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Supplemental Information

LAG-3xPD-L1 bispecific antibody potentiates antitumor responses of T cells through dendritic cell activation

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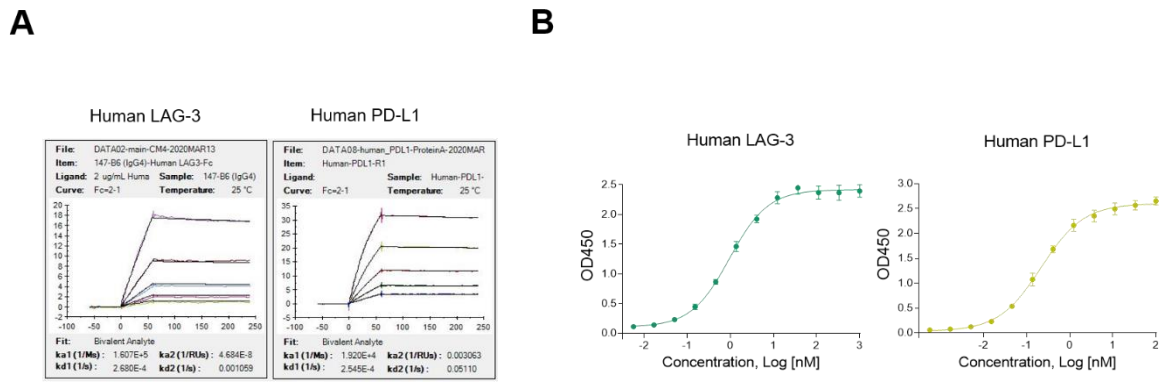


Figure S1. Measurement of the LAG-3 or PD-L1 binding affinity of ABL501

(A) SPR analysis of ABL501 binding against human recombinant LAG-3 (left) and PD-L1 (right). The binding affinity (K_D) of ABL501 was 2.0 nM to human LAG-3 and 16 nM to human PD-L1. (B) ELISA of ABL501 binding to human recombinant LAG-3 (left, EC_{50}=0.958 nM) and PD-L1 (right, EC_{50}=0.532 nM). Data were pooled from three independent experiments.

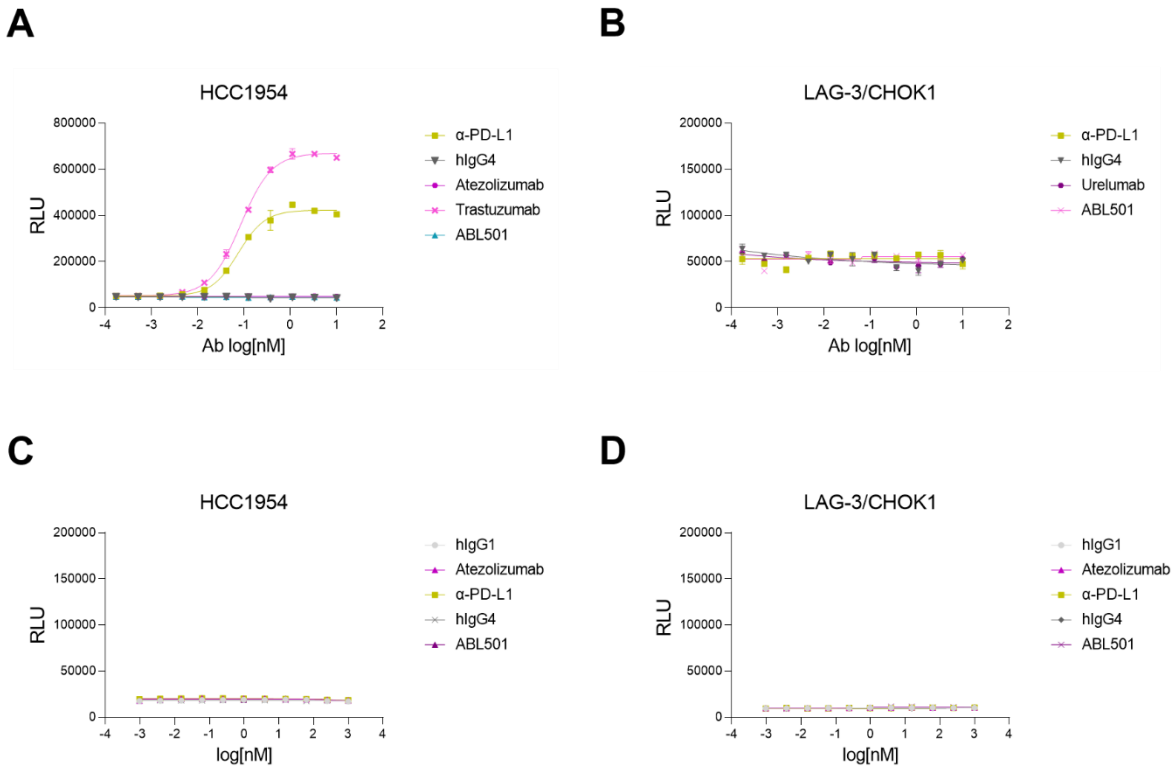


Figure S2. Measurement of Fc-mediated effector functions of ABL501

(A-B) ADCC activity of ABL501 was evaluated by co-incubation of NFAT-reporter Jurkat cells expressing hFcγRIIIa-V158 variant with tumor cells expressing PD-L1 or LAG-3. Measurement of ADCC activity of the indicated antibodies against (A) HCC1954 cells expressing PD-L1 or (B) CHOK1 cells expressing LAG-3. (C-D) CDC activity of ABL501 was evaluated in tumor cells expressing PD-L1 or LAG-3 via co-incubation with human complement serum. Measurement of CDC activity of the indicated antibodies in (C) HCC1954 cells expressing PD-L1 or (D) CHOK1 cells expressing LAG-3. Data were pooled from three independent experiments.

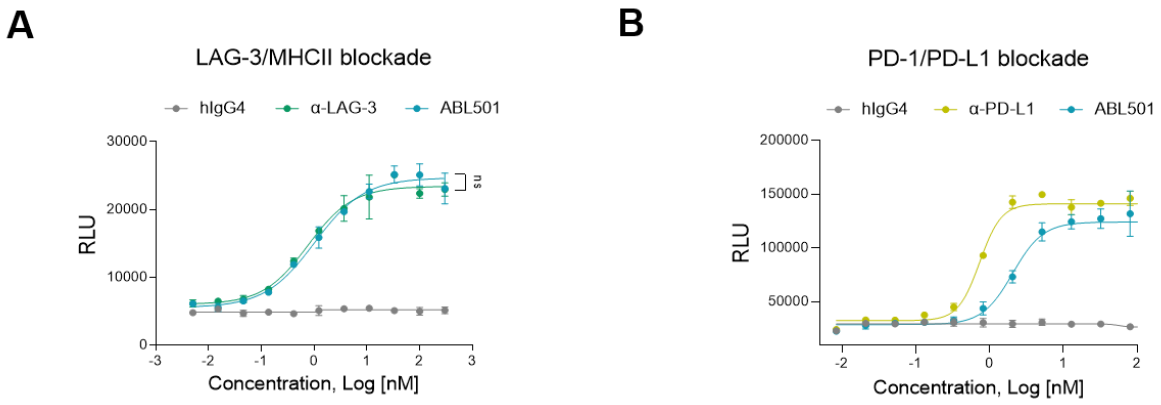


Figure S3. Inhibition of PD-1/PD-L1 or LAG-3/MHC-II signaling by ABL501

(A-B) The effect of ABL501 on LAG-3/MHC-II or PD-1/PD-L1 signaling was assessed by co-incubation of NF-κB-Luc2/LAG-3 or PD-1 reporter Jurkat cells with target cells expressing MHC-II or PD-L1 (Bioassay). (A) Blocking activity of ABL501 or anti-LAG-3 against LAG-3/MHC-II was measured by luciferase activity assay in NF-κB-Luc2/LAG-3 reporter Jurkat cells. (ABL501: EC_{50}=0.84 nM, anti-LAG-3: EC_{50}=0.60 nM). (B) Blocking activity of ABL501 or anti-PD-L1 against PD-1/PD-L1 was measured by luciferase activity assay in NF-κB-Luc2/PD-1 reporter Jurkat cells. (ABL501: EC_{50}=2.8 nM, anti-PD-L1: EC_{50}=0.72 nM). Data were pooled from three independent experiments.

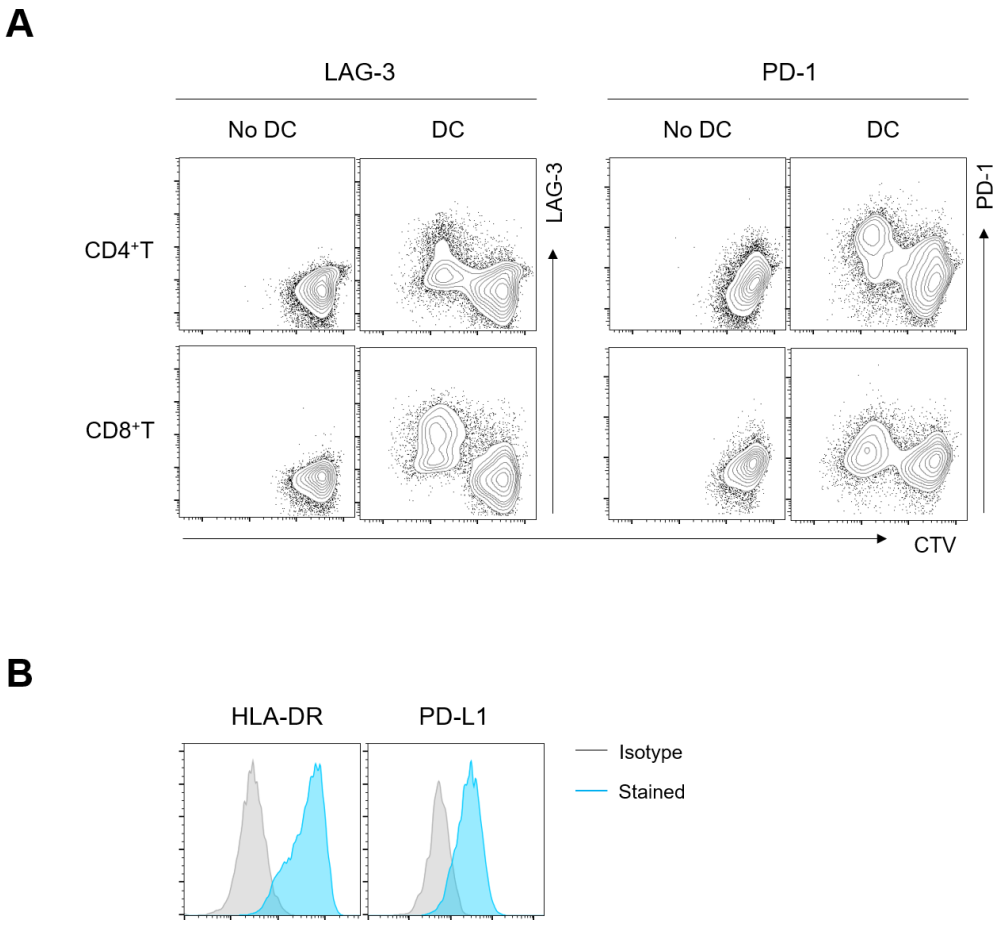


Figure S4. Upregulation of LAG-3 and PD-1 expressions by T cells upon stimulation with allogeneic mo-DCs

$CD4^+$T or $CD8^+$Tcells isolated from PBMCs were co-cultured with allogeneic mo-DCs in the presence of anti-human IgG antibody at a final concentration of 33.4 nM for 5 days. (A) FACS analysis showing LAG-3 and PD-1 expressions in CTV_{low} $CD4^+$ or $CD8^+$T cells. (B) FACS histograms showing the expression of HLA-DR and PD-L1 in mo-DCs.

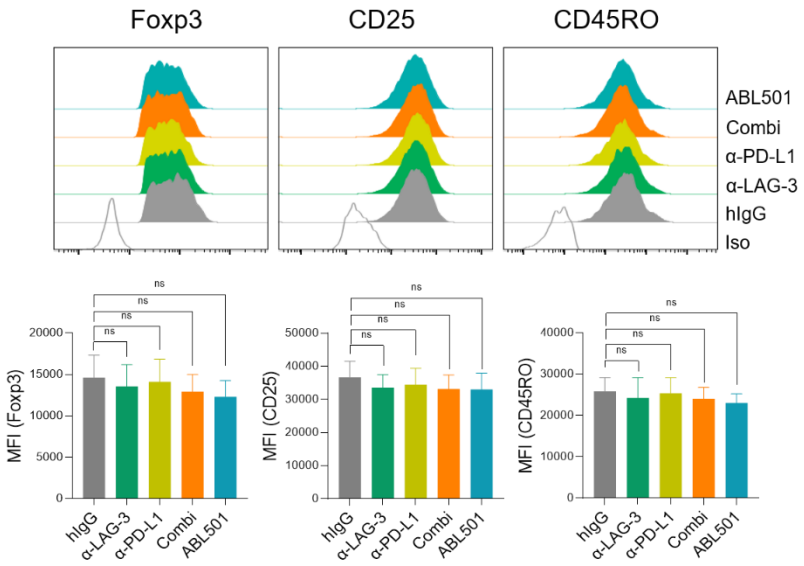


Figure S5. Effect of ABL501 on phenotypic characteristics of T_{reg} cells

Isolated and ex vivo expanded Treg cells were co-cultured with A375-OKT3 cells in the presence of the indicated antibodies at a final concentration of 66.8 nM. Representative FACS histograms (above) and summary graphs (below) showing the expression of Foxp3, CD25 and CD45RO in T_{reg} cells. Data were compiled from three independent experiments with two replicates. Statistical significance was determined by one-way ANOVA with Holm–Sidak multiple comparisons. ns: not significant.

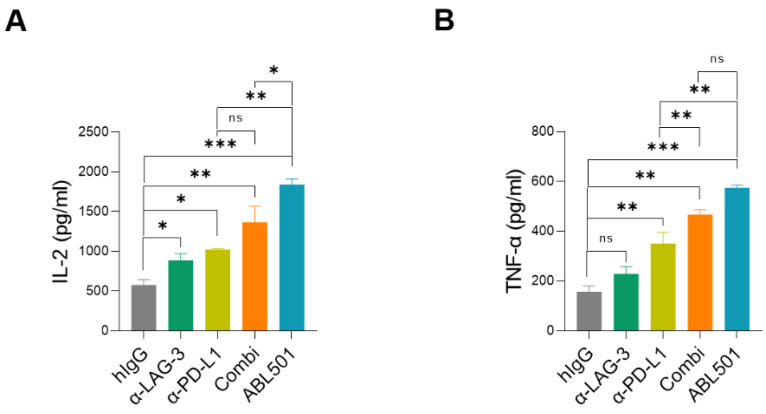


Figure S6. ABL501 increases IL-2 and TNF-α secretion by T cells upon stimulation with CMV lysate

(A-B) CTV-stained PBMCs were stimulated with CMV lysate in the presence of the indicated antibodies at a final concentration of 66.8 nM for 5 days. ELISA of IL-2 (A) or TNF-α (B) secretion by CMV-responsive T cells with the indicated antibodies. Data were pooled from three independent experiments with two replicates. Statistical significance was determined by one-way ANOVA with Holm–Sidak multiple comparisons. *p<0.05, **p<0.01, ***p<0.001, ns: not significant.

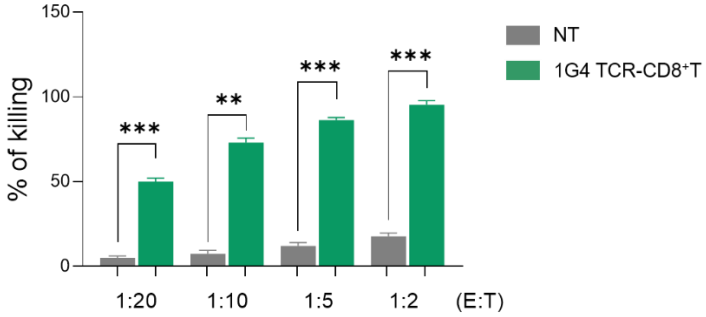


Figure S7. NY-ESO-1-specific killing of A375 cells by 1G4 TCR-CD8$^+$T cells

NT, or 1G4 TCR-CD8$^+$T cells were co-cultured with A375 cells expressing luciferase at variable E:T ratios for 2 days. A375 cell killing was measured by luciferase activity. Bar graph showing percentages of A375 cell killing by NT or 1G4 TCR-CD8$^+$T cells. Data were pooled from three independent experiments with three replicates. Statistical significance was determined by paired *t*-tests with two-tailed analysis. $^{**}p<0.01$ and $^{***}p<0.001$

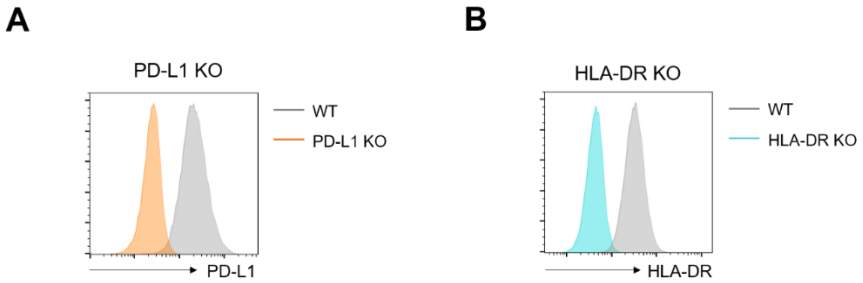


Figure S8. Establishment of HLA-DR KO or PD-L1 KO A375 cells

(A) Flow cytometric analysis of the surface expression of PD-L1 in A375-WT (parental) and A375 PD-L1 KO cells after immunostaining with a fluorochrome-conjugated anti-PD-L1 antibody. (B) Flow cytometric analysis of the surface expression of HLA-DR in A375-WT (parental) and A375 HLA-DR KO cells after immunostaining with a fluorochrome-conjugated anti-HLA-DR antibody.

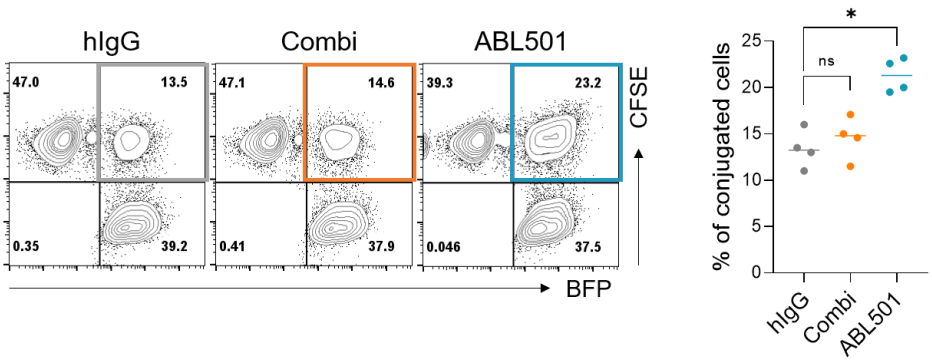


Figure S9. Effect of ABL501 on CD4^{+}T cell conjugation with tumor cells

CFSE-labeled CD4^{+}T cells expressing LAG-3 by pre-activation with anti-CD3/CD28 antibodies were incubated with A375-OKT3-PD-L1 cells for 30 min in the presence of the indicated antibodies and followed by FACS analysis. Representative FACS plots (left panel) and a summary plot (right panel) showing the percentages of BFP and CFSE double-positive populations in the presence of the indicated antibodies. Data were compiled from two independent experiments with two replicates. Statistical significance was determined by one-way ANOVA with Holm–Sidak multiple comparisons. *p<0.05, ns: not significant.

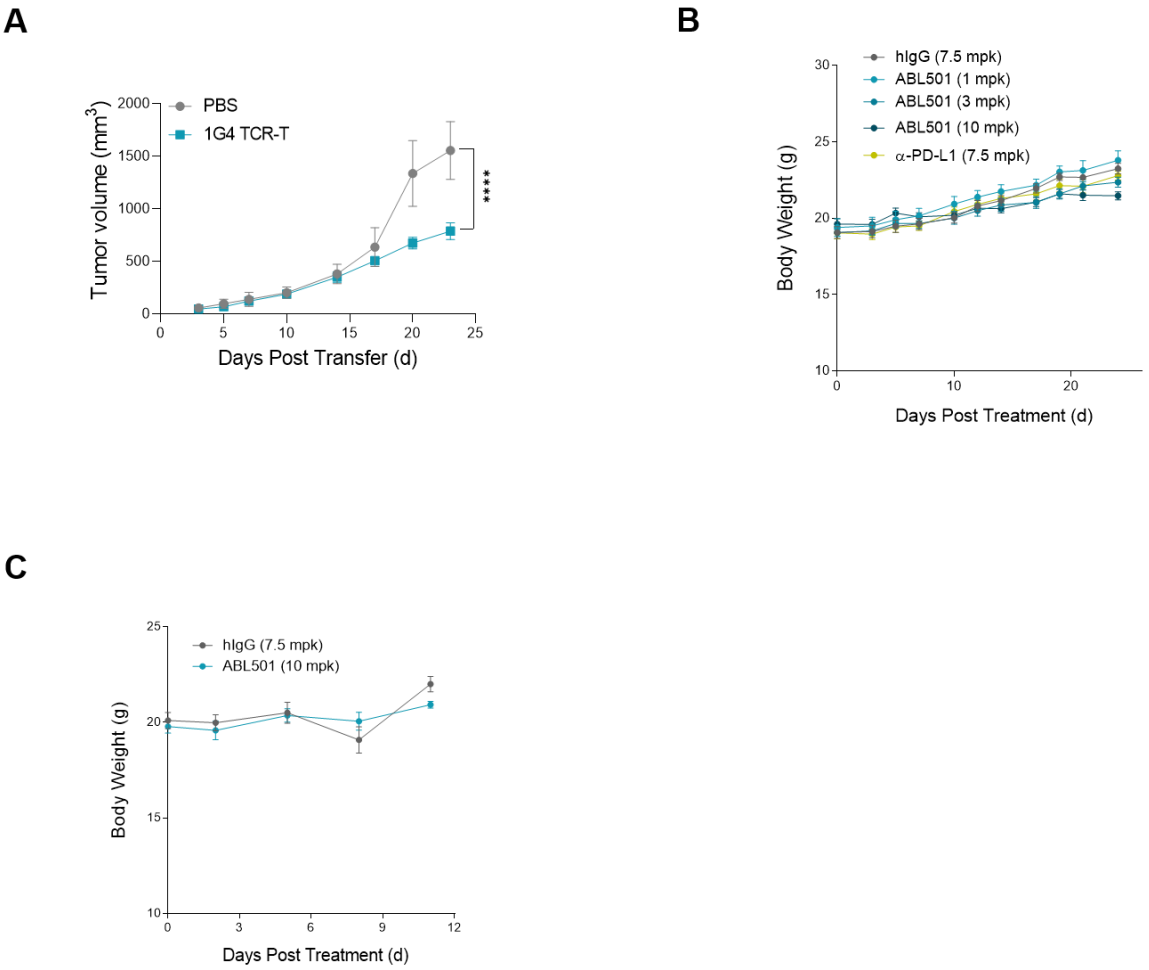
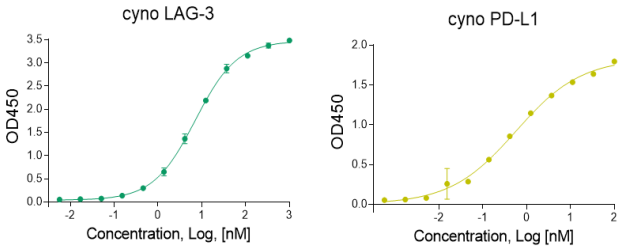


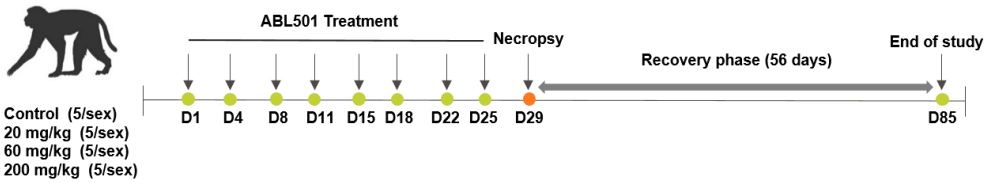
Figure S10. ABL501 inhibits tumor growth in humanized mouse models

(A) Tumor volume of NSG mice bearing A375-PD-L1 following adoptive transfer with $CD8^{+}$T cells expressing an NY-ESO-specific TCR. (B) Graphs of body weight change in human LAG-3/PD-1 knock-in BALB/c mice. (C) Graphs of body weight change in human LAG-3/PD-1/PD-L1 knock-in C57BL/6 mice. Significant differences between groups were determined by Two-way ANOVA Tukey's multiple comparison test. ****p <0.001.

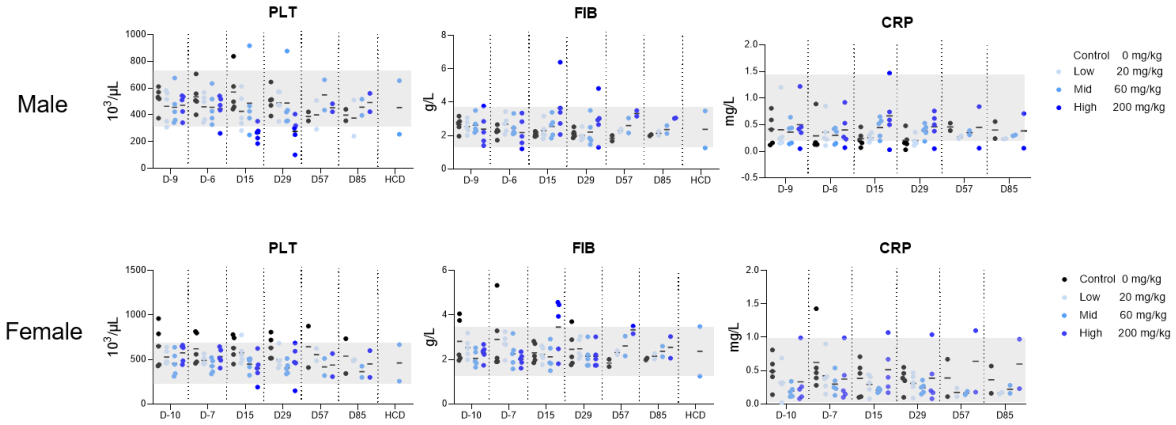
A


cyno LAG-3
OD450
Concentration, Log, [nM]
cyno PD-L1
OD450
Concentration, Log [nM]

B


ABL501 Treatment
Necropsy
Recovery phase (56 days)
End of study
D1 D4 D8 D11 D15 D18 D22 D25 D29
D85
Control (5/sex)
20 mg/kg (5/sex)
60 mg/kg (5/sex)
200 mg/kg (5/sex)

C


Male
PLT
FIB
CRP
Female
Control 0 mg/kg
Low 20 mg/kg
Mid 60 mg/kg
High 200 mg/kg

D

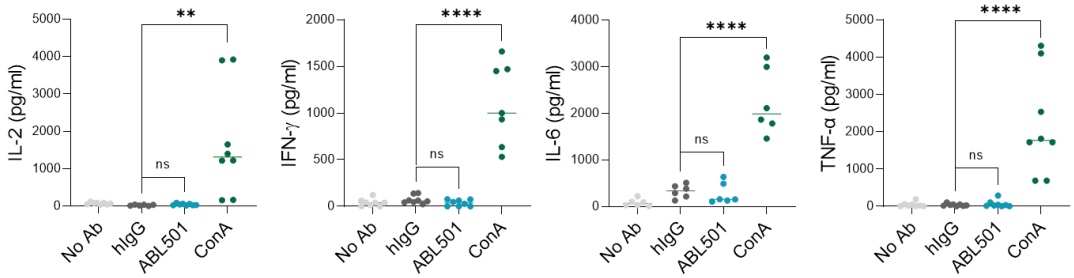

IL-2 (pg/ml)
IFN-γ (pg/ml)
IL-6 (pg/ml)
TNF-α (pg/ml)
No Ab
hIgG
ABL501
ConA
ns

Figure S11. ABL501 exhibits a safe toxicity profile in non-human primates

(A) ELISA of ABL501 binding to cynomolgus recombinant LAG-3 (left, EC50=7.05 nM) and PD-L1 (right, EC50=0.532 nM). Data were pooled from three independent experiments. (B) A schematic overview of the safety study in cynomolgus monkeys. Cynomolgus monkeys were administered twice weekly doses of ABL501 at 20, 60, and 200 mg/kg for up to 8 times. Necropsies were performed on day 29 after the first dose. (C) Platelet (PLT) number, fibrinogen (FIB), and C-reactive protein (CRP) were measured over time. Gray areas denote the historical normal range. (D) ELISA of IL-2, IFN-γ, IL-6, and TNF-α production by human PBMC cultured for 48 h in the presence or absence of hIgG1 (1 μg/well) or ABL501 (1.4 μg/well). Concanavalin A (Con A) at 5 μg/mL was used as a positive control. Each dot represents an individual human sample. Statistical significance was determined by one-way ANOVA with Holm–Sidak multiple comparisons in (D). $^{**}p<0.01$ and $^{****}p<0.0001$, ns: not significant.

Patient characteristics

Variable	Non-relapsed (N=9)	Relapsed (N=11)
Age (yr)	64 (52-75)	62.6 (53-74)
Sex		
Male	8	8
Female	1	3

Figure S12. Cholangiocarcinoma (CCA) patient characteristics

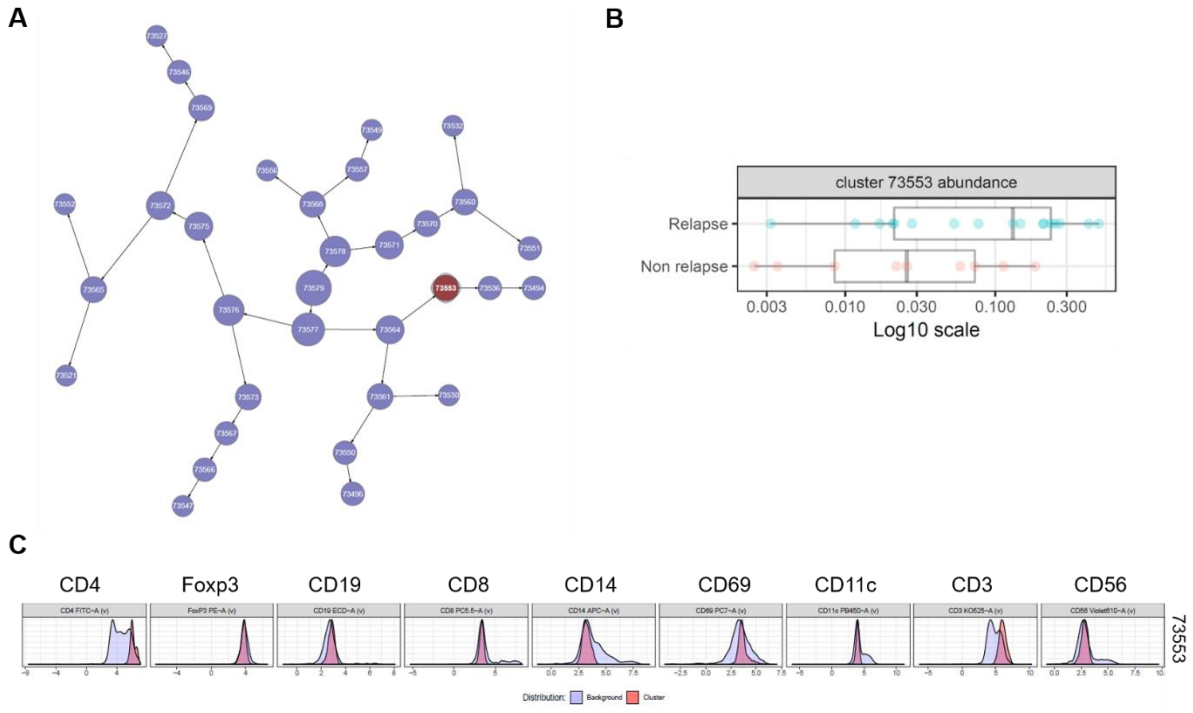


Figure S13. CITRUS analysis of peripheral blood immune cells in CCA patients

(A) CITRUS plot showing clusters from CCA patients in two groups (Relapse vs Non-relapse). The red dot represents a cluster that showed a different abundance with a statistical significance between groups. (B) Bar graphs showing the relevance of abundance for the selected cluster (red dot) between groups. (C) Histogram plots showing each marker expression by the selected cluster (red) over background (light blue).

Supplementary Methods

Expression and purification of recombinant antibodies

ExpiCHO-S cells were cultured in ExpiCHO expression medium (A2910001; Thermo Fisher) in a humidified shaking incubator at 37℃ with 8% CO_2. Transfection of ExpiCHO cells was performed using an ExpiCHO Expression System Kit (A29133; Thermo Fisher) according to the manufacture's protocol. Briefly, ExpiFectamine CHO transfection reagent and plasmid DNA were separately diluted in OptiPRO SFM (12309019; Thermo Fisher) and incubated at room temperature for 5 min. ExpiFectamine CHO-DNA-OptiPRO mixture was added to the cells (6×10^6 cells). At 18-22 h post-transfection, ExpiFectamine CHO Transfection Enhancer and ExpiFectamine CHO Transfection Feed were added, and the cells were further incubated for 7 days. Each antibody was purified from the cell culture supernatant by recombinant Protein A affinity chromatography (HiTram MabSelect SuRe, 28-4082-55; GE Healthcare). Equilibration was carried out using buffer (50 mM Tris-HCl, pH 7.4), and the sample was loaded onto the equilibrated column. The recombinant antibody was eluted with elution buffer (50 mM citrate, pH 3.4) and then neutralized to pH 6.5 using 1 M Tris-HCl (pH 9.0). Purified antibodies were concentrated by ultrafiltration using an Amicon Ultra 15 30K device (MilliporeSigma), and endotoxin levels of all antibodies were measured under 1.0 EU (endotoxin unit)/mg by Endosafe nexgen-PTS (Charles River Laboratories).

Enzyme-linked Immunosorbent assay (ELISA)

The binding properties of ABL501 against LAG-3 or PD-L1 proteins (human or monkey) was analyzed by ELISA. ELISA for PD-L1 proteins was performed at 37℃, whereas the LAG-3 binding assay was carried out at 25℃. Each well of the 96-well plate was coated with 0.5 μg/mL of human PD-L1 (10084-H08H; Sino Biological), monkey PD-L1 (90251-C08H; Sino

Biological), human LAG-3 (16498-H08H; Sino Biological), or 2 μg/mL of monkey LAG-3 (9992-L3; R&D Systems) overnight at 4℃. Diluted antibodies in blocking solution was added to the plate, which was then incubated for 1 h. After washing, diluted anti-human IgG Fc HRP conjugated antibody (31413; Pierce) in blocking solution was added, and the plate was incubated at for 1 h. The reaction was stopped by the addition of 50 μL/well of 0.5 N H_2SO_4 following 100 μL/well of tetramethylbenzidine (T0440; MilliporeSigma) treatment. The absorbance was measured with an ELISA plate reader at 450 nm (calibration: 650 nm) within 5 min, and the data were analyzed using the four-parameter logistics equation from the SoftMax Pro 5.4 software (Molecular Device).

Surface plasmon resonance (SPR)

(1) PD-L1 binding assay

ABL501 was diluted with 1xHBS-EP (BR100669; Cytiva) containing 500 mM NaCl and 2% (w/v) BSA, and then captured on the surface of the Protein A chip (29-1275-56; Cytiva) as approximately 370 RU. Two-fold serial diluted human PD-L1 (9049-B7; R&D systems) or monkey (cynomolgus) PD-L1 (10145-B7; R&D systems) with 1xHBS-EP from 500 to 31.25 nM were injected over the ABL501-captured surface for 60 s at 30 μL/min, and then 1xHBS-EP buffer was flown on the ABL501-PD-L1 complex for 180 s to monitor the complex dissociation. After that, 10 mM Glycine-HCl pH 1.5 (BR100354; Cytiva) was injected on the chip surface for 30 s at 30 μL/min. Kinetic analysis was performed using the bivalent analyte model in the BiaEvaluation software (version.01; Cytiva). All assay temperature was set as 25 ℃.

(2) LAG-3 binding assay

Human LAG-3 (2319-L3; R&D systems) or monkey (cynomolgus) LAG-3 (LA3-C5252; Acro Biosystems) were immobilized on the surface of the CM4 chip (BR100534; Cytiva) as approximately 100 RU with the amine coupling method. Two-fold serial diluted ABL501 with 50 mM sodium phosphate containing 150 mM NaCl pH 7.0 from 12.5 to 0.781 nM was injected on the LAG-3 immobilized chip surface for 60 s, and the LAG-3-ABL 501 complex was dissociated for 180 s using 50 mM sodium phosphate with 150 mM NaCl (pH 7.0) to monitor the complex dissociation. Kinetic analysis was performed using the bivalent analyte model in the BiaEvaluation software (version.01; Cytiva). All assay temperature was set as 25 ℃.

Flow cytometry-based antibody binding assay

The cell surface binding of ABL501 to tumor cells or target-positive engineered cell lines was measured by flow cytometry. Cells were incubated with varying concentrations of each antibody followed by a secondary antibody (FITC-conjugated anti-human Fc, F9512; Sigma). hIgG antibody was used as a negative control. EC_{50} value was determined using the GraphPad Prism software (version 8.3.0; GraphPad).

Antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) assays

ADCC was determined using an ADCC reporter bioassay kit (Promega) following the protocol provided by the supplier. For CDC assay, target cells (1×10^4/100 μL per well) were plated in a 96-well plate. Serially diluted antibodies were added, and 25 μL of 2-fold diluted human complement serum (Quidel) was added to the well, followed by incubation for 5 h at 37 ℃ in 5% CO_2 incubator. Next, 75 μL of CytoTox-Glo reagent (Promega) was added to each well, and luminescence was measured using a plate reader (PHERAstar).

Luciferase reporter assays

ABL501-mediated regulation of PD-1 inhibitory signaling was assessed using human PD-L1-expressing CHO-K1 cells and Jurkat effector cells (JA011, Promega). Jurkat effector cells express human PD-1 and a luciferase reporter driven by NF-κB response elements. The ability of ABL501 to block LAG-3 inhibitory signaling was tested in LAG-3/MHCII Blockade Bioassay (JA2111, Promega). The bioassay consists of two cell lines: Jurkat effector cells expressing human LAG-3 and a luciferase reporter driven by T-cell activation pathway-dependent response elements; human MHC-II^+ antigen presenting cells (APC) expressing TCR activating surface molecule. Assays were performed according to the manufacturer's instructions (Promega). Data were analyzed as RLU, and the EC_{50} value was obtained using the GraphPad Prism software (version 8.3.0; GraphPad).