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Supplemental Information

Oncolytic Virus Therapy with HSV-1 for Hematological Malignancies

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Figure S1

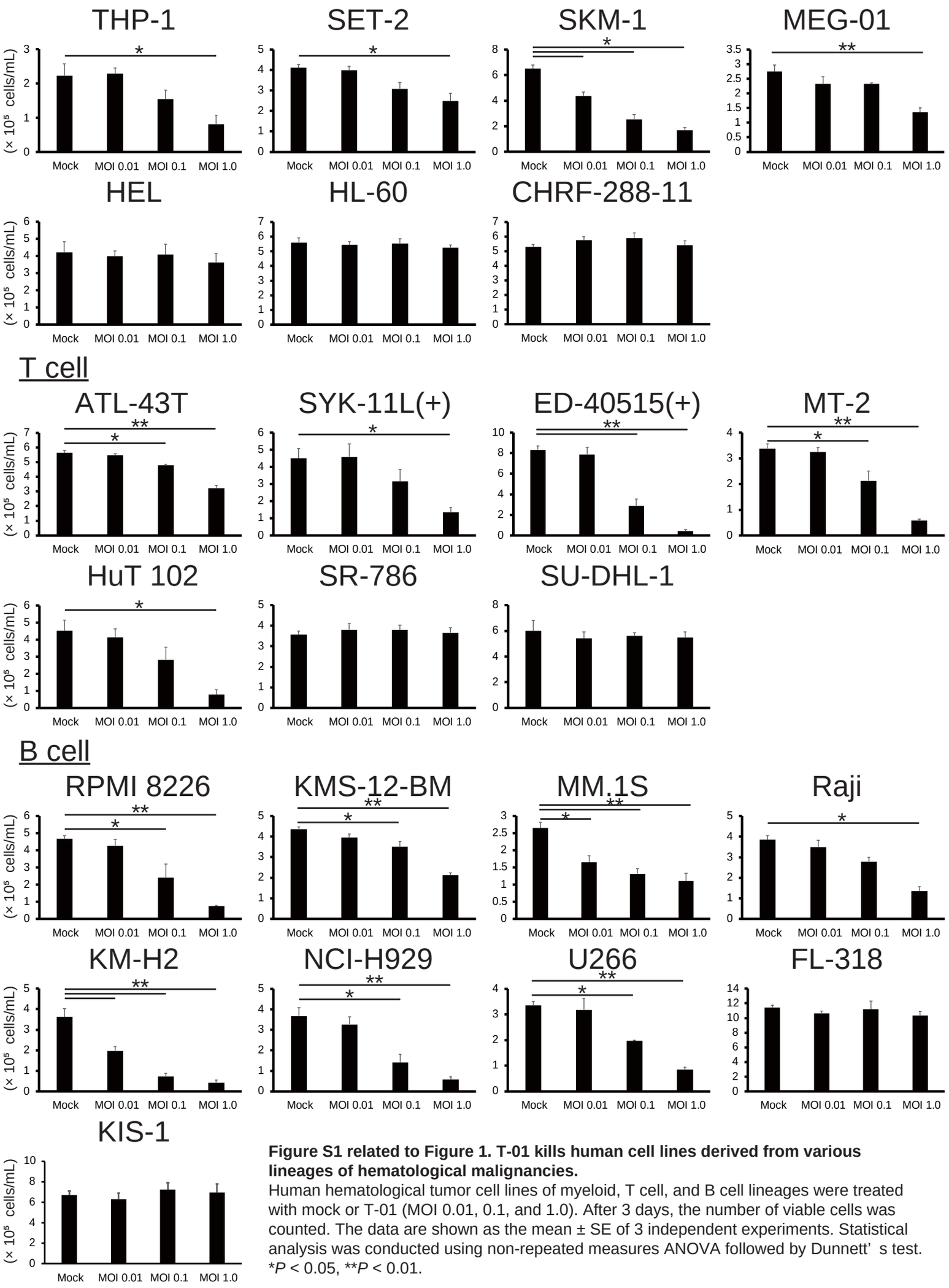
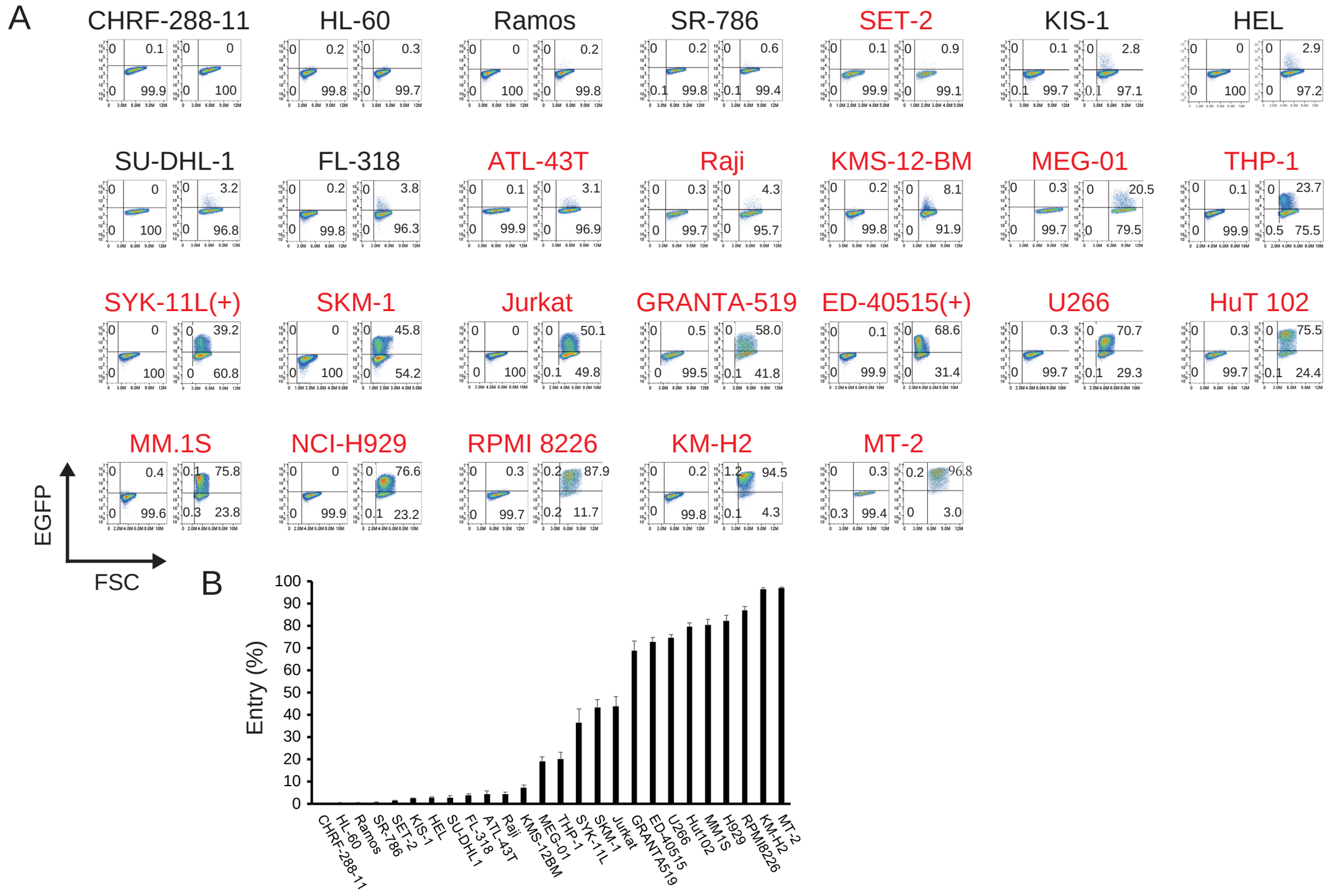


Figure S1 related to Figure 1. T-01 kills human cell lines derived from various lineages of hematological malignancies.

Human hematological tumor cell lines of myeloid, T cell, and B cell lineages were treated with mock or T-01 (MOI 0.01, 0.1, and 1.0). After 3 days, the number of viable cells was counted. The data are shown as the mean ± SE of 3 independent experiments. Statistical analysis was conducted using non-repeated measures ANOVA followed by Dunnett's test. **P* < 0.05, ***P* < 0.01.

Figure S2


A
CHRF-288-11
HL-60
Ramos
SR-786
SET-2
KIS-1
HEL
SU-DHL-1
FL-318
ATL-43T
Raji
KMS-12-BM
MEG-01
THP-1
SYK-11L(+)
SKM-1
Jurkat
GRANTA-519
ED-40515(+)
U266
HuT 102
MM.1S
NCI-H929
RPMI 8226
KM-H2
MT-2
EGFP
FSC
B
Entry (%)
CHRF-288-11
HL-60
Ramos
SR-786
SET-2
KIS-1
HEL
SU-DHL1
FL-318
ATL-43T
Raji
KMS-12BM
MEG-01
THP-1
SYK-11L
SKM-1
Jurkat
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ED-40515
U266
Hut102
MM1S
H929
RPMI8226
KM-H2
MT-2

Figure S2C

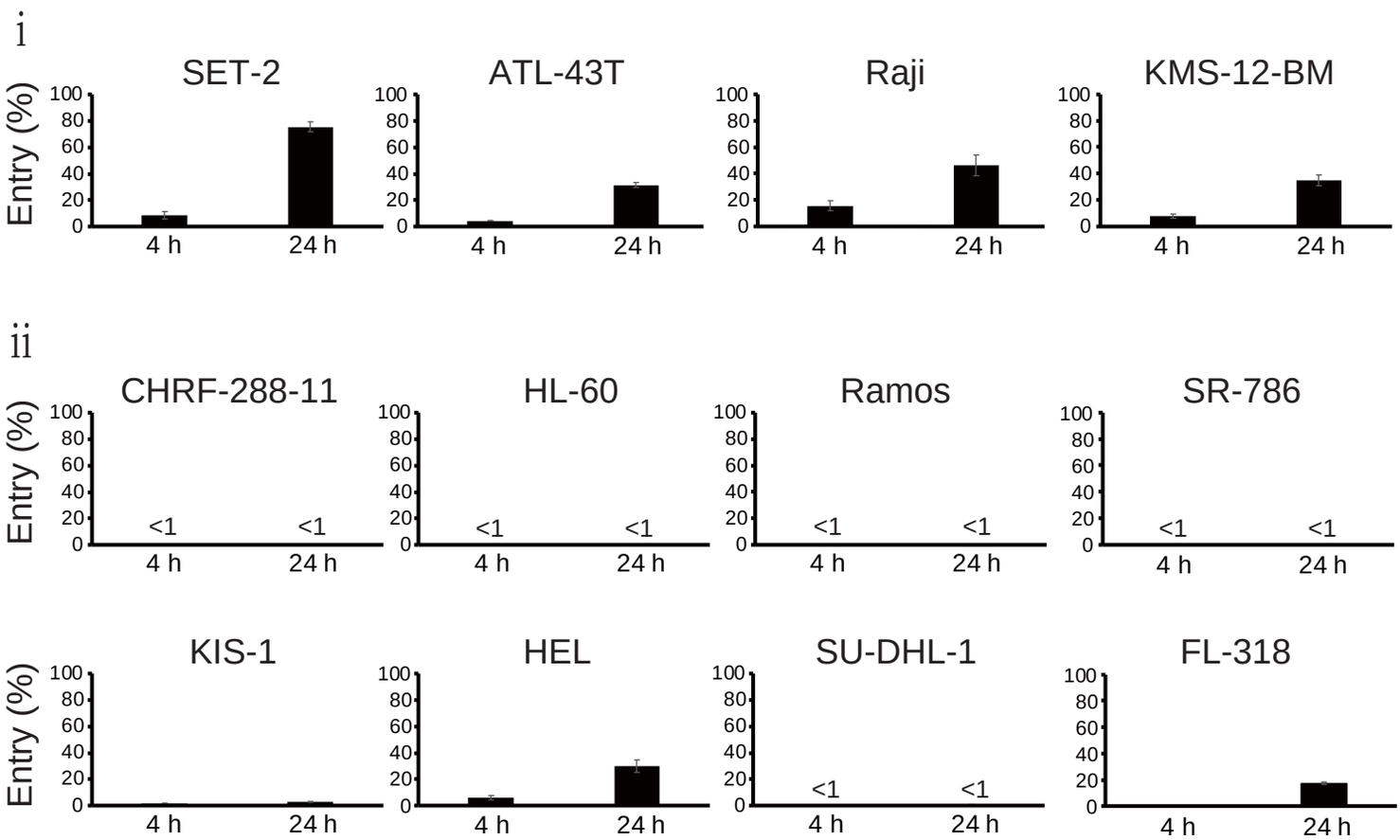


Figure S2 related to Figure 3. The degrees of viral entry are highly variable among the cell lines.

(A) Cell lines were treated with T-GFP (MOI 1.0) for 4 hours. The degrees of viral entry are shown by flow cytometry with forward scatter versus EGFP. The left and right panels of each cell line indicate cells without and with T-GFP, respectively. The percentages of EGFP-positive and negative cells are indicated on the plot. The cell lines susceptible to T-01 are labeled with red.

(B) A graph showing the percentages of cells with viral entry as detected by EGFP positivity shown in (A). The data are shown as the mean ± SE of 3 independent experiments.

(C) Graphs showing the percentages of cells with viral entry as detected by EGFP positivity at 4 and 24 hours. The cell lines with low levels of viral entry at 4 hours are shown. (i) T-01-susceptible cell lines, (ii) T-01-resistant cell lines. The data are shown as the mean ± SE of 3 independent experiments.

Figure S3

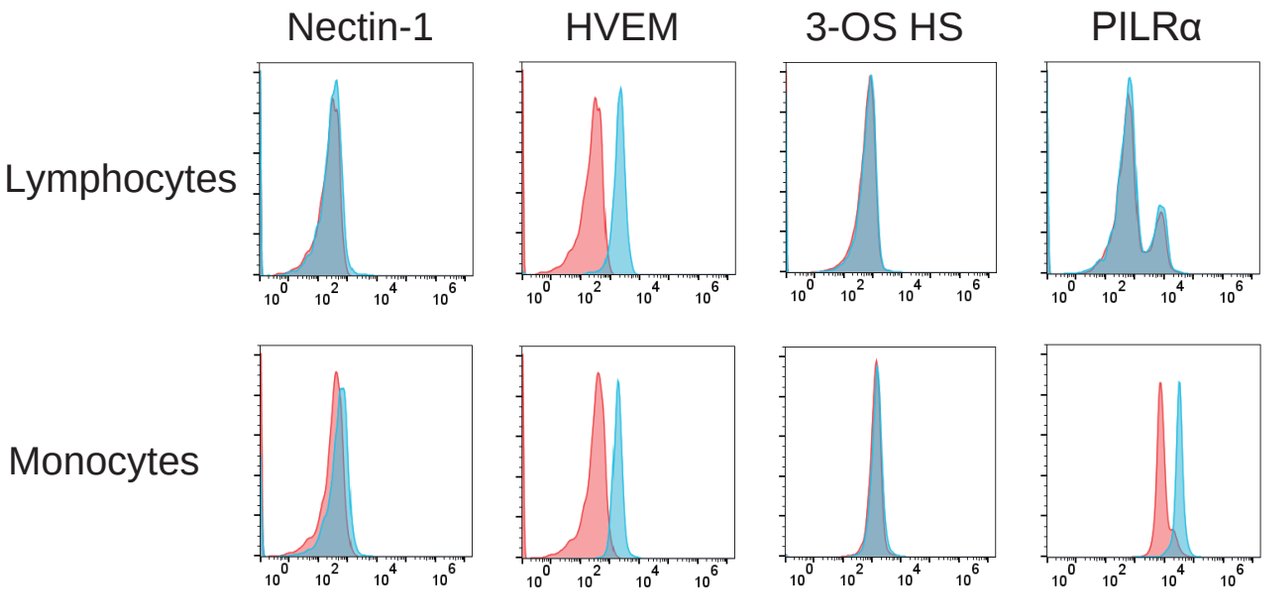
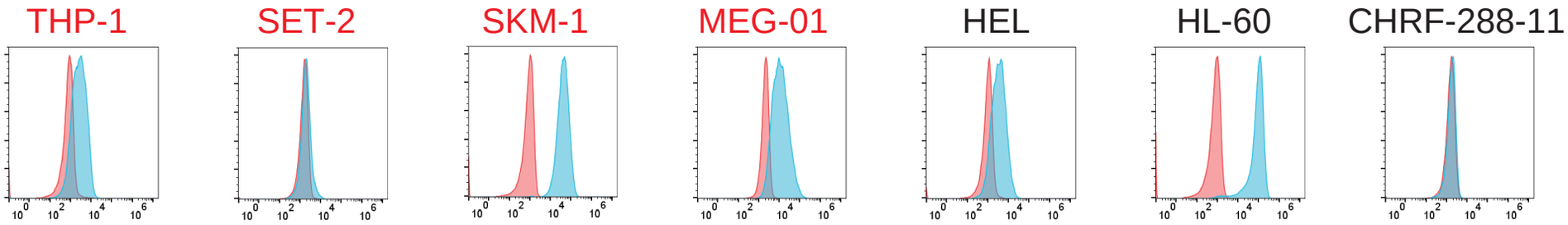


Figure S3 related to Figure 3. Expression of nectin-1, HVEM, 3-OS HS, and PILRα on lymphocytes and monocytes in human peripheral blood.

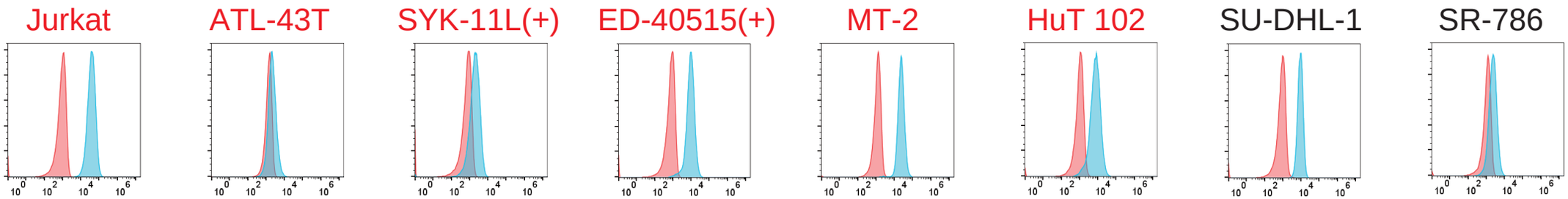
Lymphocytes and monocytes were gated based on forward and side scatters. Red histograms represent cells stained with isotype-matched control mAbs. Anti-heparan sulfate antibody HS4C3 was omitted for the negative control of 3-OS HS. The data are representative of 3 independent experiments.

Figure S4A Nectin-1 expression

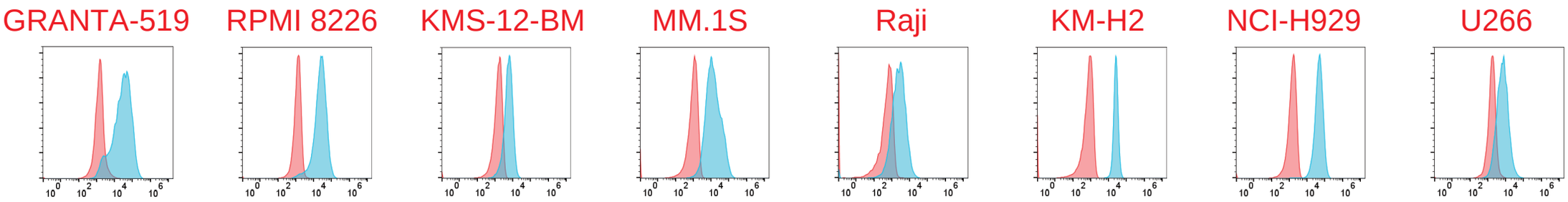
Myeloid



T cell



B cell



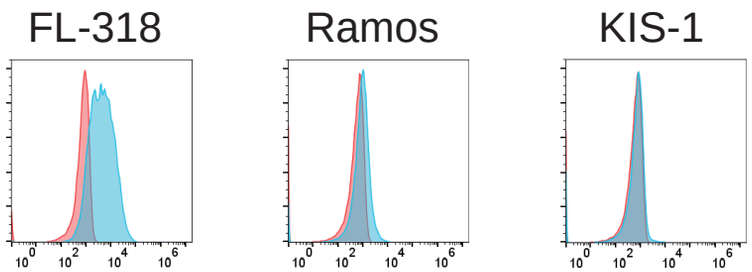
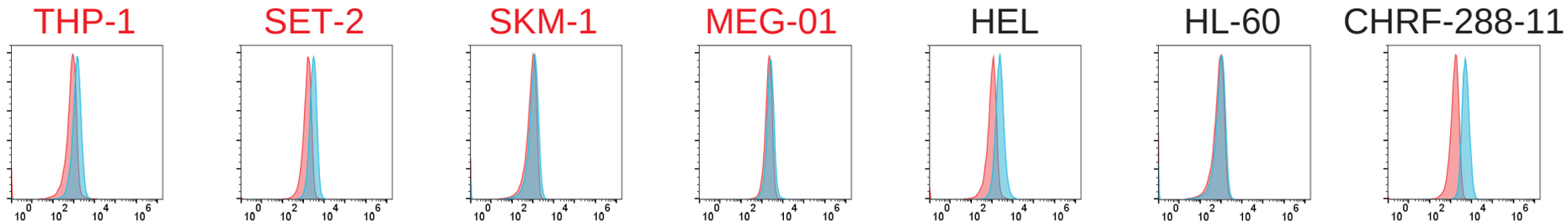


Figure S4 related to Figure 3. Expression of nectin-1 (A), HVEM (B), and 3-OS HS (C) on the cell lines.

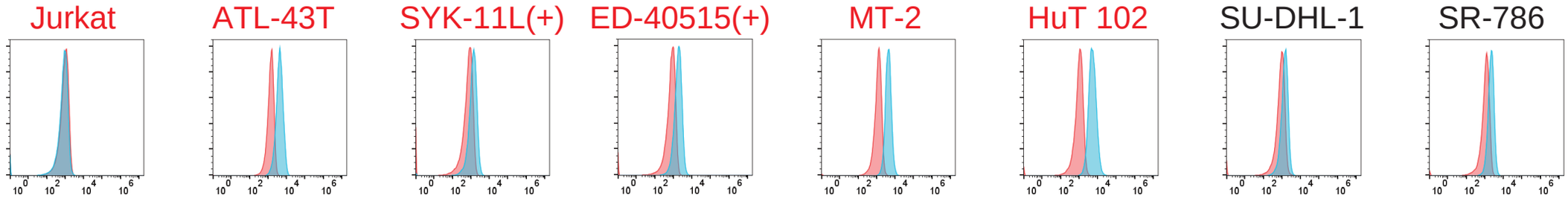
(A) Red histograms represent cells stained with isotype-matched control mAbs. The cell lines susceptible to T-01 are labeled with red. The data are representative of 3 independent experiments.

Figure S4B HVEM expression

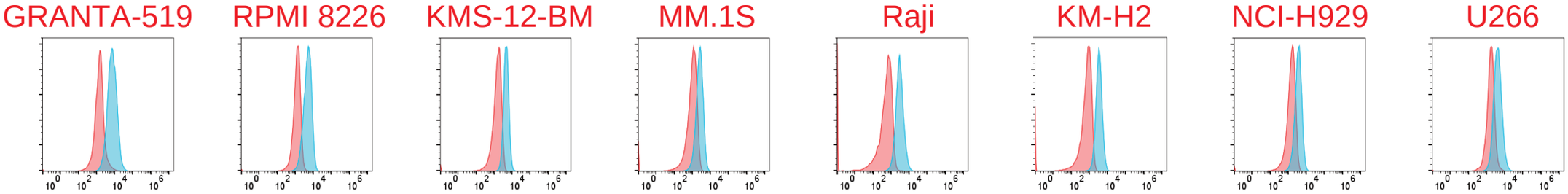
Myeloid



T cell



B cell



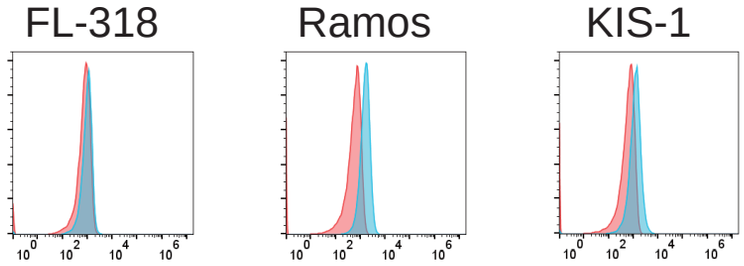
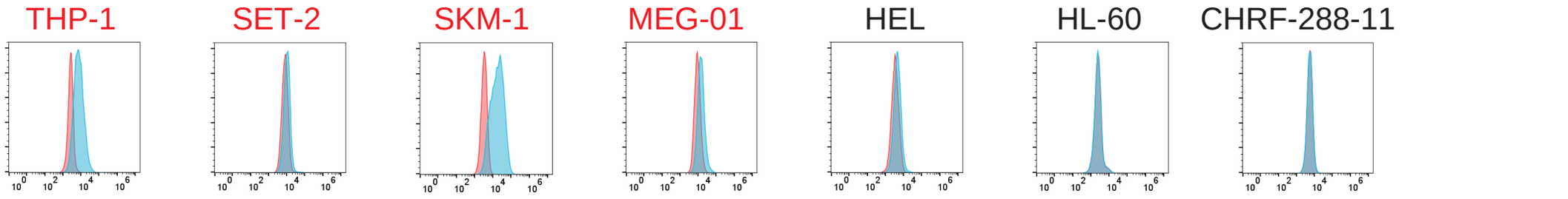


Figure S4 related to Figure 3. Expression of nectin-1 (A), HVEM (B), and 3-OS HS (C) on the cell lines.

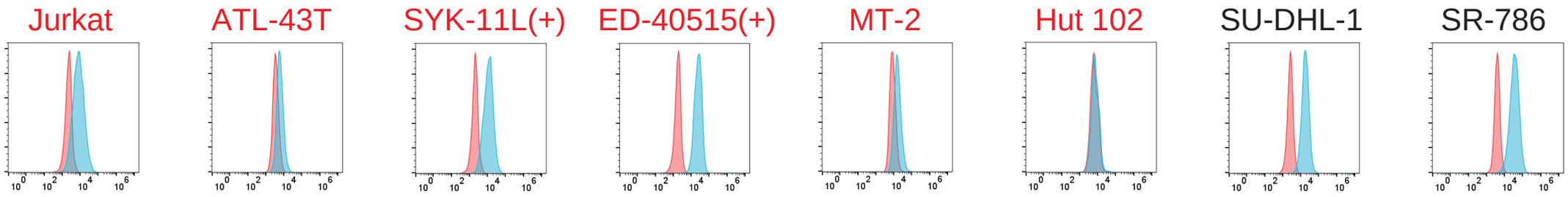
(B) Red histograms represent cells stained with isotype-matched control mAbs. The cell lines susceptible to T-01 are labeled with red. The data are representative of 3 independent experiments.

Figure S4C 3-OS HS expression

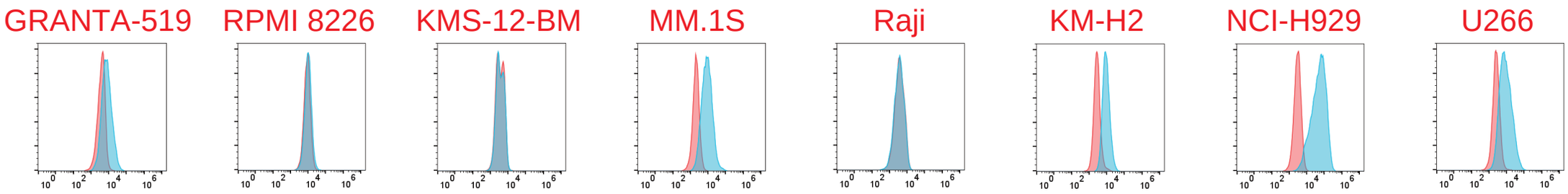
Myeloid



T cell



B cell



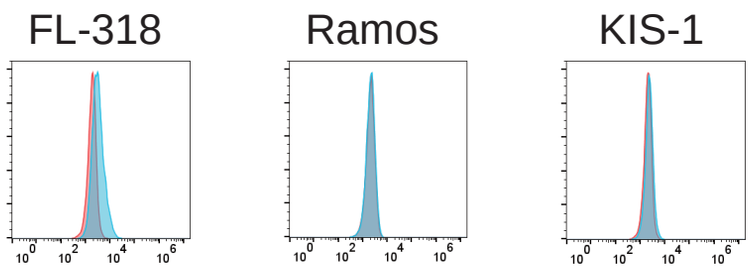


Figure S4 related to Figure 3. Expression of nectin-1 (A), HVEM (B), and 3-OS HS (C) on the cell lines.

(C) Red histograms represent cells stained by omitting anti-heparan sulfate antibody HS4C3. The cell lines susceptible to T-01 are labeled with red. The data are representative of 3 independent experiments.

Figure S5

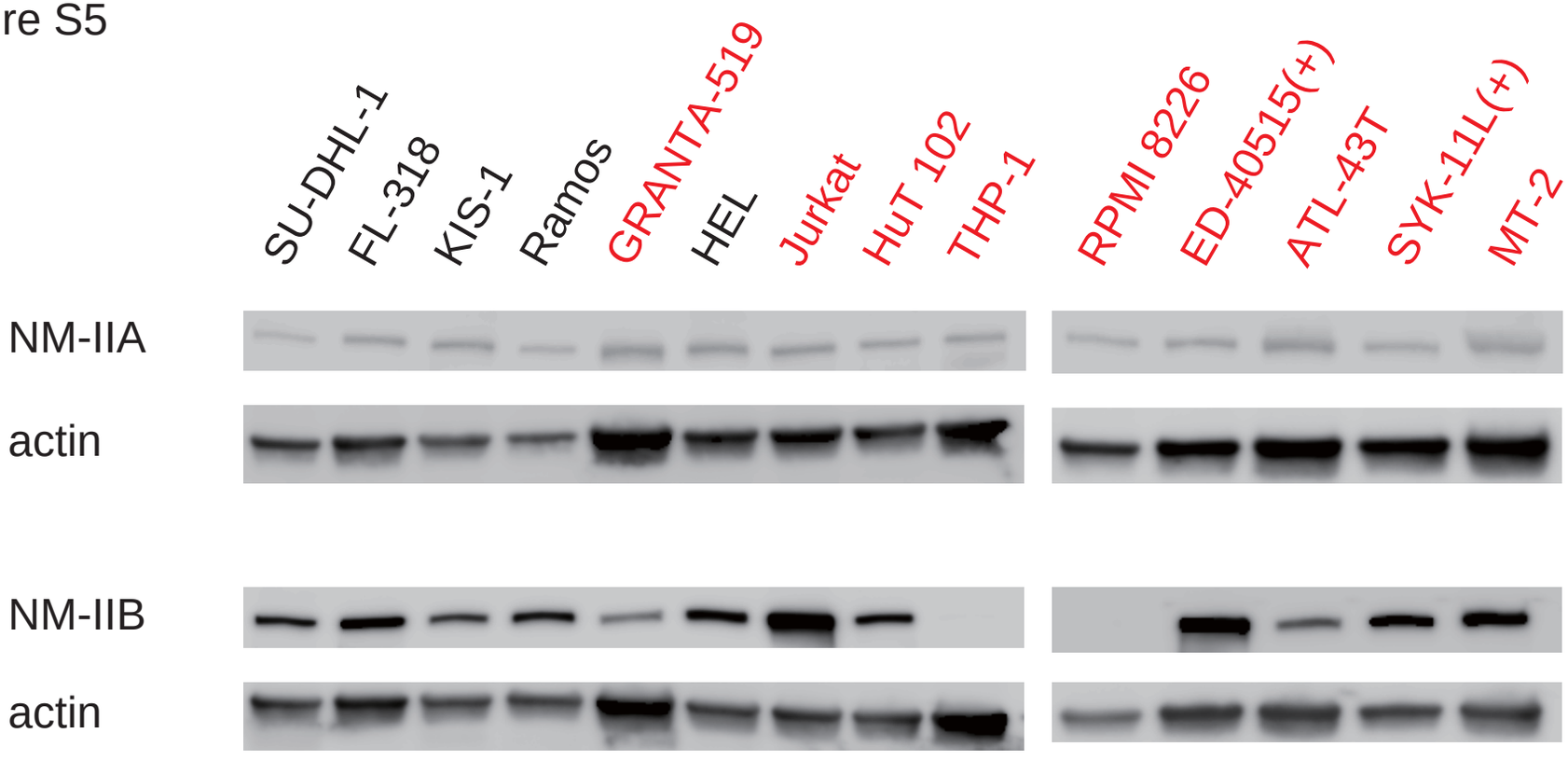


Figure S5. Expression of NMHC-IIA and IIB in the cell lines.

(A) NMHC-IIA (NM-IIA), (B) NMHC-IIB (NM-IIB), and β-actin in cell extracts were detected by western blotting. The cell lines susceptible to T-01 are labeled with red. The data are representative of 3 independent experiments.

Figure S6

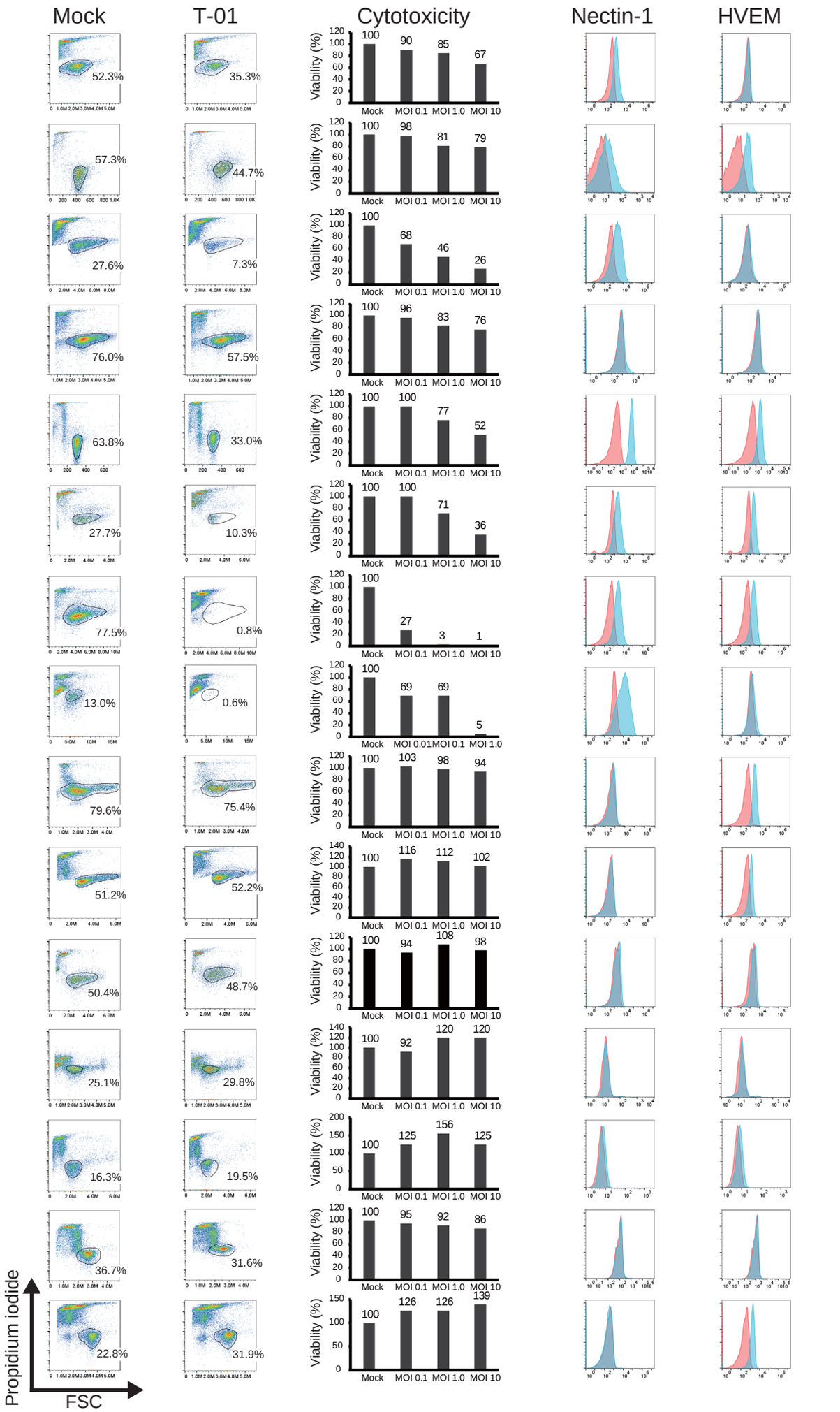


Figure S6 related to Figure 4. Susceptibility to T-01 and expression of nectin-1 and HVEM on clinical samples.
Mononuclear cells from clinical samples were stained with appropriate combinations of mAbs, based on prior flow cytometric analysis of each clinical sample. Tumor cells within each sample were gated before analysis. Left: dot plots of forward scatter and propidium iodide with gating of viable cells after treatment with mock or T-01 at the indicated MOI for 3 days. Percentages of viable cells are indicated on each plot. The samples regarded as susceptible to T-01 are labeled with red. Middle: viability after treatment with mock or T-01 (MOI 0.1, 1, or 10) for 3 days, calculated as (viable cell percentage with T-01/viable cell percentage with mock) x 100 (%). Right: expression of nectin-1 and HVEM on tumor cells. Red histograms represent cells stained with isotype-matched control mAbs.

Figure S7

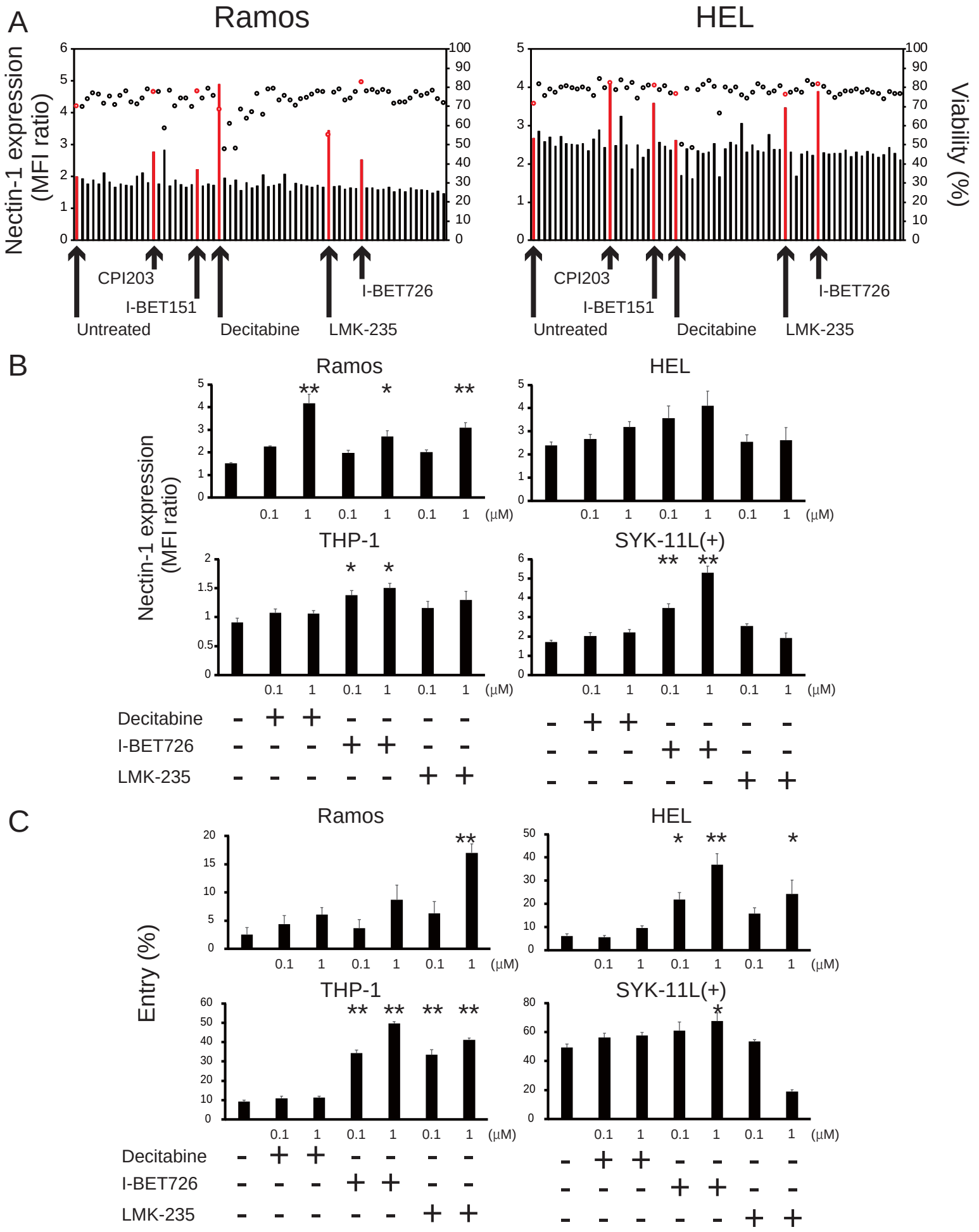


Figure S7. Nectin-1 expression and viral entry are upregulated by epigenetic regulation.
(A) Ramos and HEL cells were treated with reagents contained in a chemical library for epigenetics research at 1 μM for 24 hours. Nectin-1 expression and cell viability are shown by columns and circles, respectively (in red for the cells treated with the indicated reagents). Nectin-1 expression was detected by flow cytometry. Cell viability was detected as trypan blue exclusion. (B) Ramos, HEL, THP-1, and SYK-11L(+) cells were treated with decitabine, I-BET726, or LMK-235 at the indicated concentrations for 24 hours. Nectin-1 expression was detected by flow cytometry. The data are shown as the mean ± SE of 3 independent measurements. (C) Cells were treated with the indicated reagents for 24 hours. Thereafter, the cells were cultured with T-GFP for 4 hours. Viral entry was detected using flow cytometry. The data are shown as the mean ± SE of 3 independent measurements. Statistical significance was examined against DMSO-treated control cells. Statistical analysis was conducted using non-repeated measures ANOVA followed by Dunnett' s test. *$P < 0.05$, **$P < 0.01$.

Figure S8

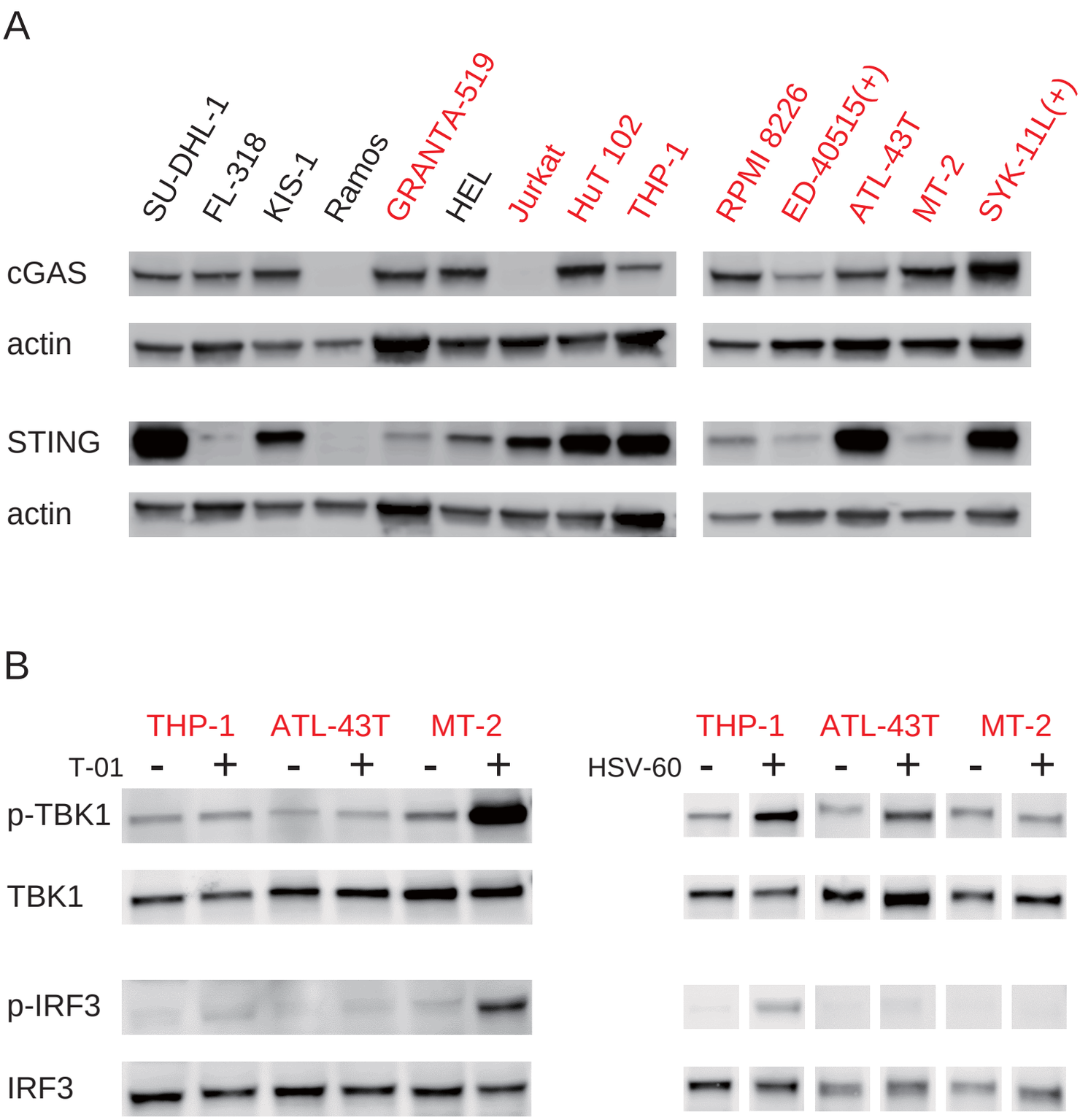


Figure S8. Expression and activity of the cGAS-STING pathway.

(A) cGAS, STING, and β-actin in cell extracts were detected by western blotting. The cell lines susceptible to T-01 are labeled with red. (B) Phosphorylated TBK1 (p-TBK1), total TBK1, p-IRF3, and total IRF3 in THP-1, ATL-43T, and MT-2 in cell extracts were detected by western blotting after stimulation with mock or T-01 (MOI 10) for 10 hours (in the left panels) or with DMSO or HSV-60 (10 μg/mL) for 3 hours (in the right panels). The cell lines susceptible to T-01 are labeled with red. The data are representative of 3 independent experiments.

Figure S9

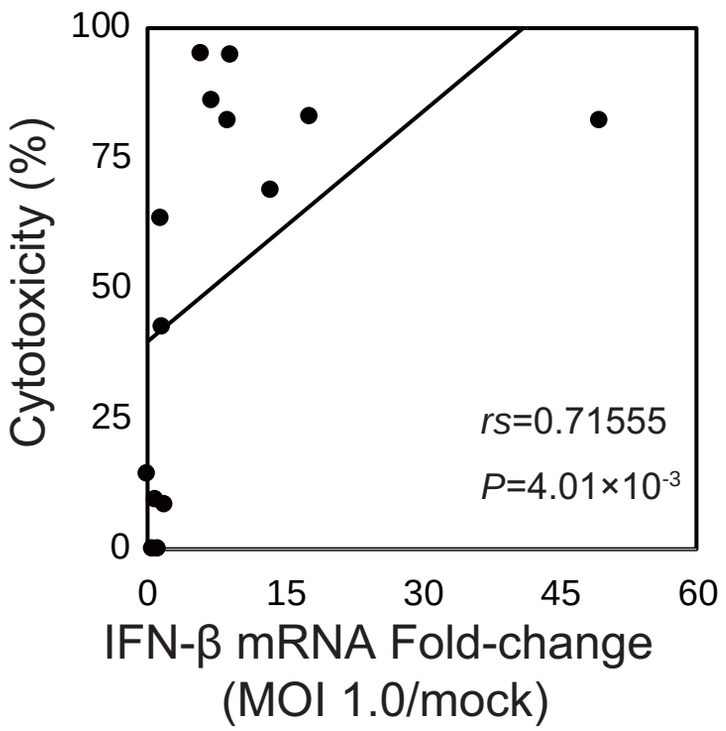


Figure S9. Correlation between IFN-β mRNA expression and cytotoxicity by T-01.

The 14 cell lines examined in Figure S8A were infected with T-01 at MOI 1.0 for 24 hours. The expression levels of IFN-β mRNA was measured by quantitative RT-PCR. Cytotoxicity was calculated as 1 - (viable cell number with T-01/viable cell number with mock) x 100 (%) after treatment with T-01 (MOI 1.0) for 3 days. Statistical analysis was conducted using Spearman's correlation.

Figure S10

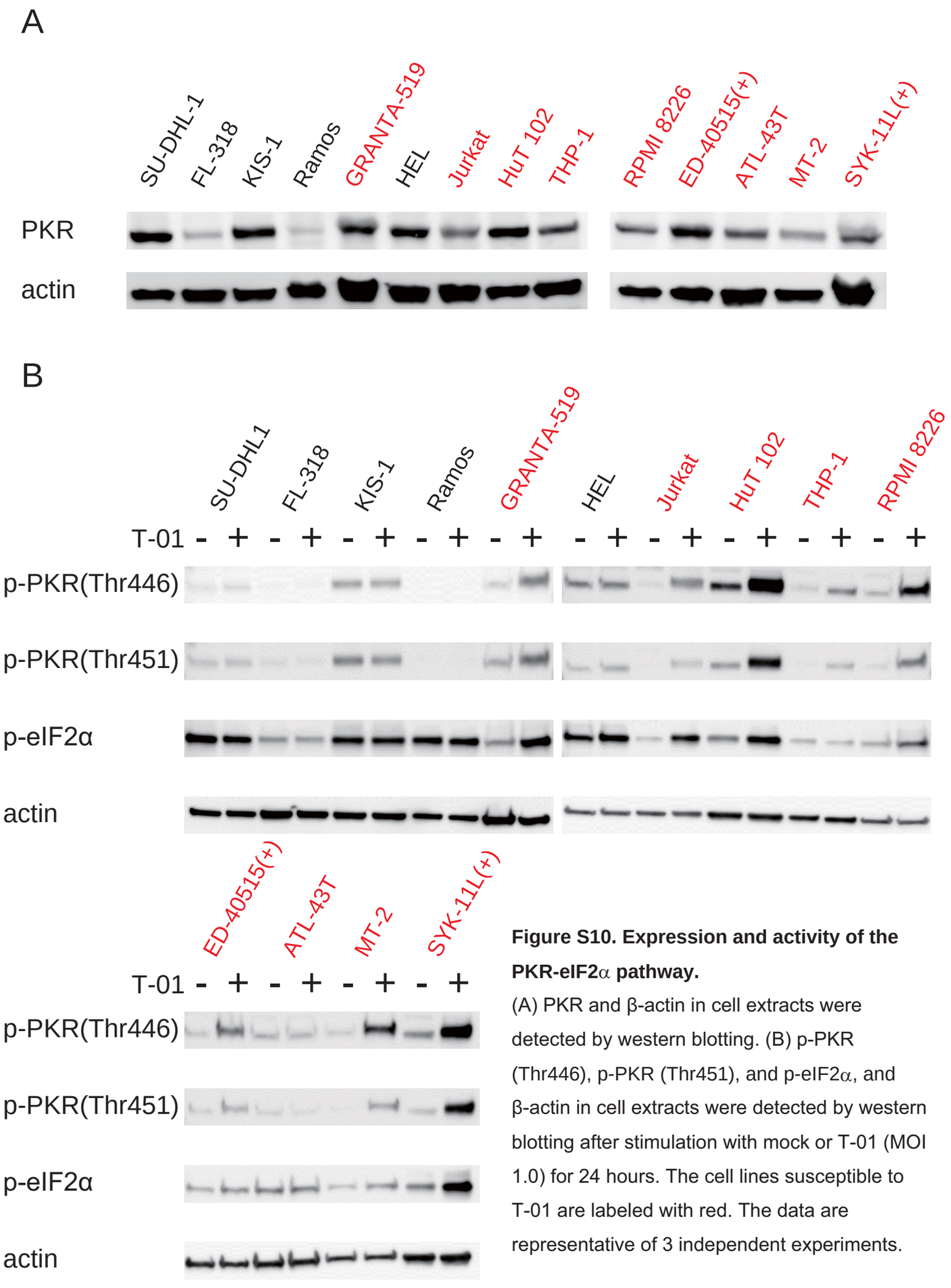


Figure S10. Expression and activity of the PKR-eIF2α pathway.

(A) PKR and β-actin in cell extracts were detected by western blotting. (B) p-PKR (Thr446), p-PKR (Thr451), and p-eIF2α, and β-actin in cell extracts were detected by western blotting after stimulation with mock or T-01 (MOI 1.0) for 24 hours. The cell lines susceptible to T-01 are labeled with red. The data are representative of 3 independent experiments.

Table S1 related to Figure 1. Human cell lines of hematological malignancies

Cell line	Origin	Source	Cytotoxicity
Myeloid-derived			
THP-1	Acute monocytic leukemia	JCRB	+
SET-2	Acute megakaryoblastic leukemia	K.Shimoda	+
SKM-1	Acute myeloid leukemia	JCRB	+
MEG-01	Chronic myeloid leukemia	ATCC	+
HEL	Acute erythroid leukemia	JCRB	-
HL-60	Acute promyelocytic leukemia	ATCC	-
CHRF-288-11	Acute megakaryoblastic leukemia	K. Shimoda	-
T cell-derived			
Jurkat	Acute T-cell leukemia	K. Imada	+
ATL-43T	Adult T-cell leukemia	M. Maeda	+
SYK-11L(+)	Adult T-cell leukemia	K. Imada	+
ED-40515(+)	Adult T-cell leukemia	M. Maeda	+
MT-2	Adult T-cell leukemia	K. Imada	+
HuT 102	Adult T-cell leukemia	M. Nishikori	+
SU-DHL-1	Anaplastic large cell lymphoma	M. Nishikori	-
SR-786	Anaplastic large cell lymphoma	M. Nishikori	-
B cell-derived			
GRANTA-519	Mantle cell lymphoma	M. Nishikori	+
Raji	Burkitt lymphoma	M. Nishikori	+
KM-H2	Hodgkin lymphoma	M. Nishikori	+
RPMI 8226	Plasma cell myeloma	JCRB	+
KMS-12-BM	Plasma cell myeloma	JCRB	+
MM.1S	Plasma cell myeloma	ATCC	+
NCI-H929	Plasmacytoma	ATCC	+
U266	Plasmacytoma	ATCC	+
Ramos	Burkitt lymphoma	M. Nishikori	-
FL-318	Diffuse large B-cell lymphoma	M. Nishikori	-
KIS-1	Diffuse large B-cell lymphoma	M. Nishikori	-

ATCC: American Type Culture Collection

JCRB: Japanese Collection of Research Bioresources Cell Bank

Cytotoxicity : with (+) or without (-) statistically significant cytotoxicity with MOI 0.01, 0.1, or 1.0 of T-01, as shown in Figure S1

Table S2 related to Figure 4. Clinical samples

Patient #	Disease	Age	Sex	Sample	Cytotoxicity	Nectin-1	HVEM
Relapsed							
1	AML	62	M	PB	+	+	-
2	AML	68	F	PB	+	+	+
3	AML	78	F	PB	+	+	-
4	AML	85	M	PB	+	+	-
5	T-LBL	31	M	PB	+	+	+
6	PTCL	77	M	Ascites	+	+	+
7	MM	68	F	Ascites	+	+	+
8	Plasmacytoma	69	M	PE	+	+	-
9	SMZL	52	M	Spleen	-	-	+
10	FL	56	F	LN	-	-	+
Untreated							
11	AML	67	F	BM	-	-	+
12	AML	83	M	BM	-	-	-
13	AML	89	M	BM	-	-	-
14	ATLL	65	F	PB	-	-	-
15	PTCL	76	F	PE	-	-	+

AML: acute myeloid leukemia; T-LBL: T-lymphoblastic leukemia; PTCL: peripheral T-cell lymphoma; MM: multiple myeloma; SMZL: splenic marginal zone lymphoma; FL: follicular lymphoma; PB: peripheral blood; PE: pleural effusion; LN: lymph node

Cytotoxicity positive (+): decrease in viability at MOI 10 $\geq$ 20% compared to mock treatment; (-): < 20% compared to mock treatment

Nectin-1, HVEM positive (+): MFI ratio $\geq$ 1.5; negative (-): MFI ratio < 1.5

Table S3. List of antibodies

Reagent	Isotype	Clone	Source	Catalog#
FITC Mouse IgG1 Isotype	Mouse IgG1, k	MOPC-21	BD Biosciences	555748
FITC anti-human CD38	Mouse IgG1, k	HB7	BD Biosciences	340927
FITC HLA-DR	Mouse IgG2b, k	B8.12.2	Beckman Coulter	IM0463U
FITC Mouse IgG1 Isotype	Mouse IgG1, k	MOPC-21	BioLegend	400110
FITC Mouse IgG2a Isotype	Mouse IgG2a, k	MOPC-173	BioLegend	400208
FITC Mouse IgG2b, k Isotype Ctrl	Mouse IgG2b, k	MPC-11	BioLegend	400309
FITC anti-human CD117(c-kit)	Mouse IgG1, k	104D2	BioLegend	313231
FITC anti-human CD19	Mouse IgG1, k	HIB19	BioLegend	302206
FITC anti-human CD138	Mouse IgG1, k	MI15	BioLegend	356508
FITC anti-human CD3	Mouse IgG2a, k	HIT3a	BioLegend	300305
FITC anti-human CD14	Mouse IgG2a, k	M5E2	BioLegend	301803
FITC anti-human CD34	Mouse IgG2a, k	561	BioLegend	343603
FITC anti-human CD45RO	Mouse IgG2a, k	UCHL1	BioLegend	304242
FITC anti-human CD4	Mouse IgG2b, k	OKT4	BioLegend	317408
Goat anti-mouse IgG(H&L),FITC conjugate	Goat IgG		Thermo Fisher	A24525
PE Mouse IgG1 Isotype	Mouse IgG1, k	679.1Mc7	Beckman Coulter	A07796
PE anti-human CD117	Mouse IgG1, k	104D2D1	Beckman Coulter	IM2732
PE Mouse IgG1, k Isotype Ctrl(FC)	Mouse IgG1, k	MOPC-21	BioLegend	400113
PE anti-human CD111(Nectin-1)	Mouse IgG1, k	R1.302	BioLegend	340404
PE anti-human CD270(HVEM, TR2)	Mouse IgG1, k	122	BioLegend	318805
PE anti-human CD3	Mouse IgG2a, k	HIT3a	BioLegend	300307
APC Mouse IgG1, k Isotype Ctrl(FC)	Mouse IgG1, k	X40	BD Biosciences	340442
APC anti-human CD34	Mouse IgG1, k	581	BD Biosciences	555824
APC anti-human CD38	Mouse IgG1, k	HB7	BD Biosciences	340439
APC Mouse IgG1, k Isotype Ctrl(FC)	Mouse IgG1, k	MOPC-21	BioLegend	400122
APC anti-human CD19	Mouse IgG1, k	HIB19	BioLegend	302212
APC Mouse IgG2a, k Isotype Ctrl	Mouse IgG2a, k	MOPC-173	BioLegend	400219
APC anti-human CD3	Mouse IgG2a, k	HIT3a	BioLegend	300311
APC anti-human CD34	Mouse IgG2a, k	561	BioLegend	343608
Alexa Fluor 488 Mouse IgG1, k Isotype Ctrl(FC)	Mouse IgG1,k	MOPC-21	BioLegend	400132
Alexa Fluor 488 anti-human CD80	Mouse IgG1,k	2D10	BioLegend	305213
Alexa Fluor 488 Mouse IgG2b, k Isotype Ctrl	Mouse IgG2b,k	MPC-11	BioLegend	400329
Alexa Fluor 488 anti-human CD86	Mouse IgG2b,k	IT2.2	BioLegend	305413
Myosin IIa Antibody	Rabbit IgG		Cell Signaling	3403
Myosin IIb XP Rabbit mAb	Rabbit IgG	D8H8	Cell Signaling	8824
Myosin IIc Rabibit mAb	Rabbit IgG	D4A7	Cell Signaling	8189
cGAS Rabbit mAb	Rabbit IgG	D1D3G	Cell Signaling	15102
STING Rabbit mAb	Rabbit IgG	D2P2F	Cell Signaling	13647
PKR	Mouse IgG2b, k	B-10	Santa Cruz	sc-6282
Anti-PKR(phospho T446)antibody	Rabbit IgG	E120	Abcam	ab32036
Anti-PKR(phospho T451)antibody	Rabbit IgG	EPR2152Y	Abcam	ab81303
Phospho-eIF2a(Ser51) XP Rabbit mAb	Rabbit IgG	D9G8	Cell Signaling	3398
TBK1/NAK Rabbit mAb	Rabbit IgG	D1B4	Cell Signaling	3504
Phospho-TBK1/NAK(Ser172)XP Rabbit mAb	Rabbit IgG	D52C2	Cell Signaling	5483
IRF-3(D6I4C)XP Rabbit mAb	Rabbit IgG	D6I4C	Cell Signaling	11904
Phospho-IRF-3(Ser386)XP Rabbit mAb	Rabbit IgG	E7J8G	Cell Signaling	37829
Human HMGB1/HMG-1 antibody	Mouse IgG2b	115603	R&D systems	MAB1690
Anti-rabbit IgG,HRP-linked Antibody	Goat		Cell Signaling	7074
Anti-Mouse IgG–Peroxidase antibody	Rabbit		Sigma-Aldrich	A9044
Anti-VSV-G tag mAb	Mouse	P5D4	Abcam	ab50549

Supplemental Methods

Cell lines

Cells except for the following cell lines were cultured in RPMI-1640 (Sigma-Aldrich) containing 10% heat-inactivated fetal bovine serum (FBS) (Corning, Corning, NY) and 5 mM HEPES (Nacalai Tesque, Kyoto, Japan). SET-2 cells were cultured in DMEM (Sigma-Aldrich) containing 10% FBS, 10 μM 2-mercaptoethanol (Fujifilm Wako), and MEM non-essential amino acid solution (Fujifilm Wako). CHRF-288-11 and HL-60 cells were cultured in IMDM (Fujifilm Wako) containing 10% and 20% FBS, respectively. ED-40515(+), ATL-43T, and SYK-11L(+) cells were cultured in RPMI-1640 containing 10% FBS, 5mM HEPES, and 8 U/mL human IL-2 (BioLegend). Vero and HEK293T cells were cultured in DMEM containing 10% FBS.

Plasmids and transfection

Human nectin-1 shRNA (#245117), control shRNA (#246995), human nectin-1 expression (#281558), and pLX304 control (#25890) lentiviral vector plasmids were purchased from Dharmacon (Lafayette, CO). QIAprep Spin Miniprep Kit (Qiagen) was used for purification of the plasmid. Approximately 24 hours before transfection, HEK293T cells (5×10^5) were seeded into 6-well culture plates in 2 mL growth medium (DMEM + 10% FBS) and incubated at 37 °C, 5% CO_2 overnight. When cells were about 85–95% confluent, half of the medium was replaced. Transfection of DNA mixture of packing plasmids and lentivirus vectors was performed by using Lipofectamine 3000 (Thermo Fisher) according to the manufacturer's instructions. About 16-20 hours after transfection, the transfection medium was replaced with 2 mL fresh complete growth medium (high glucose DMEM + 10% FBS) and cells were incubated at 37 °C for additional 48 hours. Thereafter, lentiviral supernatants were harvested and centrifuged at 1600g, 4 °C for 10 min. The supernatant was stored in -80°C until used. Cell lines (Jurkat, THP-1, Ramos, and E.G7-OVA) were infected by adding half the volume with the lentiviral supernatants plus polybrene (Nacalai Tesque) at a 5 μg/mL final concentration. The infected cells were incubated for 24 hours and then given fresh growth media for 24–48 hours before beginning selection. The infected cells were propagated in medium containing 10 μg/mL puromycin (InvivoGen, San Diego, CA) or 10 μg/mL blasticidin S (Fujifilm Wako).

Detection of 3-OS HS

After blocking with 5% human IgG and 1% goat serum for 15 min on ice, cells were stained with a vesicular stomatitis virus (VSV)-tagged single chain variable fragment antibody (HS4C3) that recognizes 3-*O*-sulfated oligosaccharide structures[1] for 30 min on ice. After washing, cells were stained with mouse anti-VSV-G tag mAb (Abcam, Cambridge, UK) for 30 min on ice, followed by staining with FITC-conjugated goat anti-mouse IgG (H+L) (Thermo Fisher) for 30 min on ice. Negative control cells were stained by omitting HS4C3. Cells were analyzed by flow cytometry.

Western blotting

Equal numbers of cells were resolved on SDS–PAGE (Criterion™ TGX™). Trans-Blot Turbo™ Transfer System was used for the transfer of proteins to PVDF membranes (all reagents from Bio-Rad, Hercules, CA). The membrane was blocked in 5% Difco™ skim milk (BD Biosciences) or 5% bovine serum albumin (Roche Diagnostics, Rotkreuz, Switzerland). The signal was detected using an ImageQuant LAS-4010 (GE Healthcare, Chicago, IL). HSV-60 (InvivoGen) was added to cells with Lipofectamine 3000 (Thermo Fisher).

Epigenetic regulation

A chemical library for epigenetics research (containing 80 compounds) was purchased from Sigma-Aldrich (S990043-EPI1). Decitabine, I-BET 726, and LMK-235 were purchased from Selleck (Houston, TX) and dissolved in DMSO. Cells were seeded in a 96-well plate at 1×10^5 cells per well, and each compound was added at the indicated concentrations. After incubation for 24 hours, cells were collected and analyzed.

Reverse transcription and real-time PCR for IFN-β mRNA

Total RNA was isolated using an RNeasy Mini Kit (Qiagen). PrimeScript RT Master Mix (Takara Bio Inc., Kusatsu, Japan) was used for cDNA synthesis. The samples were run on a ViiA7 Real-Time PCR System (Applied Biosystems). Amplification was performed using SYBR Premix Ex Taq II (Takara Bio Inc.) as follows: 95°C for 30 seconds followed by 40 cycles of 95°C for 5 seconds and 60°C for 30 seconds. All the experiments were performed in triplicate. RPL13A was used as an endogenous control. The ΔΔCt method was used to quantify the relative amount of mRNA in each sample in comparison with the control. Primer sequences used were: RPL13A (forward: 5'-TGTTGGACTTTCCACCTG-3', reverse: 5'-AACCCCTTGGTTGTGC-3'), IFN-β (forward: 5'-CGACACTGTTCGTGTTGTCA-3', reverse: 5'-GAAGCACAACAGGAGAGCAA-3')

Animal experiments

Four-week-old female SCID Beige mice were purchased from Charles River Laboratories Japan, Inc. and were used in experiments at five weeks of age. GRANTA-519 or ED-40515(+) cells (5×10^6) in a mixture of 50 μL of RPMI-1640 without serum and 50 μL of Matrigel (BD Biosciences) were implanted subcutaneously into the left flank.[2] When tumors reached about 5 mm in diameter, mice were randomized, and T-01 (2×10^5 or 1×10^6 pfu) or mock in 20μL PBS containing 10% glycerol was injected into the tumors on days 0 and 3. The mice were killed when the maximum diameter of tumors exceeded 20 mm. The tumor size was measured using Vernier calipers every 2 or 3 days. The tumor volume was calculated using the formula $1/2 \times [\text{long axis}] \times [\text{short axis}]^2$.

Four-week-old female C57BL/6 mice were purchased from Charles River Laboratories Japan, Inc. and were used in experiments at five weeks of age. E.G7-OVA-nectin-1 cells (1×10^6) in a mixture of 50 μL of RPMI-1640 without serum and 50 μL of Matrigel were implanted subcutaneously into the right and left flanks.[2] When tumors reached about 5 mm in diameter, mice were randomized, and T-01 (2×10^6 pfu) or mock in 20 μL PBS containing 10% glycerol was injected into the right-side tumors on days 0 and 3. The mice were killed 10 days after the second injection of T-01 or mock, and TILs and spleen cells were isolated. The cells were stained with FITC-conjugated rat anti-mouse CD8 mAb, biotinylated and streptavidin-PE-bound H-2K^b OVA tetramer or negative (SIY) tetramer (MBL), and 7-aminoactinomycin D (BioLegend). OVA tetramer$^+$CD8$^+$ T cells were detected by flow cytometry.

Supplemental References

1. ten Dam, G.B., Kurup, S., van de Westerlo, E.M.A., Versteeg, E.M.M., Lindahl, U., Spillmann, D., et al. (2006). 3-O-Sulfated Oligosaccharide Structures Are Recognized by Anti-heparan Sulfate Antibody HS4C3. J. Biol. Chem. *281*, 4654-4662.
2. Mezencev, R., and McDonald, J.F. (2011). Subcutenous xenografts of human T-lineage acute lymphoblastic leukemia Jurkat cells in nude mice. In Vivo *25*, 603-607.