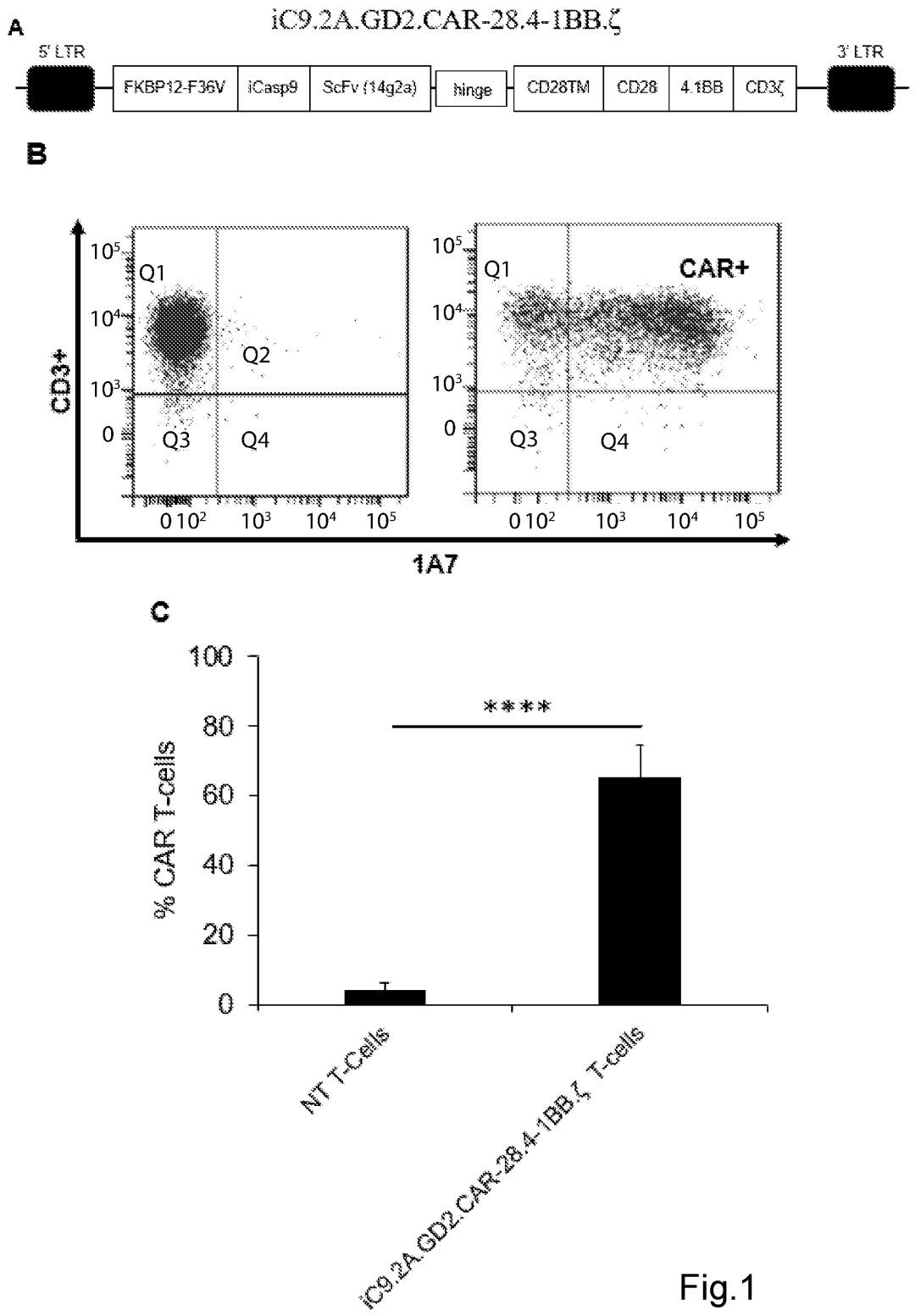
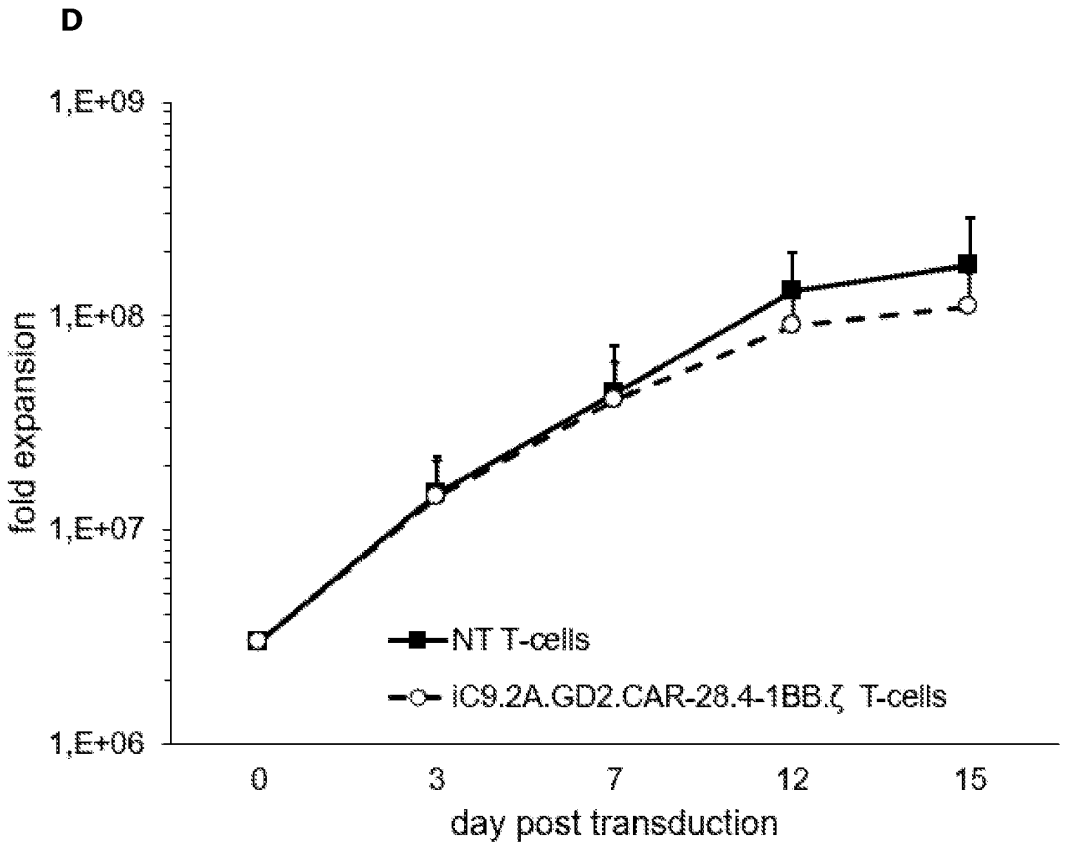

A
iC9.2A.GD2.CAR-28.4-1BB.ζ
5' LTR
FKBP12-F36V
iCasp9
ScFv (14g2a)
hinge
CD28TM
CD28
4.1BB
CD3ζ
3' LTR
B
CD3+
1A7
Q1
Q2
Q3
Q4
CAR+
C
% CAR T-cells
****
NT T-Cells
iC9.2A.GD2.CAR-28.4-1BB.ζ T-cells

Fig.1


D
1,E+09
1,E+08
1,E+07
1,E+06
fold expansion
NT T-cells
iC9.2A.GD2.CAR-28.4-1BB.ζ T-cells
0
3
7
12
15
day post transduction

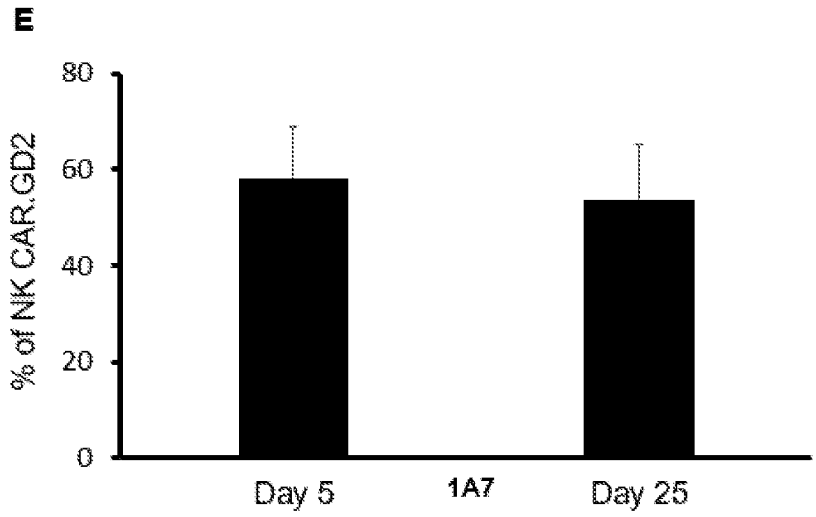

E
80
60
40
20
0
% of NK CAR.GD2
Day 5
1A7
Day 25

Fig.1

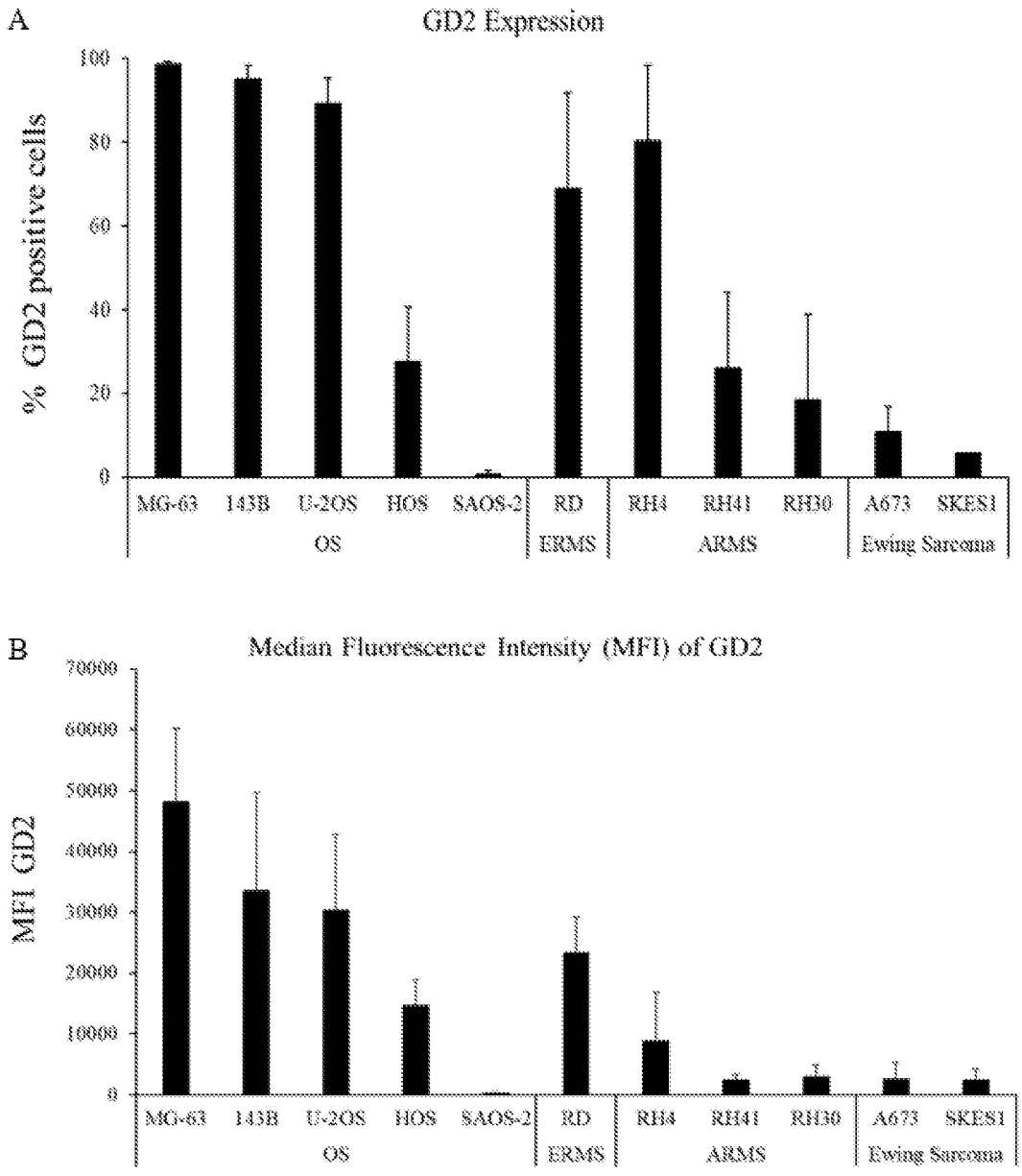
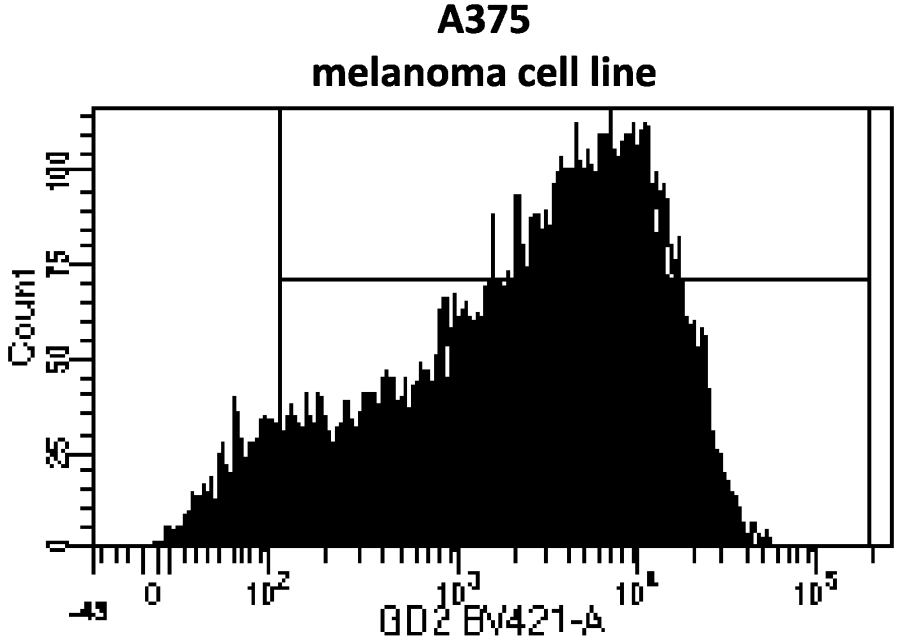
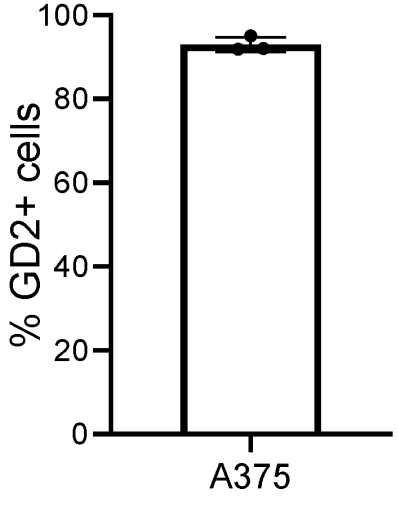

A
GD2 Expression
% GD2 positive cells
100
80
60
40
20
0
MG-63
143B
U-2OS
HOS
SAOS-2
RD
RH4
RH41
RH30
A673
SKES1
OS
ERMS
ARMS
Ewing Sarcoma
B
Median Fluorescence Intensity (MFI) of GD2
MFI GD2
70000
60000
50000
40000
30000
20000
10000
0
MG-63
143B
U-2OS
HOS
SAOS-2
RD
RH4
RH41
RH30
A673
SKES1
OS
ERMS
ARMS
Ewing Sarcoma

Fig.2

C


A375
melanoma cell line
Count
GD2 BV421-A

D


% GD2+ cells
A375

E

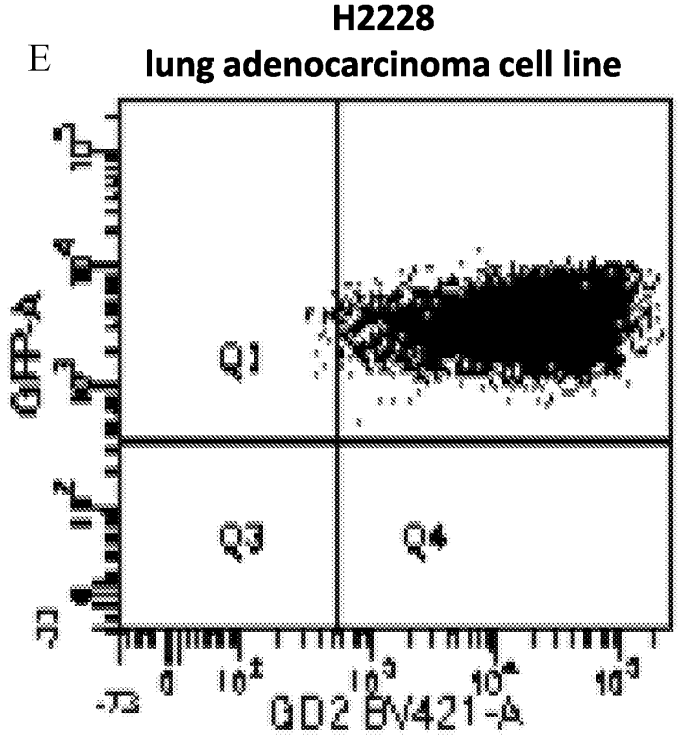
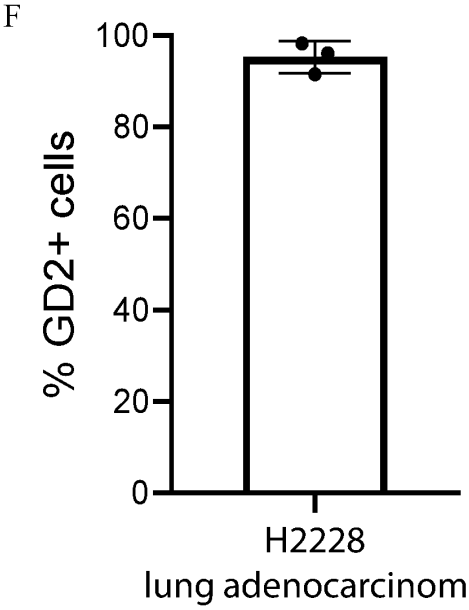

H2228
lung adenocarcinoma cell line
GRP-A
Q1
Q3
Q4
GD2 BV421-A

Fig.2

F


100
80
60
40
20
0
% GD2+ cells
H2228
lung adenocarcinoma

G

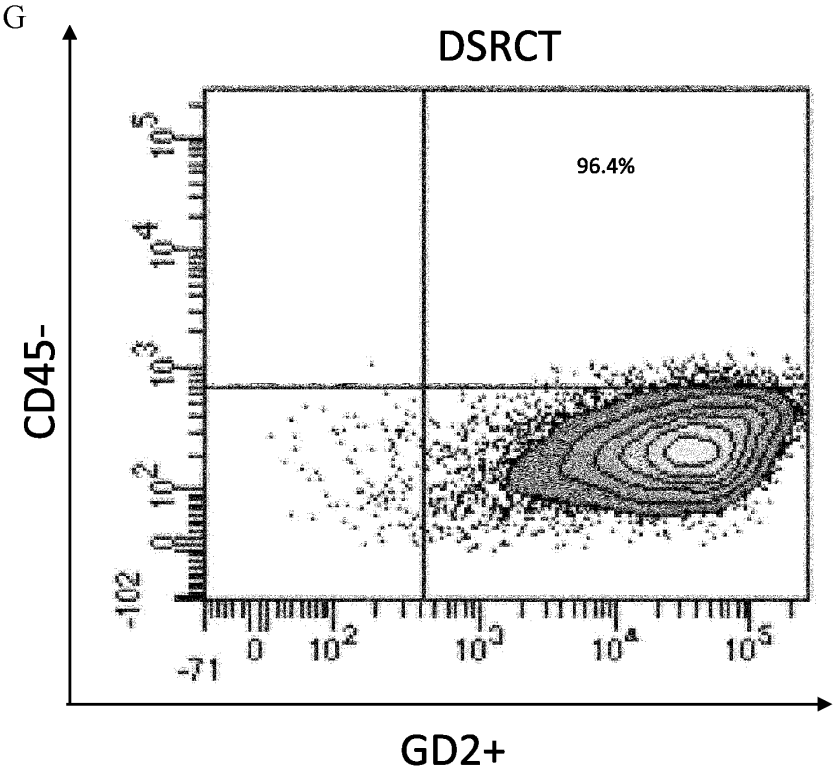

DSRCT
96.4%
CD45-
GD2+
-102
0
10^2
10^3
10^4
10^5
-71

Fig.2

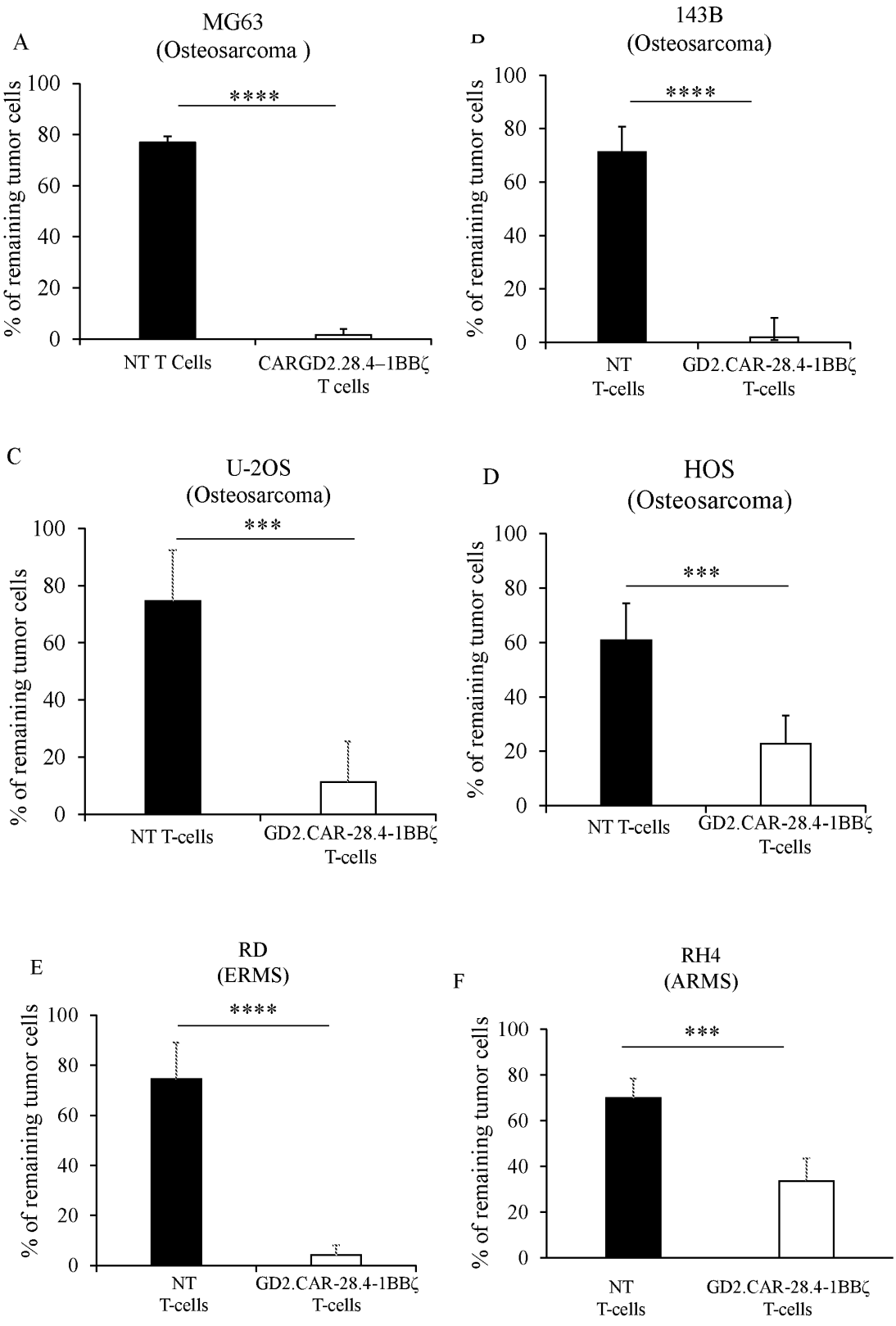

A
MG63
(Osteosarcoma)
% of remaining tumor cells
100
80
60
40
20
0
****
NT T Cells
CARGD2.28.4–1BBζ
T cells
B
143B
(Osteosarcoma)
% of remaining tumor cells
****
NT
T-cells
GD2.CAR-28.4-1BBζ
T-cells
C
U-2OS
(Osteosarcoma)
% of remaining tumor cells
***
NT T-cells
GD2.CAR-28.4-1BBζ
T-cells
D
HOS
(Osteosarcoma)
% of remaining tumor cells
***
NT T-cells
GD2.CAR-28.4-1BBζ
T-cells
E
RD
(ERMS)
% of remaining tumor cells
****
NT
T-cells
GD2.CAR-28.4-1BBζ
T-cells
F
RH4
(ARMS)
% of remaining tumor cells
***
NT
T-cells
GD2.CAR-28.4-1BBζ
T-cells

Fig.3

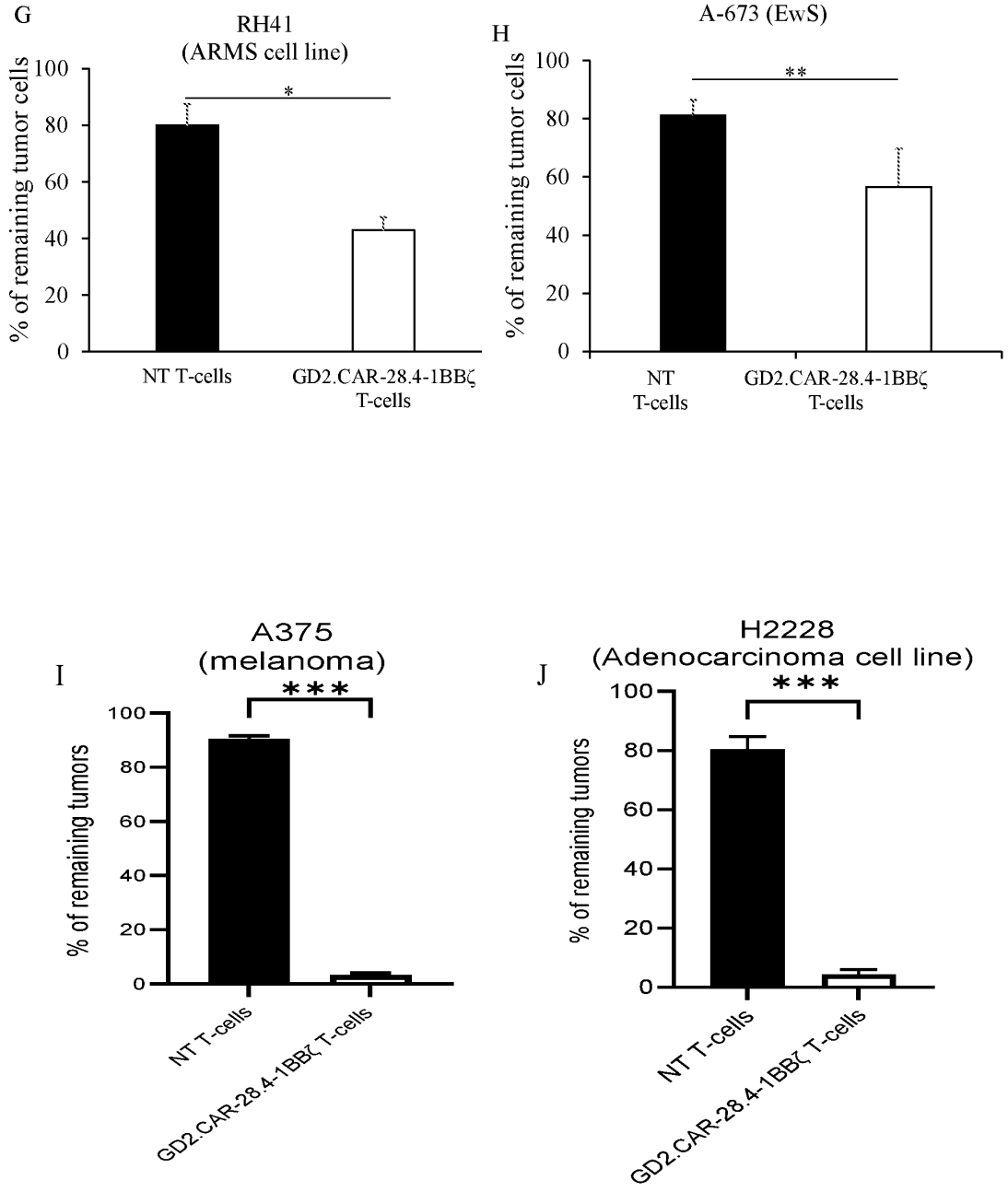

G
RH41
(ARMS cell line)
% of remaining tumor cells
100
80
60
40
20
0
*
NT T-cells
GD2.CAR-28.4-1BBζ
T-cells
H
A-673 (EwS)
% of remaining tumor cells
100
80
60
40
20
0
**
NT
T-cells
GD2.CAR-28.4-1BBζ
T-cells
I
A375
(melanoma)
***
% of remaining tumors
100
80
60
40
20
0
NT T-cells
GD2.CAR-28.4-1BBζ T-cells
J
H2228
(Adenocarcinoma cell line)
***
% of remaining tumors
100
80
60
40
20
0
NT T-cells
GD2.CAR-28.4-1BBζ T-cells

Fig.3

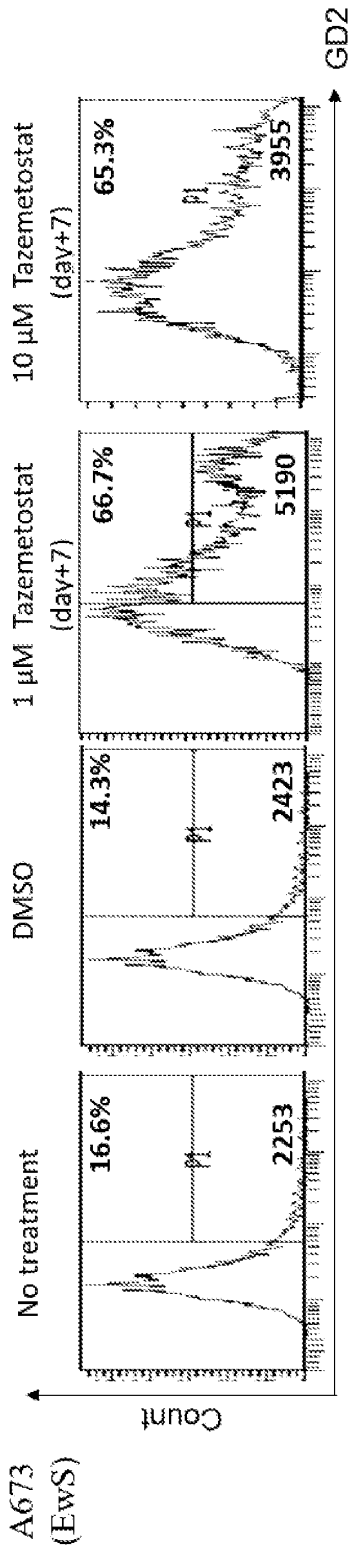

A673
(EwS)
Count
GD2
No treatment
DMSO
1 µM Tazemetostat
(day+7)
10 µM Tazemetostat
(day+7)
16.6%
2253
14.3%
2423
66.7%
5190
65.3%
3955
P1

Fig.4

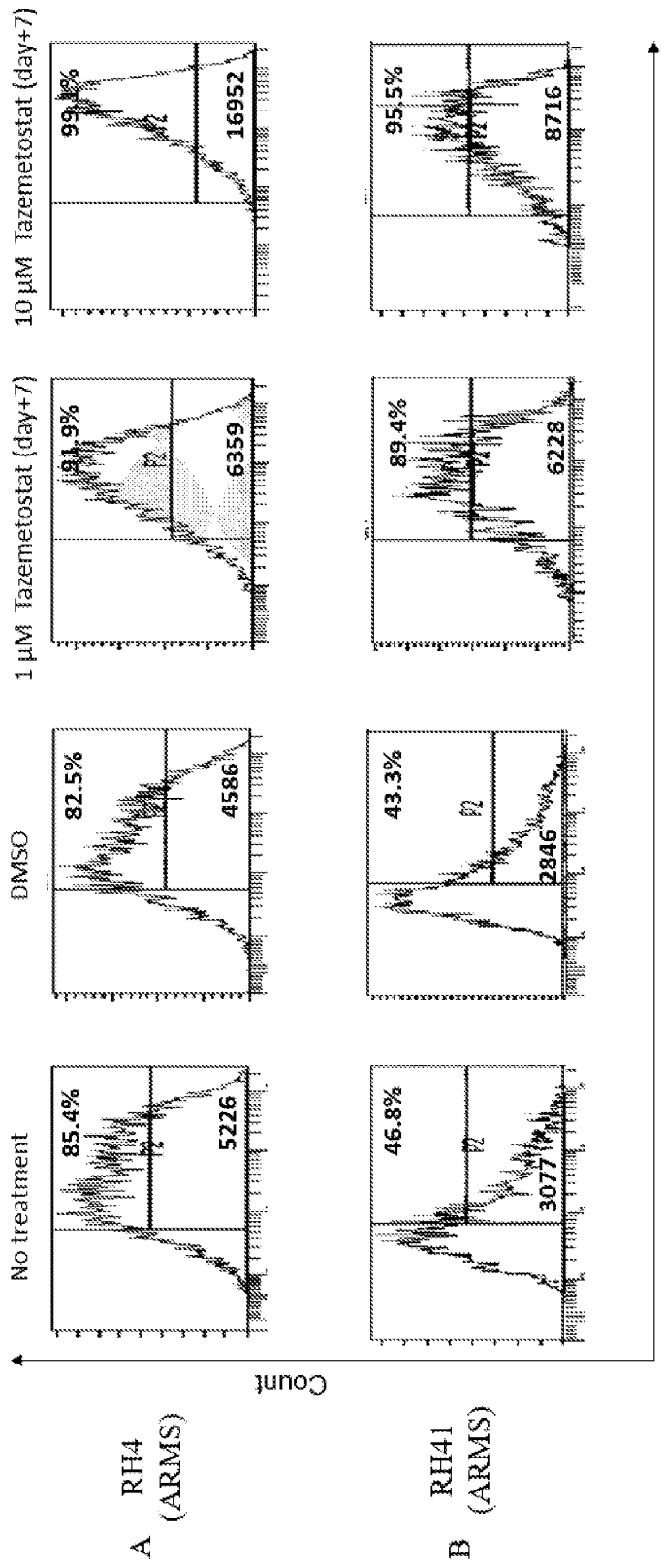

No treatment
DMSO
1 µM Tazemetostat (day+7)
10 µM Tazemetostat (day+7)
A RH4 (ARMS)
B RH41 (ARMS)
Count
GD2
85.4%
5226
82.5%
4586
91.9%
6359
99.8%
16952
46.8%
3077
43.3%
2846
89.4%
6228
95.5%
8716

Fig.5

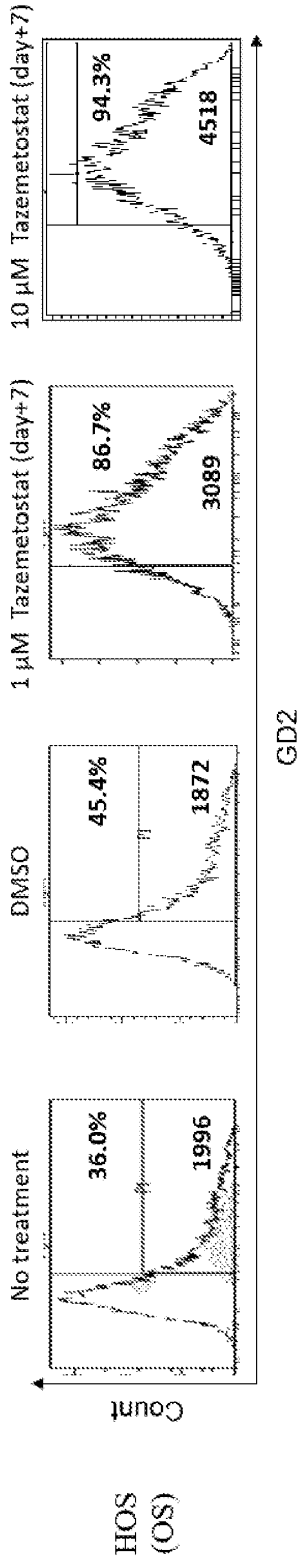

HOS (OS)
Count
GD2
No treatment
36.0%
1996
DMSO
45.4%
1872
1 µM Tazemetostat (day+7)
86.7%
3089
10 µM Tazemetostat (day+7)
94.3%
4518

Fig.6

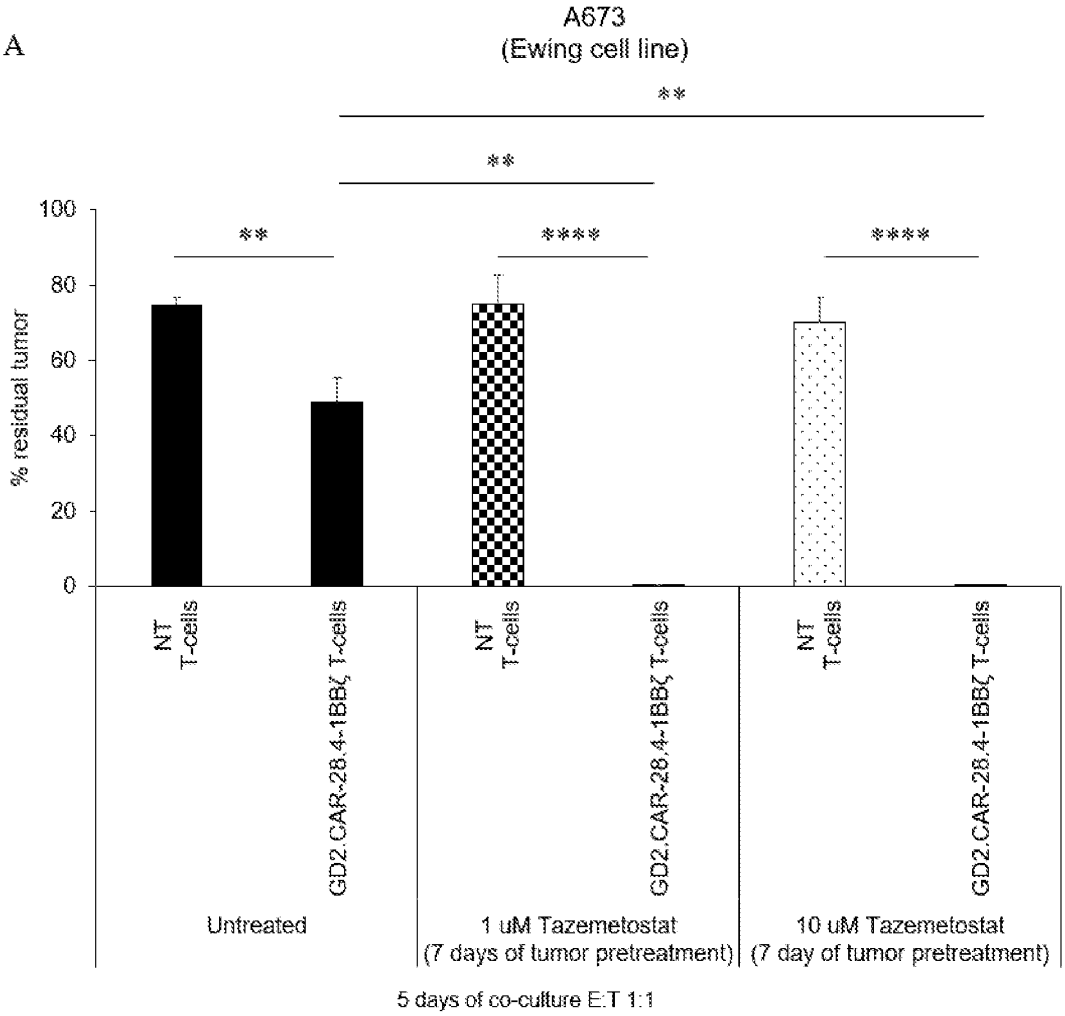

A
A673
(Ewing cell line)
**
**
**
****
****
100
80
60
40
20
0
% residual tumor
NT T-cells
GD2.CAR-28.4-1BBζ T-cells
NT T-cells
GD2.CAR-28.4-1BBζ T-cells
NT T-cells
GD2.CAR-28.4-1BBζ T-cells
Untreated
1 uM Tazemetostat
(7 days of tumor pretreatment)
10 uM Tazemetostat
(7 day of tumor pretreatment)
5 days of co-culture E:T 1:1

Fig.7

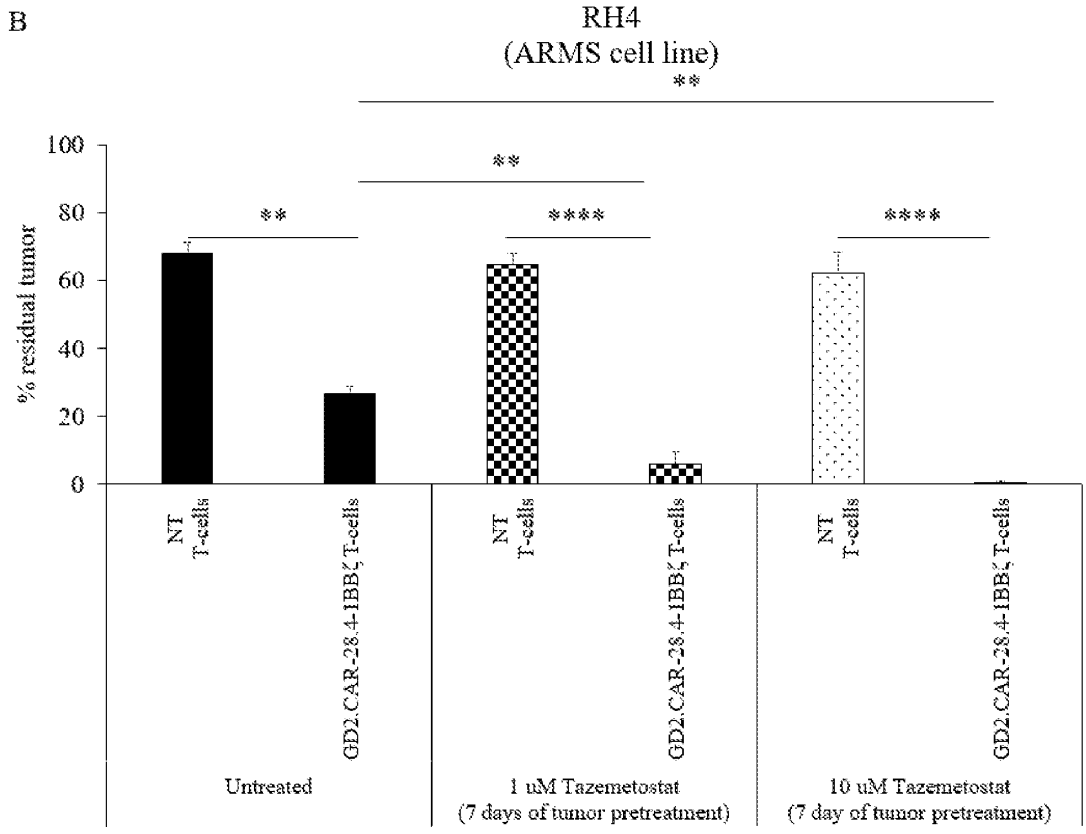

B
RH4
(ARMS cell line)
**
**
**
****
****
% residual tumor
100
80
60
40
20
0
NT T-cells
GD2.CAR-28.4-IBBζ T-cells
NT T-cells
GD2.CAR-28.4-IBBζ T-cells
NT T-cells
GD2.CAR-28.4-IBBζ T-cells
Untreated
1 uM Tazemetostat
(7 days of tumor pretreatment)
10 uM Tazemetostat
(7 day of tumor pretreatment)
5 days of co-culture E:T 1:1

Fig.7

C

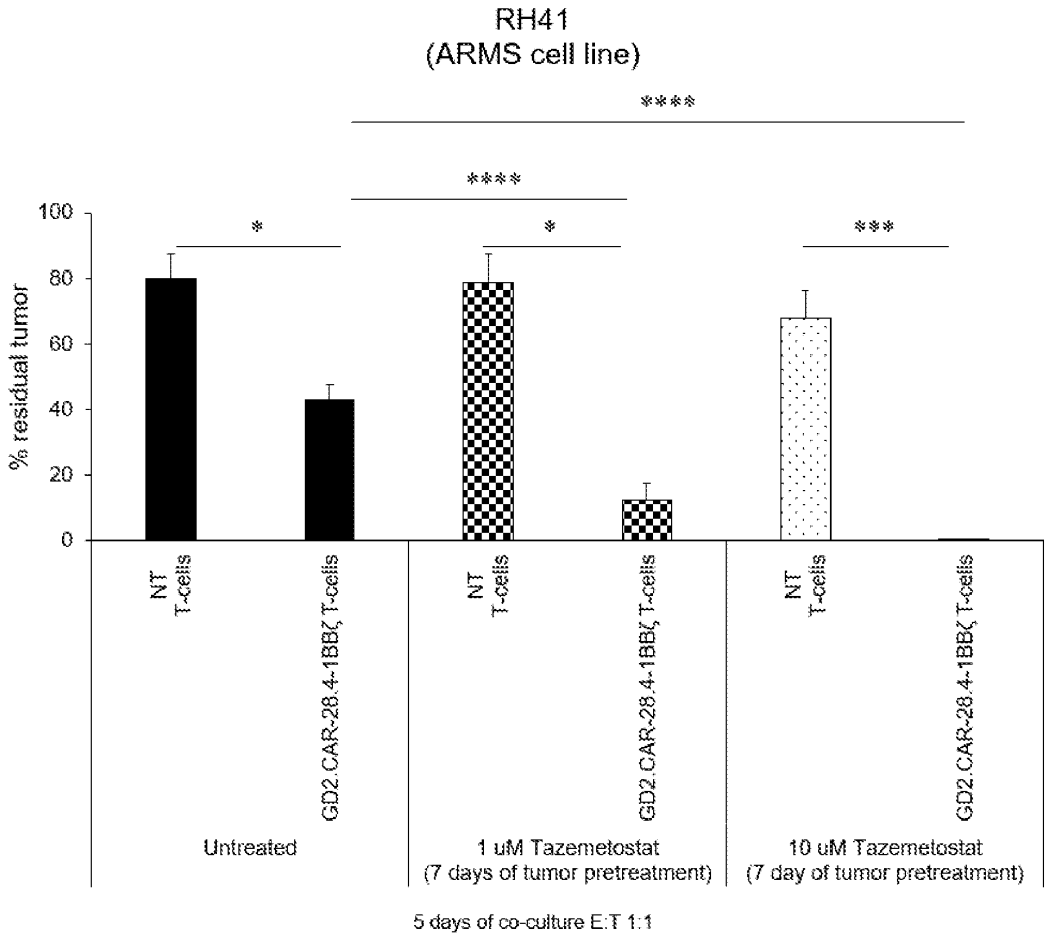

RH41
(ARMS cell line)
****
****
*
*
***
100
80
60
40
20
0
% residual tumor
NT T-cells
GD2.CAR-28.4-1BBζ T-cells
NT T-cells
GD2.CAR-28.4-1BBζ T-cells
NT T-cells
GD2.CAR-28.4-1BBζ T-cells
Untreated
1 uM Tazemetostat
(7 days of tumor pretreatment)
10 uM Tazemetostat
(7 day of tumor pretreatment)
5 days of co-culture E:T 1:1

Fig.7

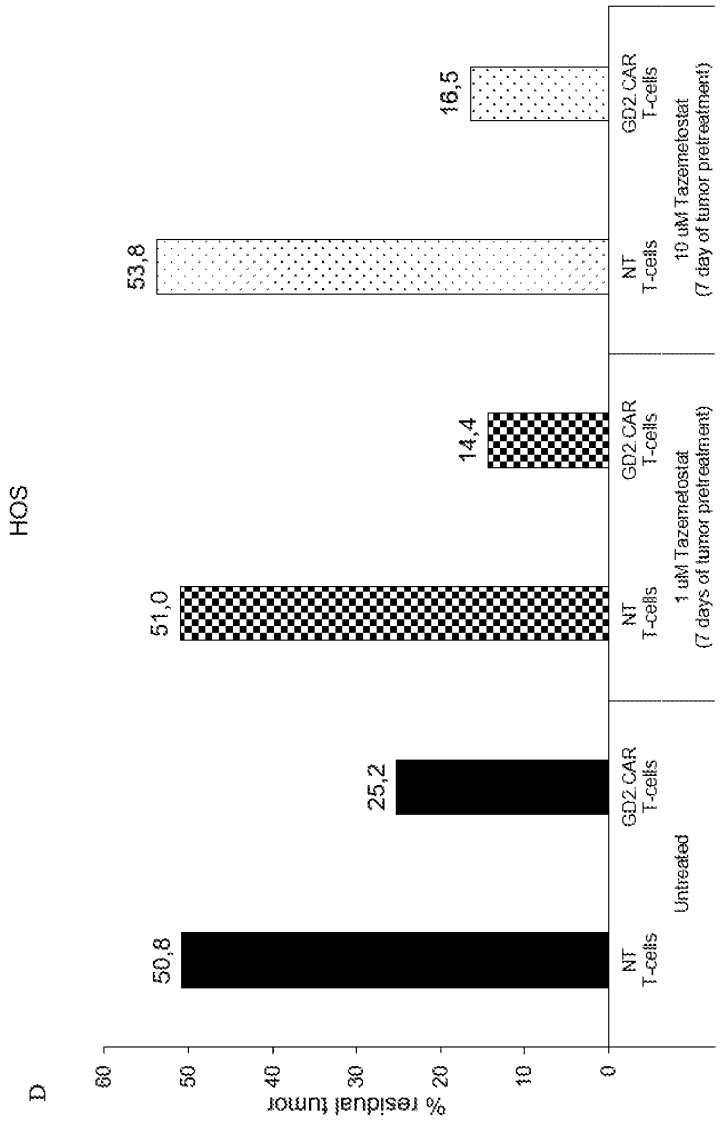
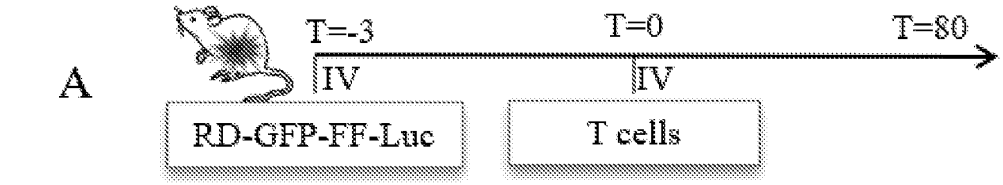
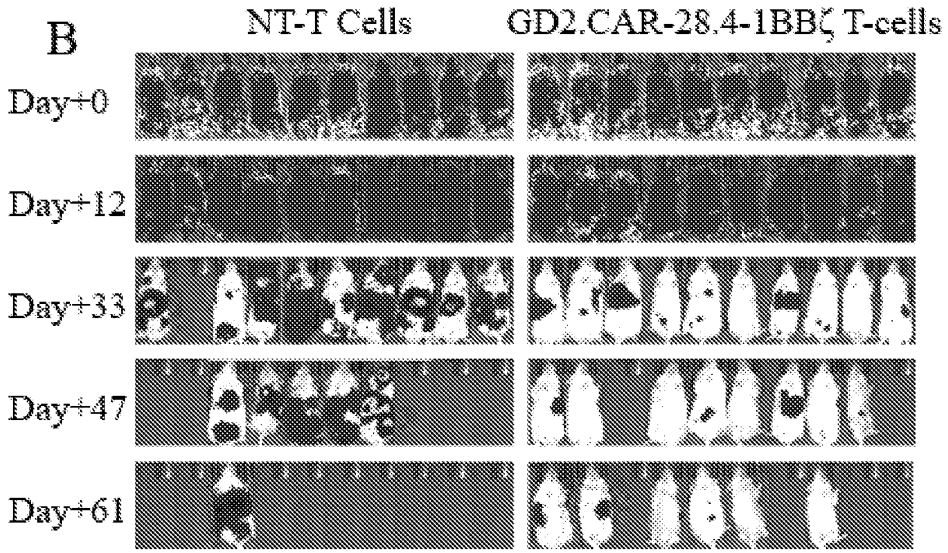

D
HOS
% residual tumor
60
50
40
30
20
10
0
50,8
25,2
51,0
14,4
53,8
16,5
NT T-cells
GD2.CAR T-cells
NT T-cells
GD2.CAR T-cells
NT T-cells
GD2.CAR T-cells
Untreated
1 uM Tazemetostat (7 days of tumor pretreatment)
10 uM Tazemetostat (7 day of tumor pretreatment)
5 days of co-culture E:T 1:1

Fig.7


A
T=-3
T=0
T=80
IV
IV
RD-GFP-FF-Luc
T cells


B
NT-T Cells
GD2.CAR-28.4-1BBζ T-cells
Day+0
Day+12
Day+33
Day+47
Day+61

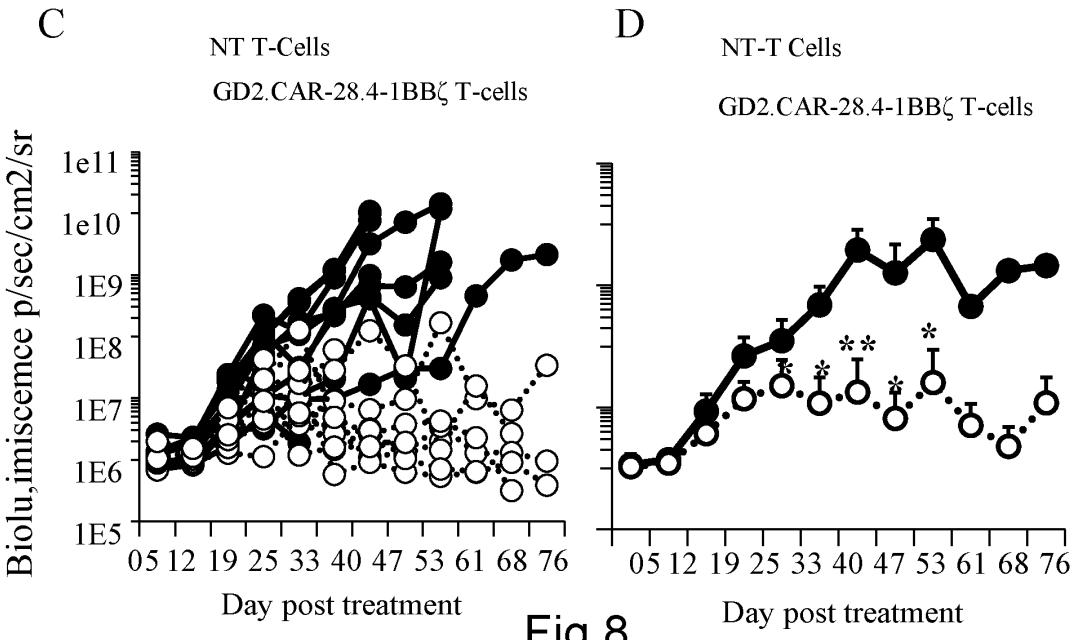

C
NT T-Cells
GD2.CAR-28.4-1BBζ T-cells
Biolu,imiscemce p/sec/cm2/sr
1e11
1e10
1E9
1E8
1E7
1E6
1E5
05 12 19 25 33 40 47 53 61 68 76
Day post treatment
D
NT-T Cells
GD2.CAR-28.4-1BBζ T-cells
05 12 19 25 33 40 47 53 61 68 76
Day post treatment

Fig.8

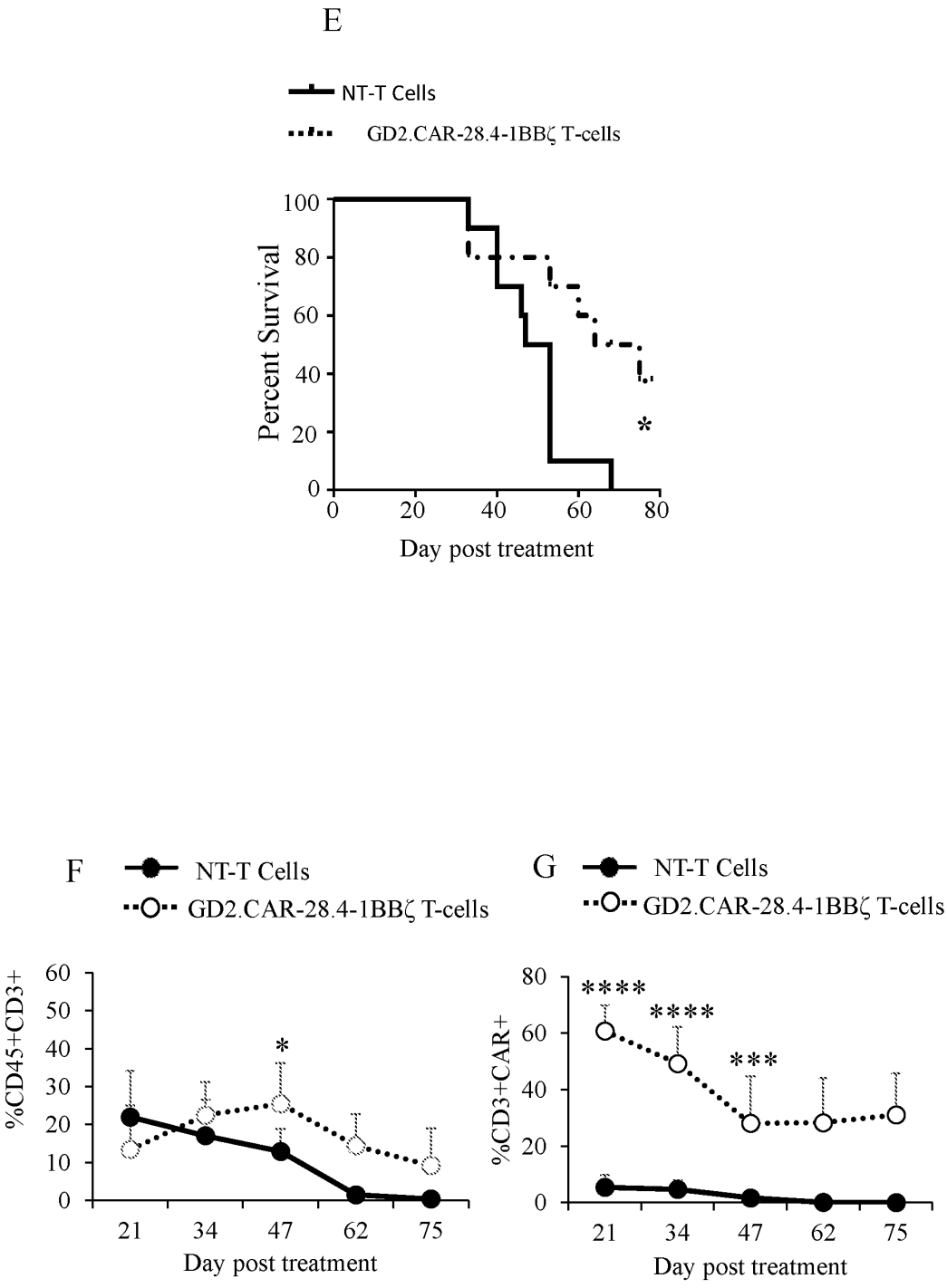

E
NT-T Cells
GD2.CAR-28.4-1BBζ T-cells
Percent Survival
100
80
60
40
20
0
0
20
40
60
80
*
Day post treatment
F
NT-T Cells
GD2.CAR-28.4-1BBζ T-cells
%CD45+CD3+
60
50
40
30
20
10
0
*
21
34
47
62
75
Day post treatment
G
NT-T Cells
GD2.CAR-28.4-1BBζ T-cells
%CD3+CAR+
80
60
40
20
0
****
****
***
21
34
47
62
75
Day post treatment

Fig.8

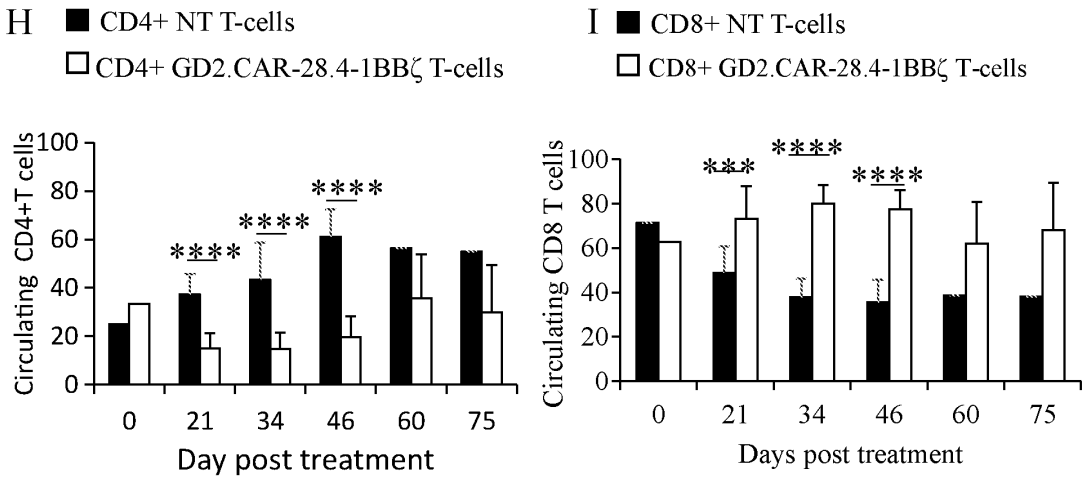

H
CD4+ NT T-cells
CD4+ GD2.CAR-28.4-1BBζ T-cells
Circulating CD4+T cells
100
80
60
40
20
0
****
****
****
0
21
34
46
60
75
Day post treatment
I
CD8+ NT T-cells
CD8+ GD2.CAR-28.4-1BBζ T-cells
Circulating CD8 T cells
100
80
60
40
20
0
***
****
****
0
21
34
46
60
75
Days post treatment

Fig.8

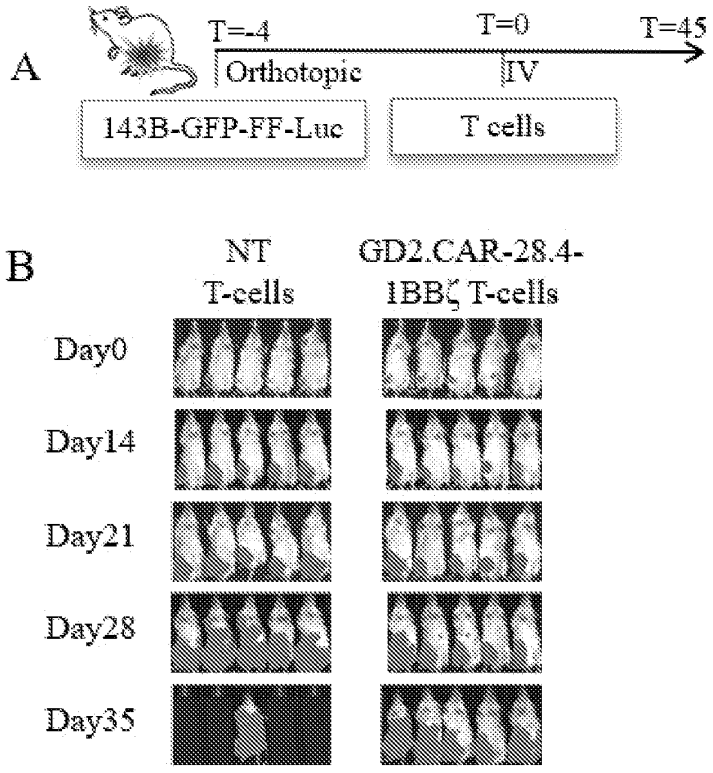
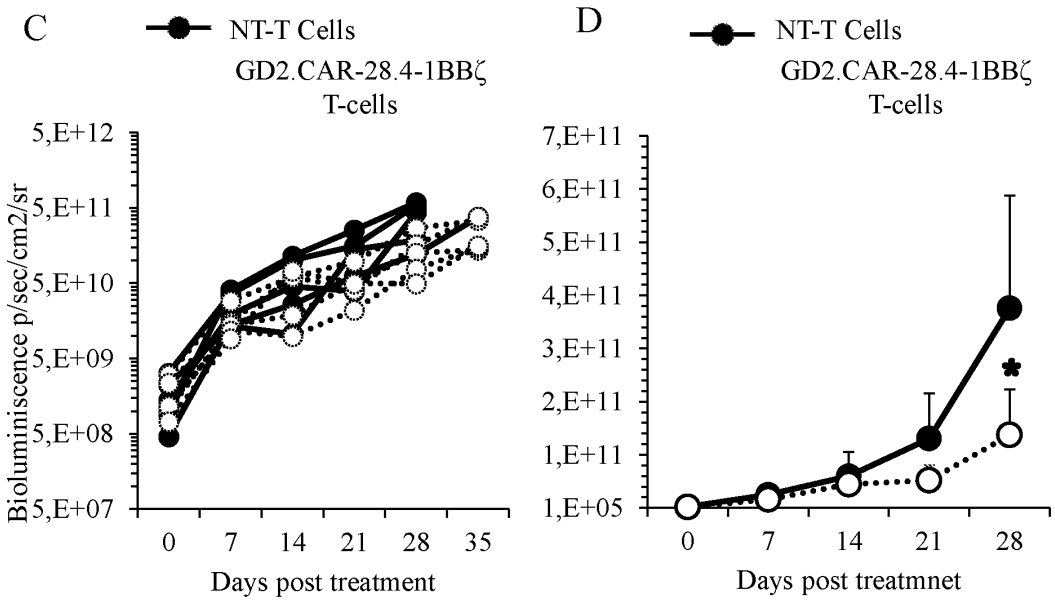

A
T=-4
T=0
T=45
Orthotopic
IV
143B-GFP-FF-Luc
T cells
B
NT
T-cells
GD2.CAR-28.4-
1BBζ T-cells
Day0
Day14
Day21
Day28
Day35

Fig.9


C
NT-T Cells
GD2.CAR-28.4-1BBζ
T-cells
Bioluminiscence p/sec/cm2/sr
5,E+12
5,E+11
5,E+10
5,E+09
5,E+08
5,E+07
0 7 14 21 28 35
Days post treatment
D
NT-T Cells
GD2.CAR-28.4-1BBζ
T-cells
7,E+11
6,E+11
5,E+11
4,E+11
3,E+11
2,E+11
1,E+11
1,E+05
0 7 14 21 28
Days post treatmnet
*

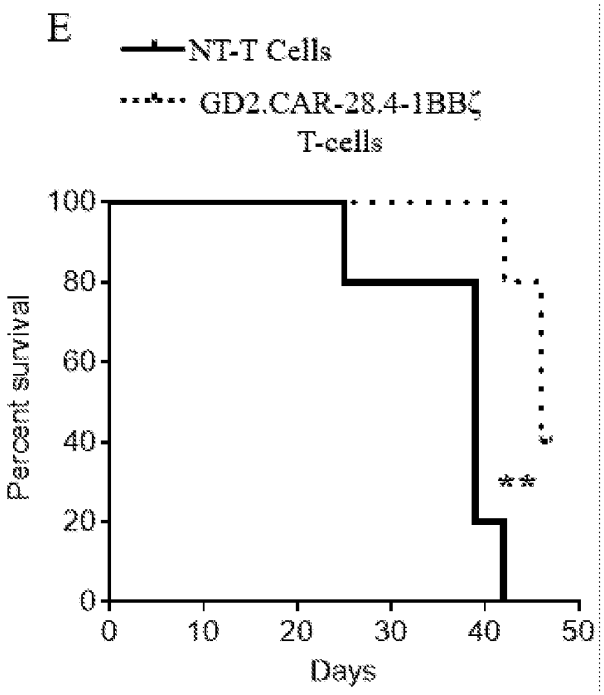

E
NT-T Cells
GD2.CAR-28.4-1BBζ
T-cells
Percent survival
100
80
60
40
20
0
0 10 20 30 40 50
Days
**

Fig.9

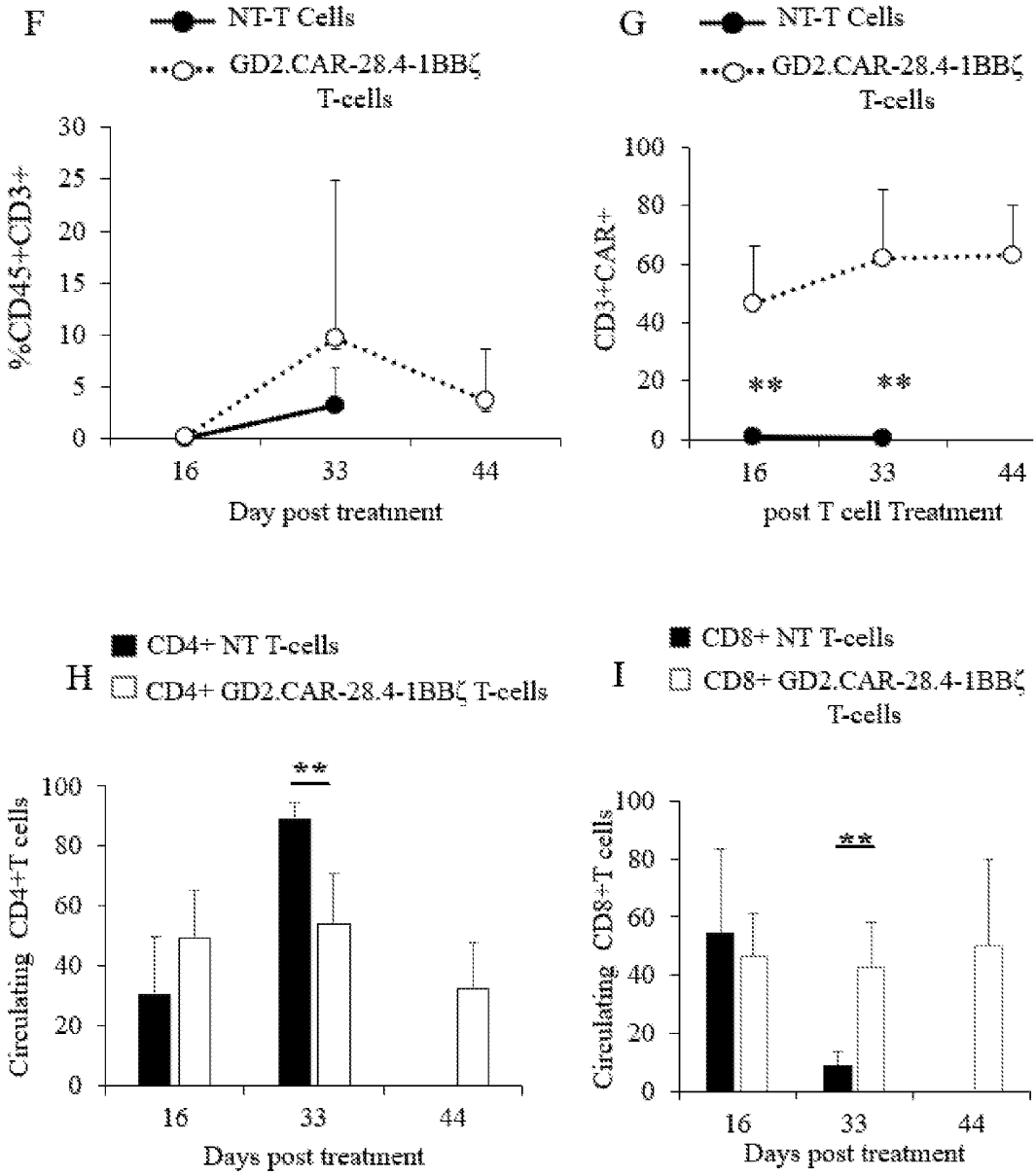
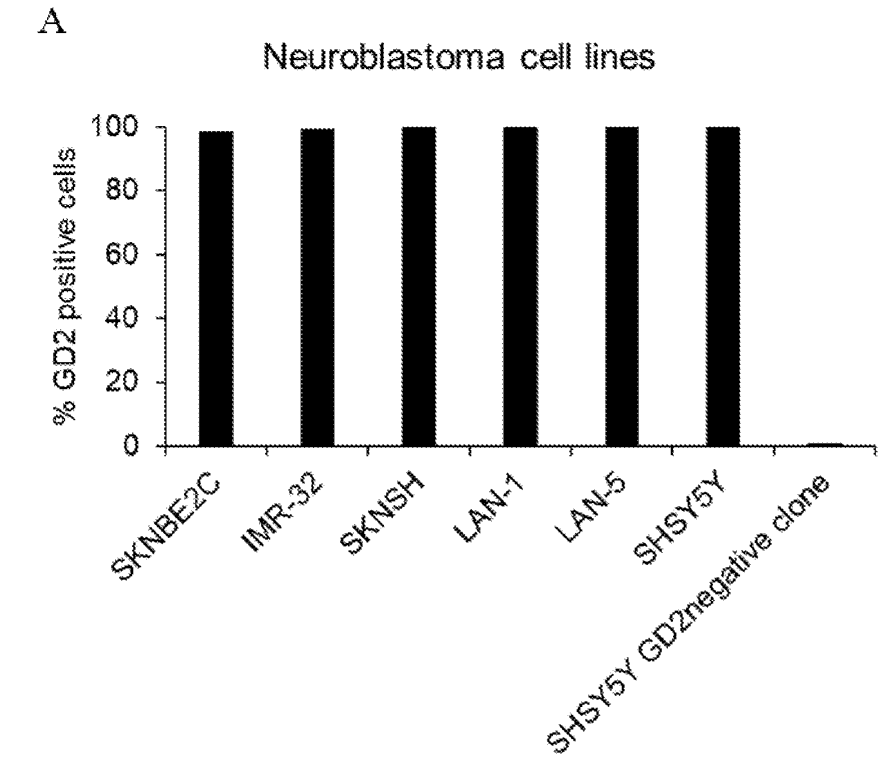

F
NT-T Cells
GD2.CAR-28.4-1BBζ T-cells
%CD45+CD3+
30
25
20
15
10
5
0
16
33
44
Day post treatment
G
NT-T Cells
GD2.CAR-28.4-1BBζ T-cells
CD3+CAR+
100
80
60
40
20
0
**
**
16
33
44
post T cell Treatment
H
CD4+ NT T-cells
CD4+ GD2.CAR-28.4-1BBζ T-cells
**
Circulating CD4+T cells
100
80
60
40
20
0
16
33
44
Days post treatment
I
CD8+ NT T-cells
CD8+ GD2.CAR-28.4-1BBζ T-cells
**
Circulating CD8+T cells
100
80
60
40
20
0
16
33
44
Days post treatment

Fig.9


A
Neuroblastoma cell lines
% GD2 positive cells
100
80
60
40
20
0
SKNBE2C
IMR-32
SKNSH
LAN-1
LAN-5
SHSY5Y
SHSY5Y GD2negative clone

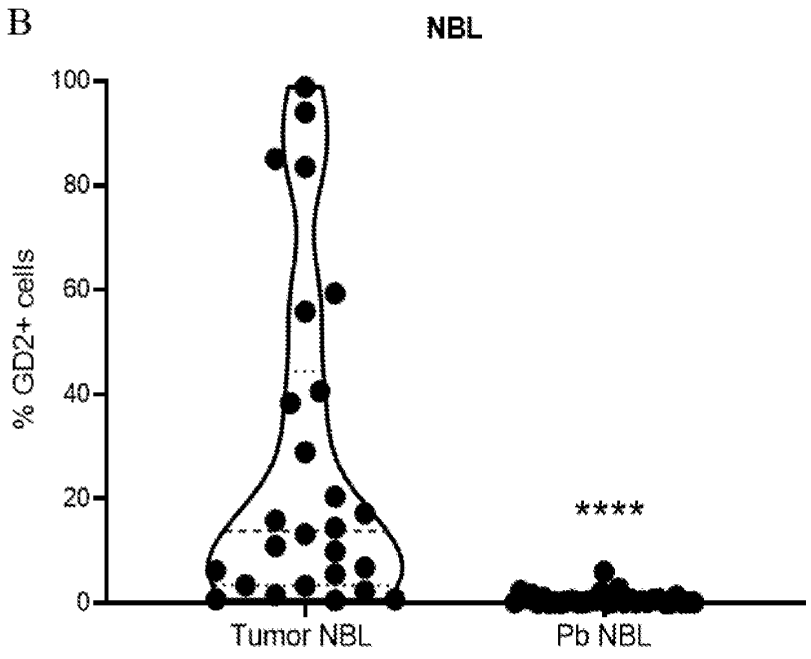

B
NBL
% GD2+ cells
100
80
60
40
20
0
****
Tumor NBL
Pb NBL

Fig.10

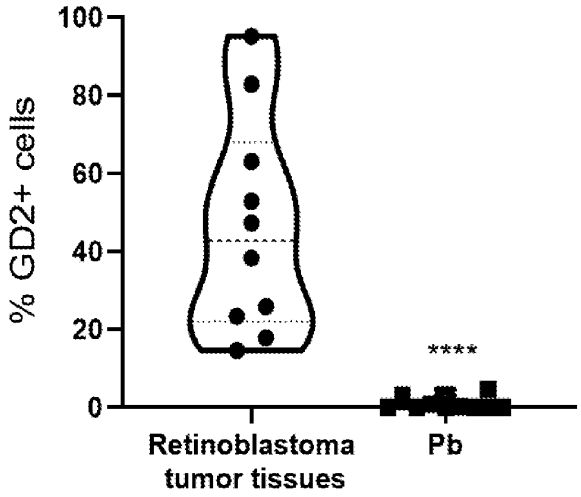

% GD2+ cells
100
80
60
40
20
0
****
Retinoblastoma tumor tissues
Pb

Fig.11

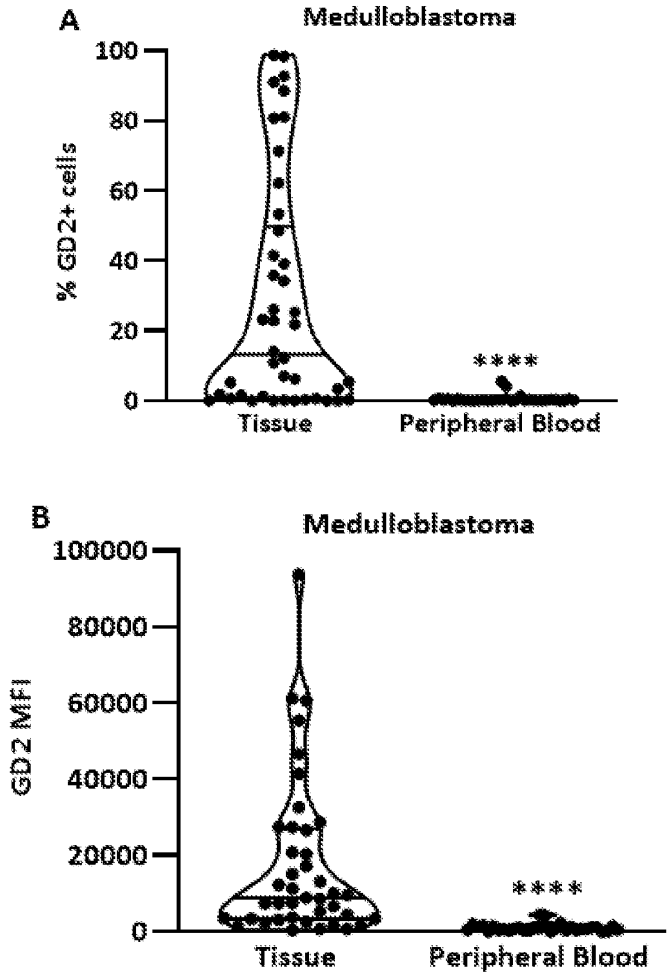

A
Medulloblastoma
% GD2+ cells
100
80
60
40
20
0
****
Tissue
Peripheral Blood
B
Medulloblastoma
GD2 MFI
100000
80000
60000
40000
20000
0
****
Tissue
Peripheral Blood

Fig.12

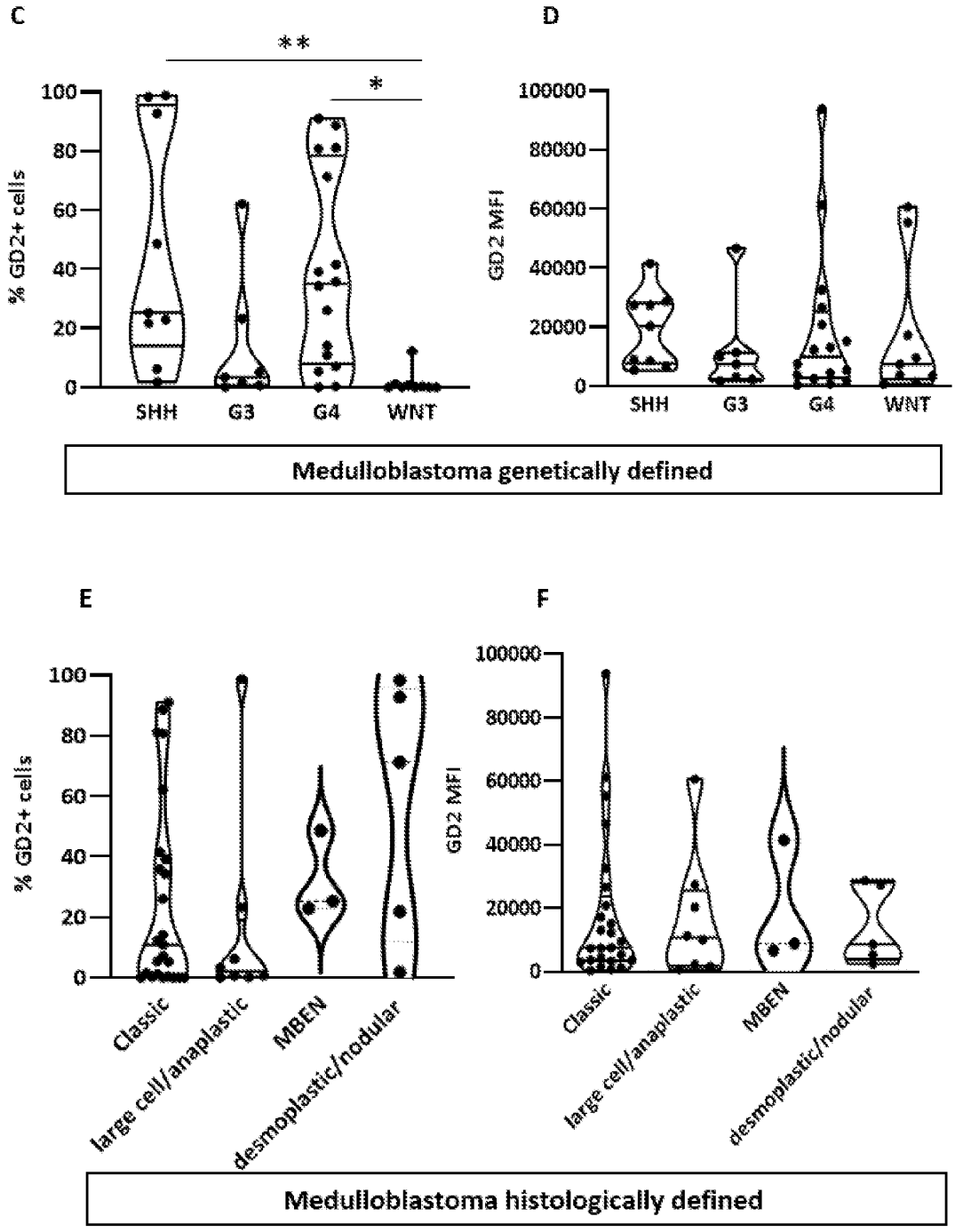

C
D
**
*
% GD2+ cells
GD2 MFI
SHH
G3
G4
WNT
Medulloblastoma genetically defined
E
F
Classic
large cell/anaplastic
MBEN
desmoplastic/nodular
Medulloblastoma histologically defined

Fig.12

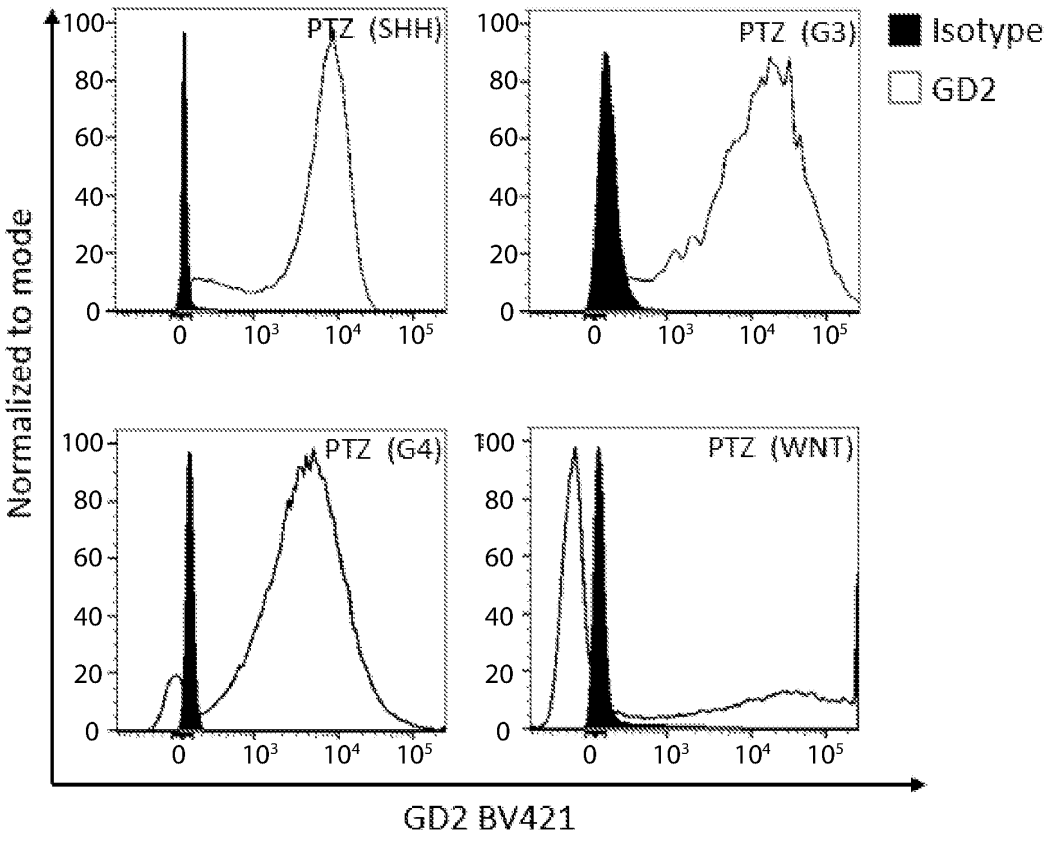

PTZ (SHH)
PTZ (G3)
PTZ (G4)
PTZ (WNT)
Isotype
GD2
Normalized to mode
GD2 BV421
100
80
60
40
20
0
0
10³
10⁴
10⁵

Fig.13

A

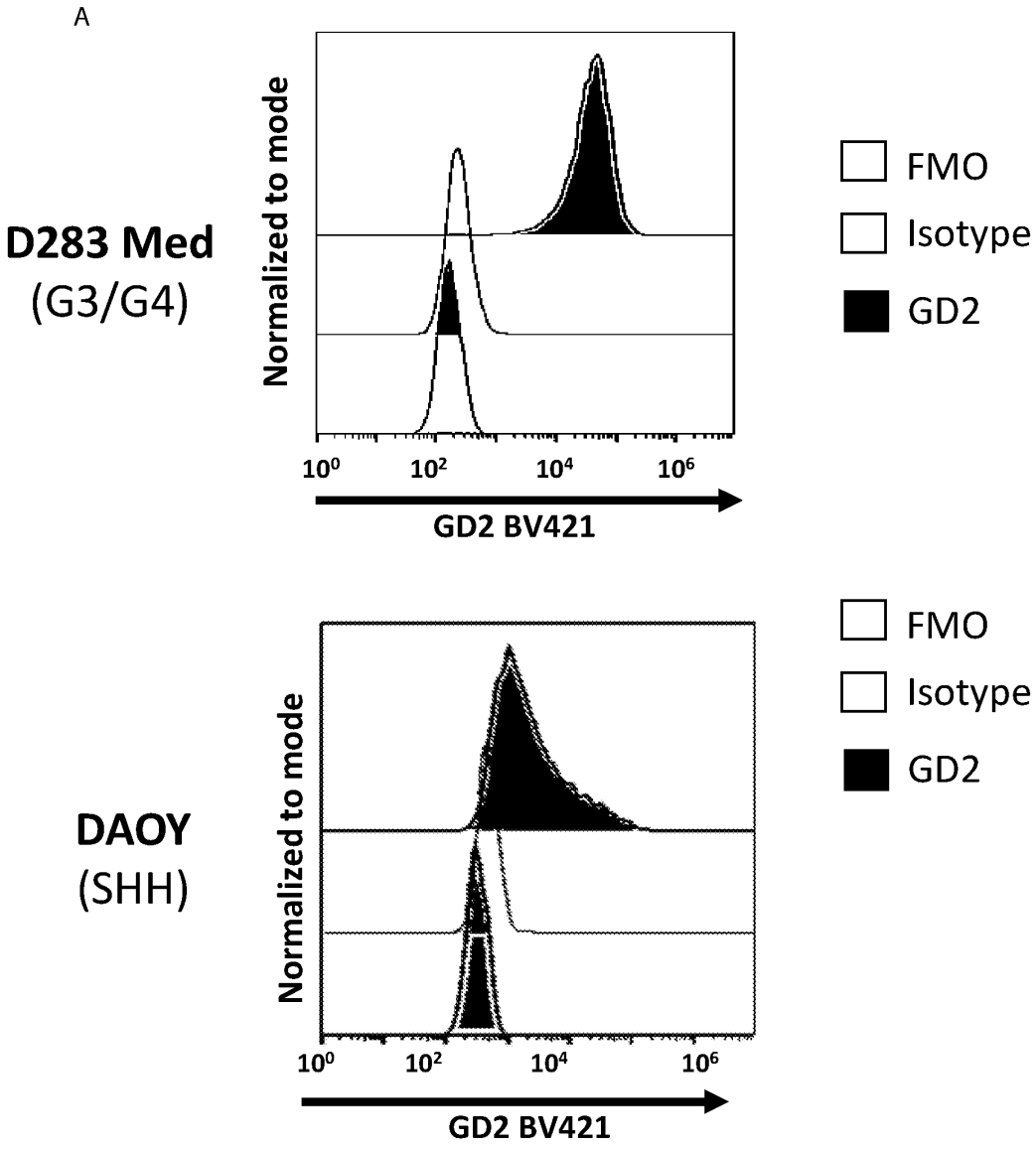
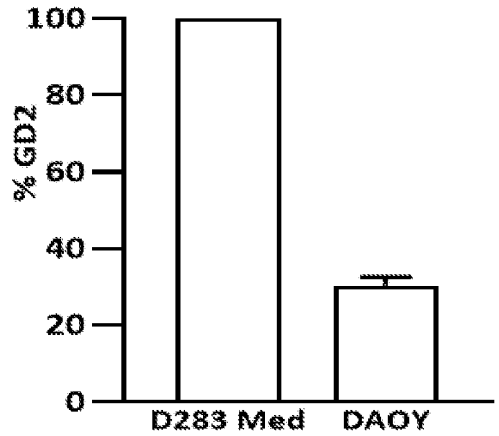

D283 Med
(G3/G4)
Normalized to mode
10^0
10^2
10^4
10^6
GD2 BV421
FMO
Isotype
GD2
DAOY
(SHH)
Normalized to mode
10^0
10^2
10^4
10^6
GD2 BV421
FMO
Isotype
GD2

Fig.14

B


100
80
60
40
20
0
% GD2
D283 Med
DAOY

C

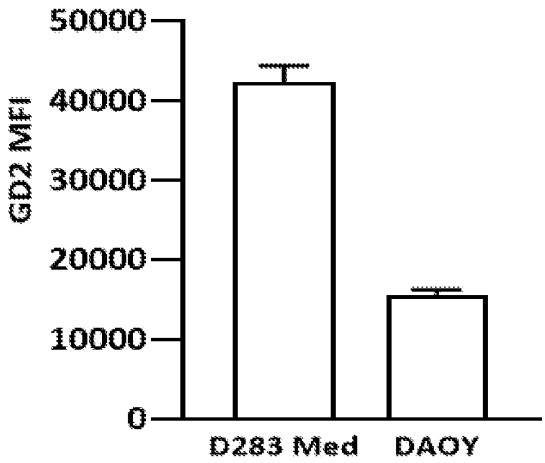

50000
40000
30000
20000
10000
0
GD2 MFI
D283 Med
DAOY

Fig.14

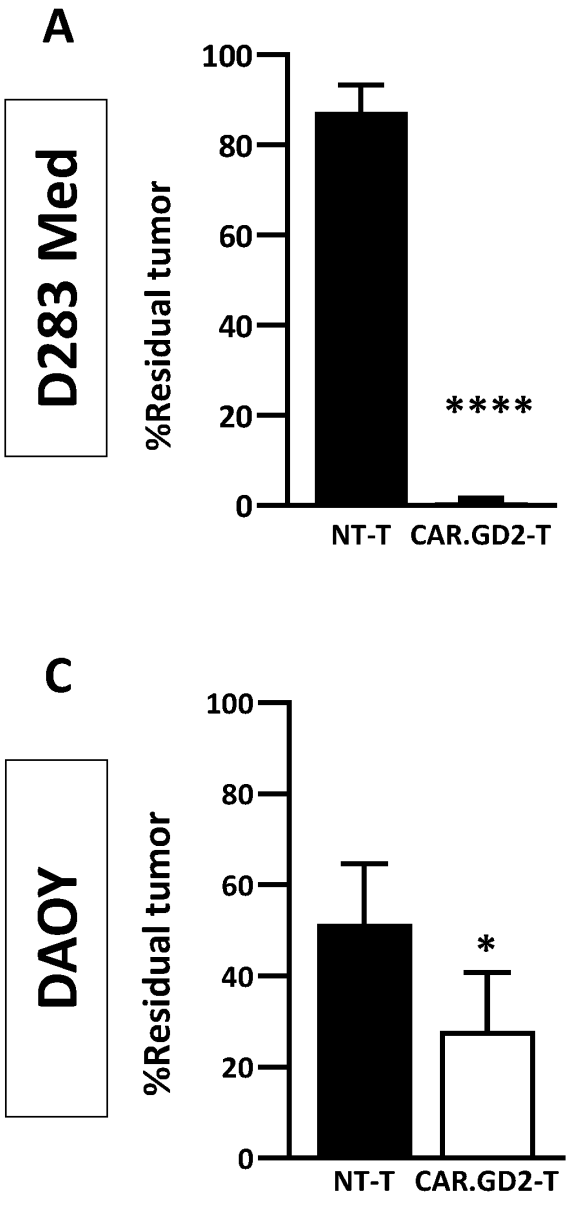

A
D283 Med
%Residual tumor
100
80
60
40
20
0
****
NT-T
CAR.GD2-T
C
DAOY
%Residual tumor
100
80
60
40
20
0
*
NT-T
CAR.GD2-T

Fig.15

B
D283 Med
Granzyme B (pg/mL)
50000
40000
30000
20000
10000
0
NT-T
CAR.GD2-T
TUMOR
*
*
INF-γ (pg/mL)
2000
1500
1000
500
0
*
*
IL-2 (pg/mL)
200
150
100
50
0
**
**
TNF-α (pg/mL)
1000
800
600
400
200
0
***
***
D
DAOY
Granzyme B (pg/mL)
50000
40000
30000
20000
10000
0
*
*
INF-γ (pg/mL)
2000
1500
1000
500
0
*
**
IL-2 (pg/mL)
200
150
100
50
0
****
****
TNF-α (pg/mL)
1000
800
600
400
200
0
****
****

Fig.15

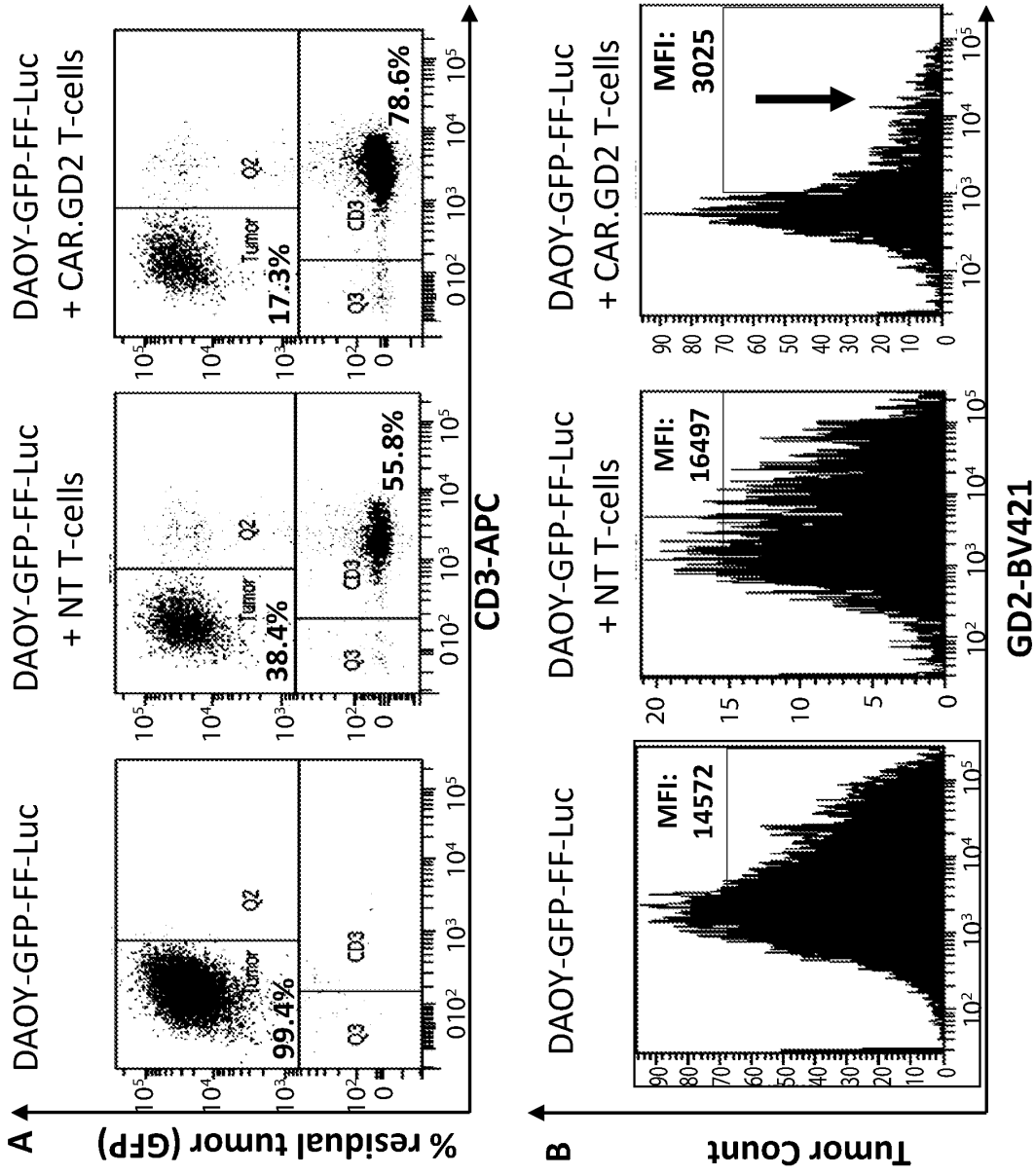

A
DAOY-GFP-FF-Luc
DAOY-GFP-FF-Luc
+ NT T-cells
DAOY-GFP-FF-Luc
+ CAR.GD2 T-cells
% residual tumor (GFP)
Tumor
CD3
Q2
Q3
99.4%
38.4%
55.8%
17.3%
78.6%
CD3-APC
B
DAOY-GFP-FF-Luc
DAOY-GFP-FF-Luc
+ NT T-cells
DAOY-GFP-FF-Luc
+ CAR.GD2 T-cells
Tumor Count
MFI: 14572
MFI: 16497
MFI: 3025
GD2-BV421

Fig.16

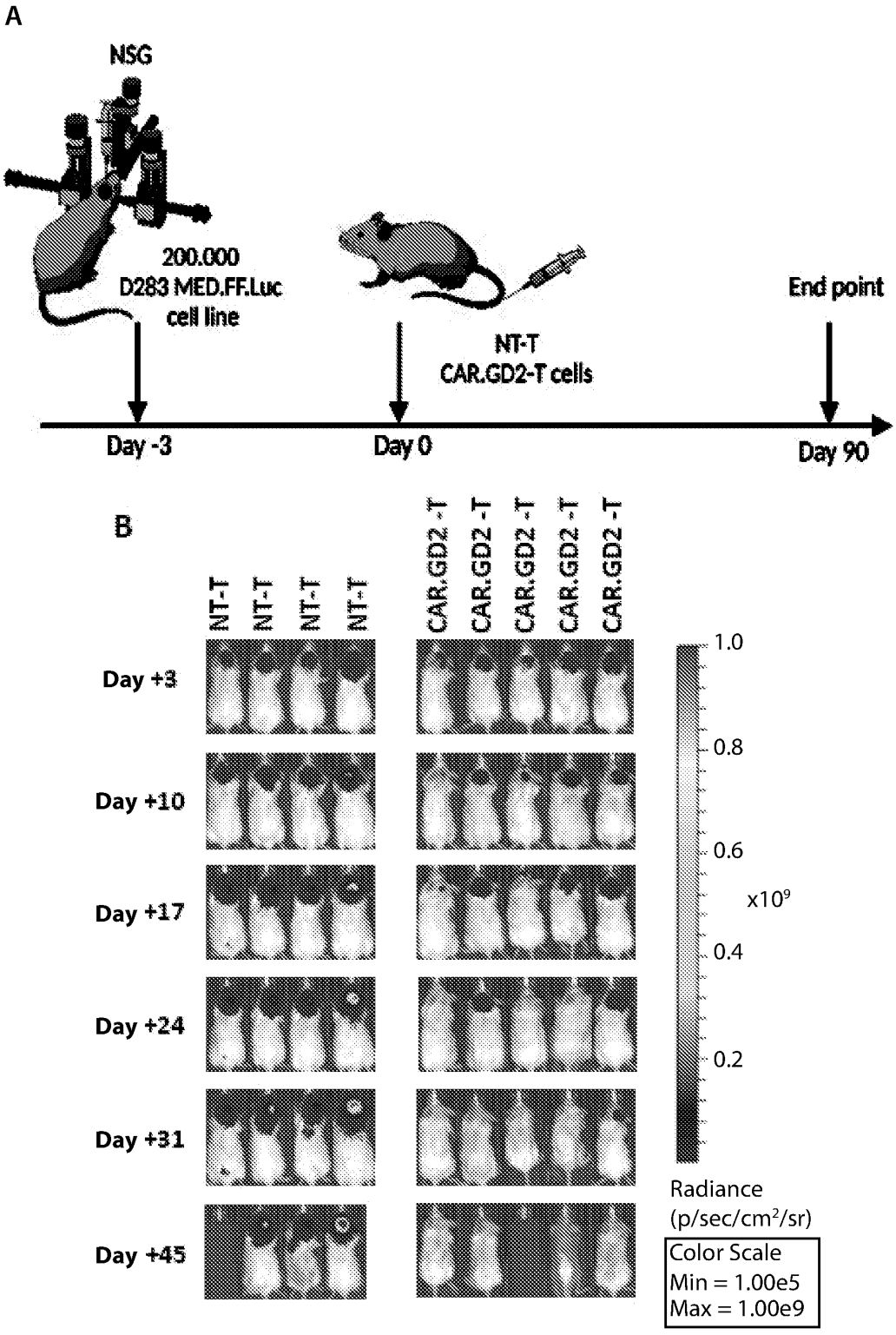

A
NSG
200.000
D283 MED.FF.Luc
cell line
NT-T
CAR.GD2-T cells
End point
Day -3
Day 0
Day 90
B
NT-T
NT-T
NT-T
NT-T
CAR.GD2 -T
CAR.GD2 -T
CAR.GD2 -T
CAR.GD2 -T
CAR.GD2 -T
Day +3
Day +10
Day +17
Day +24
Day +31
Day +45
1.0
0.8
0.6
x10⁹
0.4
0.2
Radiance
(p/sec/cm²/sr)
Color Scale
Min = 1.00e5
Max = 1.00e9

Fig.17

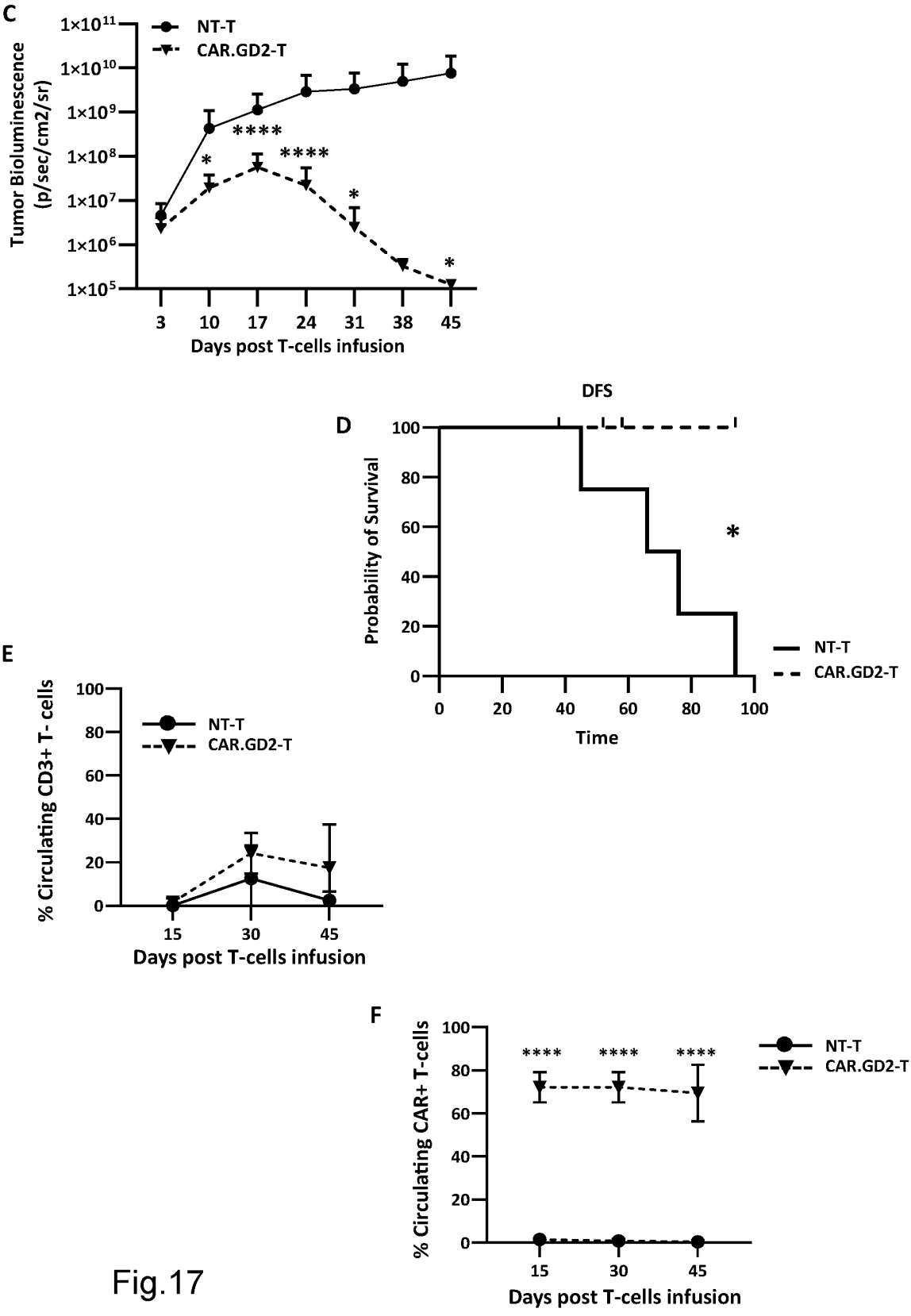

C
Tumor Bioluminescence (p/sec/cm2/sr)
1×10^11
1×10^10
1×10^9
1×10^8
1×10^7
1×10^6
1×10^5
NT-T
CAR.GD2-T
****
*
****
*
*
3
10
17
24
31
38
45
Days post T-cells infusion
DFS
D
Probability of Survival
100
80
60
40
20
0
*
0
20
40
60
80
100
Time
NT-T
CAR.GD2-T
E
% Circulating CD3+ T- cells
100
80
60
40
20
0
NT-T
CAR.GD2-T
15
30
45
Days post T-cells infusion
F
% Circulating CAR+ T-cells
100
80
60
40
20
0
****
****
****
NT-T
CAR.GD2-T
15
30
45
Days post T-cells infusion

Fig.17

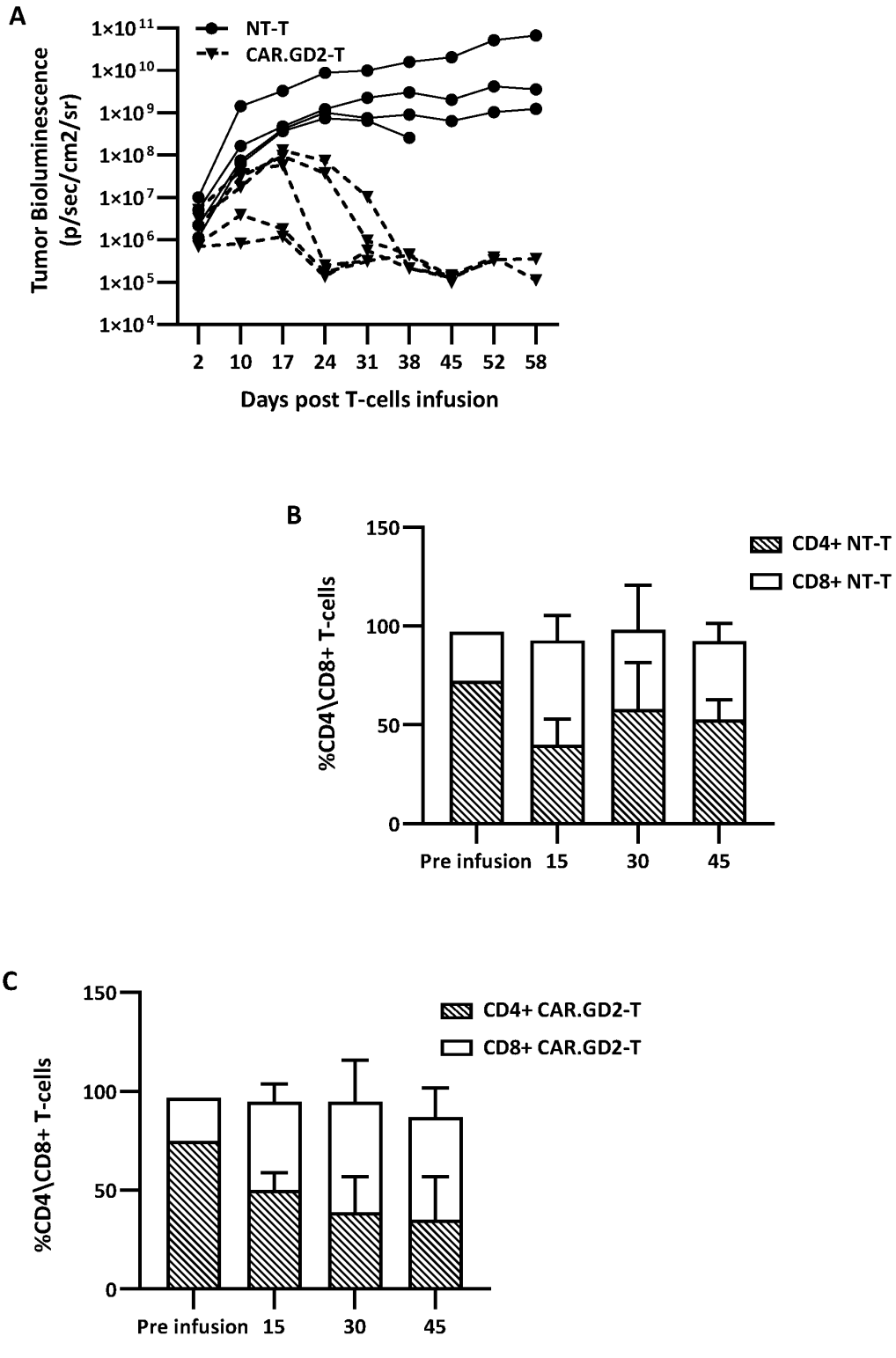
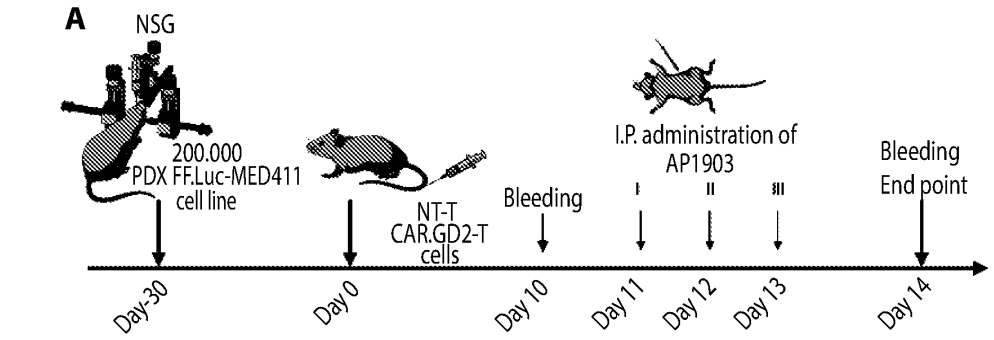

A
NT-T
CAR.GD2-T
Tumor Bioluminescence (p/sec/cm2/sr)
1×10^11
1×10^10
1×10^9
1×10^8
1×10^7
1×10^6
1×10^5
1×10^4
2 10 17 24 31 38 45 52 58
Days post T-cells infusion
B
CD4+ NT-T
CD8+ NT-T
%CD4\CD8+ T-cells
150
100
50
0
Pre infusion 15 30 45
C
CD4+ CAR.GD2-T
CD8+ CAR.GD2-T
%CD4\CD8+ T-cells
150
100
50
0
Pre infusion 15 30 45

Fig.18


A
NSG
200.000
PDX FF.Luc-MED411
cell line
NT-T
CAR.GD2-T
cells
Bleeding
I.P. administration of
AP1903
I
II
III
Bleeding
End point
Day-30
Day0
Day10
Day11
Day12
Day13
Day14

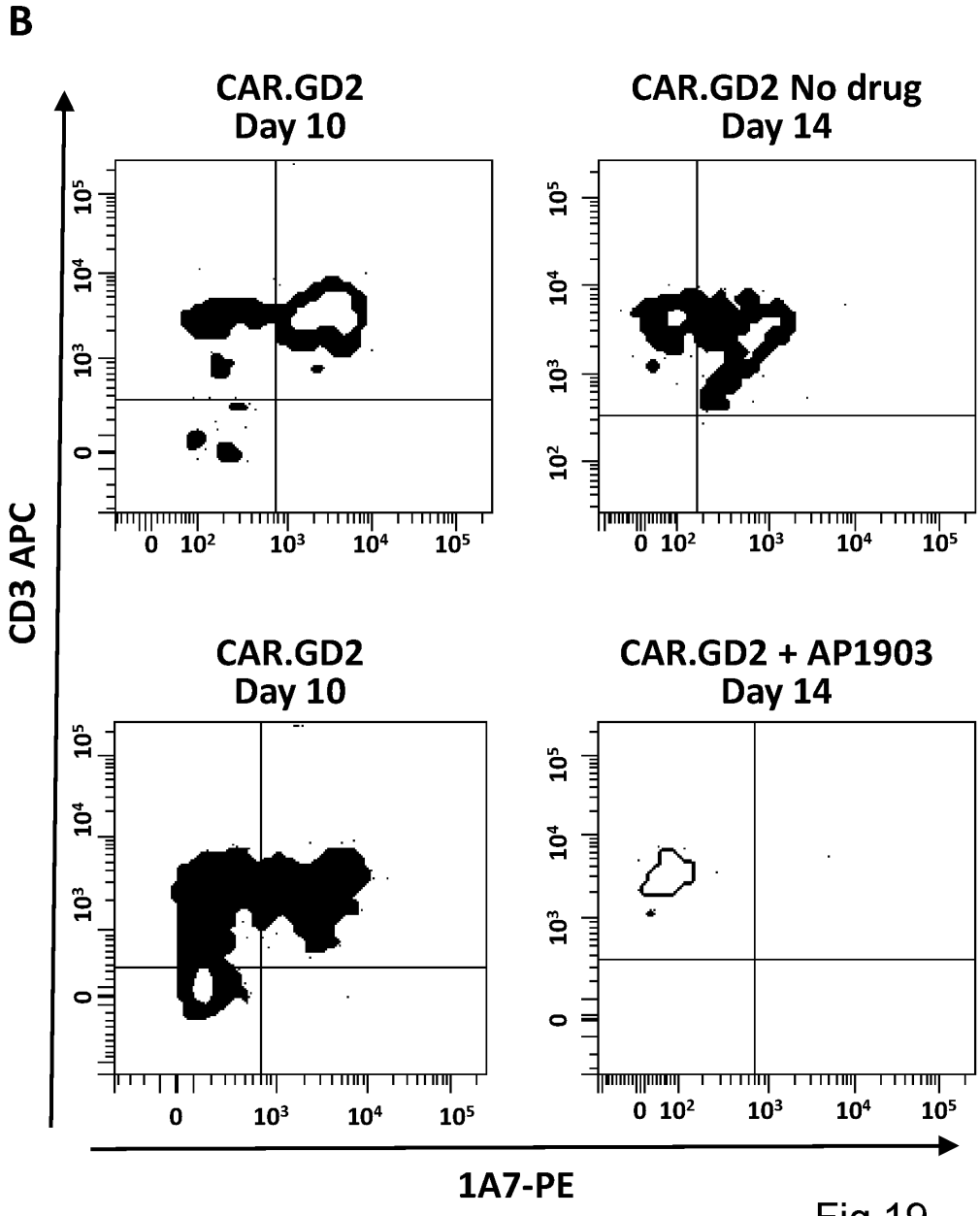

B
CAR.GD2
Day 10
CAR.GD2 No drug
Day 14
CAR.GD2
Day 10
CAR.GD2 + AP1903
Day 14
CD3 APC
1A7-PE
0
10²
10³
10⁴
10⁵

Fig.19

C

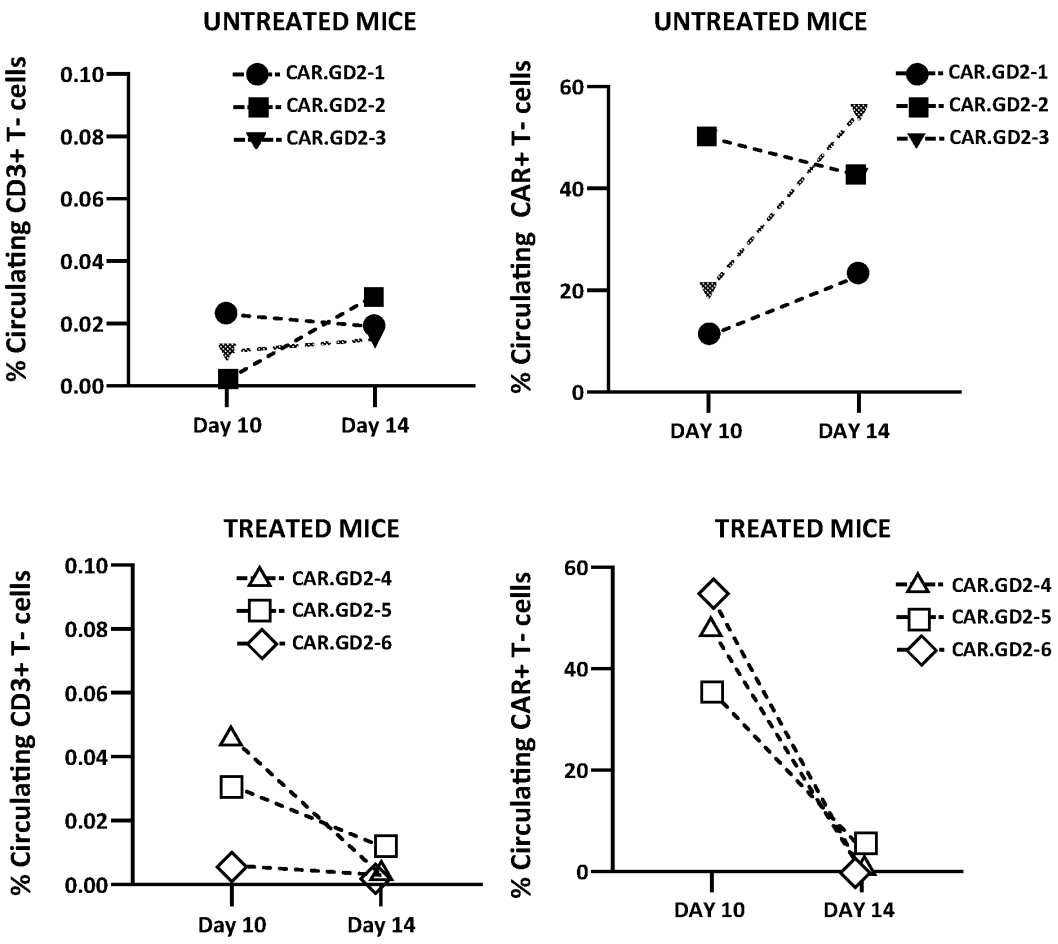

UNTREATED MICE
% Circulating CD3+ T- cells
0.10
0.08
0.06
0.04
0.02
0.00
CAR.GD2-1
CAR.GD2-2
CAR.GD2-3
Day 10
Day 14
UNTREATED MICE
% Circulating CAR+ T- cells
60
40
20
0
CAR.GD2-1
CAR.GD2-2
CAR.GD2-3
DAY 10
DAY 14
TREATED MICE
% Circulating CD3+ T- cells
0.10
0.08
0.06
0.04
0.02
0.00
CAR.GD2-4
CAR.GD2-5
CAR.GD2-6
Day 10
Day 14
TREATED MICE
% Circulating CAR+ T- cells
60
40
20
0
CAR.GD2-4
CAR.GD2-5
CAR.GD2-6
DAY 10
DAY 14

Fig.19

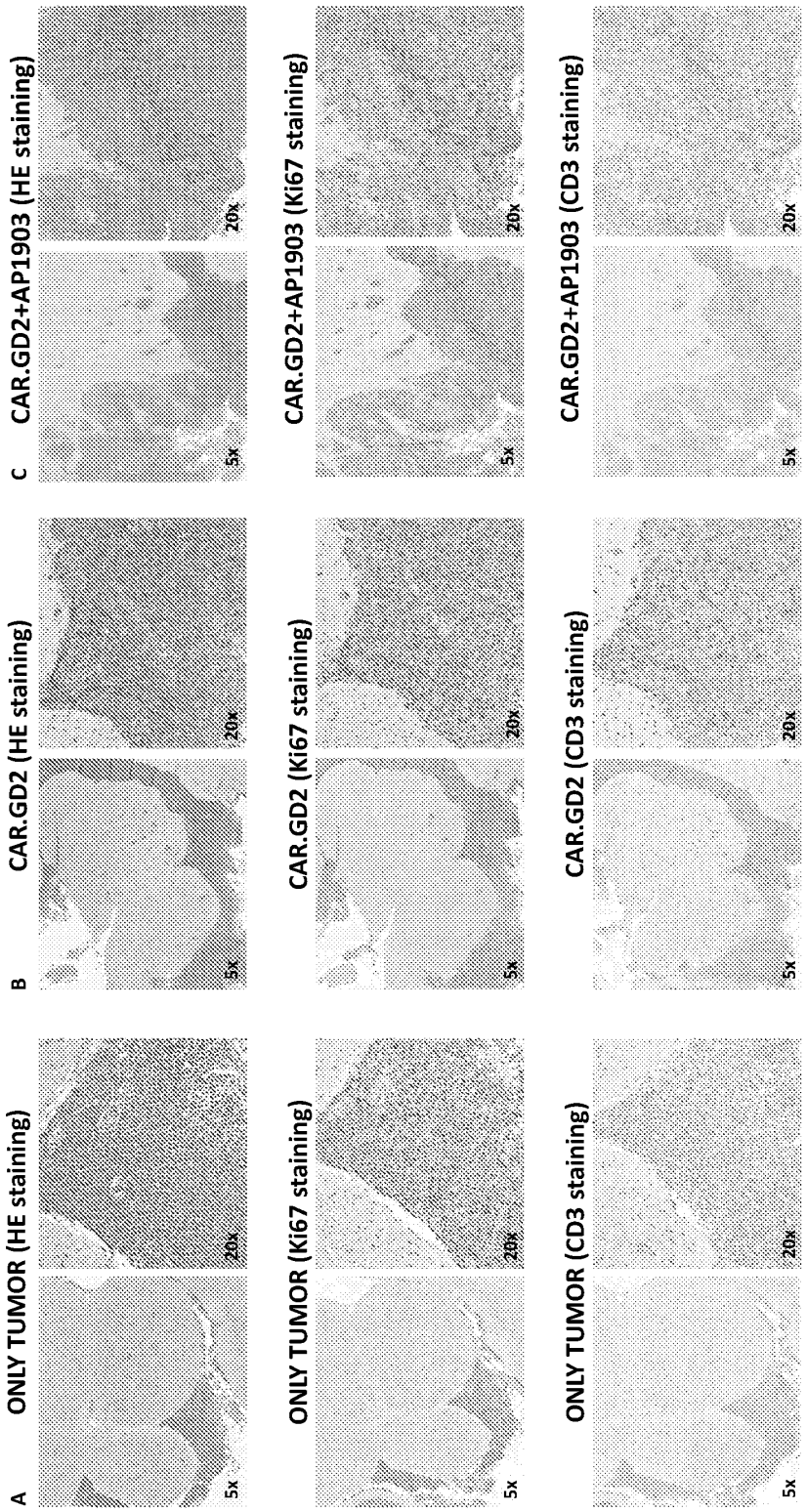

A
ONLY TUMOR (HE staining)
B
CAR.GD2 (HE staining)
C
CAR.GD2+AP1903 (HE staining)
ONLY TUMOR (Ki67 staining)
CAR.GD2 (Ki67 staining)
CAR.GD2+AP1903 (Ki67 staining)
ONLY TUMOR (CD3 staining)
CAR.GD2 (CD3 staining)
CAR.GD2+AP1903 (CD3 staining)
5x
20x

Fig.20

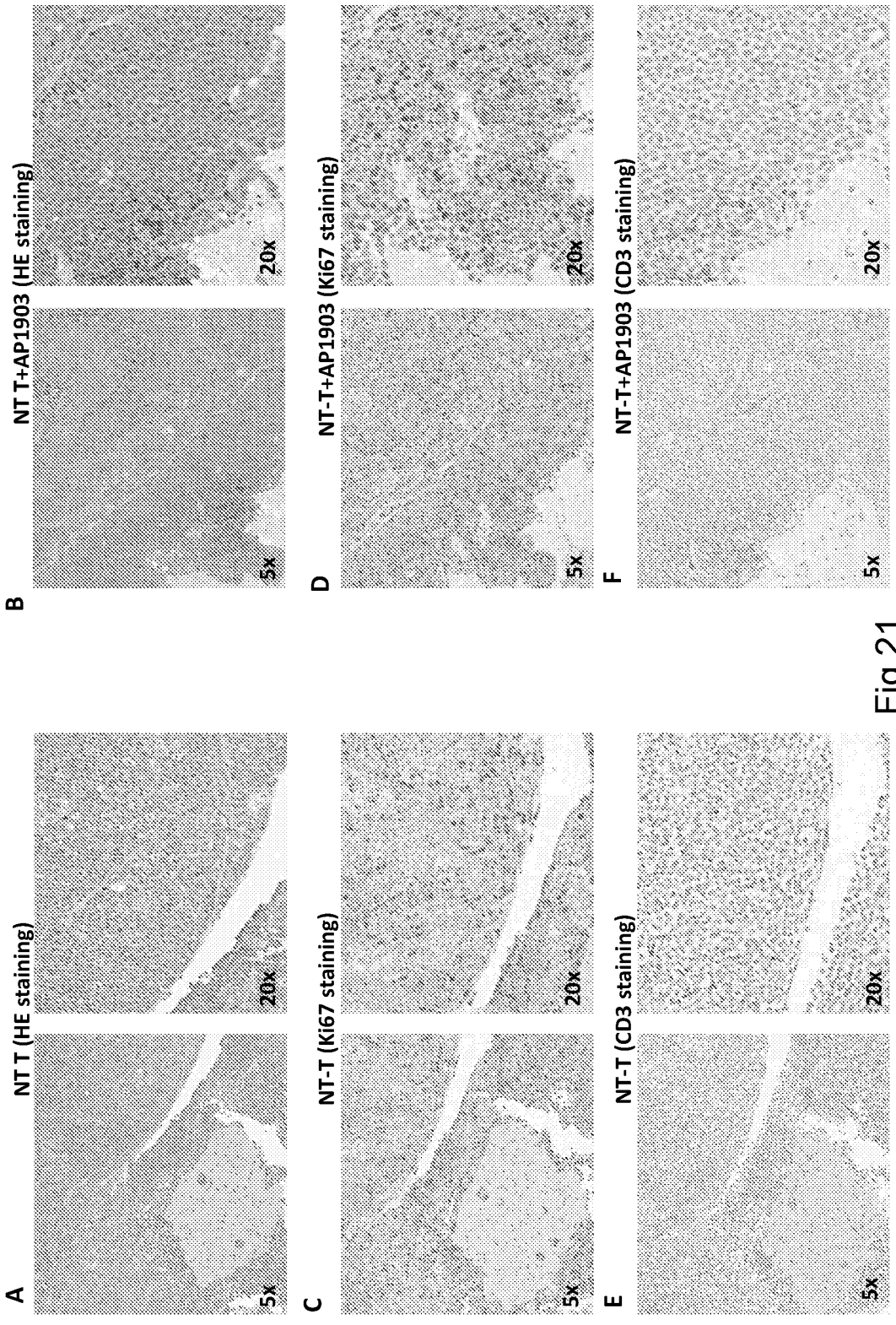

A
NT T (HE staining)
5x
20x
B
NT T+AP1903 (HE staining)
5x
20x
C
NT-T (Ki67 staining)
5x
20x
D
NT-T+AP1903 (Ki67 staining)
5x
20x
E
NT-T (CD3 staining)
5x
20x
F
NT-T+AP1903 (CD3 staining)
5x
20x

Fig.21

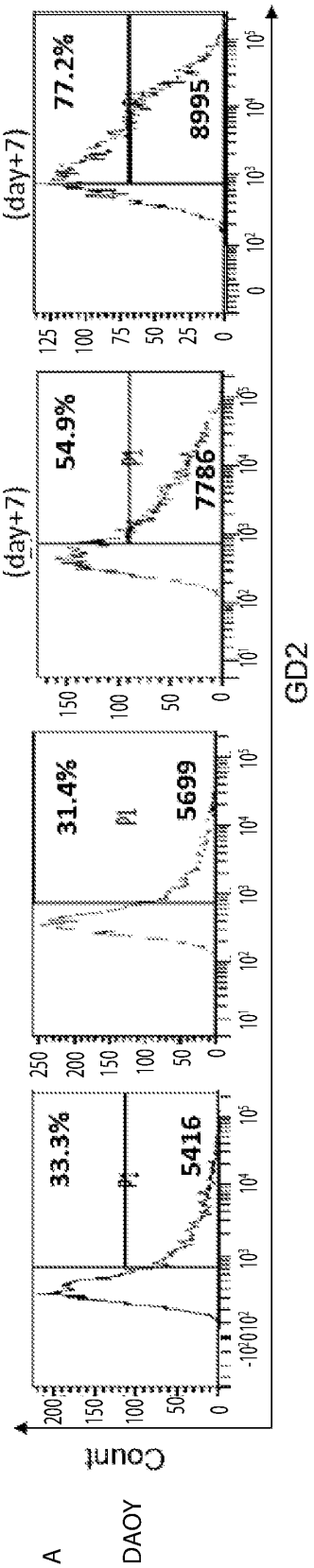

No treatment
DMSO
1 µM Tazemetostat (day+7)
10 µM Tazemetostat (day+7)
Count
DAOY
A
GD2
33.3%
5416
31.4%
5699
54.9%
7786
77.2%
8995

Fig.22

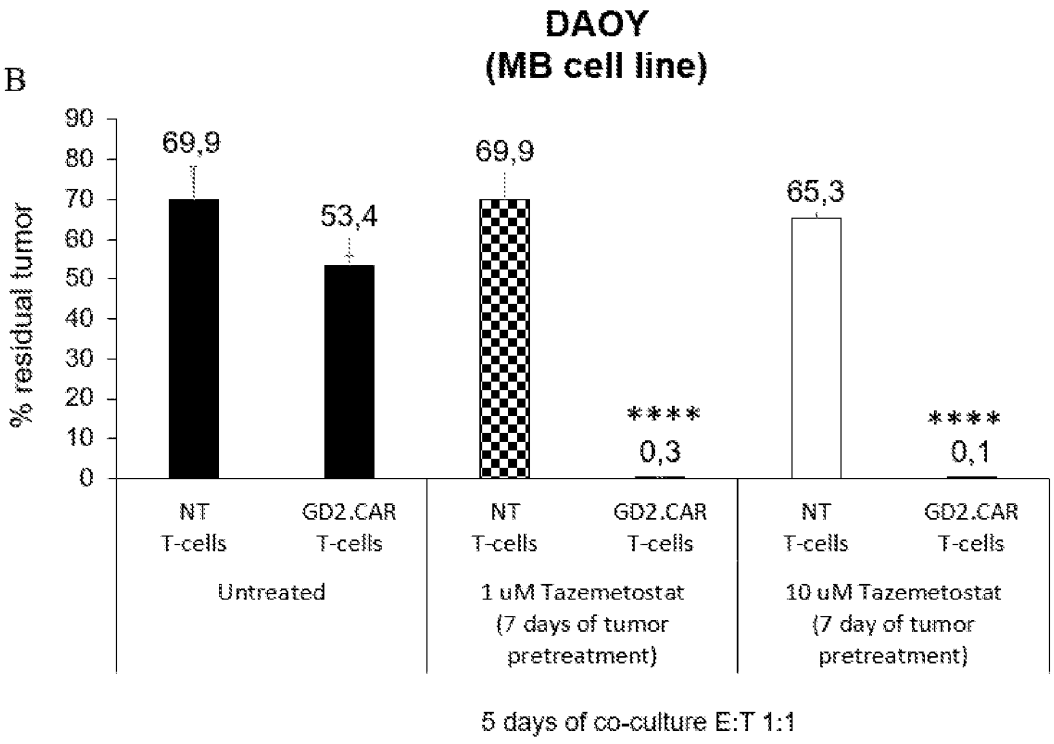
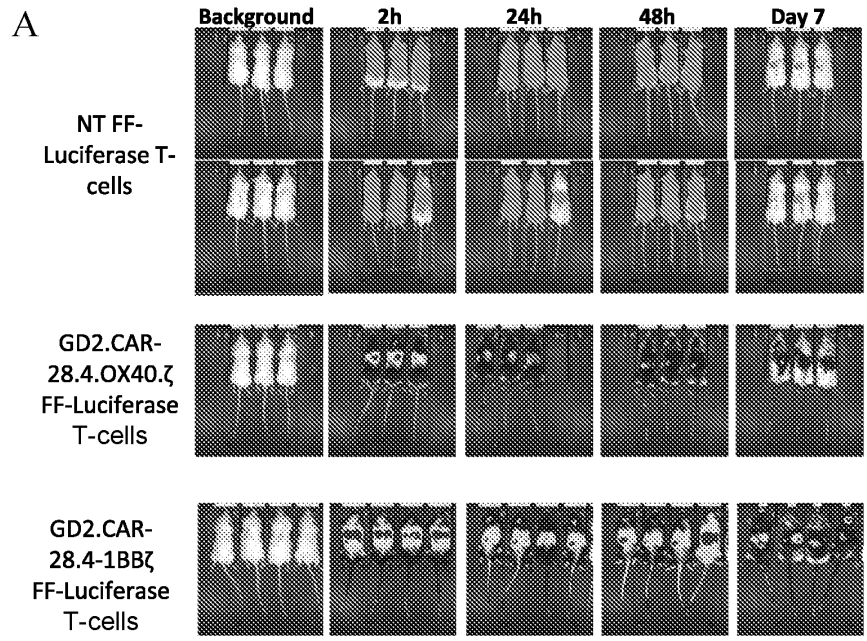

B
DAOY
(MB cell line)
% residual tumor
90
80
70
60
50
40
30
20
10
0
69,9
53,4
69,9
****
0,3
65,3
****
0,1
NT T-cells
GD2.CAR T-cells
NT T-cells
GD2.CAR T-cells
NT T-cells
GD2.CAR T-cells
Untreated
1 uM Tazemetostat (7 days of tumor pretreatment)
10 uM Tazemetostat (7 day of tumor pretreatment)
5 days of co-culture E:T 1:1

Fig.22


A
Background
2h
24h
48h
Day 7
NT FF-Luciferase T-cells
GD2.CAR-28.4.OX40.ζ FF-Luciferase T-cells
GD2.CAR-28.4-1BBζ FF-Luciferase T-cells

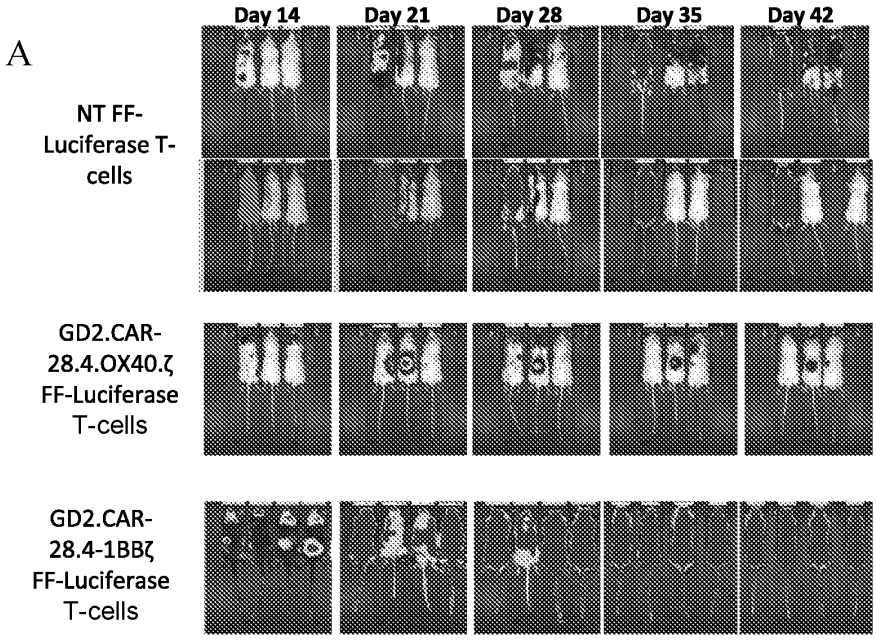

A
Day 14
Day 21
Day 28
Day 35
Day 42
NT FF-Luciferase T-cells
GD2.CAR-28.4.OX40.ζ FF-Luciferase T-cells
GD2.CAR-28.4-1BBζ FF-Luciferase T-cells

Fig. 23

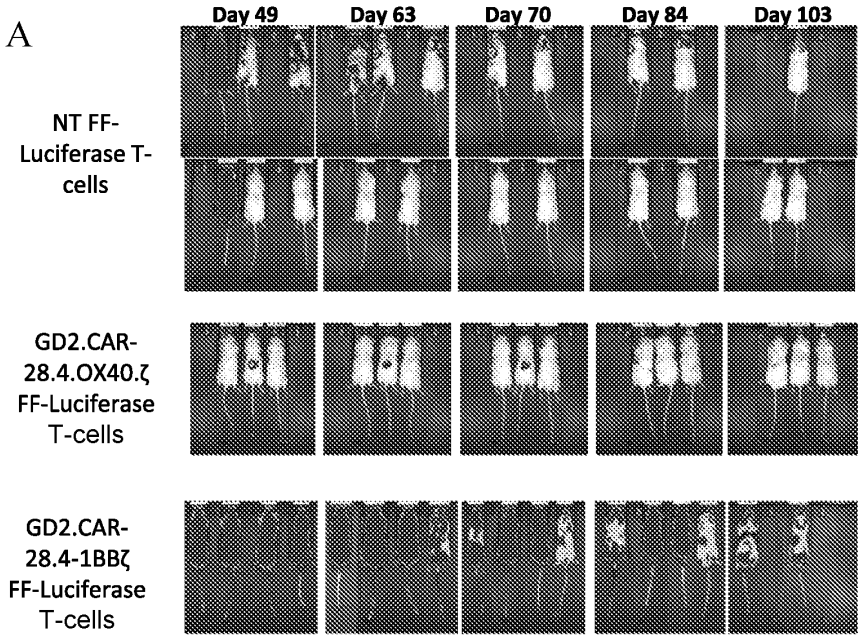
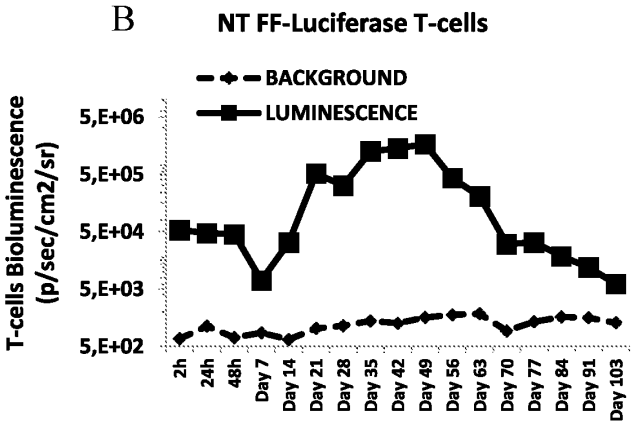
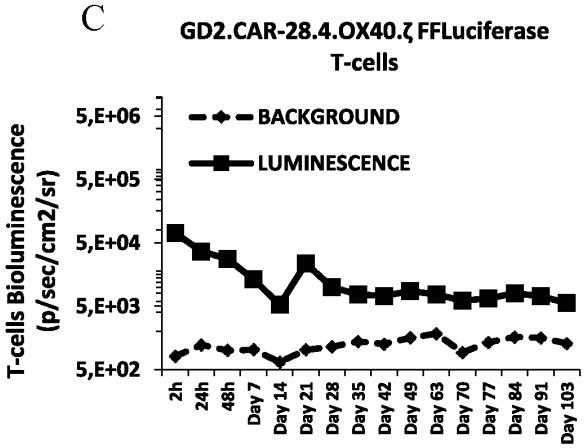

A
Day 49
Day 63
Day 70
Day 84
Day 103
NT FF-Luciferase T-cells
GD2.CAR-28.4.OX40.ζ FF-Luciferase T-cells
GD2.CAR-28.4-1BBζ FF-Luciferase T-cells

Fig. 23


B
NT FF-Luciferase T-cells
BACKGROUND
LUMINESCENCE
T-cells Bioluminescence (p/sec/cm2/sr)
5,E+06
5,E+05
5,E+04
5,E+03
5,E+02
2h
24h
48h
Day 7
Day 14
Day 21
Day 28
Day 35
Day 42
Day 49
Day 56
Day 63
Day 70
Day 77
Day 84
Day 91
Day 103


C
GD2.CAR-28.4.OX40.ζ FFLuciferase T-cells
BACKGROUND
LUMINESCENCE
T-cells Bioluminescence (p/sec/cm2/sr)
5,E+06
5,E+05
5,E+04
5,E+03
5,E+02
2h
24h
48h
Day 7
Day 14
Day 21
Day 28
Day 35
Day 42
Day 49
Day 63
Day 70
Day 77
Day 84
Day 91
Day 103

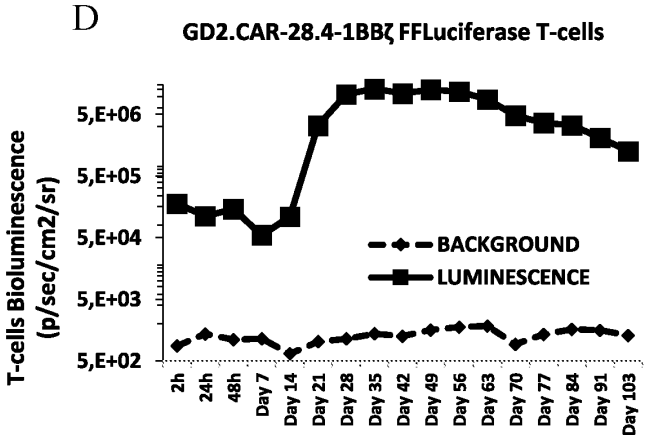

D
GD2.CAR-28.4-1BBζ FFLuciferase T-cells
BACKGROUND
LUMINESCENCE
T-cells Bioluminescence (p/sec/cm2/sr)
5,E+06
5,E+05
5,E+04
5,E+03
5,E+02
2h
24h
48h
Day 7
Day 14
Day 21
Day 28
Day 35
Day 42
Day 49
Day 56
Day 63
Day 70
Day 77
Day 84
Day 91
Day 103

Fig. 23