Cell Reports Medicine, Volume *5*

Supplemental information

Venetoclax acts as an immunometabolic modulator to potentiate adoptive NK cell immunotherapy against leukemia

Yan Wang, Beibei Huang, Tingting Liang, Lai Jiang, Mingming Wu, Xinru Liu, Mingming Zhu, Xian Song, Na Zhao, Haiming Wei, Changcheng Zheng, and Fang Ni

Supplemental Figures and Figure Legends

Figure S1

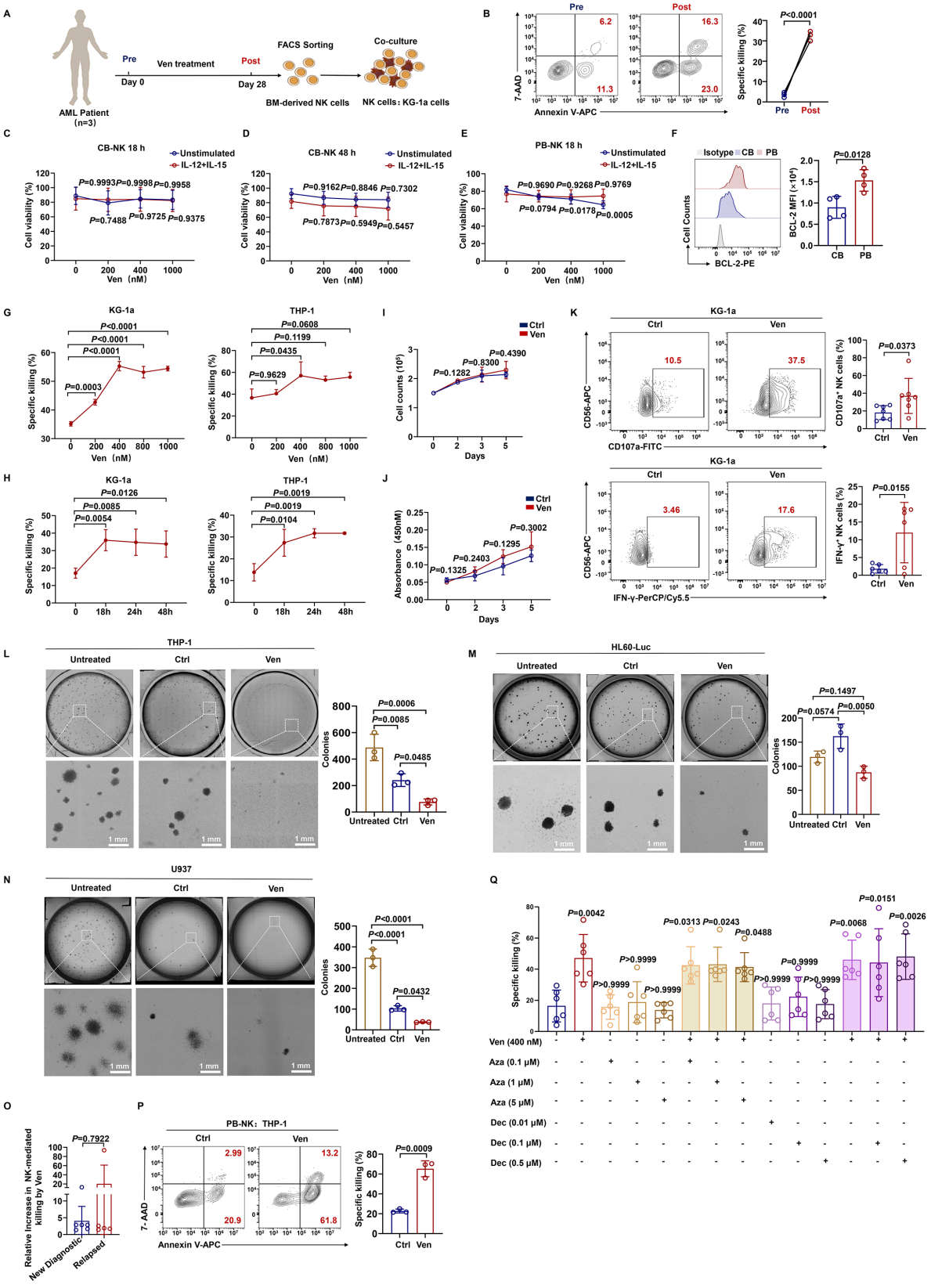


Figure S1. Venetoclax enhances NK-mediated killing of AML cells without compromising NK cell viability, related to Figure 1.

(A) Schematic strategy for isolating NK cells from the bone marrow of AML patients pre- and post-venetoclax treatment for cytotoxicity assays.

(B) Representative flow cytometry plots and quantification of the specific killing ability of NK cells (5×10^4 cells per well) derived from frozen bone marrow samples of AML patients pre- and post-venetoclax treatment against KG-1a cells at a 2.5:1 ratio for 4 h (n=3, biological replicates). The results represent three independent experiments.

(C) Sensitivity of resting or IL-12 (10 ng/mL) + IL-15 (50 ng/mL)-activated cord blood NK cells (CB-NK cells) to venetoclax for 18 h (n=4, biological replicates). NK cell viability was assessed by Annexin V/7-AAD co-staining. The results represent two independent experiments.

(D) Sensitivity of resting or IL-12 (10 ng/mL) + IL-15 (50 ng/mL)-activated CB-NK cells to venetoclax for 48 h (n=4, biological replicates), and the viability of NK cells were determined as in (C). The results represent two independent experiments.

(E) Sensitivity of resting or IL-12 (10 ng/mL) + IL-15 (50 ng/mL)-activated peripheral blood NK cells (PB-NK cells) to venetoclax for 18 h (n=4, biological replicates), and the viability of NK cells was determined as in (C). The results represent two independent experiments.

(F) Flow cytometric detection of BCL-2 expression in resting PB-NK or CB-NK cells (n=4, biological replicates).

(G) CB-NK cells (5×10^4 cells per well) treated with venetoclax (18 h), co-cultured with KG-1a or THP-1 cells (2.5:1 ratio, 4 h, n=3, biological replicates). AML cell viability assessed by flow cytometry. The results represent two independent experiments using NK cells from different donors.

(H) CB-NK cells (5×10^4 cells per well) treated with 400 nM venetoclax (18 h, 24 h, 48 h) or untreated. Cytotoxicity against KG-1a (left, n=4, biological replicates) and THP-1 (right, n=3, biological replicates) cells were determined at a 2.5:1 ratio. The results represent two independent experiments using NK cells from different donors.

(I-J) Proliferation of CB-NK cells treated with or without venetoclax for the indicated time, as measured by cell counting (I, n=3, biological replicates) and CCK-8 assay (J, n=4, biological replicates).

(K) Representative flow cytometry plots (left) and quantification (right) of CD107a (n=7, biological replicates) and IFN-γ (n=6, biological replicates) production in CB-NK cells pretreated with 400 nM venetoclax or untreated, upon stimulation with KG-1a cells in vitro. The results represent three independent experiments.

(L-N) Colony formation assay using THP-1, HL60-Luc, and U937 cells co-cultured with venetoclax-treated or untreated CB-NK cells (2.5:1 ratio, 4 h, n=3, biological replicates). Equal cell numbers (2×10^3 cells per dish) were

used. Colonies counted after 14 days.

(O) Impact of venetoclax on NK cell cytotoxicity against AML cells in newly diagnosed or relapsed AML patients (newly diagnosed n=6, biological replicates, relapsed n=5, biological replicates).

(P) Representative flow cytometry plots and quantification of venetoclax-pretreated (400 nM,18 h) PB-NK cell (5×10^4 cells per well) killing of THP-1 cells (2.5:1 ratio,4 h, n=3, biological replicates).

(Q) NK cells (5×10^4 cells per well) from different donors were treated with 400 nM venetoclax, azacitidine (Aza, 0.1-5 μM), decitabine (Dec, 0.01-0.5 μM) alone, or their combination for 18 h, and then co-cultured with KG-1a cells for 4 h at a 2.5:1 ratio (n=6, biological replicates). The viability of KG-1a cells was assessed by flow cytometry using an Annexin V/7-AAD co-staining assay. The results represent three independent experiments.

Statistical significance was calculated by the unpaired Student's *t*-test (F, I, J, K, and P), paired Student's *t*-test (B), Mann-Whitney test (O), or one-way ANOVA with Tukey's multiple comparisons test (C, D, E, G, H, L, M, N, and Q). The data are represented as mean ± SD.

Figure S2

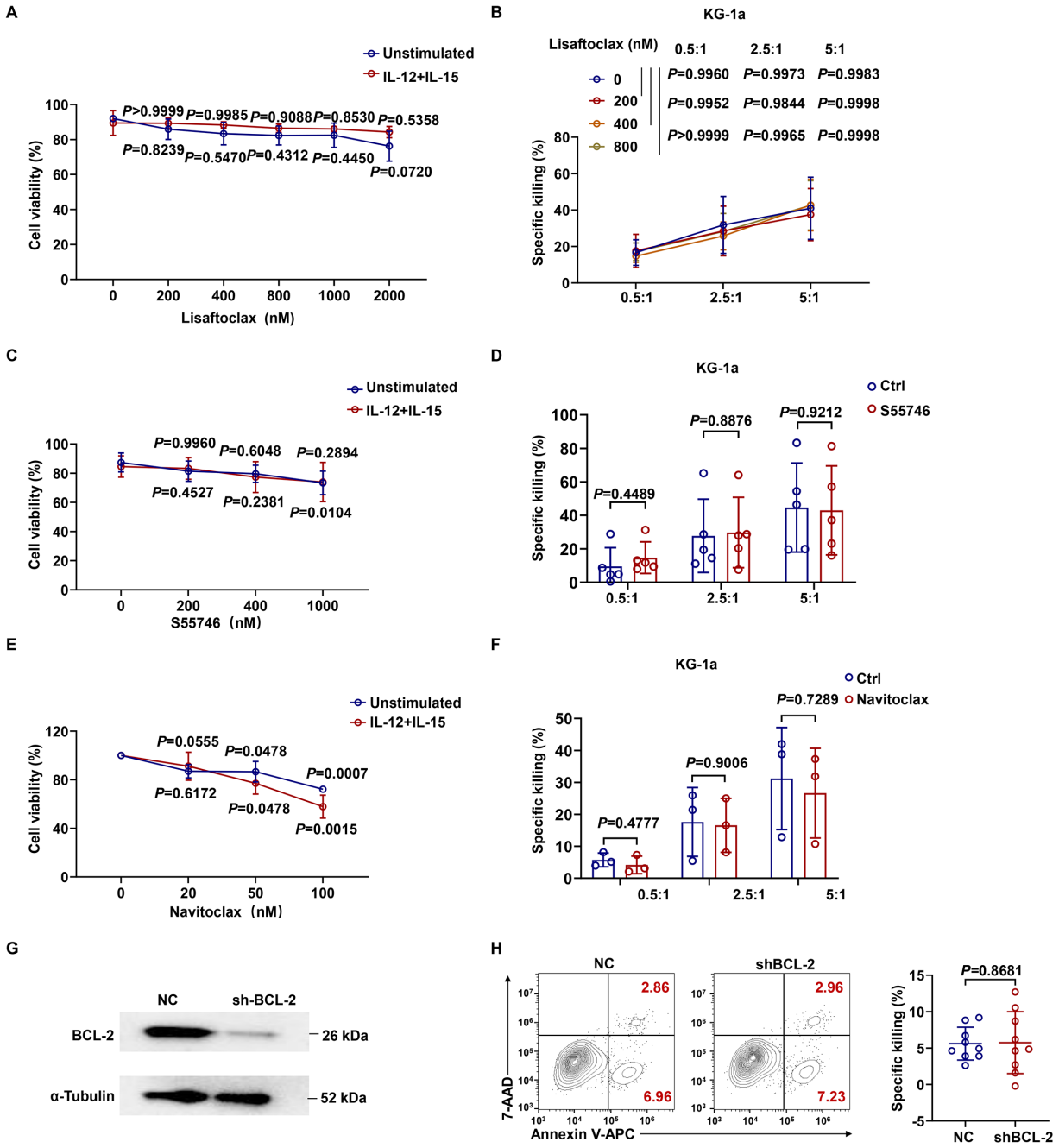

A
Unstimulated
IL-12+IL-15
Cell viability (%)
Lisaftoclax (nM)
P>0.9999 P=0.9985 P=0.9088 P=0.8530 P=0.5358
P=0.8239 P=0.5470 P=0.4312 P=0.4450
P=0.0720
B
KG-1a
Lisaftoclax (nM)
0.5:1 2.5:1 5:1
0
200
400
800
P=0.9960 P=0.9973 P=0.9983
P=0.9952 P=0.9844 P=0.9998
P>0.9999 P=0.9965 P=0.9998
Specific killing (%)
C
Unstimulated
IL-12+IL-15
Cell viability (%)
S55746 (nM)
P=0.9960 P=0.6048 P=0.2894
P=0.4527 P=0.2381 P=0.0104
D
KG-1a
Ctrl
S55746
Specific killing (%)
P=0.4489 P=0.8876 P=0.9212
E
Unstimulated
IL-12+IL-15
Cell viability (%)
Navitoclax (nM)
P=0.0555 P=0.0478 P=0.0007
P=0.6172 P=0.0478 P=0.0015
F
KG-1a
Ctrl
Navitoclax
Specific killing (%)
P=0.4777 P=0.9006 P=0.7289
G
NC
sh-BCL-2
BCL-2
26 kDa
α-Tubulin
52 kDa
H
NC
shBCL-2
2.86
6.96
2.96
7.23
7-AAD
Annexin V-APC
Specific killing (%)
P=0.8681
NC
shBCL-2

Figure S2. Venetoclax enhances NK cell cytotoxicity against AML, likely independent of BCL-2 inhibition, related to Figure 1.

(A) Survival of CB-NK cells after 18 h of treatment with various concentrations of Lisaftoclax (a selective BCL-2 inhibitor), assessed by an Annexin V/7-AAD co-staining assay (n=3, biological replicates).

(B) Cytotoxicity of NK cells (5×10^4 cells per well) from different donors treated with Lisaftoclax for 18 h or left untreated, and co-cultured with KG-1a cells for 4 h at different E: T ratios (n=4, biological replicates). The results represent two independent experiments.

(C) Survival of CB-NK cells treated with different concentrations of S55746 (a selective BCL-2 inhibitor) for 18 h, as assessed by an Annexin-V/7-AAD co-staining assay (n=6, biological replicates). The results represent two independent experiments.

(D) Cytotoxicity of NK cells (5×10^4 cells per well) from different donors treated with 200 nM S55746 for 18 h or left untreated, followed by 4 h co-culture with KG-1a cells at different E: T ratios (n=5, biological replicates). The viability of AML cells was assessed by an Annexin V/7-AAD co-staining assay. The results represent three independent experiments.

(E) Survival of CB-NK cells treated with different concentrations of navitoclax (a BCL-2/BCL-xL inhibitor) for 18 h, as assessed by an Annexin-V/7-AAD co-staining assay (n=3, biological replicates). The results represent two independent experiments.

(F) Cytotoxicity of NK cells (5×10^4 cells per well) from different donors treated with 20 nM Navitoclax or left untreated for 18 h, followed by 4 h co-culture with KG-1a cells at different E: T ratios. The viability of KG1a cells was assessed by an Annexin V/7-AAD co-staining assay (n=3, biological replicates). The results represent two independent experiments.

(G) Western blot analysis showing BCL-2 knockdown in CB-NK cells using negative control shRNA (NC) or BCL-2 shRNA (sh-BCL-2), with α-Tubulin as a loading control.

(H) Cytotoxicity assay with CB-NK cells (2×10^4 cells per well) infected with control shRNA or BCL-2 shRNA against KG-1a cells at a 1:1 ratio for 4 h (n=9, biological replicates). The results represent three independent experiments using NK cells from different donors.

Statistical significance was calculated by unpaired Student's t-test (D, F, and H) or one-way ANOVA with Tukey's multiple comparisons test (A, B, C, and E). The data are represented as mean ± SD. For B, the data are presented as mean ± SEM.

83 **Figure S3**

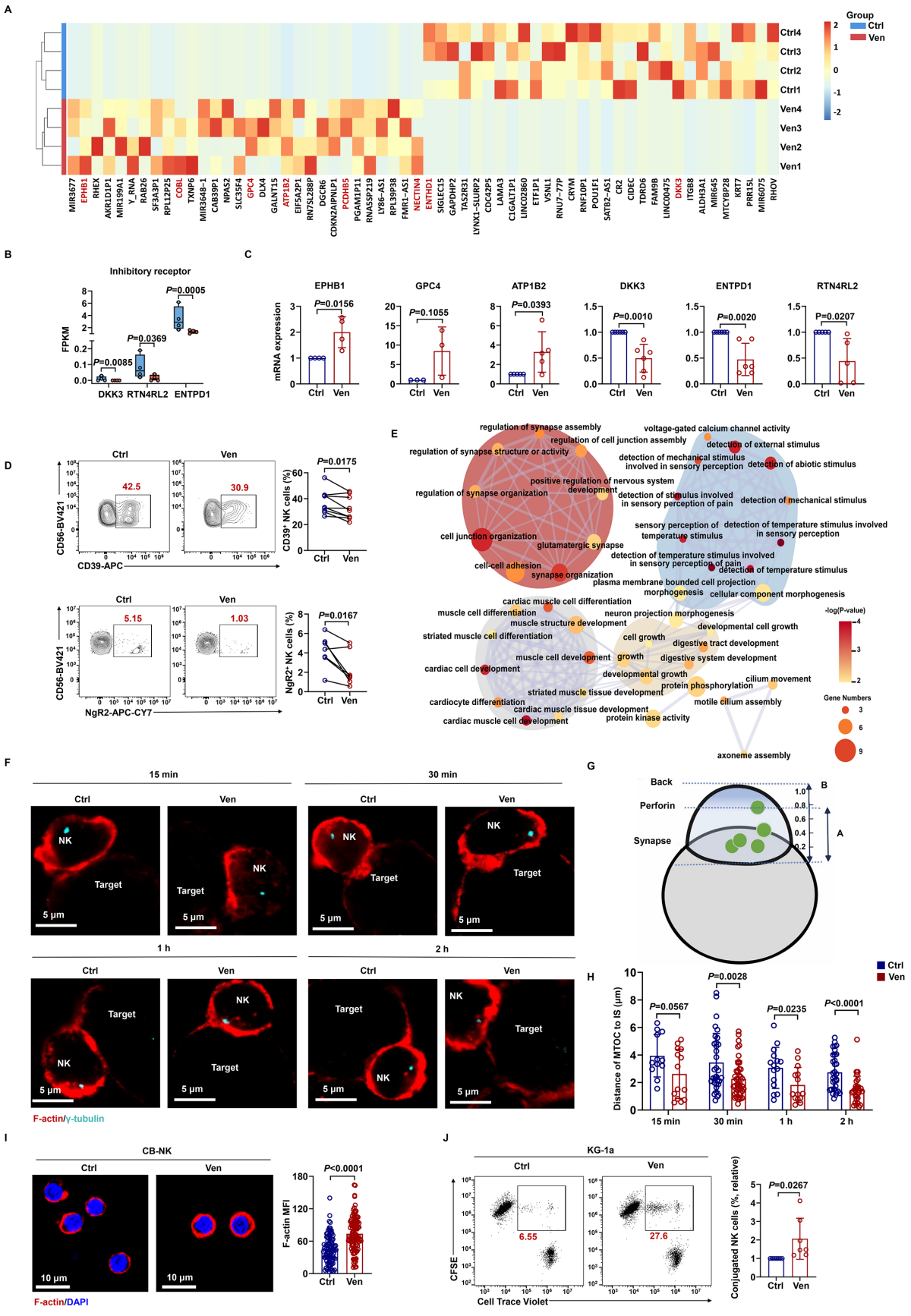

A
Group
Ctrl
Ven
Ctrl4
Ctrl3
Ctrl2
Ctrl1
Ven4
Ven3
Ven2
Ven1
MIR3677
EPHB1
RHEX
AKR1D1P1
MIR199A1
Y_RNA
RAB26
SF3A3P1
RPL12P25
COBL
TXNP6
MIR3648-1
CAB39P1
NPAS2
SLC35F4
GPC4
DLX4
GALNT15
ATP1B2
EIF5A2P1
RN7SL288P
DGCR6
CDKN2AIPNLP1
PCDHB5
PGAM1P11
RNA5SP219
LY86-AS1
RPL39P38
FMR1-AS1
NECTIN4
ENTHD1
SIGLEC15
GAPDHP2
TAS2R31
LYNX1-SLURP2
CDC42P5
LAMA3
C1GALT1P1
LINC02860
ETF1P1
VSNL1
RNU7-77P
CRYM
RNF10P1
POU1F1
SATB2-AS1
CR2
CIDEC
TDRD6
FAM9B
LINC00475
DKK3
ITGB8
ALDH3A1
MIR645
MTCYBP28
KRT7
PRR15L
MIR6075
RHOV
B
Inhibitory receptor
FPKM
P=0.0005
P=0.0369
P=0.0085
DKK3 RTN4RL2 ENTPD1
C
EPHB1
GPC4
ATP1B2
DKK3
ENTPD1
RTN4RL2
mRNA expression
P=0.0156
P=0.1055
P=0.0393
P=0.0010
P=0.0020
P=0.0207
Ctrl Ven
D
Ctrl
Ven
42.5
30.9
CD56-BV421
CD39-APC
CD39+ NK cells (%)
P=0.0175
5.15
1.03
NgR2-APC-CY7
NgR2+ NK cells (%)
P=0.0167
E
regulation of synapse assembly
regulation of cell junction assembly
regulation of synapse structure or activity
positive regulation of nervous system development
regulation of synapse organization
cell junction organization
glutamatergic synapse
cell-cell adhesion
synapse organization
voltage-gated calcium channel activity
detection of external stimulus
detection of mechanical stimulus involved in sensory perception
detection of abiotic stimulus
detection of stimulus involved in sensory perception of pain
detection of mechanical stimulus
sensory perception of temperature stimulus
detection of temperature stimulus involved in sensory perception
detection of temperature stimulus involved in sensory perception of pain
detection of temperature stimulus
plasma membrane bounded cell projection morphogenesis
cellular component morphogenesis
cardiac muscle cell differentiation
muscle cell differentiation
striated muscle cell differentiation
neuron projection morphogenesis
muscle structure development
developmental cell growth
cell growth
digestive tract development
muscle cell development
growth
digestive system development
cardiac cell development
developmental growth
cilium movement
protein phosphorylation
striated muscle tissue development
motile cilium assembly
cardiocyte differentiation
cardiac muscle tissue development
protein kinase activity
cardiac muscle cell development
axoneme assembly
-log(P-value)
Gene Numbers
F
15 min
30 min
1 h
2 h
Ctrl
Ven
NK
Target
5 μm
F-actin/γ-tubulin
G
Back
Perforin
Synapse
A
B
H
Distance of MTOC to IS (μm)
P=0.0567
P=0.0028
P=0.0235
P<0.0001
15 min 30 min 1 h 2 h
I
CB-NK
F-actin MFI
P<0.0001
10 μm
F-actin/DAPI
J
KG-1a
6.55
27.6
CFSE
Cell Trace Violet
Conjugated NK cells (%, relative)
P=0.0267

Figure S3. Venetoclax modulates NK cell actin polymerization, microtubule dynamics, and conjugate formation with AML cells, related to Figure 3.

(A) Heatmap of the top 30 differentially expressed genes between venetoclax-treated (Ven) and control (Ctrl) NK cells.

(B) The distribution of DEG (inhibitory receptor) values in each sample from RNA-seq.

(C) Quantitative real-time PCR verification of randomly selected DEGs in venetoclax (400 nM for 18 h)-treated or untreated CB-NK cells. *GAPDH* was used as an internal reference gene. The results represent two independent experiments.

(D) Flow cytometric detection of CD39 (n=9, biological replicates) and NgR2 (n=7, biological replicates) protein expression in venetoclax (400 nM for 18 h)-treated or untreated CB-NK cells. The results represent three independent experiments.

(E) Network plots of GO-enriched terms, highlighting upregulated terms in the venetoclax-treated group.

(F) Representative confocal images of NK-KG-1a conjugates stained for γ-tubulin (cyan) and F-actin (red). Untreated or venetoclax-treated (400 nM for 18 h) NK cells (1×10^5 cells per well) were incubated with KG-1a cells at a 1:1 ratio for varying time durations.

(G) Schematic illustrating the measurement of the polarization distance from perforin to the IS.

(H) Graphs showing the distance between the MTOC and the IS. The results represent two independent experiments.

(I)Left: representative confocal images of venetoclax (400 nM for 18 h)-treated or untreated NK cells stained with DAPI (blue) and F-actin (red). Right: graphs showing the mean F-actin staining intensity of each cell (control group, n=95; venetoclax-treated group, n=110). The results represent three independent experiments.

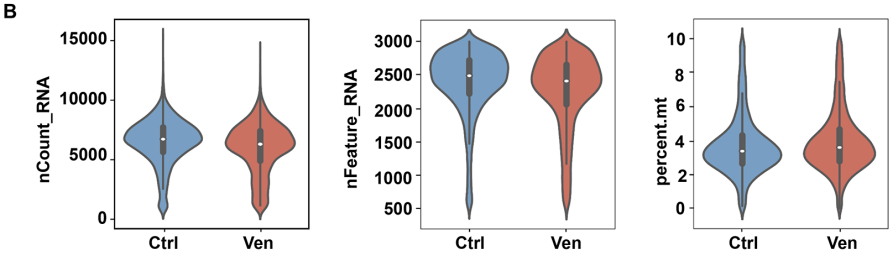
(J) Representative flow cytometry plots (left) and quantification (right) of the conjugation of venetoclax (400 nM for 18 h)-treated or untreated NK cells with target KG-1a cells at 1:4 ratio for 1 h (n=7, biological replicates). Numbers represent the percentages of NK cells conjugated to target cells within the whole NK cell population. The statistical graph shows the relative values of the venetoclax-treated group compared to the untreated group. The results represent three independent experiments.

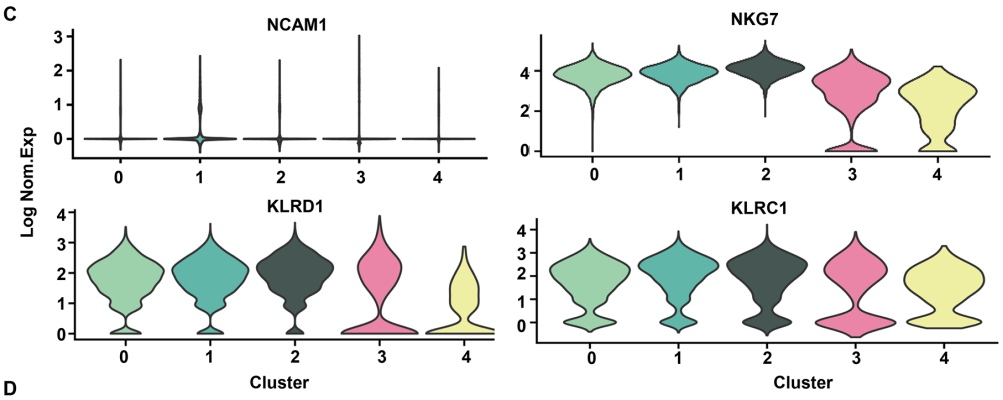
Statistical significance was calculated by unpaired Student's *t*-test (B, C, H, and I), paired Student's *t*-test (D and J), and hypergeometric tests (E). The data are represented as mean ± SD.

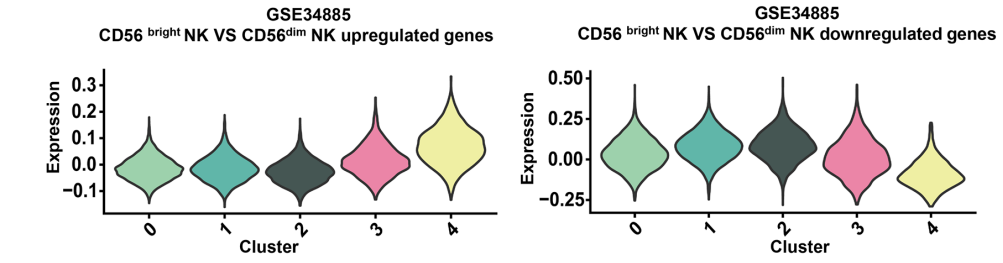
Figure S4

A

Group name	Number of cells	Median genes per cells	Median UMIs per cells	Median mt% per cells
Ctrl	6050	2485	6677	3.39
Ven	8996	2384	6253	3.6







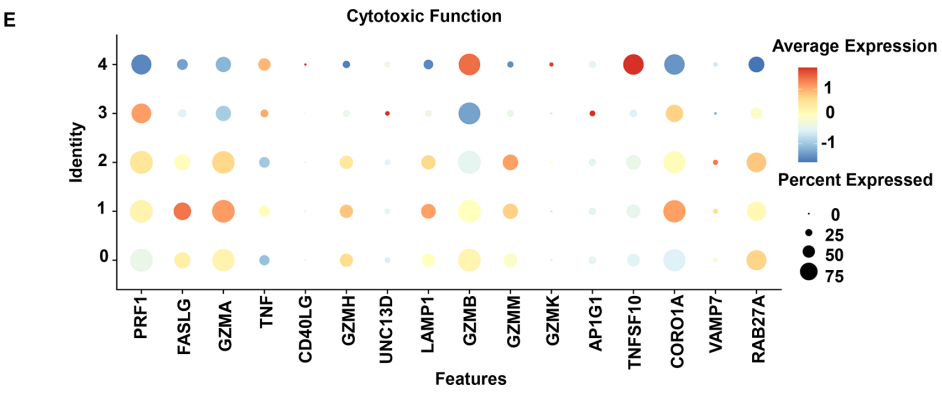


Figure S4. Quality control and phenotypic characteristics of scRNA-seq data obtained from venetoclax-treated or untreated CB-NK cells, related to Figure 4.

(A) Overview of median genes per cell, median UMIs per cell, median mt% per cell, and the number of cells passing the quality control criteria for scRNA-seq data.

(B) Violin plots depicting the nCount, nFeature, and percent.mit (percentage of mitochondrial genes in the transcriptome) single-cell data parameters of the control and venetoclax-treated NK cells.

(C) Violin plots displaying the expression of genes defining NK cells in each cluster.

(D) Violin plots depicting the expression levels of upregulated (left)/downregulated (right) genes in $CD56^{bright}$ vs. $CD56^{dim}$ NK cells in each subcluster.

(E) Bubble plot illustrating representative genes related to NK cell cytotoxicity.

Figure S5

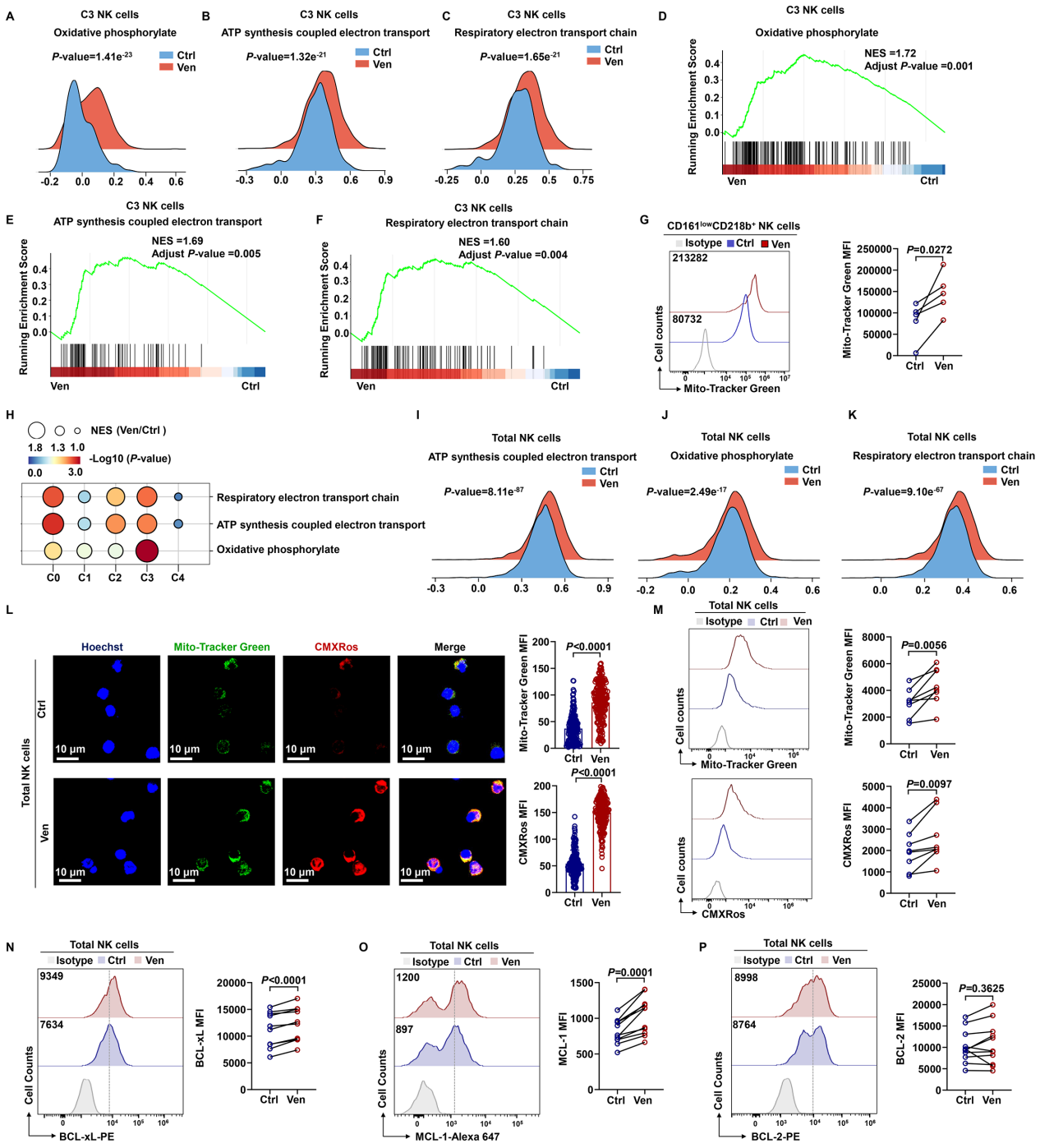

A
C3 NK cells
Oxidative phosphorylate
P-value=1.41e-23
Ctrl
Ven
B
C3 NK cells
ATP synthesis coupled electron transport
P-value=1.32e-21
C
C3 NK cells
Respiratory electron transport chain
P-value=1.65e-21
D
C3 NK cells
Oxidative phosphorylate
NES =1.72
Adjust P-value =0.001
Running Enrichment Score
E
C3 NK cells
ATP synthesis coupled electron transport
NES =1.69
Adjust P-value =0.005
F
C3 NK cells
Respiratory electron transport chain
NES =1.60
Adjust P-value =0.004
G
CD161lowCD218b+ NK cells
Isotype Ctrl Ven
213282
80732
Cell counts
Mito-Tracker Green
Mito-Tracker Green MFI
P=0.0272
H
NES (Ven/Ctrl)
-Log10 (P-value)
Respiratory electron transport chain
ATP synthesis coupled electron transport
Oxidative phosphorylate
C0 C1 C2 C3 C4
I
Total NK cells
ATP synthesis coupled electron transport
P-value=8.11e-87
J
Total NK cells
Oxidative phosphorylate
P-value=2.49e-17
K
Total NK cells
Respiratory electron transport chain
P-value=9.10e-67
L
Hoechst
Mito-Tracker Green
CMXRos
Merge
Total NK cells
Ctrl
Ven
10 μm
Mito-Tracker Green MFI
P<0.0001
CMXRos MFI
P<0.0001
M
Total NK cells
Isotype Ctrl Ven
Mito-Tracker Green
CMXRos
P=0.0056
P=0.0097
N
Total NK cells
9349
7634
BCL-xL-PE
BCL-xL MFI
P<0.0001
O
Total NK cells
1200
897
MCL-1-Alexa 647
MCL-1 MFI
P=0.0001
P
Total NK cells
8998
8764
BCL-2-PE
BCL-2 MFI
P=0.3625

Figure S5. Venetoclax treatment upregulates mitochondrial metabolism and the expression of BCL-xL and MCL-1 in NK cells, related to Figure 5.

(A-C) Elevated expression of gene signatures for OXPHOS (A), ATP synthesis-coupled electron transport (B), and respiratory electron transport chain (ETC) (C) in C3 NK cells of the venetoclax-treated group.

(D-F) GSEA plots illustrating enrichment of the response to OXPHOS (D), ATP synthesis-coupled electron transport (E), and respiratory ETC (F) in C3 NK cells of the venetoclax-treated group. NES, normalized enrichment score.

(G) MitoTracker Green staining of C3 NK cells treated with or without venetoclax (400 nM, 18 h) analyzed by flow cytometry (n=5, biological replicates). The results represent two independent experiments.

(H) GSEA using mitochondrial metabolism-related gene sets from MSigDB in the indicated subpopulations.

(I-K) Elevated expression of ATP synthesis-coupled electron transport (I), OXPHOS (J), and respiratory ETC (K) signature genes in venetoclax-treated total NK cells.

(L) Representative images and quantification of the mitochondrial phenotype in total NK cells treated with or without 400 nM venetoclax for 18 h (control group, n=161; venetoclax-treated group, n=199; MitoTracker Green, green; MitoTracker Red CMXRos, CMXRos, red; blue, Hoechst). The results represent three independent experiments using NK cells from different donors.

(M) Representative histograms and quantification of the mean fluorescence intensity of MitoTracker Green (n=8, biological replicates) and MitoTracker Red CMXRos (CMXRos, n=8, biological replicates) in total NK cells treated with or without 400 nM venetoclax for 18 h. The results represent three independent experiments using NK cells from different donors.

(N-P) Flow cytometric analysis of BCL-xL(N), MCL-1(O), and BCL-2 (P) expression in venetoclax (400 nM for 18 h)-treated or untreated CB-NK cells (n=10-11, biological replicates). The results represent three independent experiments.

Statistical significance was calculated by paired Student's *t*-test (G, M, N, O, and P), unpaired Student's t-tests (A, B, C, I, J, K, and L), multiple hypothesis tests (D, E, and F), or hypergeometric tests (H). The data are represented as mean ± SD.

Figure S6

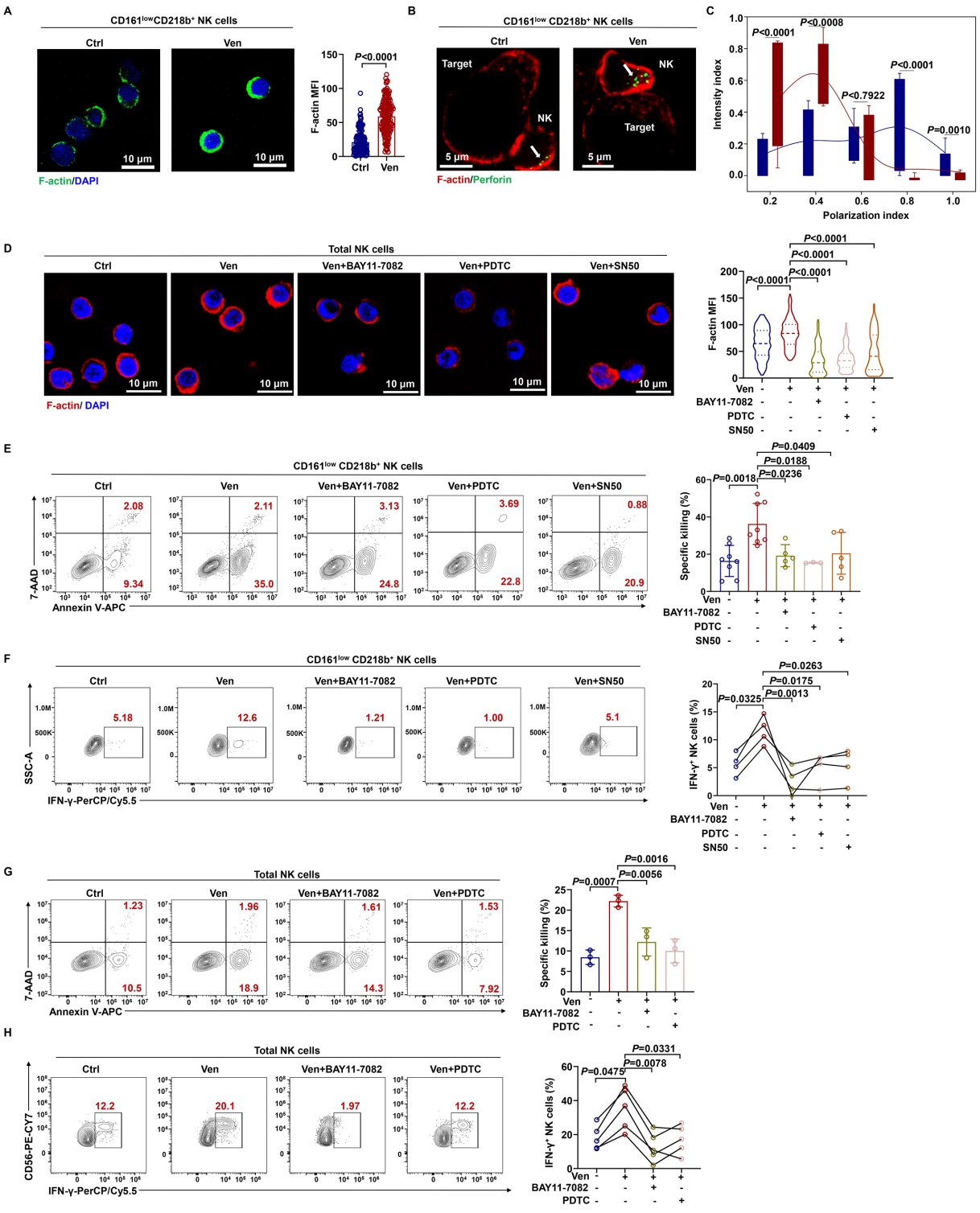

A
CD161low CD218b+ NK cells
Ctrl
Ven
10 μm
F-actin/DAPI
F-actin MFI
P<0.0001
B
CD161low CD218b+ NK cells
Target
NK
5 μm
F-actin/Perforin
C
Intensity index
Polarization index
P<0.0001
P<0.0008
P<0.0001
P<0.7922
P=0.0010
D
Total NK cells
Ven+BAY11-7082
Ven+PDTC
Ven+SN50
F-actin/ DAPI
BAY11-7082
PDTC
SN50
E
CD161low CD218b+ NK cells
7-AAD
Annexin V-APC
2.08
9.34
2.11
35.0
3.13
24.8
3.69
22.8
0.88
20.9
Specific killing (%)
P=0.0409
P=0.0188
P=0.0236
P=0.0018
F
SSC-A
IFN-γ-PerCP/Cy5.5
5.18
12.6
1.21
1.00
5.1
IFN-γ+ NK cells (%)
P=0.0263
P=0.0175
P=0.0013
P=0.0325
G
Total NK cells
1.23
10.5
1.96
18.9
1.61
14.3
1.53
7.92
P=0.0016
P=0.0056
P=0.0007
H
CD56-PE-CY7
12.2
20.1
1.97
12.2
P=0.0331
P=0.0078
P=0.0475

Figure S6. Inhibition of NF-κB signaling attenuates venetoclax-induced effects on NK cell function and cytoskeleton remodeling, related to Figure 7.

(A) Confocal microscopy images (left) and statistical analyses (right) of C3 NK cells treated with or without venetoclax (400 nM for 18 h), stained with DAPI (nuclei, blue) and F-actin (green). The results represent two independent experiments.

(B) Confocal microscopy images showing F-actin (red) and Perforin (green) in cell conjugates formed 1 h after co-incubating C3 NK cells (1×10^5 cells per well) treated with or without 400 nM venetoclax, with KG-1a cells at a ratio of 1:1.

(C) Measurement of granule-to-synapse distances in 32-51 conjugates per condition from B. The results represent three independent experiments.

(D) Left: representative confocal images of total NK cells treated with venetoclax (400 nM) alone or in combination with NF-κB inhibitors (BAY 11-7082 1 μM; PDTC 5 μM; SN50 25 μg/ml), stained with DAPI (blue) and phalloidin (F-actin, red). Right: graphs showing the mean intensity of F-actin for each cell (control group, n=263; venetoclax-treated group, n=263; venetoclax + BAY 11-7082 group n=120; venetoclax + PDTC group n=121; venetoclax + SN50 group n=107). The results represent three independent experiments.

(E) Representative flow cytometry plots and quantification of specific killing of C3 NK cells (2×10^4 cells per well) after venetoclax treatment alone or in combination with NF-κB inhibitors against KG-1a cells (1:1 ratio, 4 h, n=3-8 biological replicates). The results represent three independent experiments.

(F) Representative flow cytometry plots and quantification of IFN-γ expression in C3 NK cells after venetoclax treatment alone or in combination with NF-κB inhibitors (n=4, biological replicates). The results represent three independent experiments.

(G) Representative flow cytometry plots and quantification of specific killing of total NK cells (5×10^4 cells per well) treated with venetoclax alone or in combination with NF-κB inhibitors against KG-1a cells (2.5:1 ratio,4 h, n=3, biological replicates). The results represent two independent experiments.

(H) Representative flow cytometry plots and quantification of IFN-γ expression in total NK cells treated with venetoclax alone or in combination with NF-κB inhibitors (n=5, biological replicates). The results represent three independent experiments.

For all assays involving venetoclax and NF-κB inhibitors presented from Figures E to H, the concentrations follow those specified in (D). Statistical significance was calculated by unpaired Student's t-tests (A and C); one-way ANOVA with Tukey's multiple comparisons test (D, E, F, G, and H). The data are represented as mean ± SD.

Figure S7

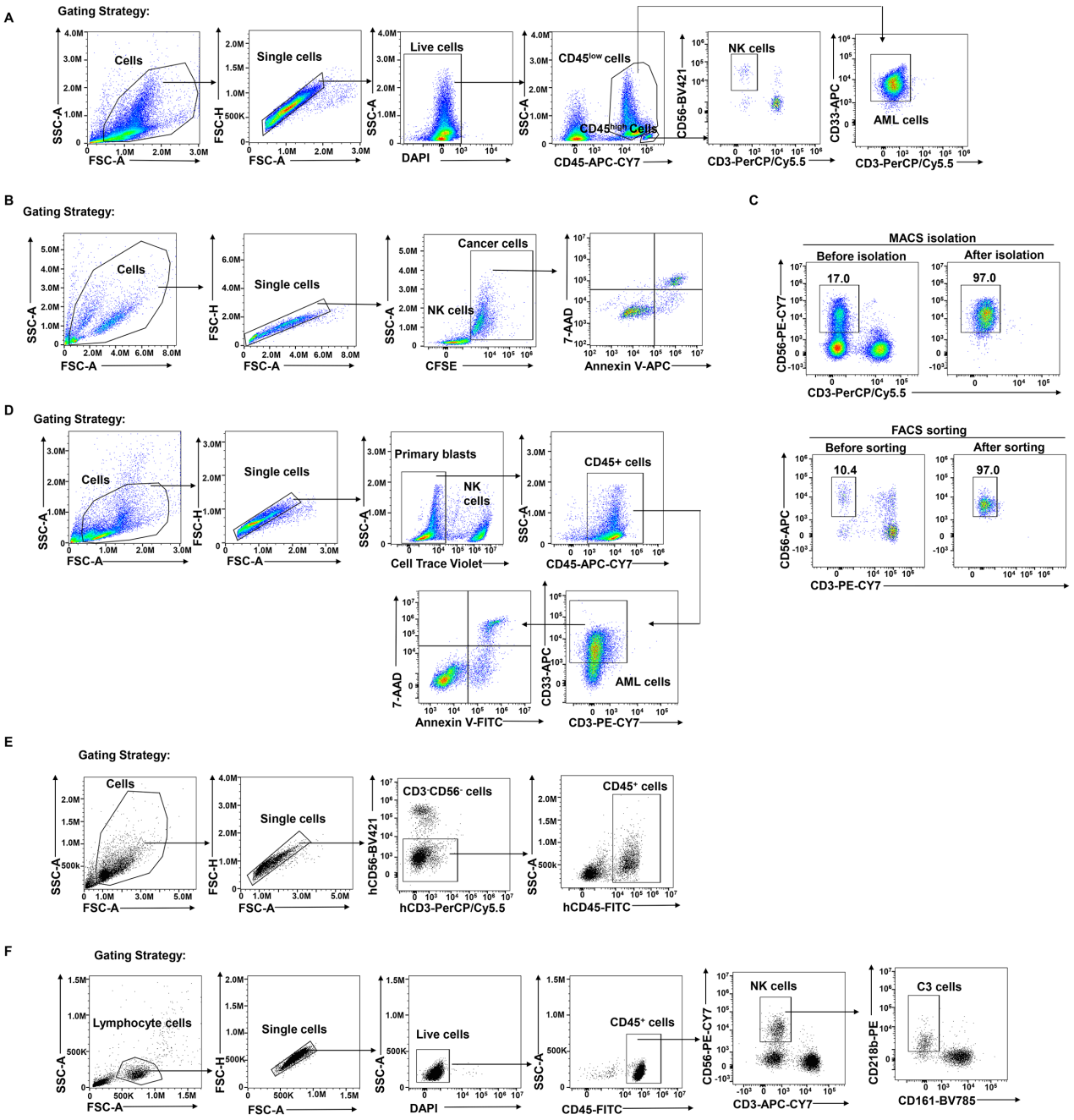


Figure S7. Gating strategies in the multiparameter flow cytometry and cell sorting experiments, related to STAR METHODS.

(A) Gating strategy for separating AML-NK cells and primary AML cells.

(B) Gating strategy for NK cells and AML cell line co-culture experiments. AML cells were labeled with CFSE (see STAR METHODS).

(C) Isolation of NK cells from CBMCs and PBMCs using the NK Cell Isolation Kit, and isolation of NK cells from the bone marrow of AML patients using flow sorting. The $CD3^{-}CD56^{+}$ cell subset was detected by flow cytometry before and after cell sorting to assess sorted NK cell purity.

(D) Gating strategy for NK cells and primary AML cells co-culture experiments.

(E) Gating strategy for detection of leukemia burden in the bone marrow of the KG-1a transplanted mouse model.

(F) Gating strategy for detecting the $CD45^{+}CD3^{-}CD56^{+}CD161^{low}CD218b^{+}$ subset within CB-NK cells.