Supplementary figures

Supplemental Figure 1

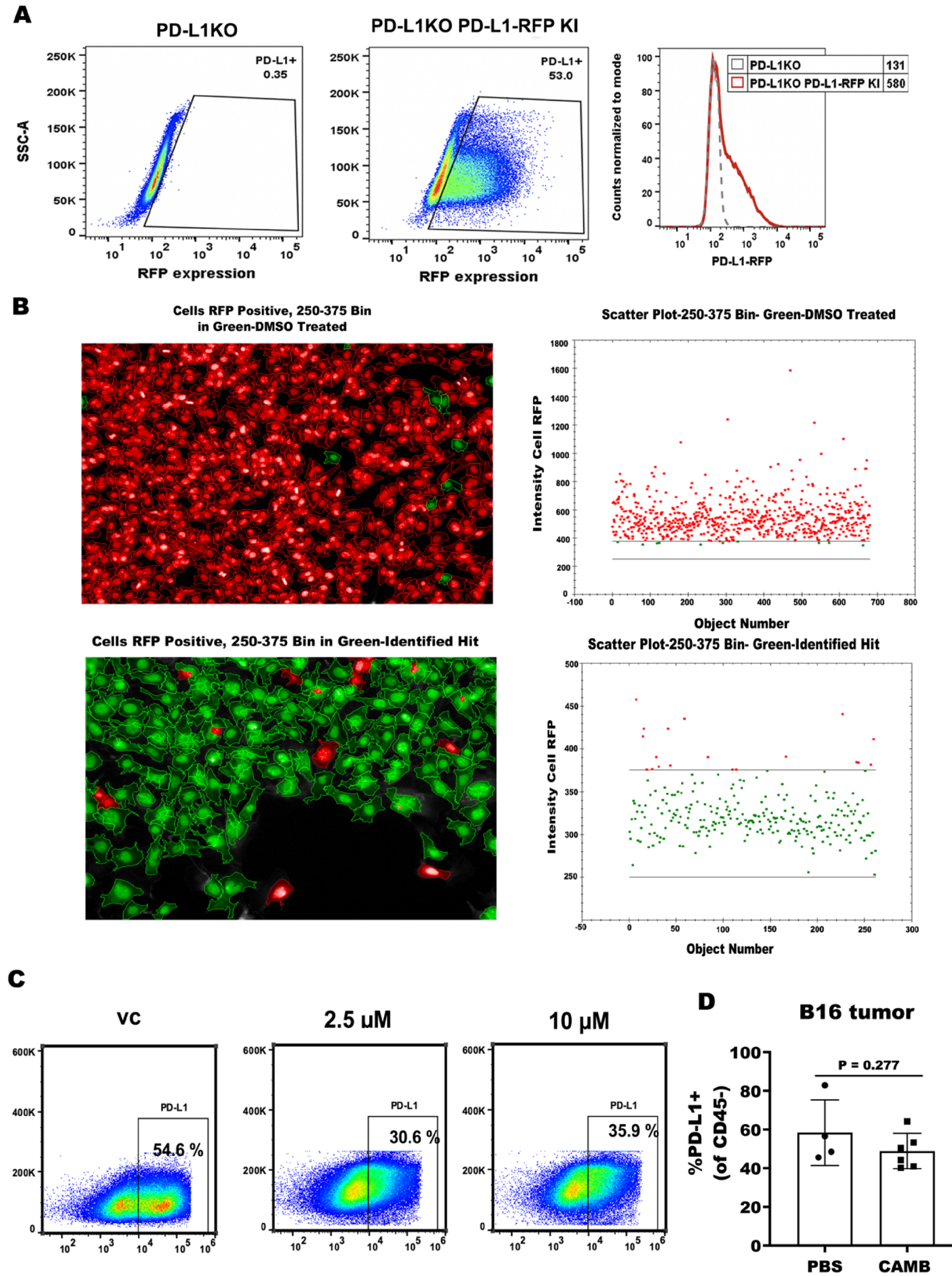


Figure S1. High throughput screening identified PDL1 depletion drugs.

(A) Flow cytometry for PDL1 expression in PDL1^{KO} B16 with or without PDL1-RFP knock-in (RFP^{KI}). **(B)** PDL1^{KO} B16 with RFP-PDL1^{KI} cells incubated with compounds from the Prestwick and LOPAC libraries (1200 and 1280 compounds, respectively) at 2.5 and 10 μM using the Agilent Bravo liquid pipetting platform and then imaged by Operetta High Content system after 48 hours incubation. **(C)** Flow cytometry for PDL1 expression in OVCAR5 with or without PMEG treatment. VC, vehicle control. **(D)** WT mice challenged with B16 cells, treated with 3 mg/kg CAMB as described in methods and sacrificed on day 19 after tumor challenged, 1 day after final CAMB. Tumors were collected for flow cytometry analysis. Summary graph of PDL1 percentage in the CD45^{-} gates. P-value by unpaired *t*-test.

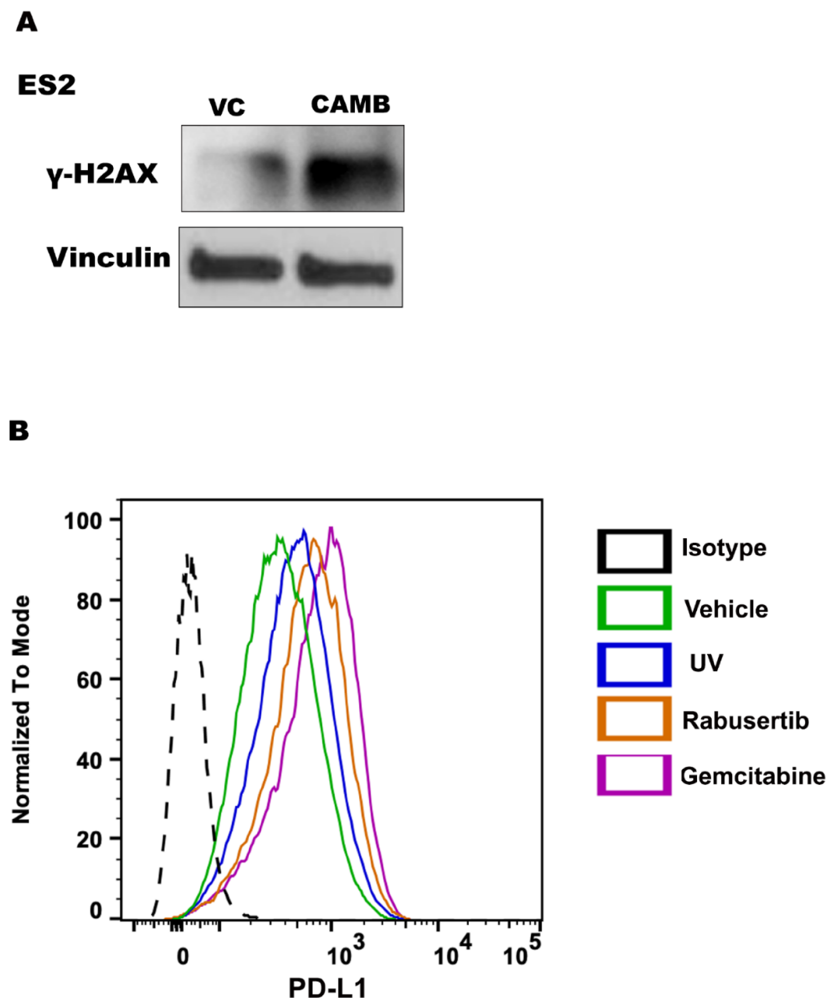


Figure S2. Chlorambucil induces DNA damage.

(A) Western blots for γ-H2AX expression in ES2 cells incubated *in vitro* with (+) or without (-) chlorambucil (10 μM) for 48 hours. **(B)** Flow cytometry comparing PDL1 expression in OVCAR5 cells with or without ultraviolet (UV) irradiation (2 Gy, assessed 24 hours later), gemcitabine (10 μM, 24 hours incubation), rabusertib (1 μM, 48 hours incubation).

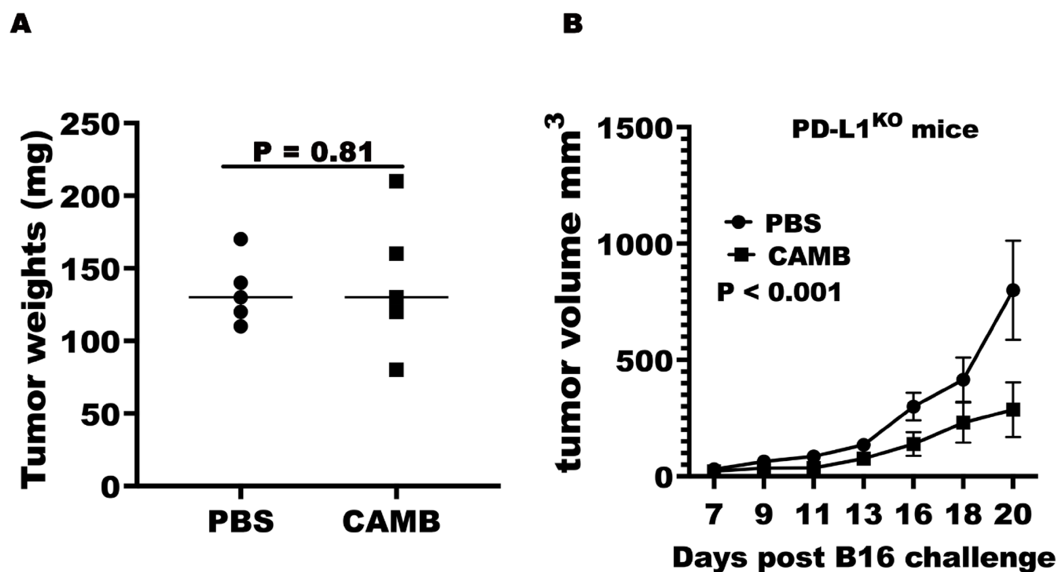


Figure S3. Chlorambucil efficacy is independent of host PDL1 expression.

(A) WT mice challenged with PDL1^{lo} ID8agg cells and treated as described in Fig. 3**A**. Mice body weight measured weekly, and increased is relative to baseline. N = 5 for PBS and 6 for chlorambucil (CAMB). At the end of experiment, mice were sacrificed, and tumor weights measured. Each symbol is an individual tumor, P-value, unpaired *t*-test. **(B)** Tumor volume of PDL1^{KO} mice challenged with B16 cells and treated as described in Fig. 3E. N = 4 for both PBS and CAMB. P-value, two-way ANOVA.

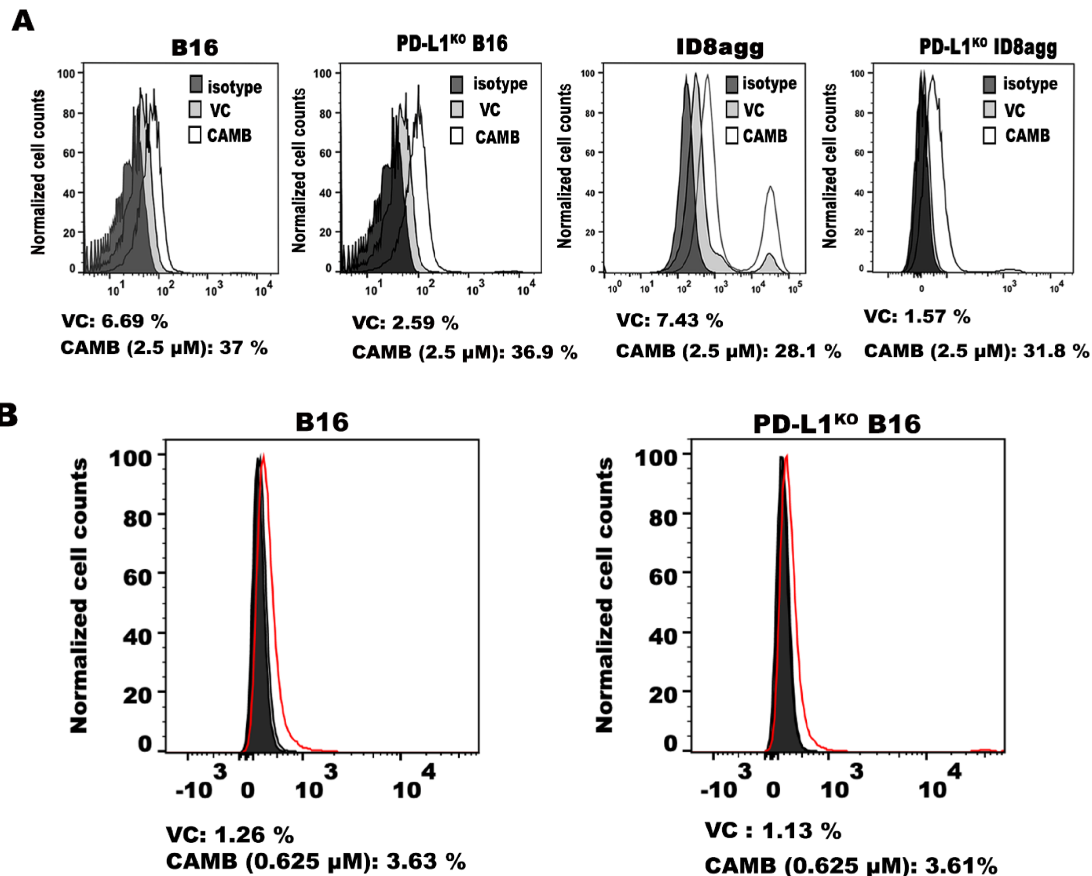
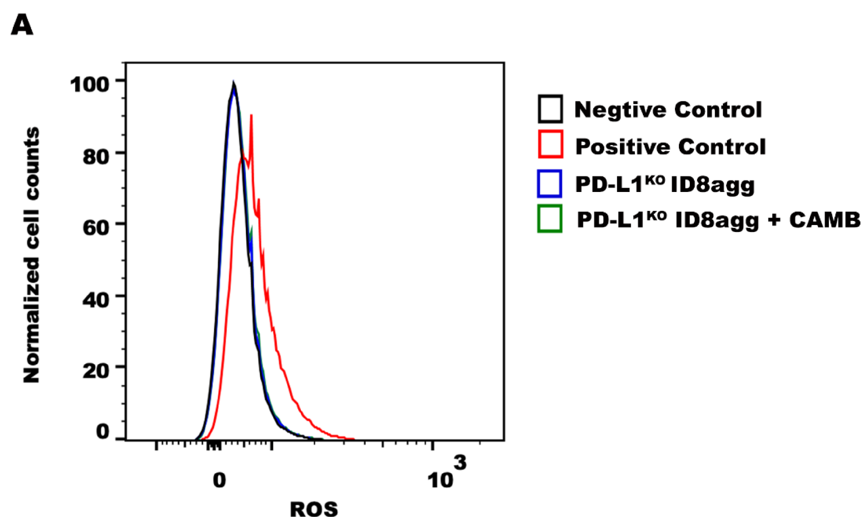


Figure S4. Chlorambucil promotes ICD in selected tumor cells

(A) Flow cytometry analysis of calreticulin content in vehicle control (VC) or chlorambucil (CAMB)-treated ID8agg or PDL1^{KO} ID8agg (2.5 μM), B16 or PDL1^{KO} B16 (2.5 μM) for 48 hours. Isotype: isotype control for anti-calreticulin antibody. Data represent 1 of 3 independent experiments with similar results. (B) Flow cytometry analysis of calreticulin content in vehicle control (VC) or chlorambucil (CAMB)-treated B16 or PDL1^{KO} B16 (0.625 μM). Isotype: isotype control for anti-calreticulin antibody. Data represent 1 of 3 independent experiments with similar results.

Supplemental Figure 5



Positive control: 18.8 %

PD-L1^{KO} ID8agg: 3.93 %

PD-L1^{KO} ID8agg + CAMB: 4.21%

Figure S5. ROS pathway does not mediate chlorambucil-induced ICD.

(A) Flow cytometry analysis of ROS production in vehicle control (VC) or chlorambucil (CAMB)-treated ID8agg (2.5 μM) for 48 hours. Negative control: PDL1^{KO} ID8agg cells only labeled with H2DCFDA; Positive control: PDL1^{KO} ID8agg cells pretreat with 50 μM tert-butyl hydrogen peroxide for 2 hours. Data represent 1 of 3 independent experiments with similar results. Percent positive cells shown at the bottom of the figure.

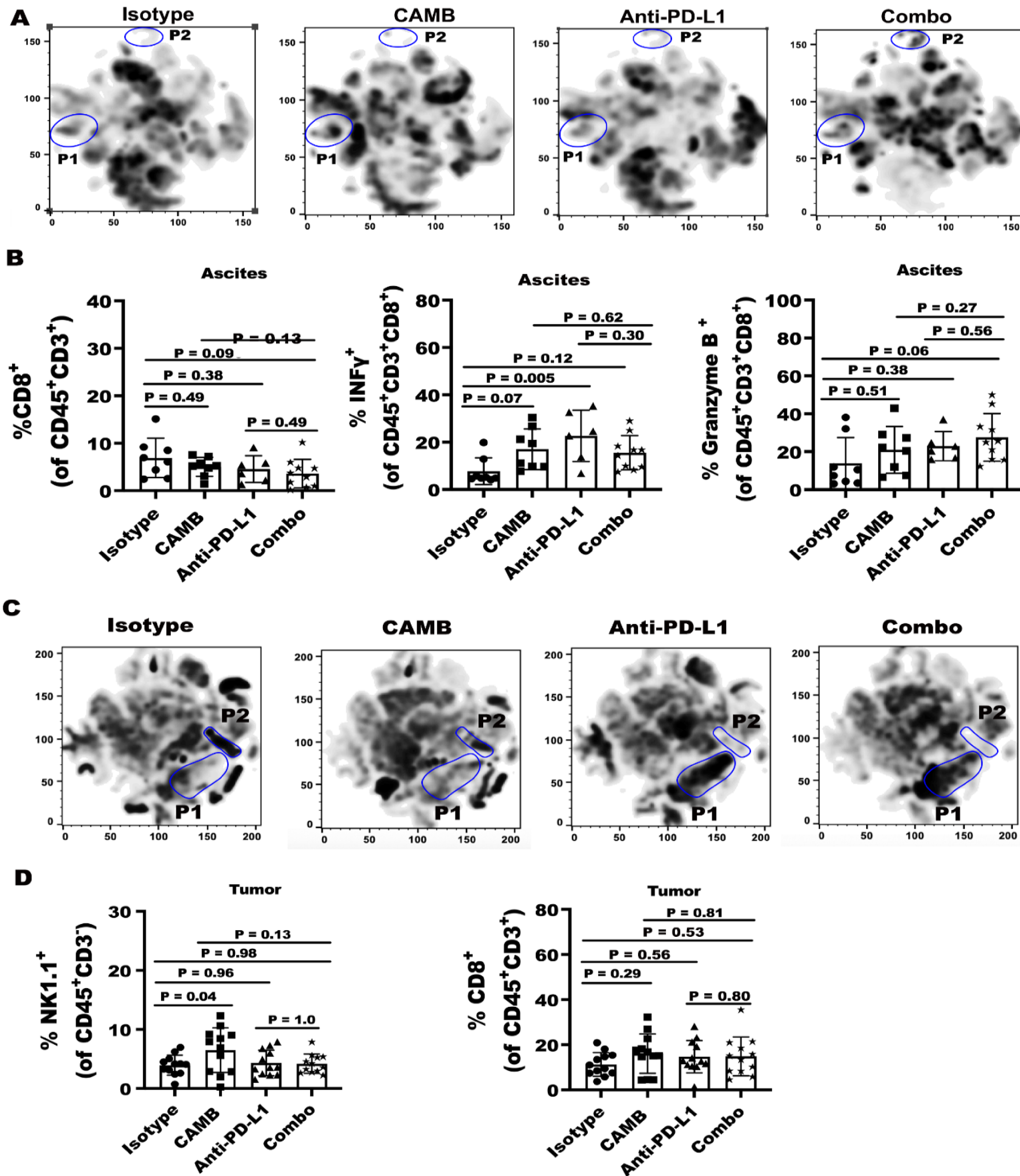

Supplemental Figure 6
A
Isotype
CAMB
Anti-PD-L1
Combo
P1
P2
B
Ascites
%CD8+ (of CD45+CD3+)
% INFγ+ (of CD45+CD3+CD8+)
% Granzyme B+ (of CD45+CD3+CD8+)
P = 0.13
P = 0.09
P = 0.38
P = 0.49
P = 0.49
P = 0.62
P = 0.30
P = 0.12
P = 0.005
P = 0.07
P = 0.27
P = 0.56
P = 0.06
P = 0.38
P = 0.51
C
D
Tumor
% NK1.1+ (of CD45+CD3-)
% CD8+ (of CD45+CD3+)
P = 0.13
P = 0.98
P = 0.96
P = 0.04
P = 1.0
P = 0.81
P = 0.53
P = 0.56
P = 0.29
P = 0.80

Figure S6. Chlorambucil and αPDL1 treatments or their combination alter the tumor immune environment distinctly

(A) *t*-SNE analysis of flow cytometry data in ascites of ID8agg tumor-bearing mice sacrificed 30 days after tumor challenge with indicated treatments. The $CD8^+$ T cell cluster is P1 and the NK cell cluster is P2. CAMB, chlorambucil. Combination, chlorambucil plus anti-PDL1. Treatment dose and schedule as in Fig. 4C. **(B)** $CD8^+$, $CD8^+$ $IFN\gamma^+CD8^+$ or Granzyme B^+ T cell prevalence in ascites from ID8agg tumor challenged mice after treatment as described in Fig. 4**C**. Isotype with each treatment. P-value, one-way ANOVA. Chlorambucil versus combination and anti-PDL1 versus combination using Mann-Whitney test. **(C)** *t*-SNE analysis of flow cytometry analysis of the immune landscape in $PDL1^{KO}$ B16 tumors treated as described in Figure 4A. **(D)** Percent $NK1.1^+$ cells and $CD8^+$ T cells in $PDL1^{KO}$ B16 tumor treated as described in figure 4A, The $CD8^+$ T cell cluster is P1 and the NK cell cluster is P2. Isotype with each treatment. P-value, one-way ANOVA. Chlorambucil versus combination and anti-PDL1 versus combination using Mann-Whitney test.

Supplemental Figure 7

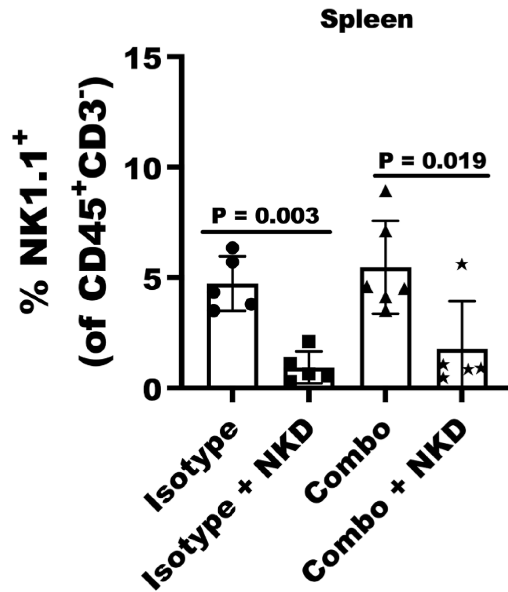


Figure S7. ***In vivo*** **NK cell depletion validation**

(A) NK cell depletion (NKD) efficiency evaluated by measuring spleen NK1.1$^+$ cells after anti-NK1.1 treatment in **Fig. 5F**. Mice sacrificed on day 28 post tumor challenge. Combo, Chlorambucil plus αPDL1.

Supplemental Figure 8

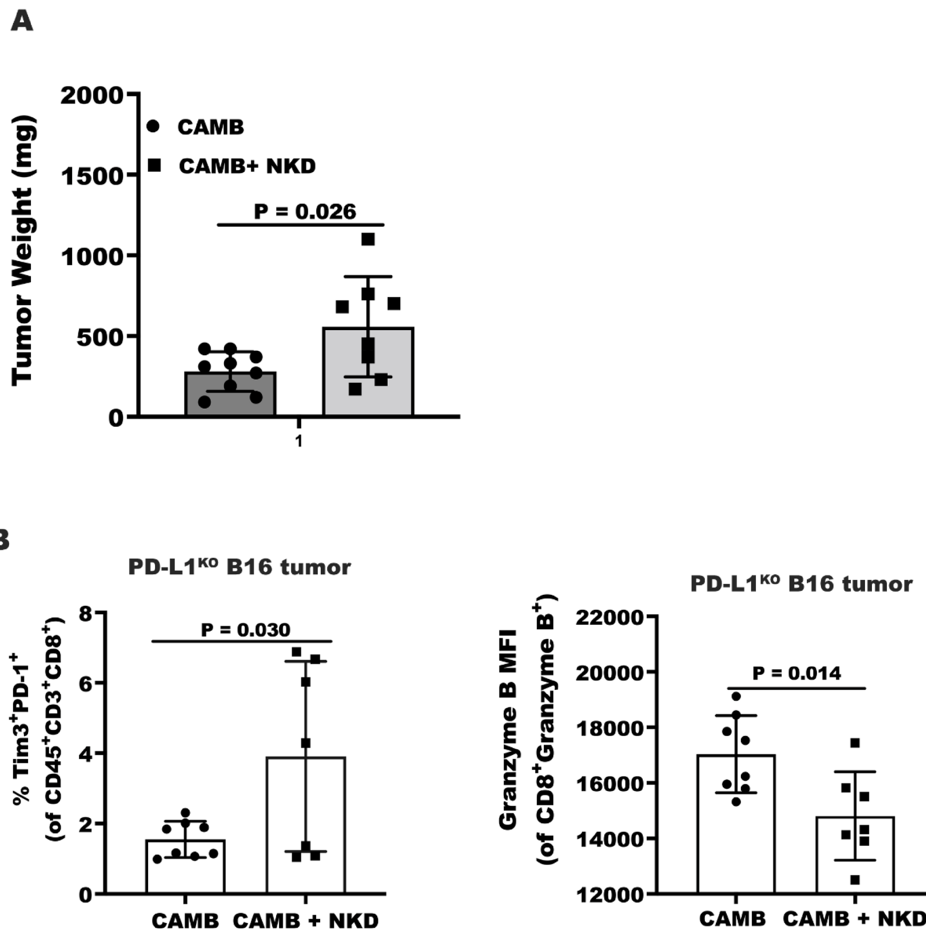


Figure S8. Chlorambucil inhibits PDL1^{KO} B16 tumor growth in an NK cell-dependent manner.

(A) Tumor weight of WT mice (N = 5/group) challenged with PDL1^{KO} B16 and treated with 3 mg/kg chlorambucil (CAMB) ± anti-NK1.1 250 µg/mouse. NKD, NK cell depletion with αNK1.1.

(B) Tim3^{+}PD-1^{+} (exhausted) CD8^{+} T cells and Granzyme B production by CD8 T cells. P-value, *t*-test.

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Supplemental Figure 9

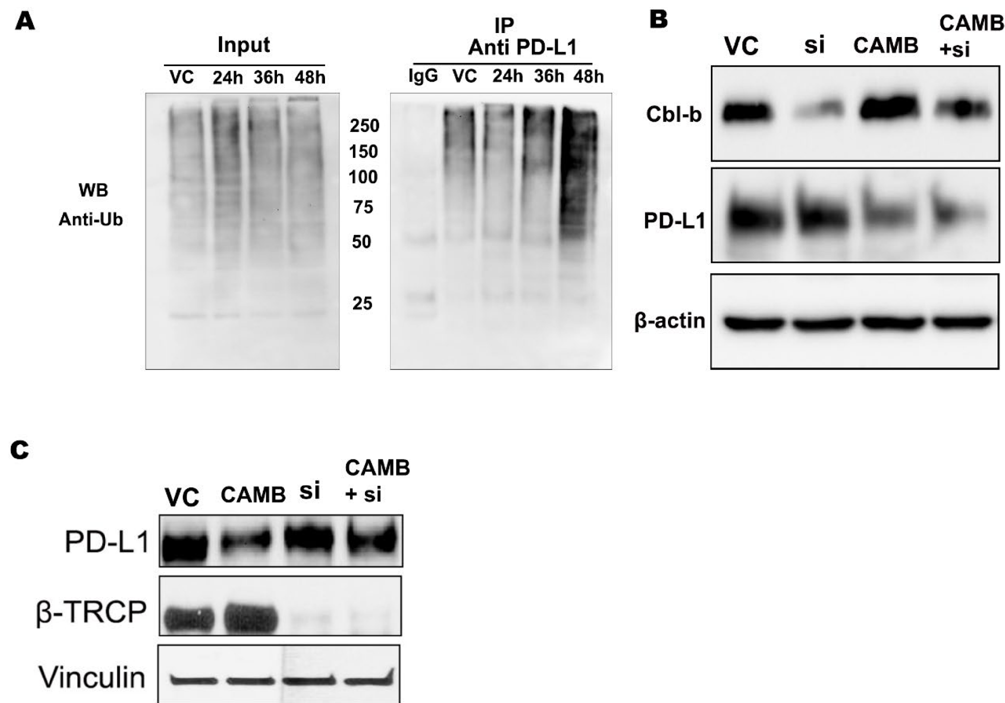

Figure S9. PDL1 degradation is dependent on the E3 ligase β-TrCP rather than CBL-B.

(A) Ubiquitinated PDL1 in ES2 cells treated with chlorambucil (CAMB, 10 μM) for indicated time was immunoprecipitated (IP) using αPDL1 antibody (1:50) and subjected to immunoblot analysis with anti-ubiquitin (Ub) antibody. Molecular weights to the left of the IP blot. Immunoblot for PDL1 with chlorambucil (2.5 μM) or siCBI-B **(B)** or siβ-TrCP **(C)** with chlorambucil (2.5 μM) in ES2 cells, both for 48 hours.