Figure 1

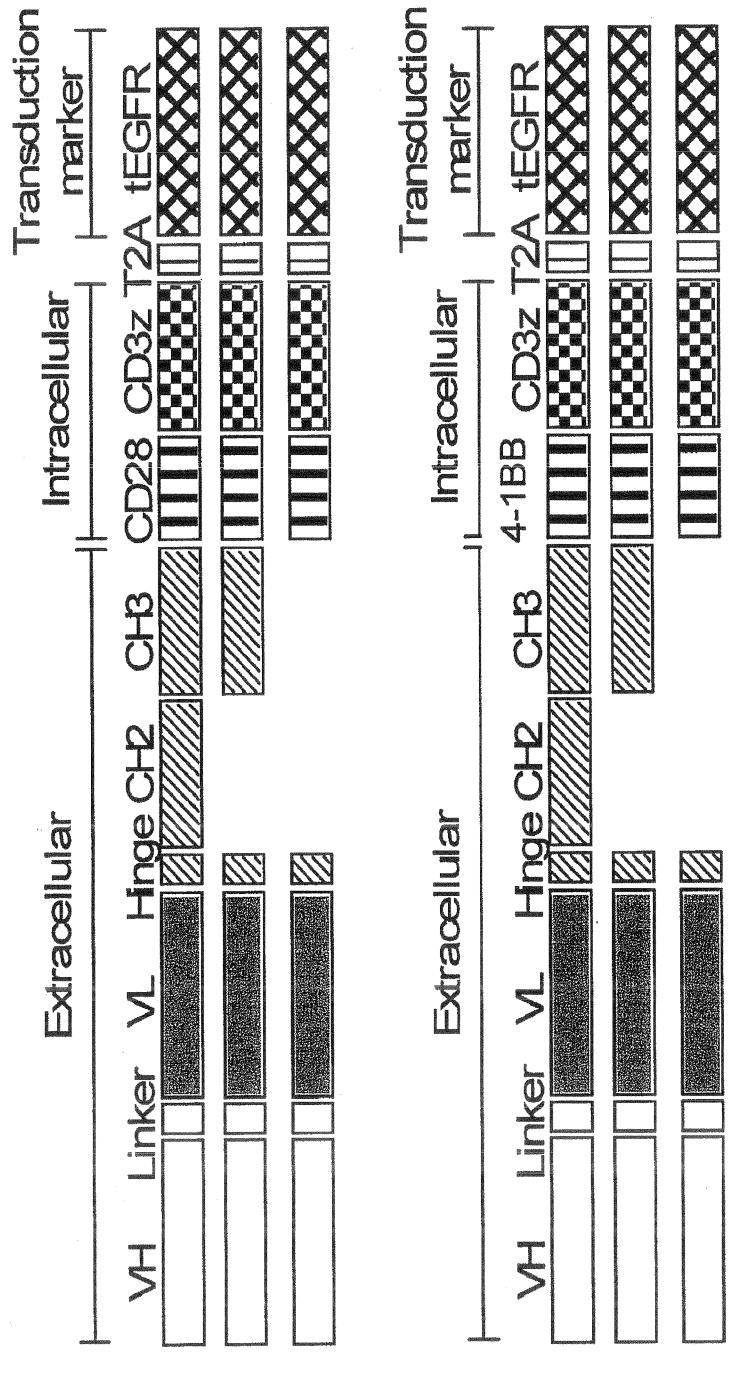

Extracellular
Intracellular
Transduction marker
VH
Linker
VL
Hinge
CH2
CH3
CD28
CD3z
T2A
tEGFR
Extracellular
Intracellular
Transduction marker
VH
Linker
VL
Hinge
CH2
CH3
4-1BB
CD3z
T2A
tEGFR

Figure 2

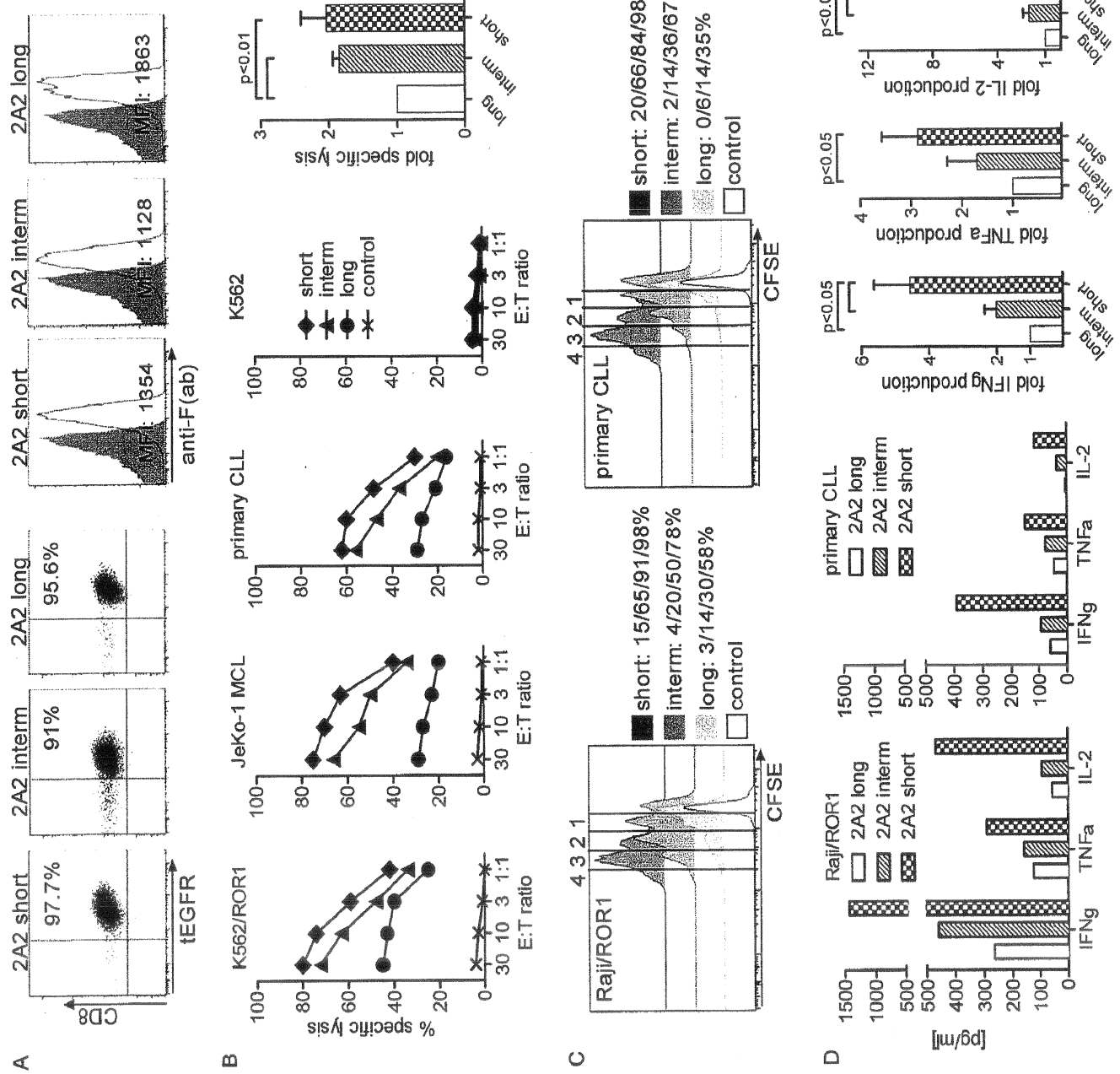

A
2A2 short
2A2 interm
2A2 long
2A2 short
2A2 interm
2A2 long
97.7%
91%
95.6%
MFI: 1354
MFI: 1128
MFI: 1863
CD8
tEGFR
anti-F(ab)
B
K562/ROR1
JeKo-1 MCL
primary CLL
K562
% specific lysis
E:T ratio
short
interm
long
control
p<0.01
fold specific lysis
C
Raji/ROR1
primary CLL
short: 15/65/91/98%
interm: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

Figure 3

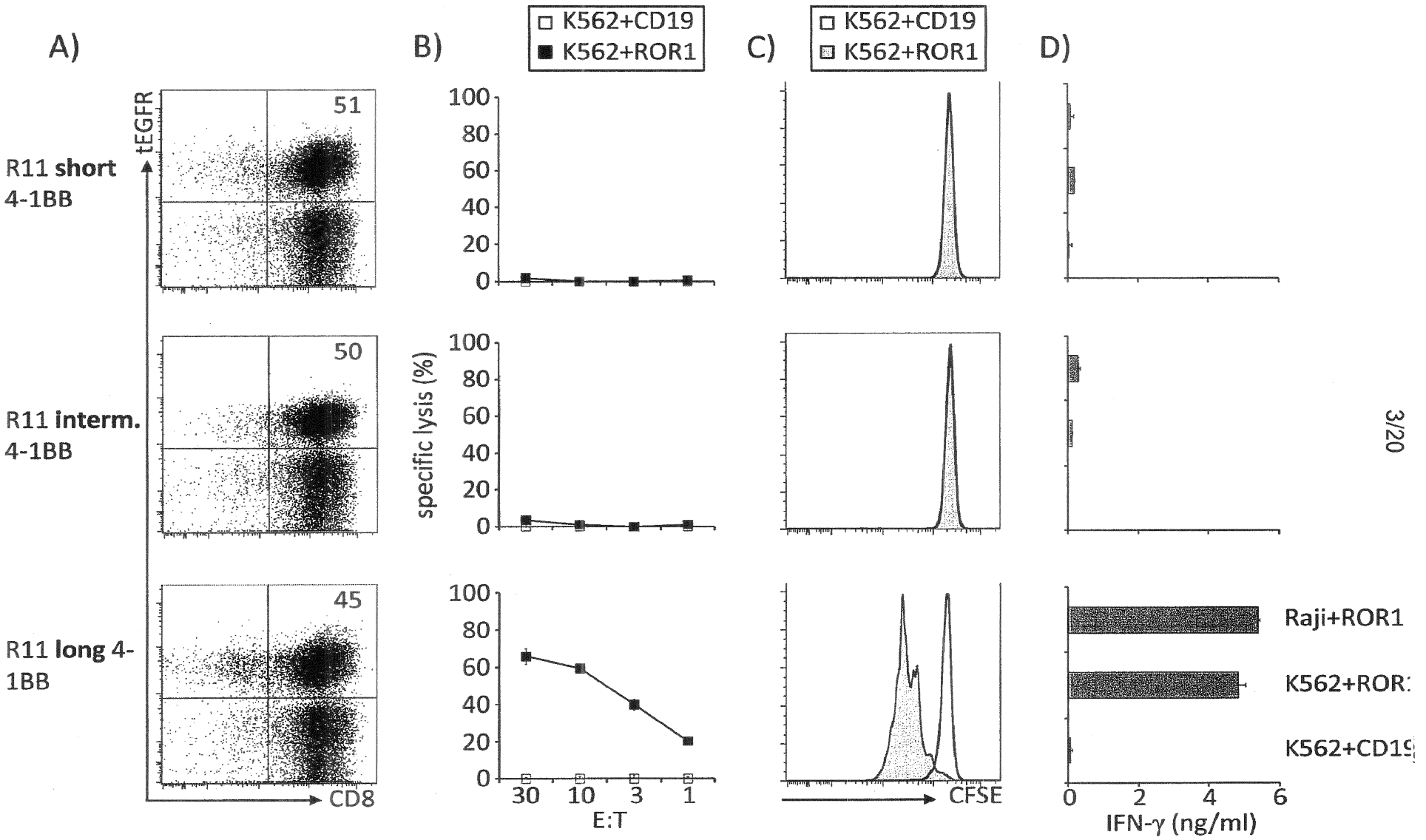

A)
B)
C)
D)
K562+CD19
K562+ROR1
tEGFR
CD8
51
50
45
R11 short 4-1BB
R11 interm. 4-1BB
R11 long 4-1BB
specific lysis (%)
100
80
60
40
20
0
30
10
3
1
E:T
CFSE
Raji+ROR1
K562+ROR1
K562+CD19
0
2
4
6
IFN-γ (ng/ml)

Figure 4

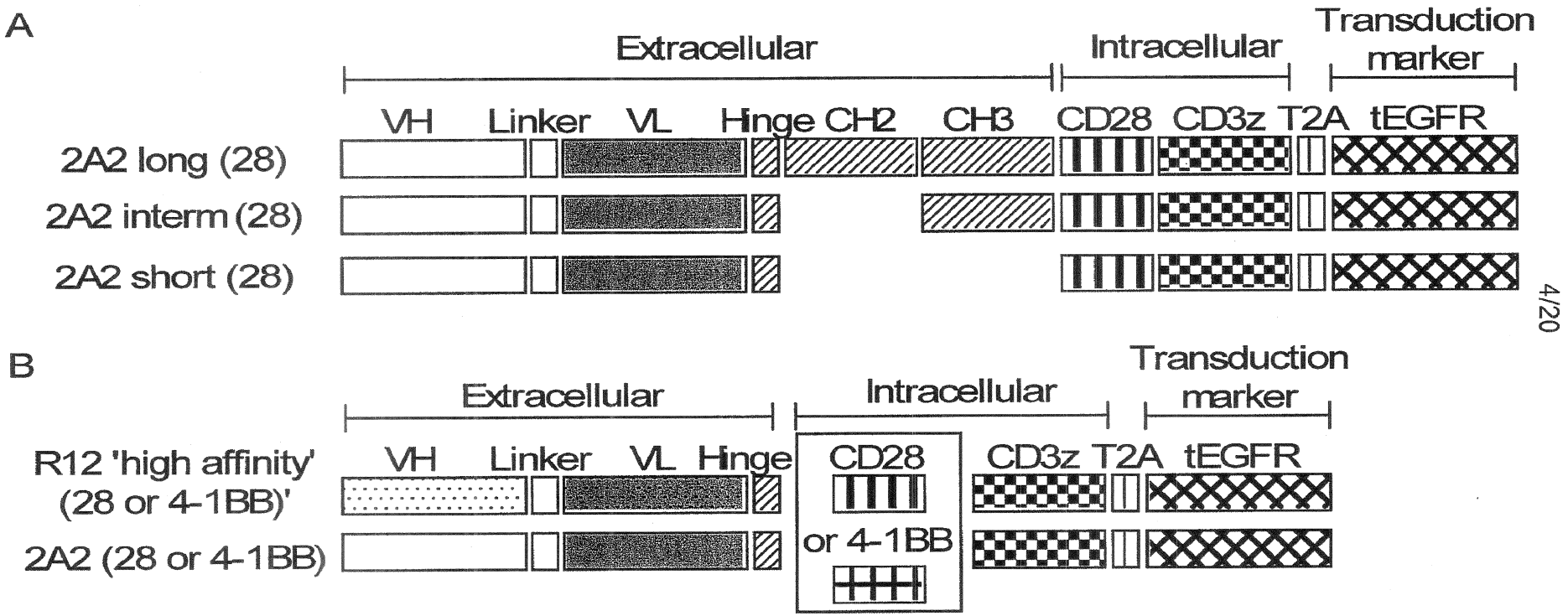

A
Extracellular
Intracellular
Transduction marker
VH
Linker
VL
Hinge
CH2
CH3
CD28
CD3z
T2A
tEGFR
2A2 long (28)
2A2 interm (28)
2A2 short (28)
B
Extracellular
Intracellular
Transduction marker
R12 'high affinity' (28 or 4-1BB)'
2A2 (28 or 4-1BB)
CD28
or 4-1BB

Figure 5

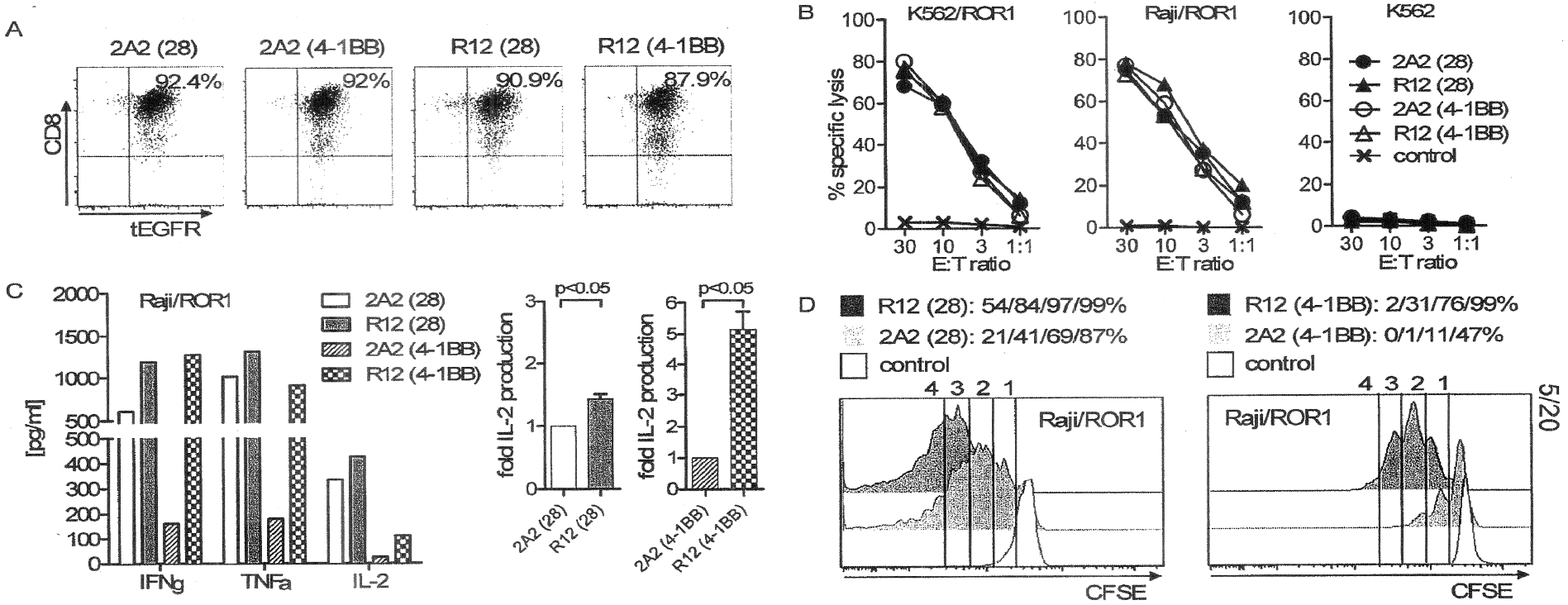
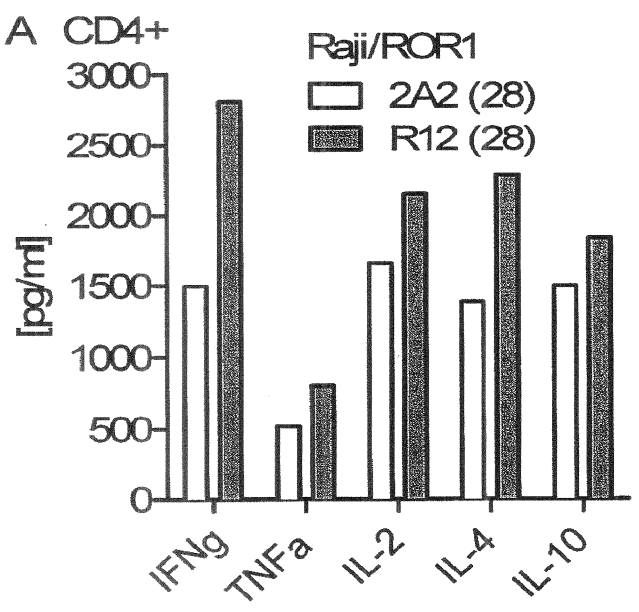

A
2A2 (28)
2A2 (4-1BB)
R12 (28)
R12 (4-1BB)
92.4%
92%
90.9%
87.9%
CD8
tEGFR
B
K562/ROR1
Raji/ROR1
K562
% specific lysis
E:T ratio
2A2 (28)
R12 (28)
2A2 (4-1BB)
R12 (4-1BB)
control
C
Raji/ROR1
[pg/ml]
IFNg
TNFa
IL-2
fold IL-2 production
p<0.05
D
R12 (28): 54/84/97/99%
2A2 (28): 21/41/69/87%
control
R12 (4-1BB): 2/31/76/99%
2A2 (4-1BB): 0/1/11/47%
CFSE
5/20

Figure 6


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

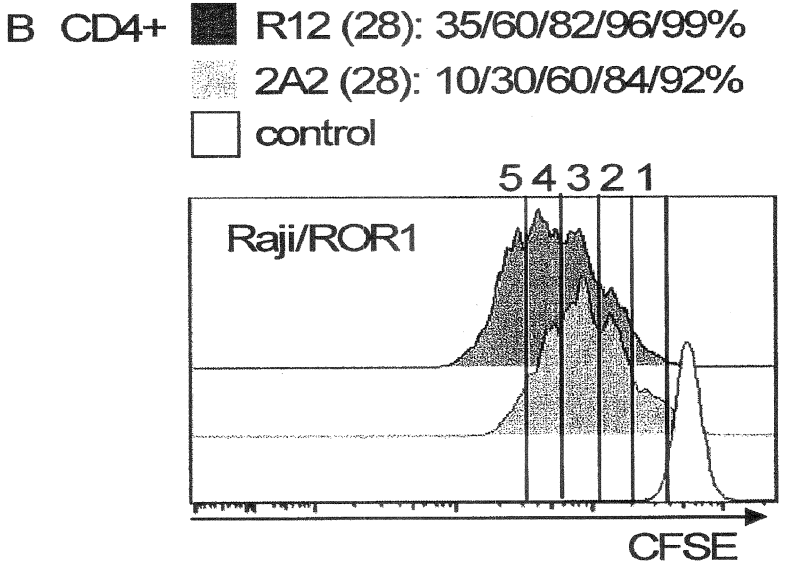

B CD4+
R12 (28): 35/60/82/96/99%
2A2 (28): 10/30/60/84/92%
control
5 4 3 2 1
Raji/ROR1
CFSE

Figure 7

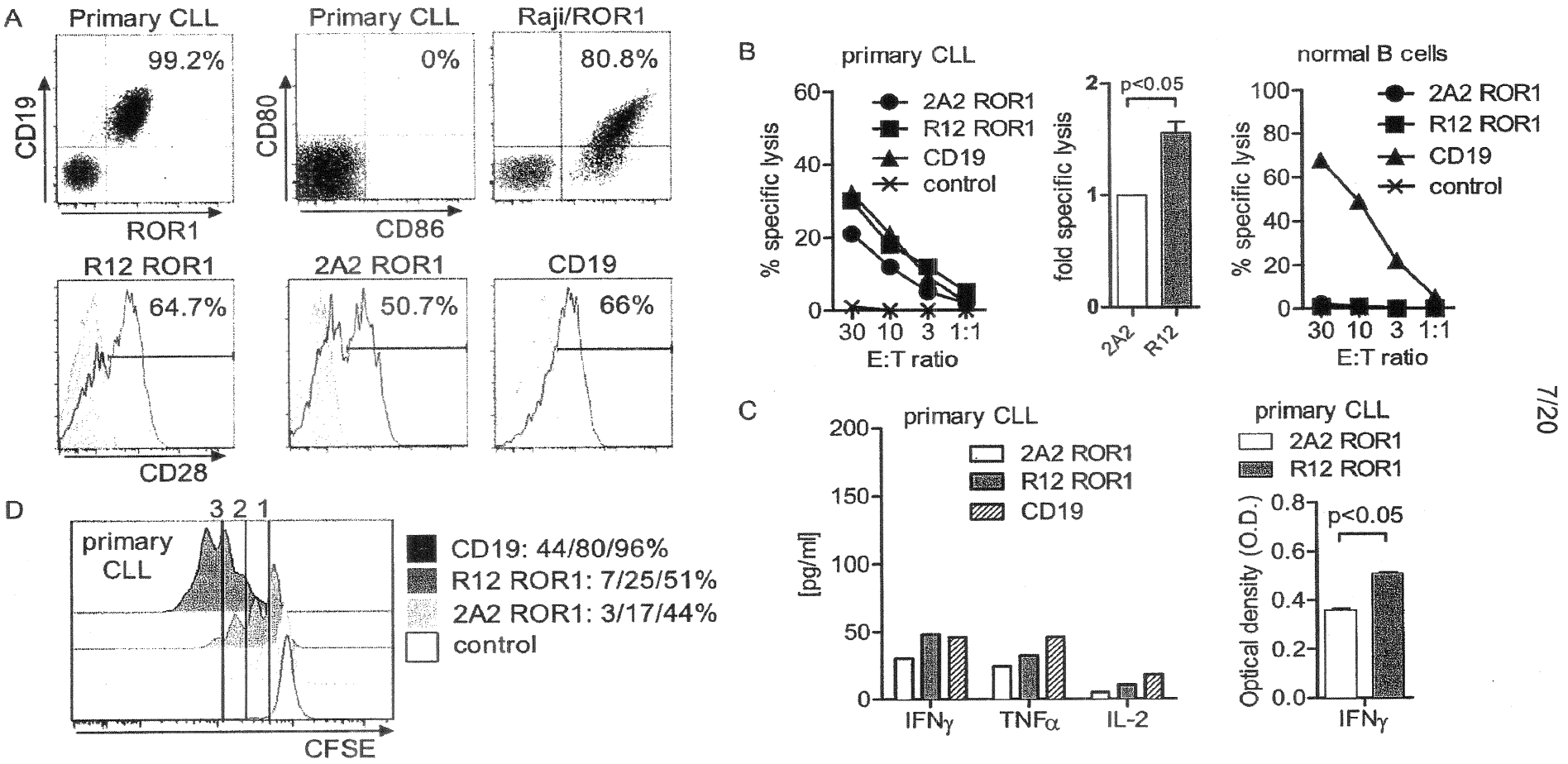

A
Primary CLL
99.2%
CD19
ROR1
Primary CLL
0%
CD80
CD86
Raji/ROR1
80.8%
R12 ROR1
64.7%
CD28
2A2 ROR1
50.7%
CD19
66%
B
primary CLL
2A2 ROR1
R12 ROR1
CD19
control
% specific lysis
E:T ratio
p<0.05
fold specific lysis
2A2
R12
normal B cells
C
primary CLL
[pg/ml]
IFNγ
TNFα
IL-2
Optical density (O.D.)
D
primary CLL
3 2 1
CFSE
CD19: 44/80/96%
R12 ROR1: 7/25/51%
2A2 ROR1: 3/17/44%
control

Figure 8

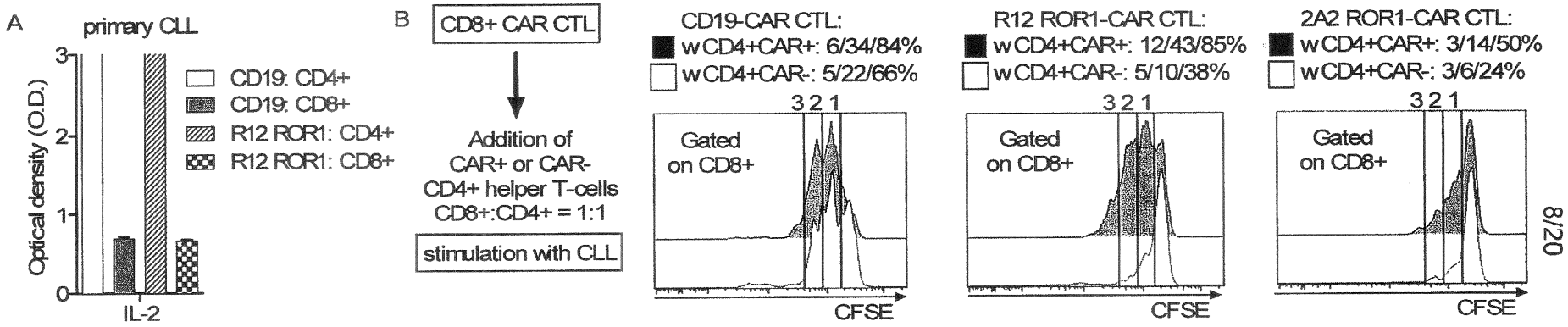

A
primary CLL
Optical density (O.D.)
3
2
1
0
IL-2
CD19: CD4+
CD19: CD8+
R12 ROR1: CD4+
R12 ROR1: CD8+
B
CD8+ CAR CTL
Addition of
CAR+ or CAR-
CD4+ helper T-cells
CD8+:CD4+ = 1:1
stimulation with CLL
CD19-CAR CTL:
wCD4+CAR+: 6/34/84%
wCD4+CAR-: 5/22/66%
3 2 1
Gated
on CD8+
CFSE
R12 ROR1-CAR CTL:
wCD4+CAR+: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

Figure 9

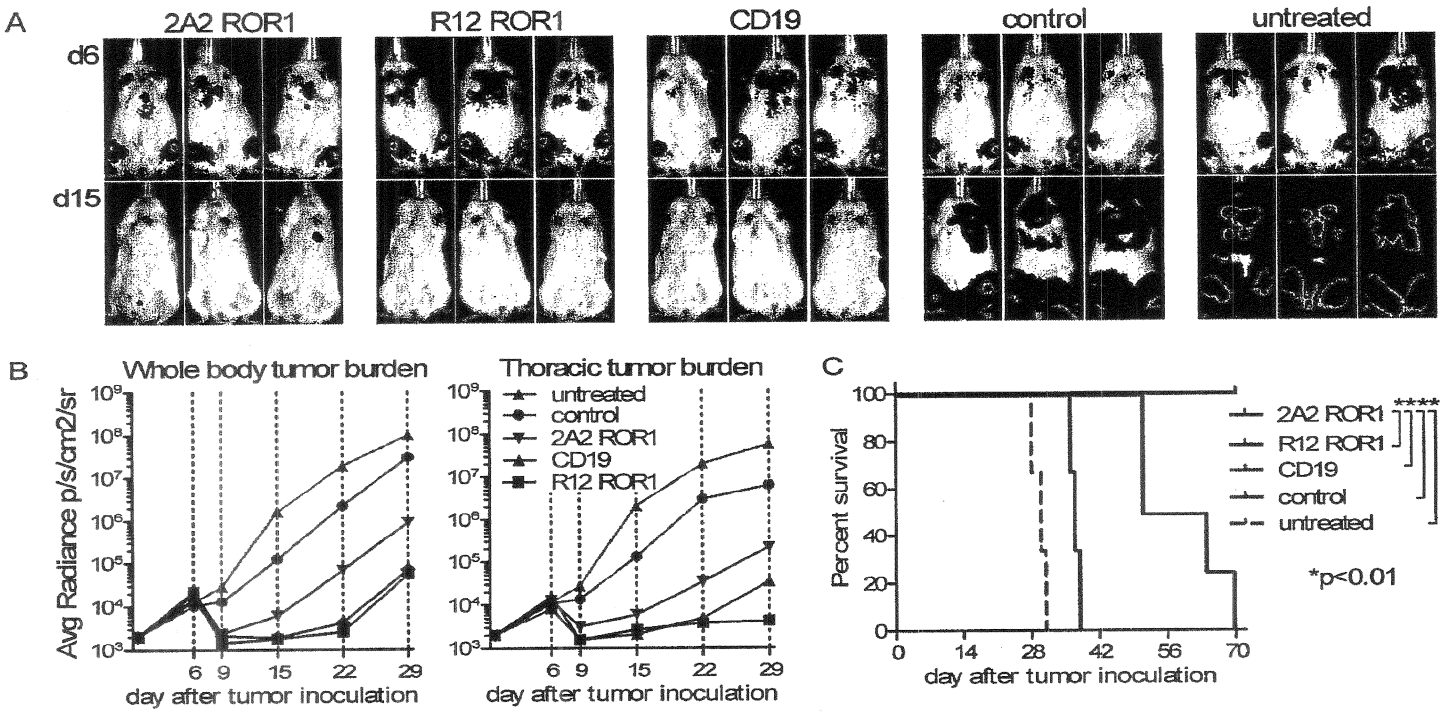

A
2A2 ROR1
R12 ROR1
CD19
control
untreated
d6
d15
B
Whole body tumor burden
Thoracic tumor burden
Avg Radiance p/s/cm2/sr
untreated
control
2A2 ROR1
CD19
R12 ROR1
day after tumor inoculation
C
Percent survival
2A2 ROR1
R12 ROR1
CD19
control
untreated
****
*p<0.01
day after tumor inoculation
D
Analysis 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%
4 3 2 1
Frequency:
CD19: 0.29%
R12: 0.15%
2A2: 0.05%
CD19: 0.43%
R12: 0.30%
2A2: 0.18%
CD19: 1.15%
R12: 0.92%
2A2: 0.42%
CFSE

Figure 10

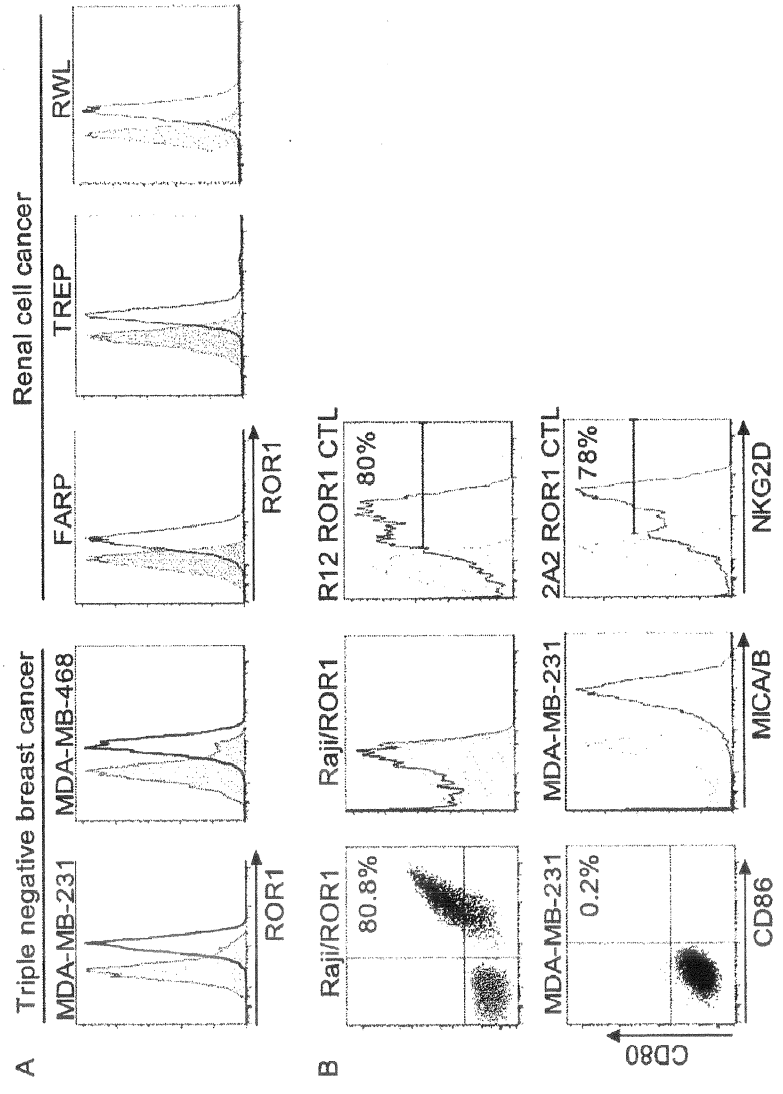

A
Triple negative breast cancer
MDA-MB-231
MDA-MB-468
ROR1
Renal cell cancer
FARP
TREP
RWL
ROR1
B
Raji/ROR1
80.8%
CD86
CD80
Raji/ROR1
R12 ROR1 CTL
80%
NKG2D
MDA-MB-231
0.2%
MDA-MB-231
MICA/B
2A2 ROR1 CTL
78%
NKG2D

Figure 11

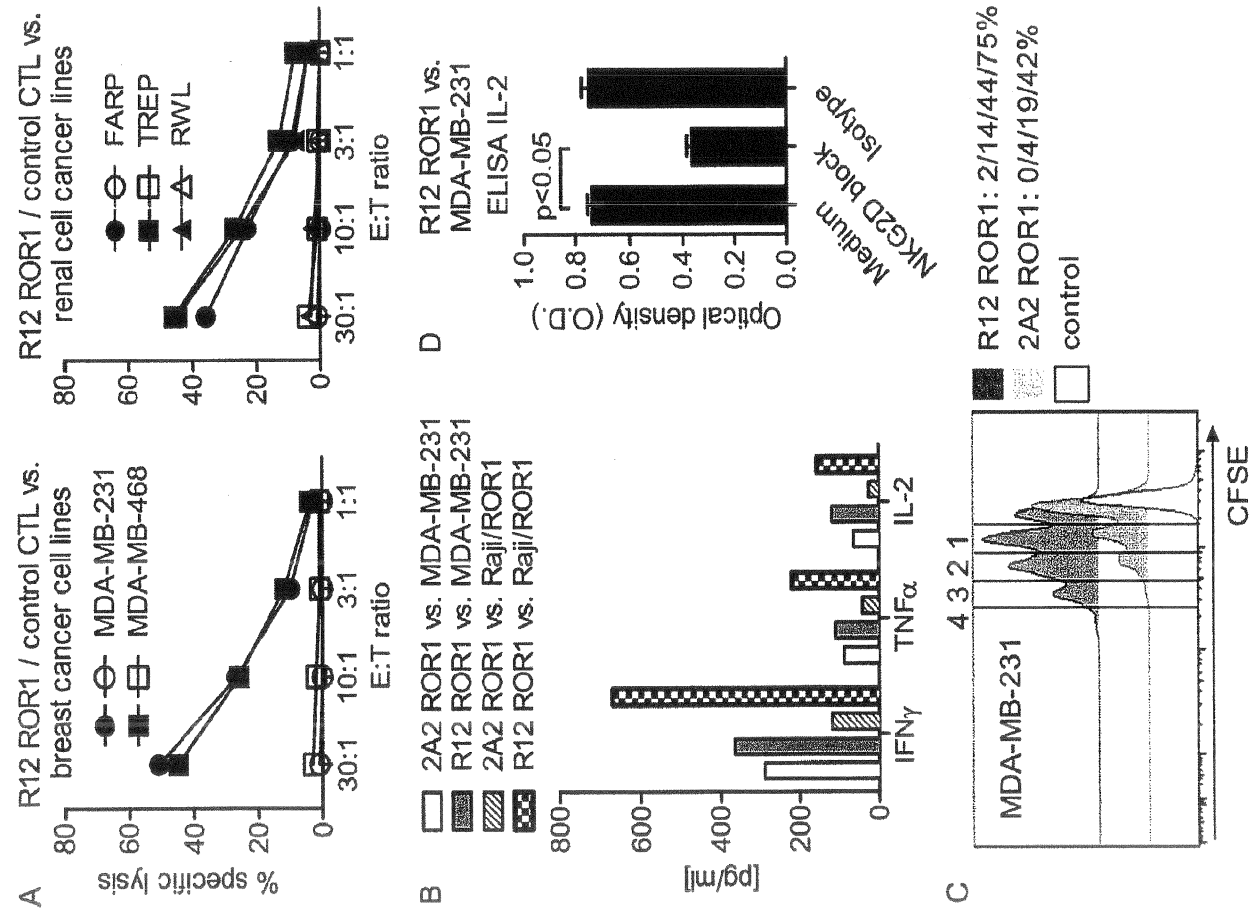

A
R12 ROR1 / control CTL vs. breast cancer cell lines
% specific lysis
MDA-MB-231
MDA-MB-468
E:T ratio
30:1
10:1
3:1
1:1
R12 ROR1 / control CTL vs. renal cell cancer lines
FARP
TREP
RWL
B
2A2 ROR1 vs. MDA-MB-231
R12 ROR1 vs. MDA-MB-231
2A2 ROR1 vs. Raji/ROR1
R12 ROR1 vs. Raji/ROR1
[pg/ml]
IFNγ
TNFα
IL-2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

Figure 12

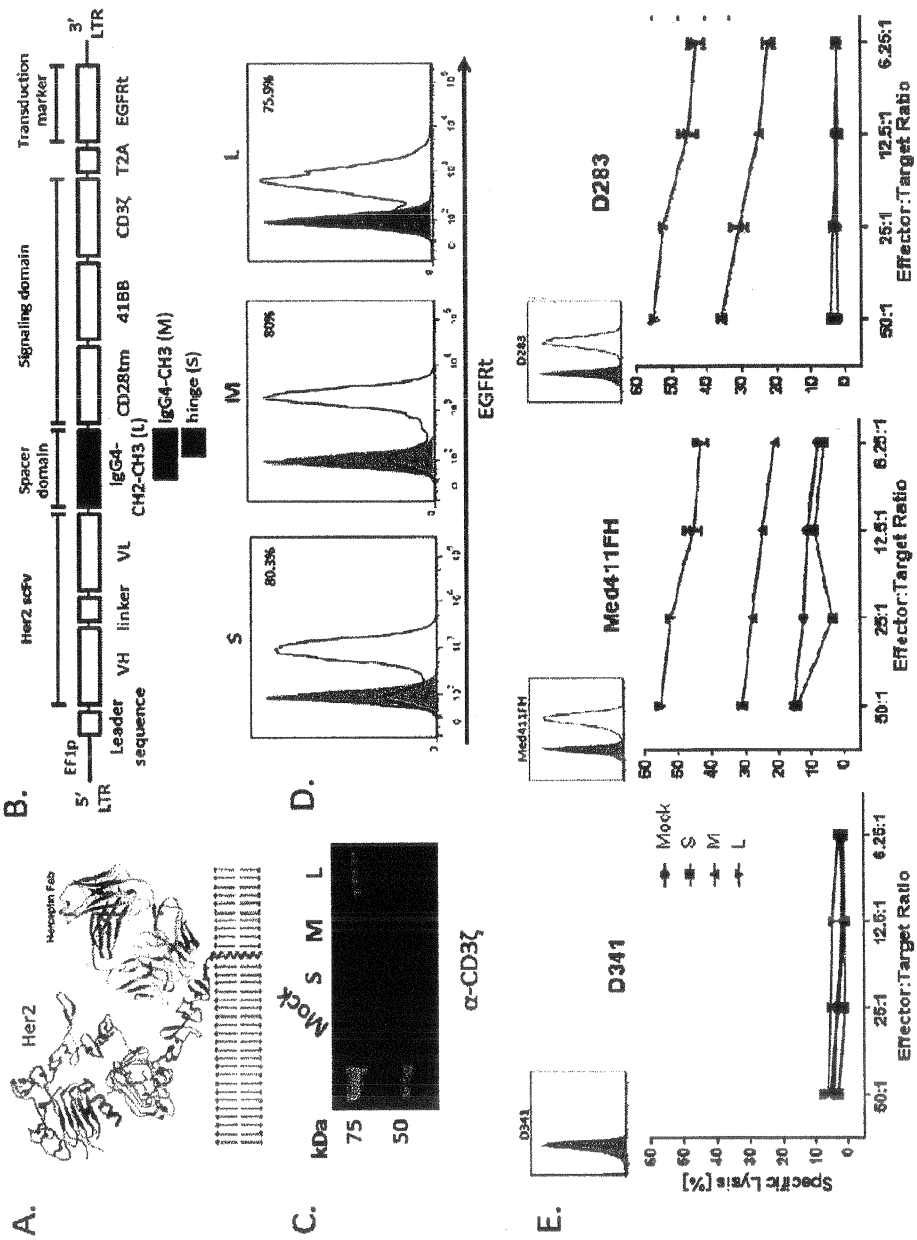

A.
Her2
Herceptin Fab
B.
Her2 scFv
Spacer domain
Signaling domain
Transduction marker
5' LTR
EF1p
Leader sequence
VH
linker
VL
IgG4-CH2-CH3 (L)
IgG4-CH2-CH3 (M)
hinge (S)
CD28tm
41BB
CD3ζ
T2A
EGFRt
3' LTR
C.
kDa
75
50
Mock
S
M
L
α-CD3ζ
D.
S
80.3%
M
80%
L
75.9%
EGFRt
E.
D341
Med411FH
D283
Mock
S
M
L
Specific Lysis [%]
Effector:Target Ratio
50:1
25:1
12.5:1
6.25:1

Figure 13

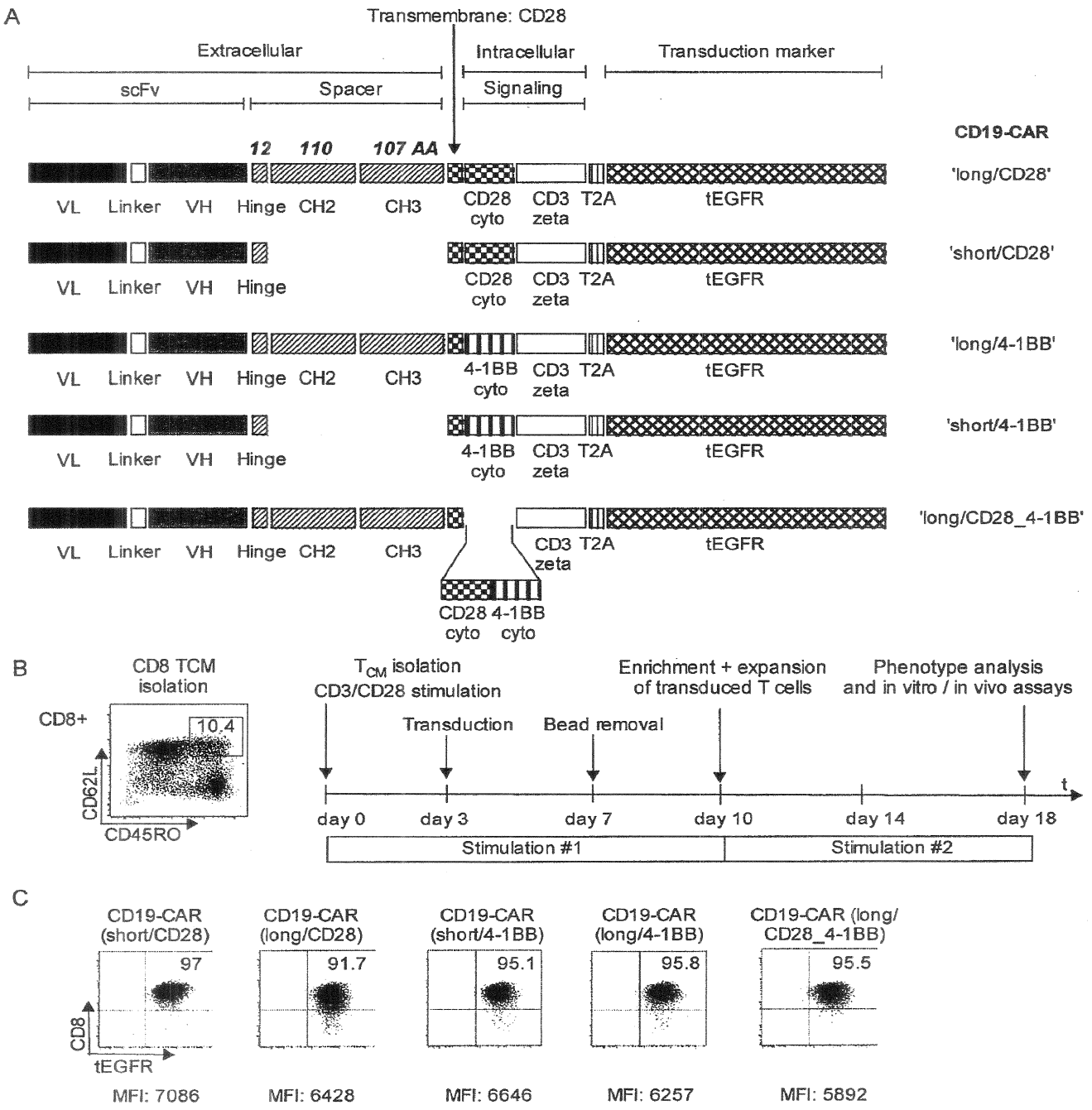

A
Transmembrane: CD28
Extracellular
Intracellular
Transduction marker
scFv
Spacer
Signaling
CD19-CAR
12
110
107 AA
VL
Linker
VH
Hinge
CH2
CH3
CD28 cyto
CD3 zeta
T2A
tEGFR
'long/CD28'
'short/CD28'
4-1BB cyto
'long/4-1BB'
'short/4-1BB'
'long/CD28_4-1BB'
B
CD8 TCM isolation
CD8+
10.4
CD62L
CD45RO
T_{CM} isolation
CD3/CD28 stimulation
Transduction
Bead removal
Enrichment + expansion of transduced T cells
Phenotype analysis and in vitro / in vivo assays
t
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

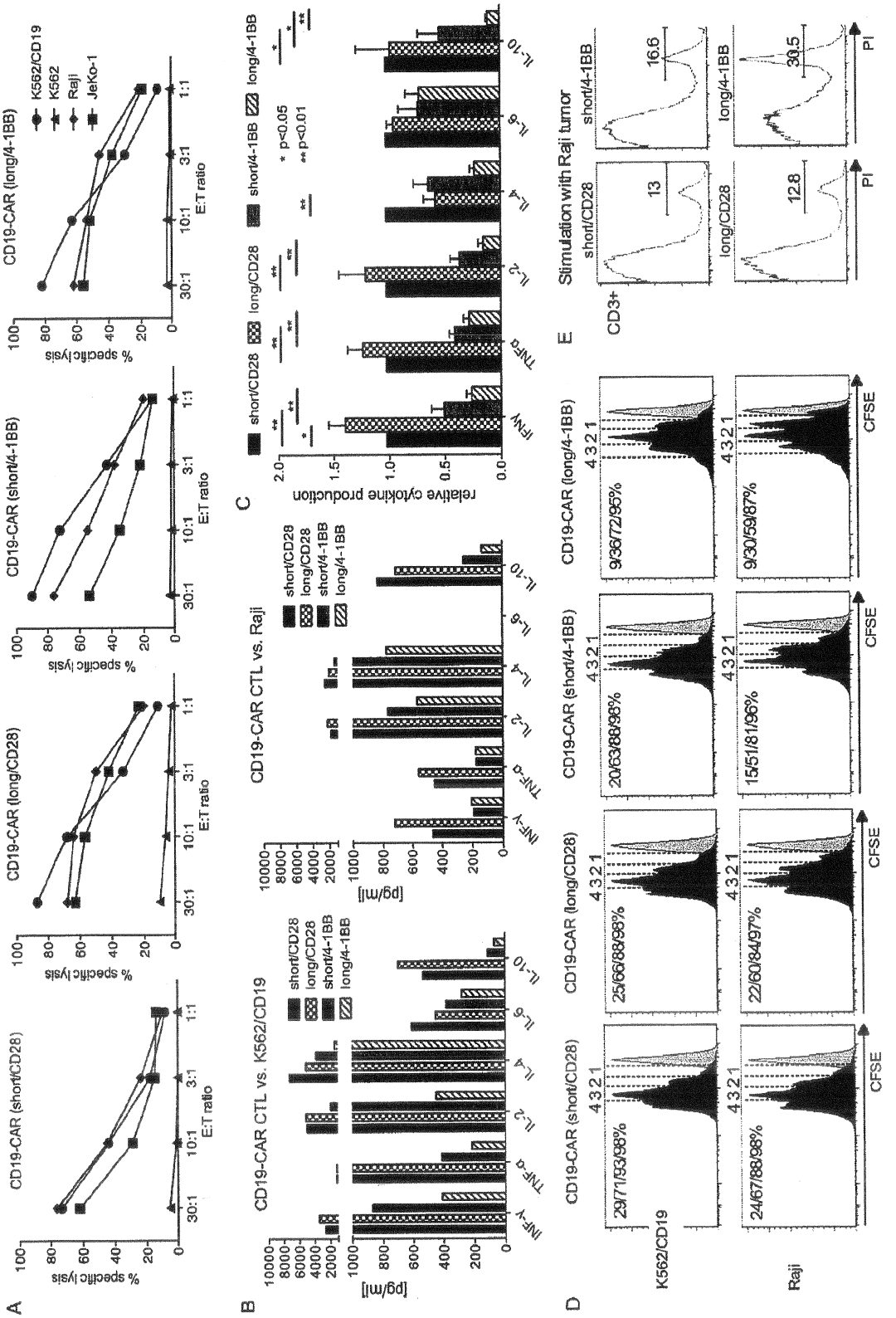

A
CD19-CAR (short/CD28)
CD19-CAR (long/CD28)
CD19-CAR (short/4-1BB)
CD19-CAR (long/4-1BB)
% specific lysis
E:T ratio
30:1
10:1
3:1
1:1
K562/CD19
K562
Raji
JeKo-1
B
CD19-CAR CTL vs. K562/CD19
CD19-CAR CTL vs. Raji
[pg/ml]
short/CD28
long/CD28
short/4-1BB
long/4-1BB
INF-γ
TNF-α
IL-2
IL-4
IL-5
IL-10
C
relative cytokine production
short/CD28
long/CD28
short/4-1BB
long/4-1BB
* 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)
K562/CD19
Raji
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%
CFSE
E
Stimulation with Raji tumor
short/CD28
long/CD28
short/4-1BB
long/4-1BB
CD3+
13
16.6
12.8
30.5
PI

Figure 15

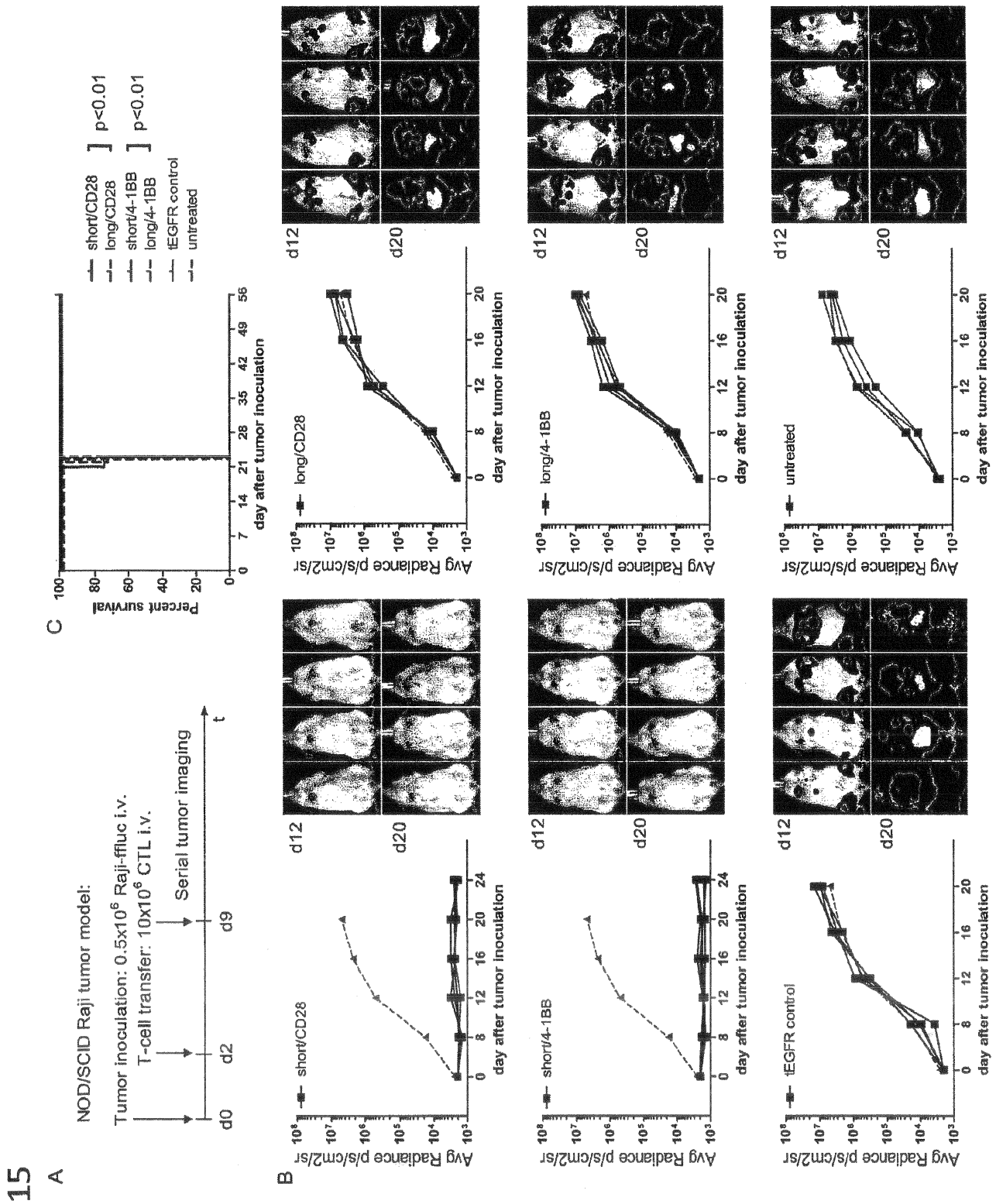

A
NOD/SCID Raji tumor model:
Tumor inoculation: 0.5x10^6 Raji-ffluc i.v.
T-cell transfer: 10x10^6 CTL i.v.
Serial tumor imaging
d0
d2
d9
t
C
Percent survival
day after tumor inoculation
short/CD28
long/CD28
short/4-1BB
long/4-1BB
tEGFR control
untreated
p<0.01
p<0.01
B
d12
d20
short/CD28
long/CD28
short/4-1BB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

Figure 16

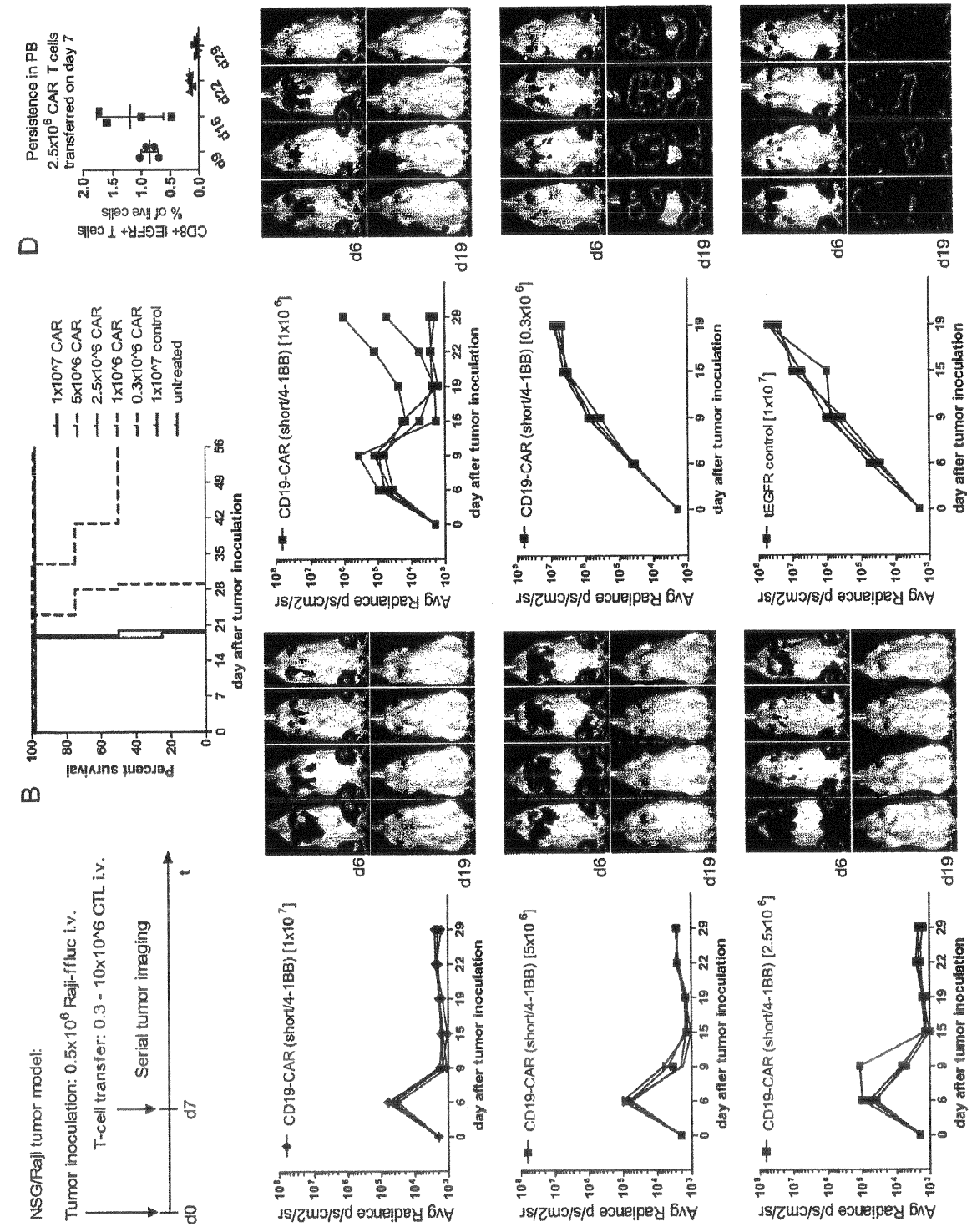

A
NSG/Raji tumor model:
Tumor inoculation: 0.5x10^6 Raji-ffluc i.v.
T-cell transfer: 0.3 - 10x10^6 CTL i.v.
Serial tumor imaging
d0
d7
t
B
Percent survival
day after tumor inoculation
1x10^7 CAR
5x10^6 CAR
2.5x10^6 CAR
1x10^6 CAR
0.3x10^6 CAR
1x10^7 control
untreated
D
Persistence in PB
2.5x10^6 CAR T cells transferred on day 7
CD8+ tEGFR+ T cells
% of live cells
6/3
2/3
1/3
6/6
C
CD19-CAR (short/4-1BB) [1x10^7]
CD19-CAR (short/4-1BB) [1x10^6]
CD19-CAR (short/4-1BB) [0.3x10^6]
tEGFR control [1x10^7]
CD19-CAR (short/4-1BB) [5x10^6]
CD19-CAR (short/4-1BB) [2.5x10^6]
Avg Radiance p/s/cm2/sr
day after tumor inoculation
d6
d19

Figure 17

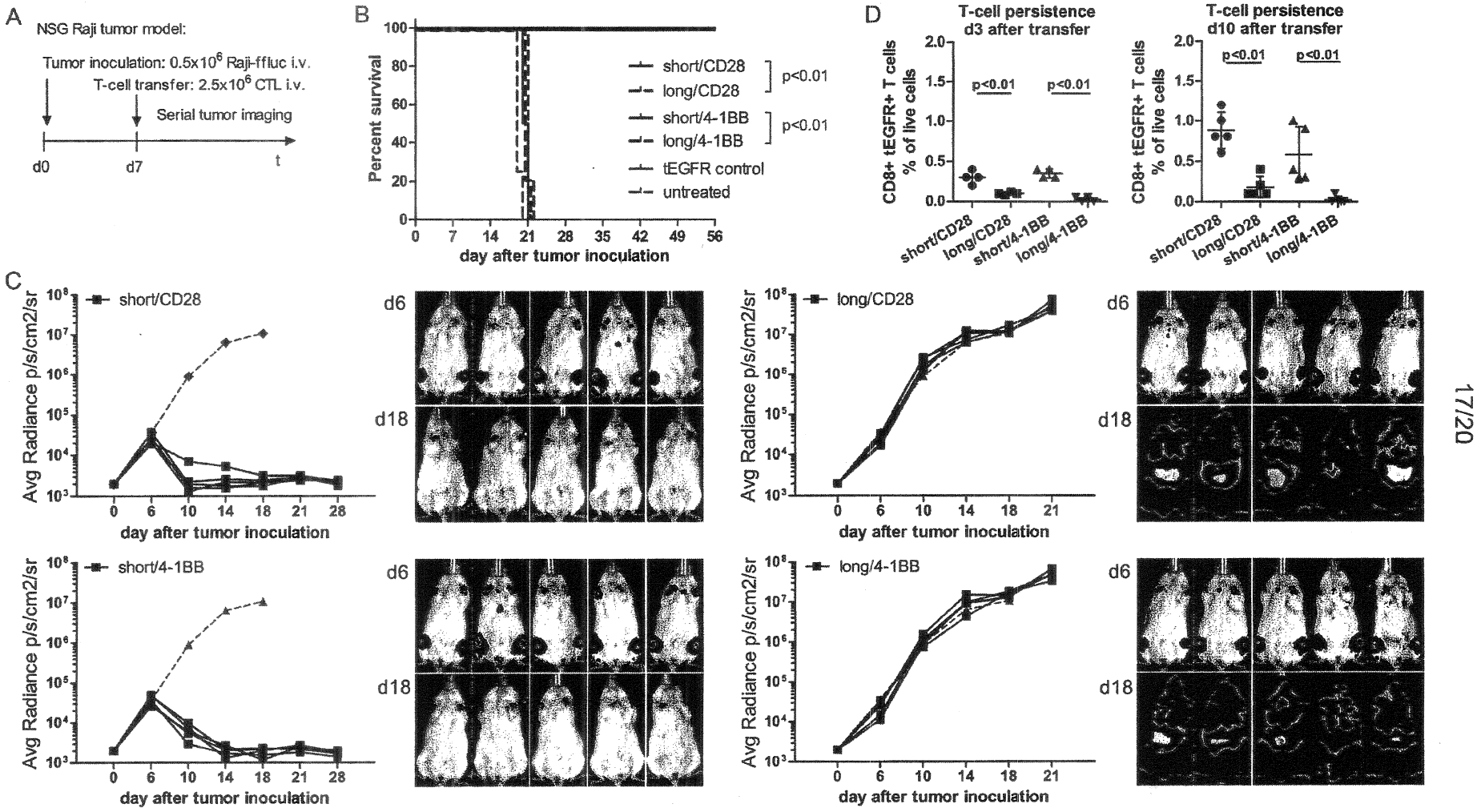

A
NSG Raji tumor model:
Tumor inoculation: 0.5x10^6 Raji-ffluc i.v.
T-cell transfer: 2.5x10^6 CTL i.v.
Serial tumor imaging
d0
d7
t
B
Percent survival
day after tumor inoculation
short/CD28
long/CD28
short/4-1BB
long/4-1BB
tEGFR control
untreated
p<0.01
p<0.01
D
T-cell persistence d3 after transfer
T-cell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

Figure 18

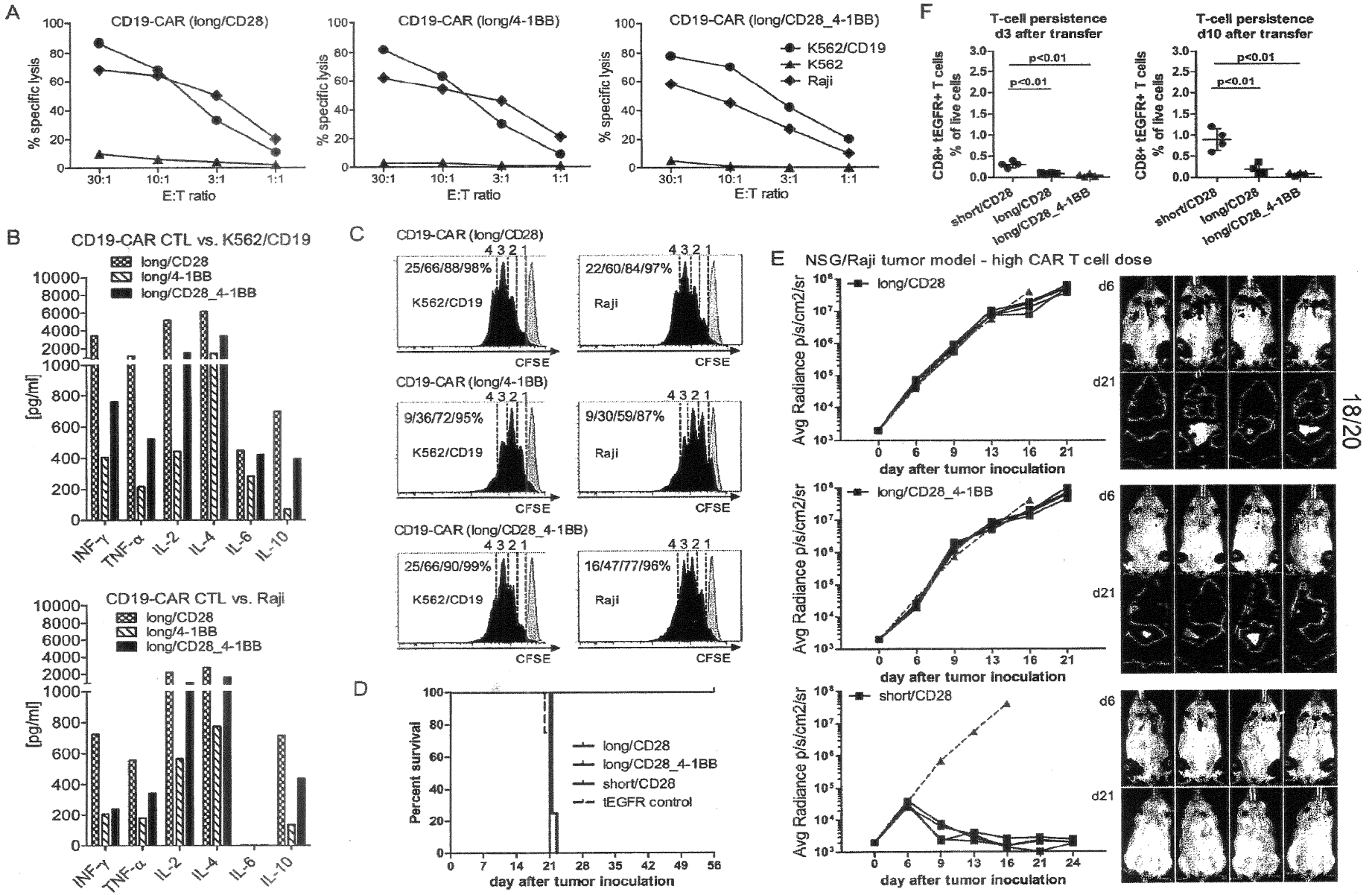
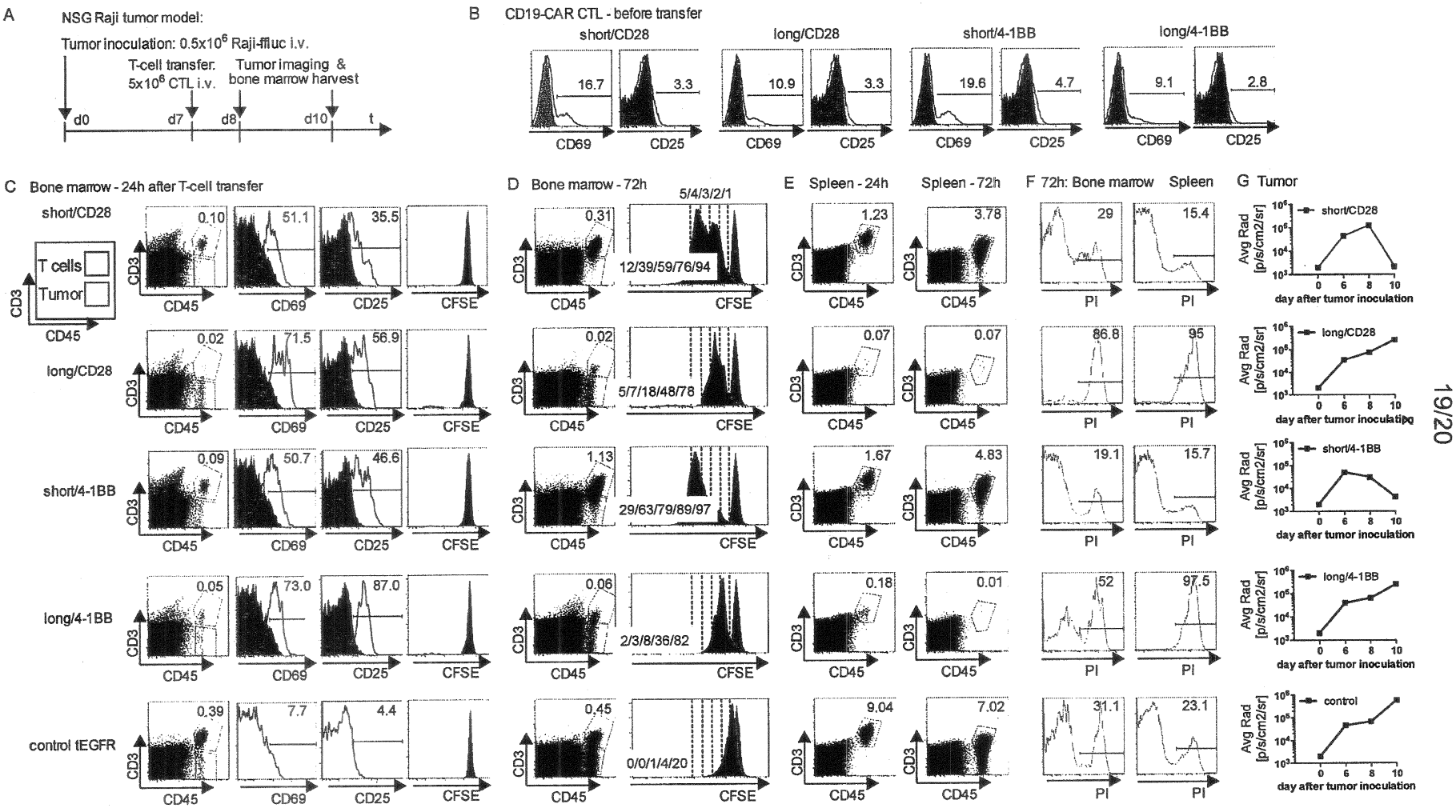
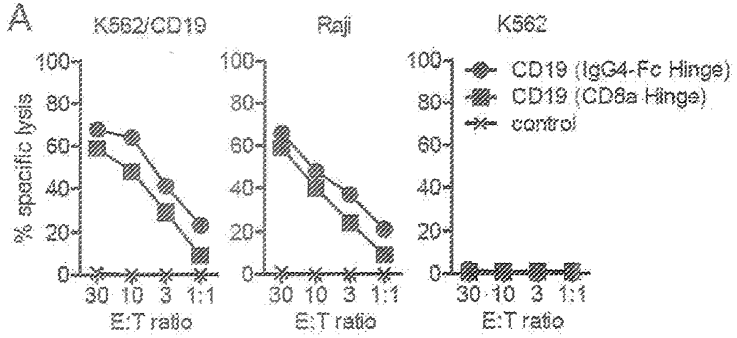

A
CD19-CAR (long/CD28)
CD19-CAR (long/4-1BB)
CD19-CAR (long/CD28_4-1BB)
% specific lysis
E:T ratio
30:1
10:1
3:1
1:1
K562/CD19
K562
Raji
B
CD19-CAR CTL vs. K562/CD19
long/CD28
long/4-1BB
long/CD28_4-1BB
[pg/ml]
INF-γ
TNF-α
IL-2
IL-4
IL-6
IL-10
CD19-CAR CTL vs. Raji
C
CD19-CAR (long/CD28)
25/66/88/98%
K562/CD19
22/60/84/97%
Raji
CFSE
CD19-CAR (long/4-1BB)
9/36/72/95%
9/30/59/87%
CD19-CAR (long/CD28_4-1BB)
25/66/90/99%
16/47/77/96%
D
Percent survival
long/CD28
long/CD28_4-1BB
short/CD28
tEGFR control
day after tumor inoculation
E
NSG/Raji tumor model - high CAR T cell dose
Avg Radiance p/s/cm2/sr
long/CD28
long/CD28_4-1BB
short/CD28
d6
d21
F
T-cell persistence
d3 after transfer
d10 after transfer
CD8+ tEGFR+ T cells
% of live cells
p<0.01
short/CD28
long/CD28
long/CD28_4-1BB

Figure 19

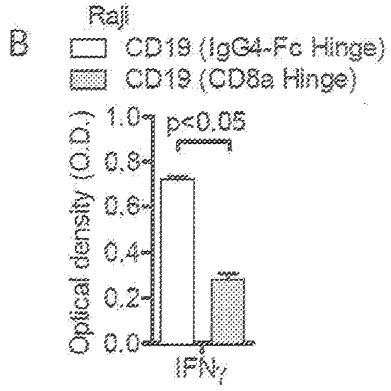

A
NSG Raji tumor model:
Tumor inoculation: 0.5x10^6 Raji-ffluc i.v.
T-cell transfer: 5x10^6 CTL i.v.
Tumor imaging & bone marrow harvest
d0
d7
d8
d10
t
B
CD19-CAR CTL - before transfer
short/CD28
long/CD28
short/4-1BB
long/4-1BB
16.7
3.3
10.9
3.3
19.6
4.7
9.1
2.8
CD69
CD25
C
Bone marrow - 24h after T-cell transfer
T cells
Tumor
CD3
CD45
CD69
CD25
CFSE
short/CD28
0.10
51.1
35.5
long/CD28
0.02
71.5
56.9
short/4-1BB
0.09
50.7
46.6
long/4-1BB
0.05
73.0
87.0
control tEGFR
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
Spleen - 72h
1.23
3.78
0.07
0.07
1.67
4.83
0.18
0.01
9.04
7.02
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



Proliferation (CFSE)

