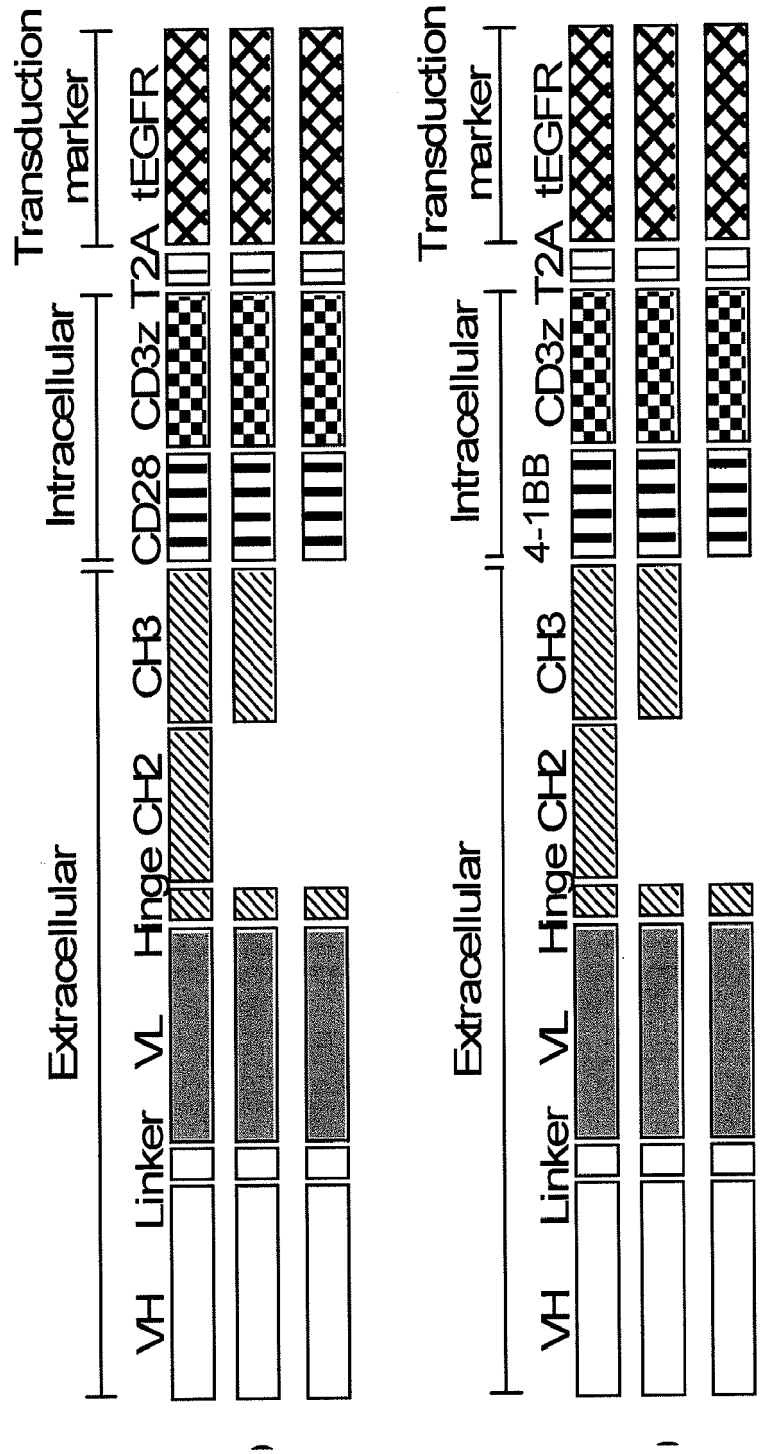

Extracellular
VH
Linker
VL
Hinge
CH2
CH3
Intracellular
CD28
CD3z
T2A
Transduction marker
tEGFR
Extracellular
VH
Linker
VL
Hinge
CH2
CH3
Intracellular
4-1BB
CD3z
T2A
Transduction marker
tEGFR

Figure 1

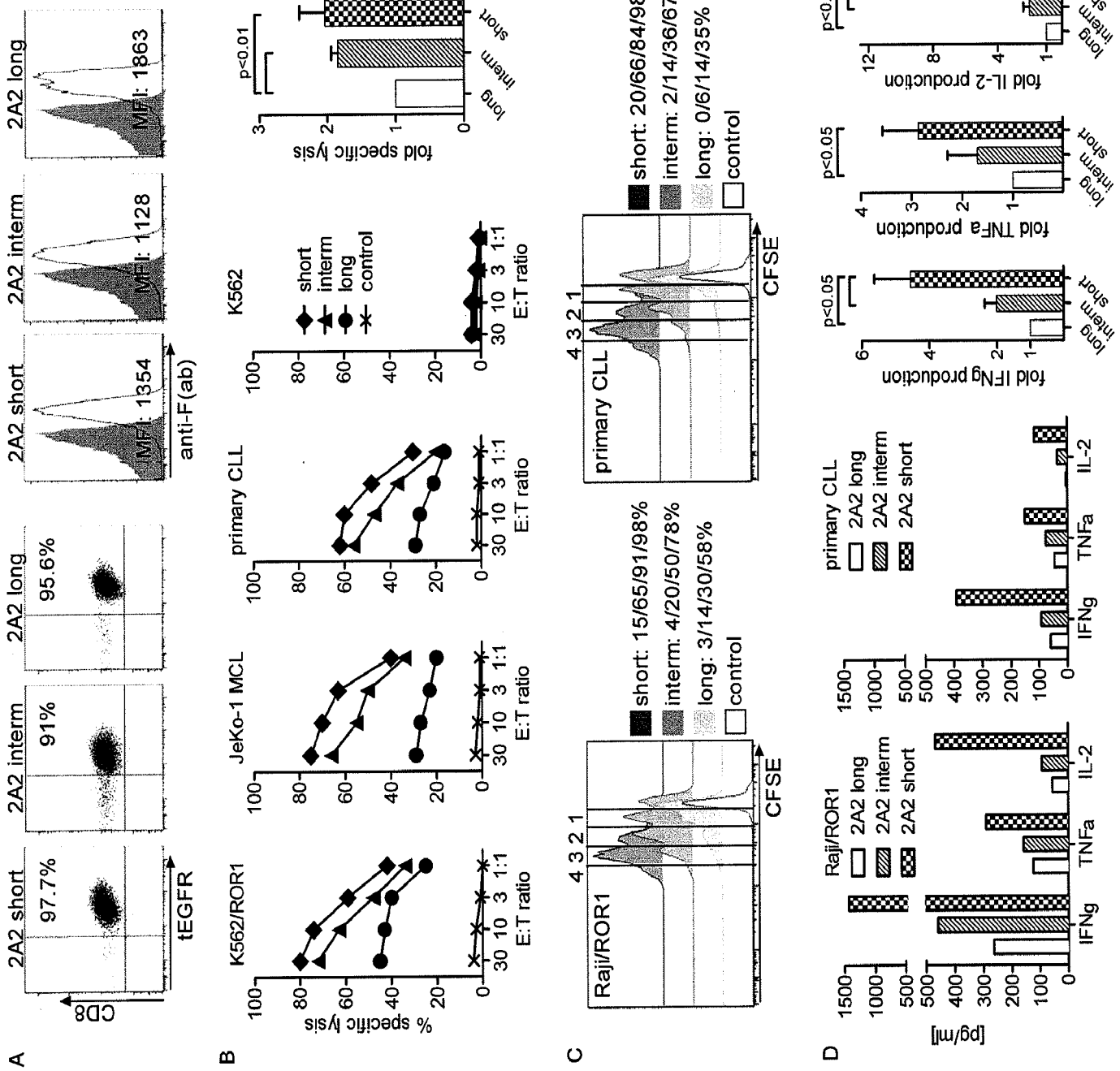

A
2A2 short
2A2 interm
2A2 long
2A2 short
2A2 interm
2A2 long
97.7%
91%
95.6%
MFI: 1354
MFI: 1128
MFI: 1863
CD8
tEGFR
anti-F(ab)
B
K562/ROR1
JeKo-1 MCL
primary CLL
K562
% specific lysis
E:T ratio
short
interm
long
control
fold specific lysis
p<0.01
C
Raji/ROR1
primary CLL
short: 15/65/91/98%
interm: 4/20/50/78%
long: 3/14/30/58%
short: 20/66/84/98%
interm: 2/14/36/67%
long: 0/6/14/35%
control
CFSE
D
Raji/ROR1
primary CLL
2A2 long
2A2 interm
2A2 short
[pg/ml]
IFNg
TNFa
IL-2
fold IFNg production
fold TNFa production
fold IL-2 production
p<0.05

Figure 2

Figure 3

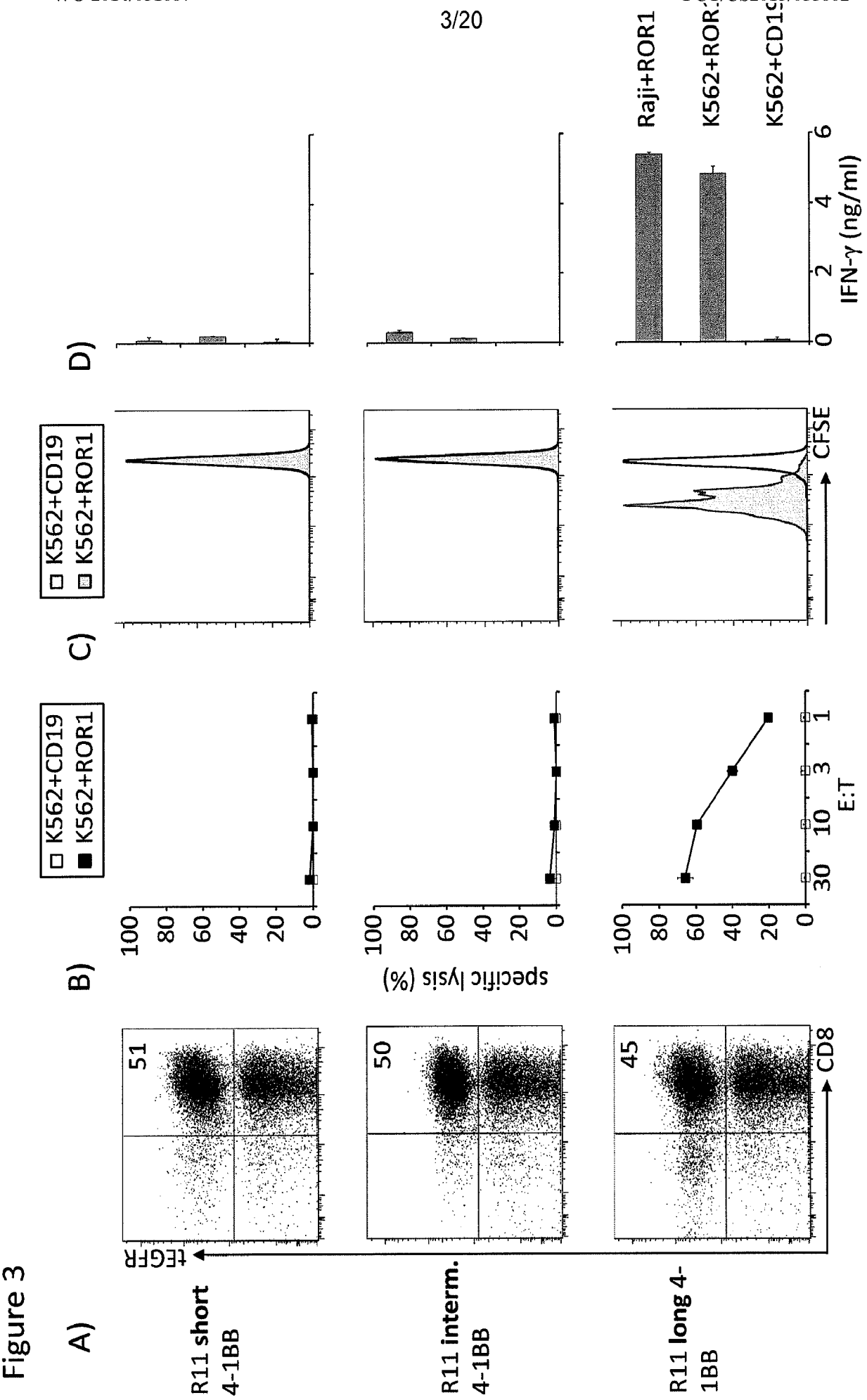

A)
R11 short 4-1BB
R11 interm. 4-1BB
R11 long 4-1BB
tEGFR
CD8
51
50
45
B)
K562+CD19
K562+ROR1
specific lysis (%)
E:T
C)
CFSE
D)
Raji+ROR1
K562+ROR1
K562+CD19
IFN-γ (ng/ml)

Figure 4

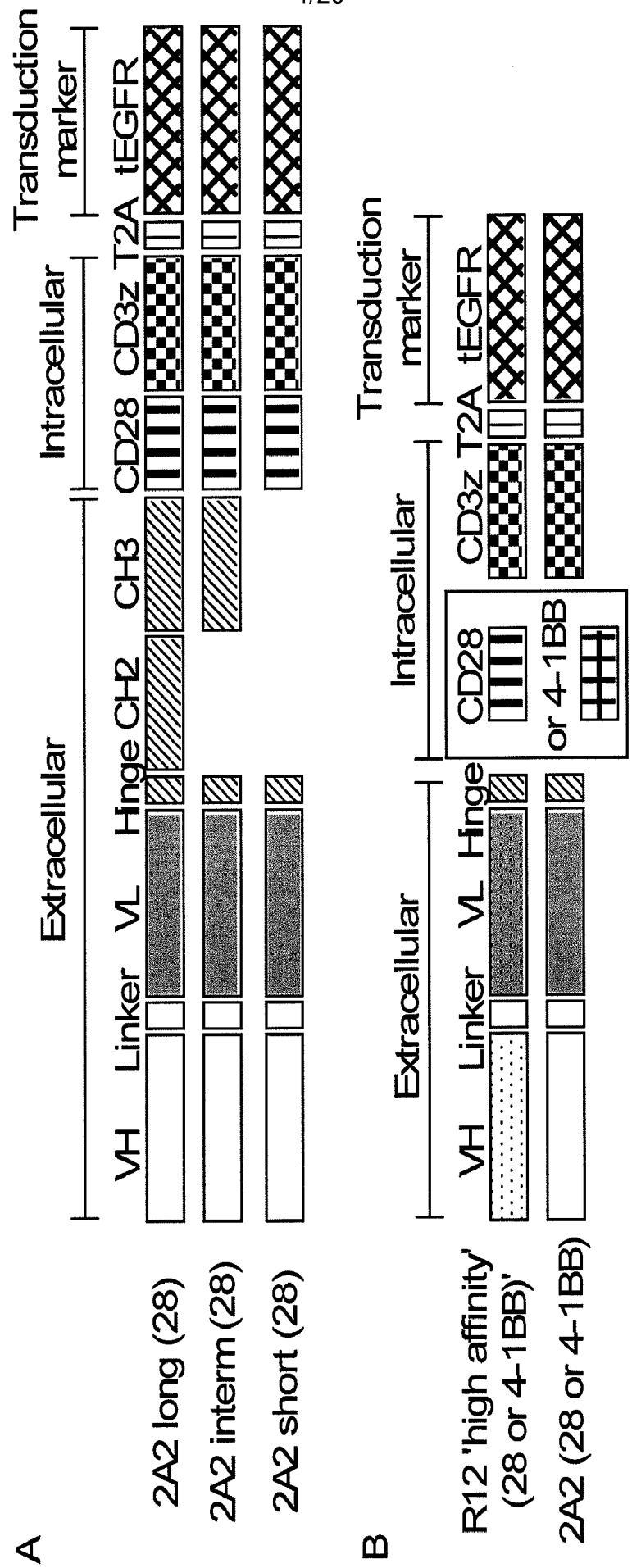
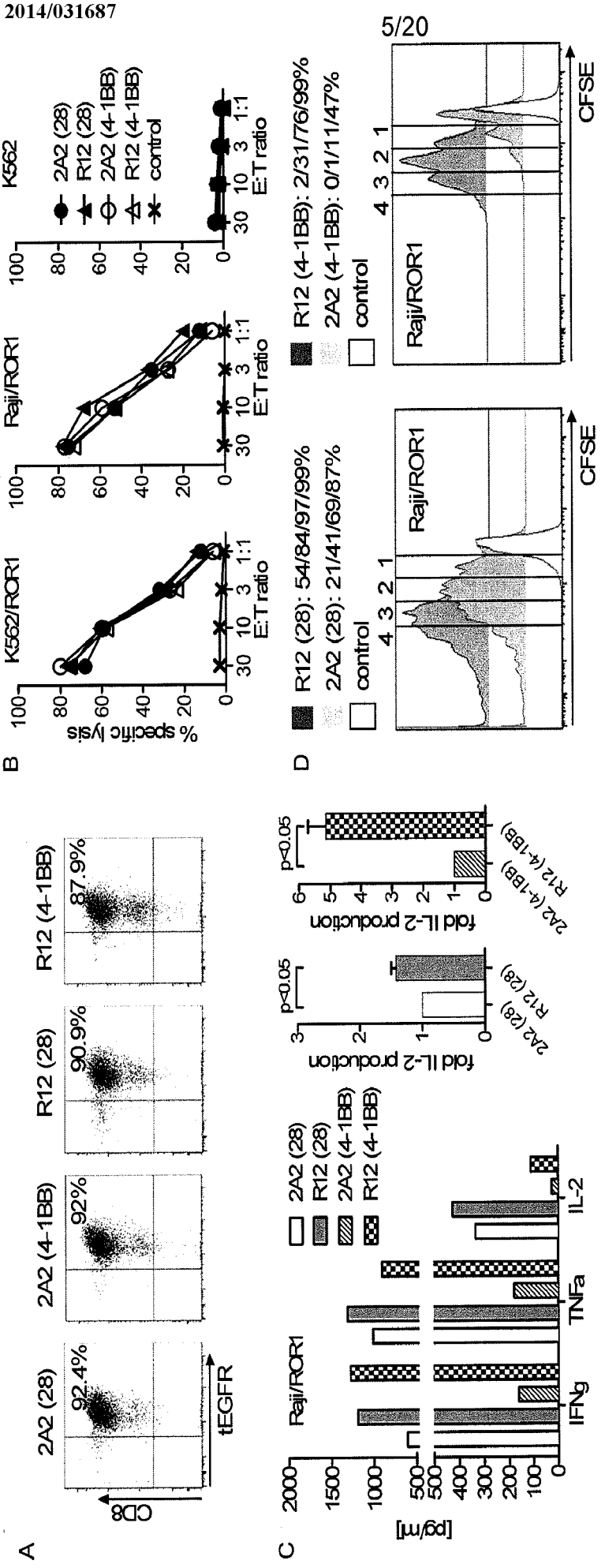

A
Extracellular
Intracellular
Transduction marker
VH
Linker
VL
Hinge
CH2
CH3
CD28
CD3z
T2A
tEGFR
2A2 long (28)
2A2 interm (28)
2A2 short (28)
B
Extracellular
Intracellular
Transduction marker
VH
Linker
VL
Hinge
CD28
or 4-1BB
CD3z
T2A
tEGFR
R12 'high affinity' (28 or 4-1BB)'
2A2 (28 or 4-1BB)

Figure 5


A
2A2 (28)
92.4%
2A2 (4-1BB)
92%
R12 (28)
90.9%
R12 (4-1BB)
87.9%
CD8
tEGFR
B
K562/ROR1
Raji/ROR1
K562
% specific lysis
E:T ratio
2A2 (28)
R12 (28)
2A2 (4-1BB)
R12 (4-1BB)
control
C
Raji/ROR1
2A2 (28)
R12 (28)
2A2 (4-1BB)
R12 (4-1BB)
[pg/ml]
IFNg
TNFa
IL-2
fold IL-2 production
p<0.05
2A2 (28)
R12 (28)
2A2 (4-1BB)
R12 (4-1BB)
D
R12 (28): 54/84/97/99%
2A2 (28): 21/41/69/87%
control
R12 (4-1BB): 2/31/76/99%
2A2 (4-1BB): 0/1/11/47%
control
Raji/ROR1
CFSE

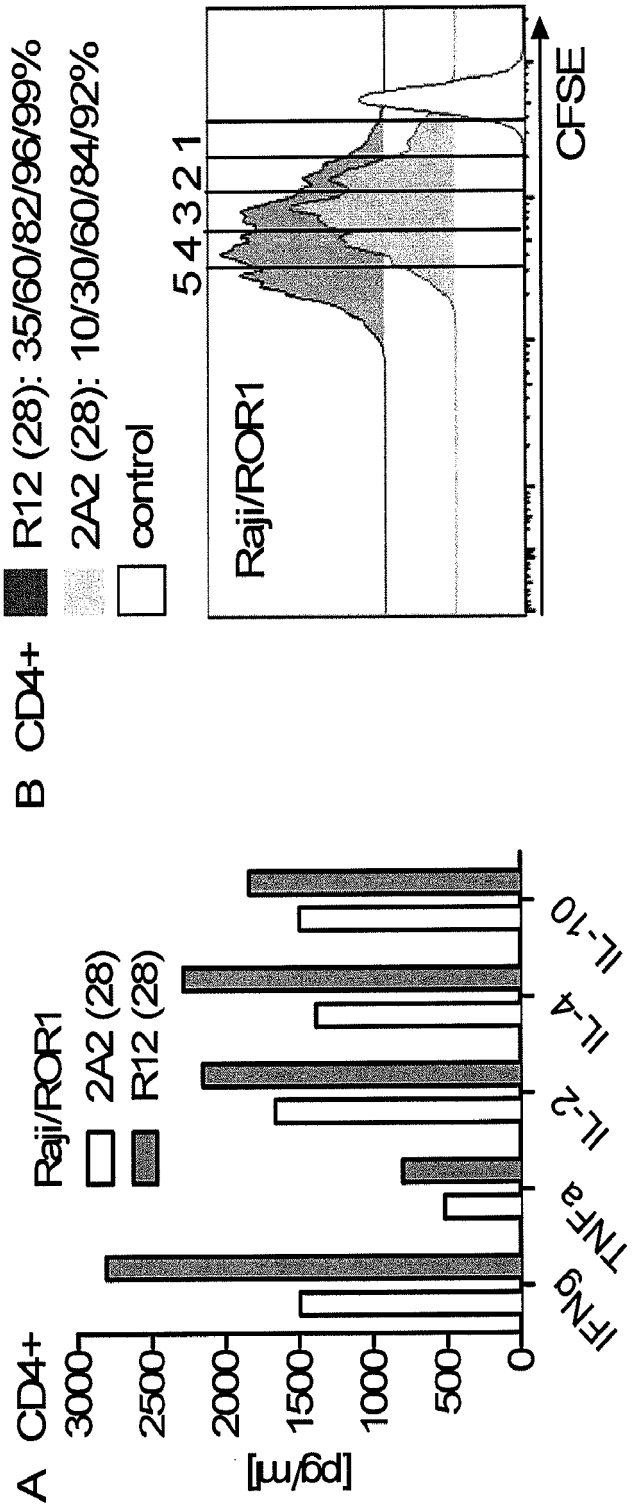

A CD4+
Raji/ROR1
2A2 (28)
R12 (28)
[pg/ml]
3000
2500
2000
1500
1000
500
0
IFNg
TNFa
IL-2
IL-4
IL-10
B CD4+
R12 (28): 35/60/82/96/99%
2A2 (28): 10/30/60/84/92%
control
Raji/ROR1
5 4 3 2 1
CFSE

Figure 6

Figure 7

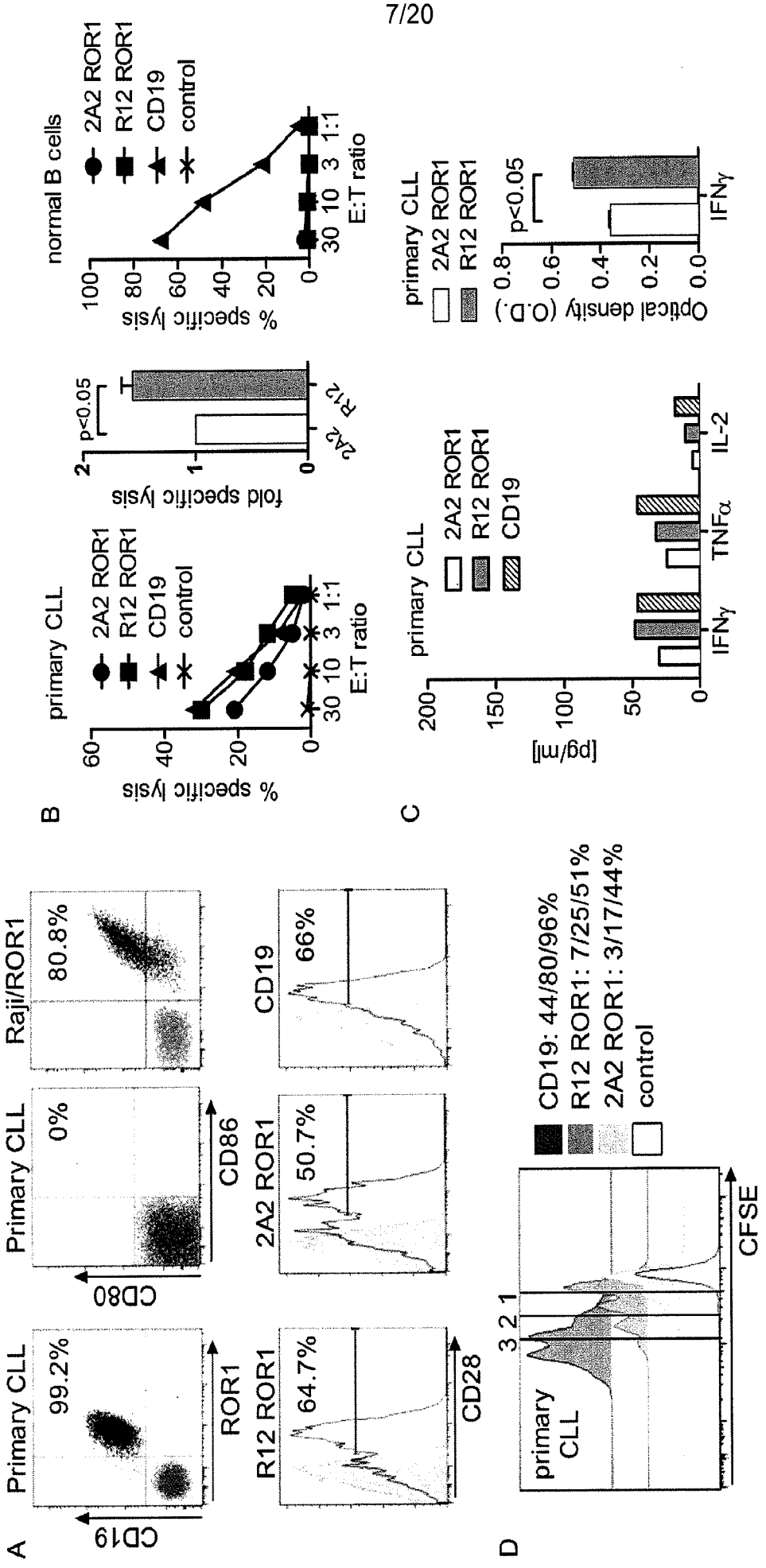

A
Primary CLL
99.2%
CD19
ROR1
R12 ROR1
64.7%
CD28
Primary CLL
0%
CD80
CD86
2A2 ROR1
50.7%
Raji/ROR1
80.8%
CD19
66%
B
primary CLL
2A2 ROR1
R12 ROR1
CD19
control
% specific lysis
E:T ratio
fold specific lysis
p<0.05
C
primary CLL
2A2 ROR1
R12 ROR1
CD19
[pg/ml]
IFNγ
TNFα
IL-2
Optical density (O.D.)
p<0.05
D
CD19: 44/80/96%
R12 ROR1: 7/25/51%
2A2 ROR1: 3/17/44%
control
primary CLL
CFSE

Figure 8

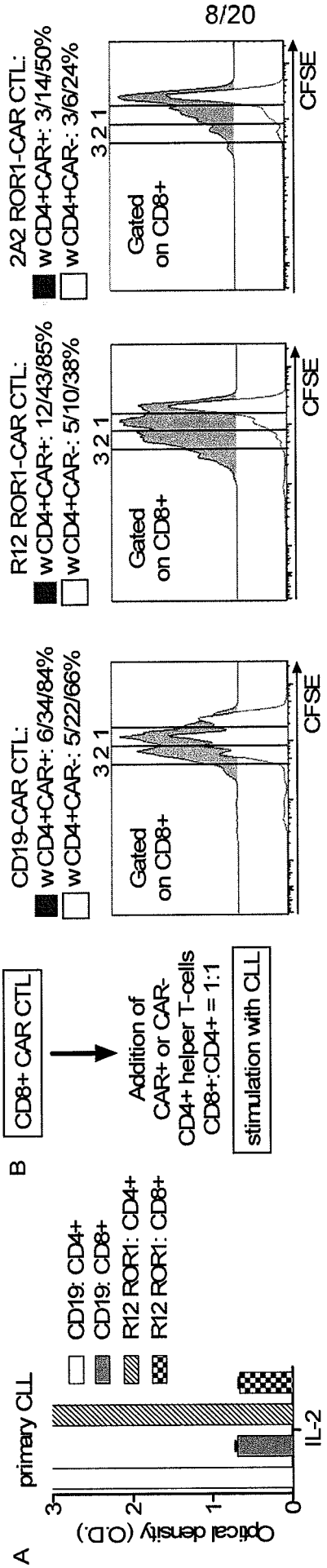
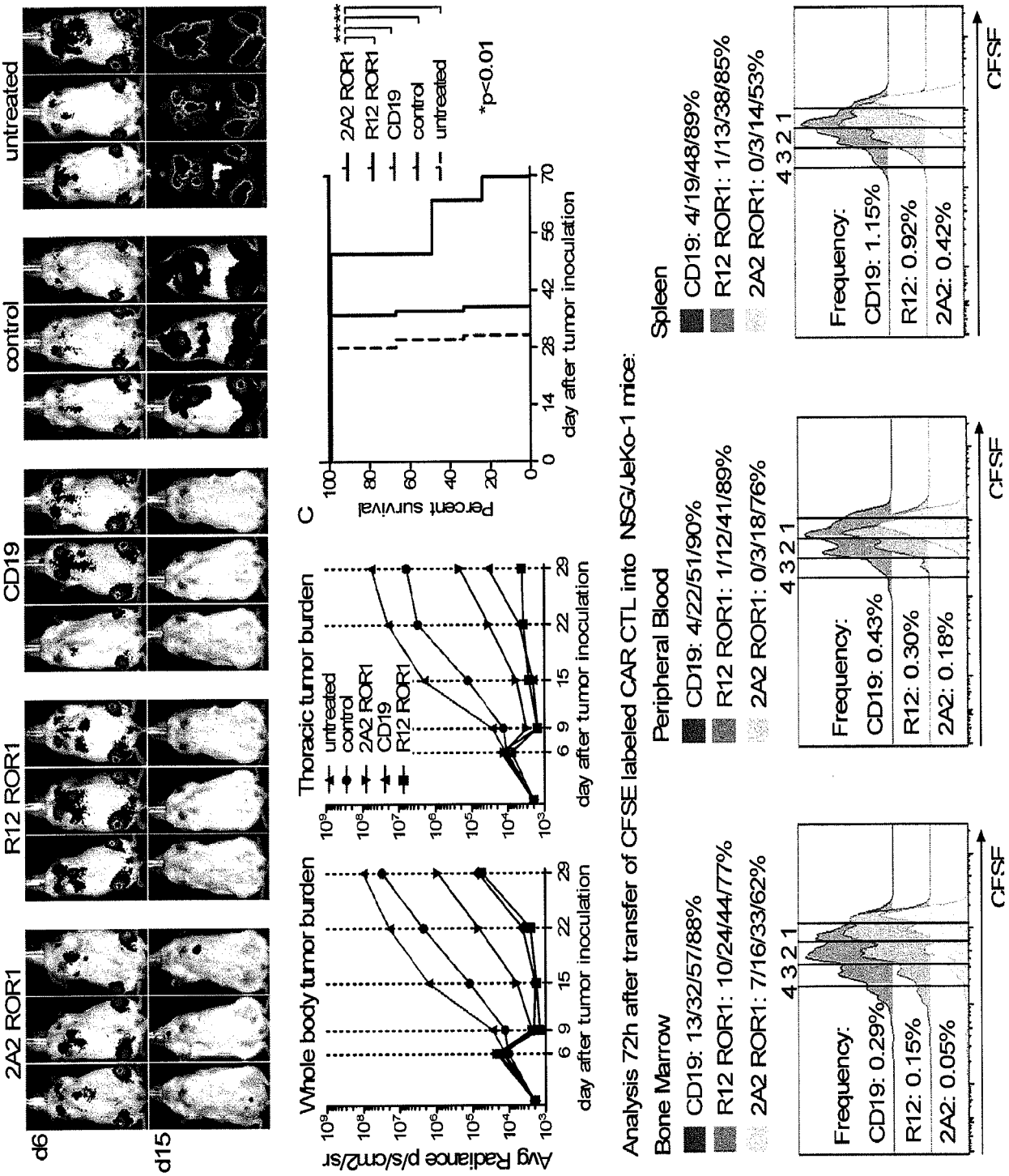

A
primary CLL
Optical density (O.D.)
3
2
1
0
IL-2
CD19: CD4+
CD19: CD8+
R12 ROR1: CD4+
R12 ROR1: CD8+
B
CD8+ CAR CTL
Addition of CAR+ or CAR- CD4+ helper T-cells CD8+:CD4+ = 1:1
stimulation with CLL
CD19-CAR CTL:
wCD4+CAR+: 6/34/84%
wCD4+CAR-: 5/22/66%
R12 ROR1-CAR CTL:
wCD4+CAR+: 12/43/85%
wCD4+CAR-: 5/10/38%
2A2 ROR1-CAR CTL:
wCD4+CAR+: 3/14/50%
wCD4+CAR-: 3/6/24%
Gated on CD8+
3 2 1
CFSE

Figure 9


A
2A2 ROR1
R12 ROR1
CD19
control
untreated
d6
d15
B
Whole body tumor burden
Thoracic tumor burden
Avg Radiance p/s/cm2/sr
day after tumor inoculation
untreated
control
2A2 ROR1
CD19
R12 ROR1
C
Percent survival
day after tumor inoculation
2A2 ROR1 ****
R12 RORI
CD19
control
untreated
*p<0.01
D
Analysis 72h after transfer of CFSE labeled CAR CTL into NSG/JeKo-1 mice:
Bone Marrow
CD19: 13/32/57/88%
R12 ROR1: 10/24/44/77%
2A2 ROR1: 7/16/33/62%
Peripheral Blood
CD19: 4/22/51/90%
R12 ROR1: 1/12/41/89%
2A2 ROR1: 0/3/18/76%
Spleen
CD19: 4/19/48/89%
R12 ROR1: 1/13/38/85%
2A2 ROR1: 0/3/14/53%
Frequency:
CD19: 0.29%
R12: 0.15%
2A2: 0.05%
Frequency:
CD19: 0.43%
R12: 0.30%
2A2: 0.18%
Frequency:
CD19: 1.15%
R12: 0.92%
2A2: 0.42%
CFSE

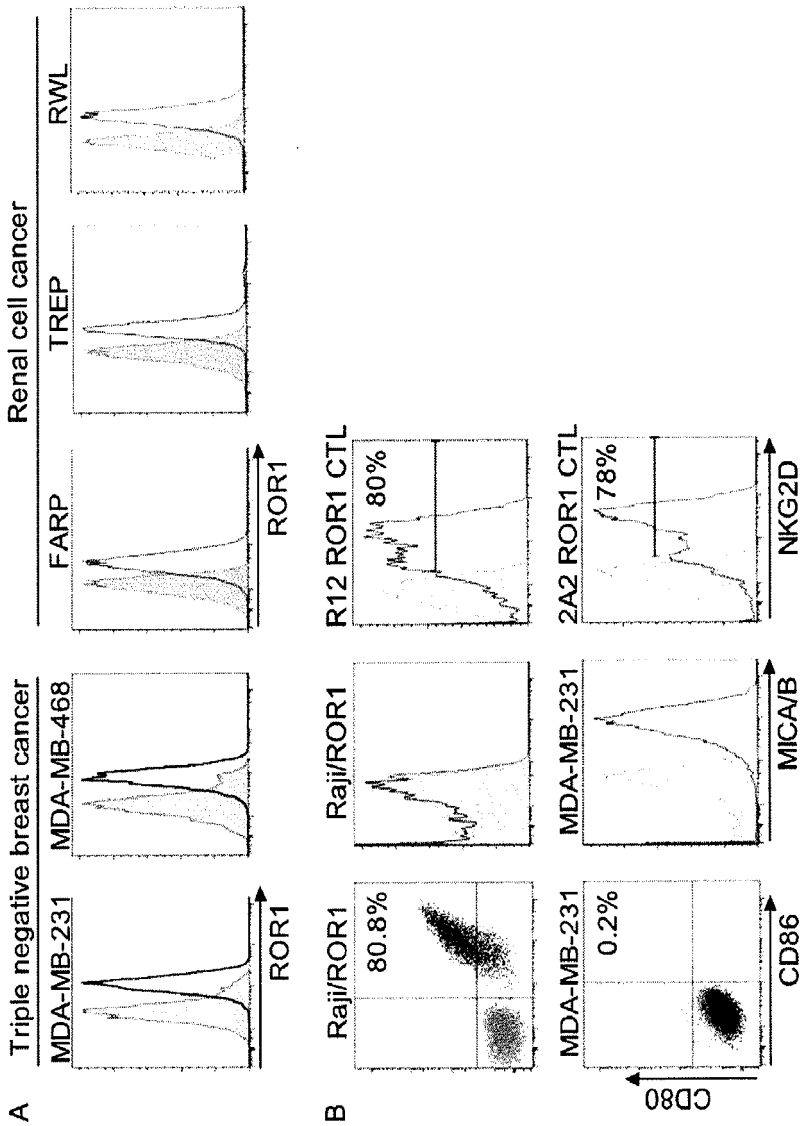

A
Triple negative breast cancer
MDA-MB-231
MDA-MB-468
ROR1
Renal cell cancer
FARP
TREP
RWL
ROR1
B
Raji/ROR1
80.8%
CD86
CD80
Raji/ROR1
R12 ROR1 CTL
80%
MDA-MB-231
0.2%
MDA-MB-231
MICA/B
2A2 ROR1 CTL
78%
NKG2D

Figure 10

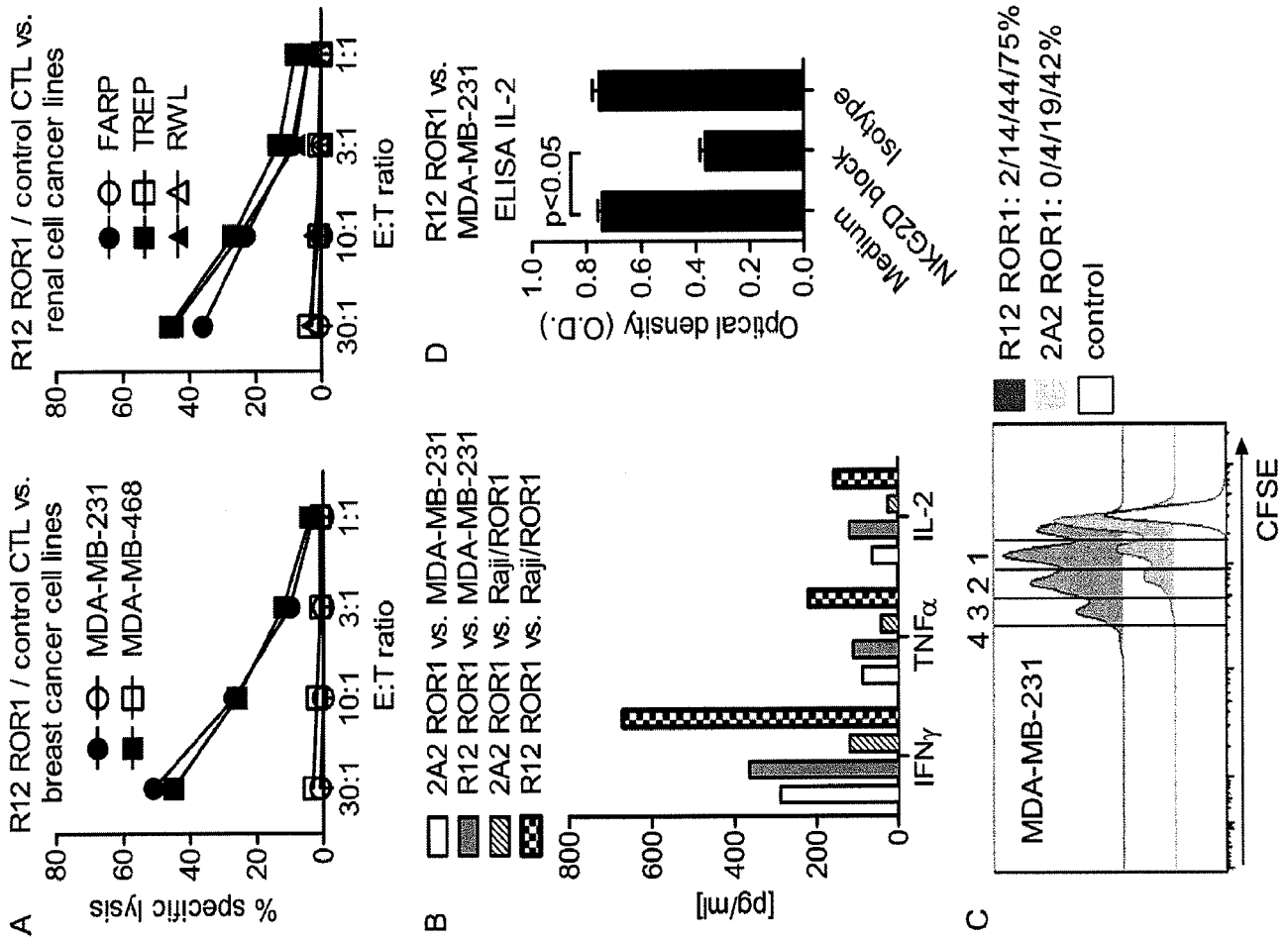

A
R12 ROR1 / control CTL vs. breast cancer cell lines
MDA-MB-231
MDA-MB-468
% specific lysis
E:T ratio
30:1
10:1
3:1
1:1
R12 ROR1 / control CTL vs. renal cell cancer lines
FARP
TREP
RWL
B
2A2 ROR1 vs. MDA-MB-231
R12 ROR1 vs. MDA-MB-231
2A2 ROR1 vs. Raji/ROR1
R12 ROR1 vs. Raji/ROR1
[pg/ml]
IFNγ
TNFα
IL-2
C
MDA-MB-231
4 3 2 1
CFSE
R12 ROR1: 2/14/44/75%
2A2 ROR1: 0/4/19/42%
control
D
R12 ROR1 vs. MDA-MB-231
ELISA IL-2
p<0.05
Optical density (O.D.)
Medium
NKG2D block
Isotype

Figure 11

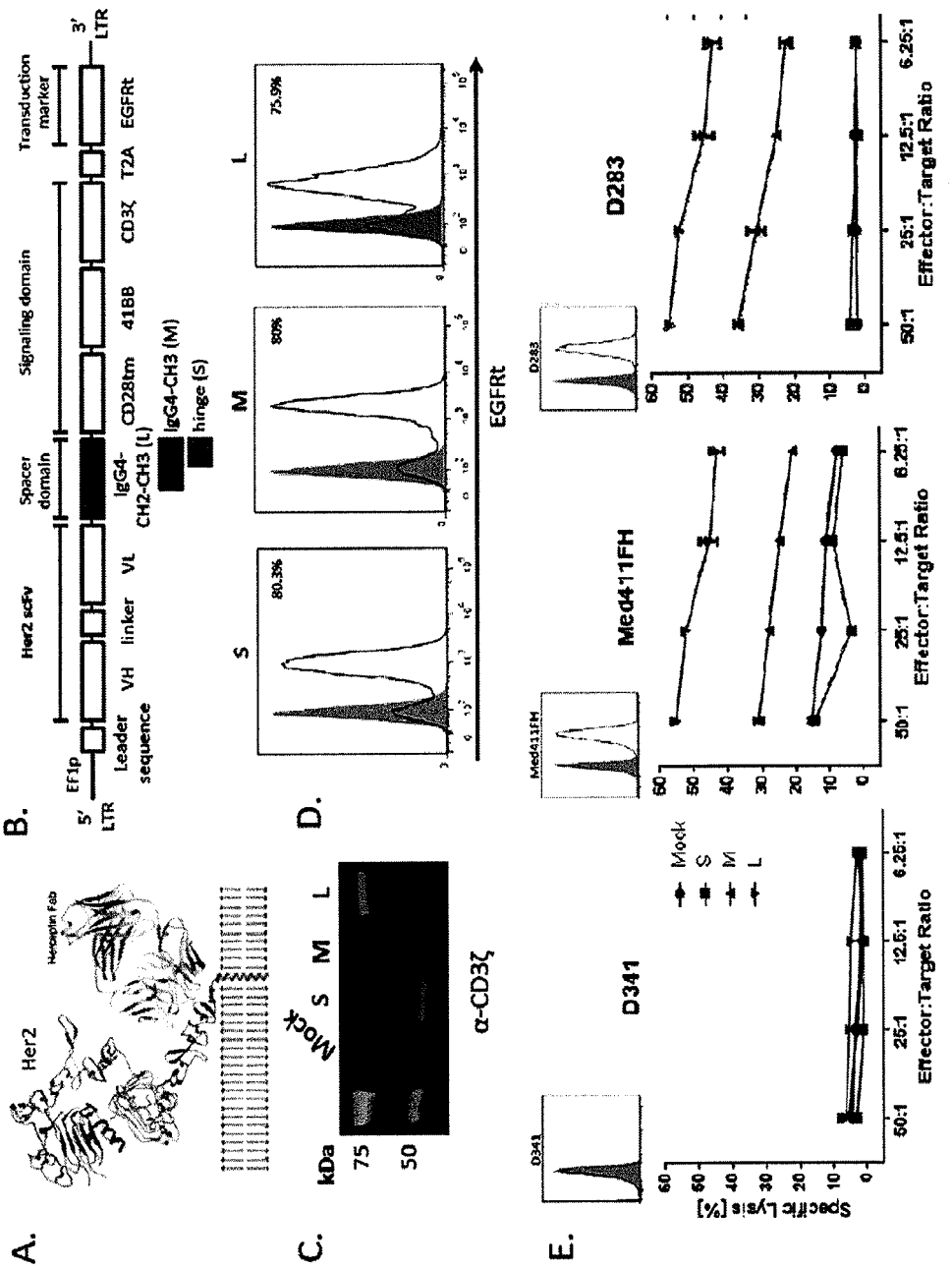

A.
Her2
Herceptin Fab
B.
5' LTR
Ef1p
Leader sequence
Her2 scFv
VH
linker
VL
Spacer domain
IgG4-CH2-CH3 (L)
IgG4-CH3 (M)
hinge (S)
Signaling domain
CD28tm
41BB
CD3ζ
Transduction marker
T2A
EGFRt
3' LTR
C.
kDa
75
50
Mock
S
M
L
α-CD3ζ
D.
S
80.3%
M
80%
L
75.9%
EGFRt
E.
D341
Med411FH
D283
Mock
S
M
L
Specific Lysis [%]
Effector:Target Ratio
50:1
25:1
12.5:1
6.25:1

Figure 12

Figure 13

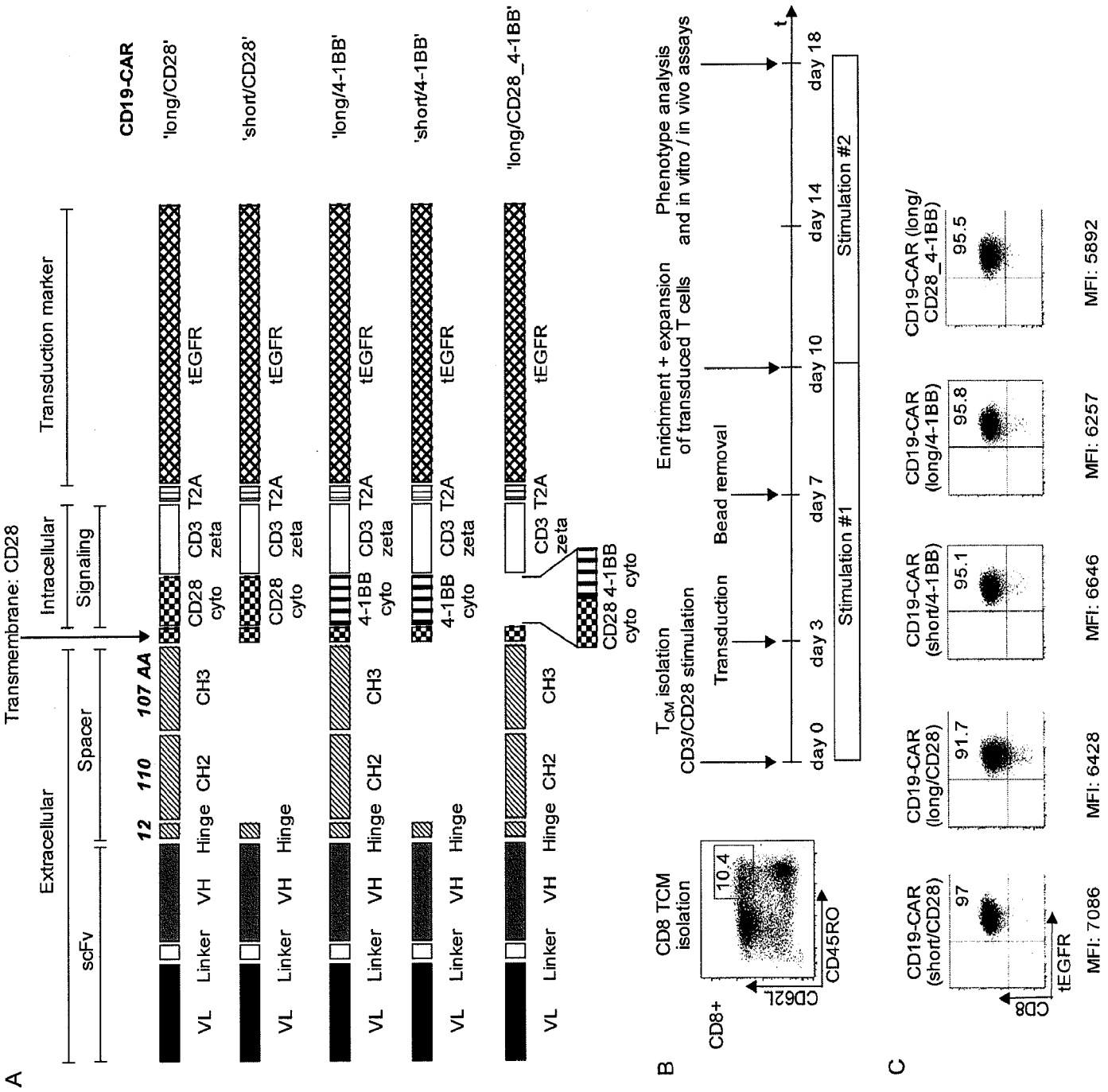

A
Transmembrane: CD28
Extracellular
Intracellular
Transduction marker
scFv
Spacer
Signaling
CD19-CAR
12 110 107 AA
'long/CD28'
'short/CD28'
'long/4-1BB'
'short/4-1BB'
'long/CD28_4-1BB'
VL Linker VH Hinge CH2 CH3
CD28 cyto
CD3 zeta
T2A
tEGFR
4-1BB cyto
CD28 4-1BB cyto cyto
B
CD8 TCM isolation
CD8+
CD62L
CD45RO
10.4
TCM isolation
CD3/CD28 stimulation
Transduction
Bead removal
Enrichment + expansion of transduced T cells
Phenotype analysis and in vitro / in vivo assays
day 0
day 3
day 7
day 10
day 14
day 18
Stimulation #1
Stimulation #2
C
CD19-CAR (short/CD28)
CD19-CAR (long/CD28)
CD19-CAR (short/4-1BB)
CD19-CAR (long/4-1BB)
CD19-CAR (long/CD28_4-1BB)
97
91.7
95.1
95.8
95.5
CD8
tEGFR
MFI: 7086
MFI: 6428
MFI: 6646
MFI: 6257
MFI: 5892

Figure 14

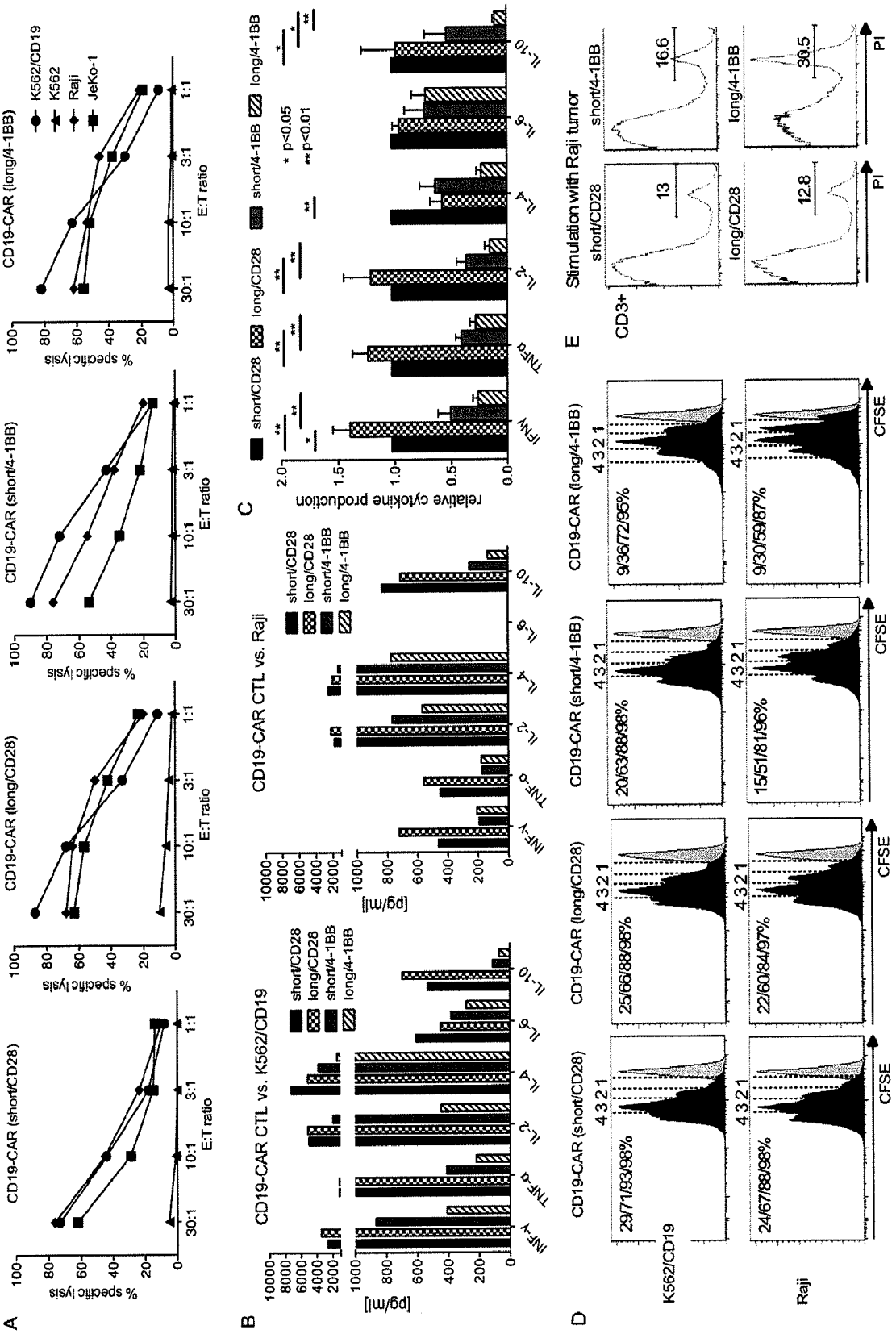

A
CD19-CAR (short/CD28)
CD19-CAR (long/CD28)
CD19-CAR (short/4-1BB)
CD19-CAR (long/4-1BB)
K562/CD19
K562
Raji
JeKo-1
% specific lysis
E:T ratio
B
CD19-CAR CTL vs. K562/CD19
CD19-CAR CTL vs. Raji
short/CD28
long/CD28
short/4-1BB
long/4-1BB
[pg/ml]
IFN-γ
TNF-α
IL-2
IL-4
IL-6
IL-10
C
relative cytokine production
* p<0.05
** p<0.01
D
CD19-CAR (short/CD28)
CD19-CAR (long/CD28)
CD19-CAR (short/4-1BB)
CD19-CAR (long/4-1BB)
29/71/93/98%
25/66/88/98%
20/63/88/98%
9/36/72/95%
24/67/88/98%
22/60/84/97%
15/51/81/96%
9/30/59/87%
K562/CD19
Raji
CFSE
E
Stimulation with Raji tumor
short/CD28
long/CD28
short/4-1BB
long/4-1BB
CD3+
13
12.8
16.6
30.5
PI

Figure 15

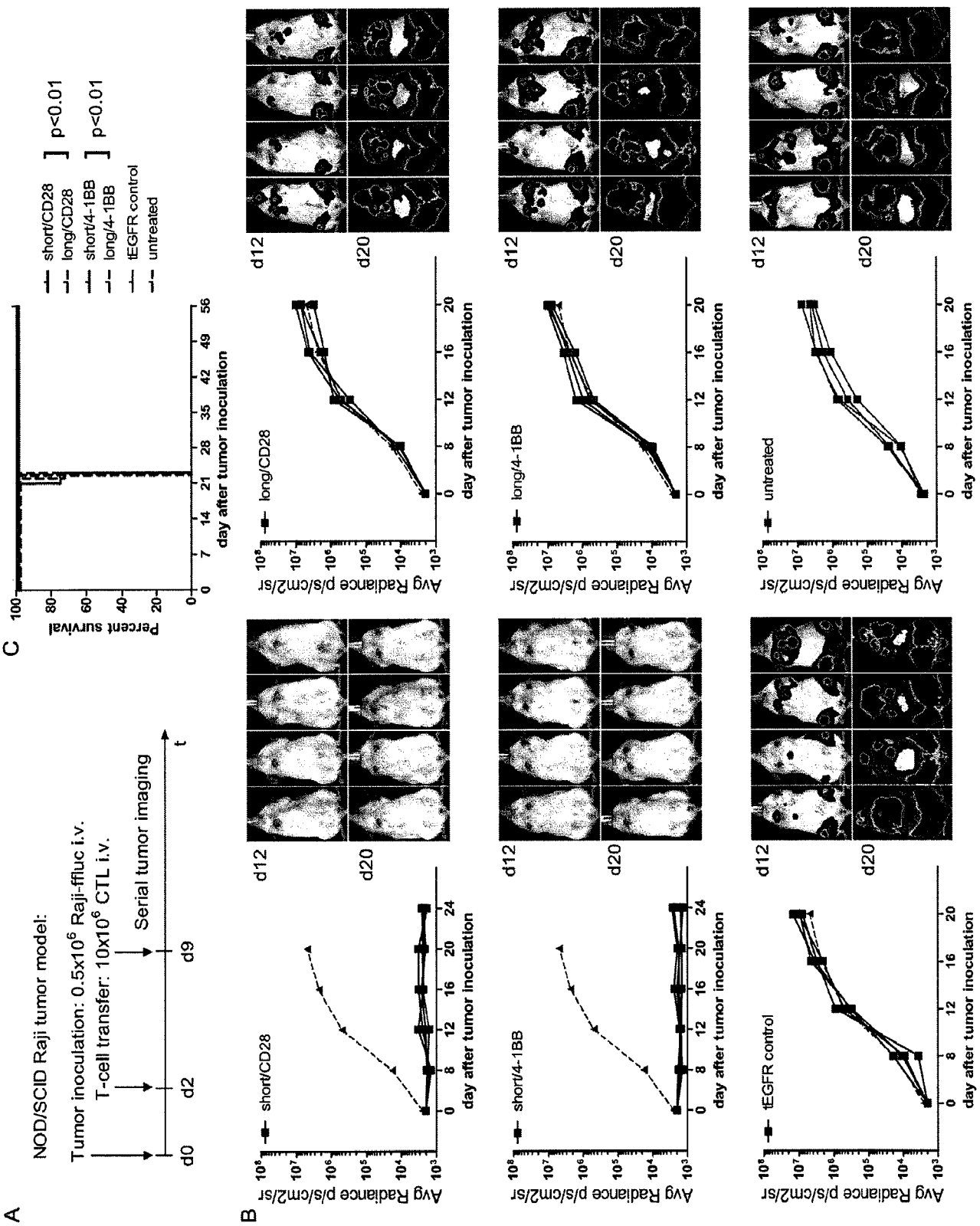

A
NOD/SCID Raji tumor model:
Tumor inoculation: 0.5x10^6 Raji-ffluc i.v.
T-cell transfer: 10x10^6 CTL i.v.
Serial tumor imaging
d0
d2
d9
t
C
Percent survival
day after tumor inoculation
short/CD28
long/CD28
short/4-1BB
long/4-1BB
tEGFR control
untreated
p<0.01
p<0.01
B
d12
d20
Avg Radiance p/s/cm2/sr
day after tumor inoculation
short/CD28
long/CD28
short/4-1BB
long/4-1BB
tEGFR control
untreated

Figure 16

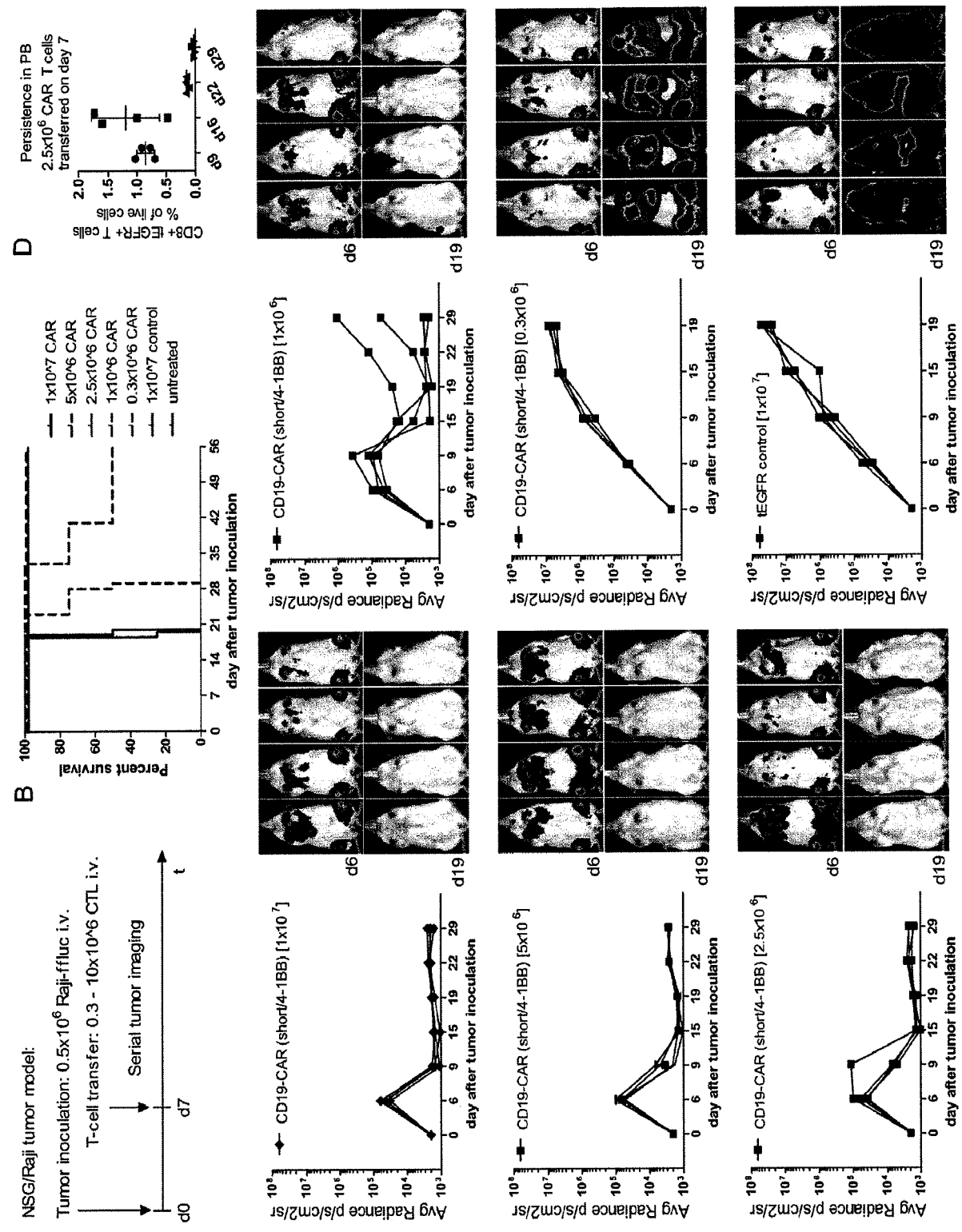

A
NSG/Raji tumor model:
Tumor inoculation: 0.5x10^6 Raji-ffluc i.v.
T-cell transfer: 0.3 - 10x10^6 CTL i.v.
Serial tumor imaging
d0
d7
t
B
Percent survival
day after tumor inoculation
1x10^7 CAR
5x10^6 CAR
2.5x10^6 CAR
1x10^6 CAR
0.3x10^6 CAR
1x10^7 control
untreated
D
Persistence in PB
2.5x10^6 CAR T cells transferred on day 7
CD8+ tEGFR+ T cells
% of live cells
C
CD19-CAR (short/4-1BB) [1x10^7]
CD19-CAR (short/4-1BB) [5x10^6]
CD19-CAR (short/4-1BB) [2.5x10^6]
CD19-CAR (short/4-1BB) [1x10^6]
CD19-CAR (short/4-1BB) [0.3x10^6]
tEGFR control [1x10^7]
Avg Radiance p/s/cm2/sr
day after tumor inoculation
d6
d19

Figure 17

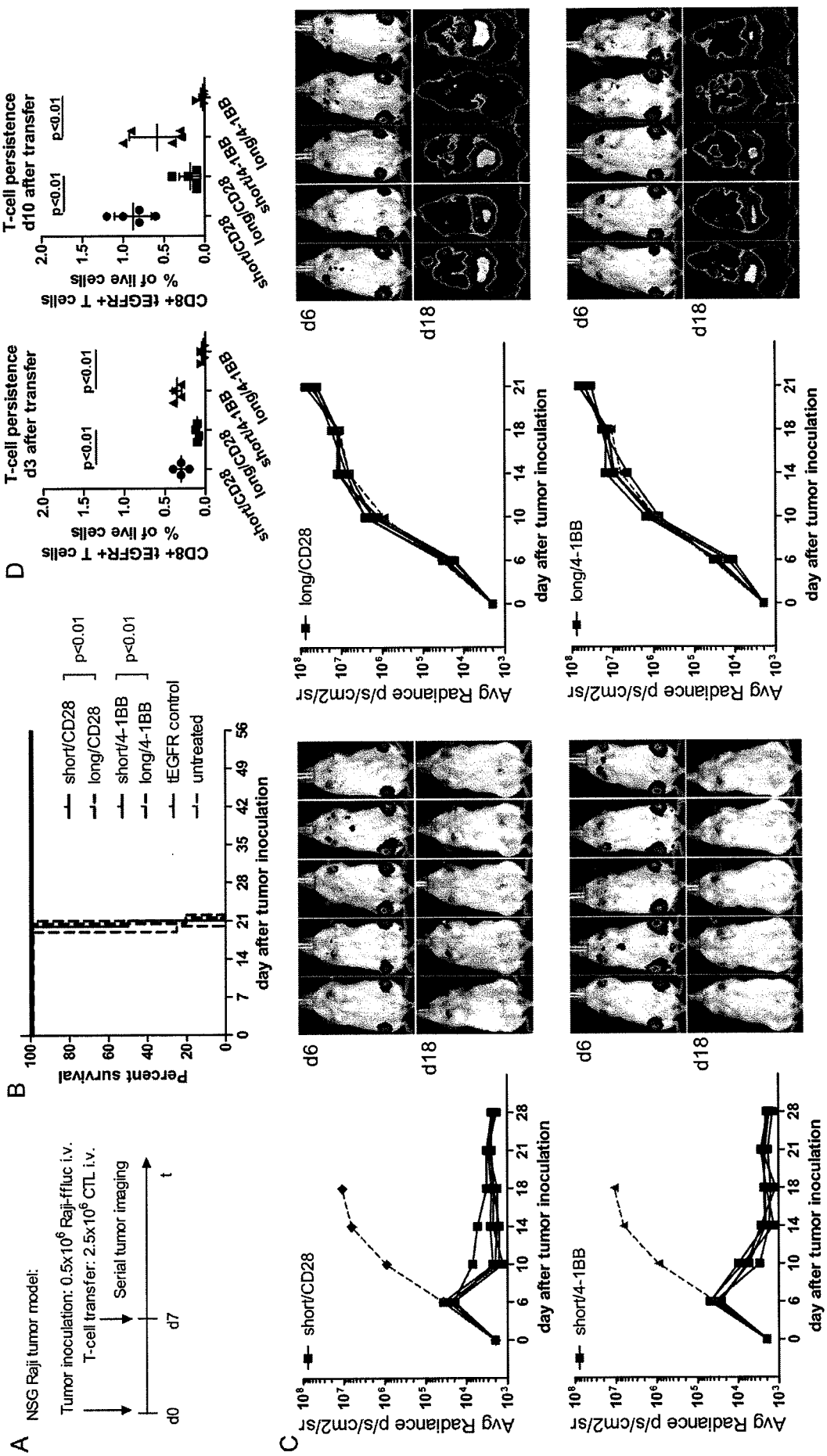

A
NSG Raji tumor model:
Tumor inoculation: 0.5x10^6 Raji-ffluc i.v.
T-cell transfer: 2.5x10^6 CTL i.v.
Serial tumor imaging
d0
d7
t
B
Percent survival
day after tumor inoculation
short/CD28
long/CD28
short/4-1BB
long/4-1BB
tEGFR control
untreated
p<0.01
p<0.01
C
short/CD28
long/CD28
short/4-1BB
long/4-1BB
Avg Radiance p/s/cm2/sr
day after tumor inoculation
d6
d18
D
T-cell persistence
d3 after transfer
T-cell persistence
d10 after transfer
CD8+ tEGFR+ T cells
% of live cells
p<0.01
p<0.01
p<0.01
p<0.01
short/CD28
long/CD28
short/4-1BB
long/4-1BB

Figure 18

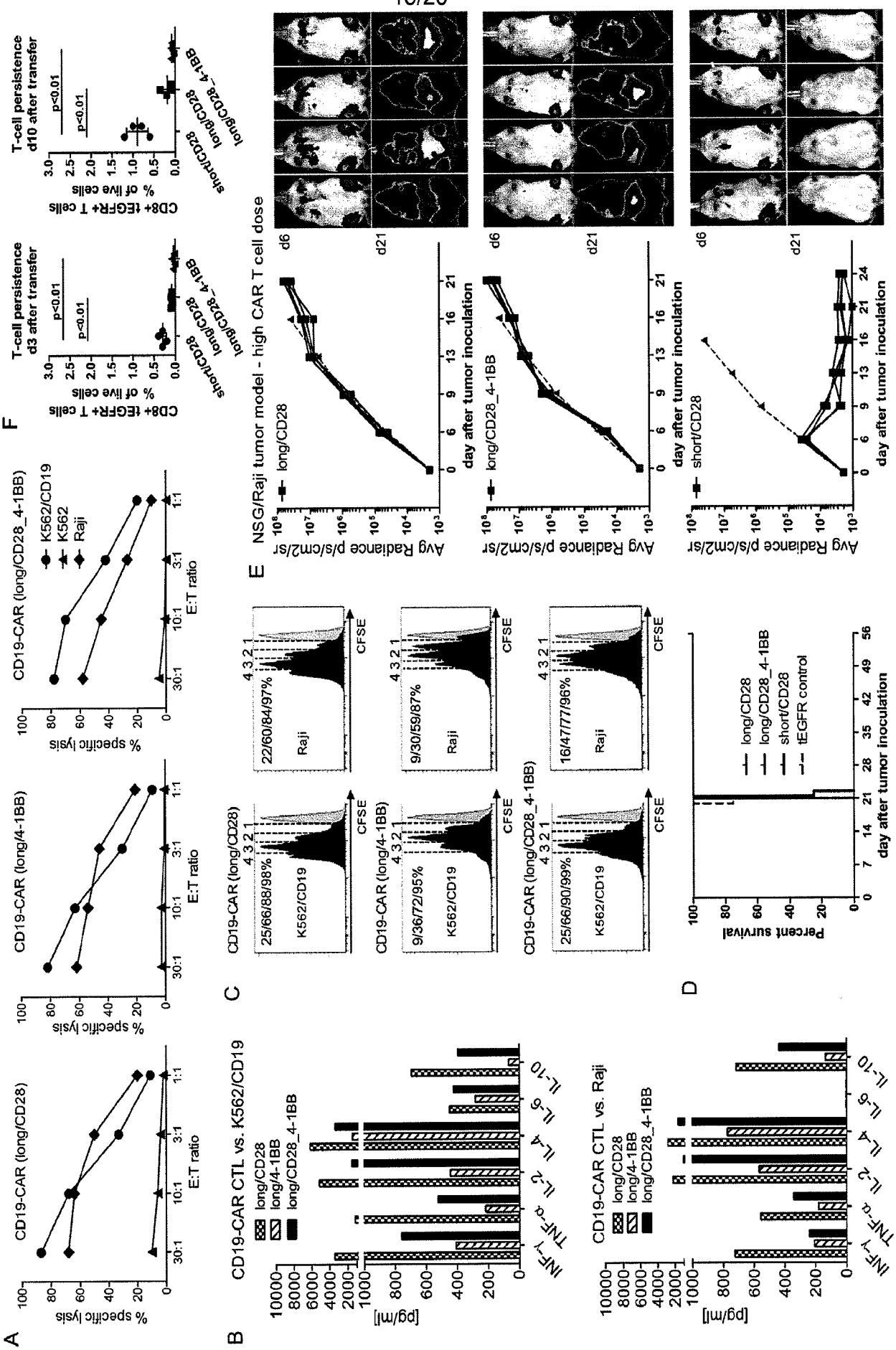
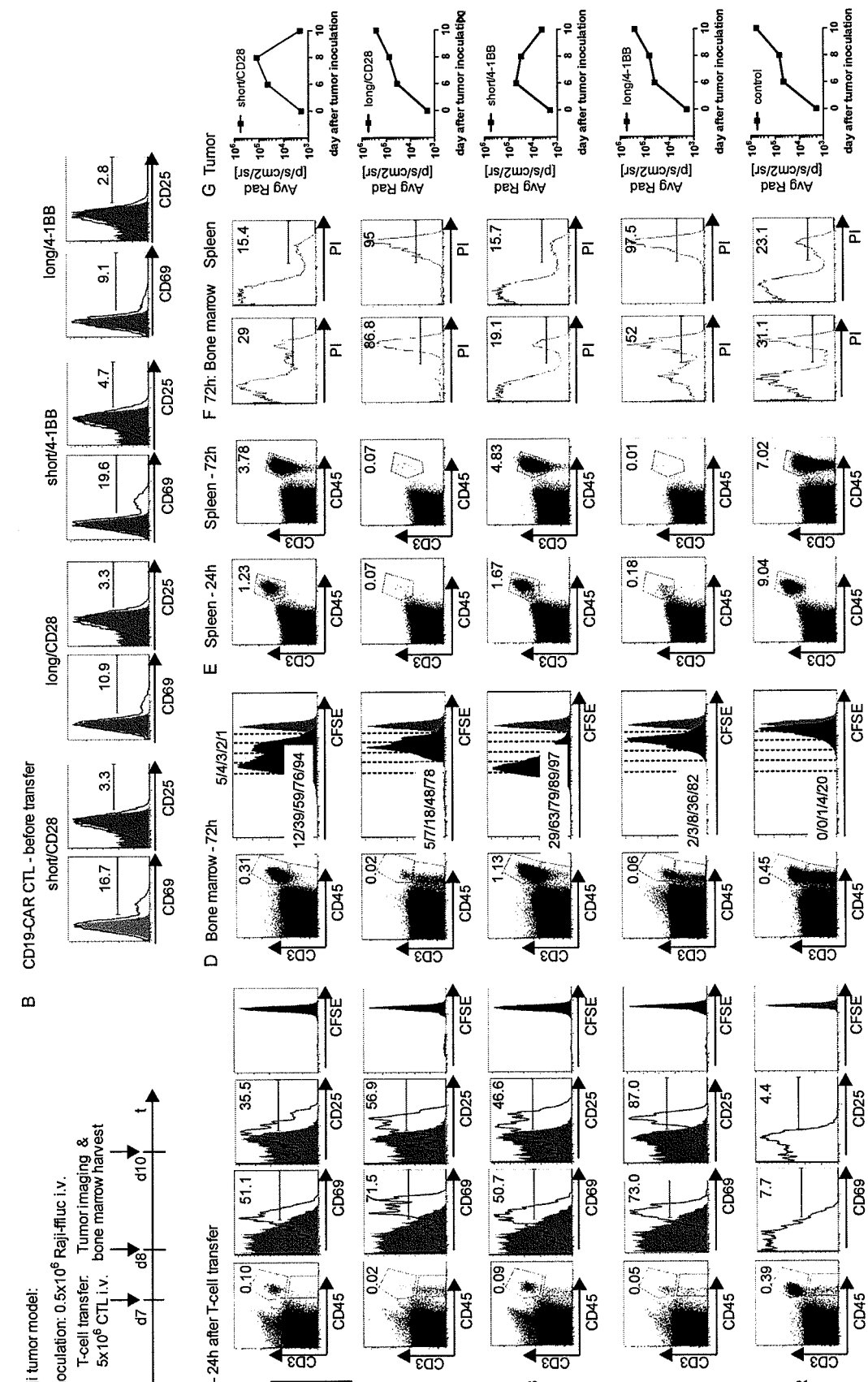
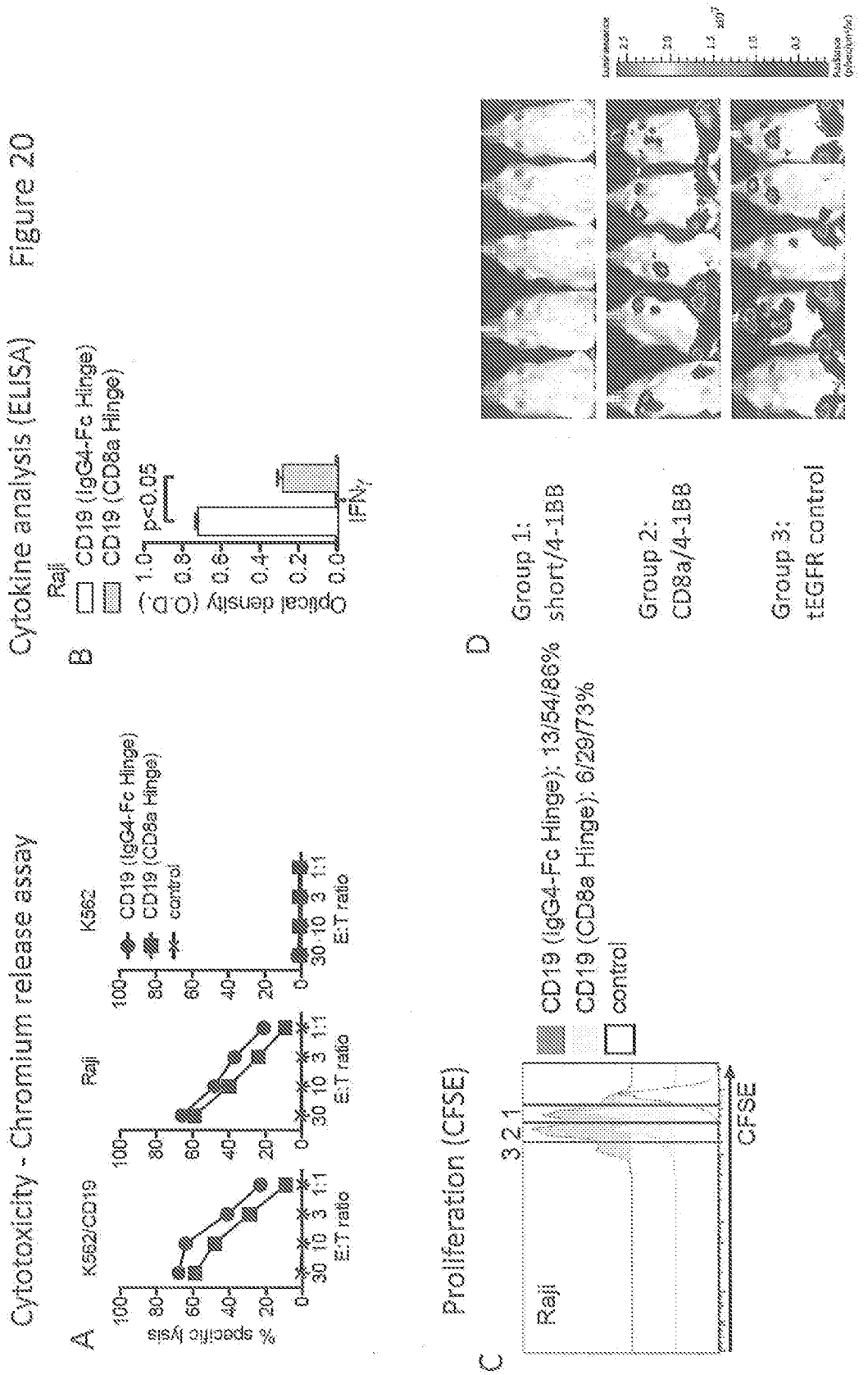

A
CD19-CAR (long/CD28)
% specific lysis
E:T ratio
CD19-CAR (long/4-1BB)
CD19-CAR (long/CD28_4-1BB)
K562/CD19
K562
Raji
B
CD19-CAR CTL vs. K562/CD19
long/CD28
long/4-1BB
long/CD28_4-1BB
[pg/ml]
INF-γ
TNF-α
IL-2
IL-4
IL-5
IL-10
CD19-CAR CTL vs. Raji
C
CD19-CAR (long/CD28)
CD19-CAR (long/4-1BB)
CD19-CAR (long/CD28_4-1BB)
4 3 2 1
25/66/88/98%
9/36/72/95%
25/66/90/99%
22/60/84/97%
9/30/59/87%
16/47/77/96%
K562/CD19
Raji
CFSE
D
Percent survival
day after tumor inoculation
long/CD28
long/CD28_4-1BB
short/CD28
tEGFR control
E
NSG/Raji tumor model - high CAR T cell dose
long/CD28
long/CD28_4-1BB
short/CD28
Avg Radiance p/s/cm2/sr
day after tumor inoculation
d6
d21
F
T-cell persistence
d3 after transfer
d10 after transfer
CD8+ tEGFR+ T cells
% of live cells
p<0.01
shortCD28
longCD28
longCD28_4-1BB

Figure 19


A
NSG Raji tumor model:
Tumor inoculation: 0.5x10^6 Raji-ffluc i.v.
T-cell transfer: 5x10^6 CTL i.v.
Tumor imaging & bone marrow harvest
d0
d7
d8
d10
t
B
CD19-CAR CTL - before transfer
short/CD28
long/CD28
short/4-1BB
long/4-1BB
CD69
CD25
16.7
3.3
10.9
3.3
19.6
4.7
9.1
2.8
C
Bone marrow - 24h after T-cell transfer
short/CD28
long/CD28
short/4-1BB
long/4-1BB
control tEGFR
T cells
Tumor
CD3
CD45
CD69
CD25
CFSE
0.10
51.1
35.5
0.02
71.5
56.9
0.09
50.7
46.6
0.05
73.0
87.0
0.39
7.7
4.4
D
Bone marrow - 72h
5/4/3/2/1
12/39/59/76/94
5/7/18/48/78
29/63/79/89/97
2/3/8/36/82
0/0/1/4/20
0.31
0.02
1.13
0.06
0.45
E
Spleen - 24h
1.23
0.07
1.67
0.18
9.04
F
72h: Bone marrow
Spleen
PI
29
15.4
86.8
95
19.1
15.7
52
97.5
31.1
23.1
G
Tumor
Avg Rad [p/s/cm2/sr]
day after tumor inoculation
short/CD28
long/CD28
short/4-1BB
long/4-1BB
control


Cytotoxicity - Chromium release assay
Cytokine analysis (ELISA)
Figure 20
A
K562/CD19
Raji
K562
% specific lysis
E:T ratio
CD19 (IgG4-Fc Hinge)
CD19 (CD8a Hinge)
control
B
Raji
CD19 (IgG4-Fc Hinge)
CD19 (CD8a Hinge)
p<0.05
Optical density (O.D.)
IFNγ
C
Proliferation (CFSE)
Raji
3 2 1
CD19 (IgG4-Fc Hinge): 13/54/86%
CD19 (CD8a Hinge): 6/29/73%
control
CFSE
D
Group 1:
short/4-1BB
Group 2:
CD8a/4-1BB
Group 3:
tEGFR control