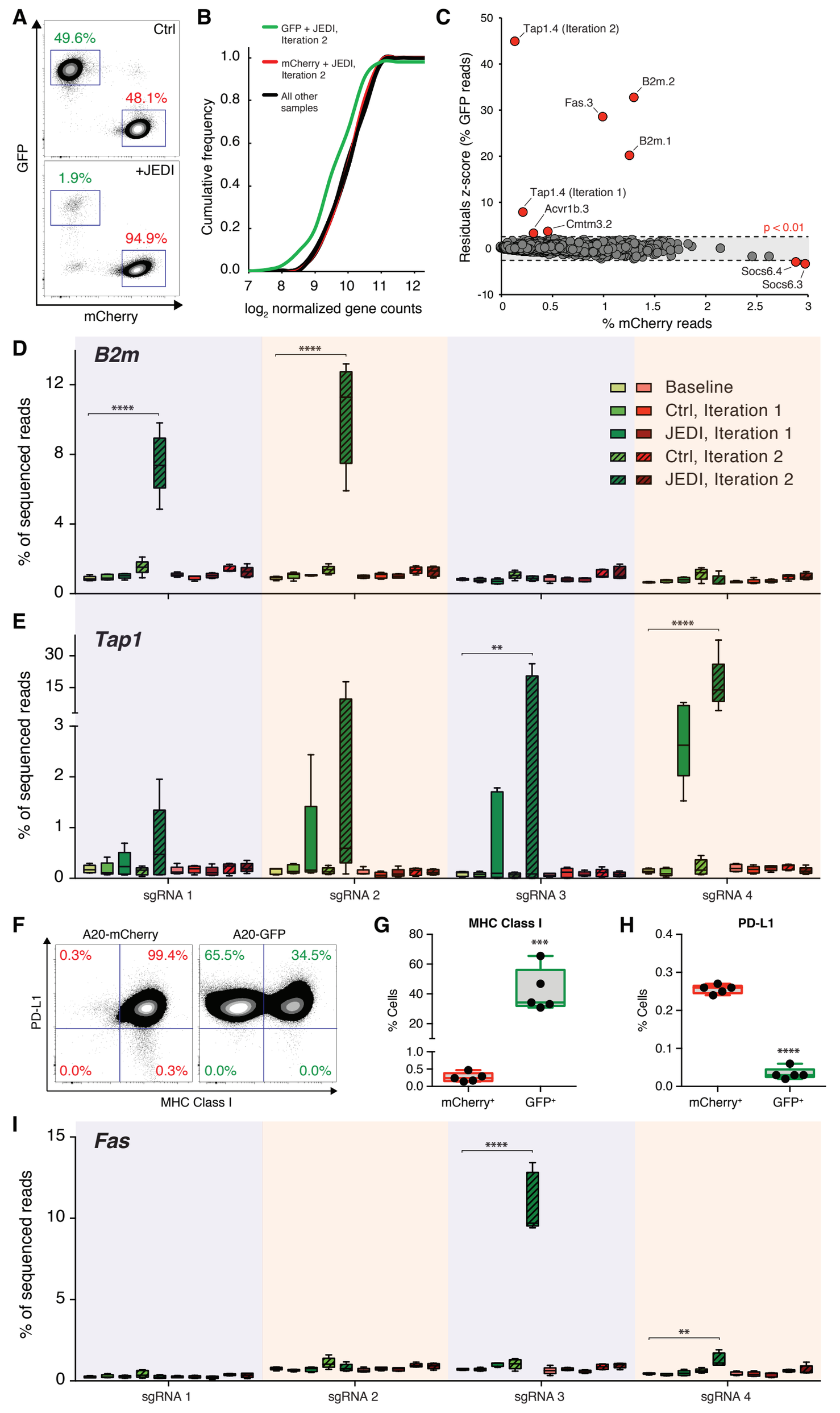
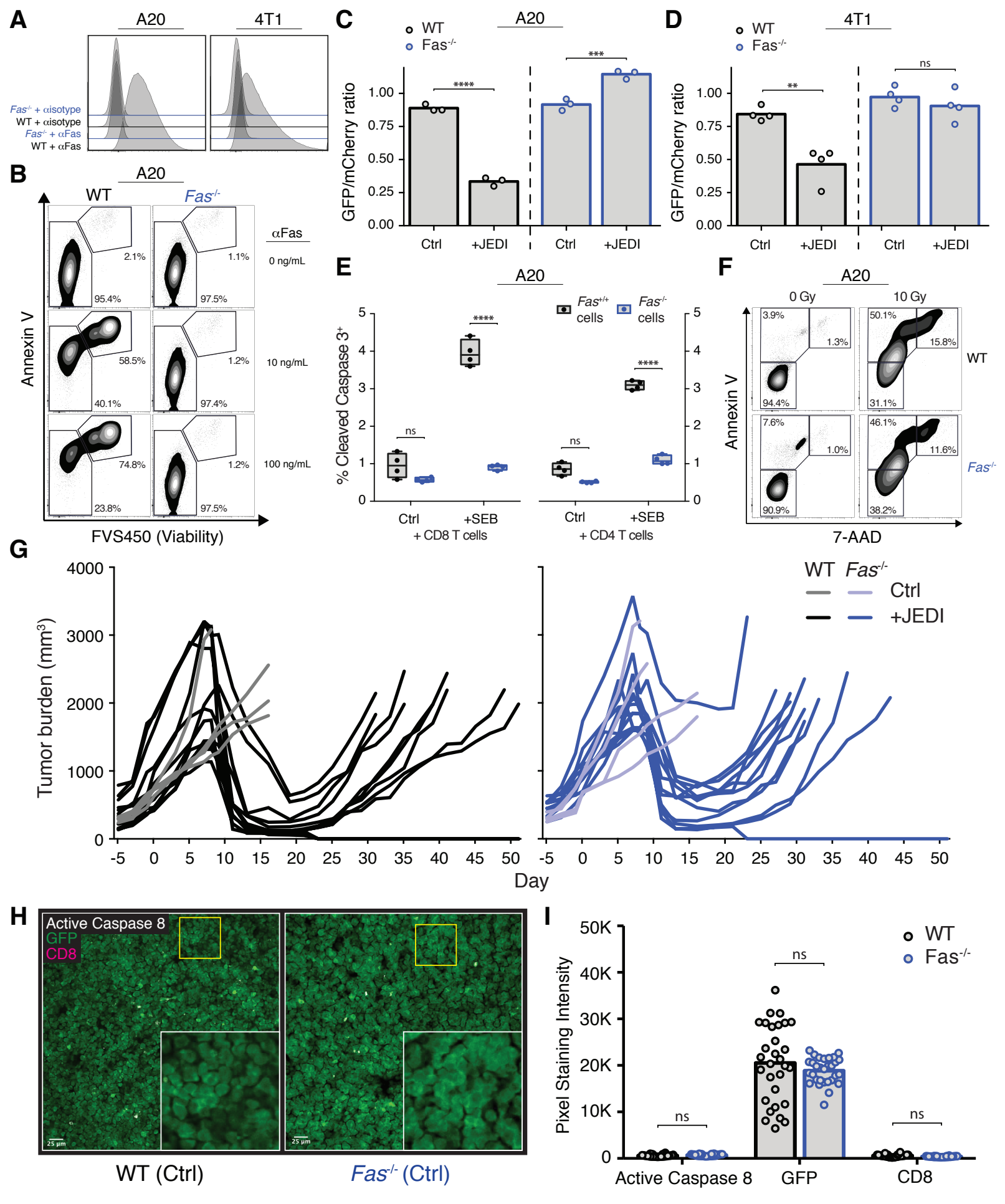
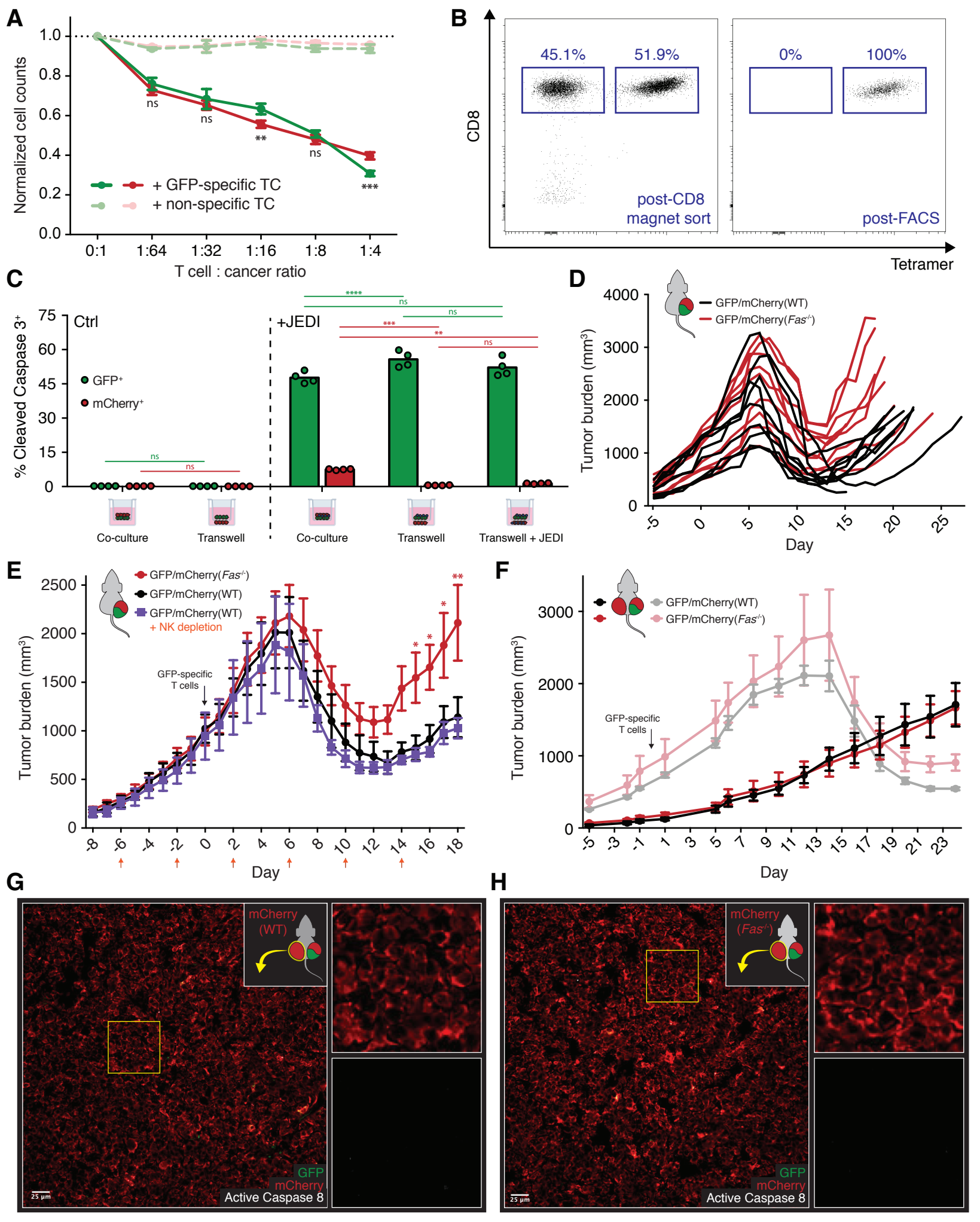

A
Ctrl
49.6%
48.1%
+JEDI
1.9%
94.9%
GFP
mCherry
B
GFP + JEDI, Iteration 2
mCherry + JEDI, Iteration 2
All other samples
Cumulative frequency
log2 normalized gene counts
C
Tap1.4 (Iteration 2)
B2m.2
Fas.3
B2m.1
Tap1.4 (Iteration 1)
Acvr1b.3
Cmtm3.2
p < 0.01
Socs6.4
Socs6.3
Residuals z-score (% GFP reads)
% mCherry reads
D
B2m
% of sequenced reads
Baseline
Ctrl, Iteration 1
JEDI, Iteration 1
Ctrl, Iteration 2
JEDI, Iteration 2
E
Tap1
% of sequenced reads
sgRNA 1
sgRNA 2
sgRNA 3
sgRNA 4
F
A20-mCherry
A20-GFP
0.3%
99.4%
0.0%
0.3%
65.5%
34.5%
0.0%
0.0%
PD-L1
MHC Class I
G
MHC Class I
% Cells
mCherry+
GFP+
H
PD-L1
% Cells
mCherry+
GFP+
I
Fas
% of sequenced reads
sgRNA 1
sgRNA 2
sgRNA 3
sgRNA 4

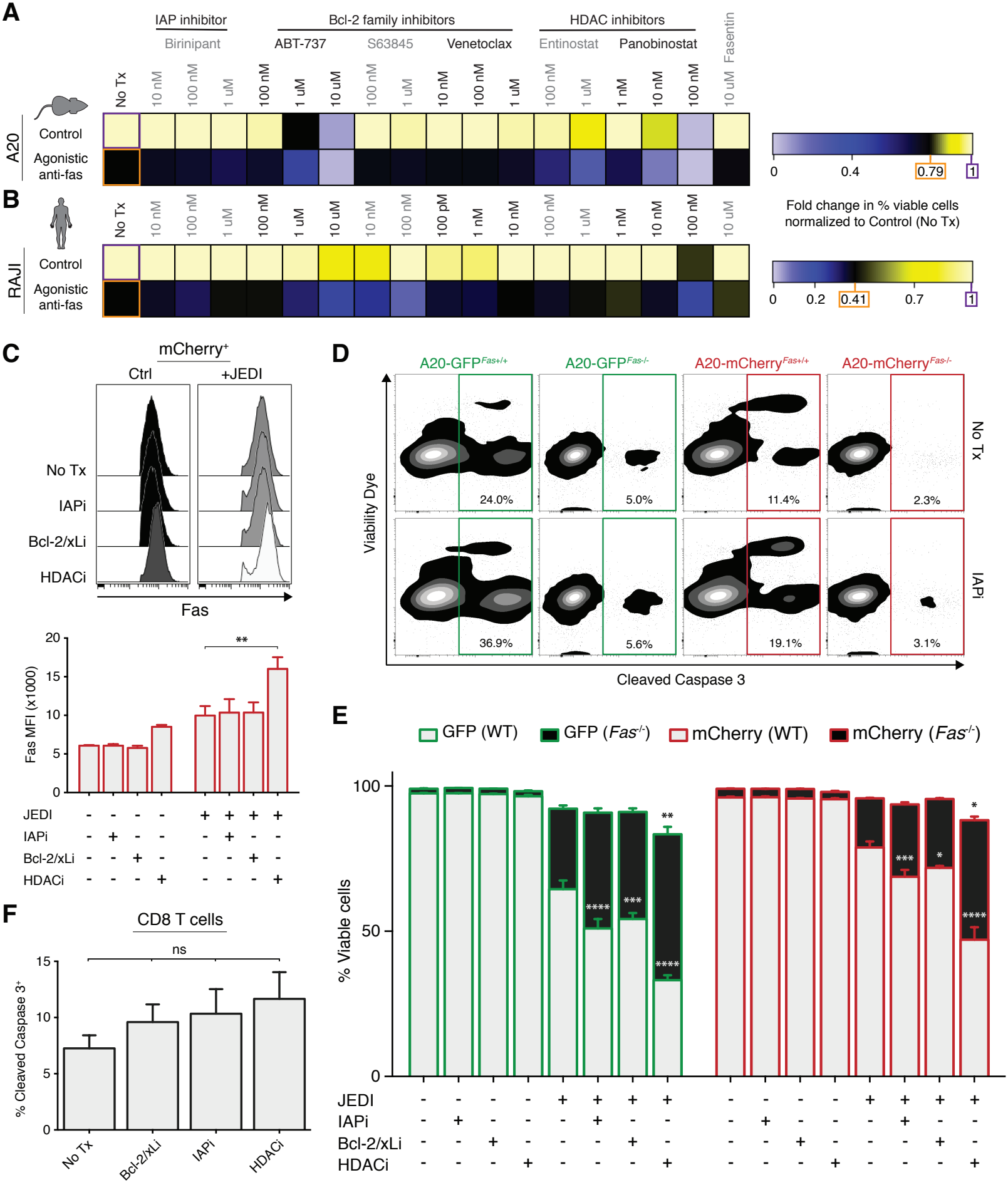
Supplementary Figure S1. CRISPR/Cas9 knockout of *B2m*, *Tap1*, and *Fas* protects A20 lymphoma from CD8 T cell killing. A, Representative changes in ratio of surviving GFP^+ and $mCherry^+$ cells after 1 iteration of selection with JEDI T cells. **B,** Cumulative frequency distribution of integrated small guide targeting RNA sequences from genomic DNA of indicated samples from a representative library; 'All other samples' include GFP^+ and $mCherry^+$ cells from Baseline, Ctrl (Iteration 1), JEDI (Iteration 1), and Ctrl (Iteration 2); data shown are medians (n=5). **C,** Standardized residuals of a linear regression function (F-statistic = 4856) of percent reads for each sgRNA target in GFP^+ population against paired reads in $mCherry^+$ population; dashed lines represent Z-score thresholds of ±2.58; select targets ($p<0.01$) out of total 5,186 data points highlighted in red. **D-E,** Percent of all sequenced reads mapping to each of 4 small guide RNA sequences (alternating purple and orange shading) targeting *B2m* (**D**) and *Tap1* (**E**) across all samples; boxplots presented as minimum to maximum; two-way ANOVA with Sidak correction for multiple comparisons, **$p<0.01$, ****$p<0.0001$ (n=5). **F,** Representative surface expression distribution of MHC Class I and PD-L1 of GFP^+ (right panel) and $mCherry^+$ (left panel) cells after 3 iterations of selection with JEDI T cells in a library containing 4 small guide RNAs targeting *B2m* and *Cd274*. **G-H,** Percent of all GFP^+ and $mCherry^+$ cells from **F** in negative gate of MHC Class I (**G**) or PD-L1 (**H**); unpaired t-test (n=5). **I,** Percent of all sequenced reads mapping to each of 4 small guide RNA sequences (alternating purple and orange shading) targeting *Fas* across all samples; boxplots presented as minimum to maximum; two-way ANOVA with Sidak correction for multiple comparisons, ****$p<0.0001$ (n=5).


A
A20
4T1
Fas-/- + αisotype
WT + αisotype
Fas-/- + αFas
WT + αFas
B
A20
WT
Fas-/-
αFas
0 ng/mL
10 ng/mL
100 ng/mL
2.1%
95.4%
1.1%
97.5%
58.5%
40.1%
1.2%
97.4%
74.8%
23.8%
1.2%
97.5%
Annexin V
FVS450 (Viability)
C
A20
WT
Fas-/-
GFP/mCherry ratio
****
***
Ctrl
+JEDI
Ctrl
+JEDI
D
4T1
WT
Fas-/-
GFP/mCherry ratio
**
ns
Ctrl
+JEDI
Ctrl
+JEDI
E
A20
Fas+/+ cells
Fas-/- cells
% Cleaved Caspase 3+
ns
****
ns
****
Ctrl
+SEB
+ CD8 T cells
Ctrl
+SEB
+ CD4 T cells
F
A20
0 Gy
10 Gy
3.9%
1.3%
94.4%
50.1%
15.8%
31.1%
WT
7.6%
1.0%
90.9%
46.1%
11.6%
38.2%
Fas-/-
Annexin V
7-AAD
G
WT Fas-/-
Ctrl
+JEDI
Tumor burden (mm3)
Day
H
Active Caspase 8
GFP
CD8
25 μm
25 μm
WT (Ctrl)
Fas-/- (Ctrl)
I
WT
Fas-/-
Pixel Staining Intensity
ns
ns
ns
Active Caspase 8
GFP
CD8

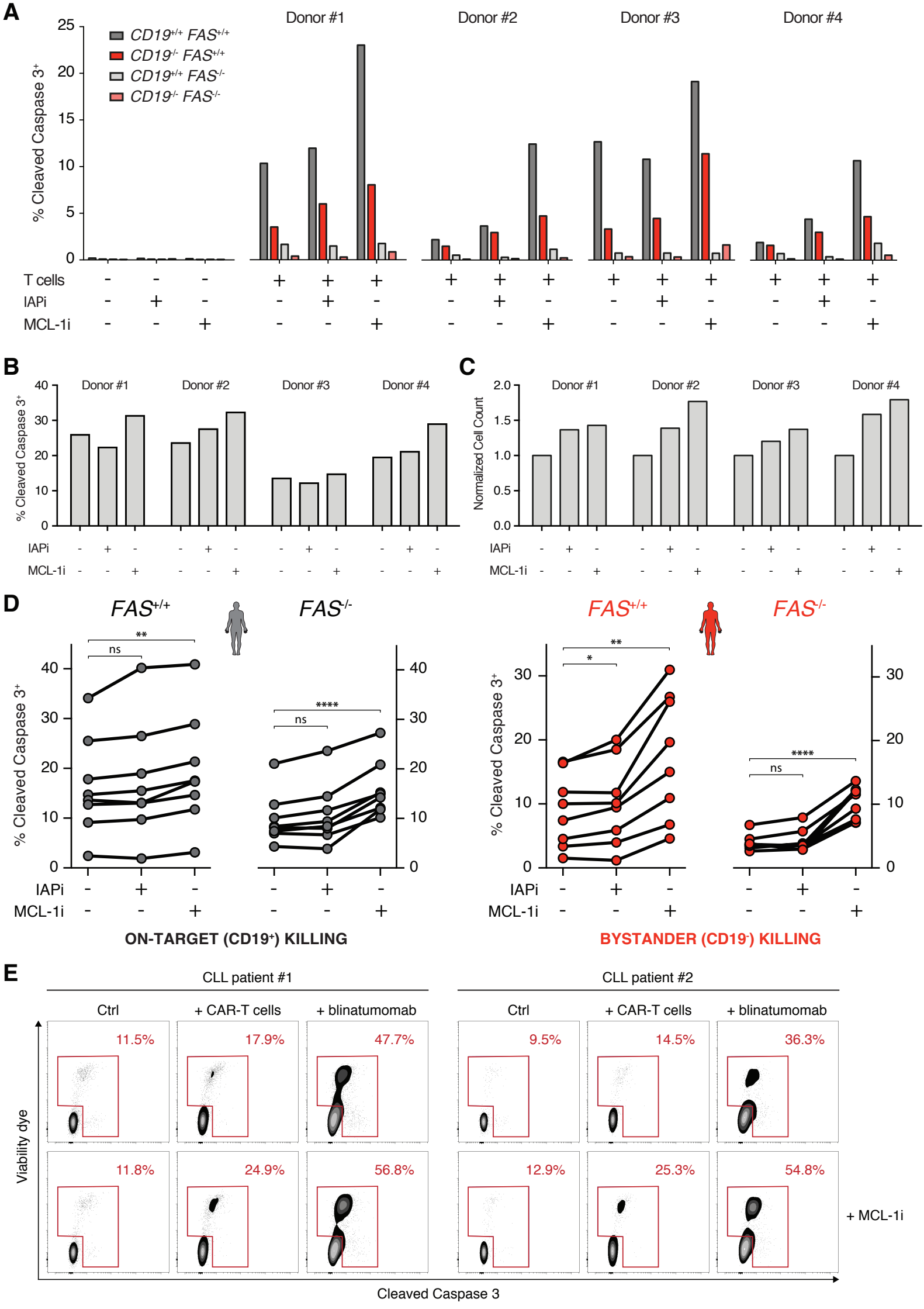
Supplementary Figure S2. *Fas*$^{-/-}$ cancer is resistant to killing by multiple model T cell systems despite comparable susceptibility to radiation-induced apoptosis. A, Relative surface staining intensity for WT (black) and *Fas*$^{-/-}$ (blue) A20 (left panel) and 4T1 (right panel) cell lines using APC-conjugated anti-Fas (clone SA367H8) or anti-IgG1k isotype control. **B,** Representative apoptosis induced in WT (left panel) and *Fas*$^{-/-}$ (right panel) A20 cells after 48-hour incubation with agonistic anti-Fas (clone Jo2) at the indicated concentrations. **C-D,** Ratio of GFP^{+}:mCherry^{+} A20 (**C**) or 4T1 (**D**) cells from Fig. 1D-E; error bars presented as mean ± SEM; two-way ANOVA with Sidak correction for multiple comparisons, **p<0.01, ***p<0.001, ****p<0.0001 (n=3-4). **E,** Percent of dying WT (black) or *Fas*$^{-/-}$ (blue) A20 lymphoma cells after co-culture with freshly isolated CD8 (left panel) or CD4 (right panel) T cells from balb/c mice in the presence of 50 ng/mL bacterial superantigen SEB; boxplots presented as minimum to maximum; two-way ANOVA with Sidak correction for multiple comparisons, ****p<0.0001 (n=4). **F,** Representative early (Annexin V^{+}, 7-AAD^{-}) and late (Annexin V^{+}, 7-AAD^{+}) apoptosis staining of WT or *Fas*$^{-/-}$ A20 lymphoma cells 48 hours after irradiation with indicated doses. **G,** Individual tumor curves from Fig. 1G; 3 out of 12 WT tumors cured (black) and 1 out of 12 *Fas*$^{-/-}$ tumors cured (blue); pooled from 2 independent experiments. **H,** Representative confocal IF of tumors cryopreserved from 'Ctrl' groups from Fig. 1G on day 8; area outlined in yellow magnified in inset. **I,** Quantification of fluorescent intensities from 30 fields of view as shown in **H**; pixel staining intensity units on a scale from 0 to 65,536 (16-bit); two-way ANOVA with Sidak correction for multiple comparisons (n=3-4 mice/group, pooled from 3 independent experiments).


A
Normalized cell counts
1.0
0.8
0.6
0.4
0.2
0.0
ns
ns
**
ns
***
+ GFP-specific TC
+ non-specific TC
0:1
1:64
1:32
1:16
1:8
1:4
T cell : cancer ratio
B
CD8
45.1%
51.9%
0%
100%
post-CD8
magnet sort
post-FACS
Tetramer
C
% Cleaved Caspase 3+
75
60
45
30
15
0
Ctrl
+JEDI
GFP+
mCherry+
****
ns
ns
***
**
ns
ns
ns
Co-culture
Transwell
Co-culture
Transwell
Transwell + JEDI
D
Tumor burden (mm3)
4000
3000
2000
1000
0
GFP/mCherry(WT)
GFP/mCherry(Fas-/-)
-5
0
5
10
15
20
25
Day
E
Tumor burden (mm3)
2500
2000
1500
1000
500
0
GFP/mCherry(Fas-/-)
GFP/mCherry(WT)
GFP/mCherry(WT)
+ NK depletion
GFP-specific
T cells
**
*
*
*
-8
-6
-4
-2
0
2
4
6
8
10
12
14
16
18
Day
F
Tumor burden (mm3)
3000
2000
1000
0
GFP/mCherry(WT)
GFP/mCherry(Fas-/-)
GFP-specific
T cells
-5
-3
-1
1
3
5
7
9
11
13
15
17
19
21
23
Day
G
mCherry
(WT)
GFP
mCherry
Active Caspase 8
25 µm
H
mCherry
(Fas-/-)
GFP
mCherry
Active Caspase 8
25 µm

Supplementary Figure S3. *In vivo* bystander killing by T cells is dependent on proximity to Ag^+ cells. A, Cell counts of *Fas*$^{-/-}$ GFP^+ (green) and WT $mCherry^+$ (red) cells co-cultured with FACS purified $tetramer^+$ (dark, GFP-specific) or $tetramer^-$ (light, non-specific) activated CD8 T cells at indicated ratios; counts relative to beads and normalized to 0:1 condition; error bars presented as mean ± SEM; two-way ANOVA with Sidak correction for multiple comparisons, **p<0.01, ***p<0.001 (n=4). **B,** Representative population of T cells used in killing assays by negative enrichment magnet sorting (left panel; 97% CD8 T cells with 52% of cells specific for GFP antigen) or after tetramer-based FACS (right panel; 100% CD8 T cells with 100% specificity for GFP antigen). **C,** Mean of cells positive for cleaved caspase 3 within GFP^+ (green) and $mCherry^+$ (red) populations from Fig. 2B; two-way ANOVA with Sidak correction for multiple comparisons, **p<0.01, ***p<0.001, ****p<0.0001 (n=4). **D,** Individual tumor curves from Fig. 2D; pooled from 2 independent experiments. **E,** Growth of mixed tumors with 50% WT GFP^+ cells and 50% WT $mCherry^+$ cells in untreated (black) or natural killer cell-depleted (purple) *Rag1*$^{-/-}$ mice adoptively transferred with tetramer-sorted GFP-specific CD8 T cells (black arrow); a cohort of mixed tumors with *Fas*$^{-/-}$ $mCherry^+$ cells is shown for comparison (red); anti-asialo-GM1 treatment for NK-depletion indicated with orange arrows; error bars presented as mean ± SEM; two-way ANOVA with Sidak correction for multiple comparisons, *p<0.05, **p<0.01 (n=4-5 mice/group). **F,** Tumor growth in *Rag1*$^{-/-}$ mice bearing mixed primary GFP^+/$mCherry^+$ tumors (light shaded curves) and distant secondary $mCherry^+$ tumors (dark shaded curves) with either WT (black) or *Fas*$^{-/-}$ (red) $mCherry^+$ cells; distant tumors inoculated 10 days after primary tumors; error bars presented as mean ± SEM; two-way ANOVA with Sidak correction for multiple comparisons (n=6 mice/group). **G-H,** Representative confocal IF images of distant tumors from **F** with insets showing magnified single channel images of area outlined in yellow; arrows point to areas of active caspase 8 staining (bottom insets) and corresponding areas in the mCherry channel (top insets).


A
IAP inhibitor
Birinipant
Bcl-2 family inhibitors
ABT-737
S63845
Venetoclax
HDAC inhibitors
Entinostat
Panobinostat
Fasentin
No Tx
10 nM
100 nM
1 uM
100 nM
1 uM
10 uM
100 nM
1 uM
10 nM
100 nM
1 uM
100 nM
1 uM
1 nM
10 nM
100 nM
10 uM
A20
Control
Agonistic anti-fas
0
0.4
0.79
1
B
No Tx
10 nM
100 nM
1 uM
100 nM
1 uM
10 uM
10 nM
100 nM
100 pM
1 nM
10 nM
100 nM
1 uM
1 nM
10 nM
100 nM
10 uM
RAJI
Control
Agonistic anti-fas
Fold change in % viable cells normalized to Control (No Tx)
0
0.2
0.41
0.7
1
C
mCherry+
Ctrl
+JEDI
No Tx
IAPi
Bcl-2/xLi
HDACi
Fas
Fas MFI (x1000)
20
15
10
5
0
**
JEDI
IAPi
Bcl-2/xLi
HDACi
D
A20-GFPFas+/+
A20-GFPFas-/-
A20-mCherryFas+/+
A20-mCherryFas-/-
No Tx
IAPi
Viability Dye
24.0%
5.0%
11.4%
2.3%
36.9%
5.6%
19.1%
3.1%
Cleaved Caspase 3
E
GFP (WT)
GFP (Fas-/-)
mCherry (WT)
mCherry (Fas-/-)
% Viable cells
100
50
0
**
****
***
****
***
*
*
****
JEDI
IAPi
Bcl-2/xLi
HDACi
F
CD8 T cells
ns
% Cleaved Caspase 3+
15
10
5
0
No Tx
Bcl-2/xLi
IAPi
HDACi

Supplementary Figure S4. Targeting inhibitors of apoptosis proteins (IAP), Bcl-2 family members, and histone deacetylases (HDACs) potentiates fas-mediated T cell cytotoxic activity. A-B, Percent of viable murine A20 (**A**) and human Raji (**B**) cells after 48 hour incubation with agonistic antibody to fas and concurrent treatment with the indicated small molecule inhibitors; viability determined by negative staining for exclusion dye FVS780; coloring bar standardized to conditions outlined in purple and orange as indicated. **C,** Surface fas expression on WT mCherry^{+} cells (top panel) from co-culture conditions in Fig. 3D and quantification of mean fluorescent intensities (bottom panel); error bars presented as mean ± SEM; two-way ANOVA with Sidak correction for multiple comparisons, **p<0.01 (n=3). **D,** Gating for data in Fig. 3D and representative percent of dying cells after 2 day co-culture of 4 cancer cell populations (columns) with JEDI T cells, untreated (top row) or with 1 μM birinapant (bottom row). **E,** Percent of WT (gray) or *Fas*$^{-/-}$ (black) GFP^{+} (green, left panel) and mCherry^{+} (red, right panel) cells negative for viability exclusion dye FVS780 after 2 day co-culture with JEDI T cells, 1 μM birinapant (IAPi), 100 nM ABT-737 (Bcl-2/xLi), and/or 1 nM panobinostat (HDACi); error bars presented as mean ± SEM; indicated statistics relative to 5th column of each panel; two-way ANOVA with Sidak correction for multiple comparisons, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 (n=3). **F,** Percent of dying CD8 T cells from co-culture conditions in Fig. 3D; error bars presented as mean ± SEM; repeated measure one-way ANOVA with Sidak correction for multiple comparisons (n=3).


A
Donor #1
Donor #2
Donor #3
Donor #4
CD19+/+ FAS+/+
CD19-/- FAS+/+
CD19+/+ FAS-/-
CD19-/- FAS-/-
% Cleaved Caspase 3+
T cells
IAPi
MCL-1i
B
% Cleaved Caspase 3+
C
Normalized Cell Count
D
FAS+/+
FAS-/-
ns
**
****
*
% Cleaved Caspase 3+
ON-TARGET (CD19+) KILLING
BYSTANDER (CD19-) KILLING
E
CLL patient #1
CLL patient #2
Ctrl
+ CAR-T cells
+ blinatumomab
11.5%
17.9%
47.7%
9.5%
14.5%
36.3%
11.8%
24.9%
56.8%
12.9%
25.3%
54.8%
Viability dye
Cleaved Caspase 3
+ MCL-1i

Supplementary Figure S5. The efficacy of on-target and bystander killing in blinatumomab and CAR-T cell therapy can be potentiated with small molecule modulators of the fas pathway. A, Percent of dying target Raji cells after co-culture with purified human CD8 T cells from healthy donors in the presence of 100 pM blinatumomab and treated with 100 nM birinapant (IAPi) or 10 nM S63845 (MCL-1i); healthy donor data individualized from aggregate data depicted in Fig. 3E and 3F. **B-C,** Percent of dying (**B**) and normalized cell counts (**C**) of CD8 T cells from co-culture conditions described in **A**. **D,** Percent of dying *CD19*$^{+/+}$ on-target (gray; left panel) and *CD19*$^{-/-}$ bystander (red; right panel) Raji cells when co-cultured with human CD19-targeting CAR-T cell product for 6 hours concurrently with 100 nM birinapant (IAPi) or 10 nM S63845 (MCL-1i); matched data points from each healthy donor CAR-T cell product connected by lines; repeated measure one-way ANOVA with Holm-Sidak correction for multiple comparisons, *p<0.05, **p<0.01, ****p<0.0001 (n=8). **E,** Percent of apoptotic chronic lymphocytic leukemia (CLL) cells from 2 independent patients (left and right panels) after 2 day co-culture with human CAR-T cell product alone or an equal number of peripheral CD8 T cells from a healthy donor and 1 nM blinatumomab; primary target cells labeled with cytoplasmic CellTrace dye for gating; MCL-1i = 10 nM S63845.