Figure S1

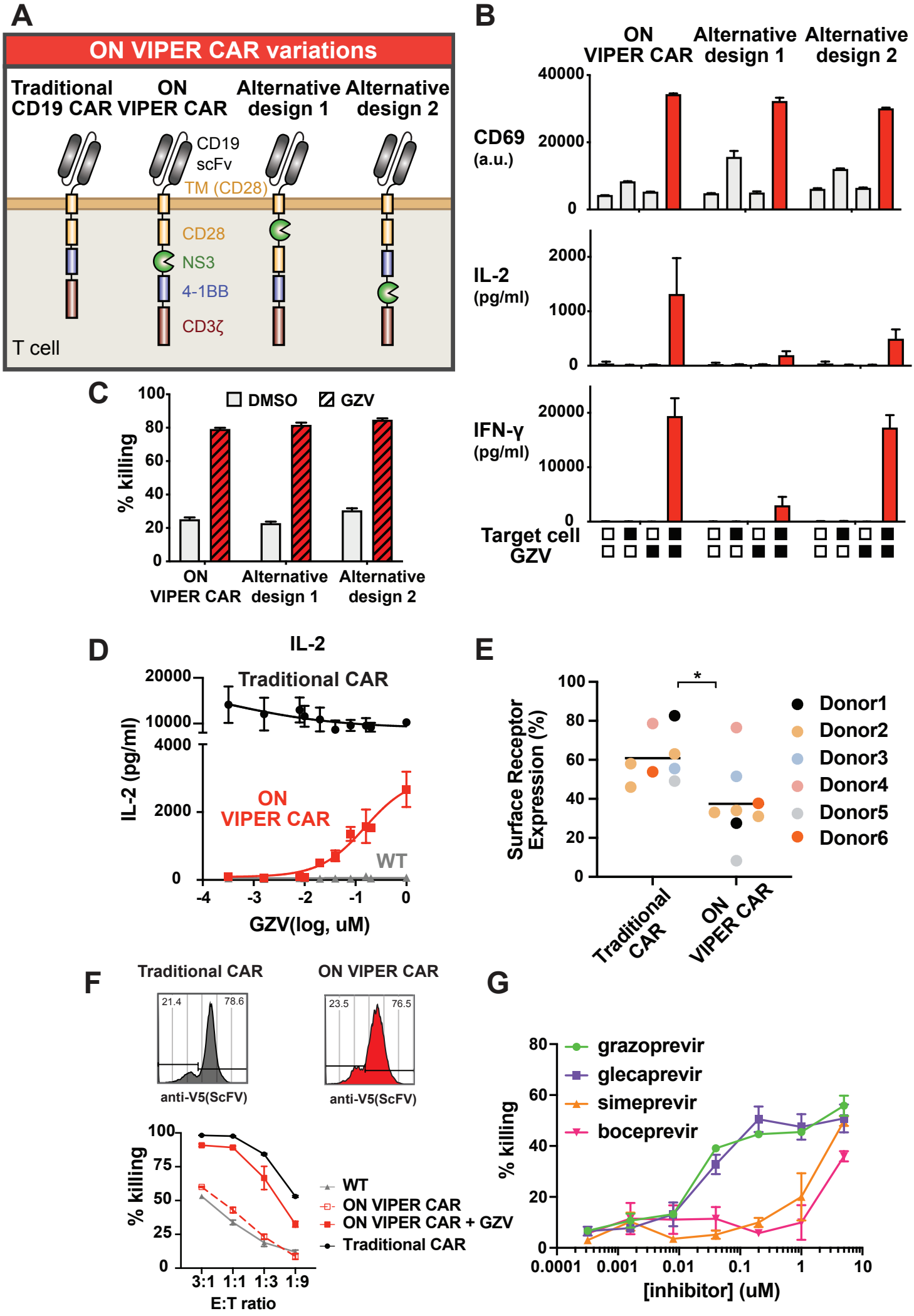

A
ON VIPER CAR variations
Traditional CD19 CAR
ON VIPER CAR
Alternative design 1
Alternative design 2
CD19 scFv
TM (CD28)
CD28
NS3
4-1BB
CD3ζ
T cell
B
ON VIPER CAR
Alternative design 1
Alternative design 2
CD69 (a.u.)
IL-2 (pg/ml)
IFN-γ (pg/ml)
Target cell
GZV
C
DMSO
GZV
% killing
ON VIPER CAR
Alternative design 1
Alternative design 2
D
IL-2
Traditional CAR
ON VIPER CAR
WT
IL-2 (pg/ml)
GZV(log, uM)
E
Surface Receptor Expression (%)
Donor1
Donor2
Donor3
Donor4
Donor5
Donor6
Traditional CAR
ON VIPER CAR
F
Traditional CAR
ON VIPER CAR
21.4
78.6
23.5
76.5
anti-V5(ScFV)
anti-V5(ScFV)
% killing
E:T ratio
WT
ON VIPER CAR
ON VIPER CAR + GZV
Traditional CAR
G
grazoprevir
glecaprevir
simeprevir
boceprevir
% killing
[inhibitor] (uM)

Figure S1: Variations of the ON VIPER CAR designs, Related to Figure 1.

A) Schematic of the variations of ON VIPER CAR designs. **B)** Regulation of the activity of ON VIPER CARs in primary T cells (measured by CD69 and cytokine levels) using combinations of target NALM6 cells and GZV. Note that the data set of cytokine levels for the final design of the ON VIPER CAR is the same as that depicted in Figure 1C. **C)** Cell killing of target NALM6 cells using primary T cells expressing ON VIPER CARs in the absence and presence of GZV. Note that the data set for the final design of the ON VIPER CAR is the same as that depicted in Figure 1D. **D)** GZV dose-response profile of VIPER CAR in primary T cells. **E)** Comparison of surface receptor expression levels for traditional CAR T cells and ON VIPER CAR T cells for various donors (mean ± s.d., n = 8, $*P < 0.05$). **F)** Traditional CAR and ON VIPER CAR expression in T cells was determined by surface staining of the V5-tagged scFV (top). Target cell (Nalm6) killing by ON VIPER CAR T cells was compared with traditional CAR and wild-type T cells at various E:T ratios (bottom). **G)** Cytotoxicity of ON VIPER CAR-expressing primary T cells in response to different NS3 inhibitors.

Figure S2

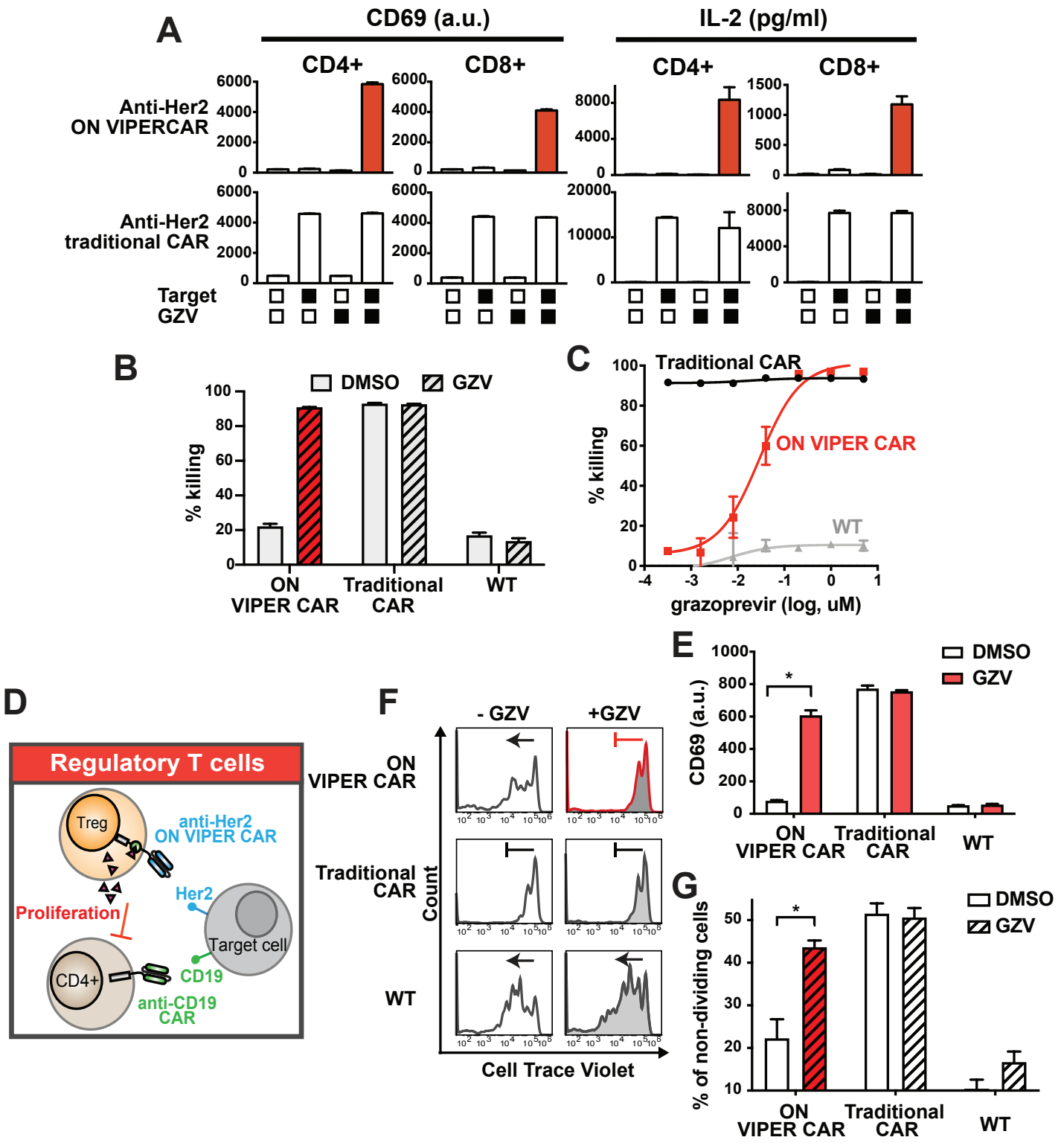

A
CD69 (a.u.)
IL-2 (pg/ml)
CD4+
CD8+
Anti-Her2 ON VIPERCAR
Anti-Her2 traditional CAR
Target
GZV
B
DMSO
GZV
% killing
ON VIPER CAR
Traditional CAR
WT
C
Traditional CAR
ON VIPER CAR
WT
% killing
grazoprevir (log, uM)
D
Regulatory T cells
Treg
anti-Her2 ON VIPER CAR
Her2
Proliferation
Target cell
CD4+
CD19
anti-CD19 CAR
E
DMSO
GZV
CD69 (a.u.)
ON VIPER CAR
Traditional CAR
WT
F
- GZV
+GZV
ON VIPER CAR
Traditional CAR
WT
Count
Cell Trace Violet
G
DMSO
GZV
% of non-dividing cells
ON VIPER CAR
Traditional CAR
WT

Figure S2: Application of ON VIPER CAR to a Her2 scFv and regulatory T cells (Treg), Related to Figure 1.

A) Response of the anti-Her2 ON VIPER CAR to combinations of target cells and GZV when expressed in CD4+ and CD8+ T cell subsets. **B)** Cell killing ability of CD8+ T cells expressing various anti-Her2 ON VIPER CAR, traditional anti-Her2 CAR, or no CAR. **C)** Dose-response of anti-Her2 ON VIPER CAR CD8+ T cells to increased amounts of GZV, as measured in cell killing efficiency. **D)** Schematic of how ON VIPER CAR-expressing Treg cells interact with CD4+ T cells and target NALM6 cells in a proliferation assay. **E)** Levels of early activation marker (CD69) expressed by ON VIPER CAR regulatory T cells in the presence and absence of NS3 inhibitor, compared with traditional CAR-expressing cells and cells with no CAR. **F)** CD4+ T cell proliferation when co-incubated with various regulatory T cell lines in the presence and absence of GZV. **G)** Quantification of non-dividing CD4+T cells from histogram proliferation data (Figure S2F).

Figure S3

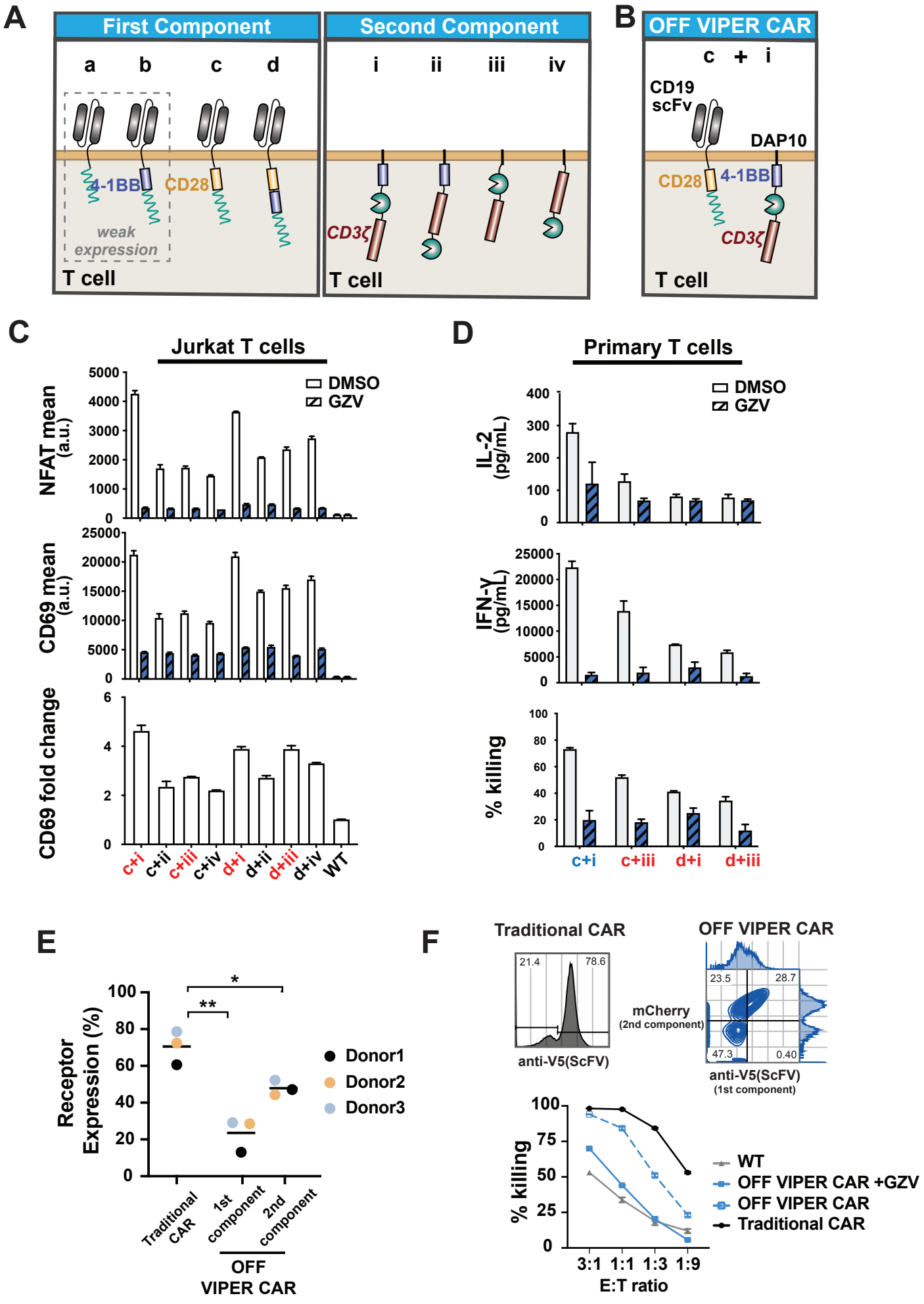

A
First Component
Second Component
a
b
c
d
i
ii
iii
iv
4-1BB
CD28
weak expression
CD3ζ
T cell
T cell
B
OFF VIPER CAR
c + i
CD19 scFv
DAP10
CD28
4-1BB
CD3ζ
T cell
C
Jurkat T cells
DMSO
GZV
NFAT mean (a.u.)
CD69 mean (a.u.)
CD69 fold change
c+i
c+ii
c+iii
c+iv
d+i
d+ii
d+iii
d+iv
WT
D
Primary T cells
DMSO
GZV
IL-2 (pg/mL)
IFN-γ (pg/mL)
% killing
c+i
c+iii
d+i
d+iii
E
Receptor Expression (%)
Donor1
Donor2
Donor3
Traditional CAR
1st component
2nd component
OFF VIPER CAR
F
Traditional CAR
OFF VIPER CAR
21.4
78.6
anti-V5(ScFV)
mCherry
(2nd component)
23.5
28.7
47.3
0.40
anti-V5(ScFV)
(1st component)
% killing
WT
OFF VIPER CAR +GZV
OFF VIPER CAR
Traditional CAR
3:1 1:1 1:3 1:9
E:T ratio

Figure S3: Variations on the OFF VIPER CAR designs, Related to Figure 2.

A) Variations on the two components that make up the OFF VIPER CAR. **B)** Schematic of the OFF VIPER CAR design. **C)** Jurkat T cell activity as measured by NFAT and CD69 levels when variations of the OFF VIPER CAR are expressed. Fold change observed in CD69 levels when GZV is added to Jurkat T cells expressing variations of the OFF CAR (mean ± s.d., n = 3). **D)** Cytokine release and cytotoxicity of primary T cells expressing different versions of the OFF VIPER CAR (mean ± s.d., n = 3). The data set for the final design of the OFF VIPER CAR (c+i) is the same as that depicted in Figure 2B-C. **E)** Comparison of receptor expression levels for traditional CAR T cells and OFF VIPER CAR T cells for various donors (mean ± s.d., n = 3, $*P < 0.05$ and $**P < 0.01$). **F)** Traditional CAR and OFF VIPER CAR expression was determined using surface staining of the V5-tagged scFV for the first component, and a mCherry fluorescence protein fused to the second component (top). Target cell (Nalm6) killing by OFF VIPER CAR T cells was compared with traditional CAR and wild-type T cells at various E:T ratios (bottom). The data indicated for wild-type and traditional CAR T cells is the same as that depicted in Figure S1F.

Figure S4

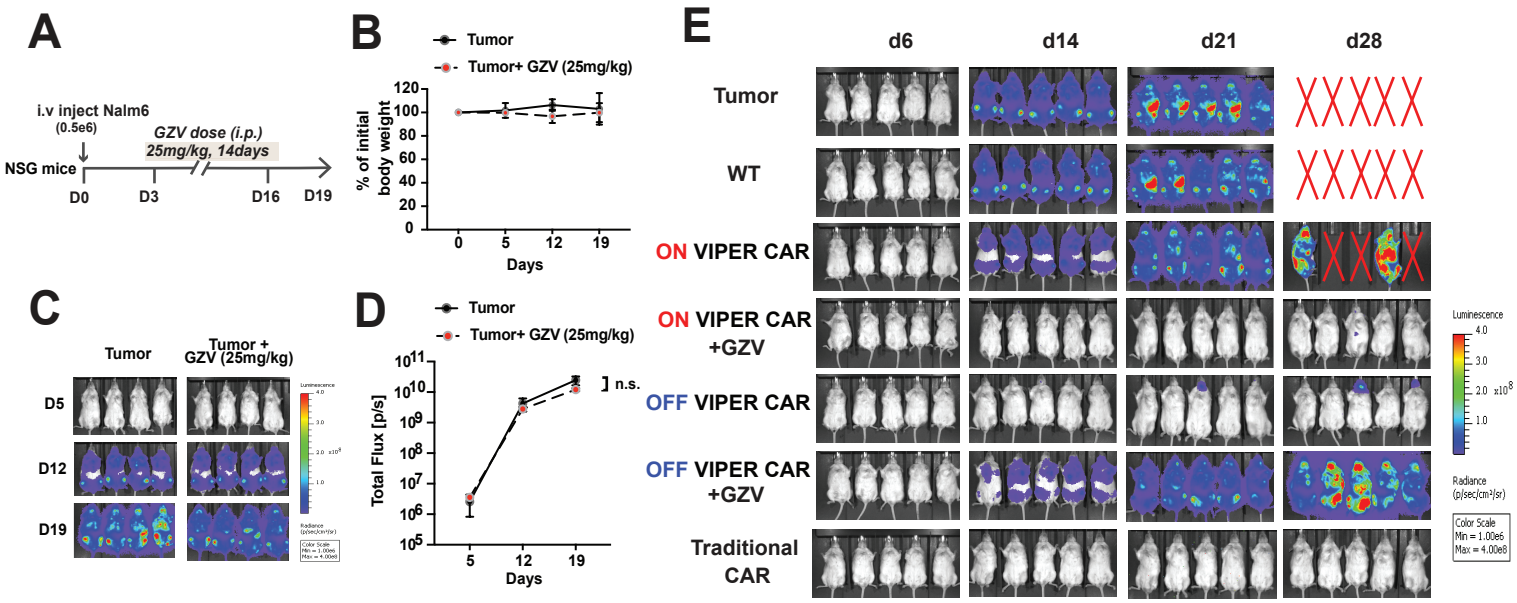
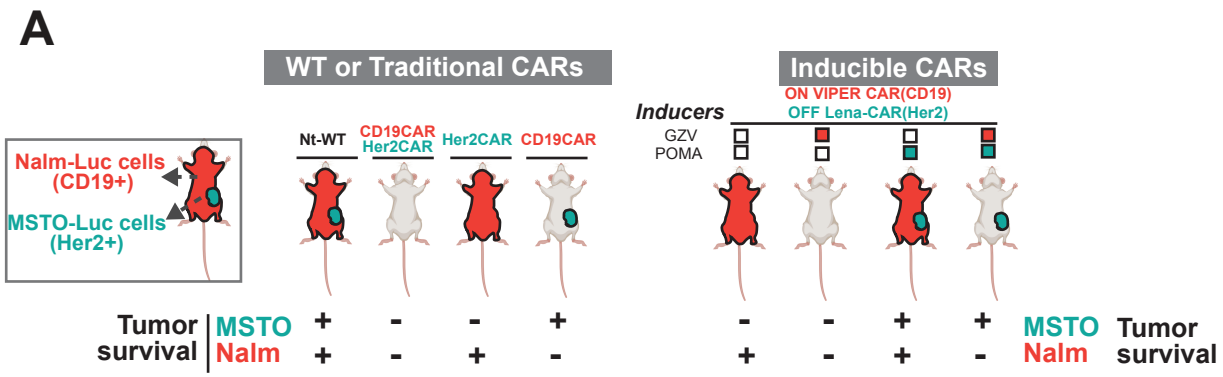

A
i.v inject Nalm6
(0.5e6)
GZV dose (i.p.)
25mg/kg, 14days
NSG mice
D0
D3
D16
D19
B
Tumor
Tumor+ GZV (25mg/kg)
% of initial body weight
Days
C
Tumor
Tumor + GZV (25mg/kg)
D5
D12
D19
D
Tumor
Tumor+ GZV (25mg/kg)
Total Flux [p/s]
n.s.
Days
E
d6
d14
d21
d28
Tumor
WT
ON VIPER CAR
ON VIPER CAR +GZV
OFF VIPER CAR
OFF VIPER CAR +GZV
Traditional CAR
Luminescence
Radiance (p/sec/cm²/sr)
Color Scale
Min = 1.00e6
Max = 4.00e8

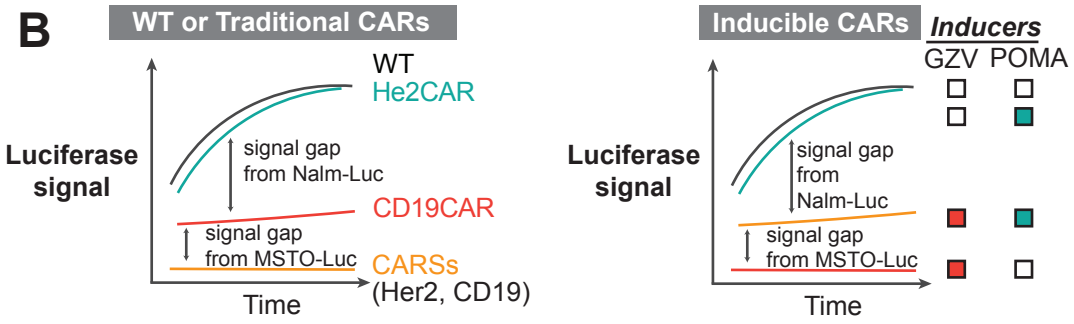
Figure S4: *In vivo* data of GZV-gated VIPER CARs, Related to Figure 3.

A) Timeline for an experiment testing the effect of GZV alone on tumor growth *in vivo*. **B)** Percentage of body weight to initial weight (day 0) was measured on days 5, 12, and 19 (mean ± s.d., n = 4). **C)** Luciferase levels from tumors imaged by IVIS for groups treated with (1) tumor alone and (2) tumor with GZV (25mg/kg for two weeks) at days 5, 12, and 19. **D)** Tumor burden was quantified as the total flux (photons/s) from the luciferase activity of each mouse using IVIS imaging (n = 4, mean ± sem). **E)** IVIS imaging of groups treated with (1) no T cells, (2) non-transduced T cell (NT-WT), (3) ON VIPER CAR T cells, (4) ON VIPER CAR T cells with GZV, (5) OFF VIPER CAR T cells, (6) OFF VIPER CAR T cells with GZV, or (7) Traditional CAR T cells at days 6, 14, 21 and 28.

Figure S5



Expected outcome



In vivo data

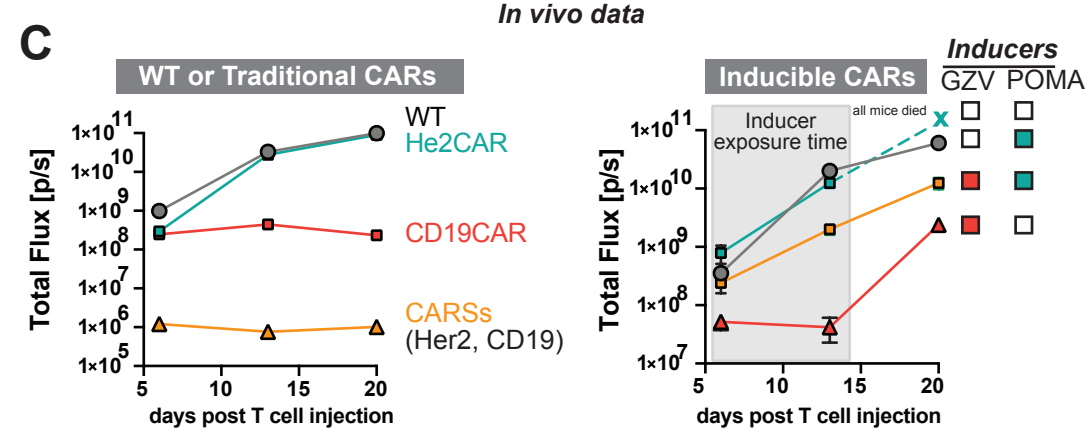


Figure S5. Characterization of dual gated-CAR T cells *in vivo,* Related to Figure 6.

A) Illustration of a hypothetical tumor burden in mice at various drug conditions. **B)** Hypothetical graph of luciferase signals over time in mice **C)** Total tumor burden was quantified as total flux (photons/s) from the luciferase activity of each mouse using IVIS imaging at d6, d13, and d20 (wild-type or traditional CARs: n=4, mean ± SEM, Inducible CARs: n=5, mean ± SEM). The dashed line indicates the expected tumor burden from the dead mice (no GZV and no POMA, on d20).

Figure S6

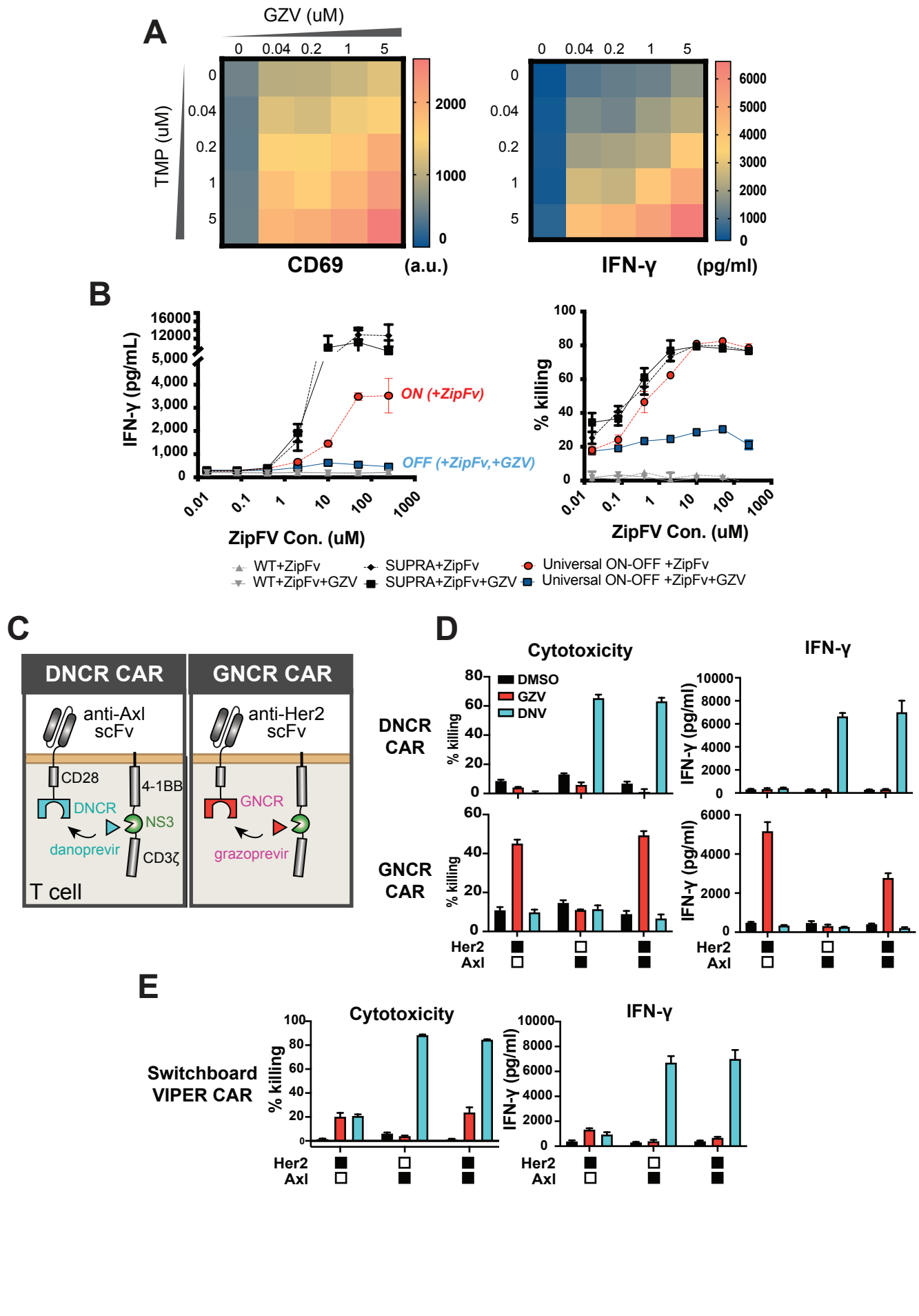

A
GZV (uM)
0 0.04 0.2 1 5
TMP (uM)
0 0.04 0.2 1 5
2000
1000
0
CD69 (a.u.)
6000
5000
4000
3000
2000
1000
0
IFN-γ (pg/ml)
B
IFN-γ (pg/mL)
16000
12000
5,000
4,000
3,000
2,000
1,000
0
ON (+ZipFv)
OFF (+ZipFv,+GZV)
0.01 0.1 1 10 100 1000
ZipFV Con. (uM)
% killing
100
80
60
40
20
0
ZipFV Con. (uM)
WT+ZipFv
WT+ZipFv+GZV
SUPRA+ZipFv
SUPRA+ZipFv+GZV
Universal ON-OFF +ZipFv
Universal ON-OFF +ZipFv+GZV
C
DNCR CAR
GNCR CAR
anti-Axl scFv
anti-Her2 scFv
CD28
4-1BB
DNCR
NS3
danoprevir
CD3ζ
GNCR
grazoprevir
T cell
D
Cytotoxicity
IFN-γ
DNCR CAR
GNCR CAR
DMSO
GZV
DNV
% killing
IFN-γ (pg/ml)
Her2
Axl
E
Switchboard VIPER CAR
Cytotoxicity
IFN-γ
% killing
IFN-γ (pg/ml)
Her2
Axl

Figure S6: Characterization of AND gate VIPER CAR, Universal ON-OFF VIPER CAR, and switchboard VIPER CARs in primary T cells, Related to Figure 7.

A) Dose-response of AND gate VIPER CAR-expressing CD4+ T cells when treated with increasing TMP and GZV. Cell activity was measured in terms of CD69 and IFN-γ (n = 3, mean values displayed). **B)** Regulation of ON state of the Universal ON-OFF-VIPER CAR and SUPRA-VIPER CAR by different concentrations of zipFv, as measured by IFN-γ (left) and cell killing ability (right). 1uM of GZV was used for all GZV conditions to turn off Universal ON-OFF-VIPER CAR functionality (mean ± s.d., n = 3). **C)** Schematic of how single NS3 reader CARs function in T cells. **D)** Cytotoxicity and cytokine levels of individual NS3 reader CARs in primary T cells when treated with various antigen-expressing target cells and with or without NS3 inhibitor (mean ± s.d., n = 3). **E)** Cytotoxicity and cytokine levels of switchboard VIPER CAR in primary T cells when treated with various antigen-expressing target cells and with or without NS3 inhibitor (mean ± s.d., n = 3).