The mechanism of anti-PD-L1 antibody efficacy against PD-L1 negative tumors identifies NK cells expressing PD-L1 as a cytolytic effector

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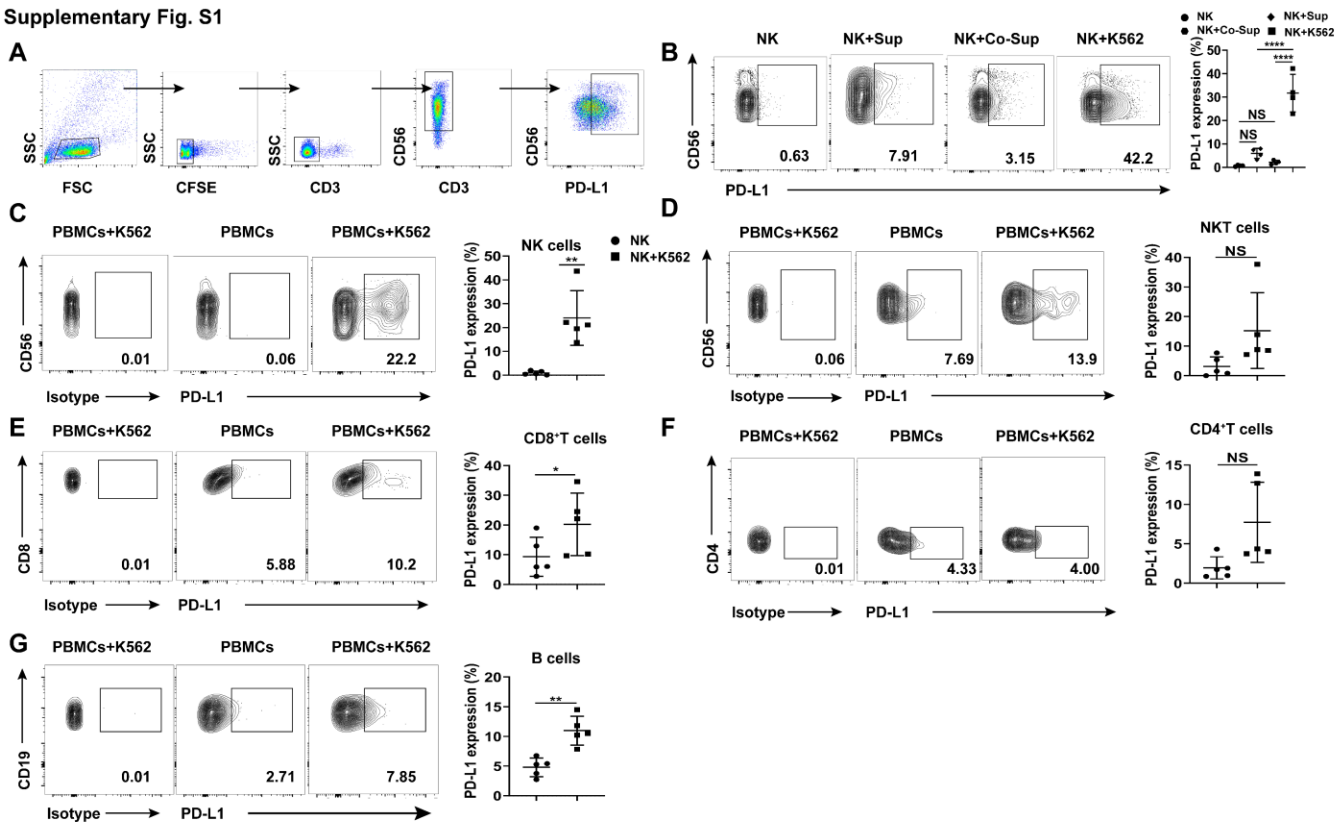
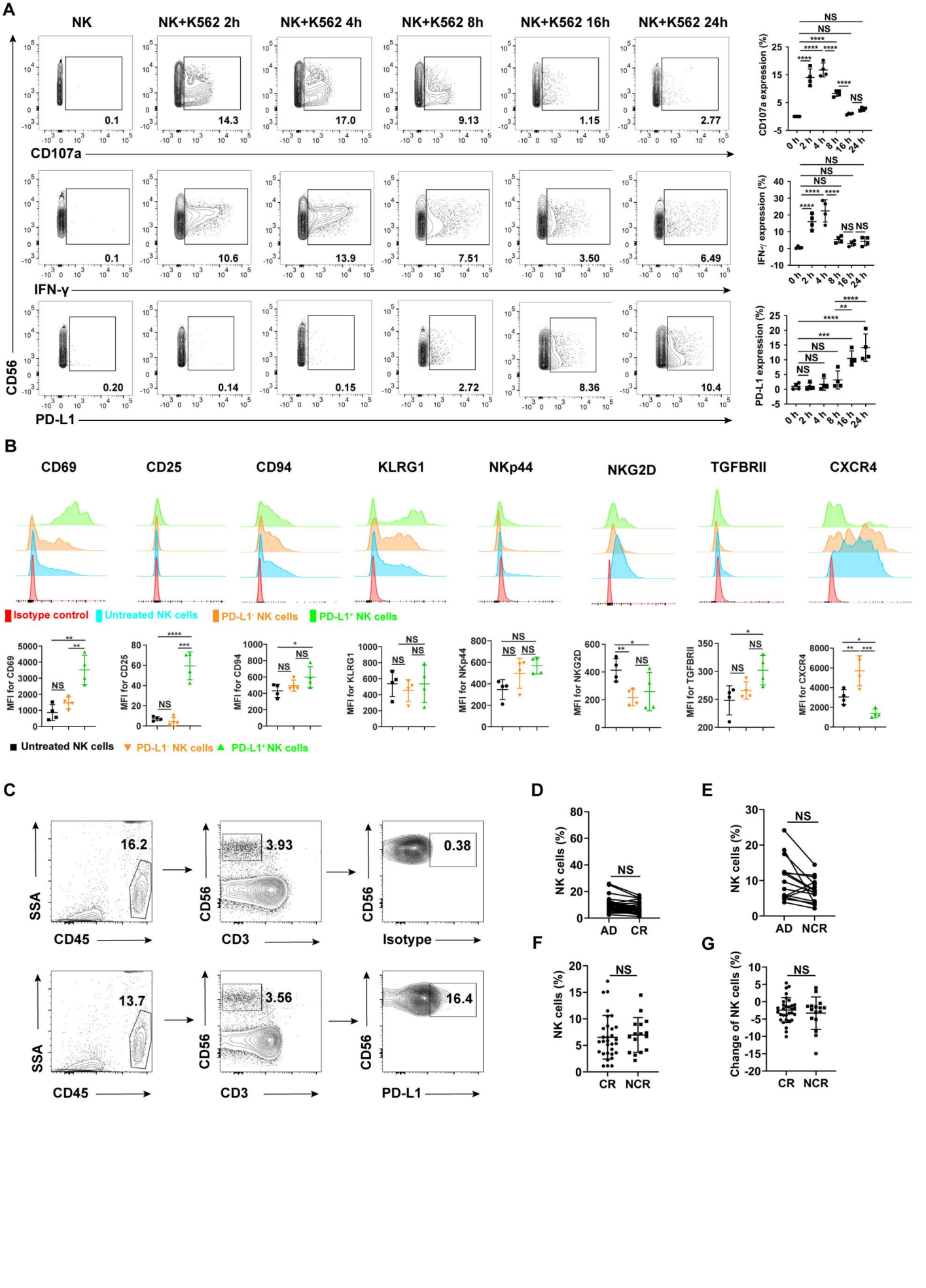


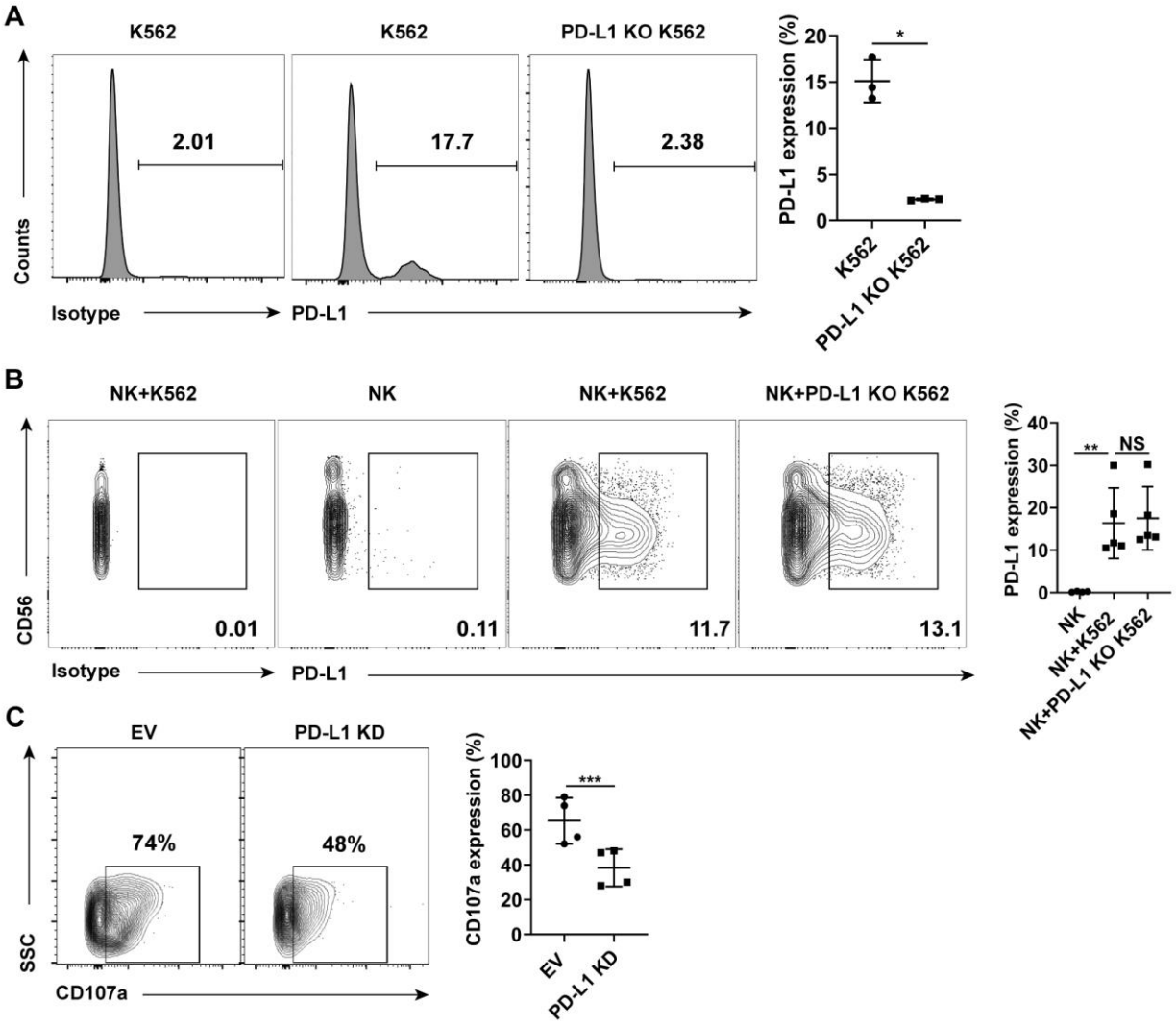
Supplementary Fig. S1. Induction of PD-L1 expression on NK cells by K562 cells and/or PBMCs in the presence of IL-2. (A) Representative flow cytometric plots illustrating the gating strategy used to gate on or to sort purified PD-L1$^+$ NK cells by fluorescence-activated cell sorting (FACS) when primary human NK cells were incubated with carboxyfluorescein succinimidyl ester (CFSE)-labeled K562 myeloid leukemia cell. The induction of PD-L1 surface expression on the NK cells following 24 h incubation with K562 cells was illustrated. (B) NK cells were incubated with IL-2 (10 ng/ml, same for all panels) alone or with the supernatant taken from with K562 cells (Sup) or with the supernatant taken from K562 cells that had been incubated with NK cells in the presence of IL-2 (Co-Sup). PD-L1 surface density expression on NK cells cultured under these conditions was then compared with PD-L1 surface density expression on primary human NK cells incubated with K562 cells plus IL-2. PD-L1 expression was measured by flow cytometry. Representative FACS plot and summary data (n = 4) are shown. (C-G) PBMCs were incubated with K562 myeloid leukemia cells for 24 h in the presence of IL-2, followed by assessment for PD-L1 surface density expression on (C) CD3$^-$CD56$^+$ NK cells, (D) CD3$^+$CD56$^+$ NKT cells, (E) CD3$^+$CD8$^+$ T cells, (F) CD3$^+$CD4$^+$ T cells, and (G) CD3$^-$CD19$^+$ B cells, as measured by flow cytometry (n = 5). Two paired groups were compared by paired t test. One-way ANOVA with repeated measures or linear mixed model was used to compare donor-matched 3 or more groups. *P* values were adjusted by the Holm-Sidak method. *, $P < 0.05$; **, $P < 0.01$; ****, $P < 0.0001$; NS, not significant. FSC, forward scatter; SSC, side scatter; PBMCs, peripheral blood mononuclear cells; Sup, supernatant; Co-Sup, Co-culture supernatant.

Supplementray Fig. S2

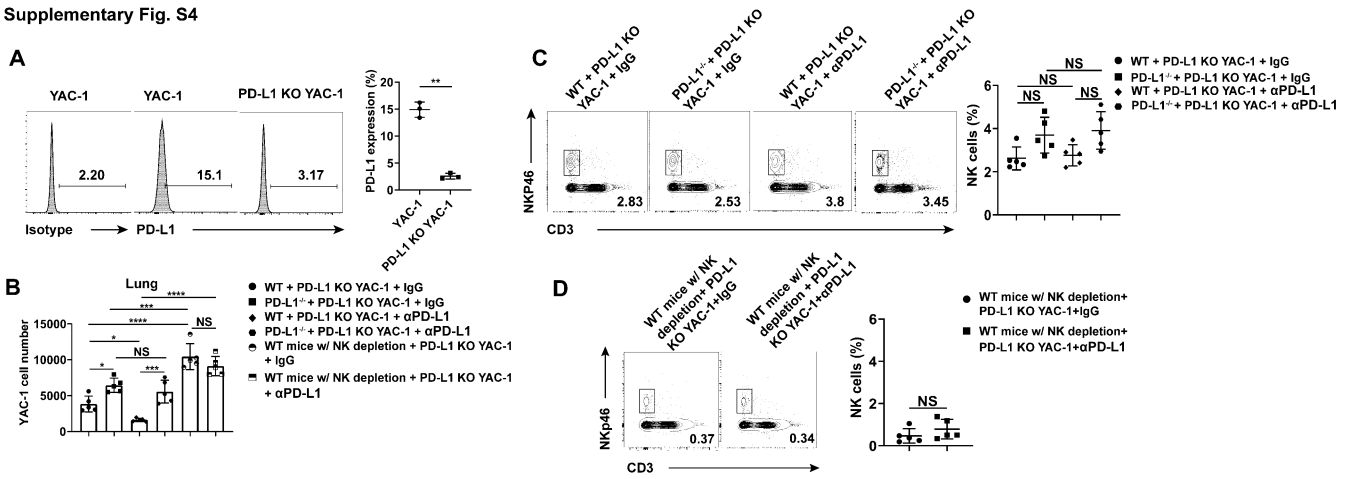


Supplementary Fig. S2. Temporal relationship between NK cell activation and PD-L1 expression during incubation with K562 myeloid leukemia cells and the correlation between PD-L1$^+$ NK cells and treatment outcomes. (A) Representative flow cytometry plots and summary data (n = 4) showing the expression of CD107a, IFN-γ, and PD-L1 in primary human NK cells when incubated with K562 myeloid leukemia cells at indicated time points in the presence of 10 ng/ml IL-2. (B) Representative flow cytometry plots and summary data (n = 4) of surface markers on primary NK cells isolated from healthy donors and incubated without or with K562 cells for 24 h. (C) Representative example of the gating strategy assessing PD-L1 induction on CD56$^+$ human NK cells upon co-culture with primary human AML blasts. PBMC from healthy donors were co-cultured with blasts isolated from AML patients for 24 h. The NK cell population was gated as CD45$^+$CD3$^-$CD56$^+$. PD-L1$^+$ cells (bottom panel) were gated according to isotype control (top panel). (D and E) Percentages of total NK cells at time of diagnosis and at time of evaluation for response following standard induction chemotherapy in AML patients who (C) achieved a CR (n = paired groups of 31) and (E) those who did not achieve a CR (NCR; n = paired groups of 16). (F) Percentages of total NK cells at time of evaluation for response following standard induction chemotherapy in AML patients who did (CR) and did not achieve a CR (NCR). (G) Percentage change of total NK cells (calculated by comparing total NK cells at diagnosis and at the time of evaluation for response following standard induction chemotherapy) in patients who achieve a CR and those who did not achieve a CR (NCR). Two paired groups were compared by paired t test. One-way ANOVA with repeated measures was used to compare donor-matched 3 or more groups. P values were adjusted by Holm-Sidak method. Multiple comparison test is adjusted by the Holm-Sidak method. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$; NS, not significant.

Supplementary Fig. S3

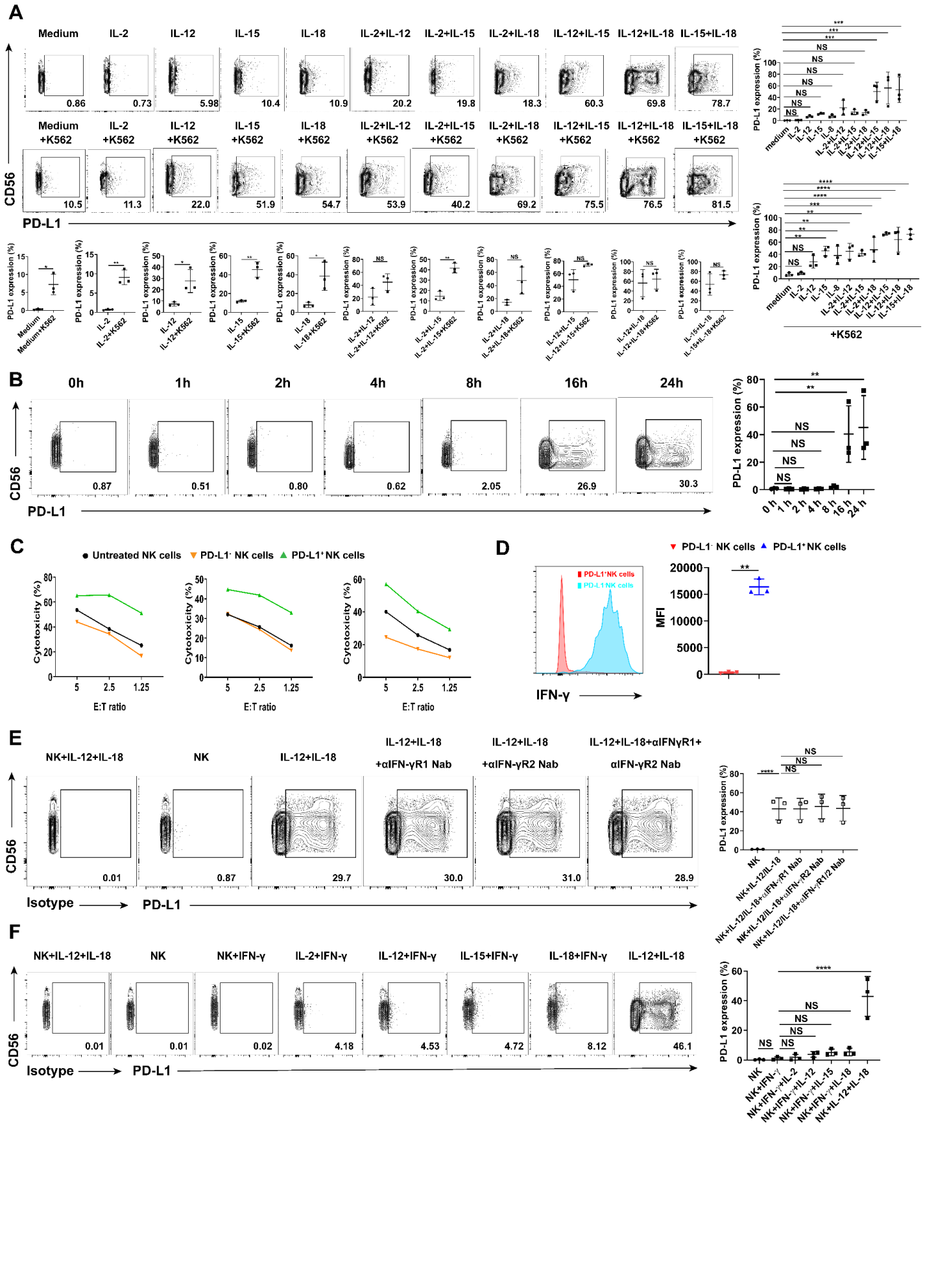


Supplementary Fig. S3. PD-L1 expression on NK cells induced by K562 cells and PD-L1 KO K562 cells. (A) Histograms assessing PD-L1 expression on WT and PD-L1 KO K562 cells by flow cytometry, confirming that the PD-L1 KO K562 cells are negative for PD-L1 expression. (B) PD-L1 expression on NK cells incubated with K562 cells or PD-L1 KO K562 cells were examined by flow cytometry and data were summarized in the right panel (n = 5). (C) NK cells were transduced by lentivius expressing shRNA targeting PD-L1 (PD-L1 KD) and empty vector (EV)-transduced NK cells as control. Representative flow cytometry plots and summary data (n = 4) show the expression of CD107a in PD-L1 KD NK cells or EV control NK cells after being incubated with PD-L1 knock-out K562 myeloid leukemia cells for 24 h and then with 20 μg/ml AZ or IgG control for additional 4 h. Two paired groups were compared by paired t test. Linear mixed model was used to compare 3 or more groups and P values were adjusted by the Holm's method. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; NS, not significant.



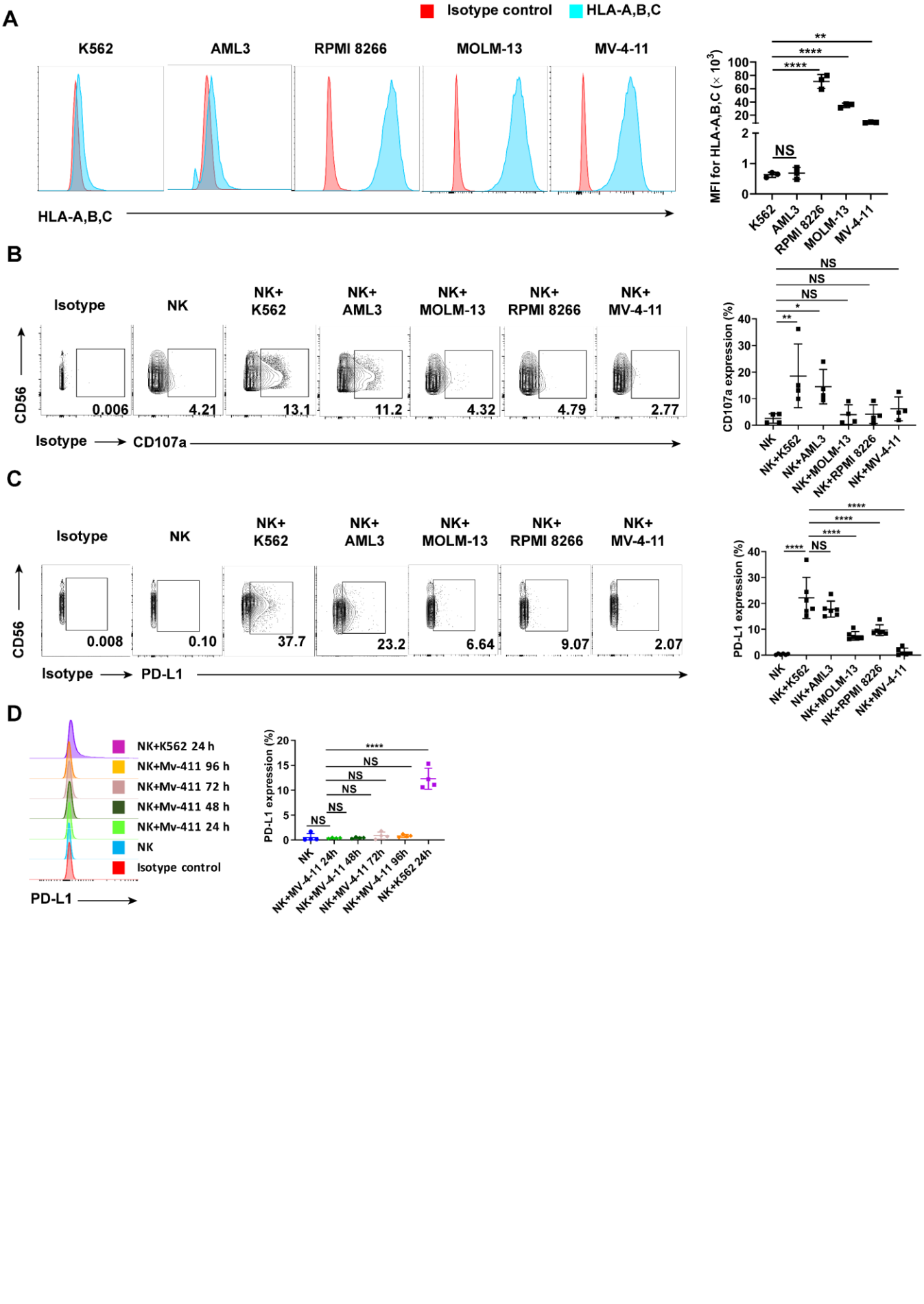
Supplementary Fig. S4. The effects of PD-L1 expressed on NK cells in PD-L1 KO YAC-1 tumor-bearing mice in the presence of a murine anti-PD-L1 antibody. (A) Histograms and summary data showing flow cytometry of PD-L1 KO YAC-1 cells, confirming the cells are negative for PD-L1 expression. (B) The number of PD-L1 KO YAC-1 tumor cells in the lungs of WT and PD-L1$^{-/-}$ mice treated without or with anti-PD-L1 mAb. Summary data are provided for n = 5. (C) The percentage of total NK cells is not significantly different in WT or PD-L1$^{-/-}$ mice, each bearing PD-L1 KO YAC- 1 tumors and each treated with either placebo or anti-PD-L1-mAb. Summary data are provided for n = 5. (D) Confirmation of NK cell depletion in PD-L1 KO YAC-1-bearingmice being treated with either placebo or anti-PD-L1-mAb. Summary data are provided for n = 5. Two paired groups were compared by paired t test. One-way ANOVA with repeated measures or linear mixed model was used to compare 3 or more groups. *P* values were adjusted by the Holm-Sidak method. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$; NS, not significant.

Supplementary Fig. S5

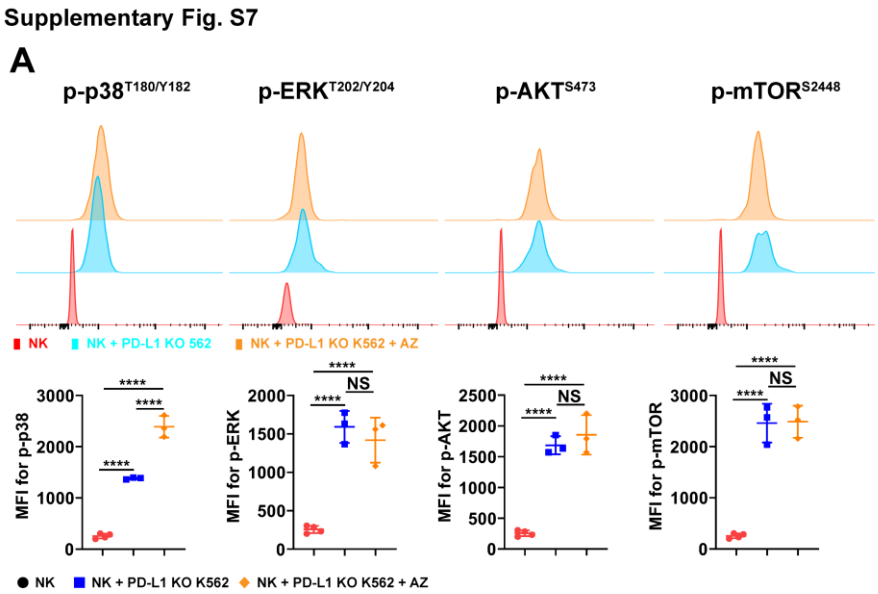


Supplementary Fig. S5. Induction of PD-L1 expression on NK cells by NK cell-activating cytokines. (A) Flow cytometry plots and summary data (n = 3) showing the percentage of PD-L1+ human NK cells under different conditions of cytokine stimulation (10 ng/ml for each cytokine) in the absence (top row) or presence (bottom row) of K562 myeloid leukemia cells. (B) Flow cytometry plots and summary data (n = 3) showing the expression of PD-L1 on human NK cells induced by IL-12 plus IL-18 (10 ng/ml for each cytokine) in a time dependent manner. (C) PD-L1^{+} and PD-L1^{-} NK cells fractionated from bulk primary human NK cells (n = 3) treated with IL-12 plus IL-18 (10 ng/ml for each) overnight were quantified for cytotoxicity using 4-hr standard 51Cr release assay. The cytotoxicity levels of total NK cells incubated with medium alone were served as control. (D) Enriched NK cells were treated with IL-12 plus IL-18 (10 ng/ml for each) for 16 h and then measured for IFN-γ expression by flow cytometry. NK cells were gated on PD-L1^{+} and PD-L1^{-} populations. (E) PD-L1 expression on NK cells treated with IL-12 plus IL-18 (10 ng/ml for each cytokine) with or without IFN-γ receptor 1 neutralizing mAb (αIFN-γR1 Nab) at 10 μg/ml or IFN-γ receptor 2 neutralizing mAb (αIFN-γR2 Nab) at 10 μg/ml or in combination of αIFN-γR1 Nab and αIFN- γR2 Nab at 10 μg/ml each. Summary graph (n = 3) is presented at the right. (F) PD-L1 expression on NK cells induced by IFN-γ or IFN-γ in combination with indicated cytokines at 10 ng/ml for 24 h. Summary graph (n = 3) is presented at the right. Two-sample t test was used for 2-group comparisons. One-way ANOVA was used to compare 3 or more groups and *P* values were adjusted by the Holm-Sidak method. **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$; NS, not significant.

Supplementary Fig. S6

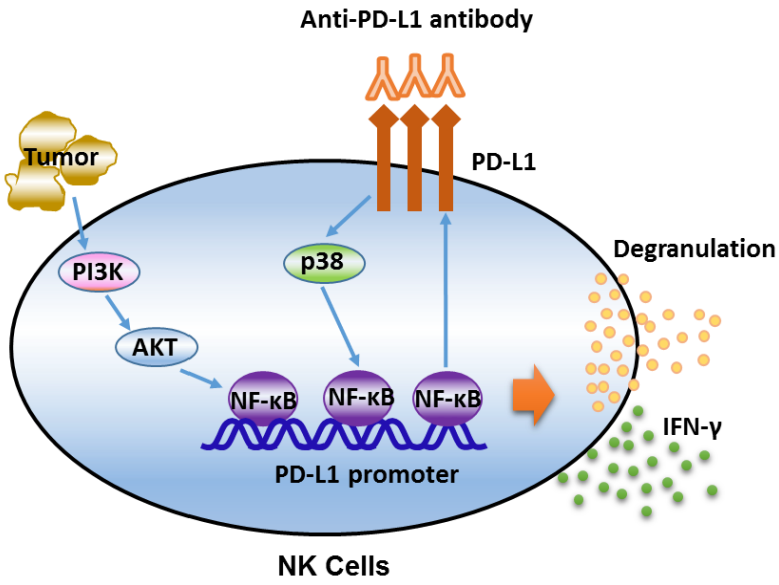


Supplementary Fig. S6. The level of PD-L1 expression is associated with the susceptibility of target cells to NK cell lysis. (A) The expression of MHC Class I (HLA-A, B, C) molecules on various human leukemic cell lines was examined by flow cytometry. The experiment was repeated three times and summarized in graphical form to the right. (B-C) Human NK cells isolated from healthy donors were incubated with indicated cell lines in the presence of 10 ng/ml IL-2 for 24 h and assessed for NK cell expression of (B, n=4) CD107a and (C, n=6) PD-L1 as measured by flow cytometry and summarized in graphical form to the right. (D) Primary human NK cells were incubated with MV- 4-11 human myeloid leukemia cells for the indicated time periods ranging from 24 h to 96 h, while the same NK cells were incubated with K562 myeloid leukemia cells for 24 h. Each culture of NK cells had their expression of PD-L1 quantified by flow cytometry, and the data are summarized (n = 4) in graphical form to the right. One-way ANOVA was used to compare 3 or more groups and *P* values were adjusted by the Holm-Sidak method. *, $P < 0.05$; **, $P < 0.01$; ****, $P < 0.0001$; NS, not significant.



Supplementary Fig. S7. Induction of p38-NF-κB signaling in primary human NK cells by the anti-PD-L1 antibody AZ. (A) Quantification of downstream kinase phosphorylation following NK cell activation when incubated with K562 myeloid leukemia cells without or with 20μg/ml anti-PD-L1 mAb in the presence of IL-2 (10 ng/ml). The histograms of provide quantification of various phosphorylated kinases expressed in NK cells, followed by a graphical summary of the data (n = 3) below each histogram. One-way ANOVA with repeated measures was used to compare donor-matched 3 groups and P values were adjusted by the Holm-Sidak method. ****, $P < 0.0001$; NS, not significant.

Supplementary Fig. S8



Supplementary Fig. S8. Schematic illustration of NK cell activation via a NK cell-susceptible tumor target such as the K562 myeloid leukemia cell line in the presence or absence of an-anti-PD-L1 mAb, which binds to PD-L1 induced by the target cell. The K562 myeloid leukemia tumor cells activate NK cells via the PI3K/AKT signaling pathway, which activates NK-κB. NK-κB binds to the PD-L1 promoter and induces the expression of PD-L1. The binding of anti-PD-L1 mAb to the induced PD-L1 activates p38, which further activates NK-κB to also induce the expression of PD-L1, in which the presence of excess anti-PD-L1 mAb forms a positive feedback signaling loop.

Supplemental table 1. Characteristics of 47 patients.

Characteristics	number
Sex	
Male	22
Female	25
Age in years, Medians (Range)	37 (13,72)
FAB	
M1	5
M2	20
M3	3
M4	9
M5	10
WBC	
$\geq 10\times10^9$/L	24
$< 10\times10^9$/L	23
PLT	
$\leq 20\times10^9$/L	17
$> 20\times10^9$/L	30
HGB	
≤60g/L	11
> 60g/L	36
ELN risk group	
Low risk group	3
Intermediate risk group	24
High risk group	20

WBC, white blood cells; FAB, French-American-British classification; ELN, European Leukemia Net; PLT, platelet and HGB, hemoglobin.

Supplemental table 2. Information of flow antibodies.

Antibody	conjugation	Company	Cat #.
CD3	APC-H7	BD Bioscience	560176
CD4	PerCP-Cy5.5	BD Bioscience	560650
CD19	BV421	Biolegend	302234
CD8	PE-Cy7	Biolegend	557750
CD56	APC	Biolegend	362504
CD56	PE-Cy7	BECKMAN COULTER	A51078
PD-L1	BV421	Biolegend	329714
PD-L1	PE	Biolegend	329706
CD107a	Alexa Fluor 488	Biolegend	328610
IFN-γ	APC	BD Bioscience	554702
Cleaved-Caspase 3	V450	BD Bioscience	560627
Ki67	PerCP-Cy5.5	Biolegend	350520
Sytox Blue	405	Thermo Fisher	S34857
Annexin V	APC	BD Bioscience	550474
CD69	PE	BECKMAN COULTER	IM1943U
CD25	APC	BD Bioscience	555434
CD94	APC	BD Bioscience	559876
KLRG1	APC	Milten Biotec	130-103-639
NKp44	PE	BECKMAN COULTER	IM3710
NKG2D	APC	BD Bioscience	558071
TGFBRII	PE	R&D	FAB2411P
CXCR4	PE	Milten Biotec	130-098-354
Granzyme B	FITC	Biolegend	372206
HLA-A,B,C	PE	Biolegend	311406
anti-Atezolizumab anitbody	biotin	GenScript	A01950
PD-L1	Biotin	Biolegend	329704
Anti-Biotin	PE	eBioscience	12-9895-82
NKp46	Alexa Fluor 647	BD Bioscience	560755
CD3	PE-Cy7	BD Bioscience	552774
PD-L1	BV421	Biolegend	124315
CD107a	PE	BD Bioscience	558661
CD45	Alexa Fluor 700	eBioscience	56-0454-82

Supplementary Methods and Materials

Cell culture

Peripheral blood samples from healthy donors were obtained from The American Red Cross. Human PBMCs were isolated by Percoll density gradient centrifugation. Primary human NK cells were enriched from the peripheral blood of healthy donors using an NK Cell Enrichment Kit (Miltenyi Biotec, Cat #.130-115-818). Enriched NK cells with purity about 90% were used immediately for *in vitro* cell culture experiments. NK cells were cultured in RPMI 1640 supplemented with 20% FBS, 100 U/ml penicillin/streptomycin, and 10 ng/ml IL-2. All cell lines were maintained in RPMI 1640 medium supplemented with 10% FBS and 100 U/ml penicillin/streptomycin. For co-culture stimulation experiments, PBMCs, enriched NK cells, or FACS-sorted NK cells were co-incubated with various cell lines including K562, MOLM-13, AML3, RPMI 8226 or MV-4-11 at an effector/target (E/T) ratio of 10:1. NK cells were cultured with 10 ng/ml IL-2 in *in vitro* co-incubation assays unless indicated otherwise in the figures or figure legends. For transwell assay experiments, 5×10^5 enriched human primary NK cells were seeded in the upper chamber of a transwell plate. The lower chamber of the transwell plate was seeded with 5×10^4 K562 myeloid leukemia cells. The transwell plate with cells was incubated at 37 °C for 20 hr.

Antibody staining and flow cytometry

Cells were suspended in 100 µl PBS with 2% FBS and incubated with the indicated mAb (Supplementary Table 2) at room temperature for 20 mins. After washing with 2% FBS for one time and PBS for another time, cells were fixed in 1% paraformaldehyde buffer for immediate analysis by flow cytometry using a LSRII flow cytometer (BD Biosciences). Cells used for sorting were re-suspended in RPMI 1640 containing 10% FBS. For intracellular flow cytometry, cells were permeabilized and fixed using the Foxp3/Transcription Factor Fixation/Permeabilization kit (eBioscience, Cat #00-5523-00). Data were analyzed by FlowJo software.

Immunostaining assay

Resting or K562-induced NK cells were seeded on a glass-bottom dish and centrifuged for 10 mins. Cells were stained with 5 μg/mL mouse anti-human CD56 antibody (Invitrogen, Cat. #MA1-35249) and rabbit anti-human PD-L1 antibody (Cell Signaling Technology, Cat. #13684) according to the manufacturer's instructions. Cells were then washed and stained with goat anti-rabbit IgG conjugated with Alexa Fluor 488 (Thermos Fisher, Cat. # A-11034) and goat anti-mouse IgG conjugated with Alexa Fluor 594 (Thermos Fisher, Cat. # A-11005). Cells were then stained with DAPI (Sigma, Cat. #D9542-1MG). The stained cells were examined under a LSM 880 Laser Scanning Microscope at 20× objective.

Immunoblotting assay

Cells were pelleted and lysed in protein extraction reagent (Thermo Fisher, Cat. #78510) supplied with proteinase inhibitors. Standard procedures were used for immunoblotting. Primary antibodies used were PD-L1 (Cell Signaling Technology, Cat. #13684), GAPDH (Cell Signaling Technology, Cat. #2118), β-actin (Cell Signaling Technology, Cat. #4967), phosphor (p)-p38 (Cell Signaling Technology, Cat. #9211), p-p65 (Cell Signaling Technology, Cat. #3033), RelB (Cell Signaling Technology, Cat. #4922), and RelC (Cell Signaling Technology, Cat. #12707). Proteins were detected using goat anti-rabbit HRP-conjugated secondary antibodies (Cell Signaling Technology, Cat. #7074).

Real-time PCR

RNA was isolated using a RNA isolation kit (QIAGEN, Cat. #74106) according to manufacturer's instructions, and cDNA was synthesized using a cDNA synthesis kit (Thermo Fisher Scientific, Cat. #18080051). Data were collected using a StepOnePlus Real-Time PCR System (Thermo Fisher Scientific) using a reaction protocol of 95°C for 1 min, followed by 40 cycles of 95°C for 10 s, 60°C for 30 s, and 72°C for 30 s. PD-L1-F: TGGCATTTGCTGAACGCATTT; PD-L1-R:

TGCAGCCAGGTCTAATTGTTTT.

ChIP assay

Chromatin immunoprecipitation (ChIP) assay was performed using Pierce™ Agarose ChIP Kit from Thermo Scientific™, followed by the manufacturer's instruction. Briefly, 293T cells were transfected with the *PD-L1* promoter alone or together with AKT, p38, p65 or empty vector control for 24 h. The cells were cross-linked at 1% formaldehyde and washed once with glycine solution. The chromatin was collected form cell lysate and digested into 20-1000 bp segments by MNase. Digested chromatin was incubated overnight with a p65 ChIP-grade antibody (Cell Signaling Technology, Cat. #8242) or IgG control antibody (Cell Signaling Technology, Cat. #3900). The enriched chromatin was analysis by Real-time PCR (RT-PCR). Primer-PD-L1 promoter- F: TCAGTCACCTTGAAGAGGCT; Primer-PD-L1 promoter- R: TTTCACCGGGAAGAGTTTCG

PD-L1-knockout cell line

PD-L1-knockout K562 and YAC-1 cells were generated using CRISPR/Cas9 knockout plasmids purchased from Santa Cruz (for K562 cells: Cat. #sc-401140-KO-2, sc-401140-HDR-2; for YAC-1 cells: Cat. #sc-425636, # sc-425636-HDR) and used according to manufacturer's instructions. K562 and YAC-1 cells were co-transfected with the homology-directed DNA repair (HDR) plasmid, which incorporates a puromycin resistance gene for selection of cells containing a successful Cas9-induced site-specific human/murine-PD-L1 knockout in genomic DNA. The cells were then selected with media containing 2 μg/ml puromycine. The expression of PD-L1 was examined by flow cytometry.

NSG mouse model

Fresh primary human NK cells (1×10^7) were injected i.v. into 8-week old NSG mice with or without PD-L1 knockout (KO) K562 myeloid leukemia cells (1×10^6) followed by i.p. injection of 1 μg IL-12 and 1 μg IL-15 per mouse every other day. The numbers of NK cells and tumor cells were

examined at day 6 post injection. For the survival experiment, 8-week old NSG mice were injected i.v. with 2×10^6 PD-L1 KO K562 myeloid leukemia cells per mouse on day 0. On day1, each mouse was injected i.v once with 2×10^7 human primary NK cells and injected i.p with IL-2 alone or the combination of IL-12, IL-15 and IL-18 at a dose of 1 μg for each cytokine per mouse. Mice in the atezolizumab (AZ)-treated or control group were also injected i.p with 200 μg AZ in 200 μl PBS or the same volume of PBS per mouse. Cytokines and AZ were injected every other day for two weeks.

YAC-1 mouse model

8-week old WT and PD-L1$^{-/-}$ BALB/c mice were i.p-injected with an anti-PD-L1 mAb or an IgG control antibody at the concentration of 500 μg per mouse. To deplete NK cells, mice were i.p-injected with 10 μl anti-asialo-GM1 antibody one day before inoculation of YAC-1 tumor cells. On the next day, mice were i.v-injected with PD-L1-knockout YAC-1 cells (PD-L1 KO YAC-1) at the dose of 1 million cells per mouse. The antibodies were administered every three days at a dose of 200 μg per mouse for four weeks. The numbers of immune cells and tumor cells were examined at day 30 post injection.

Data Availability

Microarray data can be accessed from the Gene Expression Omnibus (GEO) database (accession number: GSE132976).