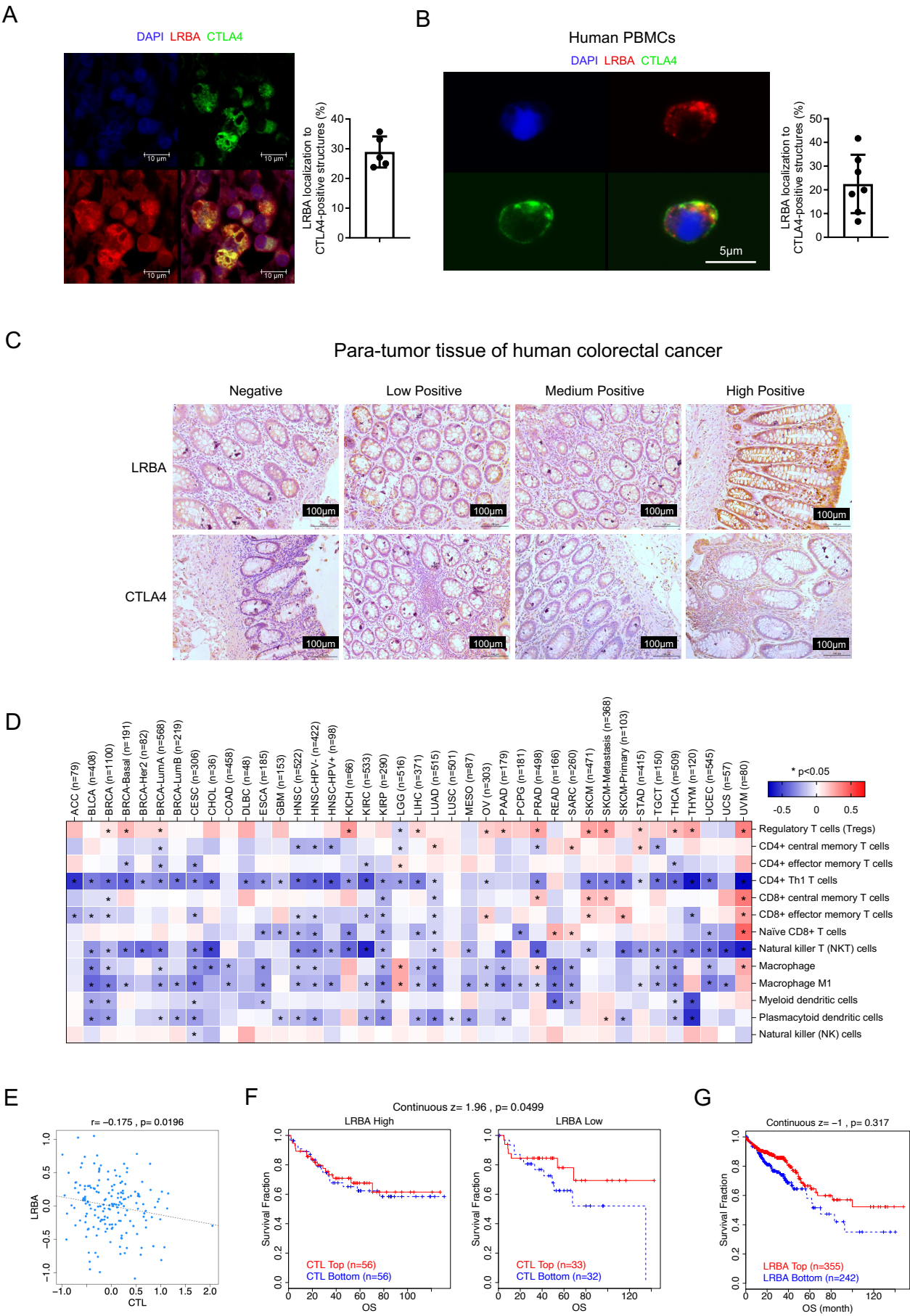


Supplementary Information



Supplementary Figure 1. LRBA is associated with CTLA4 expression and cytotoxic T cell activity in cancer patients.

(A) Representative images and quantification analysis of LRBA (red) and CTLA4 (green) immunofluorescence staining in colorectal cancer tissues (n=5 biological replicates).

(B) Representative images and quantification analysis of LRBA (red) and CTLA4 (green) immunofluorescence staining in human PBMC cells (n=7 biological replicates).

(C) Representative images of LRBA (top) and CTLA4 (bottom) IHC staining in para-tumor tissues of human colorectal cancer of colorectal cancer patients (n=94 patients).

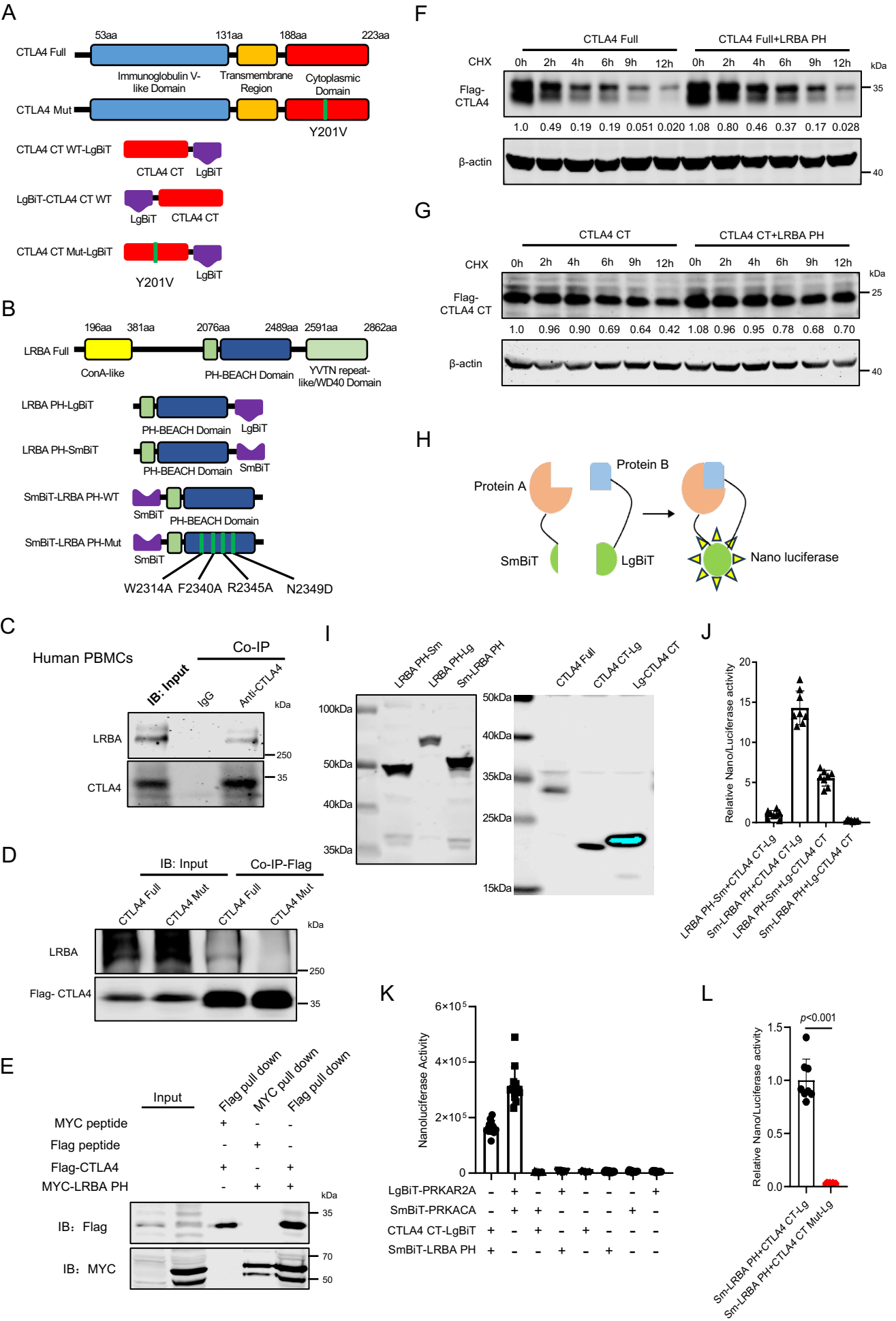
(D) Correlation analysis of LRBA expression with infiltrating immune cells in cancer patients. Linear regression lines are displayed along with Spearman correlation coefficient as heat maps, $*p < 0.05$.

(E) Scatter plot showing the spearman correlation between cytotoxic T lymphocytes (CTL) level and LRBA expression in colorectal cancer patients (n=177 patients).

(F) The association between the cytotoxic T lymphocytes (CTL) level and overall survival (OS) for colorectal cancer patients with high LRBA (left) and low LRBA (right) (n=177 patients).

(G) The association between overall survival (OS) and LRBA expression in colorectal cancer patients (n=597 patients).

Statistical significance was determined by log-rank (Mantel-Cox) test or Spearman correlation coefficient. Data is from TCGA tumors data in E, F and G. Source data are provided as a Source Data file.



Supplementary Figure 2. LRBA exhibits interaction with CTLA4 in the dual-luciferase reporter System.

(A) Domains of human CTLA4 and fusion proteins with Nano-luciferase. LgBiT, a 18kDa subunit of Nano-luciferase (2nd aa to 159th aa) from NanoBiT Kit; SmBiT, a 1kDa subunit of Nano-luciferase (160th aa to 170th aa) from NanoBiT Kit (Promega).

(B) Domains of human LRBA and fusion proteins with Nano-luciferase. LgBiT, a 18kDa subunit of Nano-luciferase (2nd aa to 159th aa) from NanoBiT Kit; SmBiT, a 1kDa subunit of Nano-luciferase (160th aa to 170th aa) from NanoBiT Kit (Promega).

(C) Co-immunoprecipitation (Co-IP) show that LRBA interacts directly with CTLA4 in human PBMCs.

(D) Co-immunoprecipitation (Co-IP) show that LRBA interacts directly with WT CTLA4, but not mutant CTLA4 (Y201V) in HEK293T cells with LRBA, WT CTLA4 or mutant CTLA4 overexpression.

(E) Detection of purified CTLA4 bound to the purified LRBA in a Flag pull-down assay.

(F, G) LRBA PH-BEACH domain (LRBA PH) increases the stabilization of full length CTLA4 (CTLA4 full) **(F)** and CTLA4 C-terminal domain (CTLA4 CT) **(G)** in HEK293 T cells with LRBA PH, CTLA4 full or CTLA4 CT overexpression.

(H) Protein-protein interaction assays using split-nano luciferase system. LgBiT, a 18kDa subunit of nano-luciferase (2nd aa to 159th aa) from NanoBiT Kit; SmBiT, a 1kDa subunit of nano-luciferase (160th aa to 170th aa) from NanoBiT Kit (Promega).

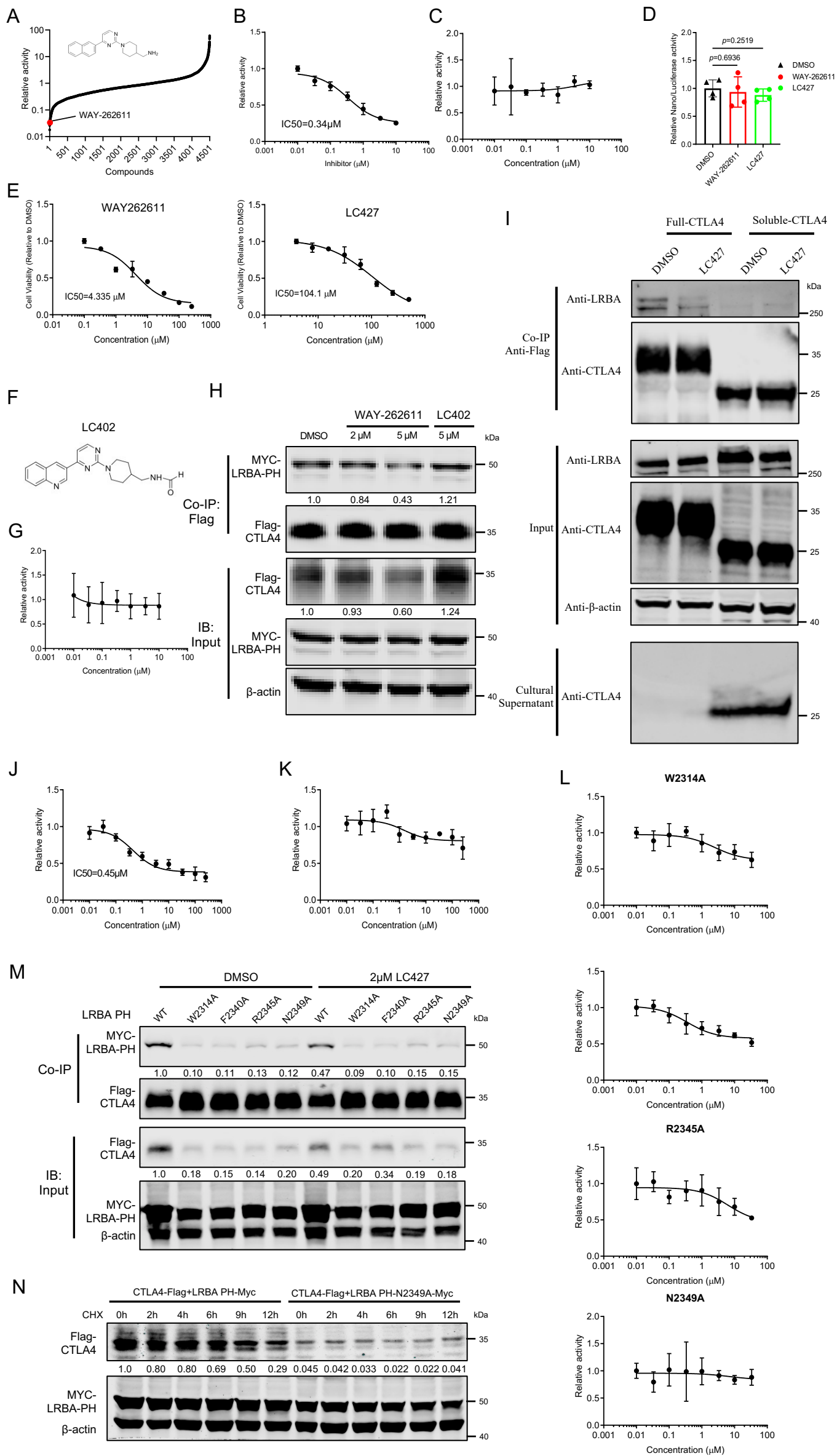
(I) Expression of LRBA-PH fusion protein with Nano-luciferase (left, LRBA PH-Sm, LRBA PH-Lg and Sm-LRBA PH) and CTLA4-CT fusion protein with Nano-luciferase (right, CTLA CT-Lg and Lg-CTLA CT) in HEK293T cells. Lg: abbreviation of LgBiT; Sm: abbreviation of SmBiT.

(J) Relative activity (a ratio of Nano to Firefly luciferase activity) in HEK293T cells transfected with LRBA PH and CTLA4-CT fusion protein with subunit of Nano-luciferase is determined by Nano-Glo® Dual-Luciferase® Reporter Assay (n=8 biological replicates). Co-expression of Sm-LRBA PH (LRBA PH tagged with SmBiT, a 1kDa subunit of Nluc, at the N-terminus) and WT CTLA4 CT-Lg (CTLA4 CT tagged with LgBiT, an 18kDa subunit of Nluc, at the C-terminus) led to a robust Nluc reporter activity.

(K) Nano-luciferase activity in HEK293T cells transfected with LRBA PH fusion protein with subunit of Nano-luciferase, CTLA4-CT fusion protein with subunit of Nano-luciferase, LgBiT-PRKAR2A, and SmBiT-PRKACA is determined by Nano-Glo® Dual-Luciferase® Reporter Assay (n=8 biological replicates). Co-expression of positive control plasmids (LgBiT-PRKAR2A and SmBiT-PRKACA, Promega) led to a robust Nluc reporter activity. Notably, co-expression of Sm-LRBA PH (LRBA PH tagged with SmBiT, a 1kDa subunit of Nluc, at the N-terminus) and WT CTLA4 CT-Lg (CTLA4 CT tagged with LgBiT, an 18kDa subunit of Nluc, at the C-terminus) also led to a robust Nluc reporter activity.

(L) Relative activity (a ratio of Nano to Firefly luciferase activity) in HEK293T cells transfected with Sm-LRBA PH and WT CTLA4 CT-Lg or mutant CTLA4 CT-Lg is determined by Nano-Glo® Dual-Luciferase® Reporter Assay (n=8 biological replicates).

The relative density values for each band beneath the corresponding Western Blot lanes and from two independent experiments (mean). Molecular weight markers (kDa) on the right of the relevant Western Blot images. All data in (J-L) represent mean $\pm$ SEM. Experiments in (C-G, and I) is representative of two independent experiments. Source data are provided as a Source Data file.



Supplementary Figure 3. High-throughput screen for small-molecule inhibitors of LRBA-CTLA4 interaction.

(A) Screening of a 4501-compound library in HEK293T cells by using Sm-LRBA PH and CTLA4 CT-Lg co-transfection assay system (left) and chemical structure of WAY-262611 (right).

(B, C) The inhibition of WAY-262611 on Sm-LRBA PH and WT CTLA4 CT-Lg **(B)** or CTLA4 CT-Lg (Y201V) **(C)** co-transfection assay system in HEK293T cells (n=4 biological replicates).

(D) The inhibition of WAY-262611 and LC427 on LgBiT-PRKAR2A and SmBiT-PRKACA co-transfection assay system in HEK293T cells (n=4 biological replicates).

(E) Cell viability of HEK293T cells after WAY262611 (left) and LC427 (right) treatment (n=4 biological replicates).

(F) Chemical structure of LC402.

(G) The inhibition of LC402 on Sm-LRBA PH and WT CTLA4 CT-Lg co-transfection assay system in HEK293T cells (n=4 biological replicates).

(H) The effects of WAY-262611 and LC402 on the interaction between LRBA and CTLA4 in HEK293T cells with LRBA and CTLA4 overexpression.

(I) The effects of LC427 on the interaction between LRBA and soluble CTLA4 in HEK293T cells with LRBA and soluble CTLA4 overexpression.

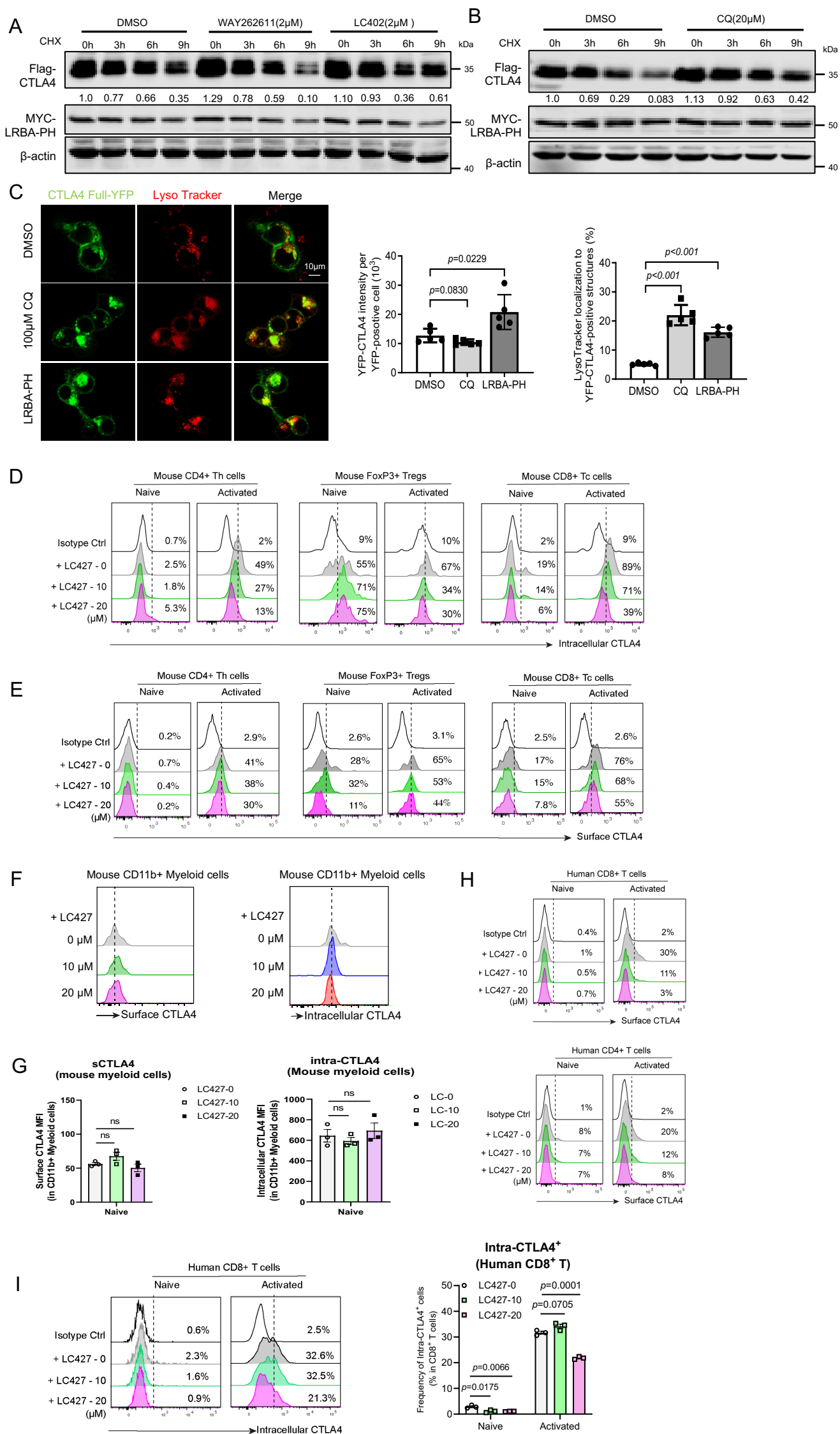
(J, K) The effects of WAY-262611 **(J)** and LC402 **(K)** on the interaction between purified LRBA and CTLA4 (n=4 biological replicates).

(L) The inhibition of LC427 in HEK293T cells with mutant Sm-LRBA PH (W2314A, F2340A, R2345A, or R2349A) and WT CTLA4 CT-Lg co-transfection (n=4 biological replicates).

(M) The effect of LC427 on the interactions between WT LRBA-PH, mutant LRBA-PH (W2314A, F2340A, R2345A, or R2349A) and WT CTLA4.

(N) The effect of WT LRBA-PH and mutant LRBA-PH (N2349A) on the stabilization of full length CTLA4.

The relative density values for each band beneath the corresponding Western Blot lanes and from two independent experiments (mean). Molecular weight markers (kDa) on the right of the relevant Western Blot images. All data represent mean $\pm$ SEM. Experiments in (H-I and M-N) were repeated twice with similar results. Statistical significance was assessed using unpaired two-tailed Student's t-test. Source data are provided as a Source Data file.



Supplementary Figure 4. LC427 enhances CTLA4 degradation in mouse and human T cells.

(A) The effects of WAY-262611 and LC402 on CTLA4 degradation in HEK293T cells with CTLA4 and LRBA overexpression (CHX, Cycloheximide).

(B) Chloroquine (CQ), a inhibitor of lysosomal degradation, blocks CTLA4 degradation in HEK293T cells with CTLA4 and LRBA PH overexpression.

(C) Confocal microscopy shows that chloroquine (CQ) and LRBA overexpression stabilize CTLA4 protein (n=5 biological replicates).

(D) The representative overlaid histogram for intracellular CTLA4 in mouse naïve or activated CD8⁺ T cells, CD4⁺ T cells or Foxp3⁺ Treg cells with LC427 treatment at the indicated concentrations (0, 10, 20μM) for 5 days.

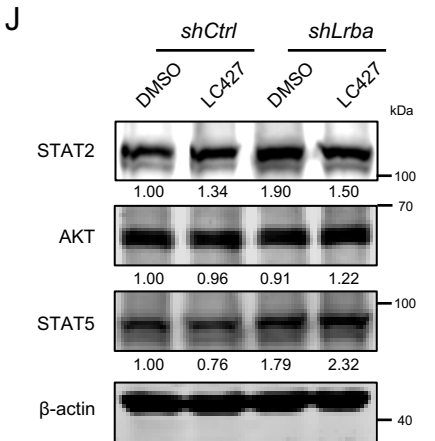
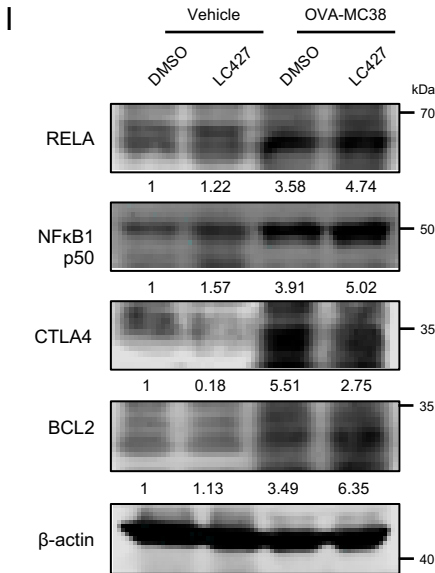
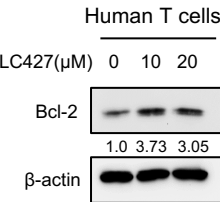
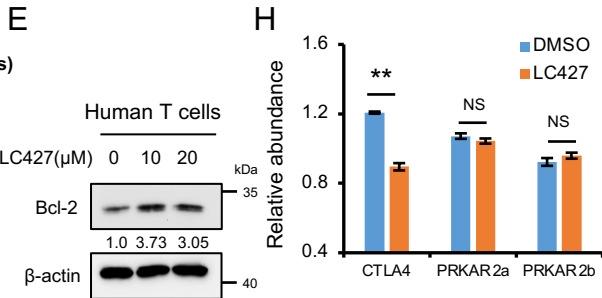
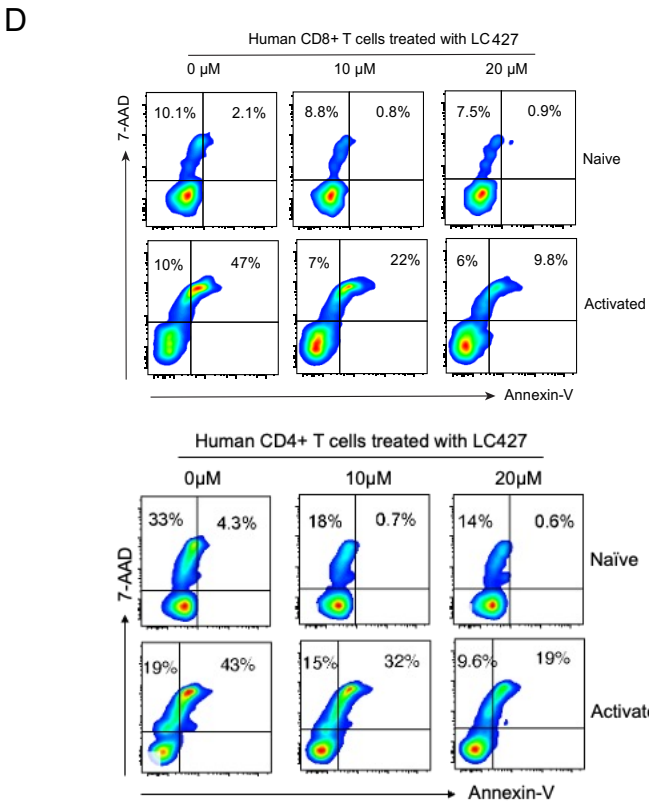
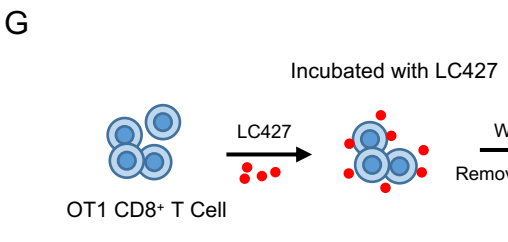
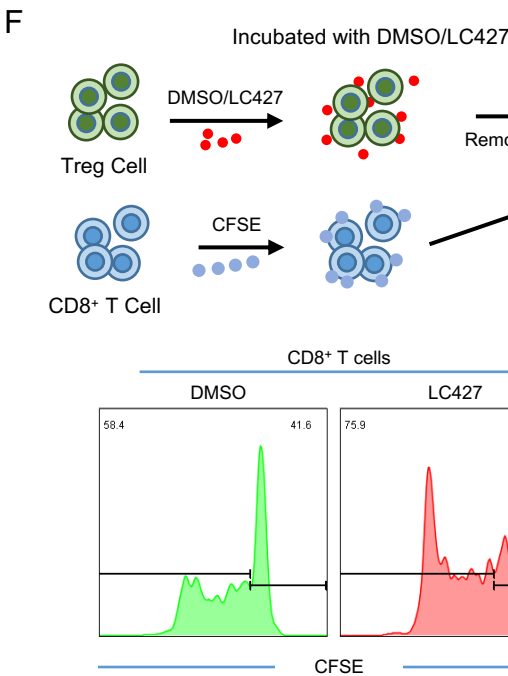
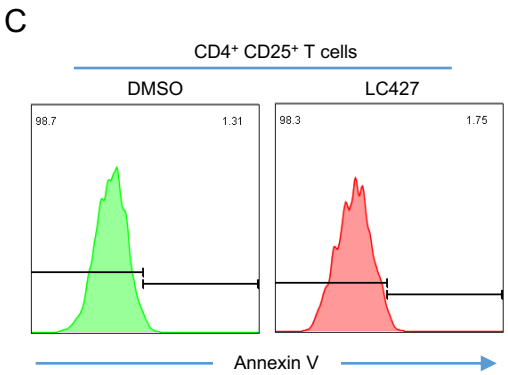
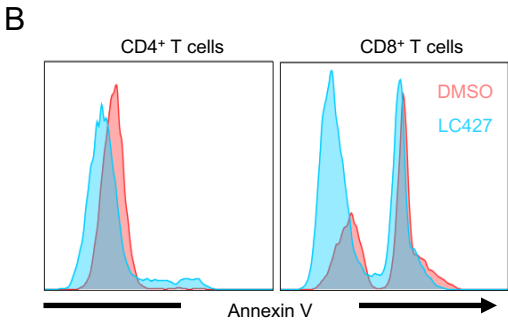
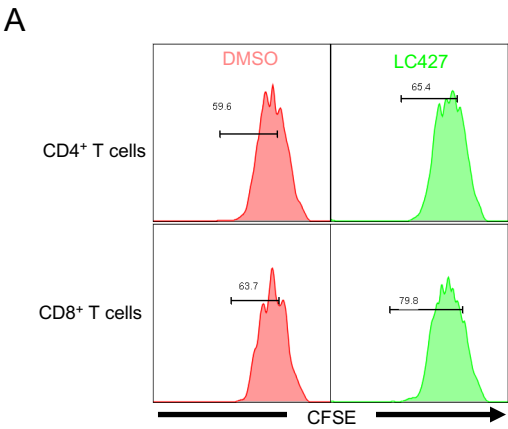
(E) The representative overlaid histogram for surface CTLA4 in mouse naïve or activated CD8⁺ T cells, CD4⁺ T cells or Foxp3⁺ Treg cells with LC427 treatment at the indicated concentrations (0, 10, 20μM) for 5 days.

(F, G) The representative overlaid histogram (**F**, n=3 biological replicates) and quantification (**G**, n=3 biological replicates) for surface (left) or intracellular (right) CTLA4 MFI in mouse CD11b⁺ myeloid cells with LC427 treatment at the indicated concentrations (0, 10, 20μM) for 5 days.

(H) The representative overlaid histogram of surface CTLA4 in human naïve or activated CD8⁺ T cells (top) and CD4⁺ T cells (bottom) with the LC427 treatment for 5 days.

(I) The representative overlaid histogram (left) and quantification (right) of intracellular CTLA4 in human naïve or activated CD8⁺ T cells with the LC427 treatment for 5 days (n=3 biological replicates).

The relative density values for each band beneath the corresponding Western Blot lanes and from two independent experiments (mean). Molecular weight markers (kDa) on the right of the relevant Western Blot images. Data are mean $\pm$ SEM. Data in (A-B) have been reproduced in two independent experiments and statistical significance was assessed using unpaired two-tailed Student's t-test and One-way ANOVA and Tukey test for multiple comparisons. Source data are provided as a Source Data file.



Supplementary Figure 5. LC427 enhances apoptosis resistance and functions in mouse and human T cells.

(A) The representative overlaid histogram for the CFSE fluorescence intensity in mouse activated CD4⁺ T cells and CD8⁺ T cells with LC427 treatment for 5 days.

(B) The representative overlaid histogram for the Annexin V fluorescence intensity in mouse activated CD4⁺ T cells and CD8⁺ T cells with LC427 treatment for 5 days.

(C) The representative overlaid histogram and quantification analysis of apoptosis in mouse Tregs after LC427 treatment for 5 days (n=3 biological replicates).

(D) The representative flow cytometry analysis of apoptosis in human CD8⁺ T cells (top) and CD4⁺ T cells (bottom) after LC427 treatment for 5 days.

(E) Immune blotting of Bcl-2 protein in human activated T cells with indicated LC427 treatment for 5 days.

(F) An in vitro coculture assay was developed that examined the effects of Tregs with DMSO or LC427 treatment on CD8⁺ T cell proliferation.

(G) Experimental strategy to evaluate the ability of LC427 to enhance activated OT-1 CD8⁺ T cells mediated tumor cell killing by co-culturing with MC38-OVA tumor cells for 48h at a 1:1 E: T (T cells: Tumor cells) ratio.

(H) The relative abundance of CTLA4, PRKAR2a and PRKAR2b proteins in activated T cells with control or LC427 treatment (n=3 biological replicates).

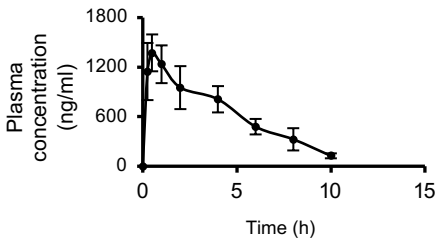
(I) Western blotting analyses determined protein expression of Lrba-dependent signaling in OT-1 CD8⁺ T cells co-cultured with vehicle or MC38-OVA cells after DMSO or LC427 treatment.

(J) Western blotting analyses determined protein expression of Lrba-dependent signaling in Lrba-Knockdown (*shLrba*) or Control-Knockdown (*shCtrl*) T cells with DMSO or LC427 treatment.

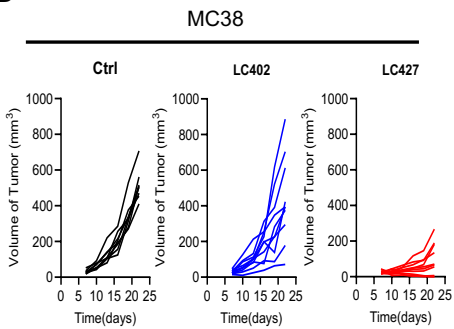
The relative density values for each band beneath the corresponding Western Blot lanes and from two independent experiments (mean). Molecular weight markers (kDa) on the right of the relevant Western Blot images. Data are mean $\pm$ SEM. Data in (I and J) have been reproduced in two independent experiments and statistical significance was assessed using One-way ANOVA and unpaired two-tailed Student's for multiple comparisons. Source data are provided as a Source Data file.

A

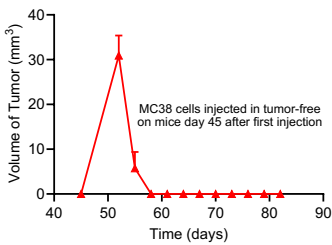
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Mean	30.000	0.263	0.375	1397.000	6512.000	6985.000	2.620
SD	0.000	0.000	0.200	66.000	1074.200	1114.200	0.500



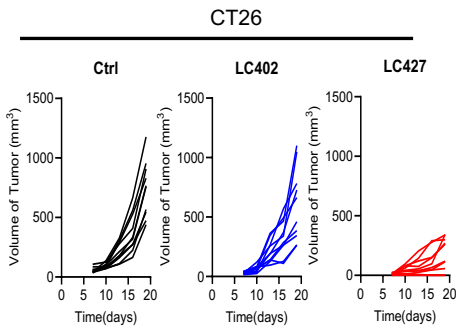
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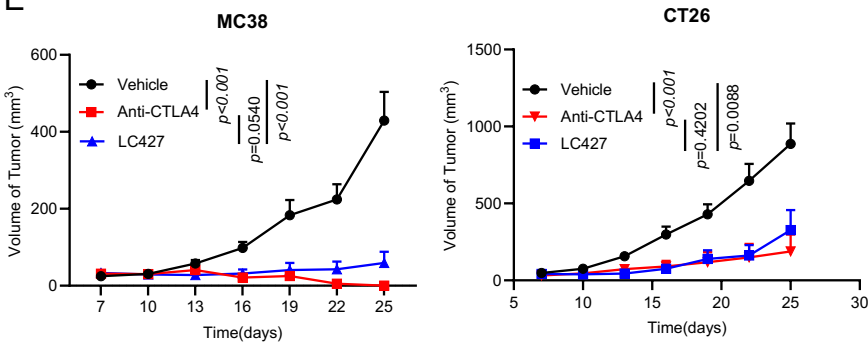
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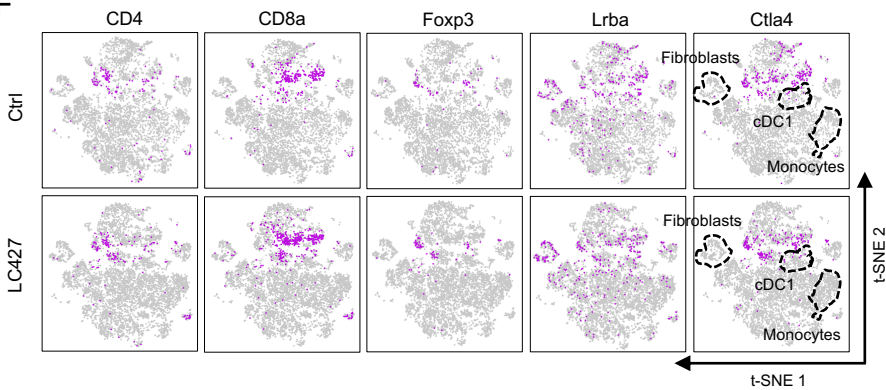
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E



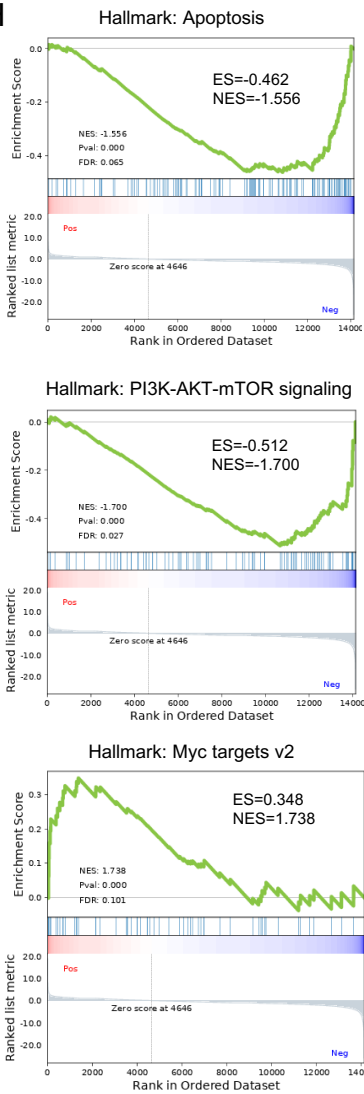
F



G

Gene	CD4	CD8a	Foxp3	Lrba	Ctla4
Ctrl	2.45	6.21	1.08	5.50	4.49
LC427	2.58	7.52	1.29	4.65	3.44

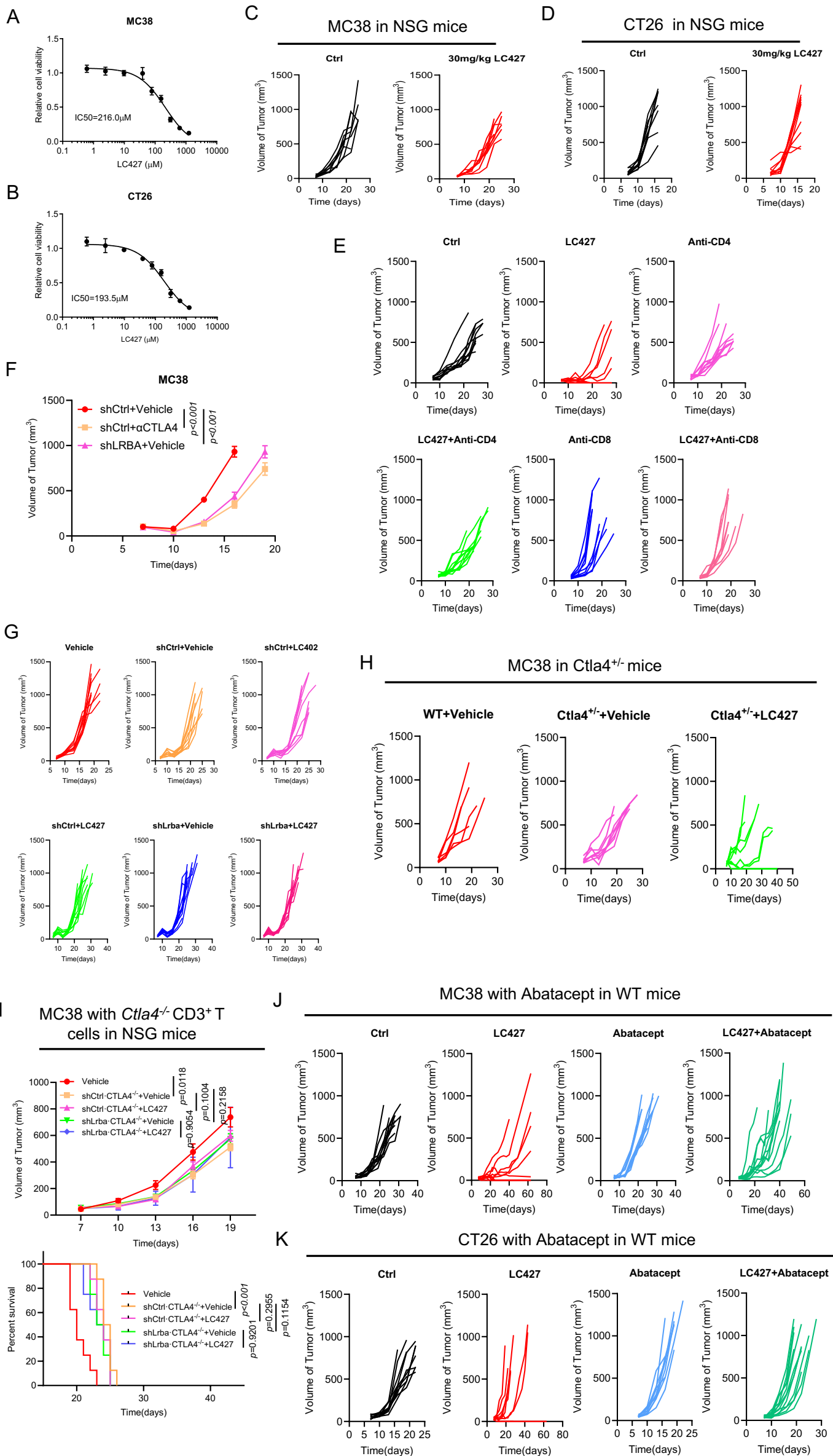
H



Supplementary Figure 6. LC427 promotes antitumor immunity in tumor models.

- (A) Single dose oral pharmacokinetics of LC427 in mice (n=6 mice).
- (B) Individual tumor volume of MC38 colorectal tumor-bearing mice with vehicle (Ctrl), or 30mg/kg LC427 or 30mg/kg LC402 treatment (n=10 mice).
- (C) Tumor measurements of tumor-ablating C57BL/6 mice rechallenged with the same number of MC38 cancer cells on day 45 after the first tumor injection (n=4 mice).
- (D) Individual tumor volume of CT26 colorectal tumor-bearing mice with vehicle (Ctrl), or 30mg/kg LC427 or 30mg/kg LC402 treatment (n=10 mice) .
- (E) Tumor volume of MC38 (left, n=10 mice) and CT26 (right, n=10 mice) colorectal tumor-bearing mice with vehicle (Ctrl), or 30mg/kg LC427 or anti-CTLA4 mAb treatment.
- (F) t-SNE plot of infiltrating cells displaying marker gene expression.
- (G) The normalized expression of marker genes in (D).
- (H) GSEA signatures in CD8⁺ T cells from MC38 colorectal tumors with LC427 treatment.

Data are mean $\pm$ SEM. The n number represents biological replicates (individual mice) obtained from one independent experiment. Statistical significance was assessed using Two-way ANOVA and Tukey test for multiple comparisons. Source data are provided as a Source Data file.



Supplementary Figure 7. LC427 promotes antitumor immunity in a T cell and LRBA-dependent manner in tumor models.

(A, B) Cell survival curve of MC38 (**A** , n=3 biological replicates) and CT26 (**B** , n=3 biological replicates) colorectal cells after LC427 treatment.

(C, D) Individual tumor volume of MC38 (**C**, n=10 mice) and CT26 (**D**, n=10 mice) colorectal tumor-bearing NSG mice with 30mg/kg LC427 treatment.

(E) Individual tumor volume of MC38 colorectal tumor-bearing WT mice with IgG2a (Ctrl), 30mg/kg LC427, anti-CD4 mAb in combination with 30mg/kg LC427 (LC427+Anti-CD4), or anti-CD8 mAb in combination with 30mg/kg LC427 (LC427+Anti-CD8) treatment (n=10 mice).

(F) Tumor volume of MC38-OVA colorectal tumor-bearing NSG mice with adoptively transferred *Lrba*-Knock down (*shLrba*) or Control-Knock down (*shCtrl*) OT-1 CD8⁺ T cells after vehicle , or anti-CTLA4 mAb treatment (n=8 mice).

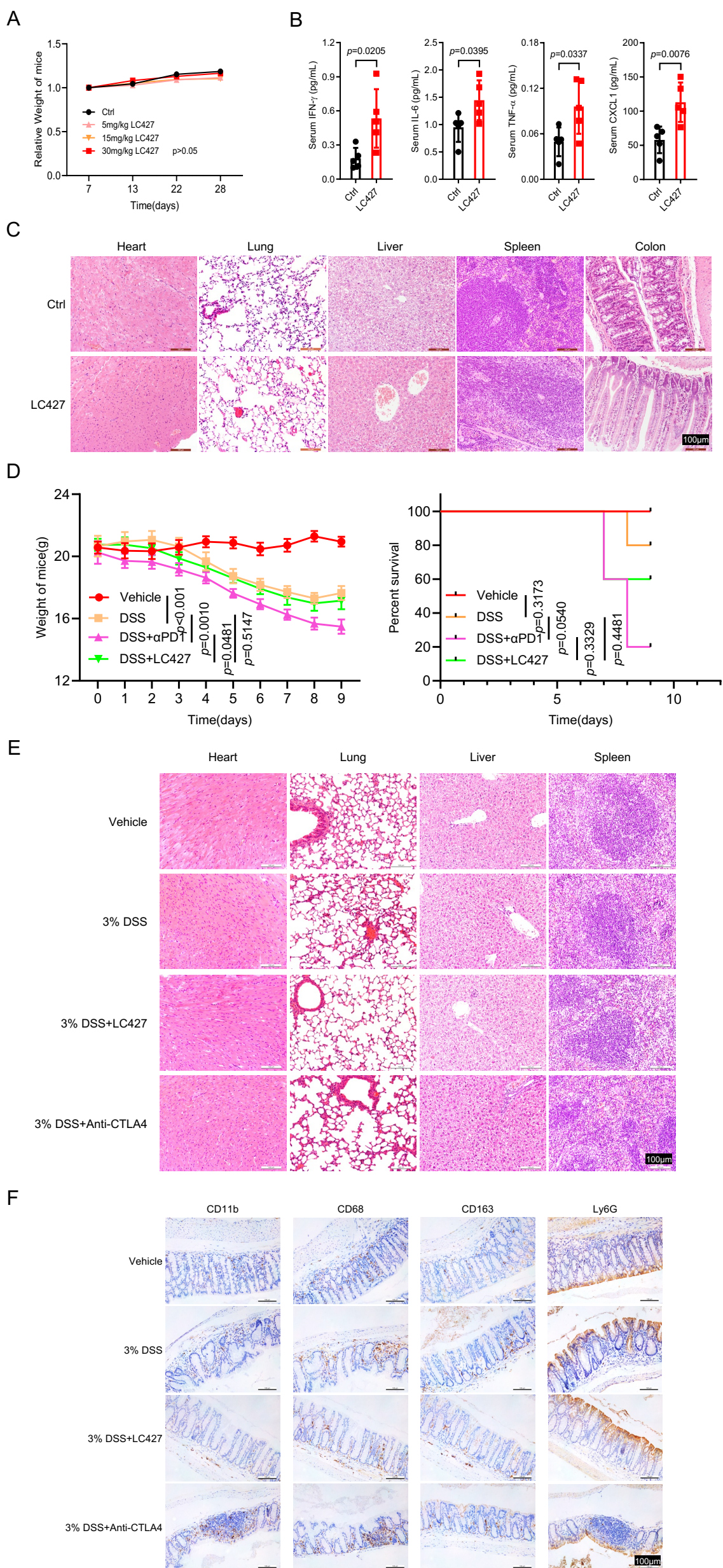
(G) Individual tumor volume of MC38-OVA colorectal tumor-bearing NSG mice with adoptively transferred *Lrba*-Knock down (*shLrba*) or Control-Knock down (*shCtrl*) OT-1 CD8⁺ T cells after vehicle , 30mg/kg LC427 or 30mg/kg LC402 treatment (n=10 mice).

(H) Individual tumor volume of MC38 colorectal tumor-bearing WT or *Ctla4*^{+/-} mice with vehicle or 30mg/kg LC427 treatment (n=8 mice).

(I) Tumor volume (top) and survival (bottom) analysis of MC38 tumor-bearing NSG mice with adoptively transferred *Lrba*-Knockdown•*Ctla4*^{-/-} (*shLrba*•*Ctla4*^{-/-}) or Control-Knockdown•*Ctla4*^{-/-} (*shCtrl*•*Ctla4*^{-/-}) CD3⁺ T cells after vehicle or 30mg/kg LC427 treatment (n=8 mice).

(J, K) Individual tumor volume of MC38 (**J** , n=10 mice) and CT26 (**K** , n=10 mice) colorectal tumor-bearing WT mice with vehicle (Ctrl), 30mg/kg LC427, Abatacept, or Abatacept in combination with 30mg/kg LC427 (LC427+Abatacept) treatment.

Data represent mean $\pm$ SEM. The n number represents biological replicates (individual mice) obtained from one independent experiment. Statistical significance was assessed using Nonlinear regression (A, B) and two-way ANOVA. Source data are provided as a Source Data file.



Supplementary Figure 8. LC427 is tolerated in mice at efficacious doses.

(A) Normalized body weight curve of mice treated with three different doses of LC427 (n=5 mice).

(B) Concentrations of CXCL1, TNF- α , IL-6, and IFN- γ in the serum of mice treated with 30mg/kg LC427 (n=5 mice).

(C) Representative H&E staining of heart, lung, liver, spleen, and colon from mice treated with vehicle (Ctrl) or 30mg/kg LC427.

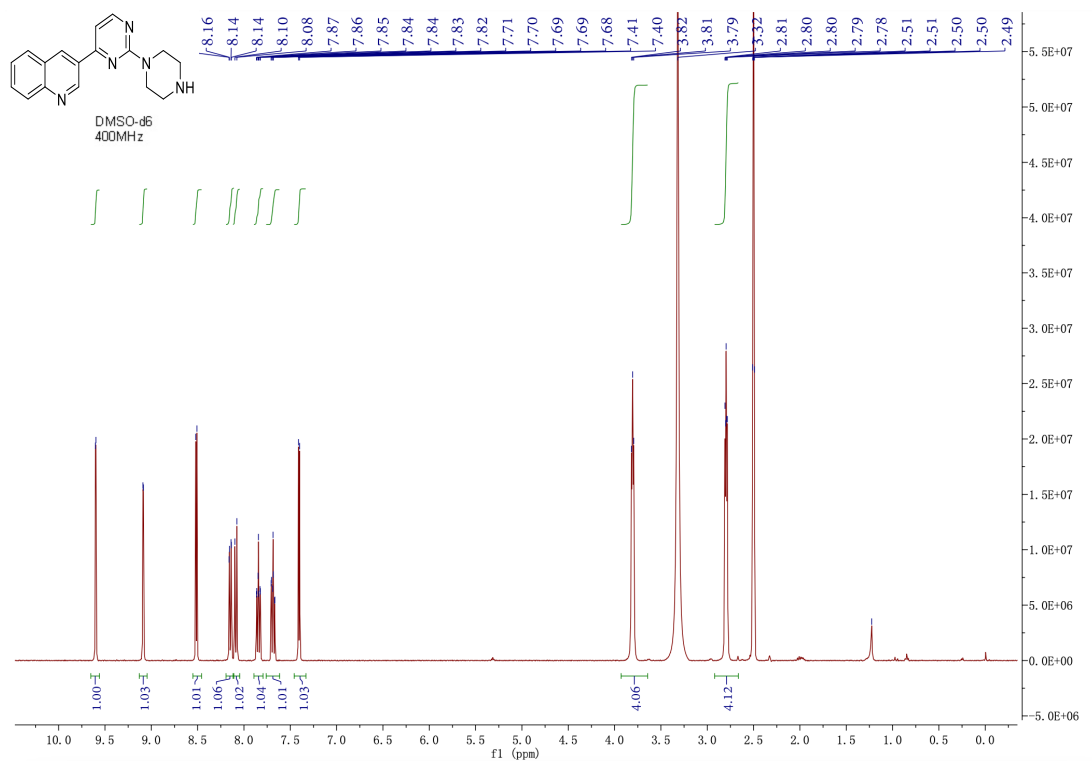
(D) Normalized body weight (left, n=5 mice) and survival (right, n=5 mice) curves of mice treated with 3% DSS and receiving LC427 or anti-PD1 mAb.

(E) Representative H&E staining of heart, lung, liver, and spleen from mice treated with 3% DSS and receiving LC427 or anti-CTLA4 mAb.

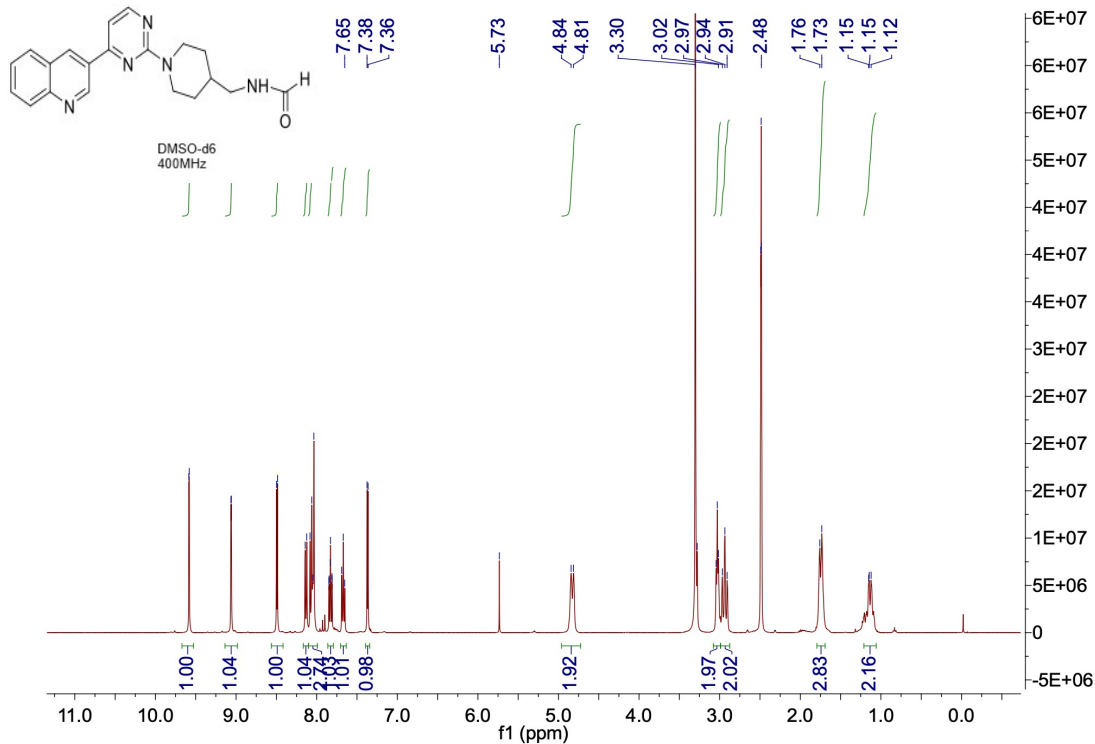
(F) Representative images of CD11b, CD68, CD163 and Ly6G IHC staining in colon from mice treated with 3% DSS and receiving LC427 or anti-CTLA4 mAb.

Data are mean $\pm$ SEM. The n number represents biological replicates (individual mice) obtained from one independent experiment. Statistical significance was assessed using one way-ANOVA and two way-ANOVA. Source data are provided as a Source Data file.

A



B



Supplementary Figure 9. The NMR spectral data of compounds.

(A) The NMR (400 MHz) spectrum of LC427. ¹H NMR (400 MHz, DMSO-d₆) δ 9.65 (d, J=4.0 Hz, 1H), 9.15 (d, J=4.0 Hz, 1H), 8.60 (d, J=8.0 Hz, 1H), 8.17-8.12 (m, 1H), 8.09 (d, J=8.0 Hz, 1H), 7.86 (t, J=8.0 Hz, 1H), 7.70 (t, J=8.0 Hz, 1H), 7.56 (d, J=8.0 Hz, 1H), 4.15-4.08 (m, 4H), 3.25-3.12 (m, 4H).

(B) The NMR (400 MHz) spectrum of LC402. ¹H NMR (400 MHz, DMSO-d₆) δ 9.58 (d, J=4.0 Hz, 1H), 9.06 (d, J=2.0 Hz, 1H), 8.49 (d, J=4.0 Hz, 1H), 8.13 (d, J=8.0 Hz, 1H), 8.06 (dd, J=12.0, 8.0 Hz, 3H), 7.87-7.79 (m, 1H), 7.67 (t, J=8.0 Hz, 1H), 7.37 (d, J=8.0 Hz, 1H), 4.83 (d, J=12.0 Hz, 2H), 3.06-3.00 (m, 2H), 2.94 (t, J=12.0 Hz, 2H), 1.75 (d, J=12.0 Hz, 2H), 1.20-1.07 (m, 2H).

Supplementary Table 1. Univariate analysis of the association of clinical factors with expression of CTLA4 and LRBA in colorectal cancer patients. Statistical significance was assessed using two-way ANOVA and two-way T-test analyses

Clinical variable	Total(n)	Univariate analysis (95% confidence interval)			
		Normalized number of CTLA4+ cells	P value	Normalized number of LRBA+ cells	P value
TNM stage					
I - II	10	-0.05591 to 0.05857	0.9632	-0.05879 to 0.06002	0.9836
III-IV	60				
Age (years)					
≥60	29	-0.05259 to 0.01630	0.2978	-0.06077 to 0.02859	0.4757
<60	34				
Nodal metastasis					
Yes	37	-0.01897 to 0.05636	0.3246	-0.01909 to 0.05821	0.3165
No	38				
Gender					
Female	26	-0.04475 to 0.02792	0.6464	-0.05644 to 0.03419	0.6267
Male	60				
Distant metastases					
Yes	11	-0.04033 to 0.06951	0.5977	-0.06182 to 0.05239	0.8696
No	59				

Supplementary Table 2. Antibodies used in this study

Anti-DYKDDDDK tag Recombinant antibody	Proteintech	Cat#180010-1-RR; RRID: AB 2882940
Anti-MYC tag Polyclonal antibody	Proteintech	Cat#16286-1-AP; RRID: AB 11182162
Anti-LRBA antibody	Thermo	Cat#PA5-53986; RRID: AB 2643453
Anti-CTLA-4 antibody (clone F-8)	Santa Cruz Biotechnology	Cat# sc-376016; RRID: None
Anti-CD4 antibody (clone MT310)	Santa Cruz Biotechnology	Cat# sc-19641; RRID: None
Anti-CD8 antibody (clone 32-M4)	Santa Cruz Biotechnology	Cat# sc-1177; RRID: None
Anti-β-Actin antibody	ABclonal	Cat#AC026; RRID:AB 2768234
IRDye® 800CW Donkey anti-Mouse IgG Secondary Antibody	LI-COR Biosciences	Cat# 925-32212; RRID: AB 2716622
IRDye® 800CW Donkey anti-Rabbit IgG Secondary Antibody	LI-COR Biosciences	Cat# 926-32213; RRID: AB 621848
HRP-conjugated Affinipure Goat Anti-Rabbit IgG(H+L)	Proteintech	Cat# SA00001-2; RRID: AB 2722564
HRP-conjugated Affinipure Goat Anti-Mouse IgG(H+L)	Proteintech	Cat# SA00001-1; RRID: AB 2722565
HRP-conjugated Affinipure Goat Anti-Rat IgG(H+L)	Proteintech	Cat# SA00001-15; RRID: AB 2864369
The combination of Rat monoclonal anti-CTLA4 antibody (clone 9H10)	Bio X Cell	Cat#BE0273; RRID:AB 2687796
The combination of Rat monoclonal anti-PD1 antibody (clone RPM1-14)	Bio X Cell	Cat#BE0164; RRID: AB 10949609
InVivoMAb anti-mouse CD8α antibody (clone YTS 169.4)	Bio X Cell	Cat#BE0117; RRID: AB 10950145
InVivoMAb anti-mouse CD4 antibody (clone YTS 191)	Bio X Cell	Cat#BE0119; RRID: AB 10950382
APC-Cy™7 Rat Anti-Mouse CD45 antibody (clone 30-F11)	BD biosciences	Cat# 557659; RRID:AB 396774
BV421 Hamster Anti-Mouse CD3e antibody (clone 145-2C11)	BD biosciences	Cat# 562600; RRID:AB 11153670