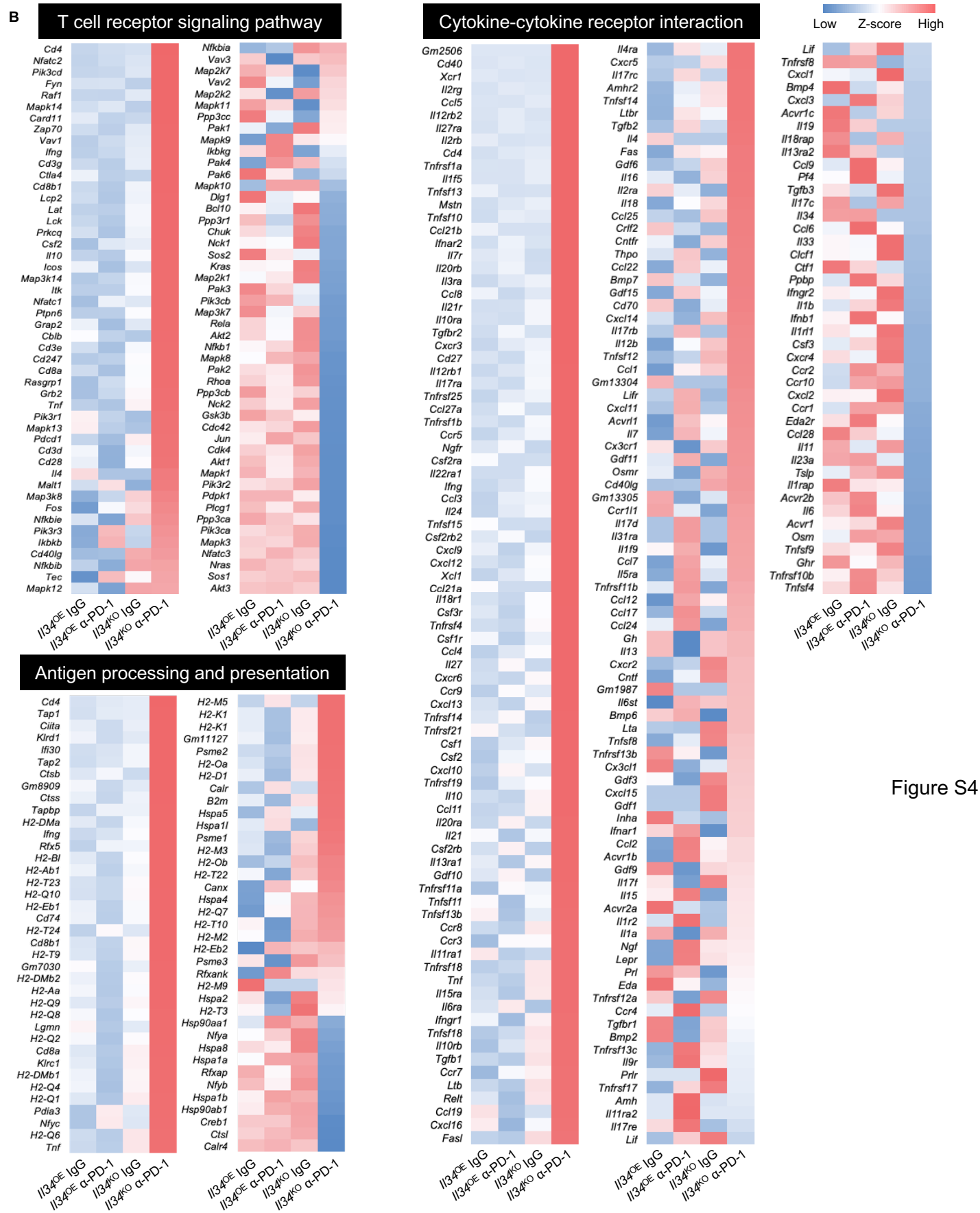

Figure S4

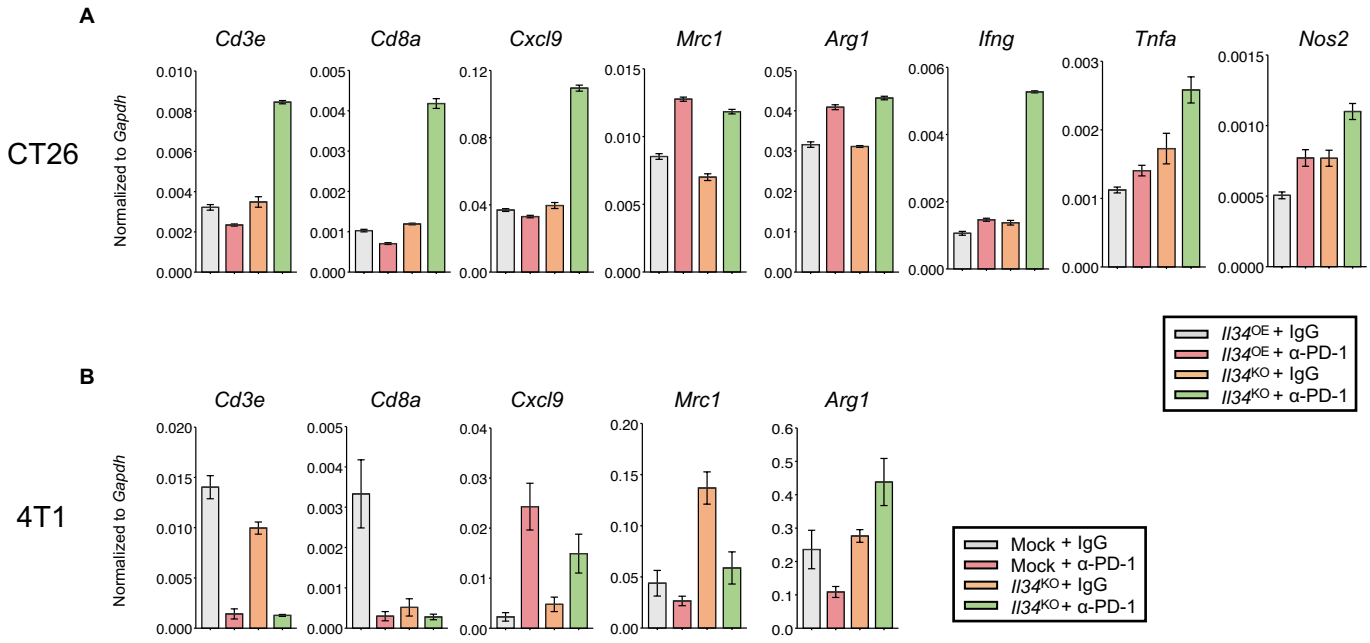

Figure S5 : qPCR analysis in CT26 and 4T1 tumors. Related to Figure 2.

(A) Expression of selected genes were evaluated by qPCR analysis in CT26 tumor samples used for NGS analysis (Fig. 2D).

(B) Gene expression displayed in (A) were analyzed by qPCR in 4T1 tumors (n=3/group).

Data represent mean ± SEM of technical triplicate.

Figure S5

Key resources table

REGENT & RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-mouse CD3ε (145-2C11) APC	BioLegend	Cat#100236; RRID: AB_2561456
Anti-mouse CD4 (RM4-5) APC-Cy7	BioLegend	Cat#00525; RRID: AB_312726
Anti-mouse CD8α (53-6.7) FITC	BioLegend	Cat#00706; RRID: AB_312745
Anti-mouse CD11b (M1/70) FITC	BioLegend	Cat#101206; RRID: AB_312789
Anti-mouse CD11c (N418) APC	BioLegend	Cat#117309; RRID: AB_313778
Anti-mouse CD45 (30-F11) Pacific blue	BioLegend	Cat#103126; RRID: AB_493535
Anti-mouse CD45 (30-F11) FITC	BioLegend	Cat#103108; RRID: AB_312973
Anti-mouse CD45 (30-F11) PE	BioLegend	Cat#103106; RRID: AB_312971
Anti-mouse CD45 (30-F11) APC	BioLegend	Cat#103112; RRID: AB_312977
Anti-mouse CD45 (30-F11) PE-Cy7	BioLegend	Cat#103114; RRID: AB_312979
Anti-mouse CD45 (30-F11) APC-Cy7	BioLegend	Cat# 103116; RRID: AB_312981
Anti-mouse F4/80 (BM8) APC	BioLegend	Cat#123116; RRID: AB_893481
Anti-mouse CD274 (MIH5) APC	BioLegend	Cat#124311; RRID: AB_10612935
Anti-mouse CD273 (TY25) PE	BioLegend	Cat#107205; RRID: AB_2299418
Anti-mouse CD80 (16-10A1) FITC	BioLegend	Cat#104705; RRID: AB_313126
Anti-mouse CD86 (GL-1) FITC	BioLegend	Cat#105005; RRID: AB_313148
Anti-mouse CD115 (AFS98) APC	BioLegend	Cat#125509; RRID: AB_2085222
Anti-mouse I-A/I-E (M5/114.15.2) FITC	BioLegend	Cat#107606; RRID: AB_313321
Anti-mouse iNOS (CXNFT) APC, eBioscience™	Invitrogen™	Cat#17-5920-80; RRID: AB_2573244
Anti-human/mouse Arginase 1 (A1exF5) PE, eBioscience™	Invitrogen™	Cat#12-3697-80; RRID: AB_2734839
Purified anti-mouse CD16/CD32 (2.4G2) (Fc Block)	TONBO bioscience	Cat#70-0161; RRID: AB_2621487
Purified anti-mouse CD45 (30-F11)	BioLegend	Cat#10302; RRID: AB_312967
Purified anti-mouse F4/80 (BM8)	BioLegend	Cat#123101; RRID: AB_893504
Purified anti-mouse Areginase-1 (D4E3M)	CST	Cat#93668; RRID: AB_2800207
Purified anti-mouse Nos2 (Rabbit polyclonal)	Abcam	Cat#ab15323; RRID: AB_301857
Purified anti-mouse PD-1 (RMP1-14)	Dr. Hideo Yagita (Juntendo University, Tokyo)	N/A

Purified anti-mouse CTLA-4 (UC10-4F10)	Dr. Hideo Yagita (Juntendo University, Tokyo)	N/A
Purified anti-mouse IL-34 (C054-35)	BioLegend	Cat#147202; RRID: AB_2563031
Purified anti-mouse CSF-1R (AFS98)	Bioxell	Cat#BE0213; RRID: AB_2687699
ChromPure Rat igG, whole molecule	Jackson Immuno Research LABORATPRIES, INC.	Cat#012-000-003; RRID: AB_2337136
Purified anti-human IL-34 (1D12)	Millipore	Cat#MABT493
Purified anti-human CD274 (E1L3N)	CST	Cat#13684; RRID: AB_2687655
Purified anti-human PD-1 (monoclonal)	Selleckcheme	Cat#A2002; RRID: AB_2810223
Purified anti-human IL-34 (E0320E7)	BioLegend	Cat#361302; RRID: AB_2563033
Cell Culture Regents		
RPMI-1640with L-Glutamine and Phenol Red	Fujifilm Wako Pure Chemical Industries	Cat#189-02025
D-MEM (high Glucose) with L-Glutamine and Phenol Red	Fujifilm Wako Pure Chemical Industries	Cat#044-29765
MEMα with L-Glutamine and Phenol Red	Fujifilm Wako Pure Chemical Industries	Cat#135-15175
Defined fetal bovine serum	Sigma Aldrich	Cat#F7524
Penicillin-Streptomycin Mixed Solution (100x)	Nacali Tesque	Cat#26253-84
MEM Non-Essential Amino Acid Solution (100x)	Nacali Tesque	Cat#06344-56
2.5g/l-Trypsin/1mmol/l-EDTA Solution, with Phenol Red	Nacali Tesque	Cat#32777-15
Critical Commercial Regents		
TransIT-X2® Dynamic Delivery System	Takara	Cat#V6104
LEGEND MAX™ Mouse IL-34 ELISA Kit	BioLegend	Cat#439107
eBioscience™ Fixation/Permeabilization Concentrate	Invitrogen™	Cat#00-5123-43
eBioscience™ Fixation/Permeabilization Diluent	Invitrogen™	Cat#00-5223-56
eBioscience™ Permeabilization Buffer (10X)	Invitrogen™	Cat#00-8333-56

BD Horizon™ Dri Tumor & Tissue Dissociation Reagent (TTDR)	BD bioscience	Cat#661563
Experimental Models: Cell Lines		
OV2944-HM-1	Japanese Collection of Research Bioresources	Cat#JCRB1208
CT26	Dr. Hidemitsu Kitamura (Hokkaido University, Hokkaido)	N/A
4T1	ATCC	Cat#CRL-2539
Experimental Models: Organisms/Strains		
B6C3F1	Japan SLC, Inc.	N/A
Balb/c	Japan SLC, Inc.	N/A
Plasmids		
pCAG-VSVG	Addgene	Cat#8454
pCMV-VSV-G-RSV-Rev	Addgene	Cat#35616
IL-34 CRISPR/Cas9 KO Plasmid (m)	Santa Cruz Biotechnology, Inc.	Cat#sc-429354
pLenti-EF1a-C-Myc-DDK-IRES-Puro	Origene	Cat#PS100085

Primer list for quantitative PCR analysis

Species	Gene	Forward (5'-3')	Reverse (5'-3')
Mouse	Gapdh	TCAAATGGGGTGAGGCCGGT	TTGCTGACAATCTTGAGTGA
	Cd3e	AAGTAATGAGCTGGCTGCGT	TCGTCACTGTCTAGAGGGCA
	Cd8a	GGATTGGACTTCGCCTGTGA	TGGGACATTTGCAAACACGC
	Mrc1	CTCTGTTCAGCTATTGGACGC	TGGCACTCCCAAACATAATTTGA
	Ifng	AAGACAATCAGGCCATCAGCA	AGCGACTCCTTTTCCGCTTC
	Tnfa	TTCTATGGCCCAGACCCTCA	CTTGGTGGTTTGCTACGACG
	Nos2	ACATCGACCCGTCCACAGTAT	CAGAGGGGTAGGCTTGTCTC
	Arg1	GTATGACGTGAGAGACCACG	CTCGCAAGCCAATGTACACG
	Cxcl9	AATGCACGATGCTCCTGCA	AGGTCTTTGAGGGATTTGTAGTG
	Cxcl10	AGTGCTG CCGTCATTTTCTG	TCCCTATGGCCCTCATTCTCA
	Cxcl11	GTAATTTACCCGAGTAACGGC	CACCTTTGTCGTTTATGAGCCTT