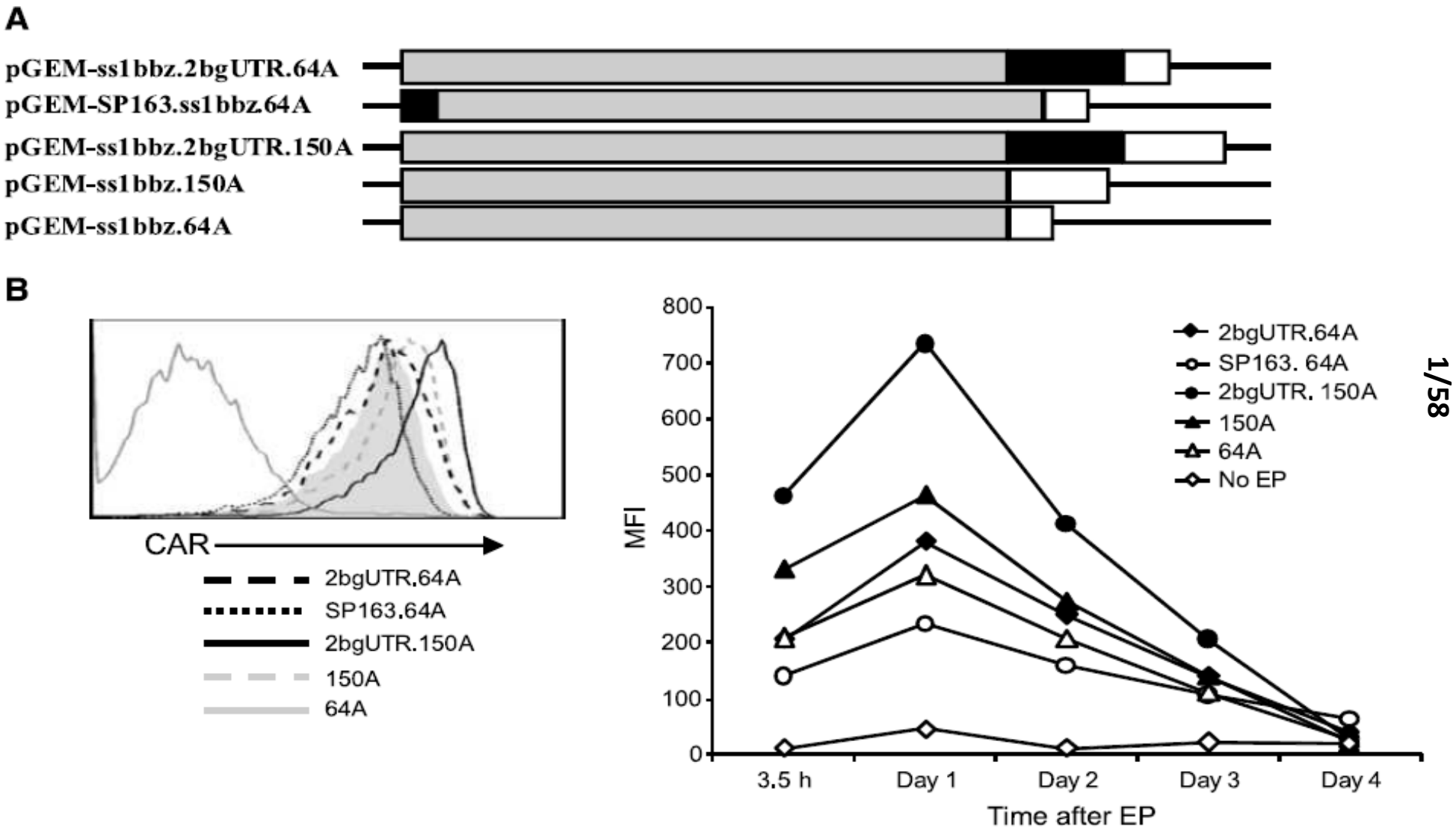

A
pGEM-ss1bbz.2bgUTR.64A
pGEM-SP163.ss1bbz.64A
pGEM-ss1bbz.2bgUTR.150A
pGEM-ss1bbz.150A
pGEM-ss1bbz.64A
B
CAR
2bgUTR.64A
SP163.64A
2bgUTR.150A
150A
64A
MFI
800
700
600
500
400
300
200
100
0
3.5 h
Day 1
Day 2
Day 3
Day 4
Time after EP
2bgUTR.64A
SP163. 64A
2bgUTR. 150A
150A
64A
No EP

Figure 1

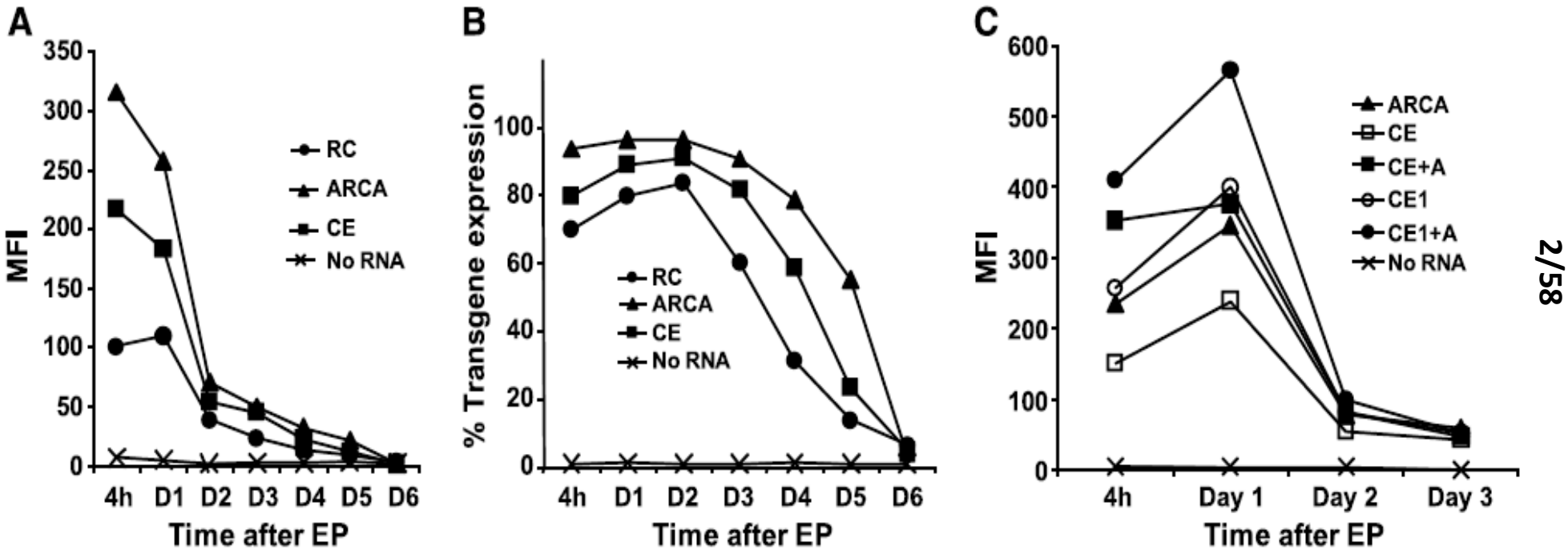
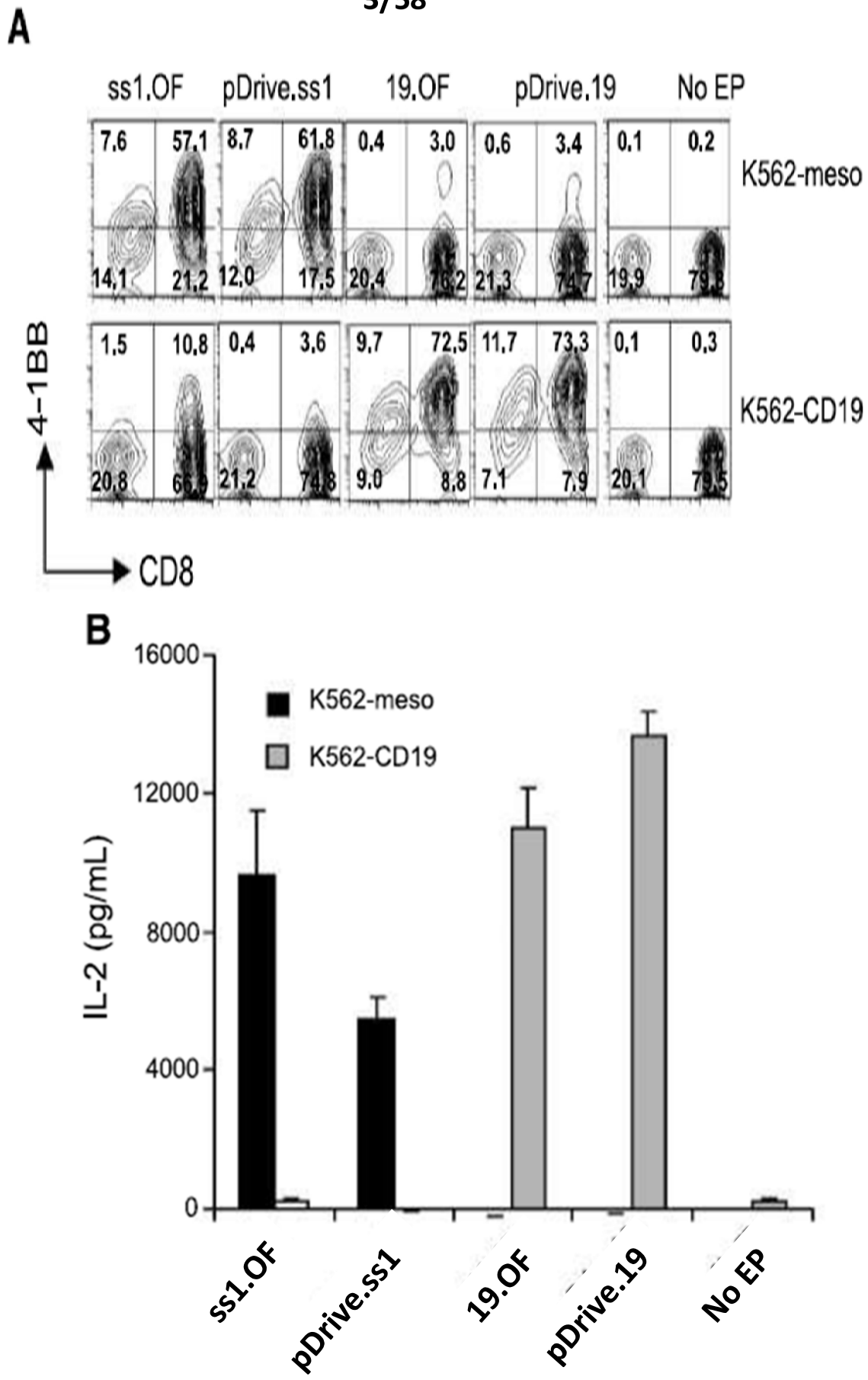
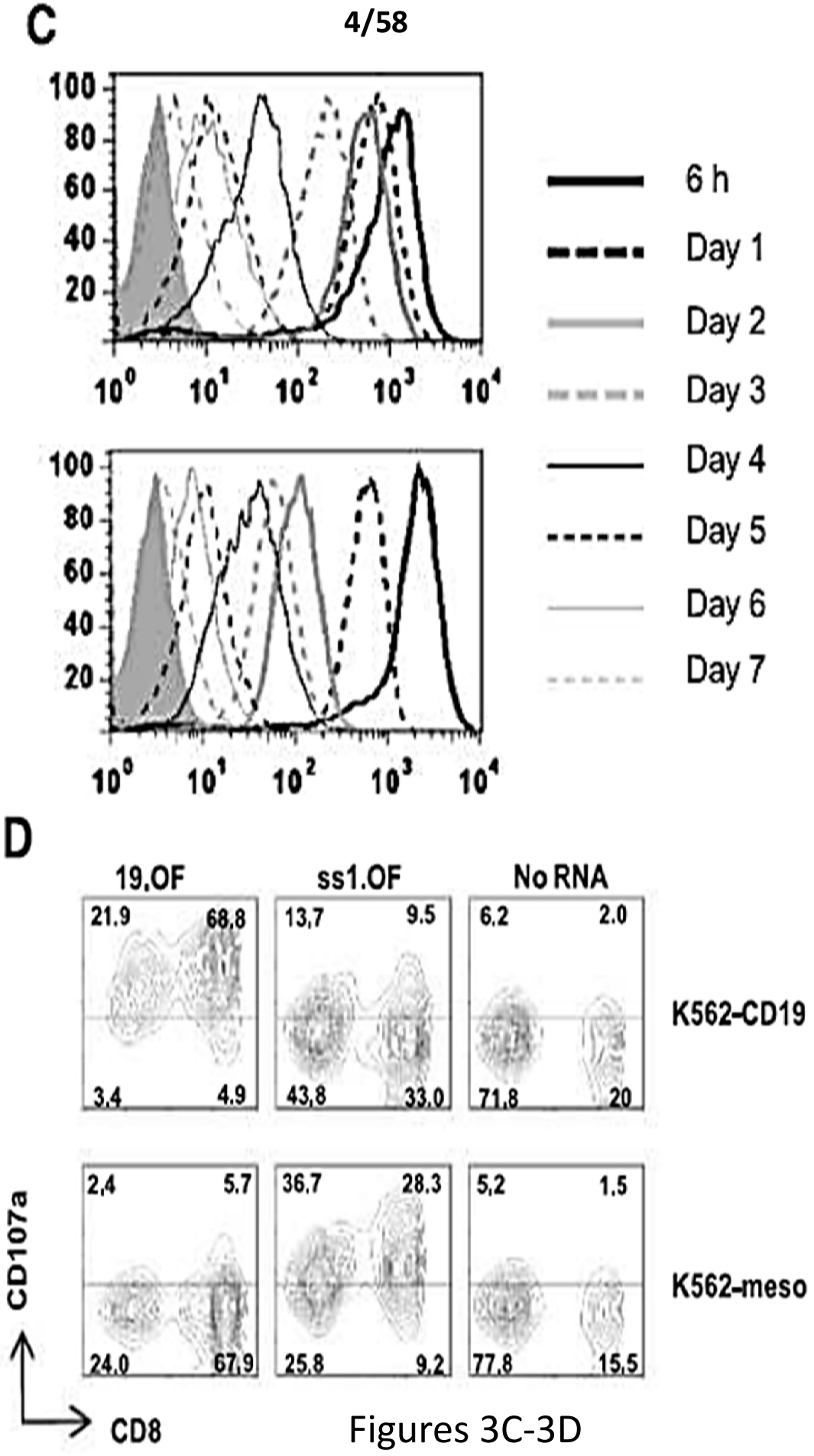

A
MFI
350
300
250
200
150
100
50
0
RC
ARCA
CE
No RNA
4h D1 D2 D3 D4 D5 D6
Time after EP
B
% Transgene expression
100
80
60
40
20
0
RC
ARCA
CE
No RNA
4h D1 D2 D3 D4 D5 D6
Time after EP
C
MFI
600
500
400
300
200
100
0
ARCA
CE
CE+A
CE1
CE1+A
No RNA
4h Day 1 Day 2 Day 3
Time after EP

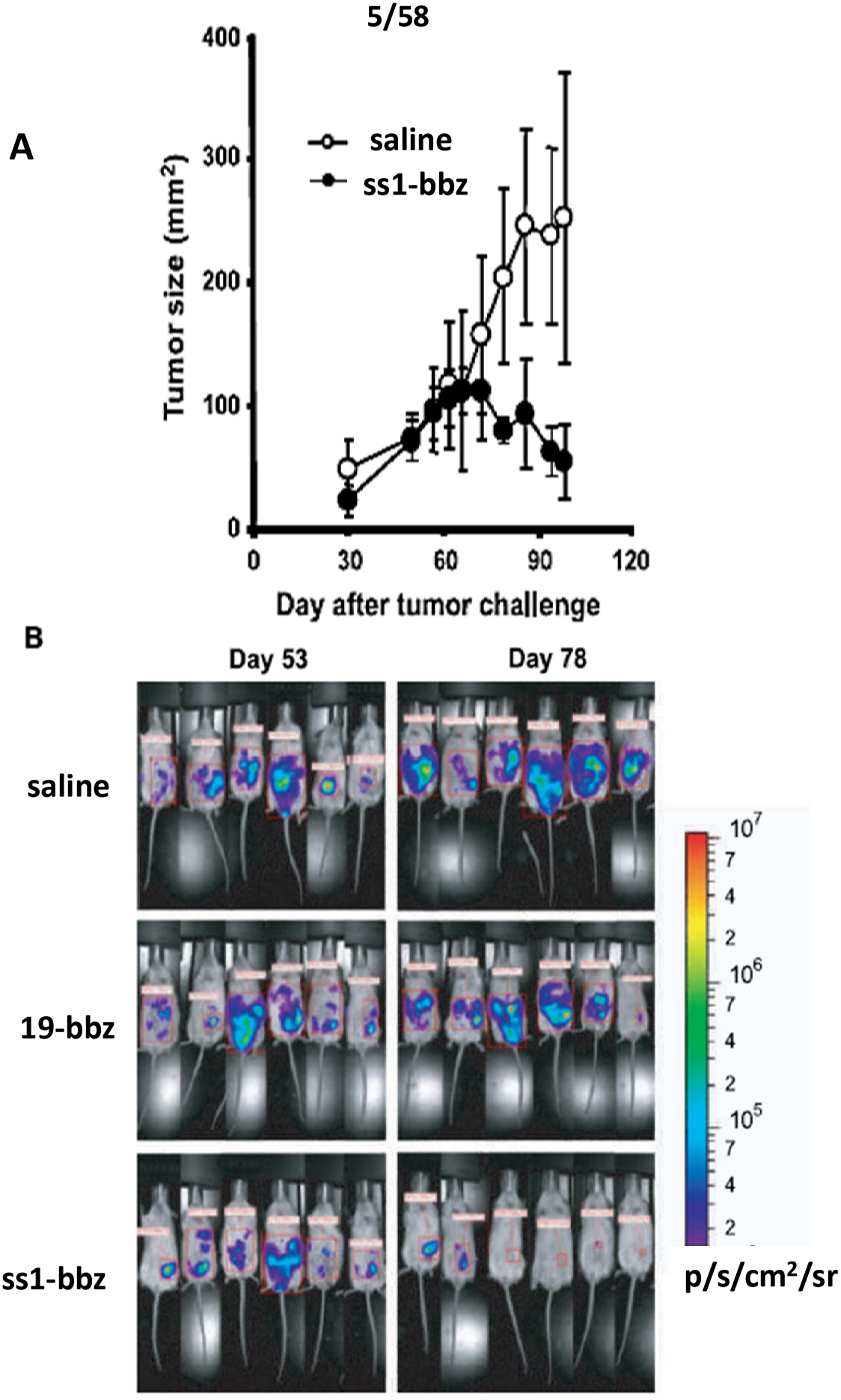
Figure 2



Figures 3A-3B



Figures 3C-3D



Figures 4A-4B

C

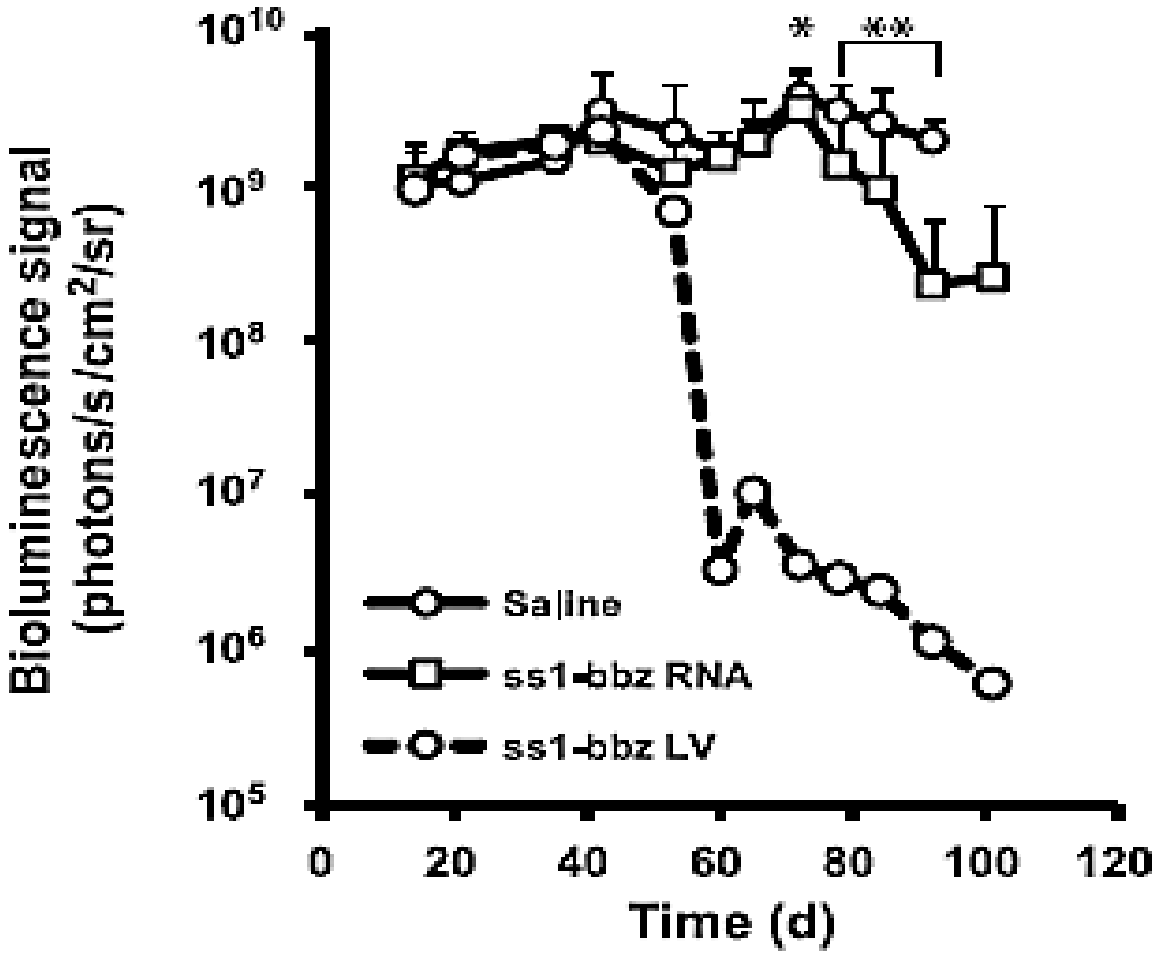

Bioluminescence signal (photons/s/cm²/sr)
10¹⁰
10⁹
10⁸
10⁷
10⁶
10⁵
*
**
Saline
ss1-bbz RNA
ss1-bbz LV
0
20
40
60
80
100
120
Time (d)

Figure 4C

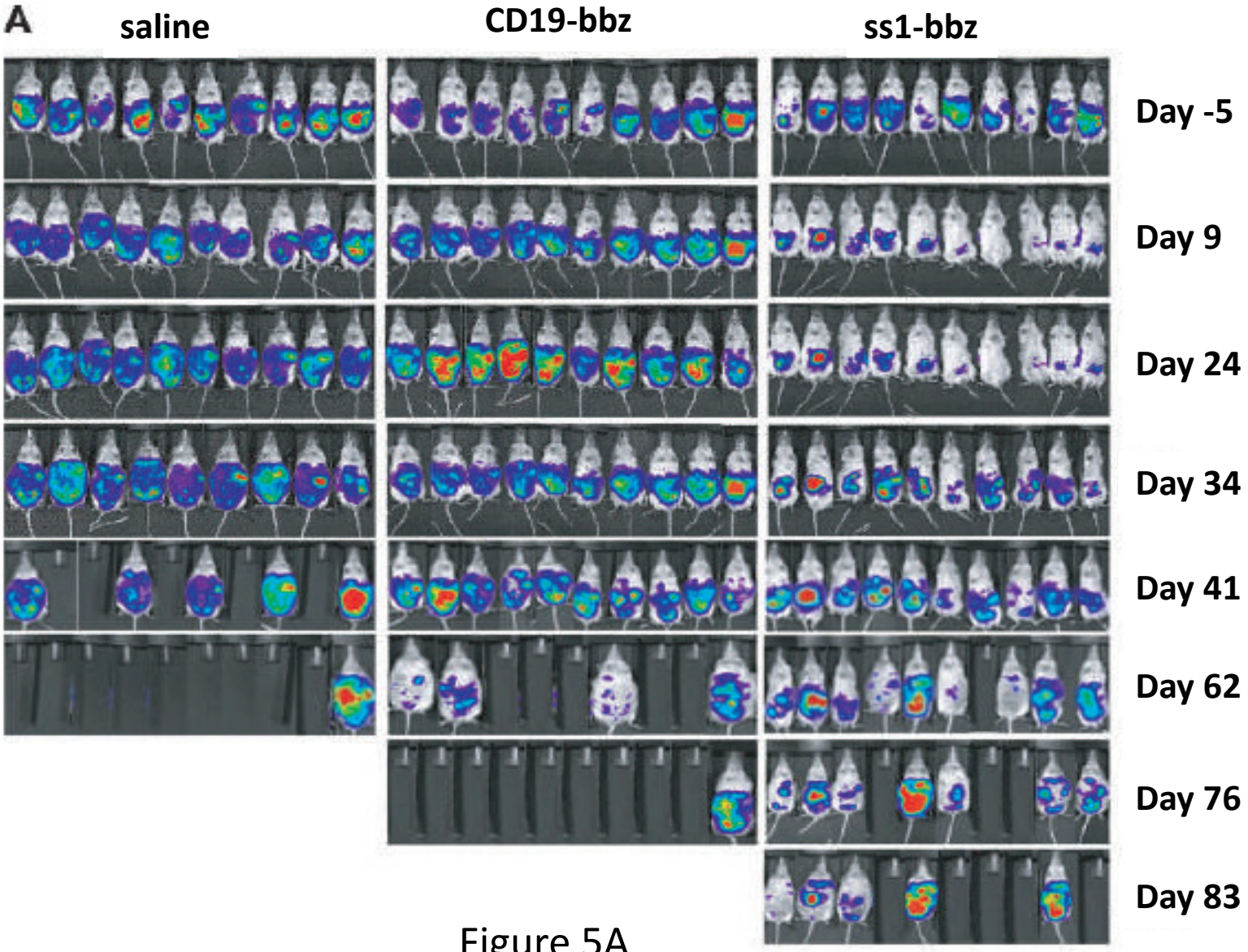
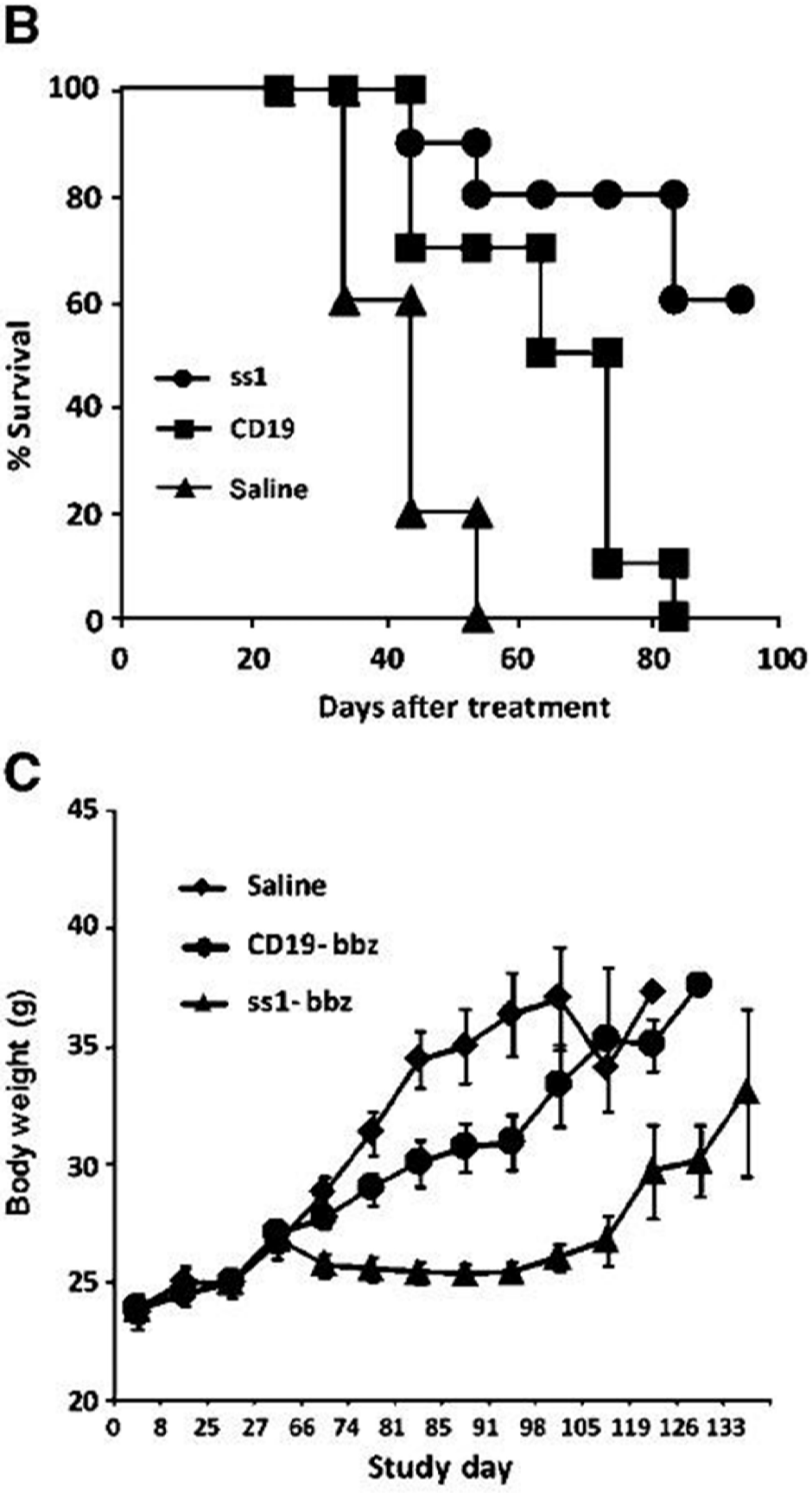

A
saline
CD19-bbz
ss1-bbz
Day -5
Day 9
Day 24
Day 34
Day 41
Day 62
Day 76
Day 83

Figure 5A



Figures 5B-5C

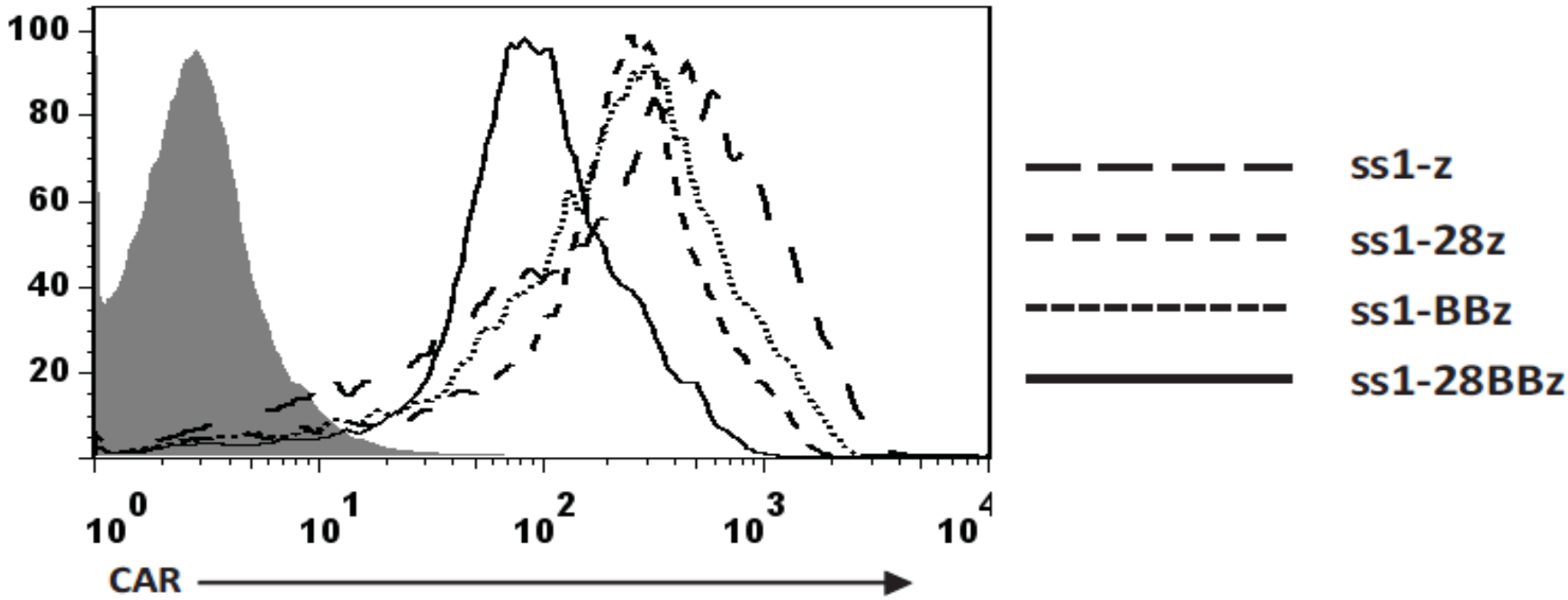

100
80
60
40
20
10^0
10^1
10^2
10^3
10^4
CAR
ss1-z
ss1-28z
ss1-BBz
ss1-28BBz

Figure 6

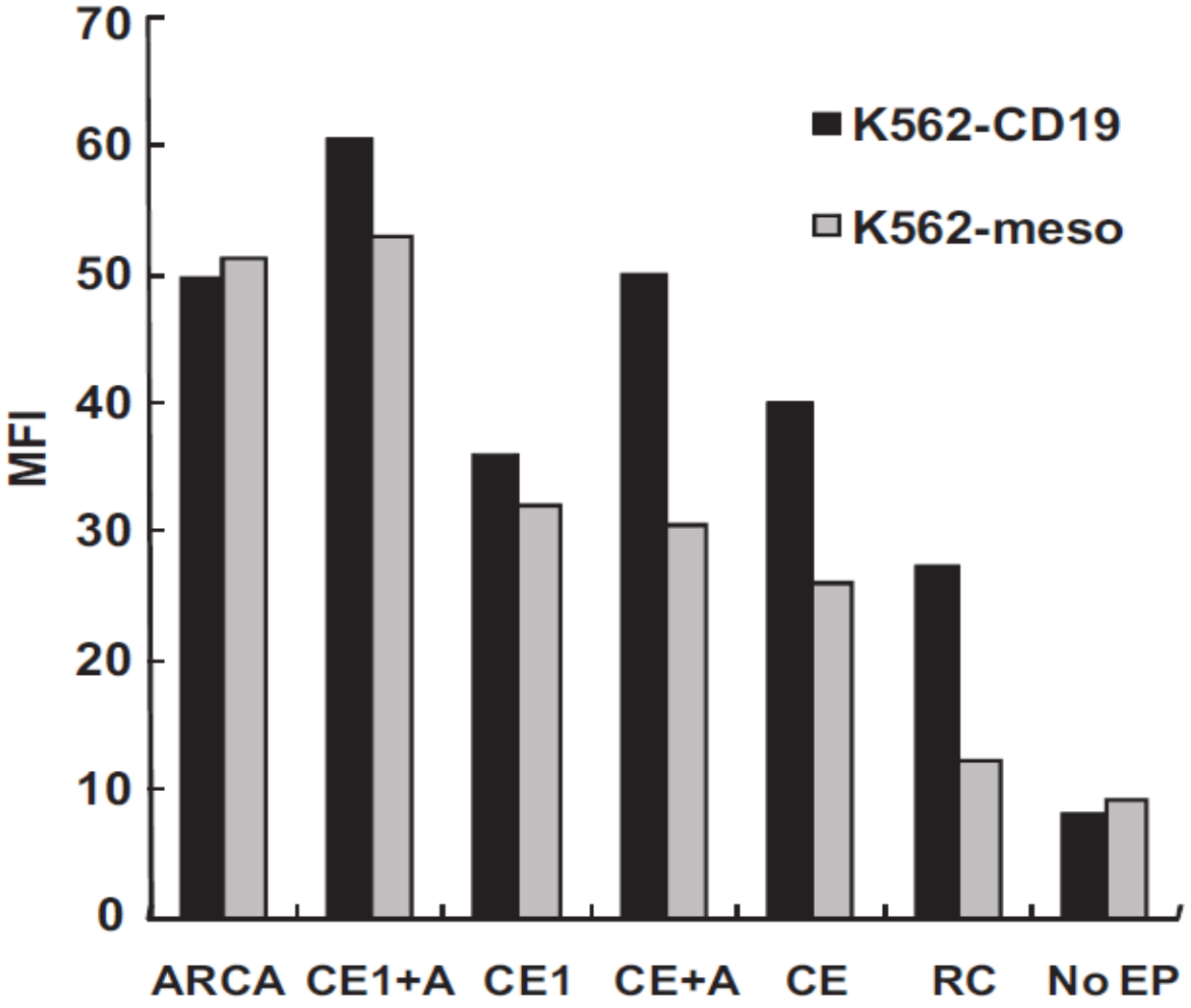

70
60
50
40
30
20
10
0
MFI
K562-CD19
K562-meso
ARCA
CE1+A
CE1
CE+A
CE
RC
No EP

Figure 7

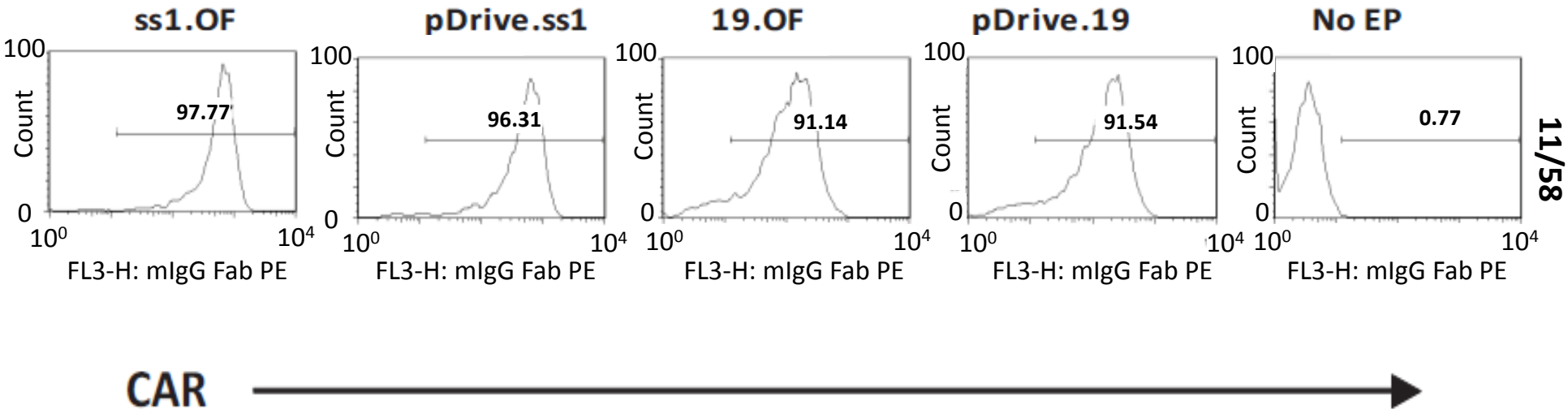

ss1.OF
pDrive.ss1
19.OF
pDrive.19
No EP
100
0
Count
97.77
96.31
91.14
91.54
0.77
10^0
10^4
FL3-H: mIgG Fab PE
11/58
CAR

Figure 8

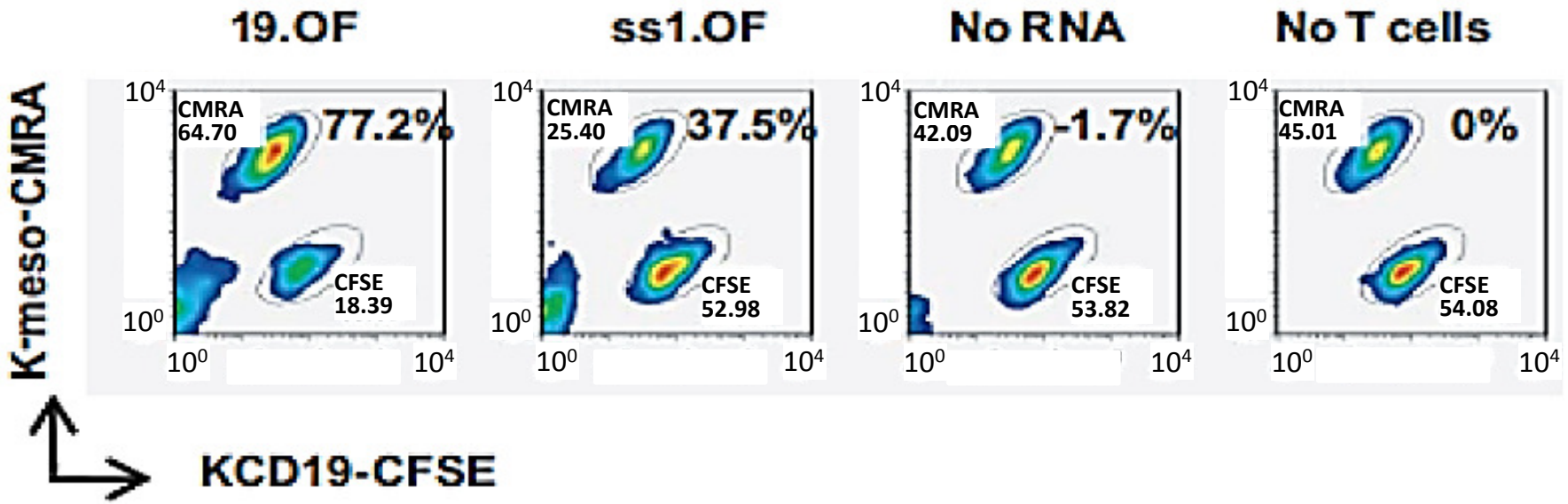

19.OF
ss1.OF
No RNA
No T cells
K-meso-CMRA
KCD19-CFSE
CMRA
64.70
77.2%
CFSE
18.39
CMRA
25.40
37.5%
CFSE
52.98
CMRA
42.09
-1.7%
CFSE
53.82
CMRA
45.01
0%
CFSE
54.08
10^4
10^0

Figure 9

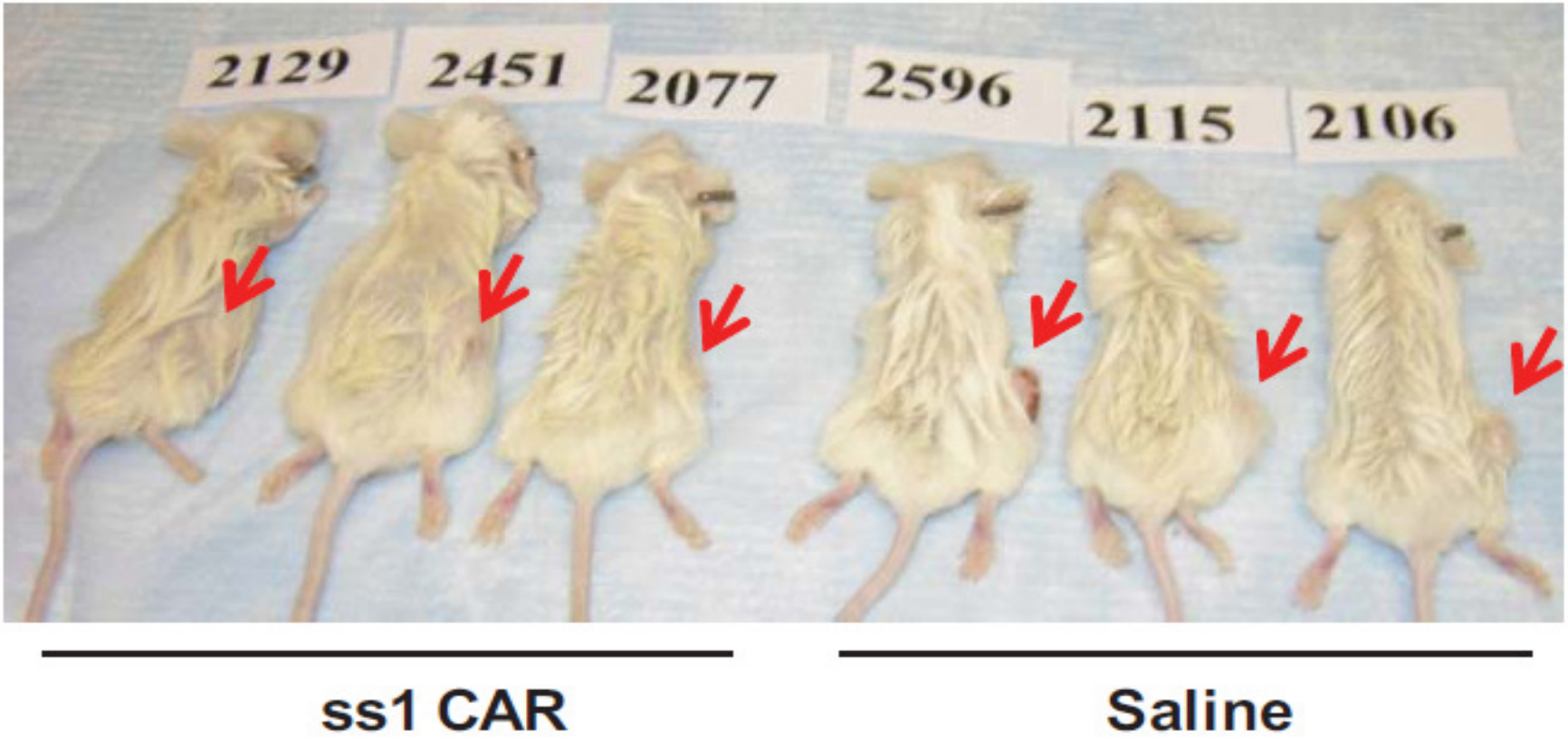
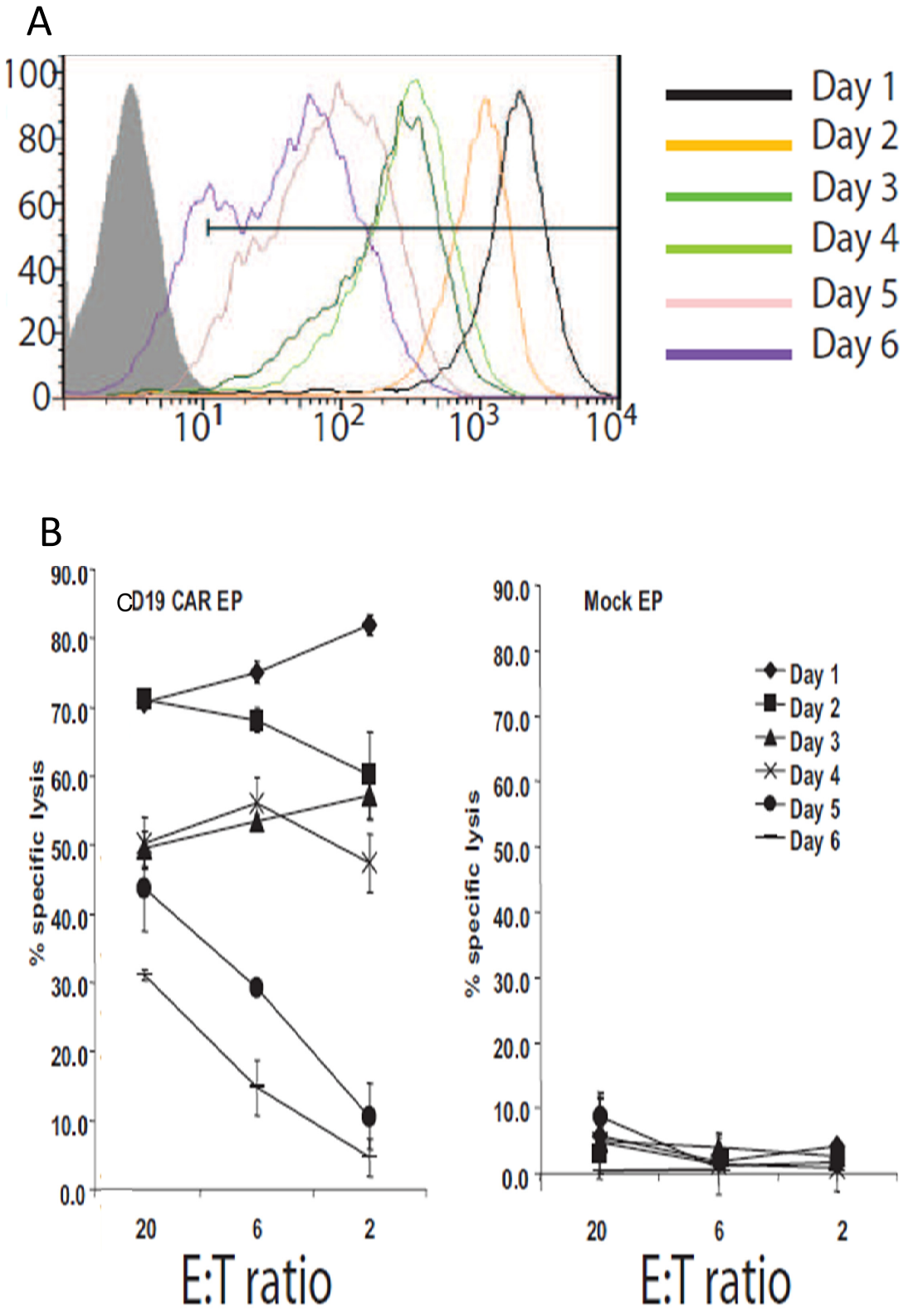


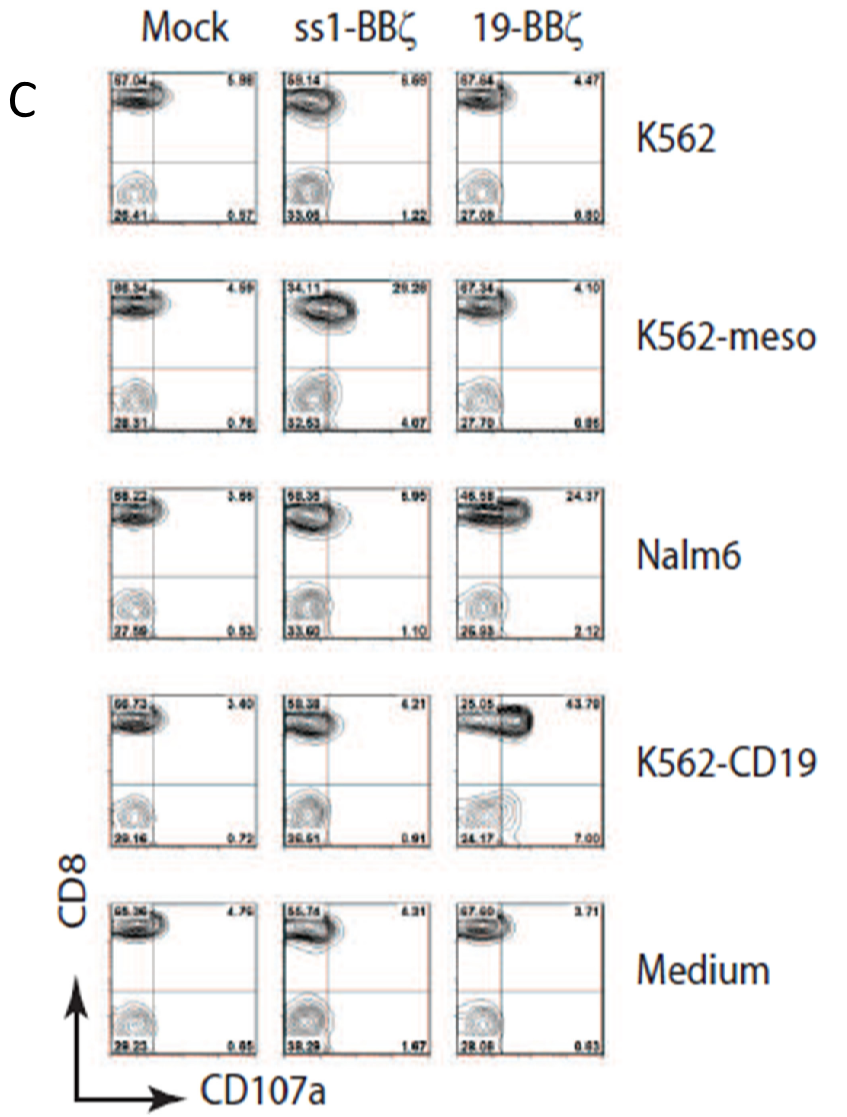
Figure 10

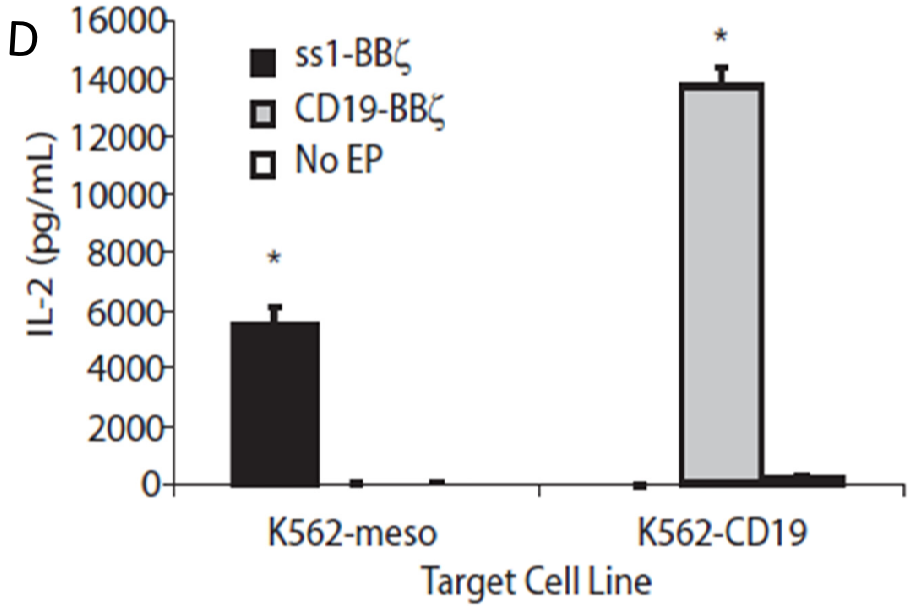
Day -56, M108-Luc
Day 0, 1st T cell Injection
Day 1, 2nd T cell Injection
Day 3, 3rd T cell Injection
Day 6, 4th T cell Injection
Day 8, 5th T cell Injection
Day 10, 6th T cell Injection
Day 22, 7th$_t$ T cell Injection
Day 24, 8th$_t$ T cell Injection
Day 27, 9th T cell Injection
Day 29, 10th T cell Injection
Day 51, 11th$_t$ T cell Injection
Day 52, 12th$_t$ T cell Injection
Day 55, 13th T cell Injection
Day 57, 14th T cell Injection
Day -5, 1st image
Day 9, 2nd image
Day 17, 3rd image
Day 24, 4th image
Day 28, 5th image
Day 34, 6th image
Day 41, 7th image
Day 48, 8th image
Day 62, 9th image
Day 76, 10th image
Day 83, 11 th image

Figure 11



Figures 12A-12B





Figures 12C-12D

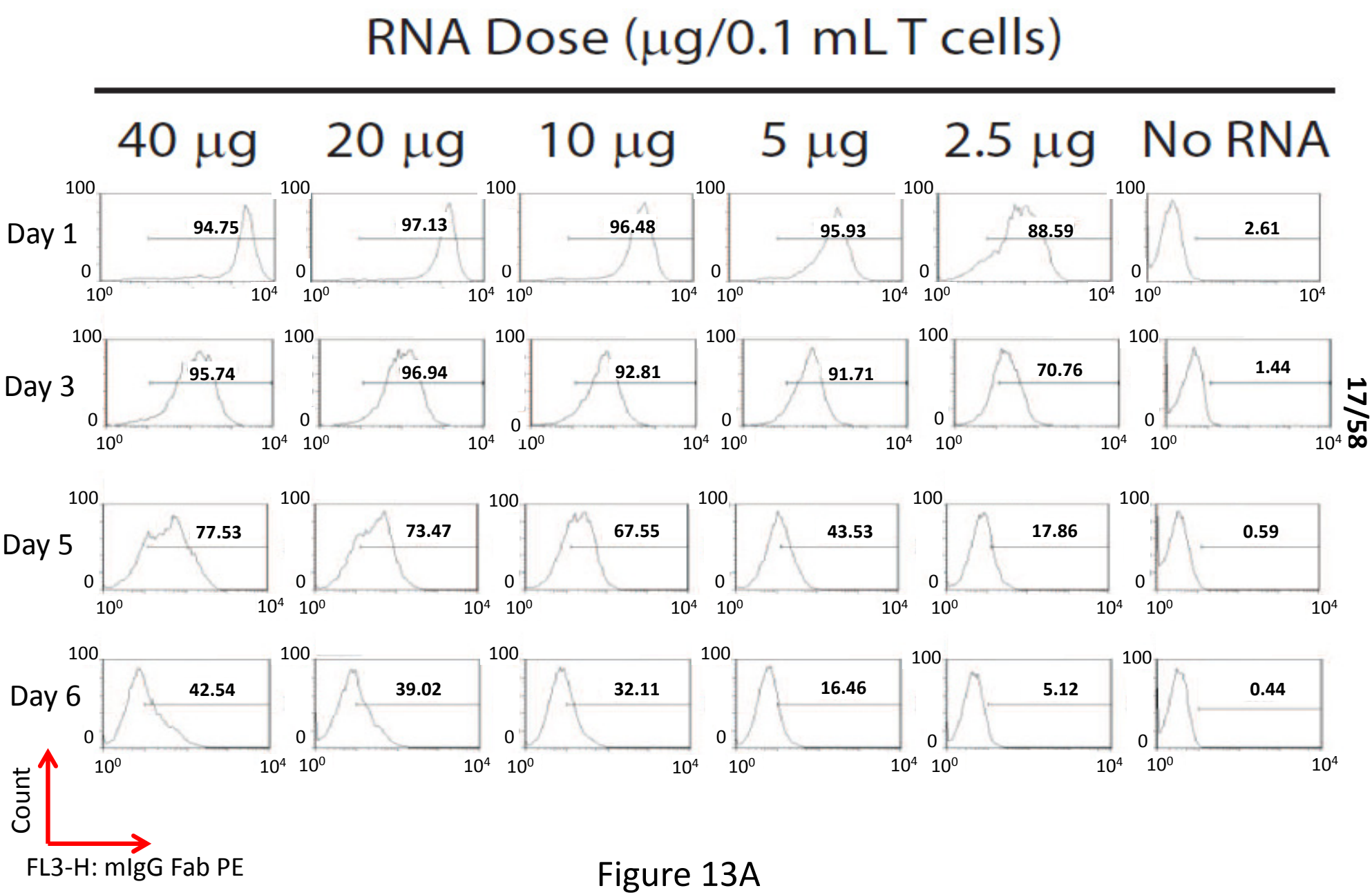
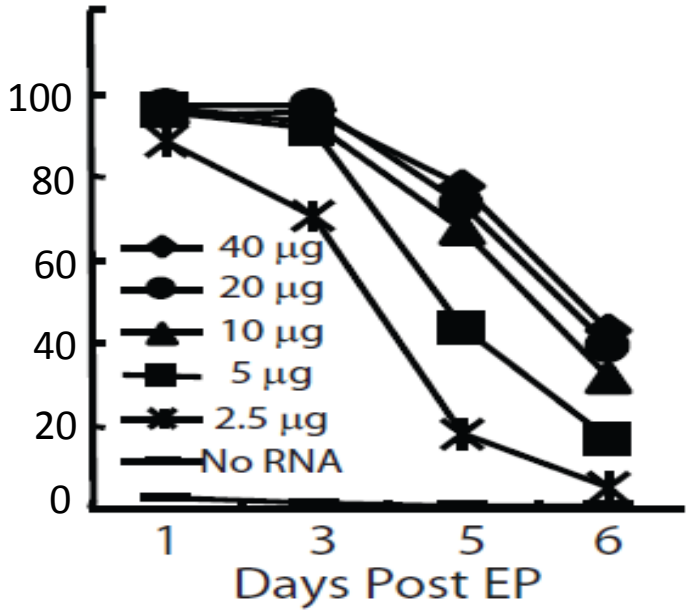
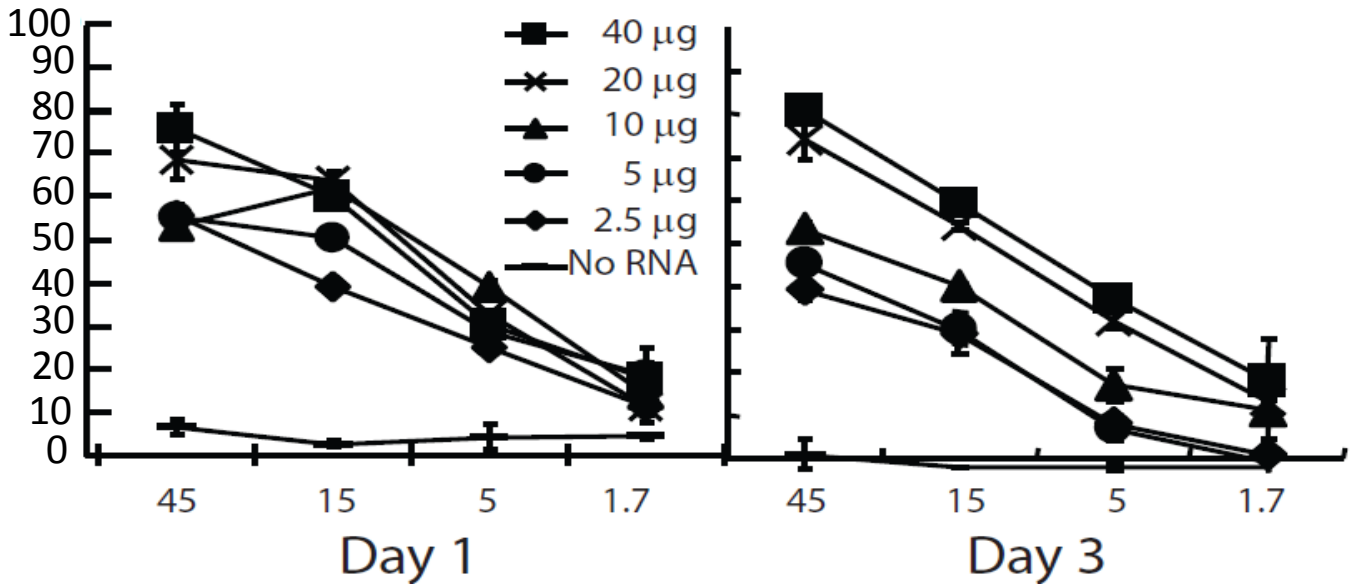

RNA Dose (µg/0.1 mL T cells)
40 µg
20 µg
10 µg
5 µg
2.5 µg
No RNA
Day 1
Day 3
Day 5
Day 6
94.75
97.13
96.48
95.93
88.59
2.61
95.74
96.94
92.81
91.71
70.76
1.44
77.53
73.47
67.55
43.53
17.86
0.59
42.54
39.02
32.11
16.46
5.12
0.44
100
0
10^0
10^4
Count
FL3-H: mIgG Fab PE

Figure 13A

B



C



Figures 13B-13C

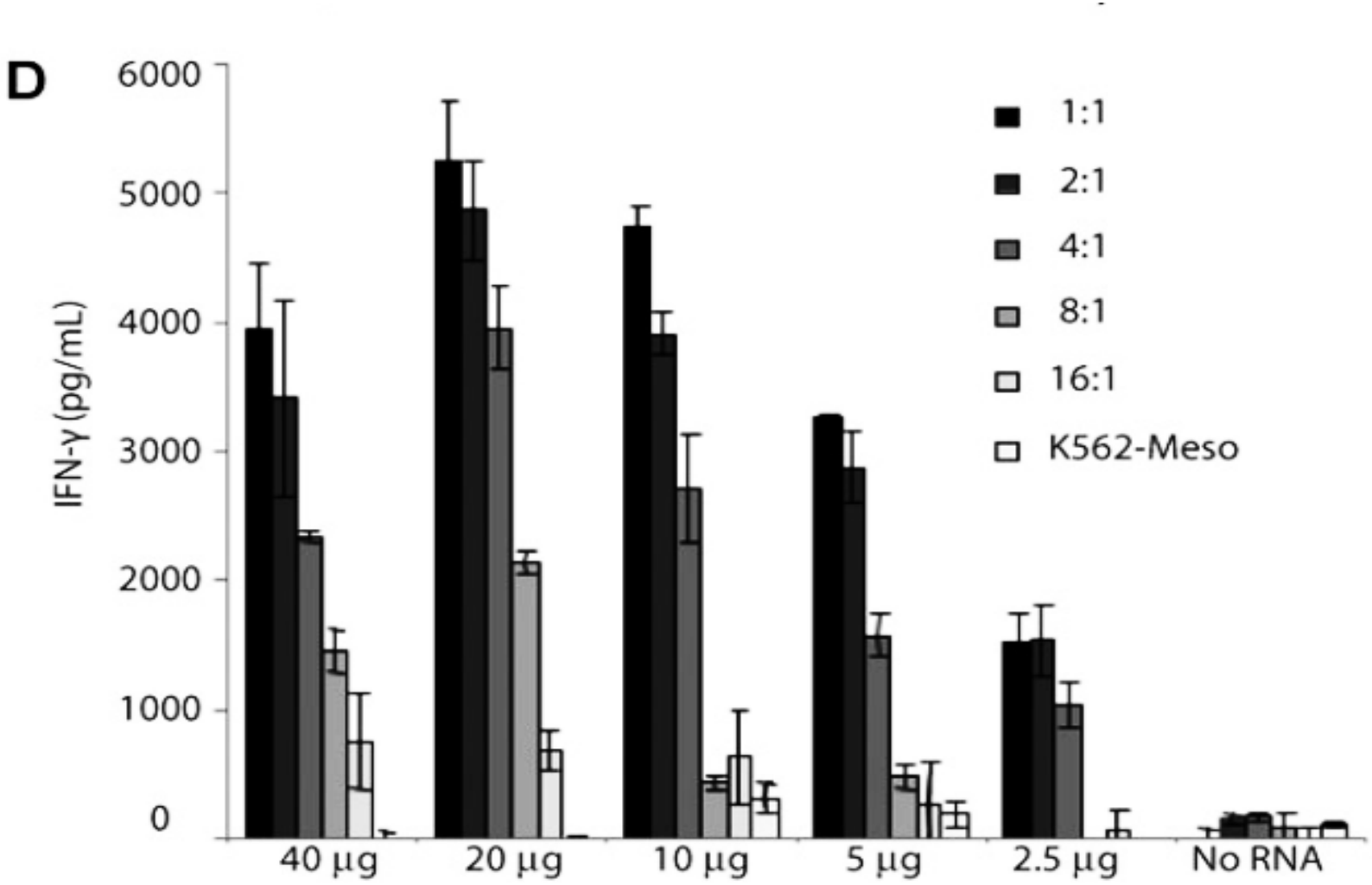

D
IFN-γ (pg/mL)
6000
5000
4000
3000
2000
1000
0
1:1
2:1
4:1
8:1
16:1
K562-Meso
40 µg
20 µg
10 µg
5 µg
2.5 µg
No RNA

Figure 13D

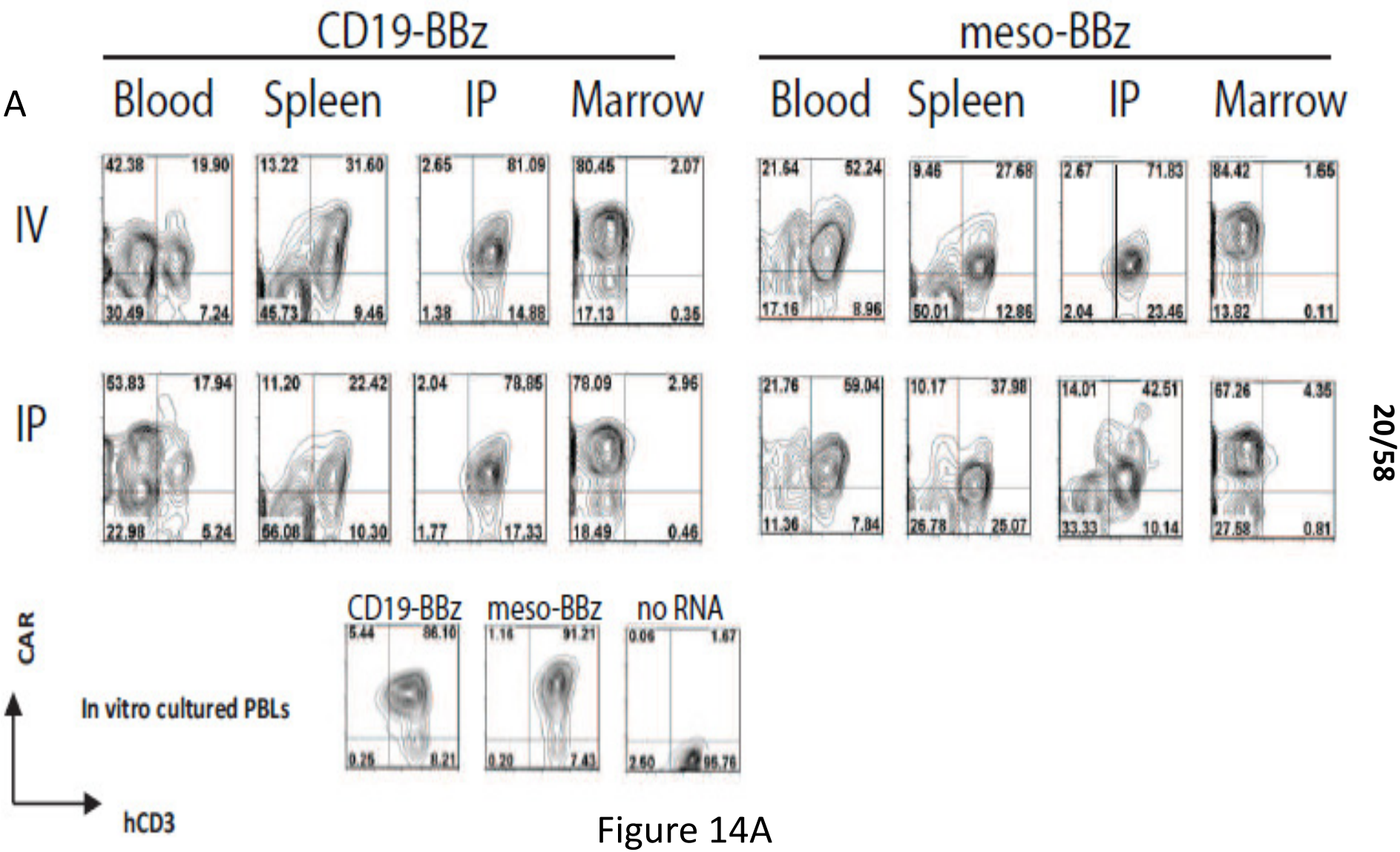

A
CD19-BBz
meso-BBz
Blood
Spleen
IP
Marrow
Blood
Spleen
IP
Marrow
IV
IP
42.38 19.90 30.49 7.24
13.22 31.60 45.73 9.46
2.65 81.09 1.38 14.88
80.45 2.07 17.13 0.35
21.64 52.24 17.16 8.96
9.46 27.68 50.01 12.86
2.67 71.83 2.04 23.46
84.42 1.65 13.82 0.11
53.83 17.94 22.98 5.24
11.20 22.42 66.08 10.30
2.04 78.85 1.77 17.33
78.09 2.96 18.49 0.46
21.76 59.04 11.36 7.84
10.17 37.98 26.78 25.07
14.01 42.51 33.33 10.14
67.26 4.35 27.58 0.81
CD19-BBz
meso-BBz
no RNA
5.44 86.10 0.25 8.21
1.16 91.21 0.20 7.43
0.06 1.67 2.60 95.76
CAR
In vitro cultured PBLs
hCD3

Figure 14A

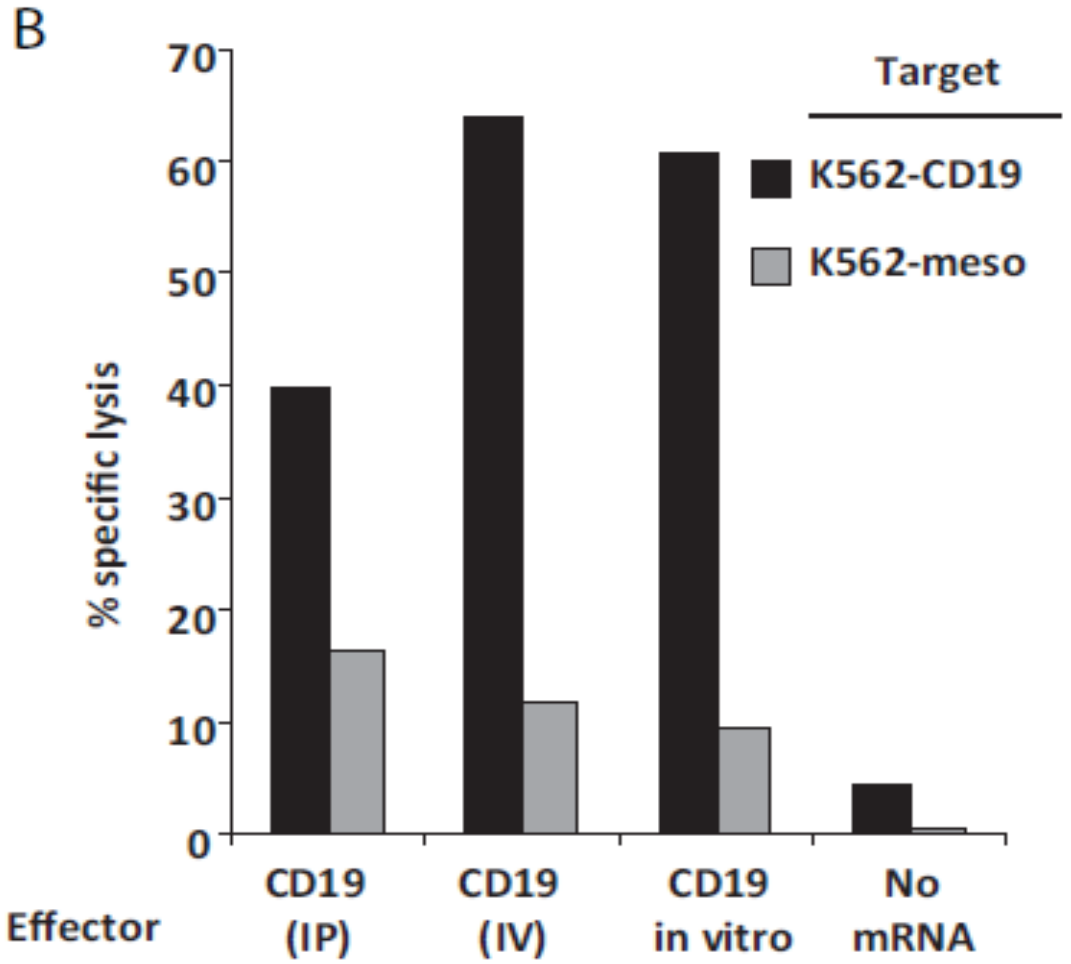

B
70
60
50
40
30
20
10
0
% specific lysis
Target
K562-CD19
K562-meso
Effector
CD19 (IP)
CD19 (IV)
CD19 in vitro
No mRNA

Figure 14B

A

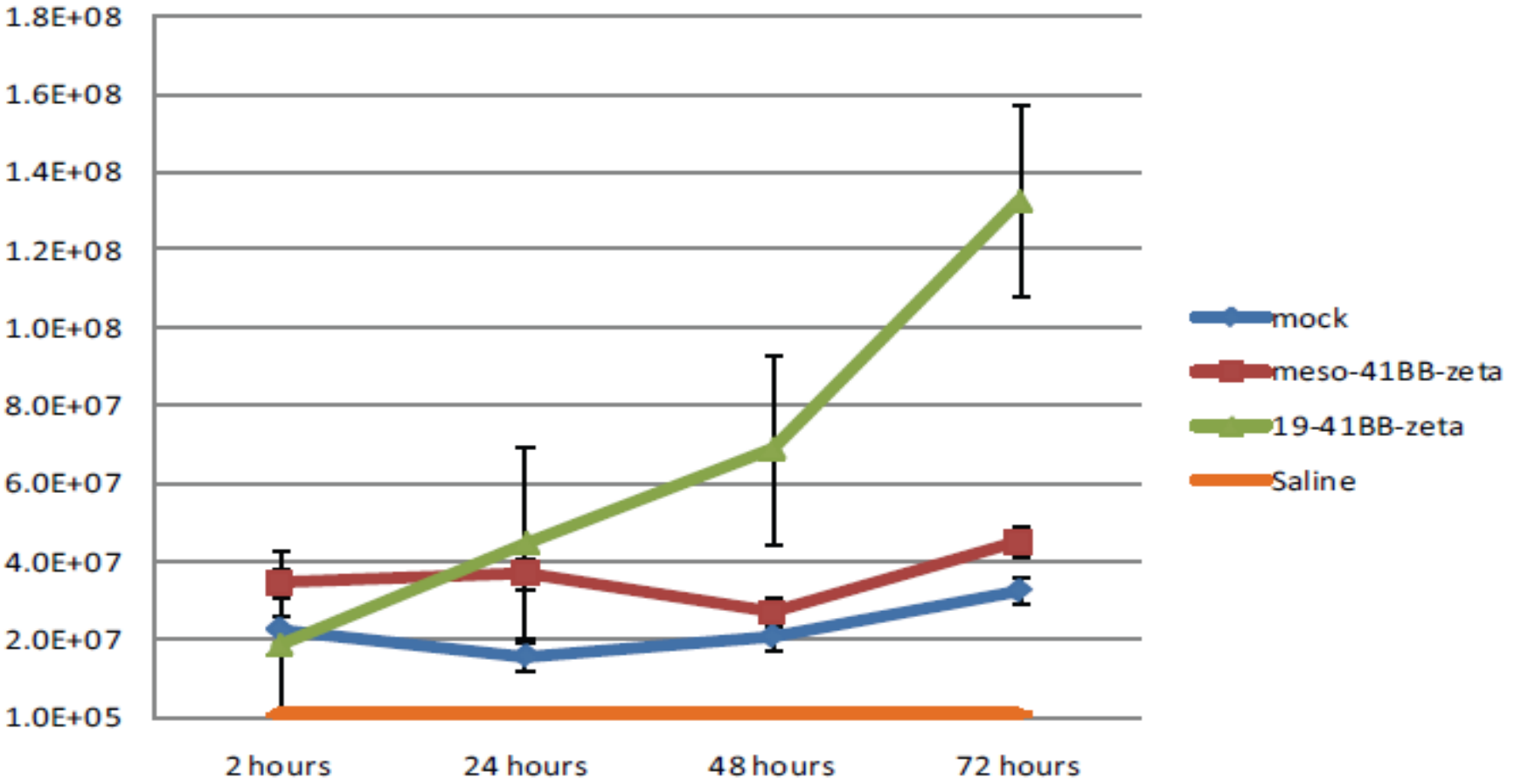

1.8E+08
1.6E+08
1.4E+08
1.2E+08
1.0E+08
8.0E+07
6.0E+07
4.0E+07
2.0E+07
1.0E+05
2 hours
24 hours
48 hours
72 hours
mock
meso-41BB-zeta
19-41BB-zeta
Saline

Figure 15A

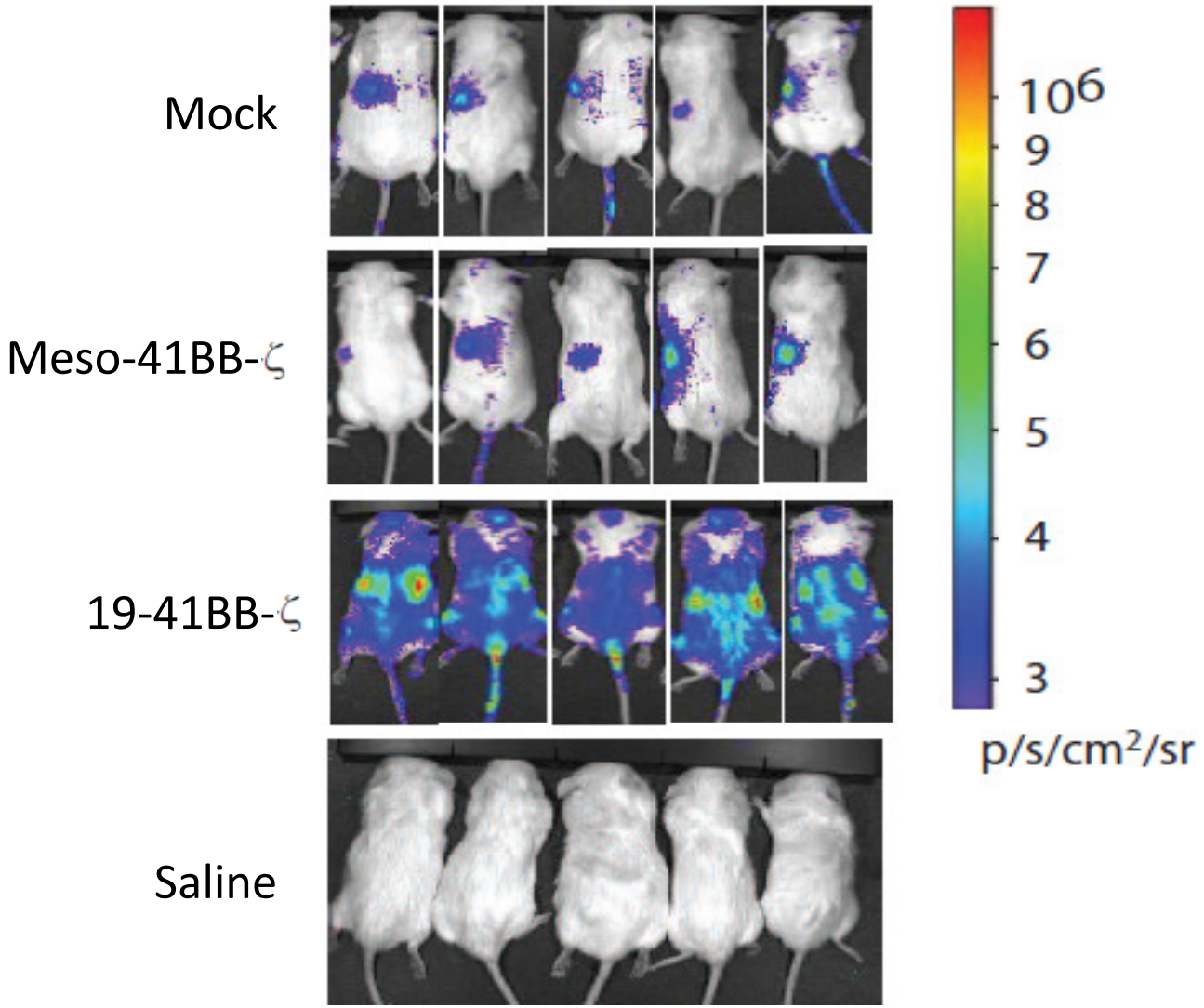


Figure 15B

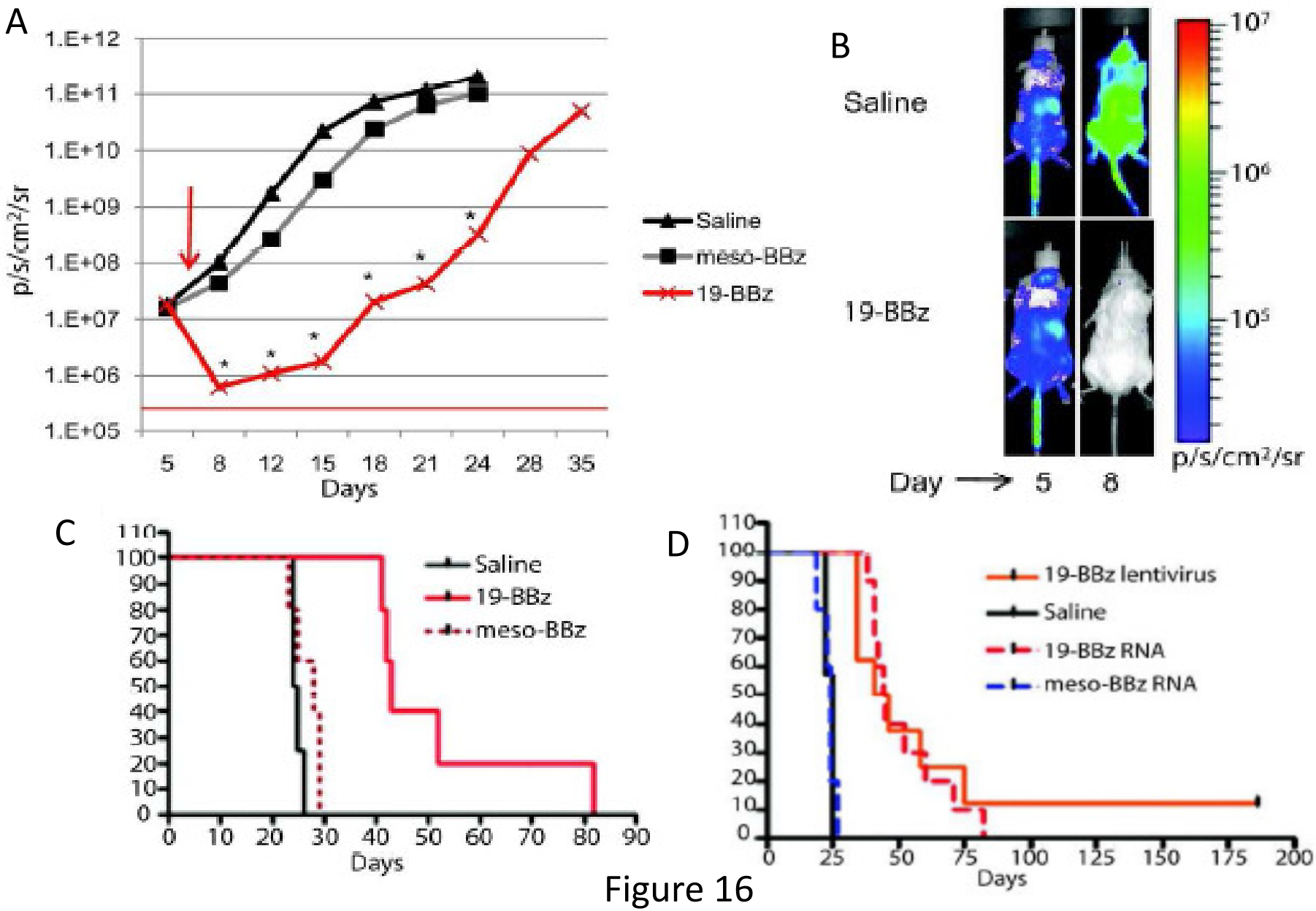

A
1.E+12
1.E+11
1.E+10
1.E+09
1.E+08
1.E+07
1.E+06
1.E+05
p/s/cm²/sr
5 8 12 15 18 21 24 28 35
Days
Saline
meso-BBz
19-BBz
B
Saline
19-BBz
10⁷
10⁶
10⁵
p/s/cm²/sr
Day → 5 8
C
110
100
90
80
70
60
50
40
30
20
10
0
0 10 20 30 40 50 60 70 80 90
Days
Saline
19-BBz
meso-BBz
D
110
100
90
80
70
60
50
40
30
20
10
0
0 25 50 75 100 125 150 175 200
Days
19-BBz lentivirus
Saline
19-BBz RNA
meso-BBz RNA

Figure 16

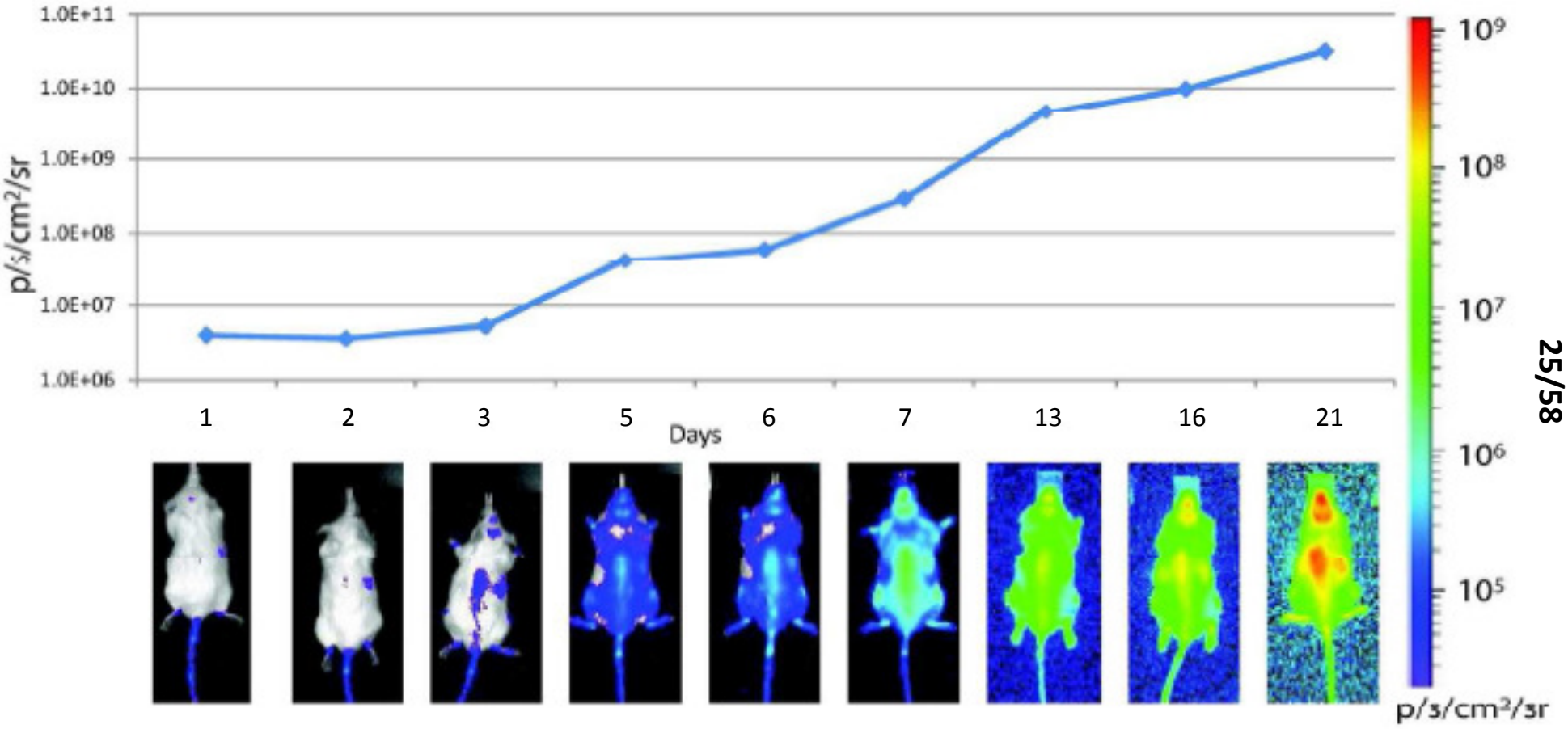

1.0E+11
1.0E+10
1.0E+09
1.0E+08
1.0E+07
1.0E+06
p/s/cm²/sr
1
2
3
5
6
7
13
16
21
Days
10⁹
10⁸
10⁷
10⁶
10⁵
p/s/cm²/sr

Figure 17

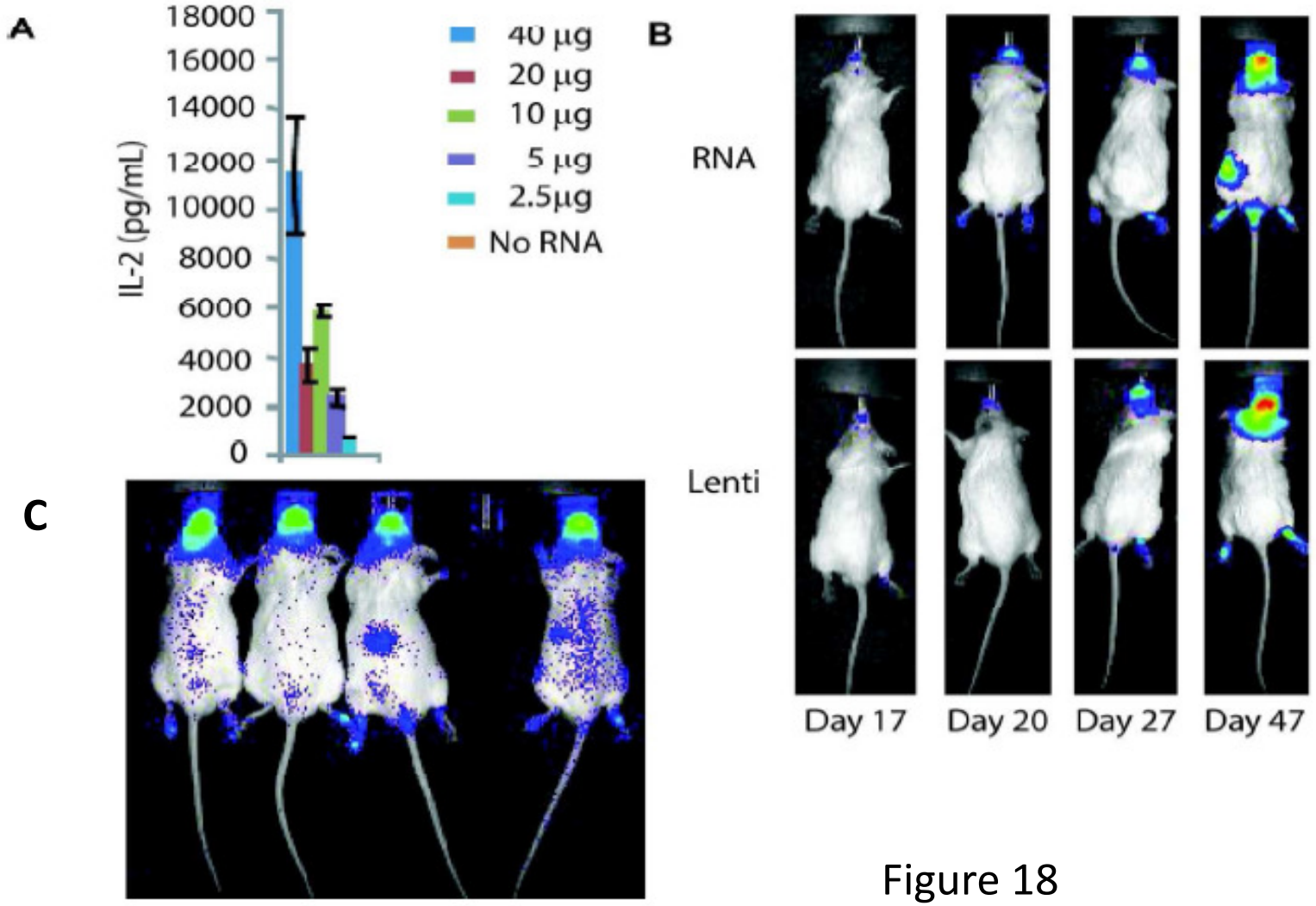
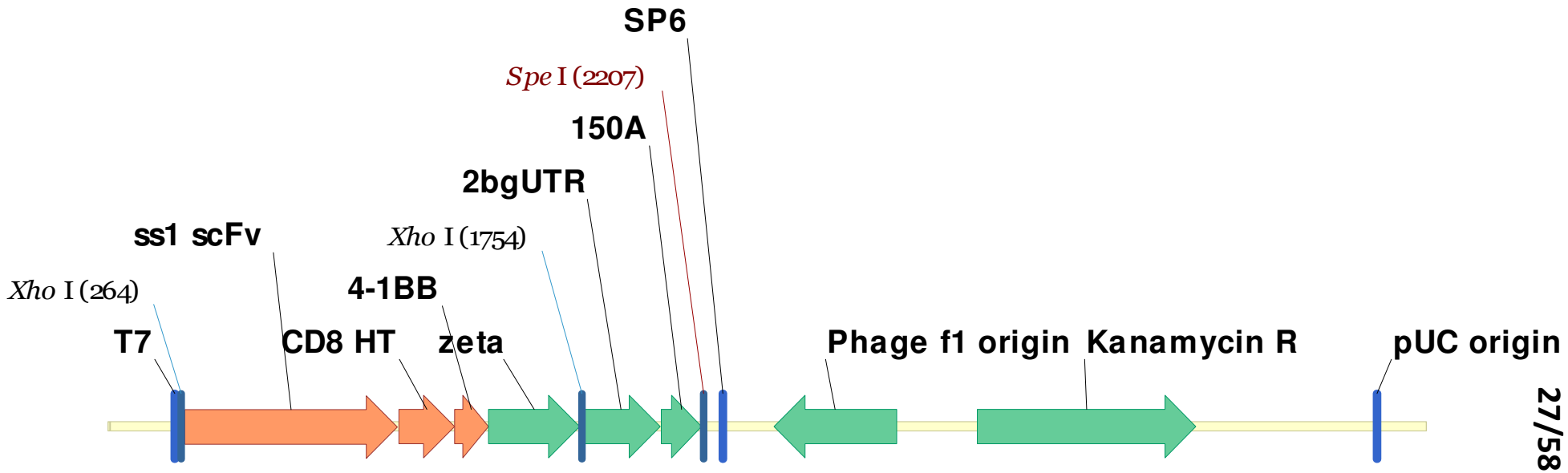

A
18000
16000
14000
12000
10000
8000
6000
4000
2000
0
IL-2 (pg/mL)
40 µg
20 µg
10 µg
5 µg
2.5µg
No RNA
B
RNA
Lenti
Day 17
Day 20
Day 27
Day 47
C

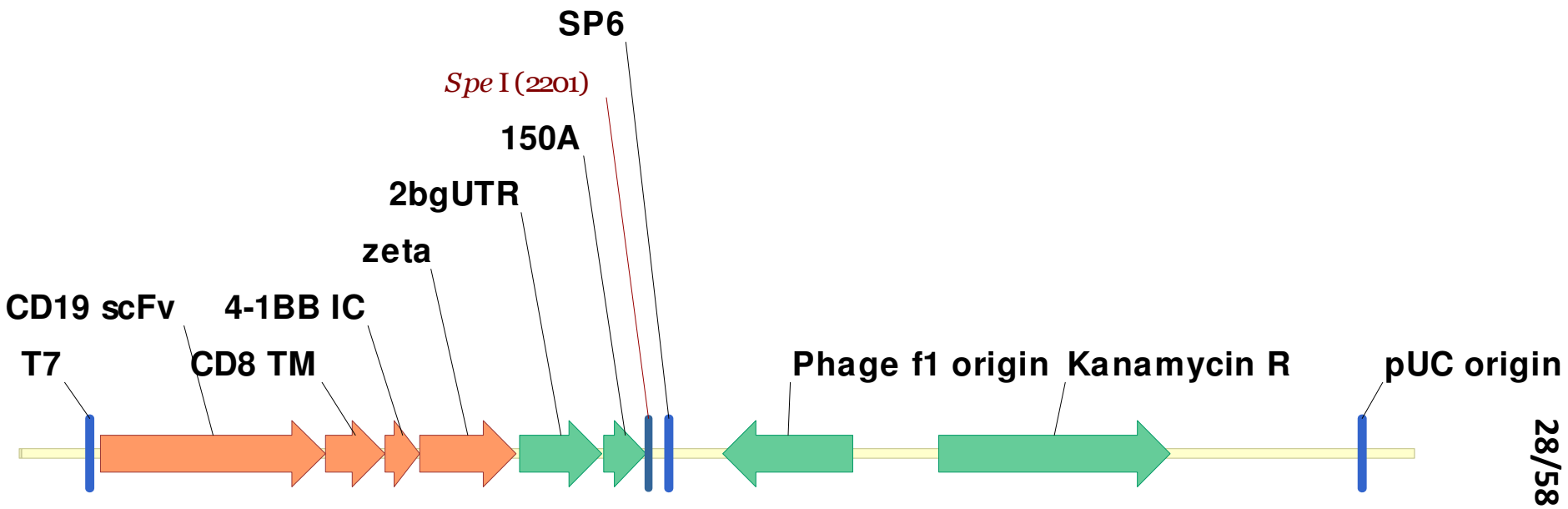
Figure 18



pD-A. ss1.OF.2bg.150A

4893 bp

Figure 19



pD-A.19.OF.2bg.150A.

4888 bp

Figure 20

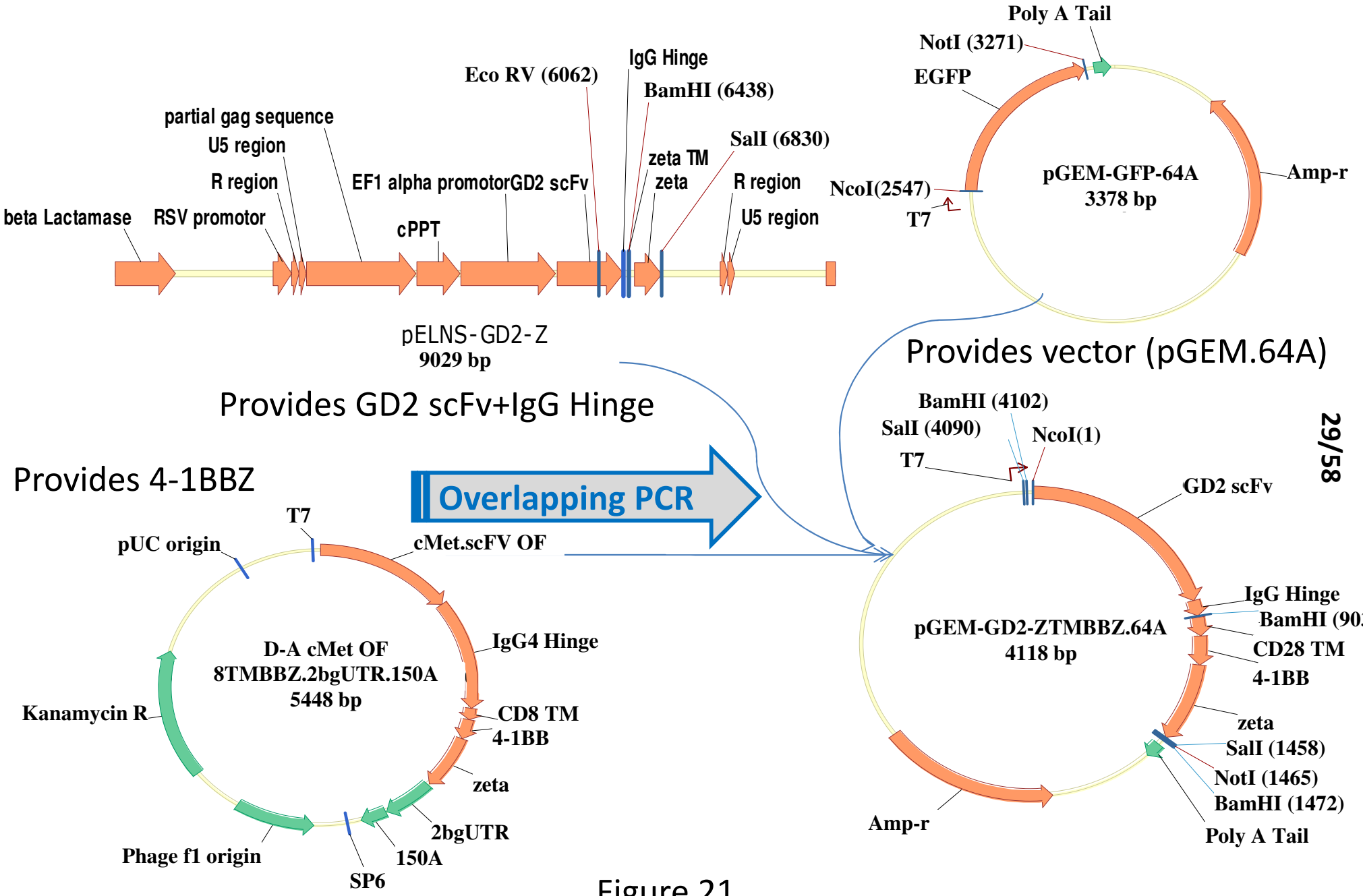

beta Lactamase
RSV promotor
partial gag sequence
U5 region
R region
cPPT
EF1 alpha promotor
GD2 scFv
Eco RV (6062)
IgG Hinge
BamHI (6438)
zeta TM
zeta
SalI (6830)
R region
U5 region
pELNS-GD2-Z
9029 bp
Provides GD2 scFv+IgG Hinge
Poly A Tail
NotI (3271)
EGFP
NcoI(2547)
T7
pGEM-GFP-64A
3378 bp
Amp-r
Provides vector (pGEM.64A)
Provides 4-1BBZ
Overlapping PCR
T7
pUC origin
cMet.scFV OF
D-A cMet OF
8TMBBZ.2bgUTR.150A
5448 bp
IgG4 Hinge
CD8 TM
4-1BB
zeta
2bgUTR
150A
SP6
Phage f1 origin
Kanamycin R
BamHI (4102)
SalI (4090)
NcoI(1)
T7
GD2 scFv
pGEM-GD2-ZTMBBZ.64A
4118 bp
IgG Hinge
BamHI (903)
CD28 TM
4-1BB
zeta
SalI (1458)
NotI (1465)
BamHI (1472)
Poly A Tail
Amp-r
29/58

Figure 21

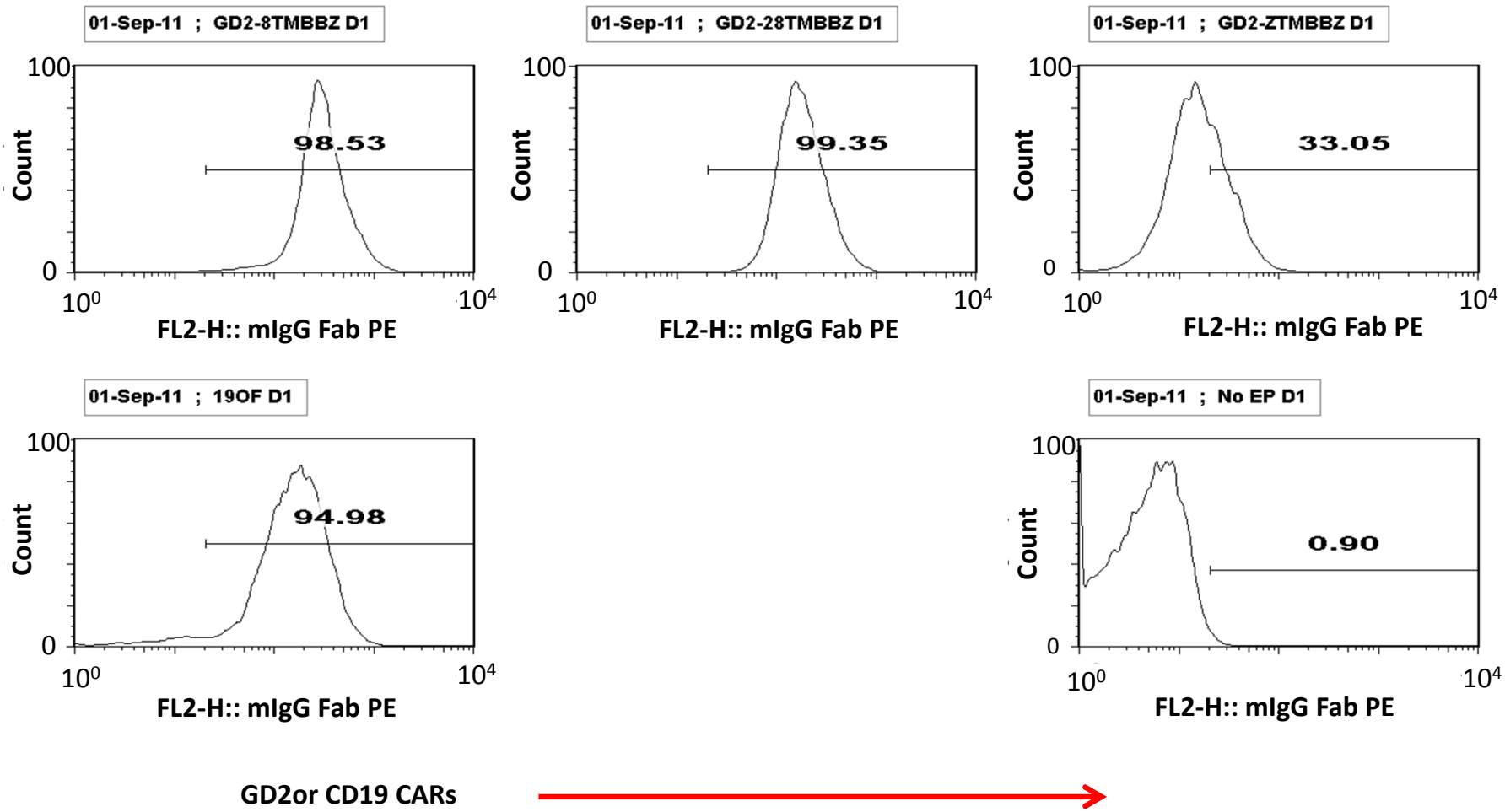

01-Sep-11 ; GD2-8TMBBZ D1
98.53
01-Sep-11 ; GD2-28TMBBZ D1
99.35
01-Sep-11 ; GD2-ZTMBBZ D1
33.05
01-Sep-11 ; 19OF D1
94.98
01-Sep-11 ; No EP D1
0.90
Count
100
0
10^0
10^4
FL2-H:: mIgG Fab PE
GD2or CD19 CARs

Figure 22

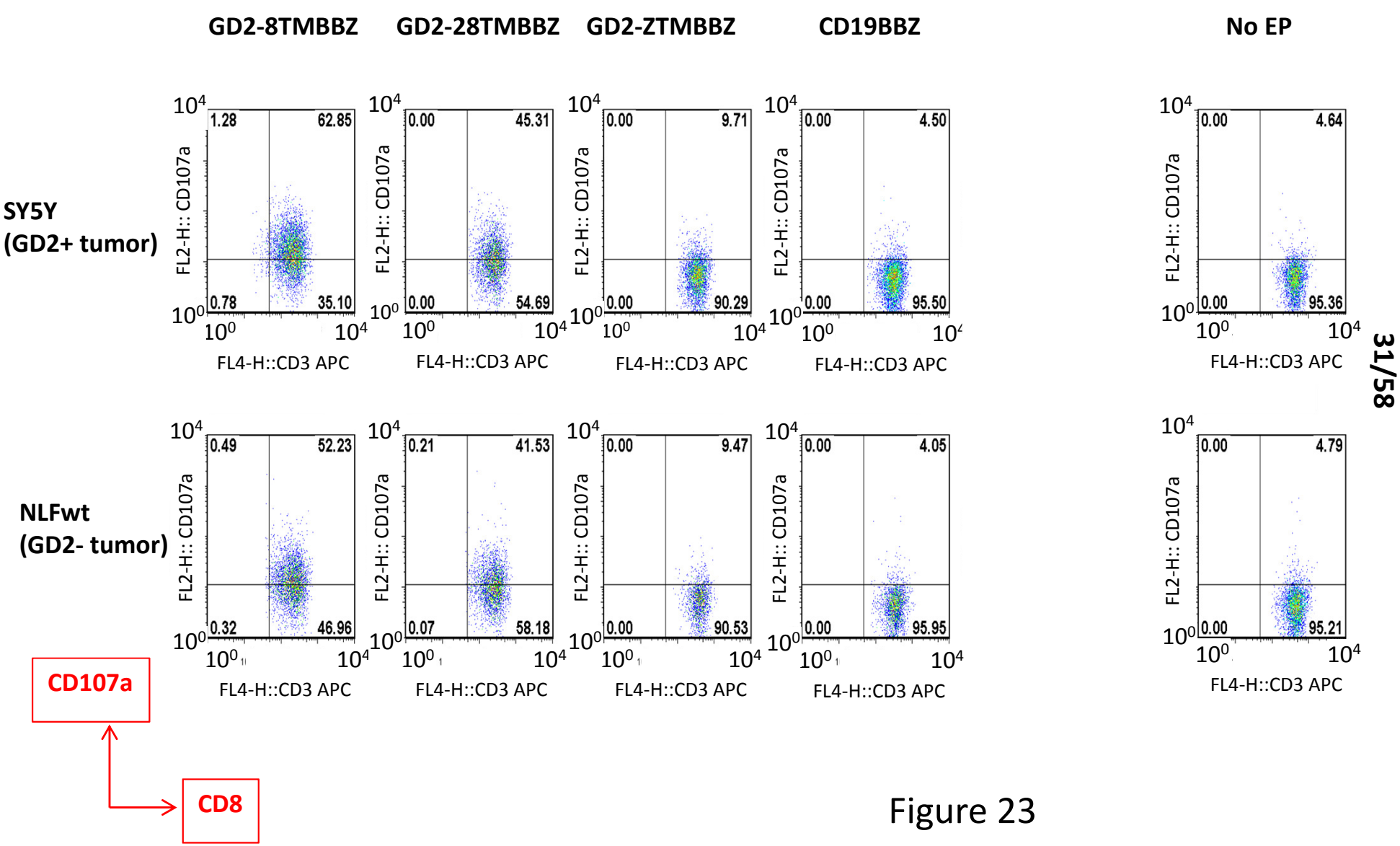

GD2-8TMBBZ
GD2-28TMBBZ
GD2-ZTMBBZ
CD19BBZ
No EP
SY5Y
(GD2+ tumor)
NLFwt
(GD2- tumor)
FL2-H:: CD107a
FL4-H::CD3 APC
1.28
62.85
0.78
35.10
0.00
45.31
0.00
54.69
0.00
9.71
0.00
90.29
0.00
4.50
0.00
95.50
0.00
4.64
0.00
95.36
0.49
52.23
0.32
46.96
0.21
41.53
0.07
58.18
0.00
9.47
0.00
90.53
0.00
4.05
0.00
95.95
0.00
4.79
0.00
95.21
CD107a
CD8

Figure 23

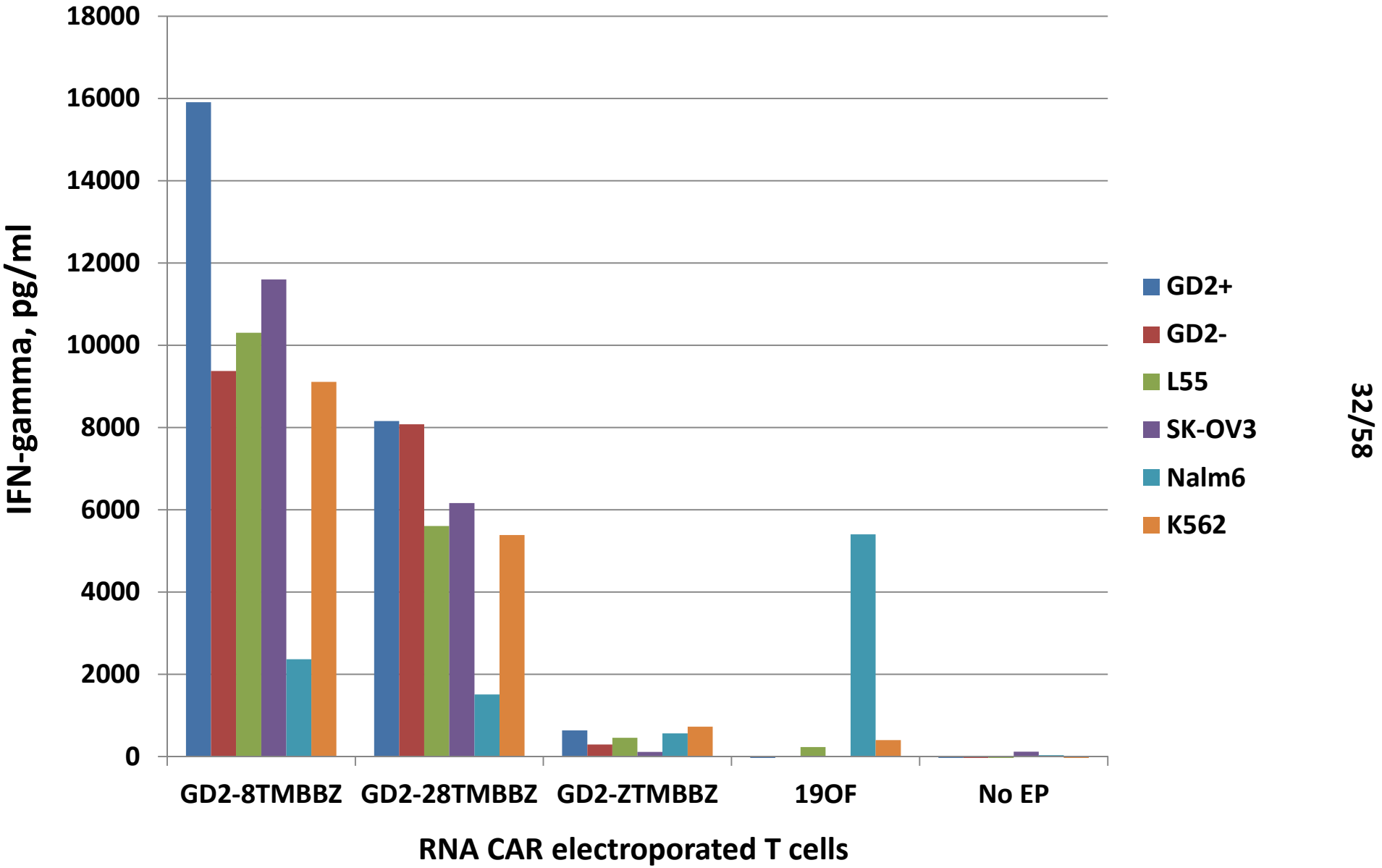
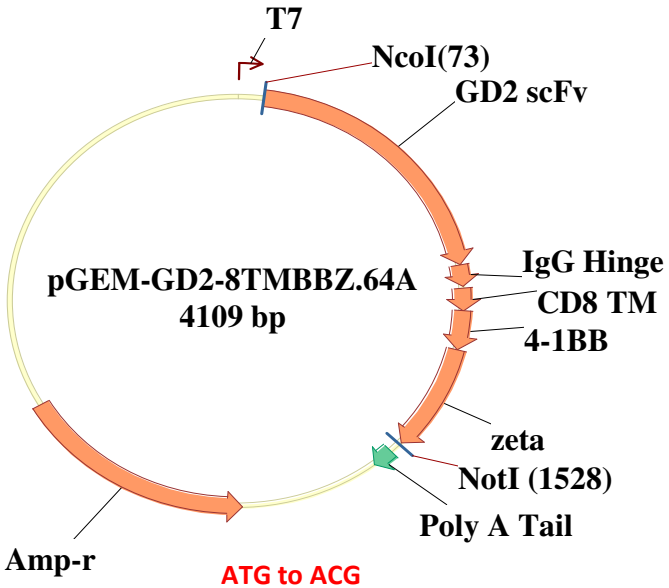
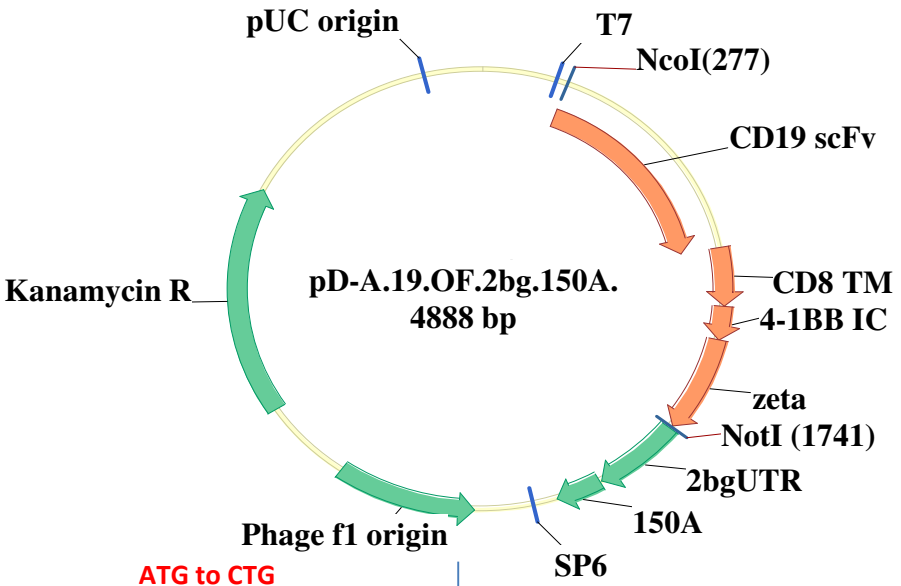

18000
16000
14000
12000
10000
8000
6000
4000
2000
0
IFN-gamma, pg/ml
GD2-8TMBBZ
GD2-28TMBBZ
GD2-ZTMBBZ
19OF
No EP
RNA CAR electroporated T cells
GD2+
GD2-
L55
SK-OV3
Nalm6
K562

Figure 24





ATG to ACG

ATG to CTG

```
GD2-8TMBBZ.seq(1>1446)   →  cccatcagtctatGgaagggtcacc-gtctcttcagcgg-agcccaaatcttgtgacaaaactcacacatgcccaccgtgc
GD2 scFv OF.seq(1>906)   →  cccatcagtctaCggaagggtcacc-gtctcttcagcgg-agcccaaatcttgtgacaaaactcacacCtgcccaccgtgc
26.2_B04_030.ab1(6>897)  →  CC
28.4_D04_026.ab1(17>902) ←  CCCATCAGTCTACGGAAGGGTCACC-GTCTCTTCAGCGG-AGCCCAAATCTTGTGACAAAACTCACACCTGCCCACCGTGC
31.7_G04_020.ab1(1>906)  →  CCCATCAGTCTACGGAAGGGTCACC-GTCTCTTCAGCGG-AGCCCAAATCTTGTGACAAAACTCACACCTGCCCACCGTGC
27.3_C04_028.ab1(5>787)  ←  CCCATCAGTCTACGGAAGGGTCACC-GTCTCTTCAGCGG-AGCCCAAATCTTGTGACAAAACTCACACCTGCCCACCGTGC
30.6_F04_022.ab1(3>911)  →                                                  CACACCTGCCCACCGTGC
```

Overlap PCR to generate internal ORF free GD2-8TMBBZ

Two internal ORFs were removed by mutations of there ATG codons without change CAR ORF

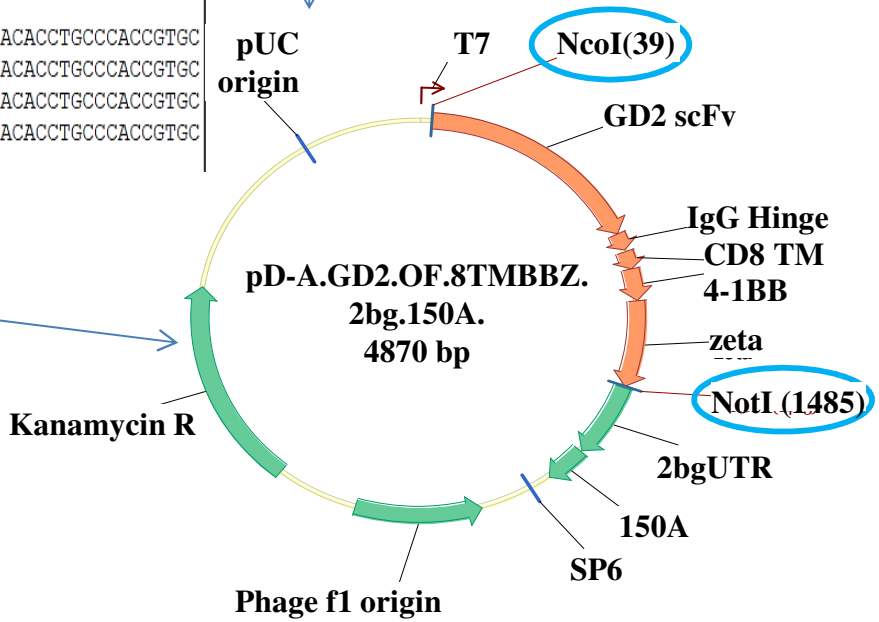
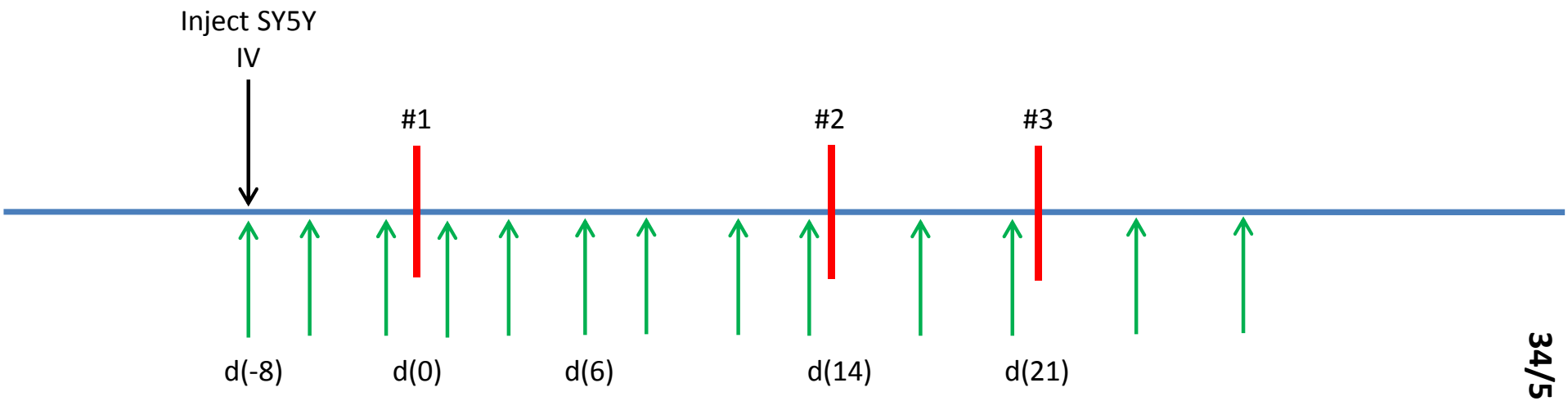


Figure 25



Study groups

Tumor	CAR	Expression	Administration	# mice
IV	GD2	RNA	IV	5
IV	CD19	RNA	IV	5
IV	PBS		IV	5

T-cell administrations, IV and IT
#1: 5M RNA T-cells
#2: 14M RNA T-cells
#3: 16M RNA T-cells

BLI

Figure 26

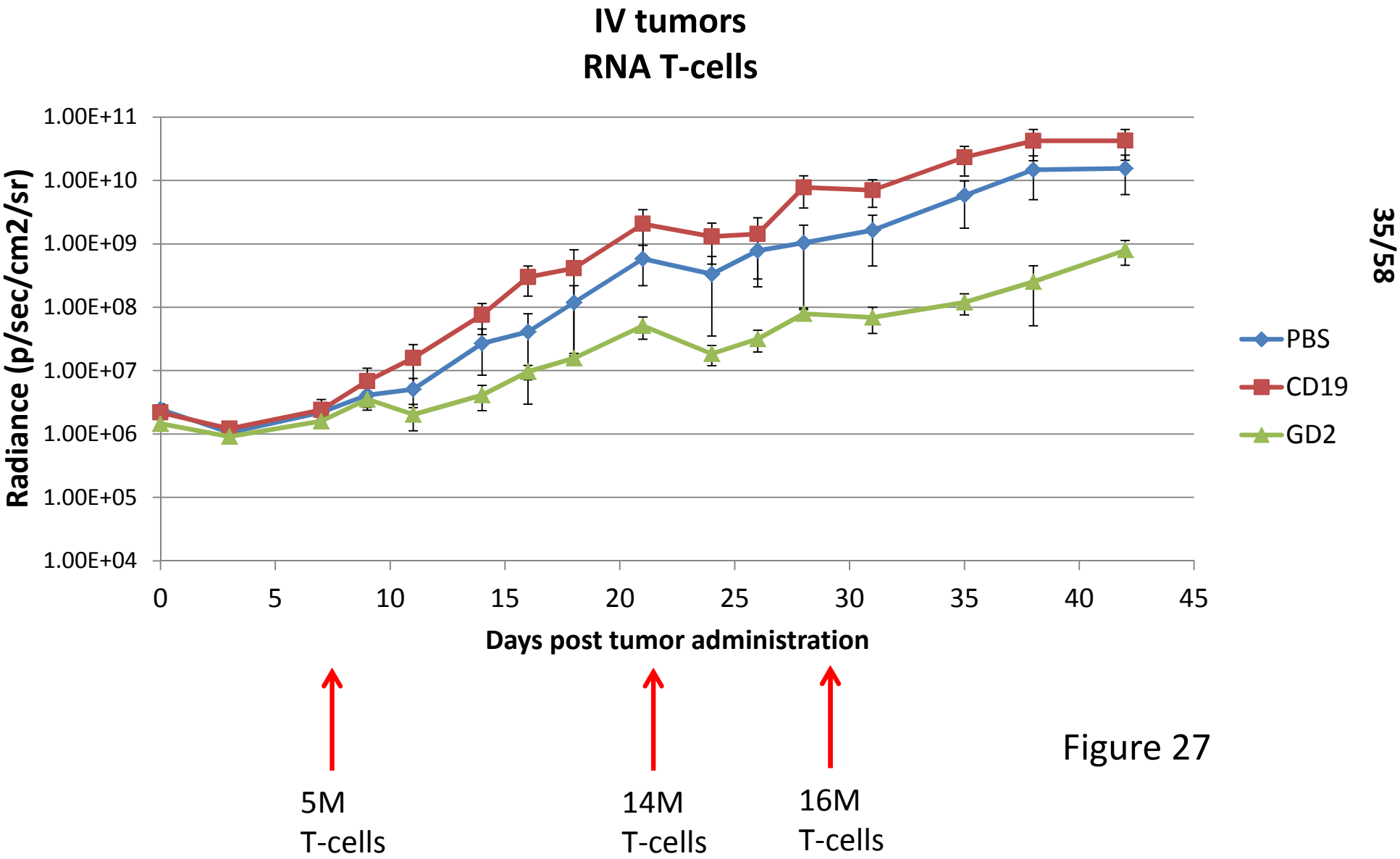
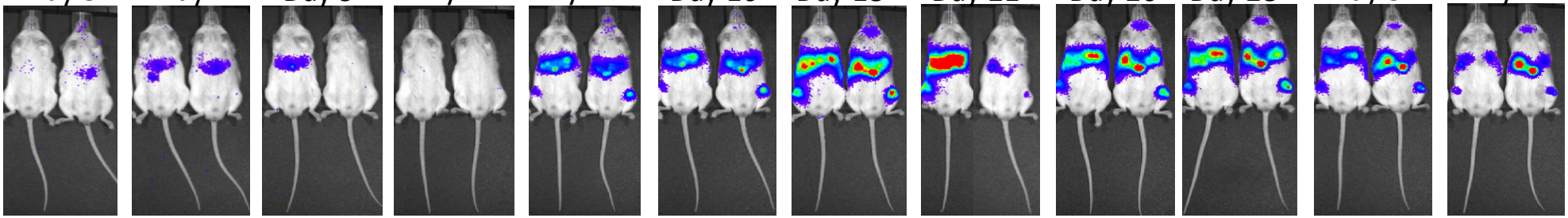

IV tumors
RNA T-cells
Radiance (p/sec/cm2/sr)
1.00E+11
1.00E+10
1.00E+09
1.00E+08
1.00E+07
1.00E+06
1.00E+05
1.00E+04
0
5
10
15
20
25
30
35
40
45
Days post tumor administration
PBS
CD19
GD2
5M
T-cells
14M
T-cells
16M
T-cells

Figure 27

GD2 mRNA T-cells

Mice 2 and 3



CD19 mRNA

T-cells

Mouse 215

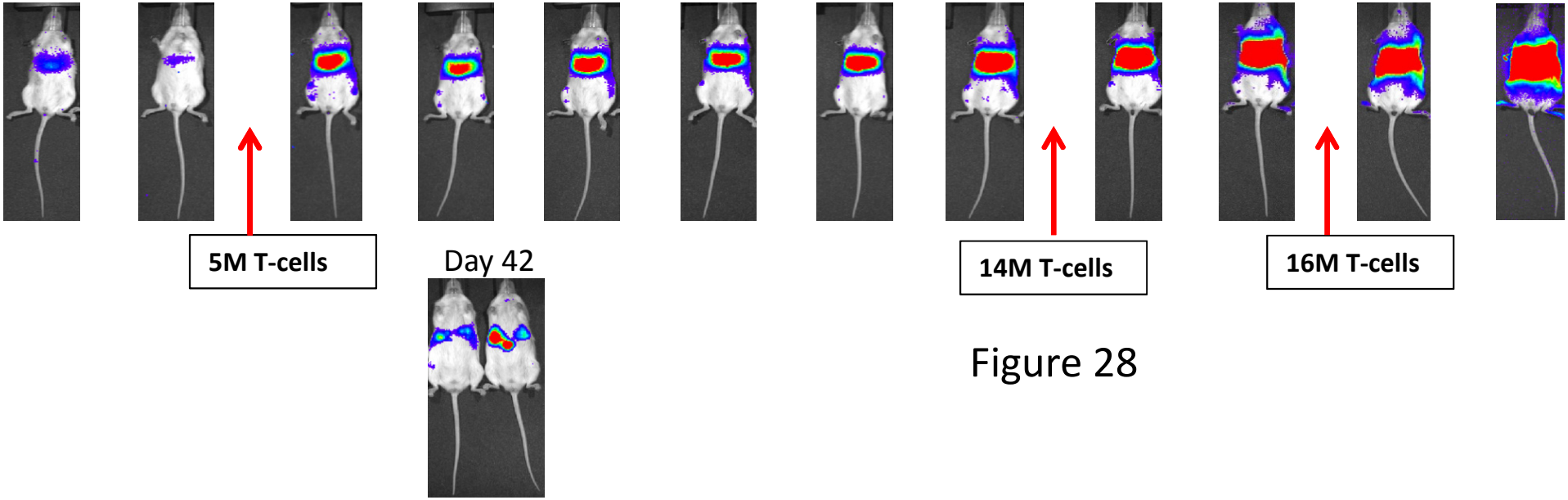


Figure 28

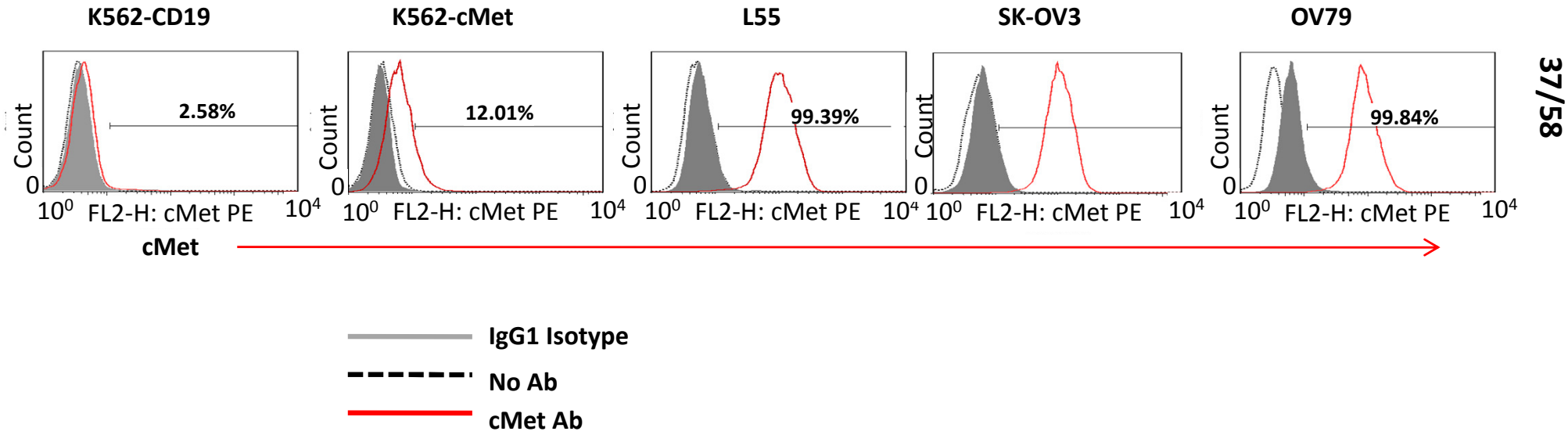

K562-CD19
K562-cMet
L55
SK-OV3
OV79
Count
0
2.58%
12.01%
99.39%
99.84%
10^0 FL2-H: cMet PE 10^4
cMet
IgG1 Isotype
No Ab
cMet Ab

Figure 29

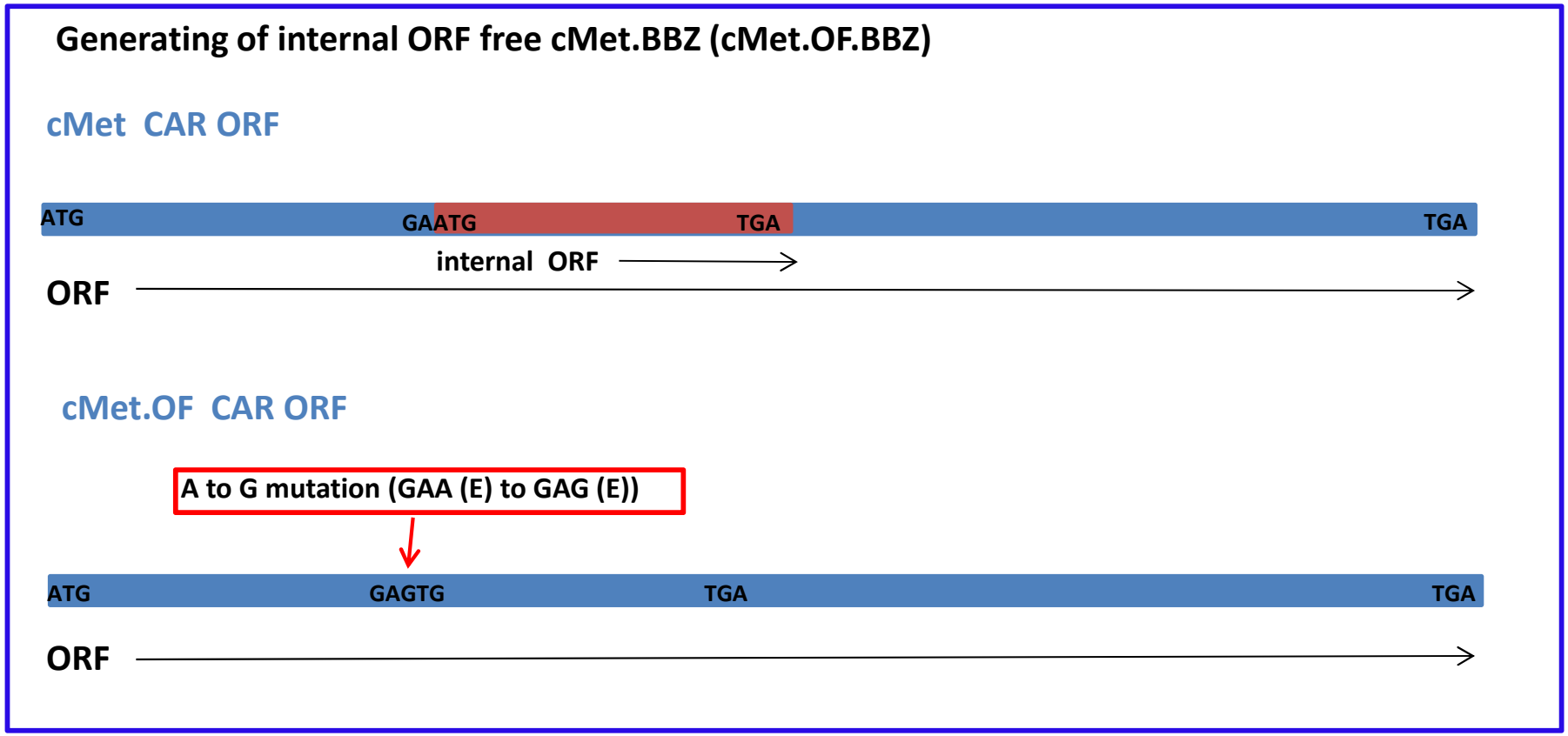

Generating of internal ORF free cMet.BBZ (cMet.OF.BBZ)
cMet CAR ORF
ATG
GAATG
TGA
TGA
internal ORF
ORF
cMet.OF CAR ORF
A to G mutation (GAA (E) to GAG (E))
ATG
GAGTG
TGA
TGA
ORF

Figure 30

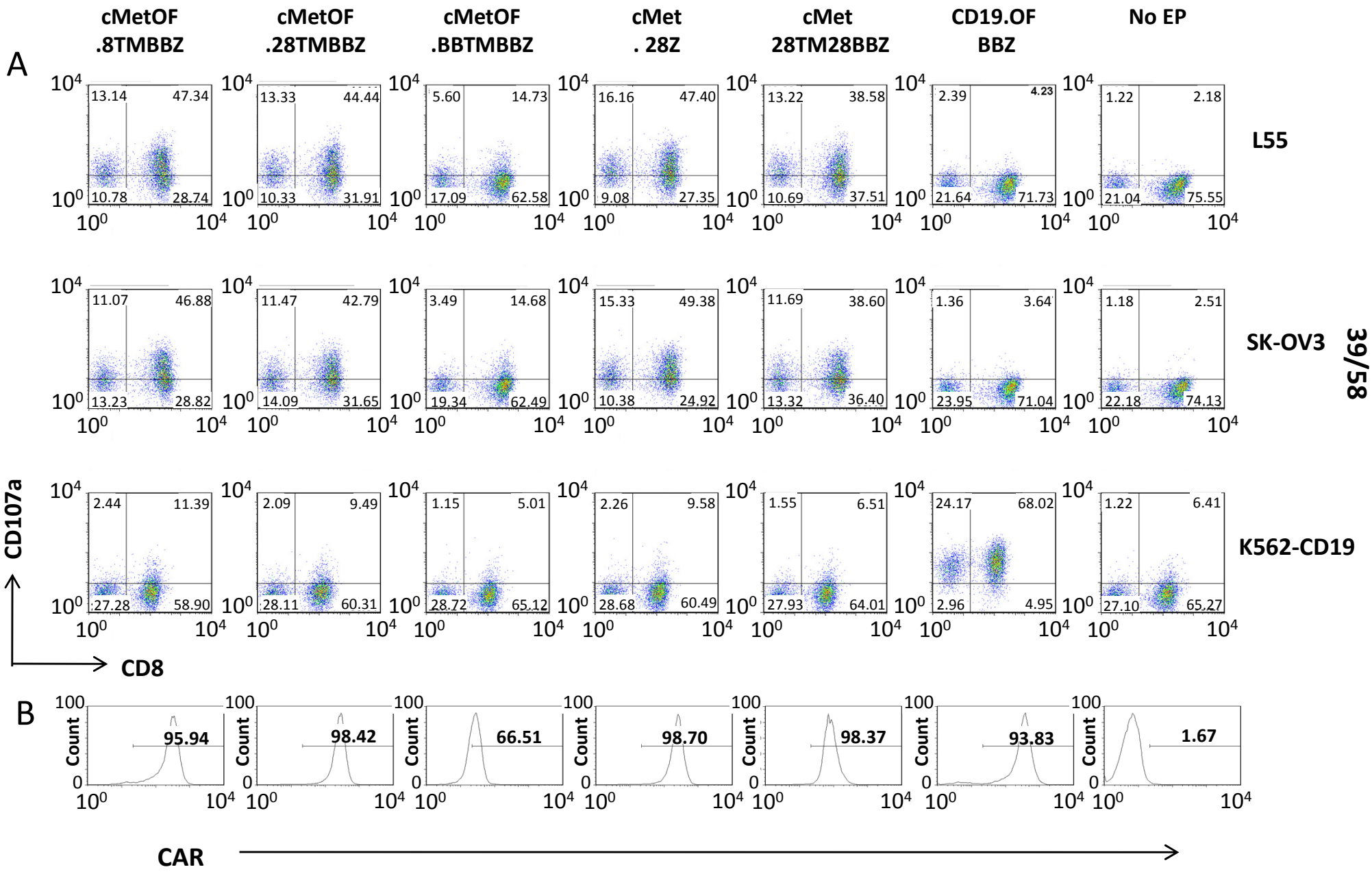
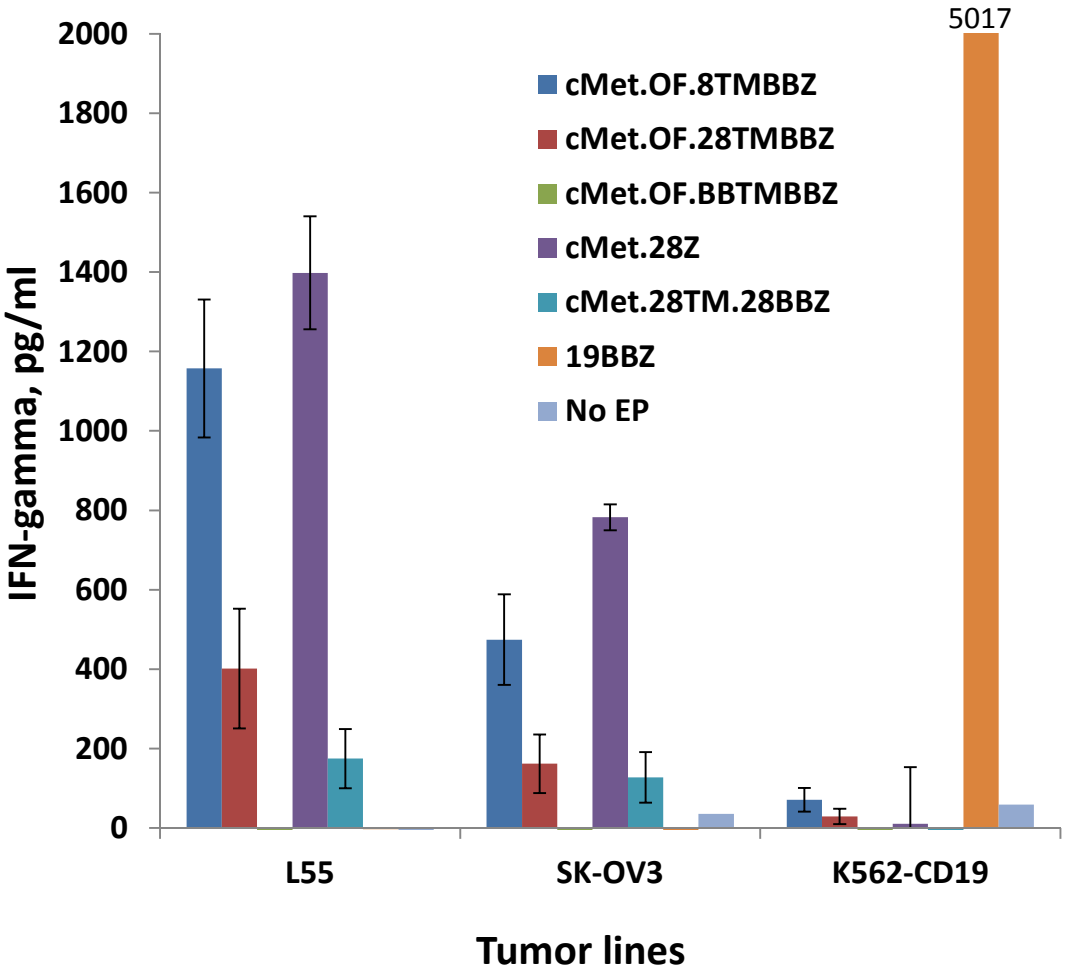

A
cMetOF .8TMBBZ
cMetOF .28TMBBZ
cMetOF .BBTMBBZ
cMet . 28Z
cMet 28TM28BBZ
CD19.OF BBZ
No EP
L55
SK-OV3
K562-CD19
CD107a
CD8
13.14 47.34 10.78 28.74
13.33 44.44 10.33 31.91
5.60 14.73 17.09 62.58
16.16 47.40 9.08 27.35
13.22 38.58 10.69 37.51
2.39 4.23 21.64 71.73
1.22 2.18 21.04 75.55
11.07 46.88 13.23 28.82
11.47 42.79 14.09 31.65
3.49 14.68 19.34 62.49
15.33 49.38 10.38 24.92
11.69 38.60 13.32 36.40
1.36 3.64 23.95 71.04
1.18 2.51 22.18 74.13
2.44 11.39 27.28 58.90
2.09 9.49 28.11 60.31
1.15 5.01 28.72 65.12
2.26 9.58 28.68 60.49
1.55 6.51 27.93 64.01
24.17 68.02 2.96 4.95
1.22 6.41 27.10 65.27
B
Count
95.94
98.42
66.51
98.70
98.37
93.83
1.67
CAR

Figure 31

A


5017
2000
1800
1600
1400
1200
1000
800
600
400
200
0
IFN-gamma, pg/ml
cMet.OF.8TMBBZ
cMet.OF.28TMBBZ
cMet.OF.BBTMBBZ
cMet.28Z
cMet.28TM.28BBZ
19BBZ
No EP
L55
SK-OV3
K562-CD19
Tumor lines

B

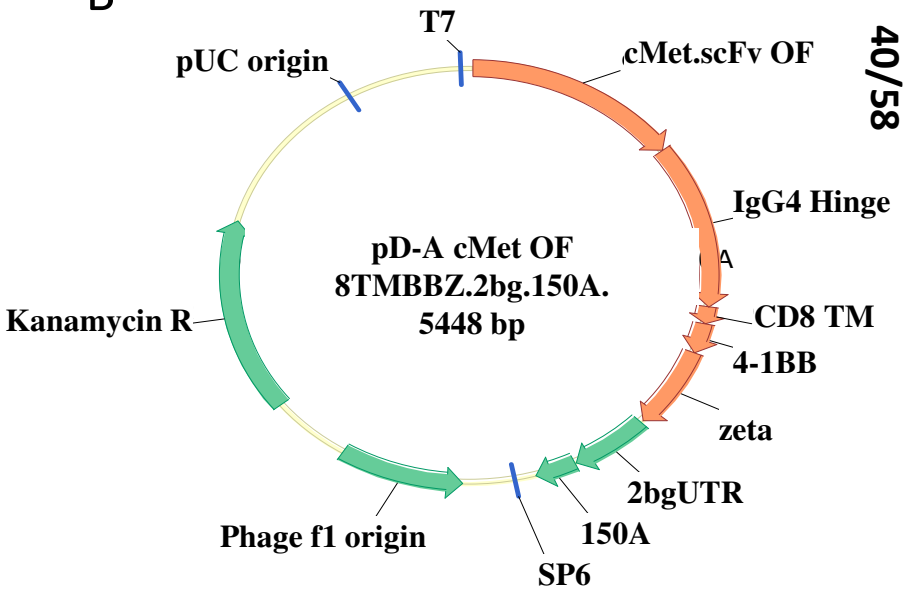

T7
pUC origin
cMet.scFv OF
IgG4 Hinge
pD-A cMet OF
8TMBBZ.2bg.150A.
5448 bp
CD8 TM
4-1BB
zeta
2bgUTR
150A
SP6
Phage f1 origin
Kanamycin R

Figure 32

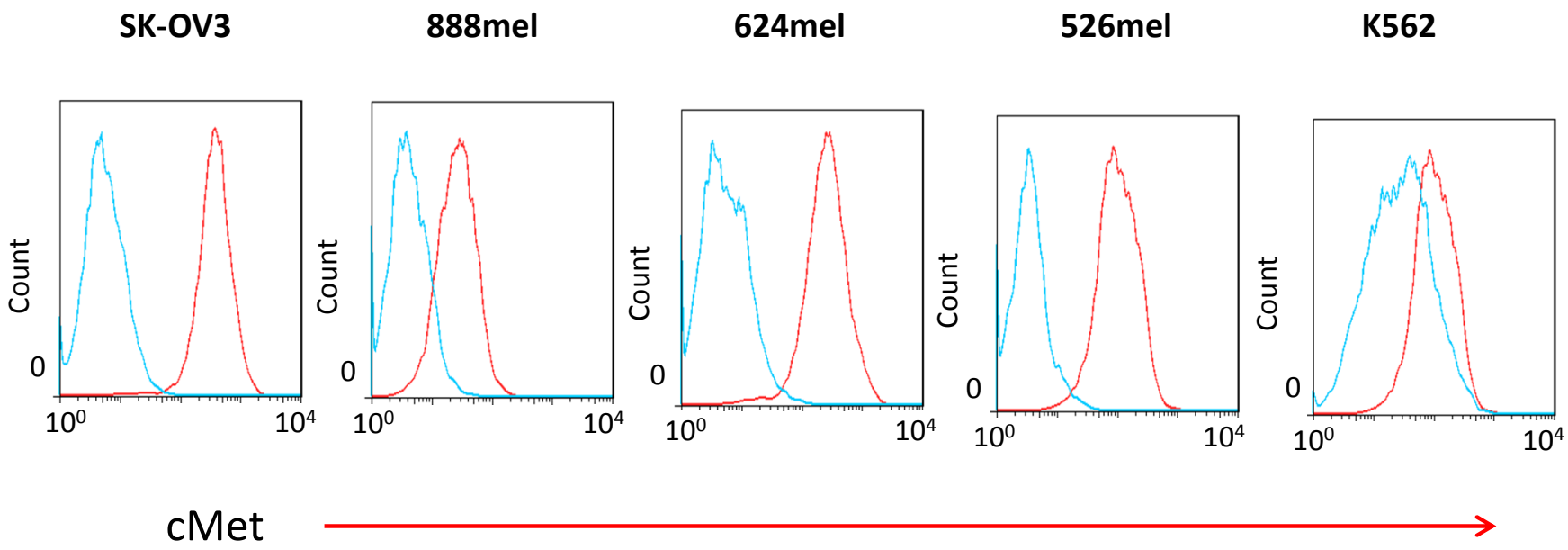


Figure 33

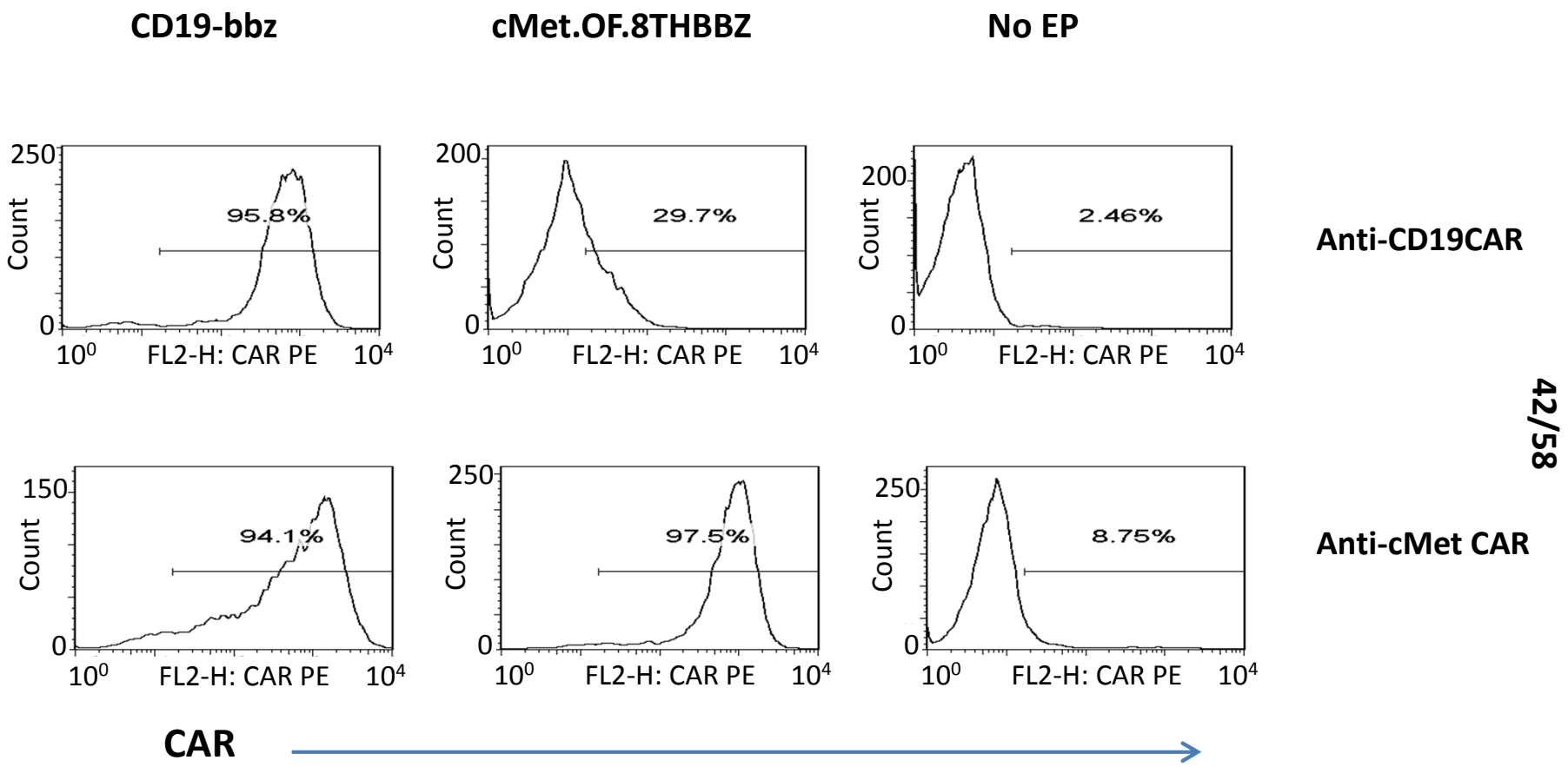

CD19-bbz
cMet.OF.8THBBZ
No EP
Anti-CD19CAR
Anti-cMet CAR
CAR
Count
FL2-H: CAR PE
95.8%
29.7%
2.46%
94.1%
97.5%
8.75%

Figure 34

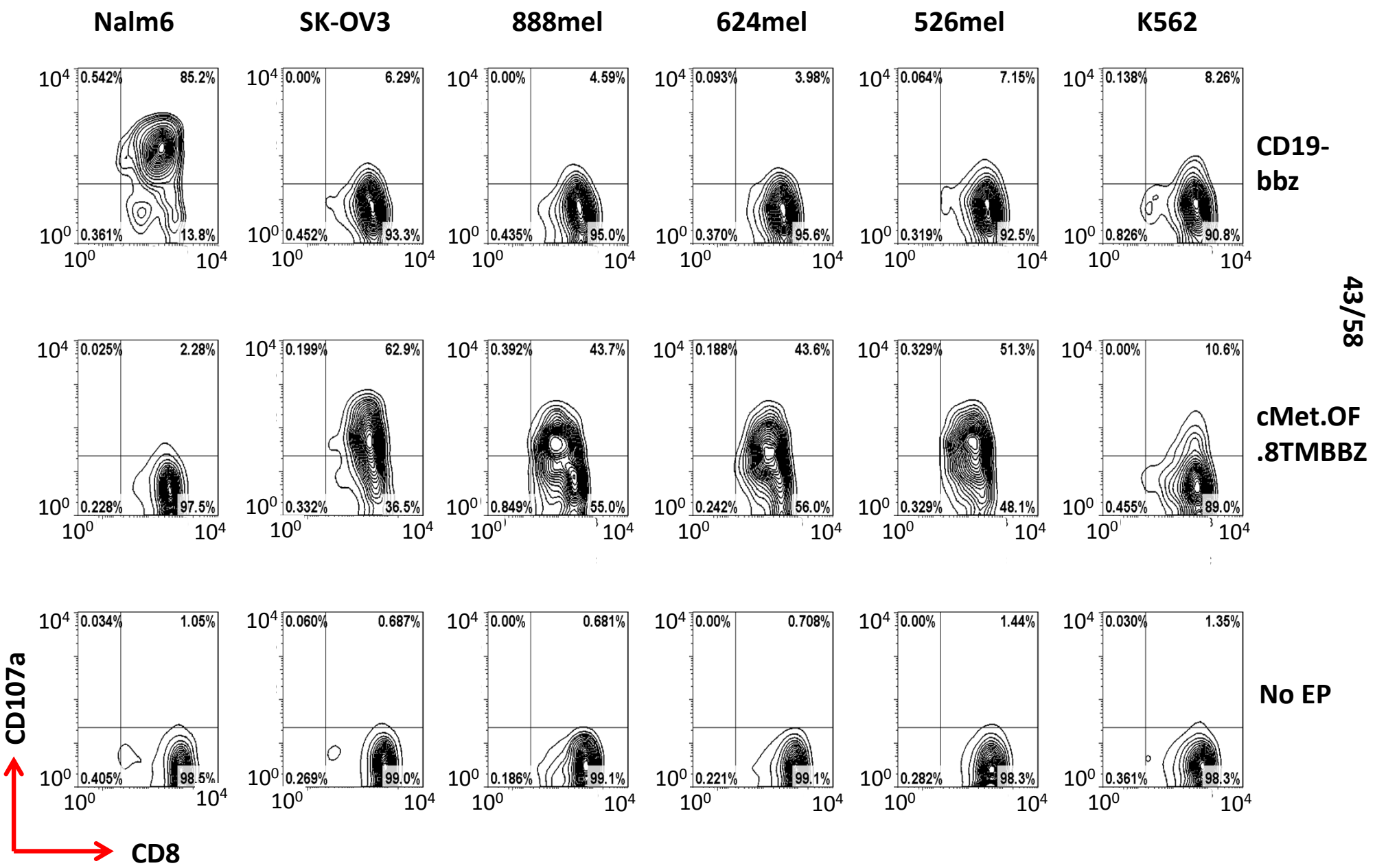

Nalm6
SK-OV3
888mel
624mel
526mel
K562
CD19-bbz
cMet.OF.8TMBBZ
No EP
CD107a
CD8
0.542% 85.2% 0.361% 13.8%
0.00% 6.29% 0.452% 93.3%
0.00% 4.59% 0.435% 95.0%
0.093% 3.98% 0.370% 95.6%
0.064% 7.15% 0.319% 92.5%
0.138% 8.26% 0.826% 90.8%
0.025% 2.28% 0.228% 97.5%
0.199% 62.9% 0.332% 36.5%
0.392% 43.7% 0.849% 55.0%
0.188% 43.6% 0.242% 56.0%
0.329% 51.3% 0.329% 48.1%
0.00% 10.6% 0.455% 89.0%
0.034% 1.05% 0.405% 98.5%
0.060% 0.687% 0.269% 99.0%
0.00% 0.681% 0.186% 99.1%
0.00% 0.708% 0.221% 99.1%
0.00% 1.44% 0.282% 98.3%
0.030% 1.35% 0.361% 98.3%

Figure 35

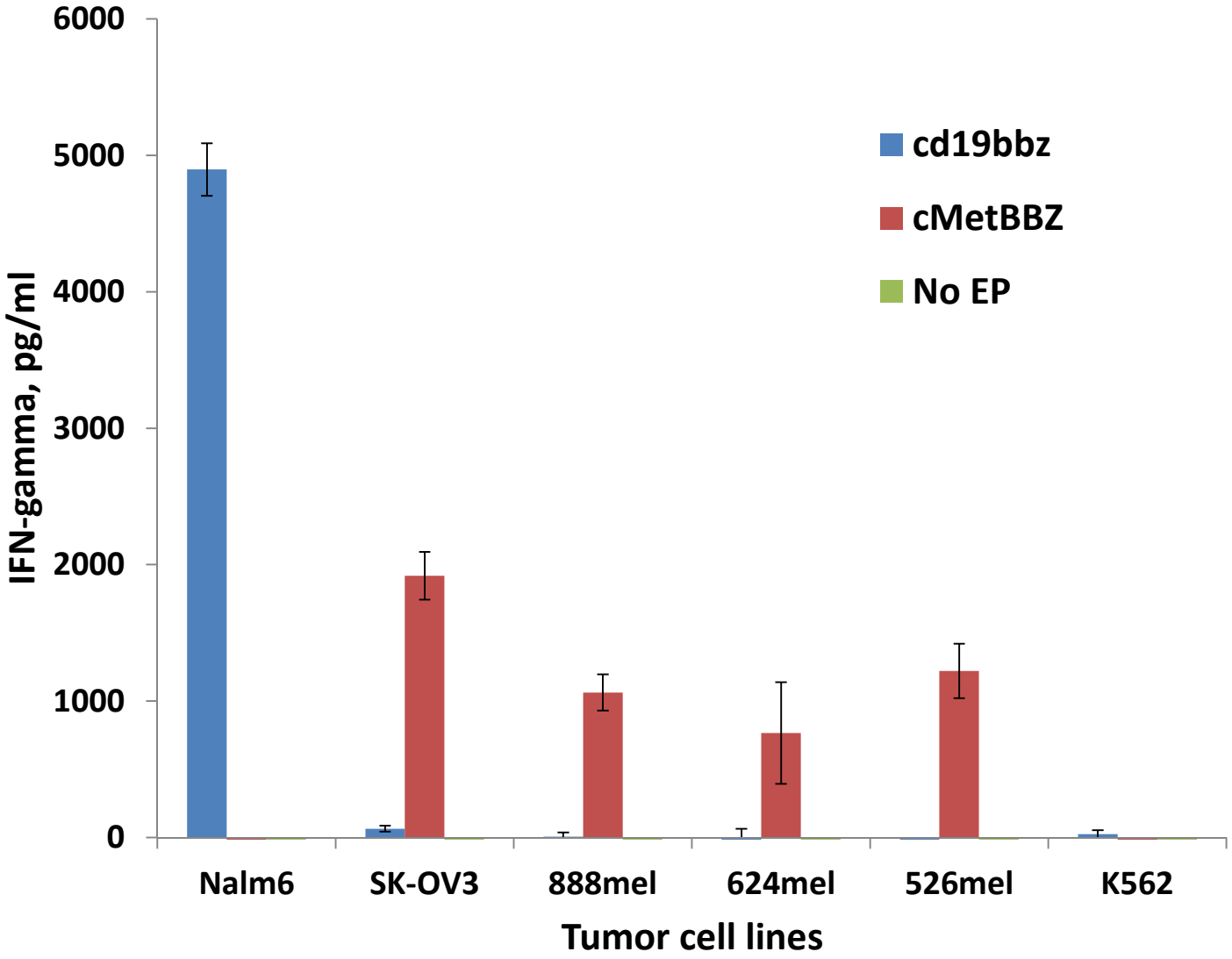

6000
5000
4000
3000
2000
1000
0
IFN-gamma, pg/ml
cd19bbz
cMetBBZ
No EP
Nalm6
SK-OV3
888mel
624mel
526mel
K562
Tumor cell lines

Figure 36

SKOV3 epithelial ovarian carcinoma cells (wild type cells express c-MET)

Group	Mice (#)	Tumor Cells s.c.	Treatment i.t. Injection T cells
cMET CAR + Cytoxan	9	SKOV3-Luc	cMET CAR + Cytoxan
CD19CAR + Cytoxan	9	SKOV3-Luc	CD19 CAR + Cytoxan
PBS	7	SKOV3-Luc	PBS

Experimental design

Establish vascularized flank xenograft tumors with SkOV3/luc tumors on day 0 in female NSG mice (n=7 to 9 mice per group)

Treatment beginning at week 6, 7, 9 and 11: 1x10e7 CAR cells injected IT
Cytoxan i.p. injection 24 hour before treatment at weeks 7, 9 and 11

Endpoints: survival and tumor burden (bioluminescence)

Figure 37

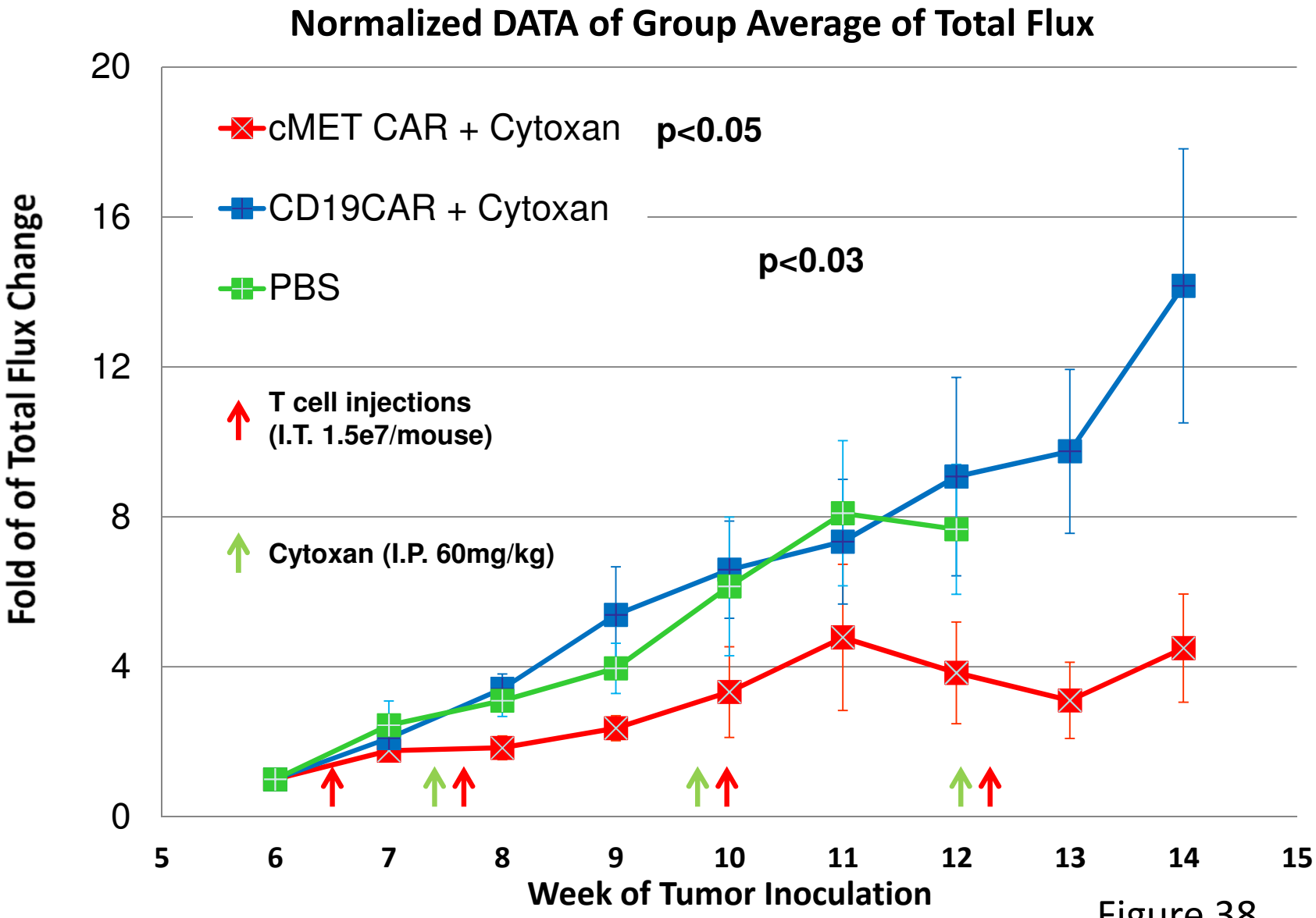

Normalized DATA of Group Average of Total Flux
cMET CAR + Cytoxan
p<0.05
CD19CAR + Cytoxan
p<0.03
PBS
T cell injections
(I.T. 1.5e7/mouse)
Cytoxan (I.P. 60mg/kg)
Fold of of Total Flux Change
Week of Tumor Inoculation
0
4
8
12
16
20
5
6
7
8
9
10
11
12
13
14
15

Figure 38

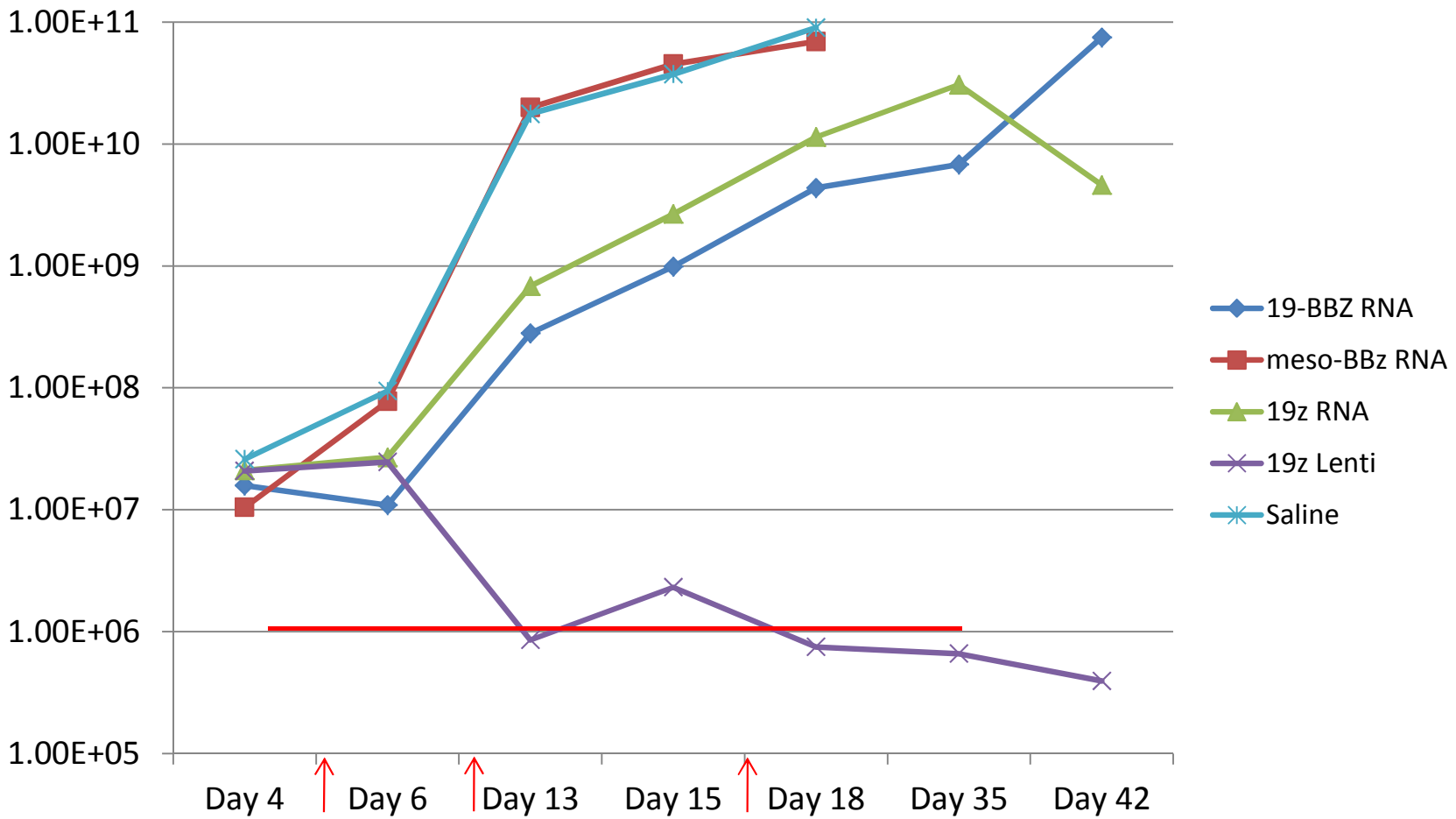
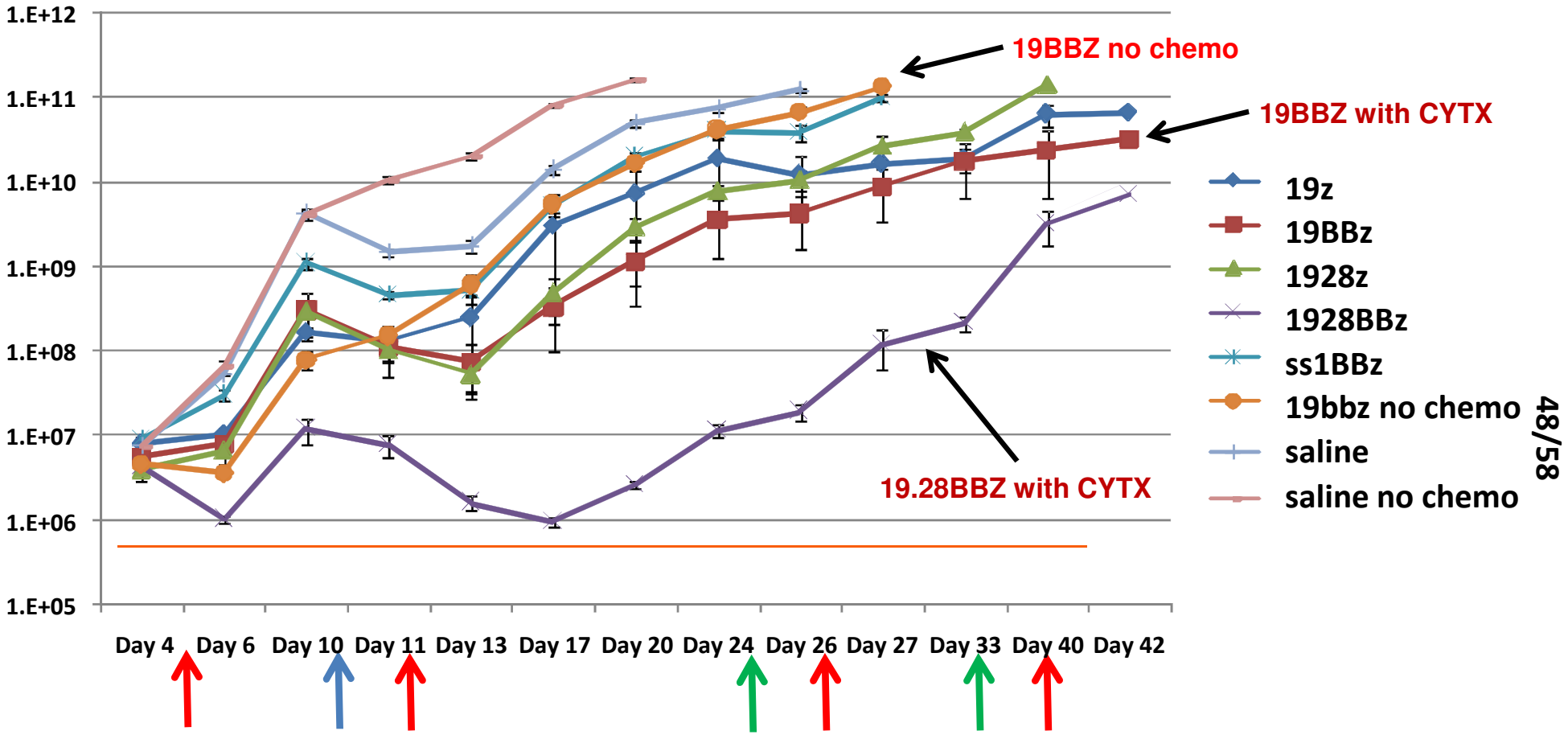

1.00E+11
1.00E+10
1.00E+09
1.00E+08
1.00E+07
1.00E+06
1.00E+05
Day 4
Day 6
Day 13
Day 15
Day 18
Day 35
Day 42
19-BBZ RNA
meso-BBz RNA
19z RNA
19z Lenti
Saline

Figure 39



10^7 CAR T cells

80 mg/kg CYTX

50 mg/kg CYTX

Figure 40

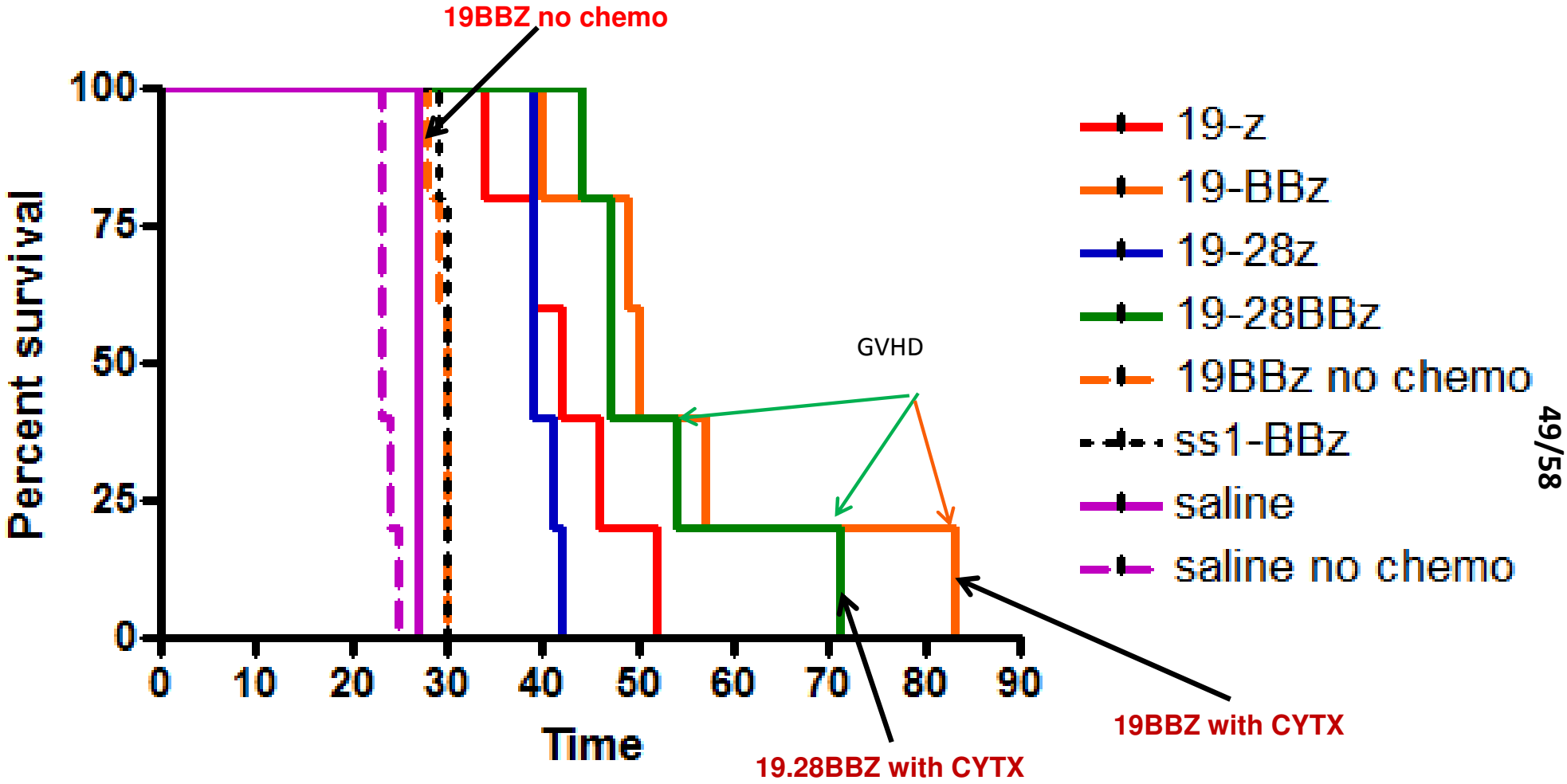

19BBZ no chemo
Percent survival
100
75
50
25
0
0
10
20
30
40
50
60
70
80
90
Time
GVHD
19-z
19-BBz
19-28z
19-28BBz
19BBz no chemo
ss1-BBz
saline
saline no chemo
19.28BBZ with CYTX
19BBZ with CYTX
49/58

Figure 41

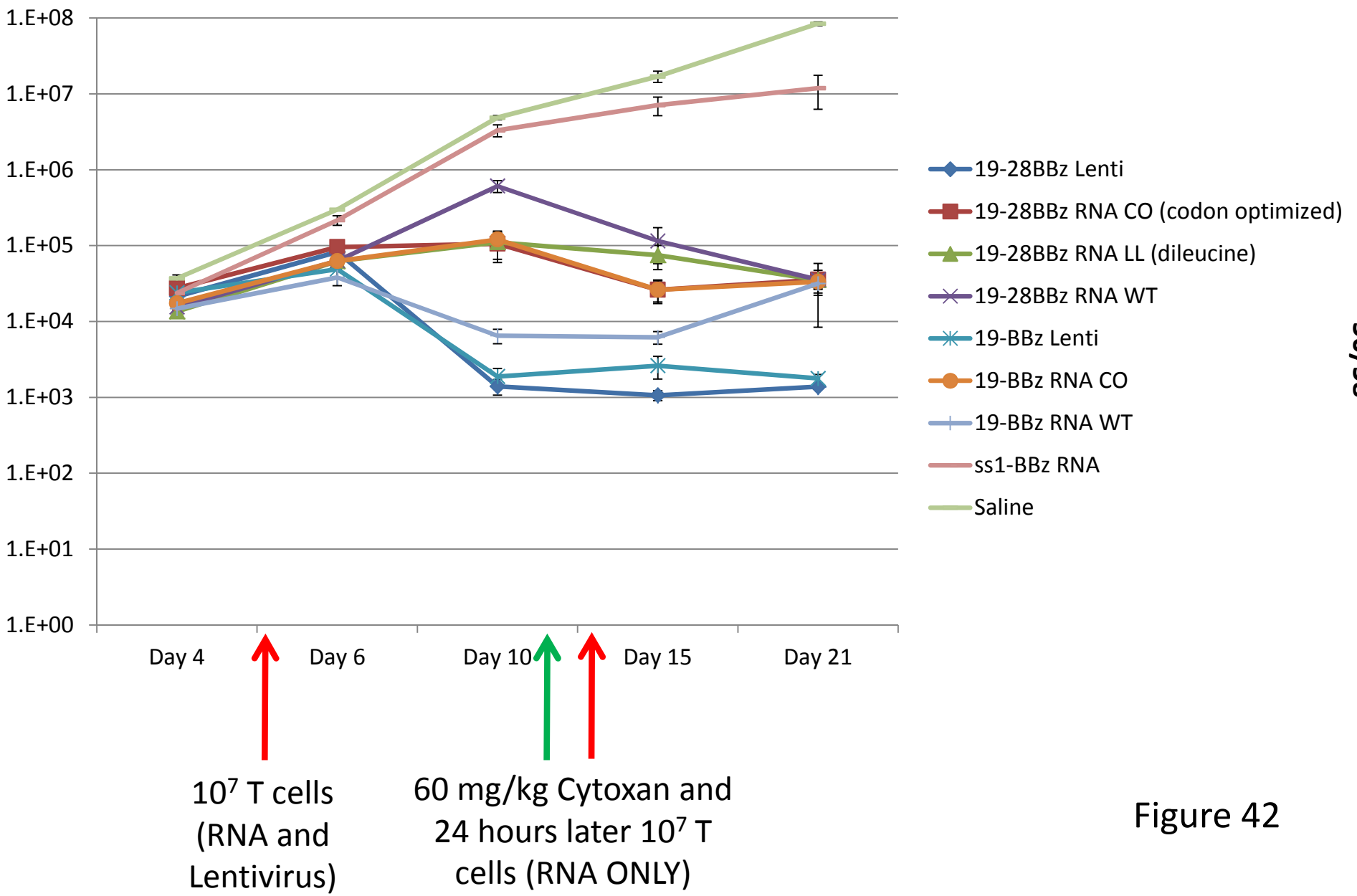

1.E+08
1.E+07
1.E+06
1.E+05
1.E+04
1.E+03
1.E+02
1.E+01
1.E+00
Day 4
Day 6
Day 10
Day 15
Day 21
19-28BBz Lenti
19-28BBz RNA CO (codon optimized)
19-28BBz RNA LL (dileucine)
19-28BBz RNA WT
19-BBz Lenti
19-BBz RNA CO
19-BBz RNA WT
ss1-BBz RNA
Saline
10⁷ T cells (RNA and Lentivirus)
60 mg/kg Cytoxan and 24 hours later 10⁷ T cells (RNA ONLY)

Figure 42

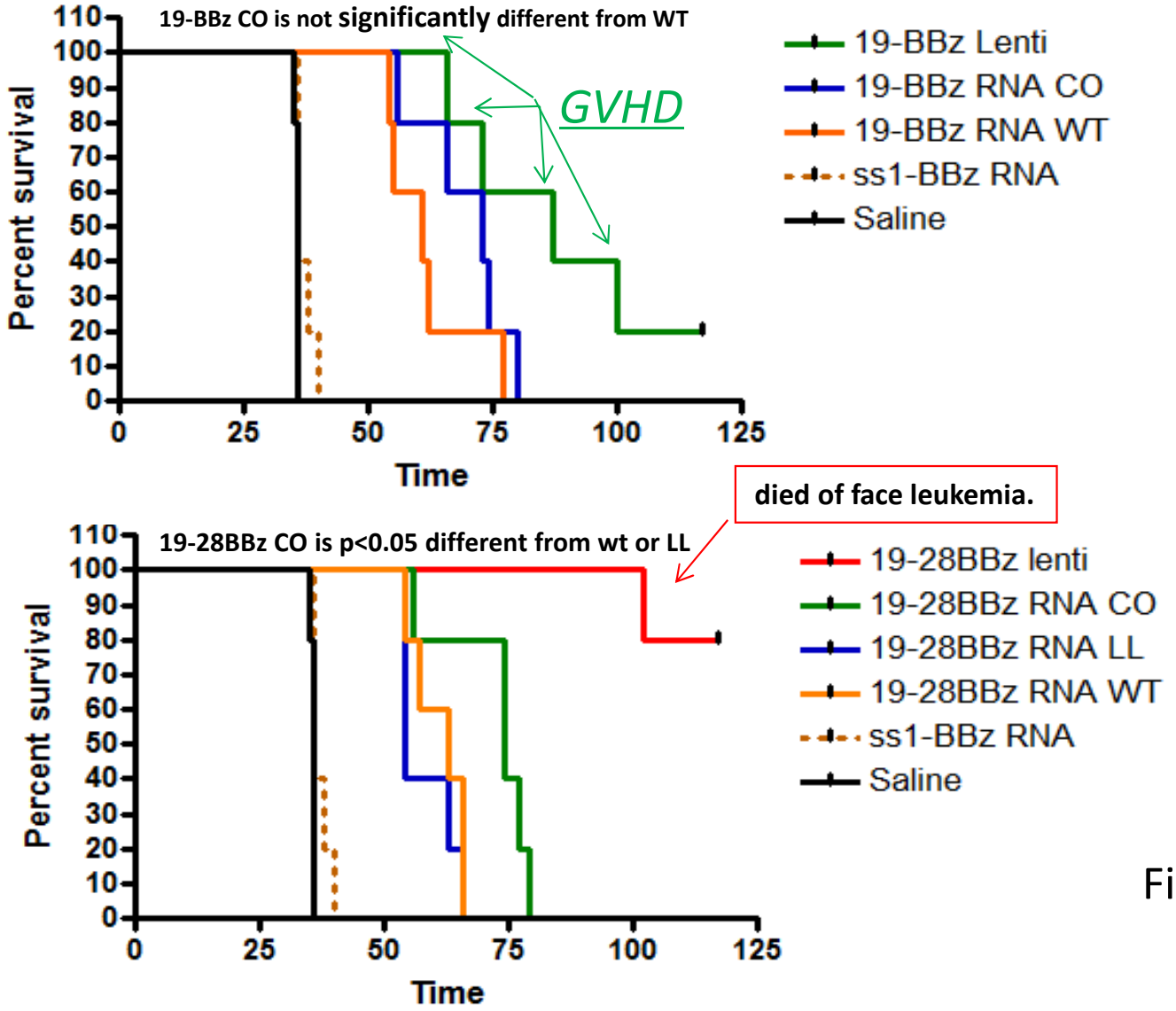
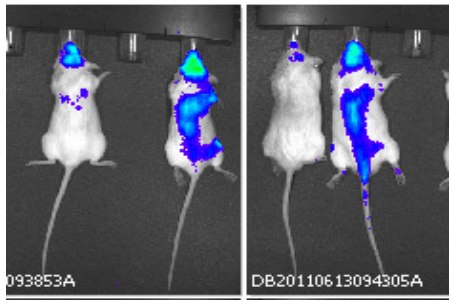

19-BBz CO is not significantly different from WT
GVHD
Percent survival
Time
19-BBz Lenti
19-BBz RNA CO
19-BBz RNA WT
ss1-BBz RNA
Saline
19-28BBz CO is p<0.05 different from wt or LL
died of face leukemia.
19-28BBz lenti
19-28BBz RNA CO
19-28BBz RNA LL
19-28BBz RNA WT
ss1-BBz RNA
Saline

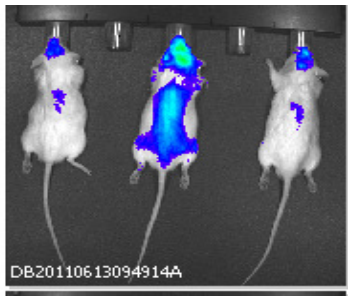
Figure 43



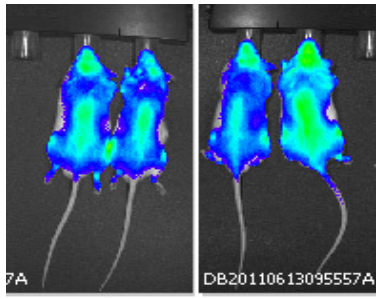
19-28BBz Lenti



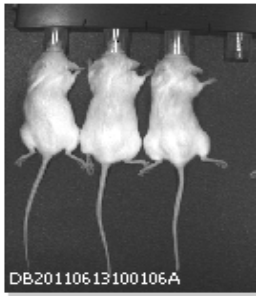
19-28BBz CO



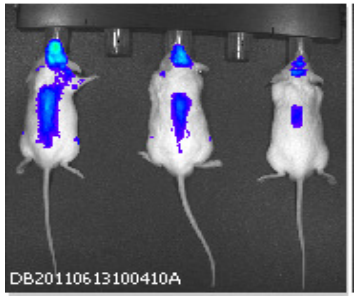
19-28BBz LL



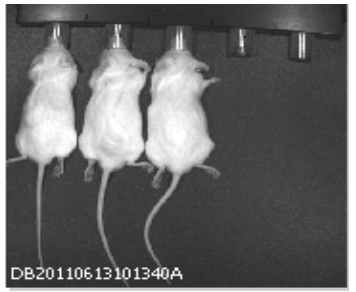
19-28BBz WT



19BBz Lenti

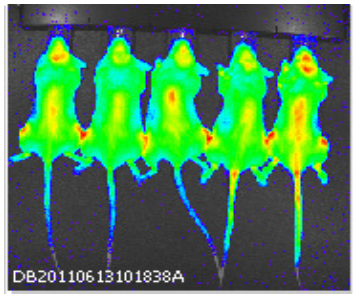


19BBz CO



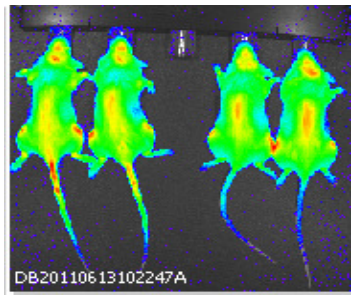
19BBz WT

Day 10 – 5 days post a single injection of 10^7 CAR T cells

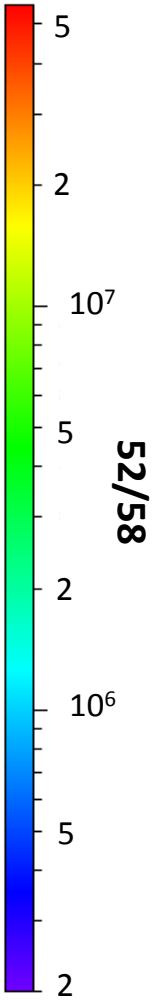


Meso BBz WT

Figure 44



Saline

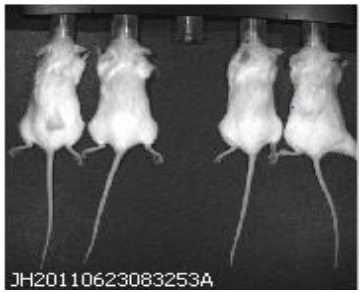


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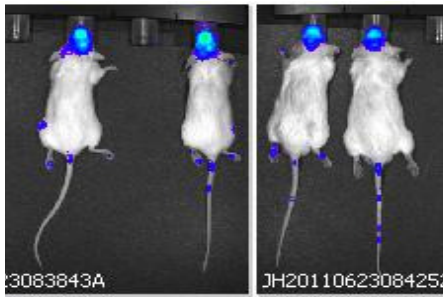
Radiance (p/s/cm²/sr)

Min = $2x10^5$

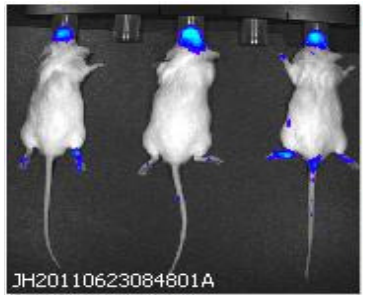
Max = $5.6x10^7$



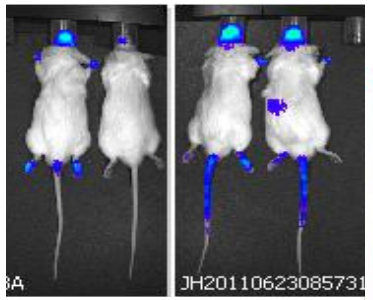
19-28BBz Lenti



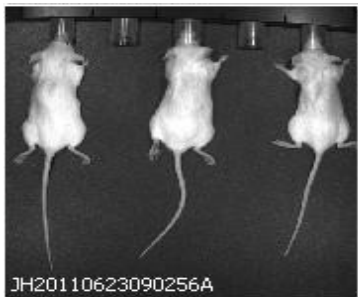
19-28BBz CO



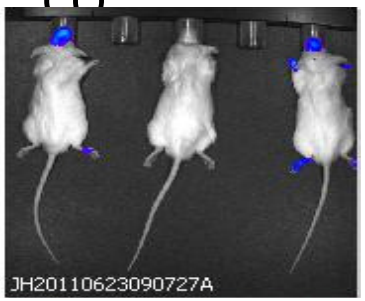
19-28BBz LL



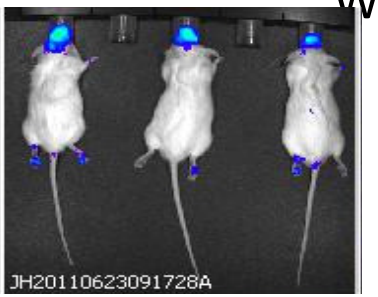
19-28BBz WT



19BBz Lenti

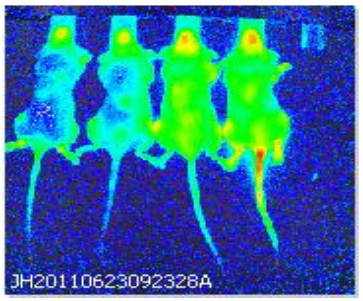


19BBz CO

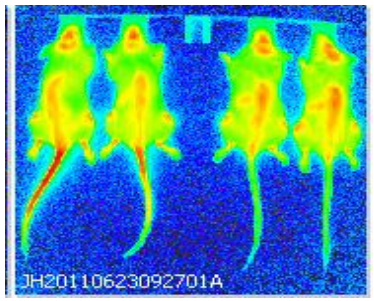


19BBz WT

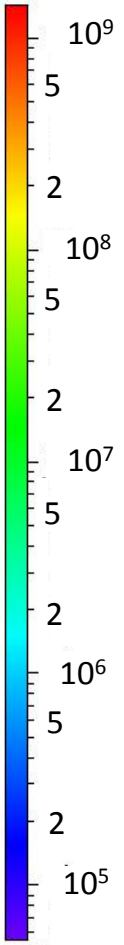
Day 21 – 7 days post cyclophosphamide and a second injection of 10^7 CAR T cells



Meso BBz WT



Saline



Radiance (p/s/cm²/sr)

Min = 5.47×10^4

Max = 1.44×10^9

Figure 45

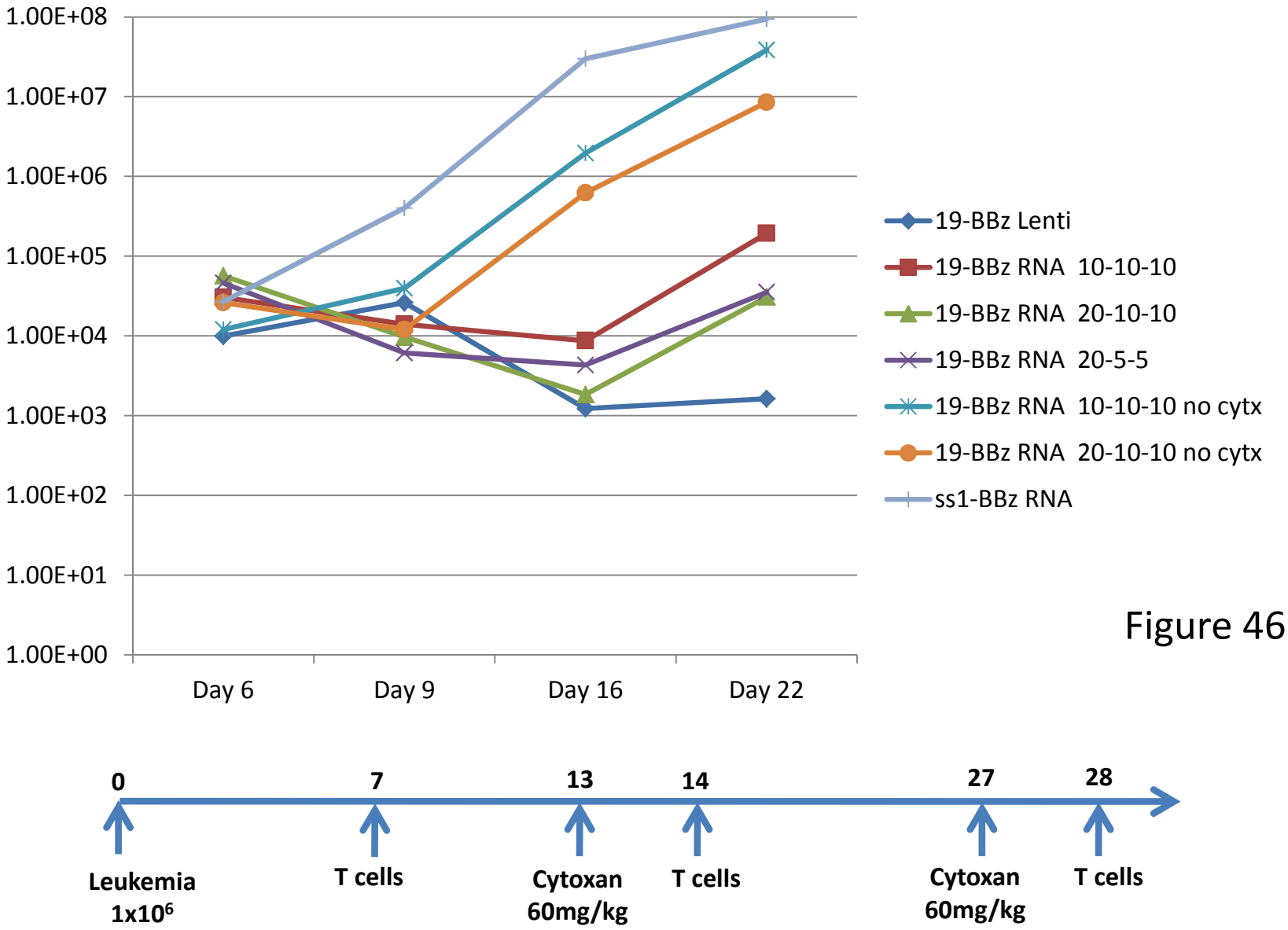
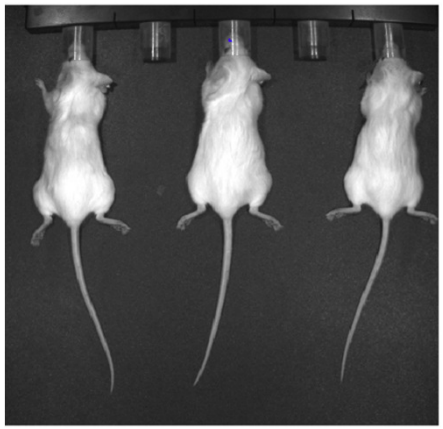
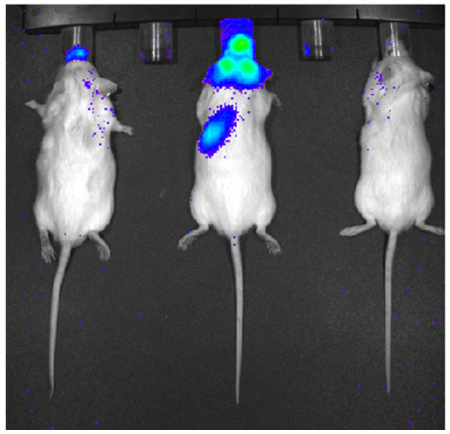

1.00E+08
1.00E+07
1.00E+06
1.00E+05
1.00E+04
1.00E+03
1.00E+02
1.00E+01
1.00E+00
Day 6
Day 9
Day 16
Day 22
19-BBz Lenti
19-BBz RNA 10-10-10
19-BBz RNA 20-10-10
19-BBz RNA 20-5-5
19-BBz RNA 10-10-10 no cytx
19-BBz RNA 20-10-10 no cytx
ss1-BBz RNA
0
7
13
14
27
28
Leukemia
1x10⁶
T cells
Cytoxan
60mg/kg
T cells
Cytoxan
60mg/kg
T cells

Figure 46

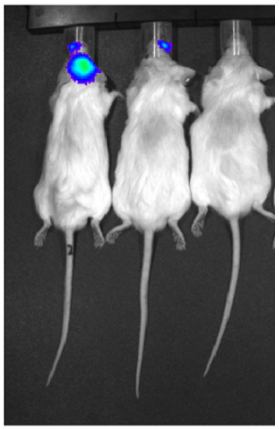
19-BBz lenti



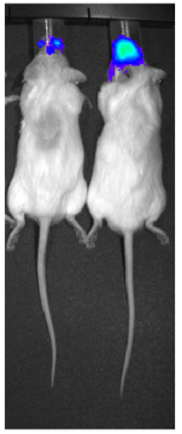
10-10-10



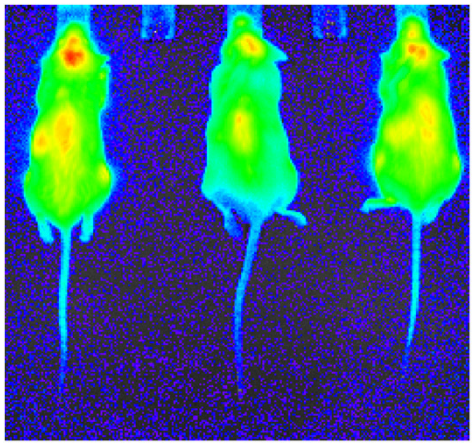
20-10-10



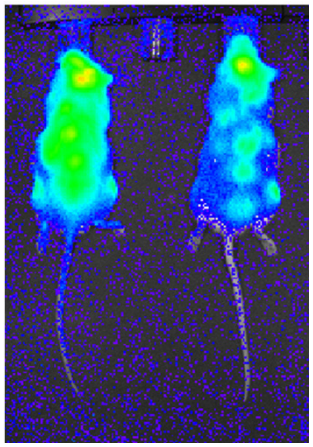
20-5-5



10-10-10 no



20-10-10 no



Ss1-BBz

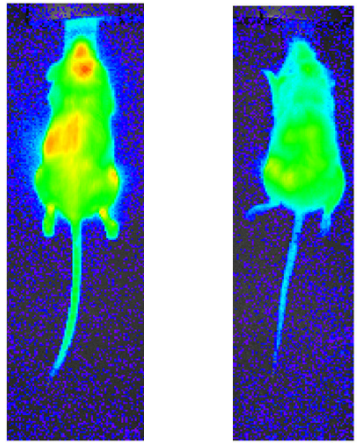


Figure 47

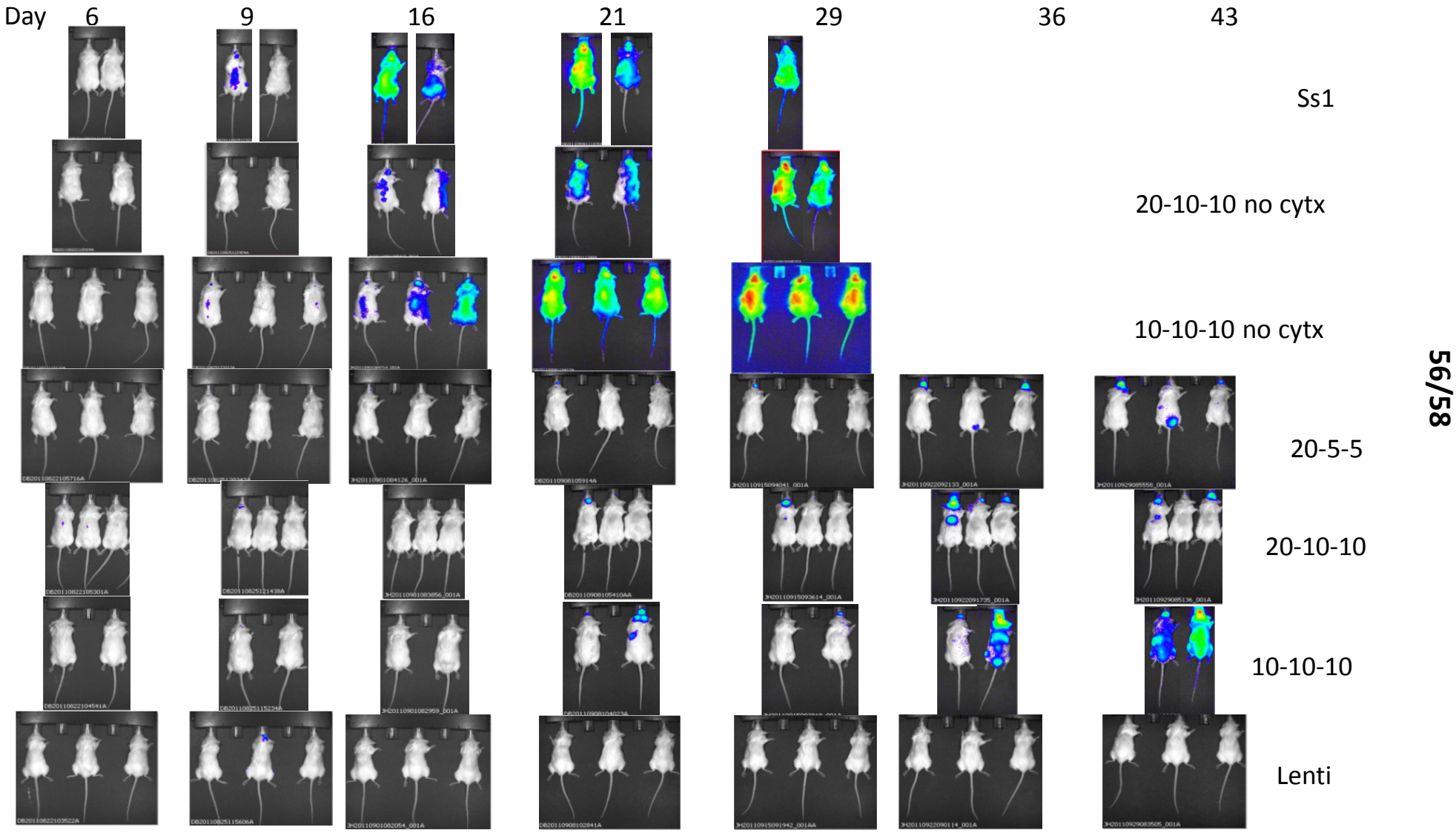

Day
6
9
16
21
29
36
43
Ss1
20-10-10 no cytx
10-10-10 no cytx
20-5-5
20-10-10
10-10-10
Lenti

Figure 48

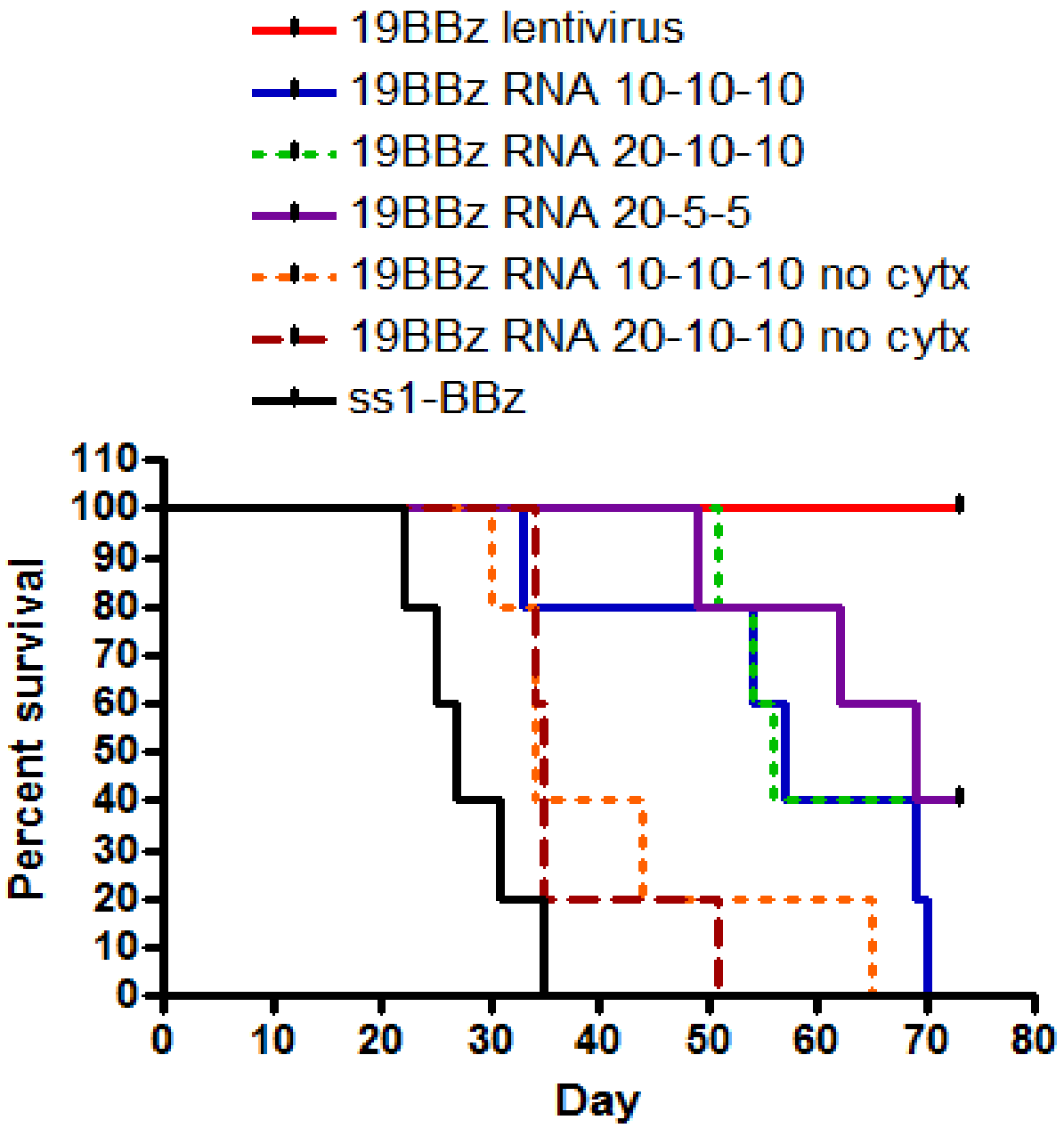

19BBz lentivirus
19BBz RNA 10-10-10
19BBz RNA 20-10-10
19BBz RNA 20-5-5
19BBz RNA 10-10-10 no cytx
19BBz RNA 20-10-10 no cytx
ss1-BBz
Percent survival
110
100
90
80
70
60
50
40
30
20
10
0
0
10
20
30
40
50
60
70
80
Day

Figure 49

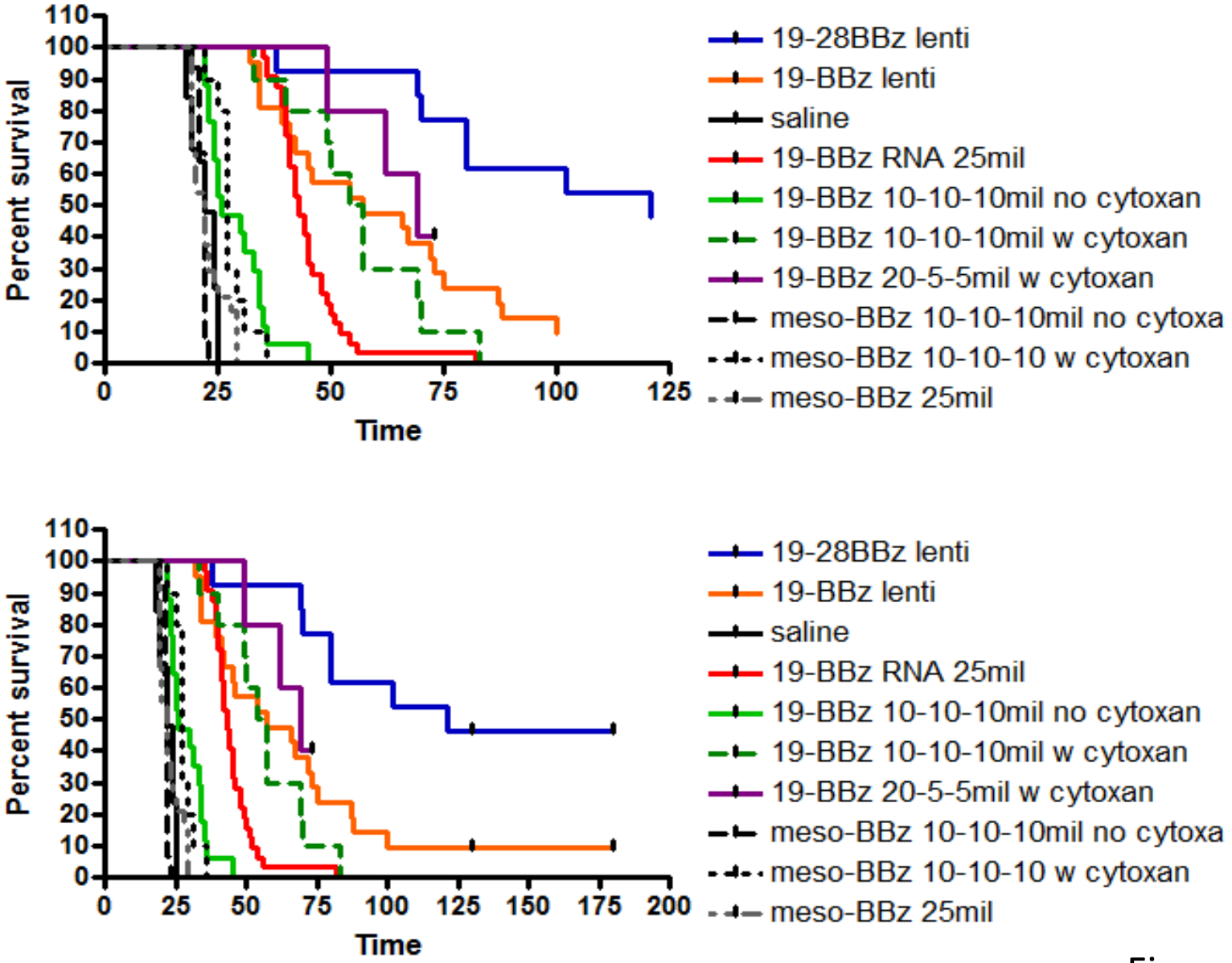

Percent survival
Time
0
25
50
75
100
125
150
175
200
10
20
30
40
50
60
70
80
90
100
110
19-28BBz lenti
19-BBz lenti
saline
19-BBz RNA 25mil
19-BBz 10-10-10mil no cytoxan
19-BBz 10-10-10mil w cytoxan
19-BBz 20-5-5mil w cytoxan
meso-BBz 10-10-10mil no cytoxa
meso-BBz 10-10-10 w cytoxan
meso-BBz 25mil

Figure 50