FIGURES

FIG. 1

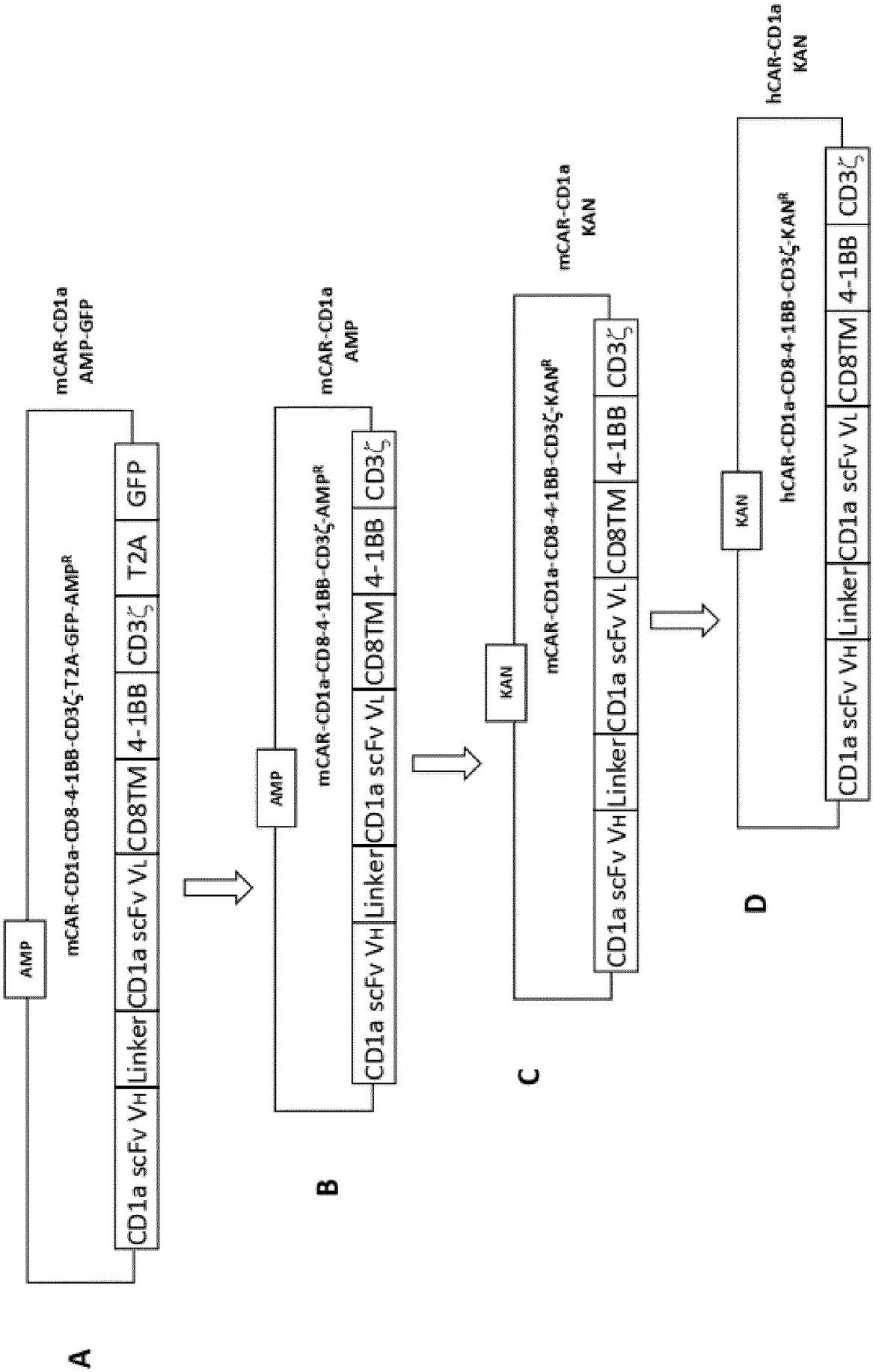


Fig. 2

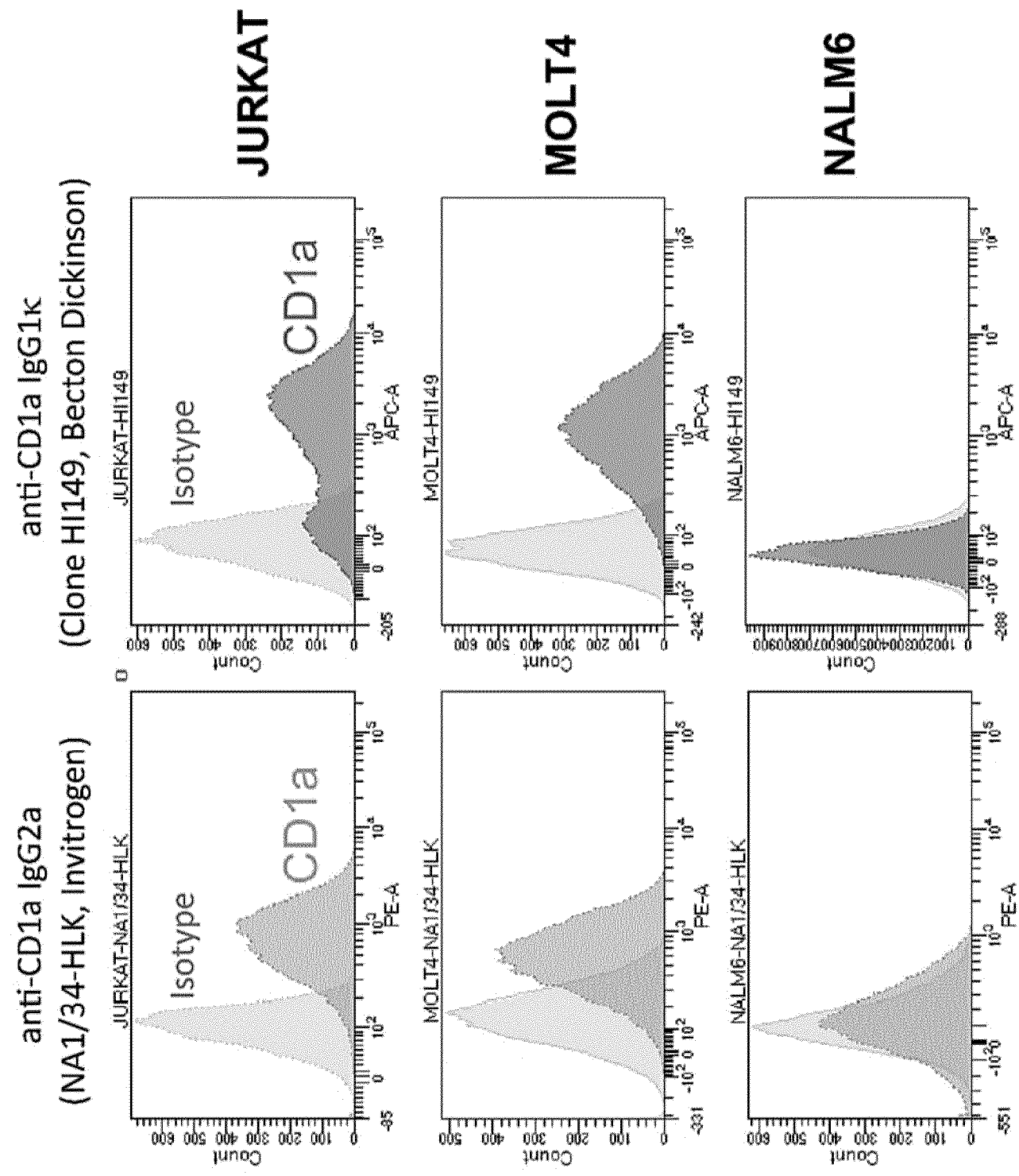

A
anti-CD1a IgG2a
(NA1/34-HLK, Invitrogen)
anti-CD1a IgG1κ
(Clone HI149, Becton Dickinson)
JURKAT
MOLT4
NALM6
Isotype
CD1a
JURKAT-NA1/34-HLK
MOLT4-NA1/34-HLK
NALM6-NA1/34-HLK
JURKAT-HI149
MOLT4-HI149
NALM6-HI149
PE-A
APC-A
Count

Fig. 2. (cont.)

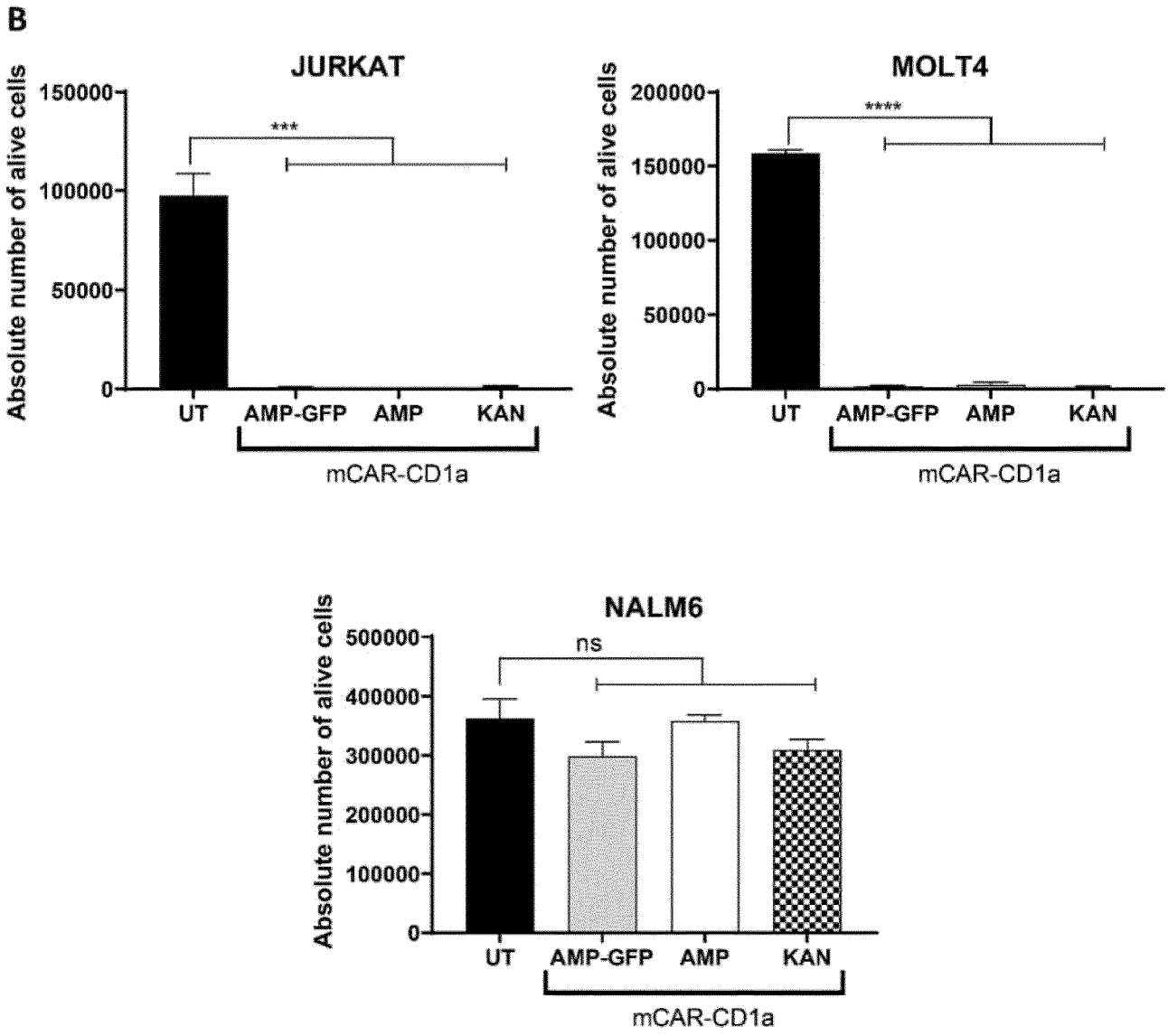
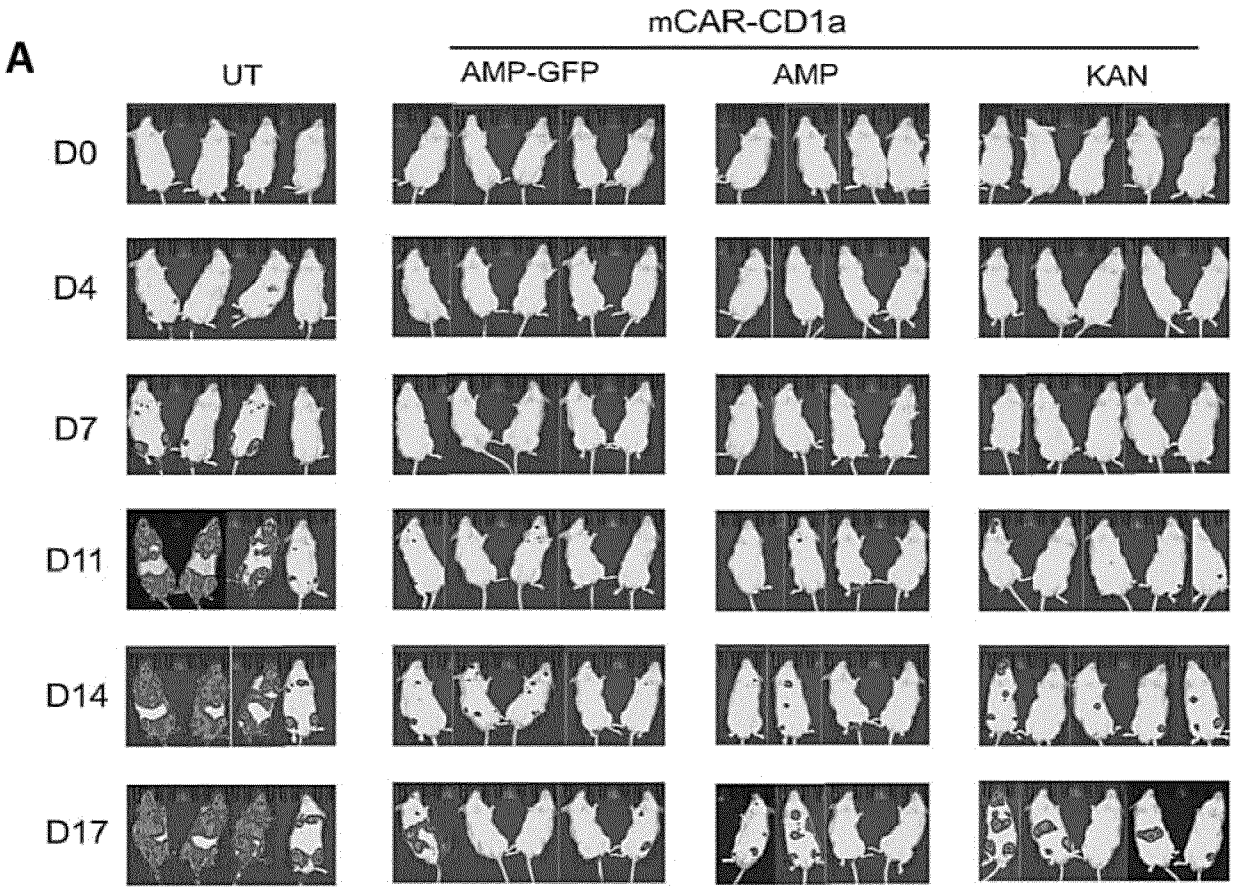

B
JURKAT
Absolute number of alive cells
150000
100000
50000
0
***
UT
AMP-GFP
AMP
KAN
mCAR-CD1a
MOLT4
Absolute number of alive cells
200000
150000
100000
50000
0
****
UT
AMP-GFP
AMP
KAN
mCAR-CD1a
NALM6
Absolute number of alive cells
500000
400000
300000
200000
100000
0
ns
UT
AMP-GFP
AMP
KAN
mCAR-CD1a

Fig. 3


A
mCAR-CD1a
UT
AMP-GFP
AMP
KAN
D0
D4
D7
D11
D14
D17

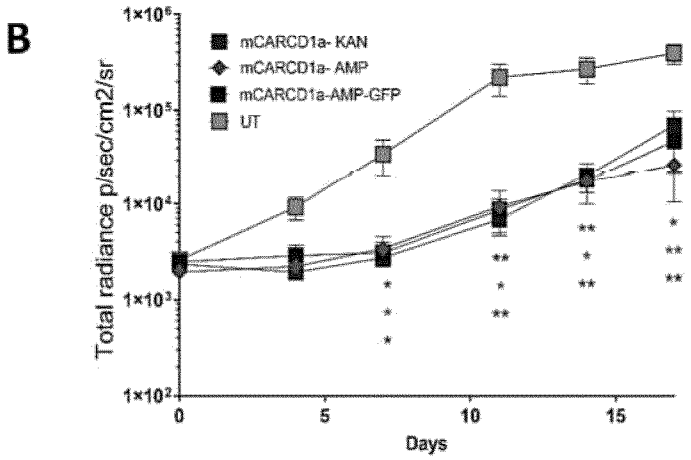
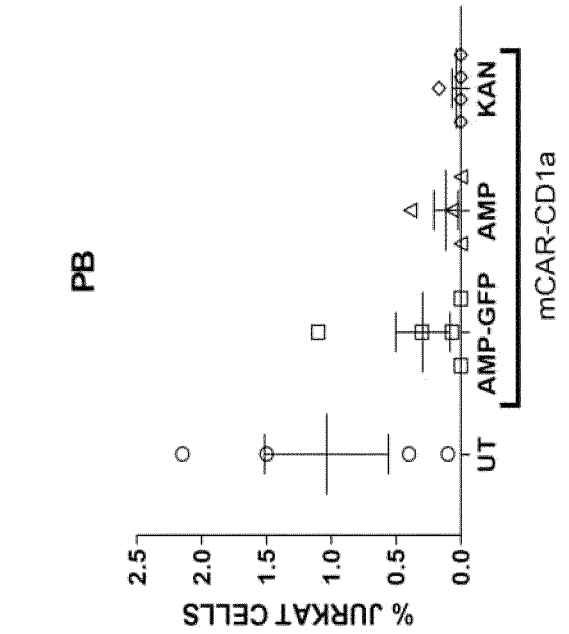
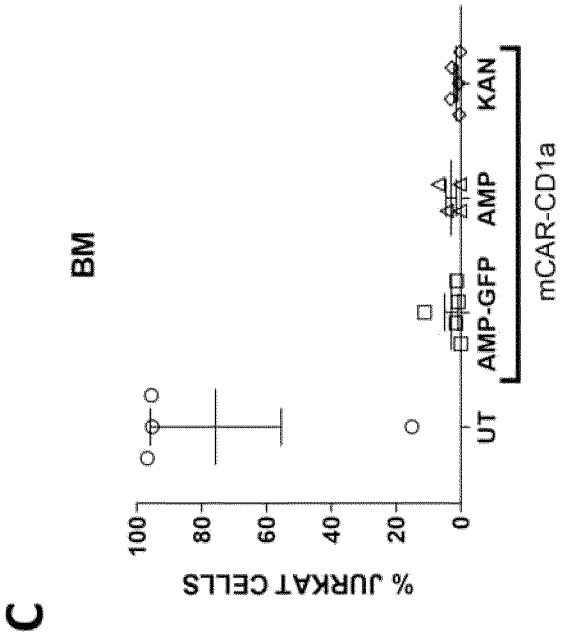

B
mCARCD1a- KAN
mCARCD1a- AMP
mCARCD1a-AMP-GFP
UT
Total radiance p/sec/cm2/sr
1×10⁶
1×10⁵
1×10⁴
1×10³
1×10²
0
5
10
15
Days

Fig. 3 (cont.)

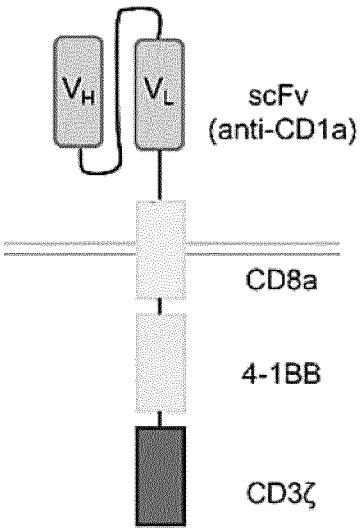

PB
% JURKAT CELLS
2.5
2.0
1.5
1.0
0.5
0.0
UT
AMP-GFP
AMP
KAN
mCAR-CD1a


BM
% JURKAT CELLS
100
80
60
40
20
0
UT
AMP-GFP
AMP
KAN
mCAR-CD1a

C

Fig. 4

A



B

HEAVY CHAIN (V_H)

```
murine          1 QVQLQQSGAELARPGASVKMSCKASGYAFSTYTMHWVKQRPRQGLEWIGY     50
                  ||||||||||:.:|||||:||||||||||||||||:|.|.||||||||
humanized       1 QVQLQQSGAEVKKPGASVKVSCKASGYAFSTYTMHWVRQAPGQGLEWMGY     50

murine         51 INPNSASTSYNENFKDKATLTADKSSNTAYMHLSSLTSEDSAVYYCARGF    100
                  ||||||||||.:.|:.:.|:|||||:|||||.||||.|||:|||||||||
humanized      51 INPNSASTSYAQKFQGRVTMTADKSTNTAYMELSSLRSEDTAVYYCARGF    100

murine        101 YTMDYWGQGTSVTVSS116
                  ||||||||||.||||||
humanized     101 YTMDYWGQGTLVTVSS116
```

CDRs + stems

H1: SGYAFSTYTMH H2: YINPNSASTS H3: ARGFYTMDY

Connector between heavy and light chains: GGGGSGGGGSGGGGSGGGGS

LIGHT CHAIN (V_L)

```
murine          1 RDIQMTQSPSSLSASLGGKVTITCQASQDINKYIAWYQFKPGKGPRLLIH     50
                  ||||||||||||||||:|.:||||||||||||||||||||||||||||||||
humanized       1 RDIQMTQSPSSLSASVGDRVTITCQASQDINKYIAWYQFKPGKGPRLLIH     50

murine         51 YTSTLQPAIPSRFSGSGSGREYSFSISNLEPEDIATYYCLHYDNLPWTFG    100
                  |||||:||||||||||||||:|:||:|:||||||||||||||||||||||
humanized      51 YTSTLEPAIPSRFSGSGSGREYTFTISSLQPEDIATYYCLHYDNLPWTFG    100

murine        101 GGTKLEIKRA    110
                  ||||:|||||
humanized     101 GGTKVEIKRA    110
```

CDRs + stems

L1: QASQDINKYIA L2: IHYTSTL L3: LHYDNLPWT

Fig. 5 A

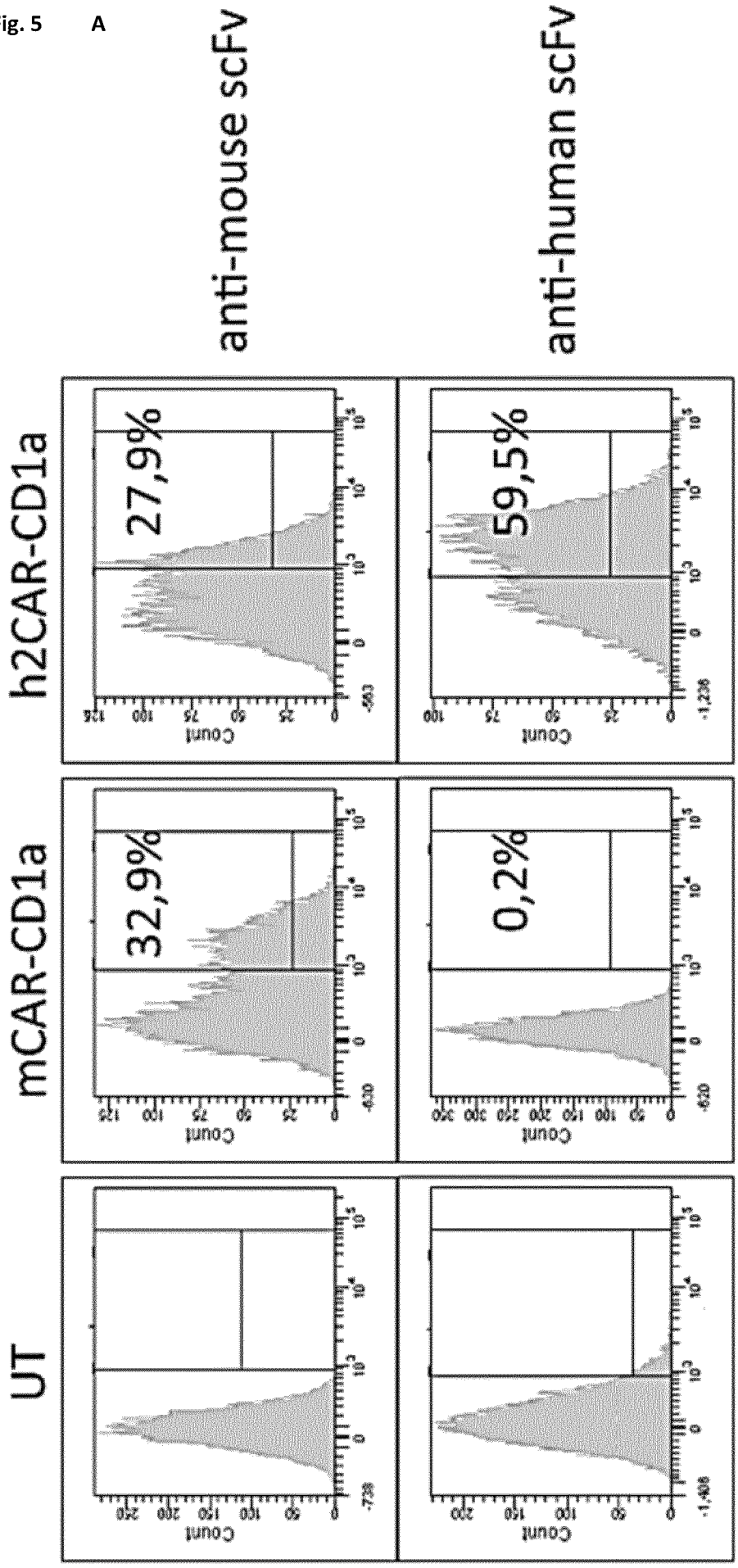
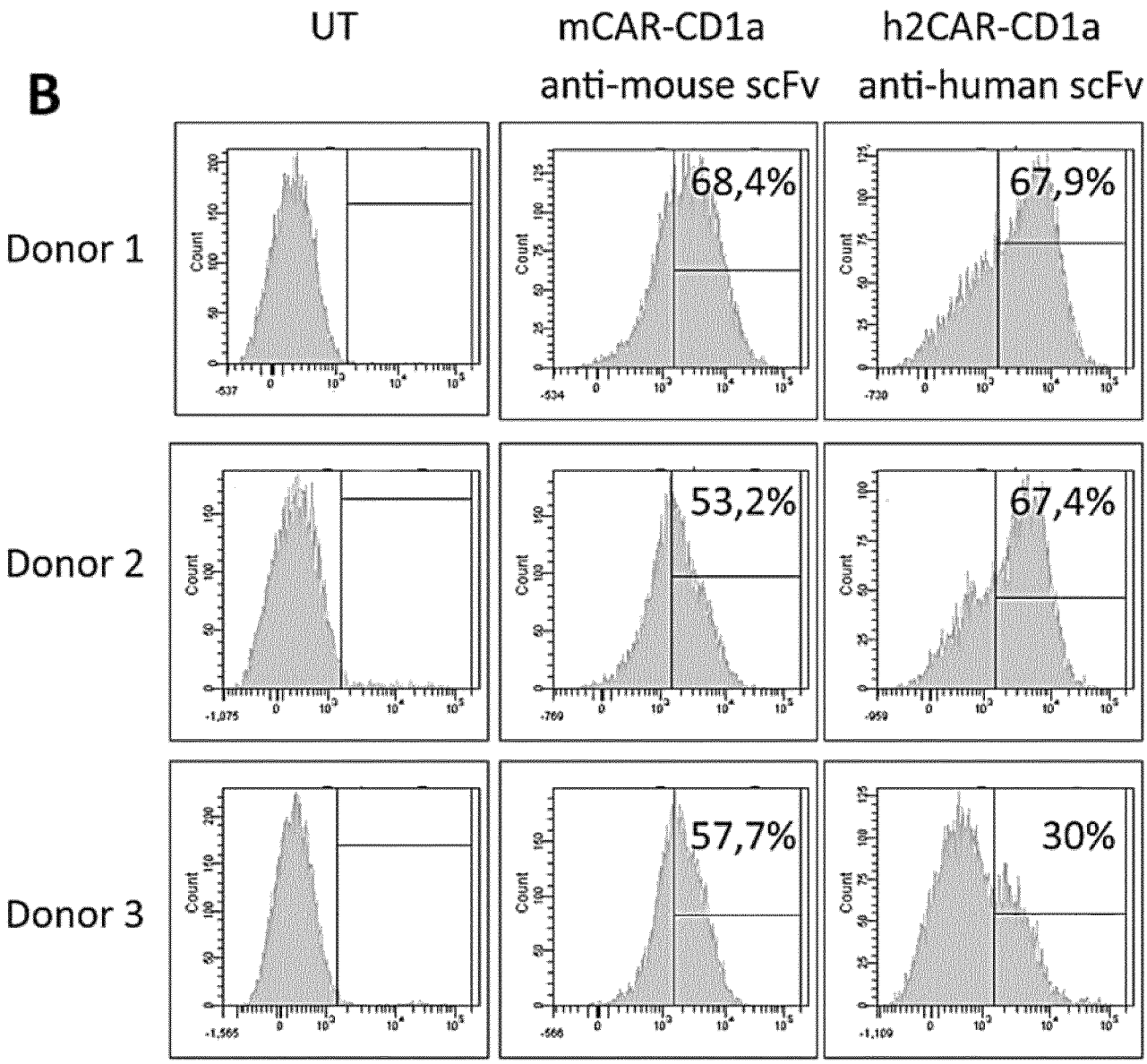

anti-mouse scFv
anti-human scFv
h2CAR-CD1a
mCAR-CD1a
UT
27,9%
59,5%
32,9%
0,2%
Count

Fig. 5 (cont.)


B
UT
mCAR-CD1a
anti-mouse scFv
h2CAR-CD1a
anti-human scFv
Donor 1
Donor 2
Donor 3
68,4%
67,9%
53,2%
67,4%
57,7%
30%
Count

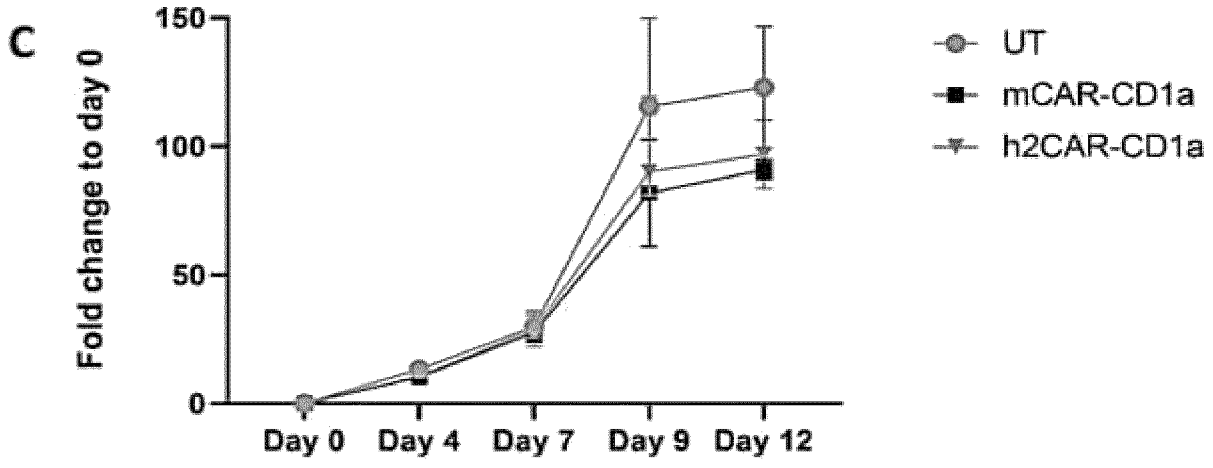

C
Fold change to day 0
150
100
50
0
Day 0
Day 4
Day 7
Day 9
Day 12
UT
mCAR-CD1a
h2CAR-CD1a

Fig. 6

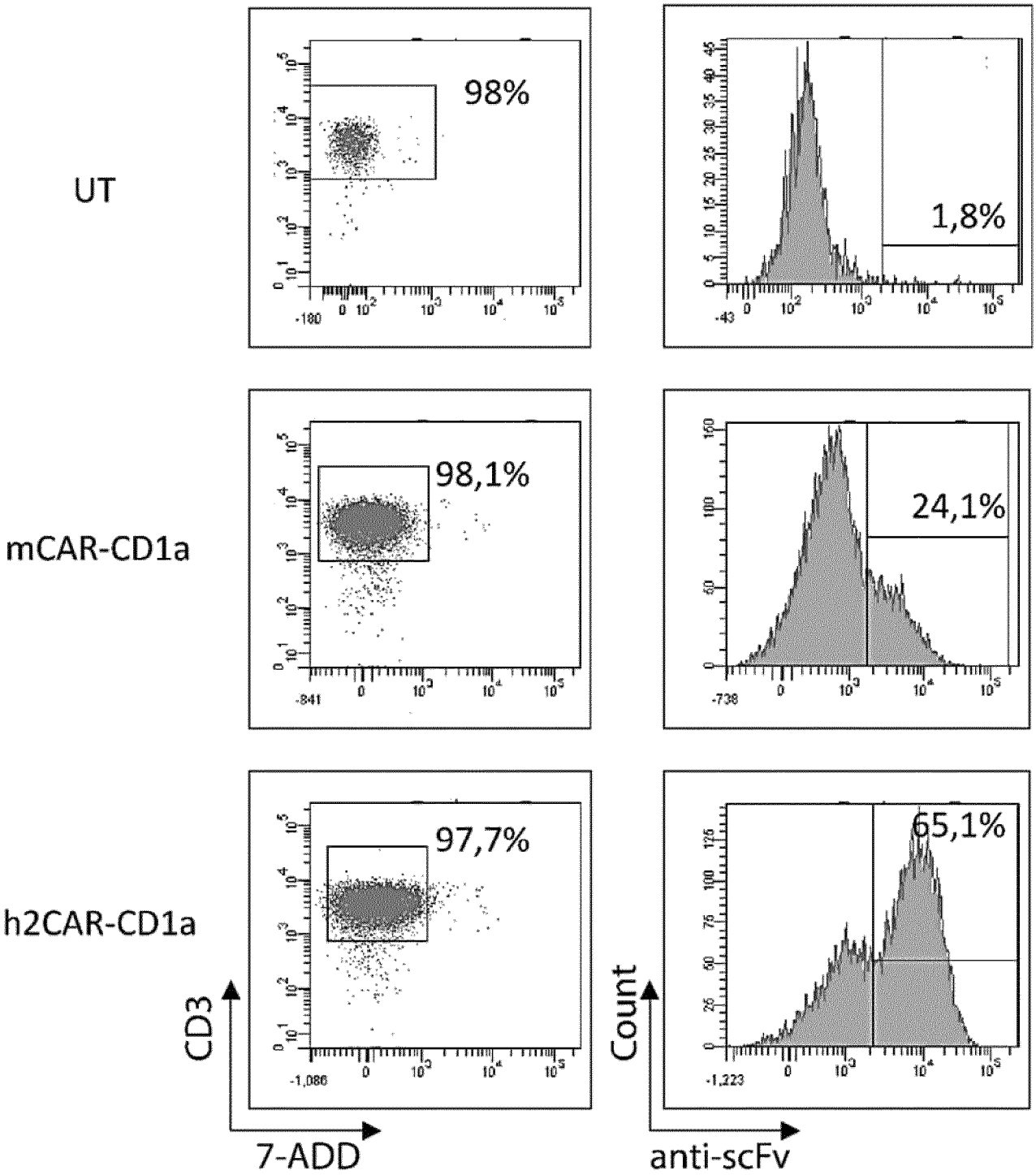

UT
98%
1,8%
mCAR-CD1a
98,1%
24,1%
h2CAR-CD1a
97,7%
65,1%
CD3
7-ADD
Count
anti-scFv

Fig. 6 (cont.)

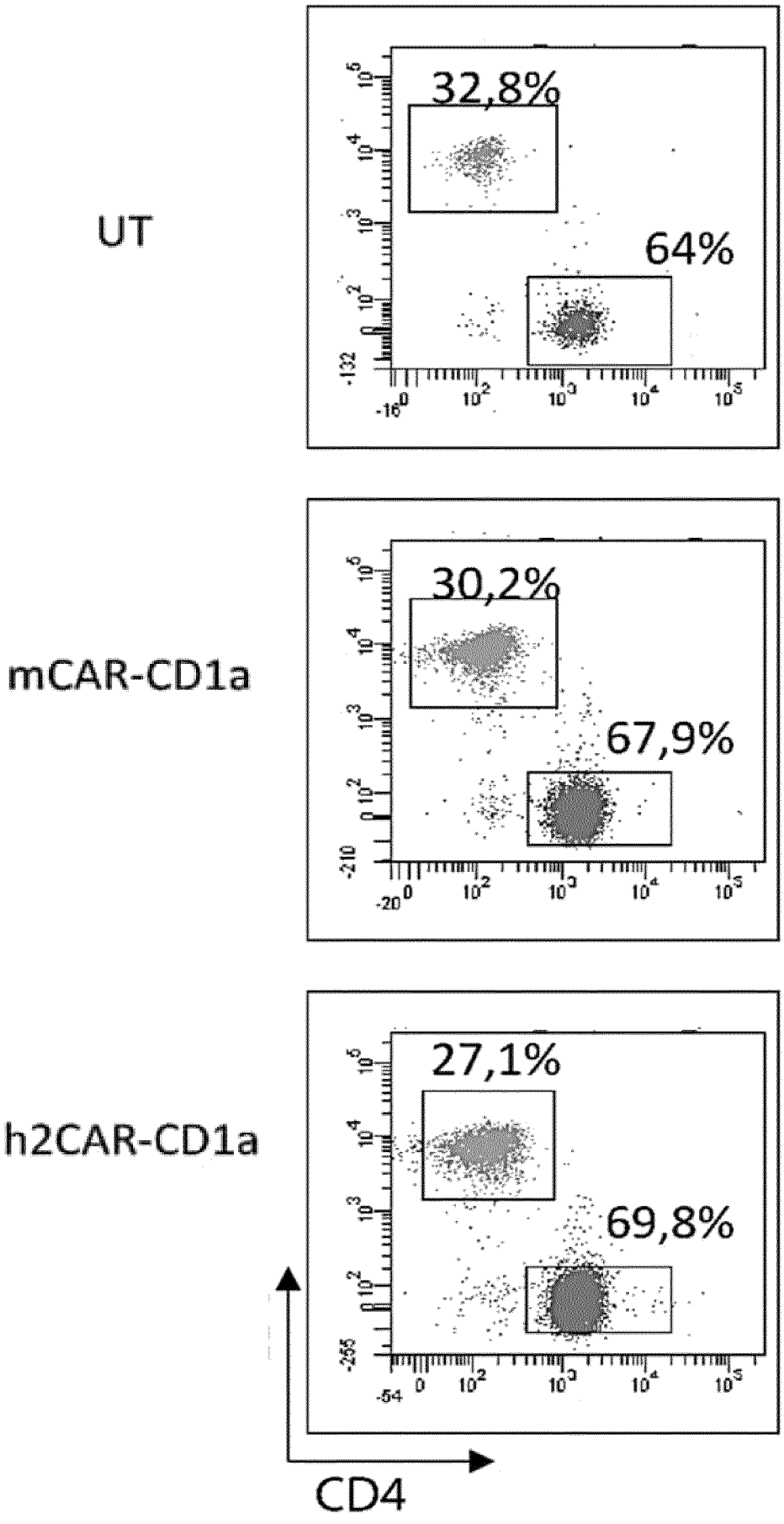


Fig. 7

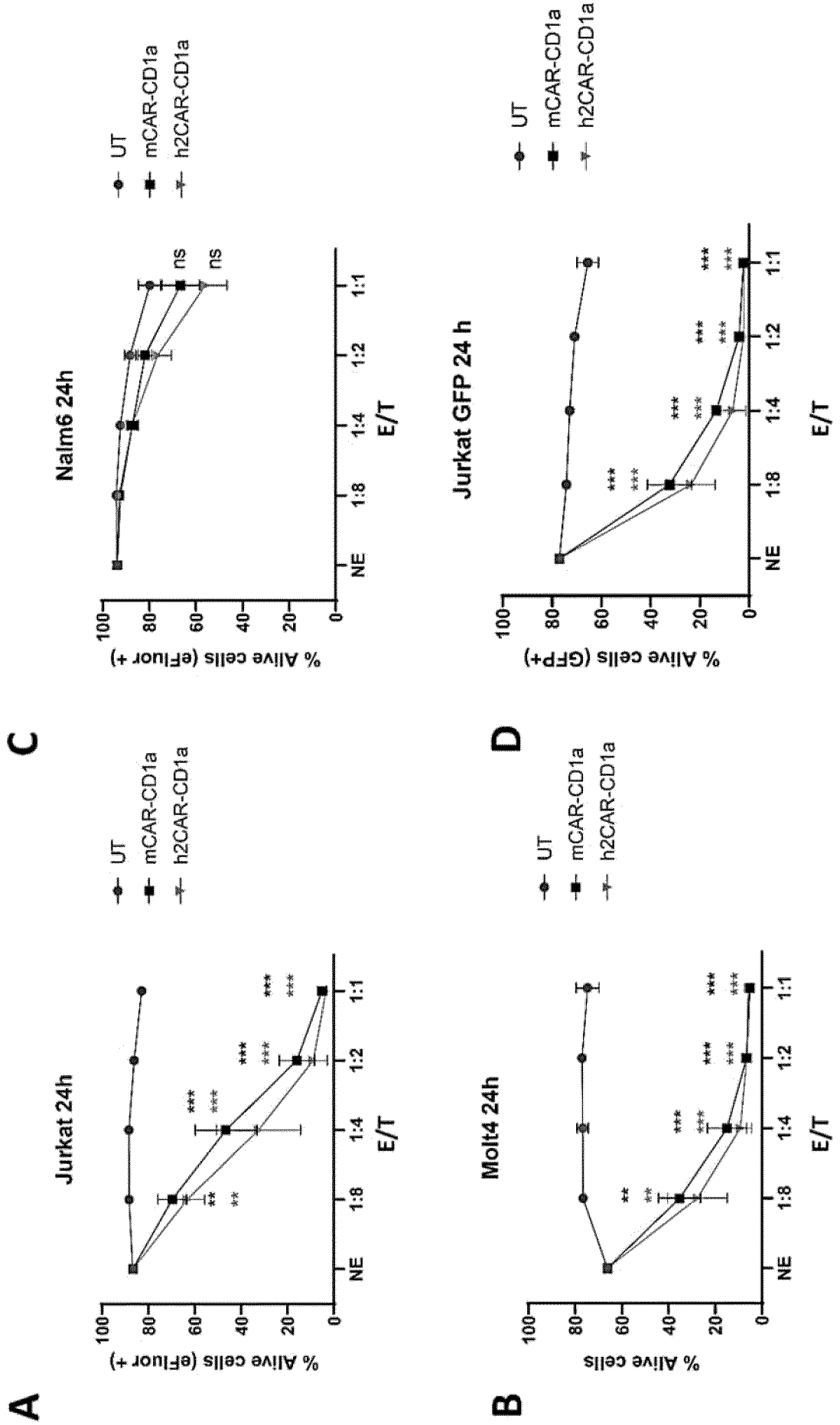

A
Jurkat 24h
% Alive cells (eFluor +)
E/T
NE
1:8
1:4
1:2
1:1
UT
mCAR-CD1a
h2CAR-CD1a
B
Molt4 24h
% Alive cells
C
Nalm6 24h
ns
D
Jurkat GFP 24 h
% Alive cells (GFP+)

Fig. 8

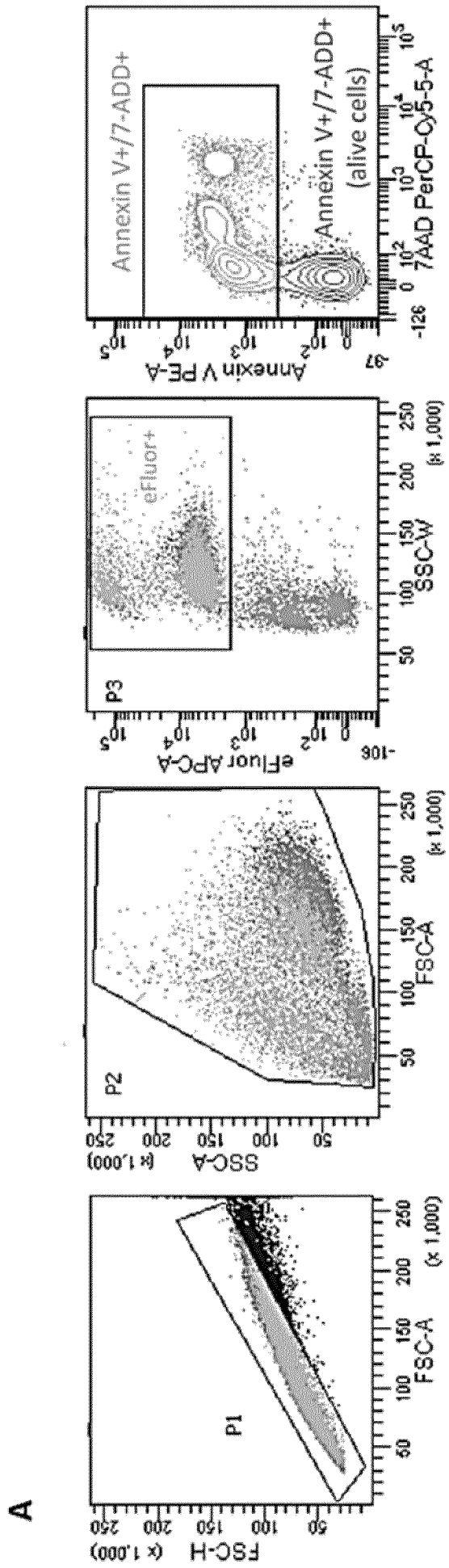

A
FSC-H (x 1,000)
FSC-A (x 1,000)
P1
SSC-A (x 1,000)
FSC-A (x 1,000)
P2
eFluor APC-A
SSC-W (x 1,000)
P3
eFluor+
Annexin V PE-A
7AAD PerCP-Cy5-5-A
Annexin V+/7-ADD+
Annexin V+/7-ADD+
(alive cells)

Fig. 8 (cont.)

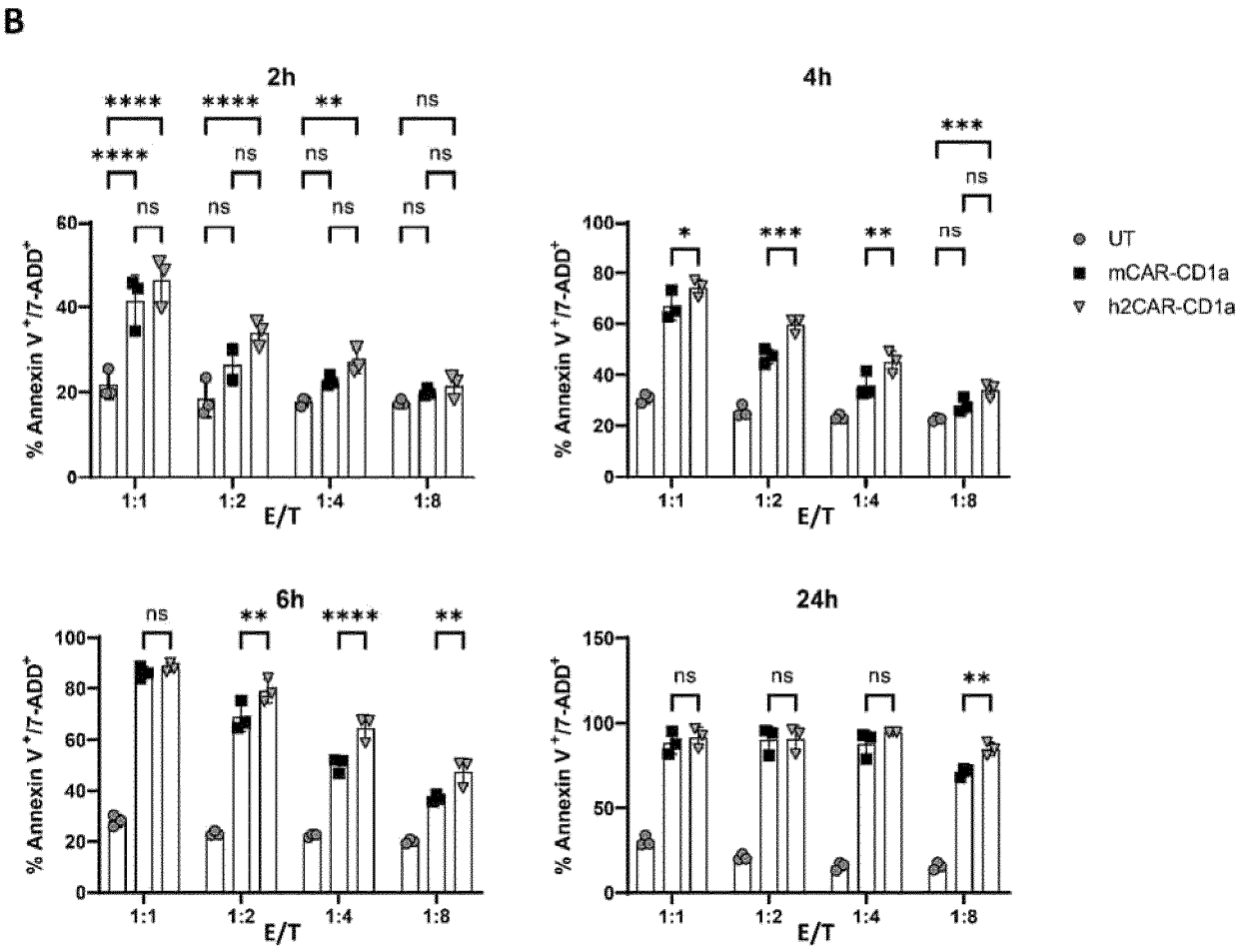

B
2h
4h
6h
24h
% Annexin V+/7-ADD+
E/T
1:1
1:2
1:4
1:8
ns
UT
mCAR-CD1a
h2CAR-CD1a

Fig. 8. (cont.)

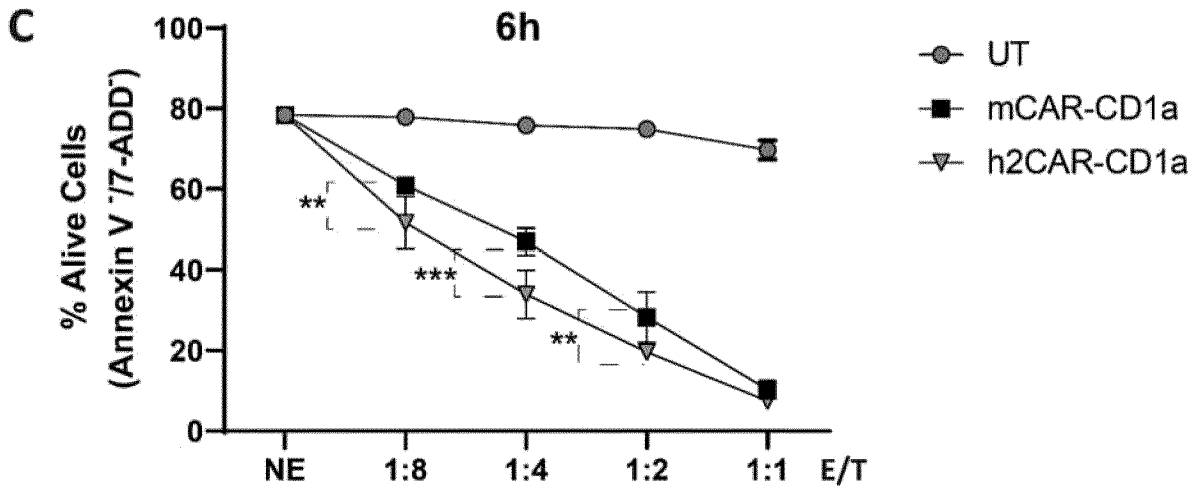

C
6h
% Alive Cells
(Annexin V-/7-ADD-)
100
80
60
40
20
0
NE
1:8
1:4
1:2
1:1
E/T
**
***
**
UT
mCAR-CD1a
h2CAR-CD1a

Fig. 9

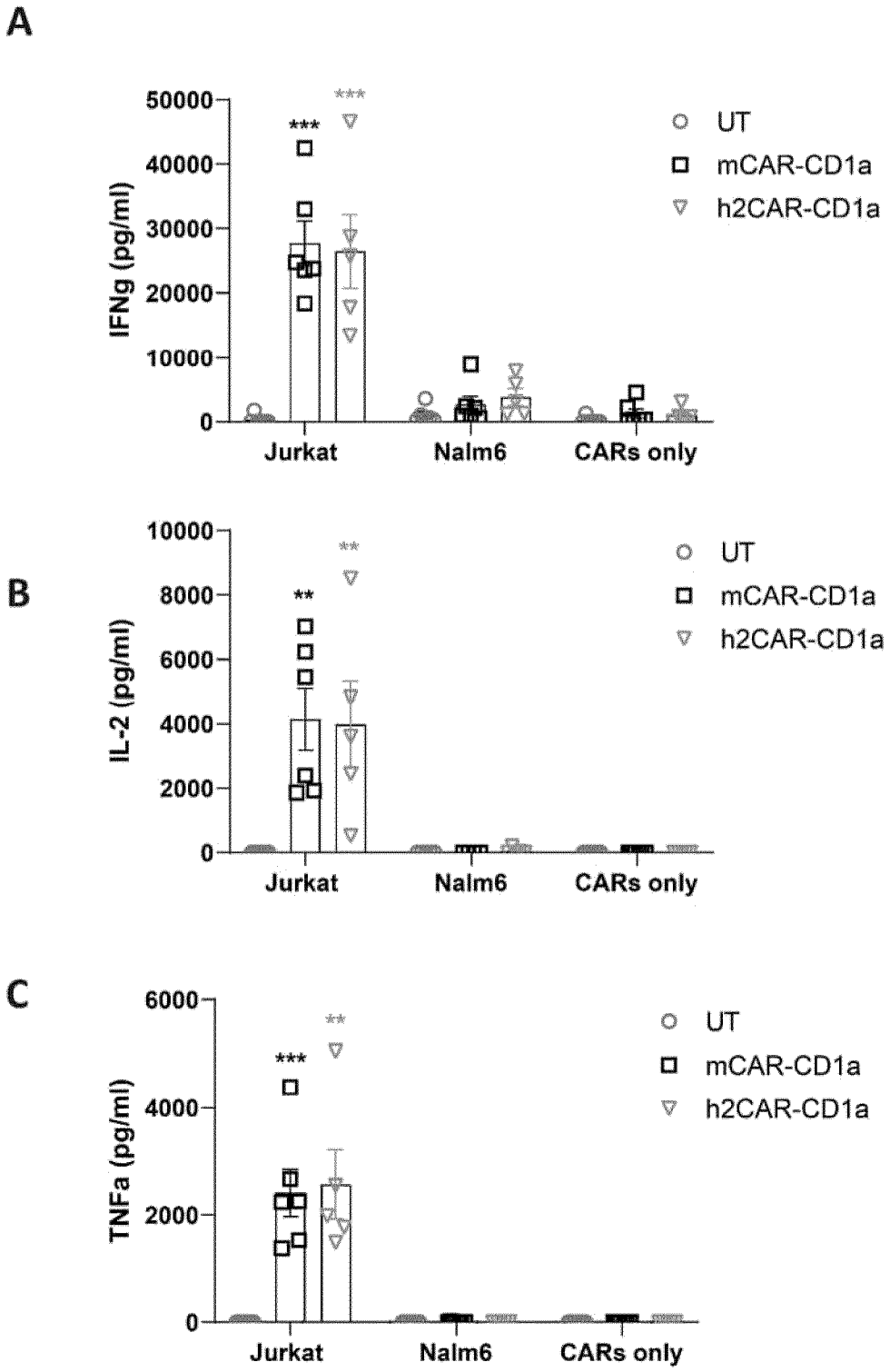
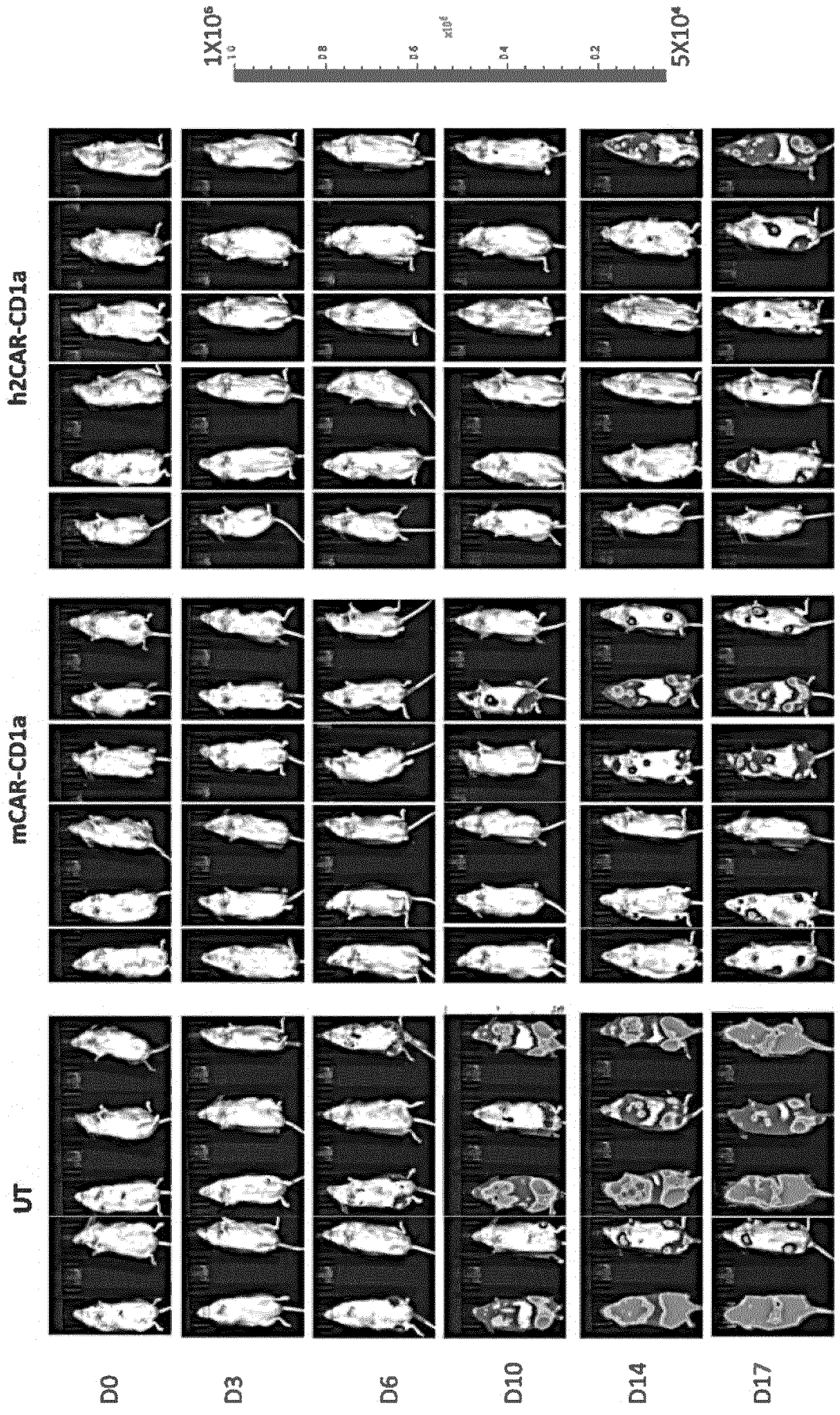

A
IFNg (pg/ml)
50000
40000
30000
20000
10000
0
***
***
Jurkat
Nalm6
CARs only
UT
mCAR-CD1a
h2CAR-CD1a
B
IL-2 (pg/ml)
10000
8000
6000
4000
2000
0
**
**
Jurkat
Nalm6
CARs only
UT
mCAR-CD1a
h2CAR-CD1a
C
TNFa (pg/ml)
6000
4000
2000
0
***
**
Jurkat
Nalm6
CARs only
UT
mCAR-CD1a
h2CAR-CD1a

Fig. 10



A

Fig. 10. (cont.)

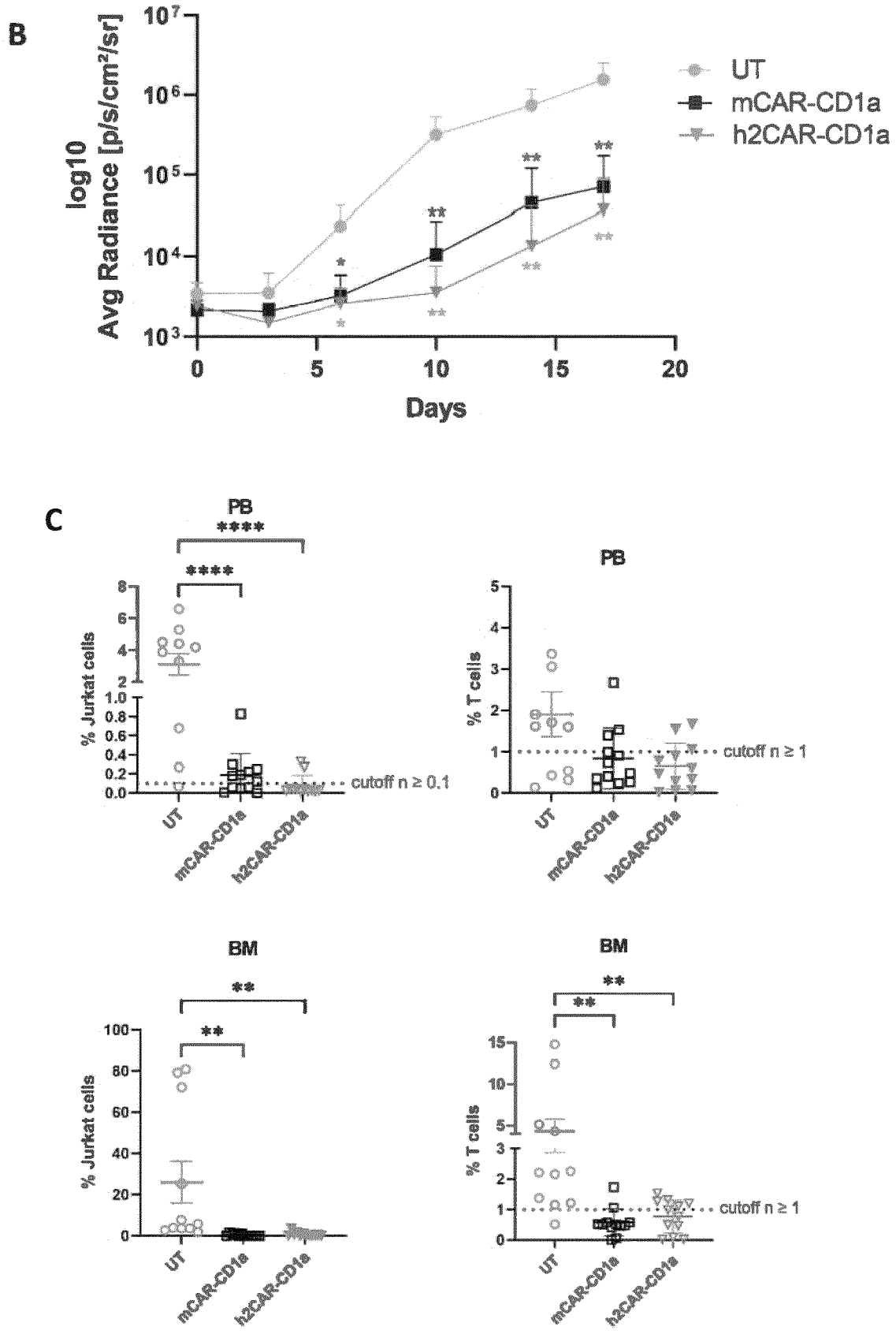

B
log10
Avg Radiance [p/s/cm²/sr]
Days
UT
mCAR-CD1a
h2CAR-CD1a
C
PB
BM
% Jurkat cells
% T cells
cutoff n ≥ 0.1
cutoff n ≥ 1
UT
mCAR-CD1a
h2CAR-CD1a

Fig. 11

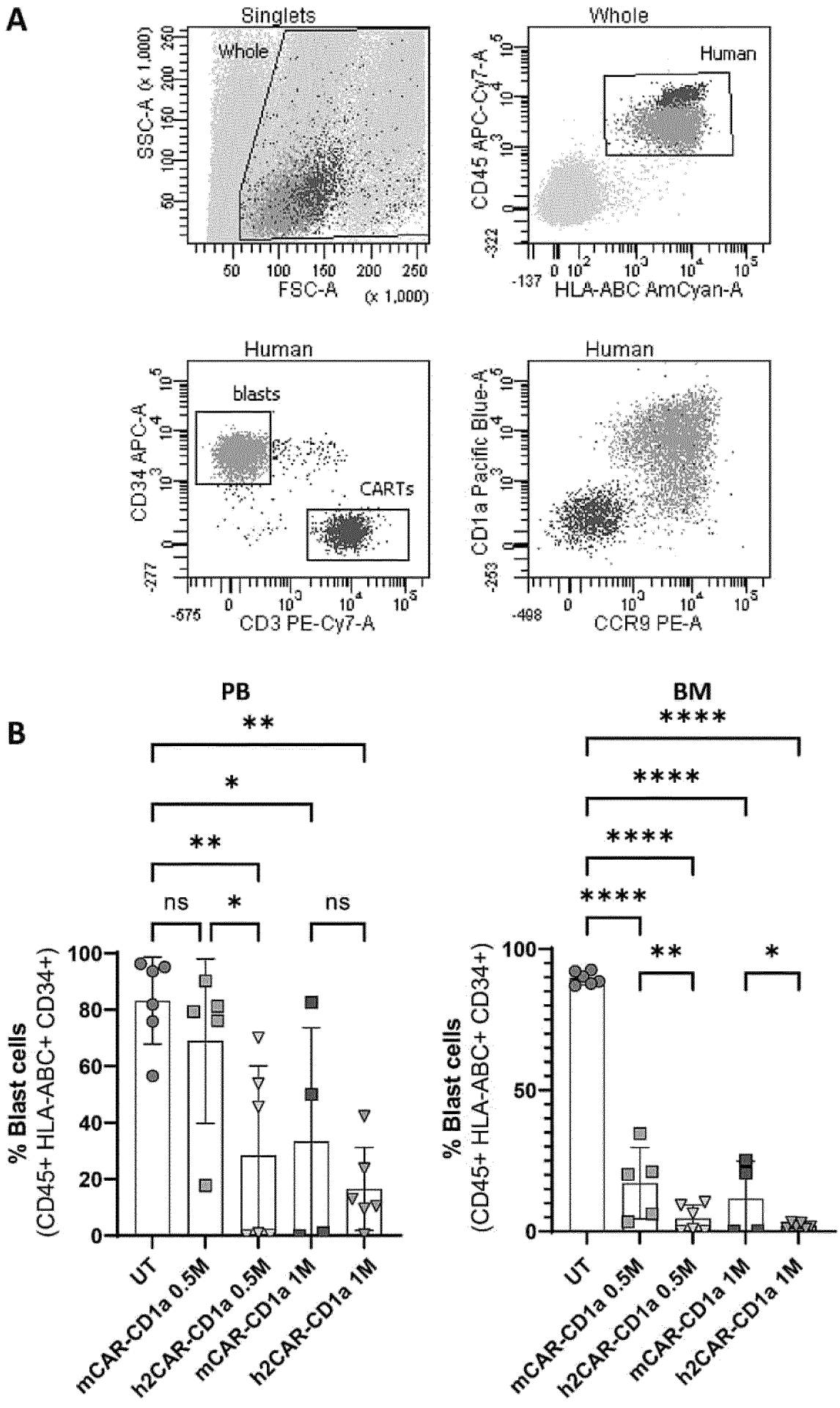

A
Singlets
Whole
SSC-A (x 1,000)
FSC-A (x 1,000)
Whole
Human
CD45 APC-Cy7-A
HLA-ABC AmCyan-A
Human
blasts
CARTs
CD34 APC-A
CD3 PE-Cy7-A
Human
CD1a Pacific Blue-A
CCR9 PE-A
B
PB
BM
% Blast cells
(CD45+ HLA-ABC+ CD34+)
UT
mCAR-CD1a 0.5M
h2CAR-CD1a 0.5M
mCAR-CD1a 1M
h2CAR-CD1a 1M
ns

Fig. 12

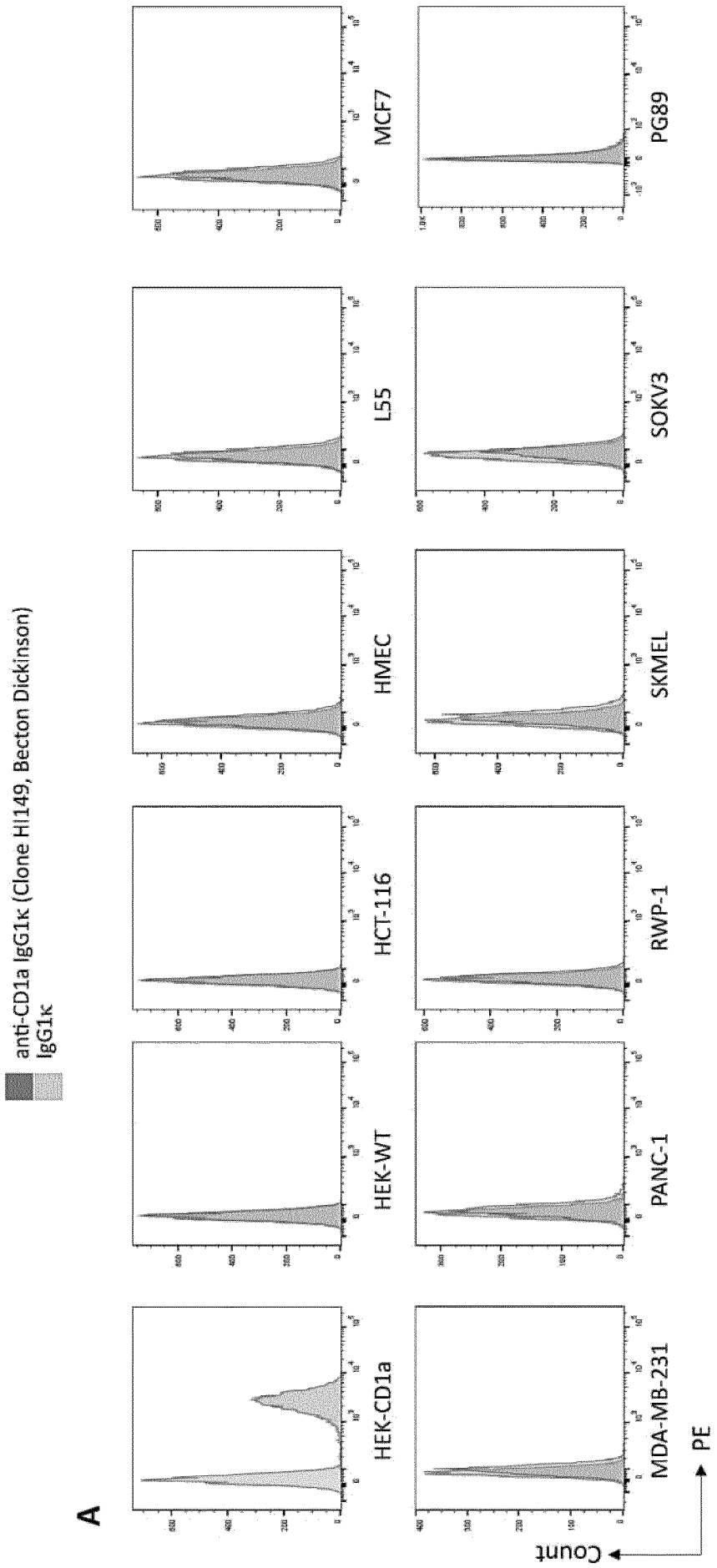

A
anti-CD1a IgG1κ (Clone HI149, Becton Dickinson)
IgG1κ
Count
PE
HEK-CD1a
HEK-WT
HCT-116
HMEC
L55
MCF7
MDA-MB-231
PANC-1
RWP-1
SKMEL
SOKV3
PG89

Fig. 12 (cont.)

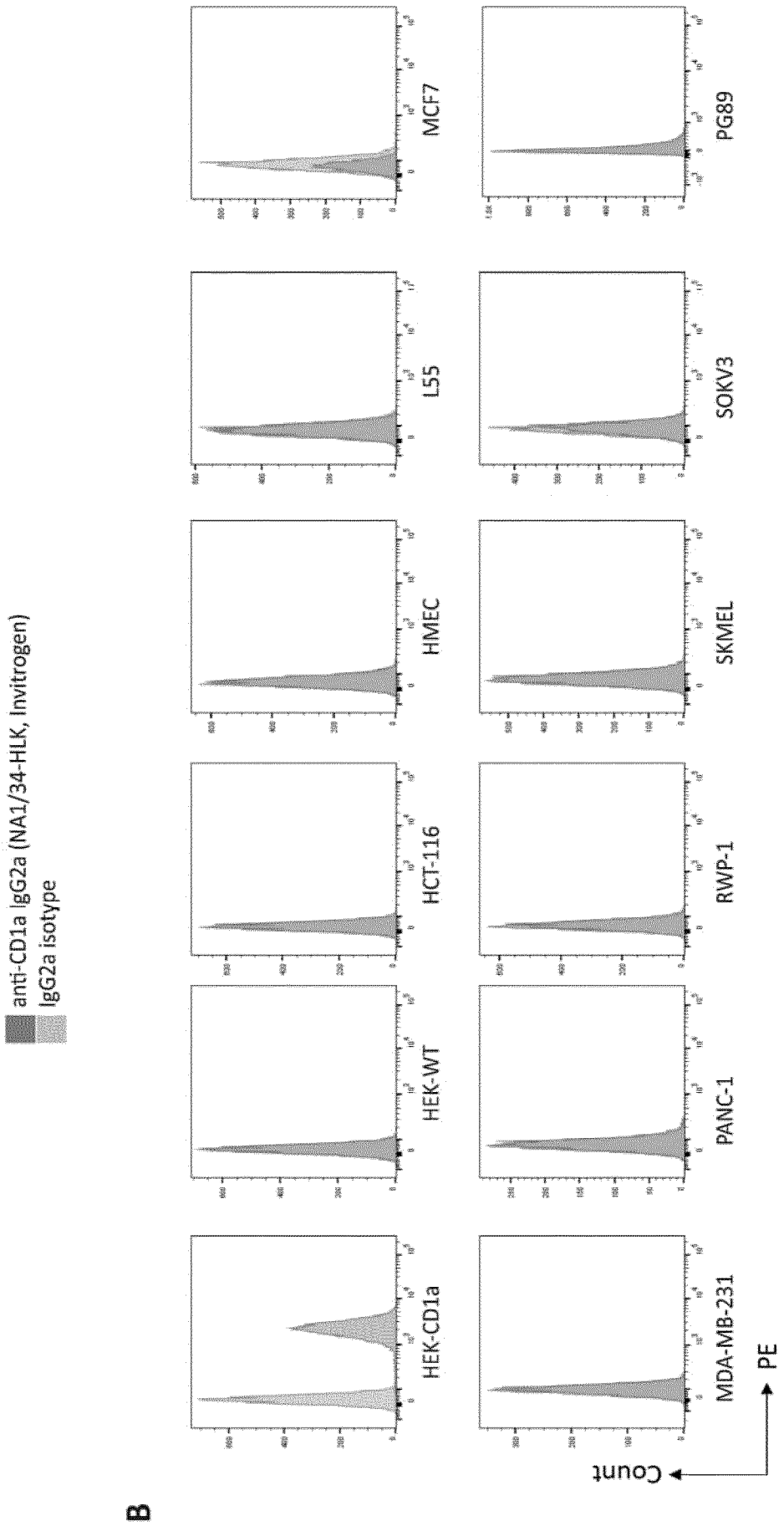

B
anti-CD1a IgG2a (NA1/34-HLK, Invitrogen)
IgG2a isotype
HEK-CD1a
HEK-WT
HCT-116
HMEC
L55
MCF7
MDA-MB-231
PANC-1
RWP-1
SKMEL
SOKV3
PG89
Count
PE

Fig. 13

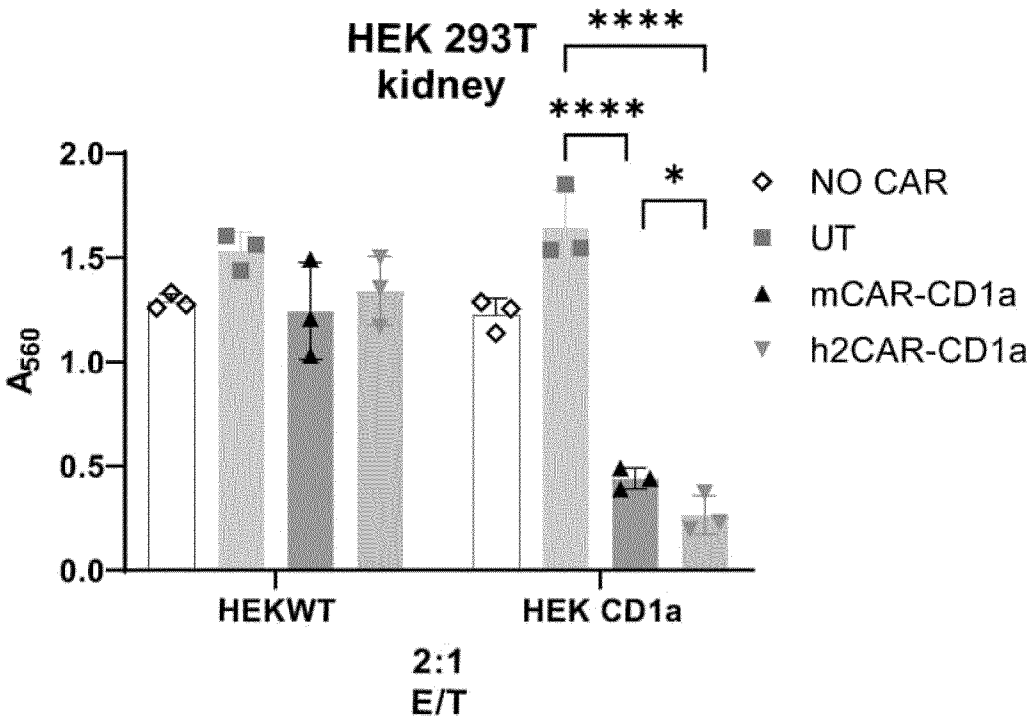

HEK 293T
kidney
****
****
*
NO CAR
UT
mCAR-CD1a
h2CAR-CD1a
2.0
1.5
1.0
0.5
0.0
A560
HEKWT
HEK CD1a
2:1
E/T

Fig. 14

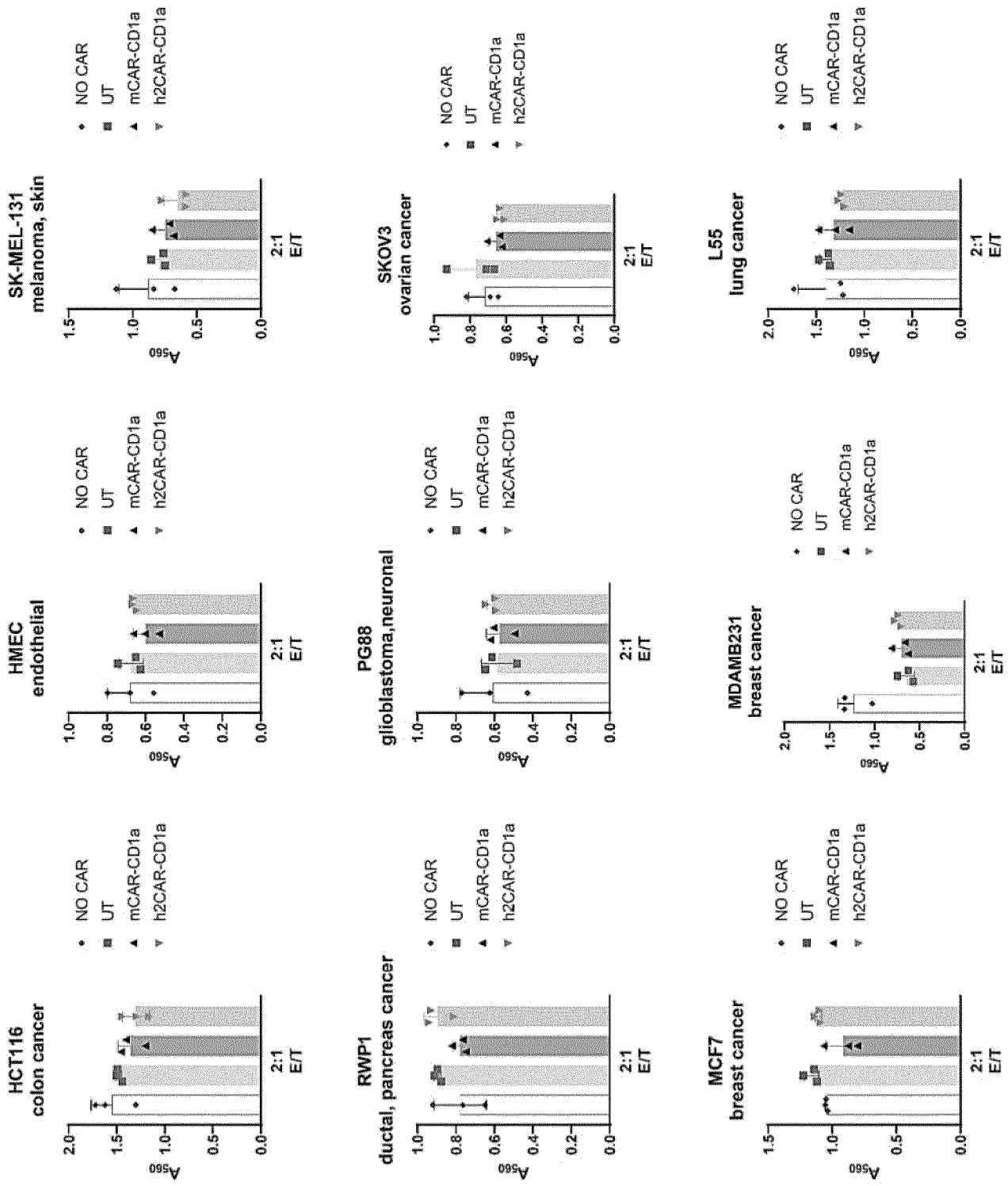

HCT116
colon cancer
HMEC
endothelial
PG88
glioblastoma,neuronal
SK-MEL-131
melanoma, skin
SKOV3
ovarian cancer
L55
lung cancer
RWP1
ductal, pancreas cancer
MDAMB231
breast cancer
MCF7
breast cancer
NO CAR
UT
mCAR-CD1a
h2CAR-CD1a
A560
2:1
E/T

Fig. 15

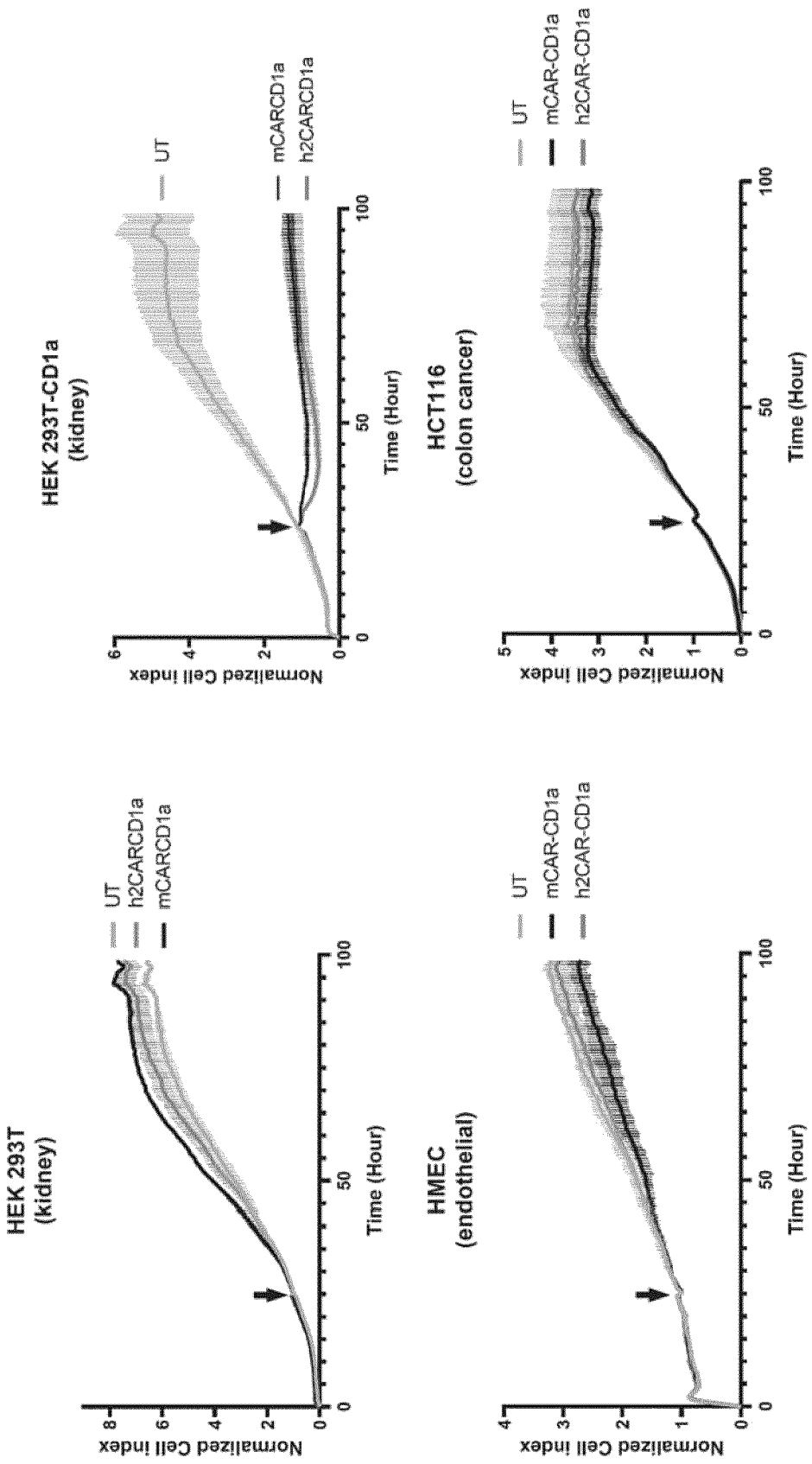

HEK 293T-CD1a
(kidney)
UT
mCARCD1a
h2CARCD1a
Normalized Cell Index
Time (Hour)
HCT116
(colon cancer)
UT
mCAR-CD1a
h2CAR-CD1a
Normalized Cell Index
Time (Hour)
HEK 293T
(kidney)
UT
h2CARCD1a
mCARCD1a
Normalized Cell Index
Time (Hour)
HMEC
(endothelial)
UT
mCAR-CD1a
h2CAR-CD1a
Normalized Cell Index
Time (Hour)

Fig. 15 (cont.)

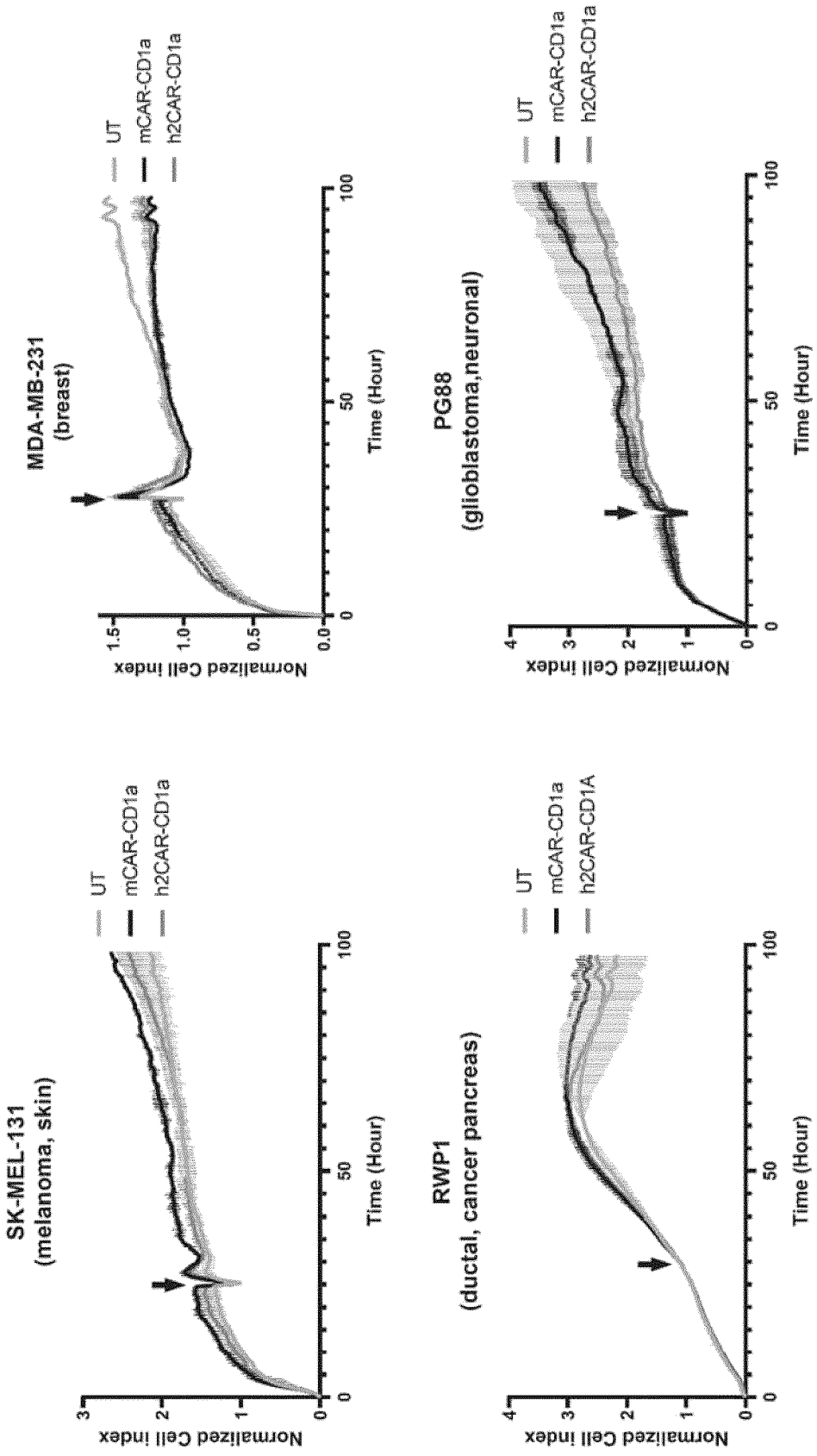

MDA-MB-231
(breast)
UT
mCAR-CD1a
h2CAR-CD1a
Normalized Cell Index
Time (Hour)
PG88
(glioblastoma,neuronal)
SK-MEL-131
(melanoma, skin)
RWP1
(ductal, cancer pancreas)
h2CAR-CD1A

Fig. 16

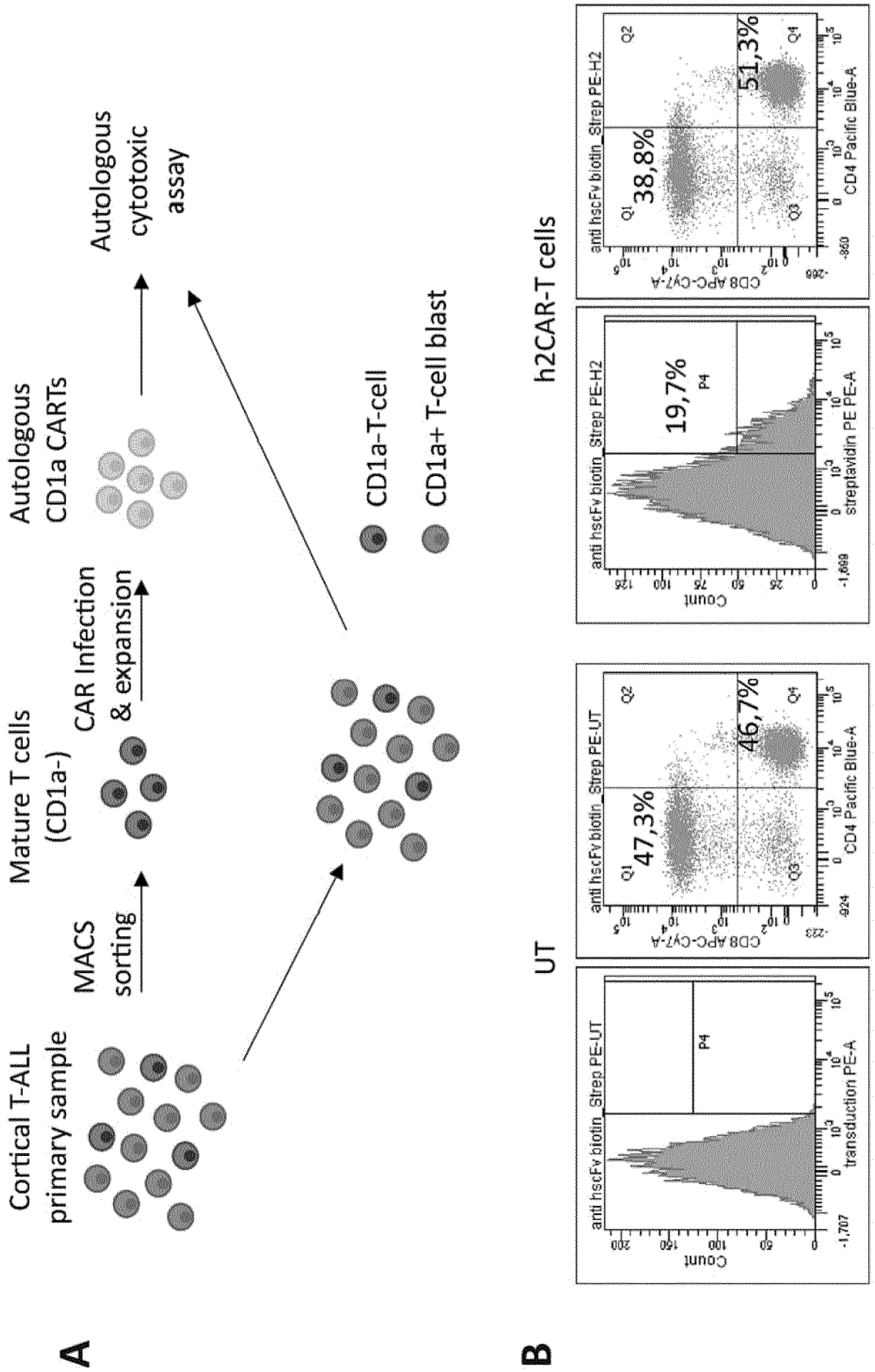

A
Cortical T-ALL primary sample
MACS sorting
Mature T cells (CD1a-)
CAR Infection & expansion
Autologous CD1a CARTs
Autologous cytotoxic assay
CD1a- T-cell
CD1a+ T-cell blast
B
UT
h2CAR-T cells
anti hscFv biotin_Strep PE-UT
transduction PE-A
Count
P4
anti hscFv biotin_Strep PE-H2
streptavidin PE PE-A
19,7%
CD8 APC-Cy7-A
CD4 Pacific Blue-A
47,3%
46,7%
38,8%
51,3%
Q1
Q2
Q3
Q4

Fig. 16 (cont.)

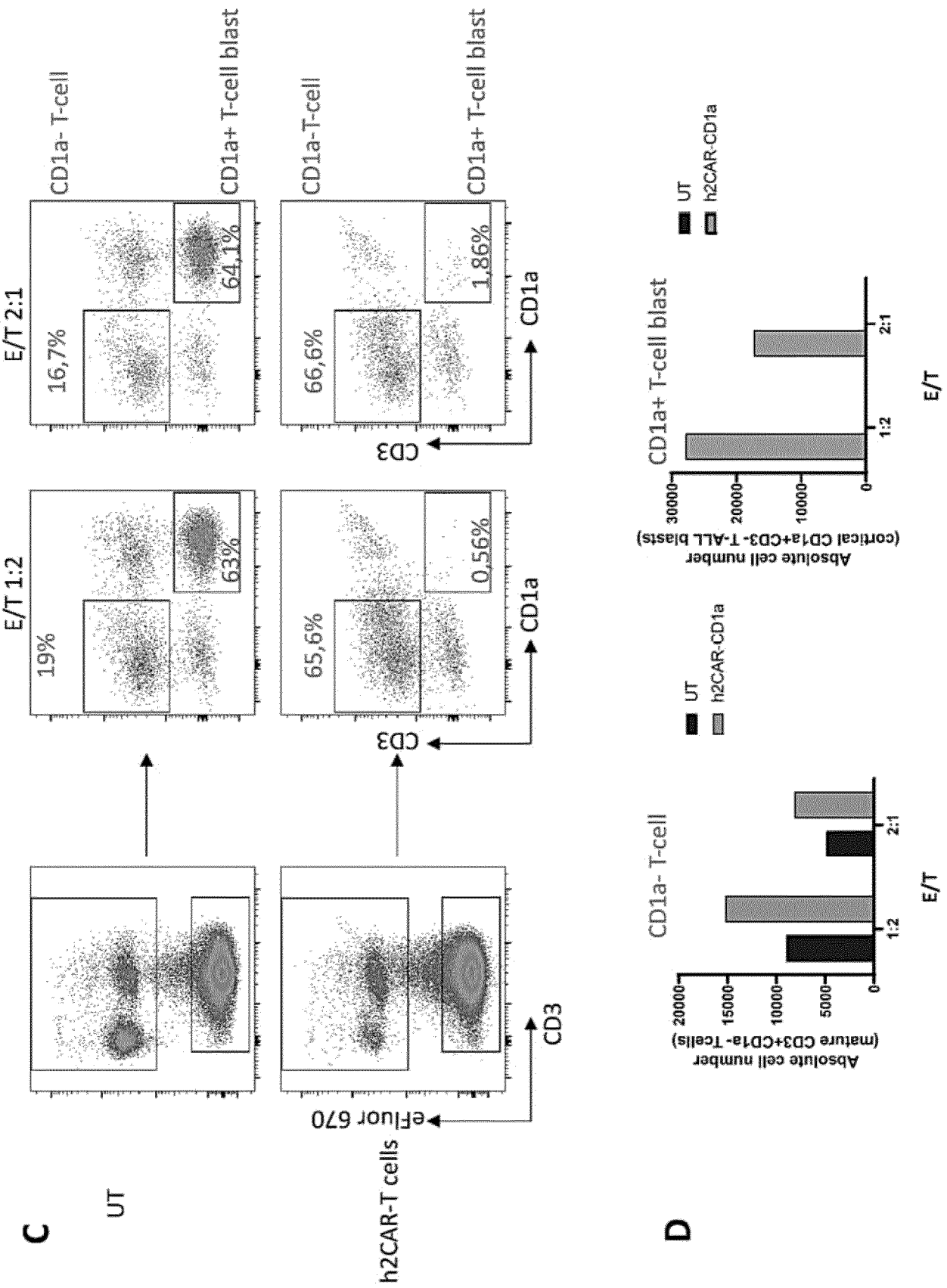

C
UT
h2CAR-T cells
eFluor 670
CD3
CD1a
E/T 1:2
19%
63%
65,6%
0,56%
E/T 2:1
16,7%
64,1%
66,6%
1,86%
CD1a- T-cell
CD1a+ T-cell blast
D
CD1a- T-cell
Absolute cell number
(mature CD3+CD1a- Tcells)
UT
h2CAR-CD1a
E/T
1:2
2:1
CD1a+ T-cell blast
Absolute cell number
(cortical CD1a+CD3- T-ALL blasts)

Fig. 17

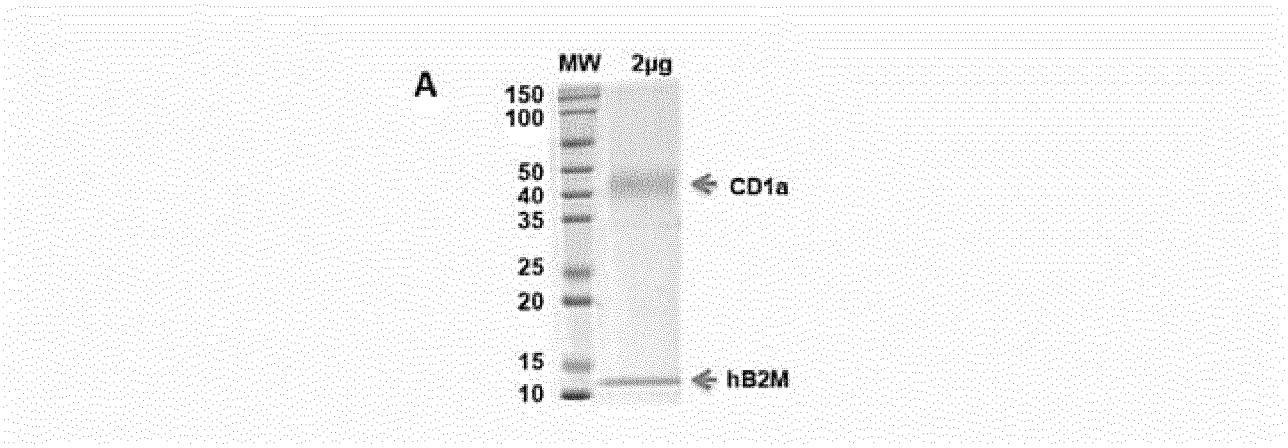


Fig. 17 (cont.)

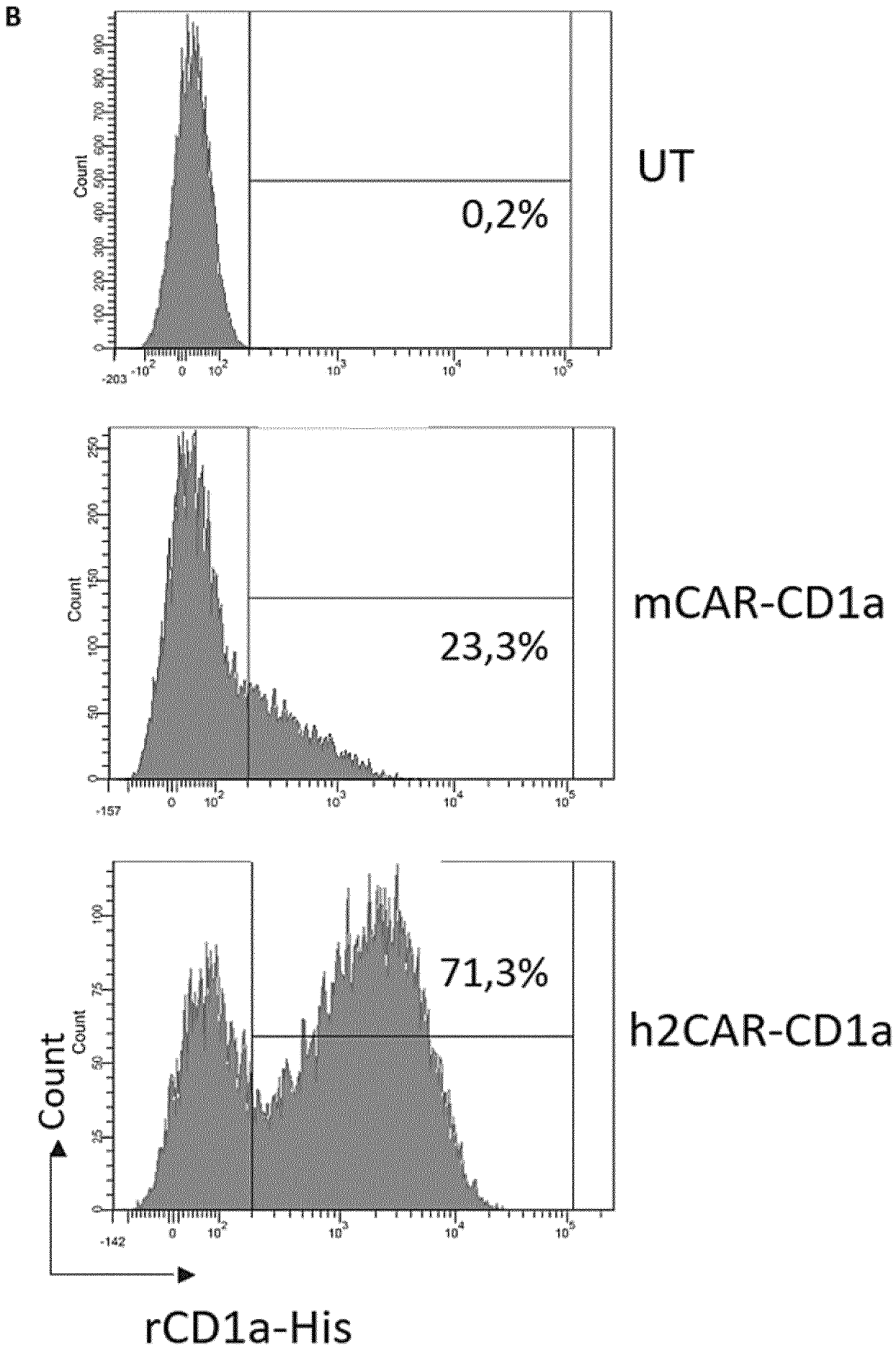


Fig. 17 (cont.)

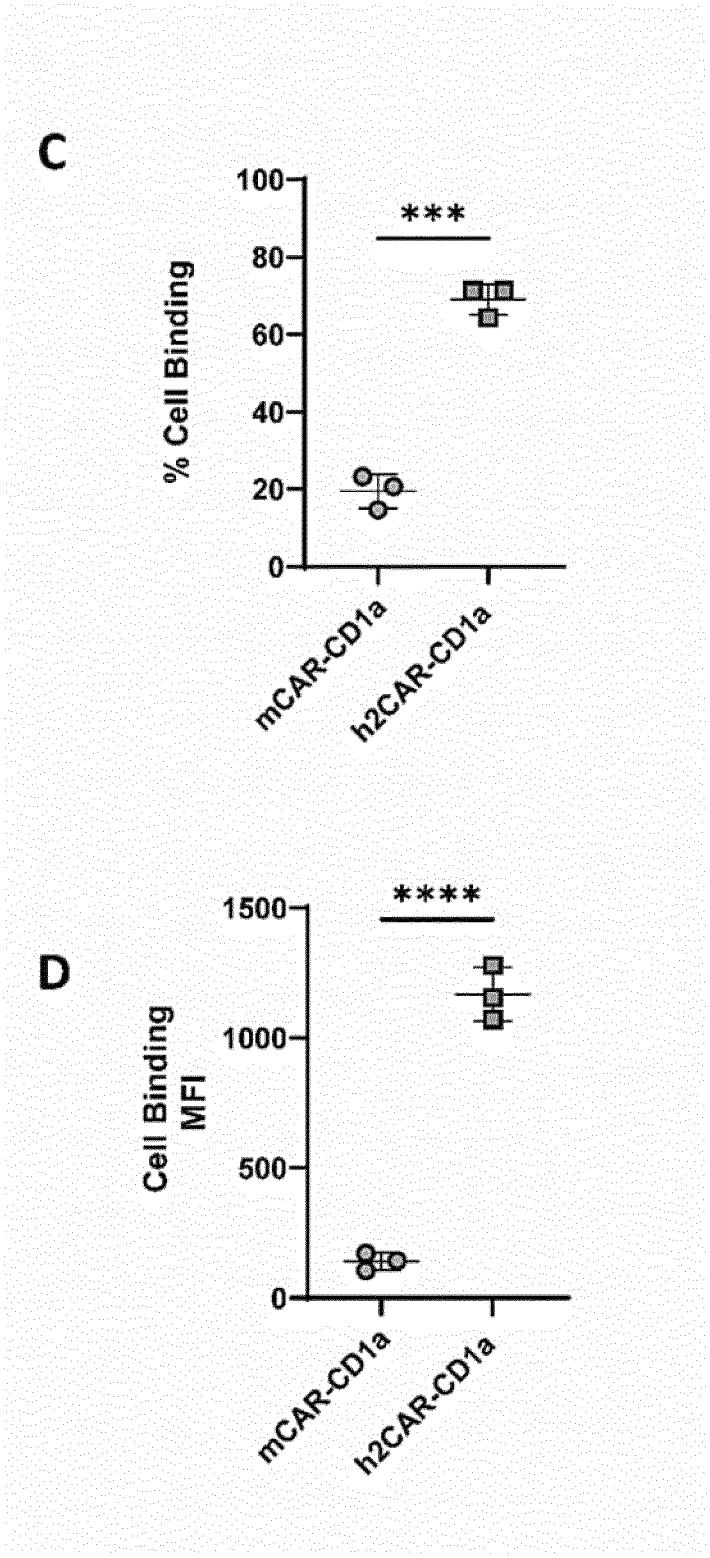

C
% Cell Binding
100
80
60
40
20
0
***
mCAR-CD1a
h2CAR-CD1a
D
Cell Binding MFI
1500
1000
500
0
****
mCAR-CD1a
h2CAR-CD1a

Fig. 18

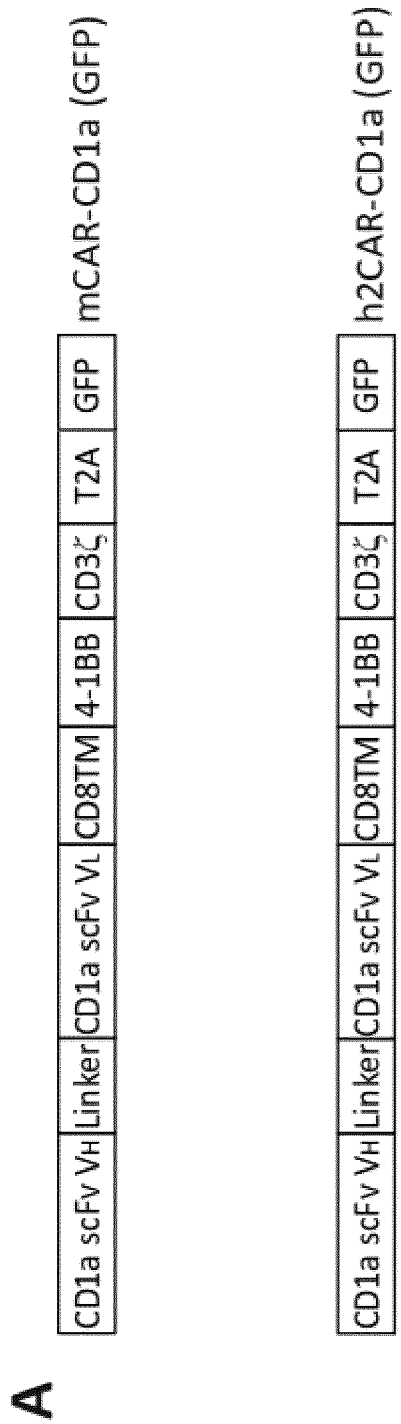

A
CD1a scFv VH
Linker
CD1a scFv VL
CD8TM
4-1BB
CD3ζ
T2A
GFP
mCAR-CD1a (GFP)
CD1a scFv VH
Linker
CD1a scFv VL
CD8TM
4-1BB
CD3ζ
T2A
GFP
h2CAR-CD1a (GFP)

Fig. 18 (cont.)

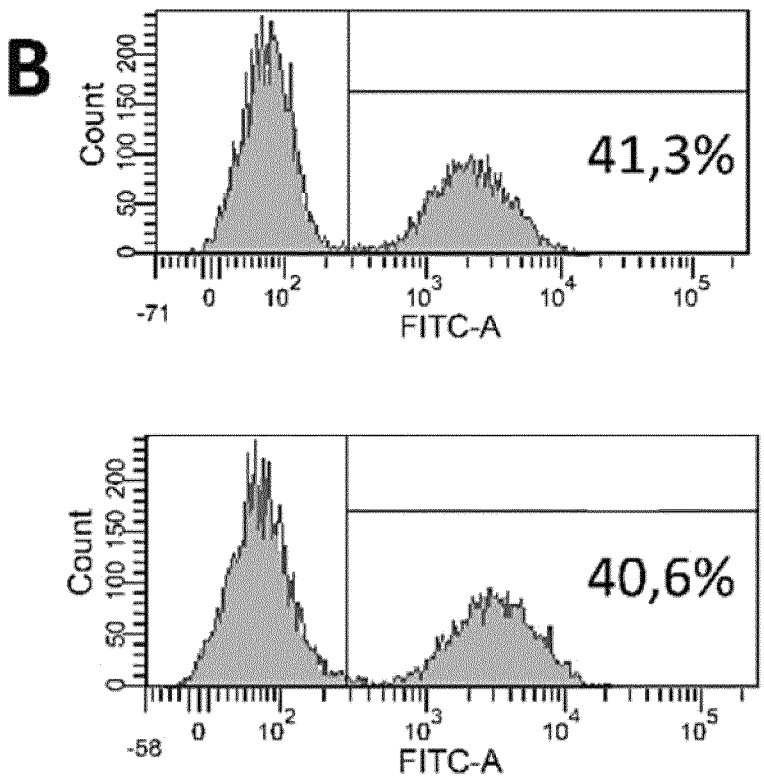
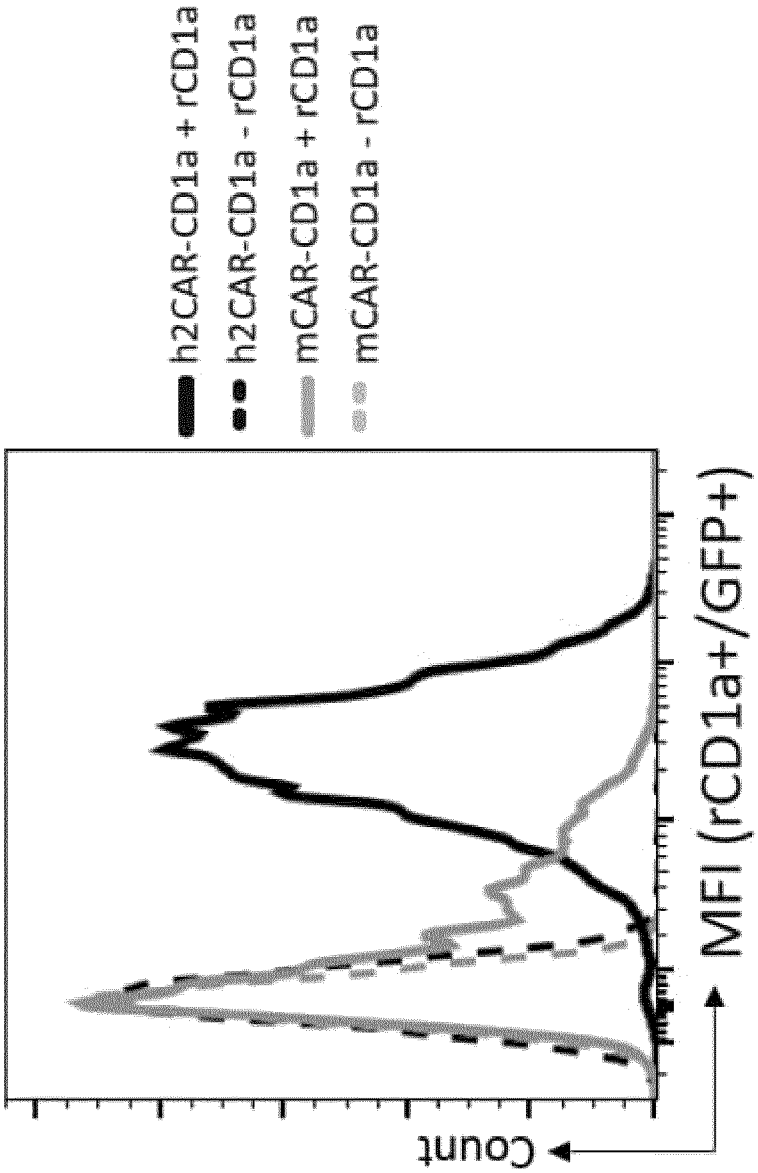

B
Count
0 50 100 150 200
41,3%
-71
0
10^2
10^3
10^4
10^5
FITC-A
Count
0 50 100 150 200
40,6%
-58
0
10^2
10^3
10^4
10^5
FITC-A

Fig. 18 (cont.)


h2CAR-CD1a + rCD1a
h2CAR-CD1a - rCD1a
mCAR-CD1a + rCD1a
mCAR-CD1a - rCD1a
Count
MFI (rCD1a+/GFP+)

C

Fig. 18 (cont.)

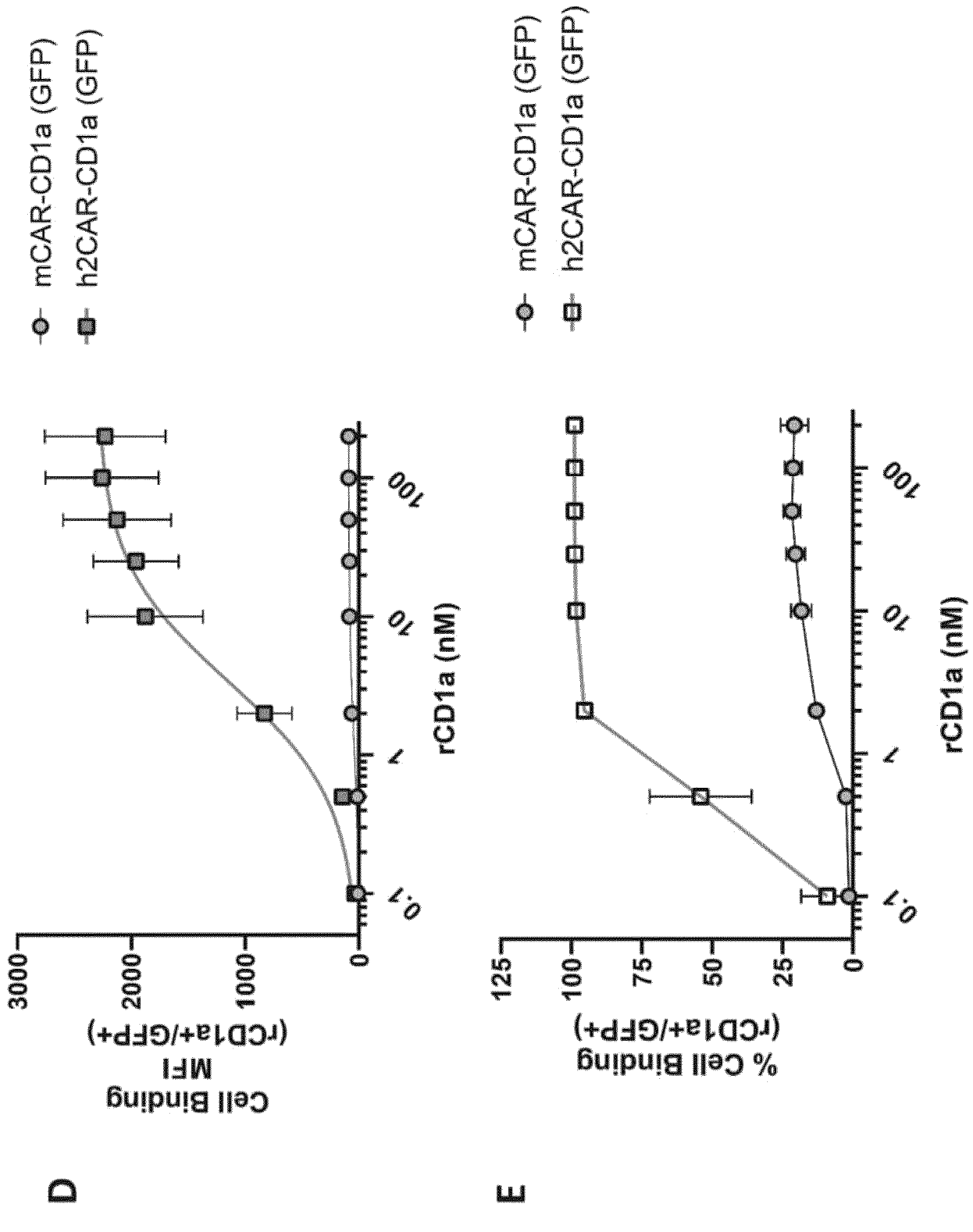

D
mCAR-CD1a (GFP)
h2CAR-CD1a (GFP)
Cell Binding
MFI
(rCD1a+/GFP+)
3000
2000
1000
0
0.1
1
10
100
rCD1a (nM)
E
mCAR-CD1a (GFP)
h2CAR-CD1a (GFP)
% Cell Binding
(rCD1a+/GFP+)
125
100
75
50
25
0
0.1
1
10
100
rCD1a (nM)

Fig. 19

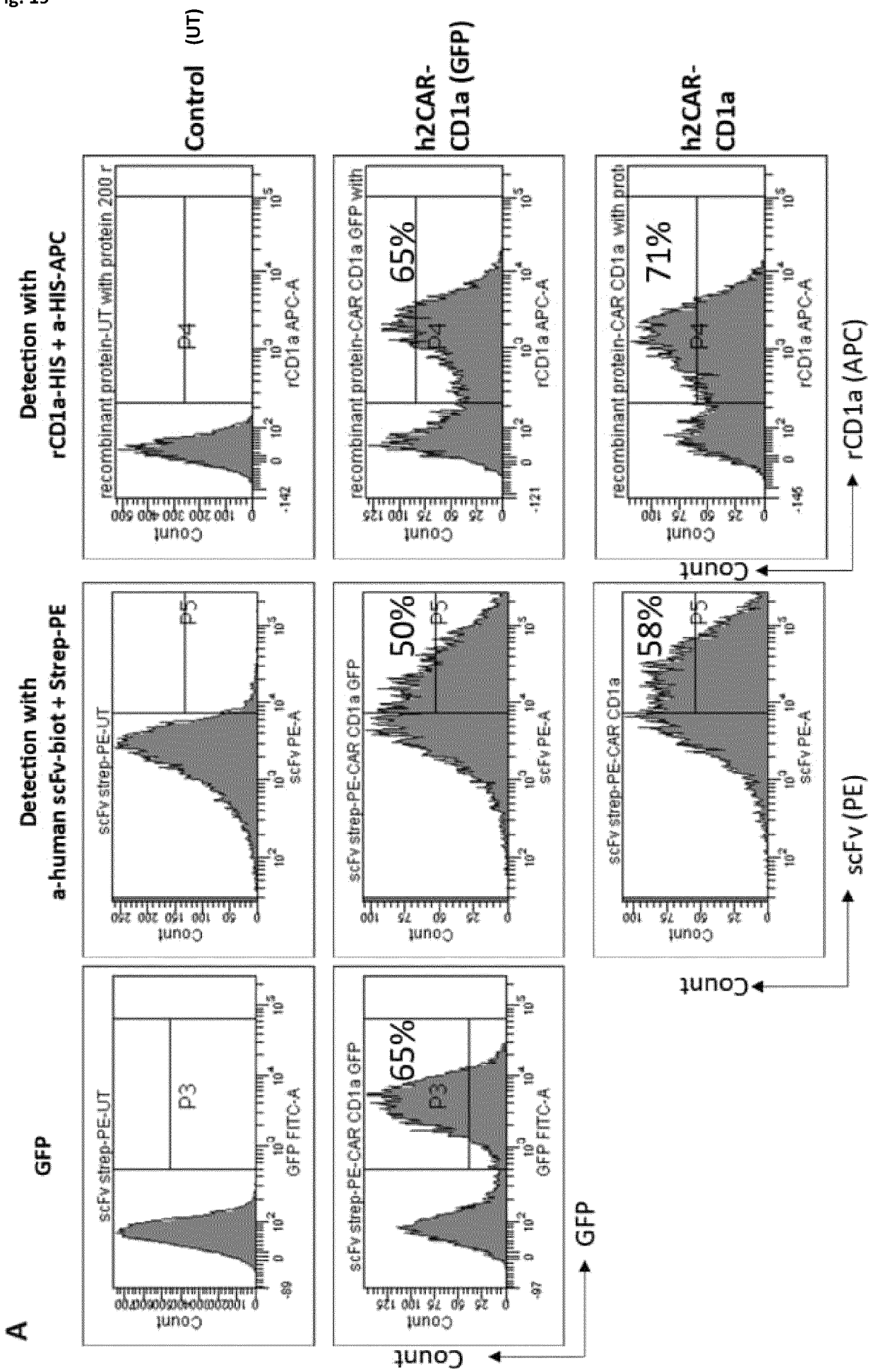

A
GFP
Detection with
a-human scFv-biot + Strep-PE
Detection with
rCD1a-HIS + a-HIS-APC
Control (UT)
h2CAR-CD1a (GFP)
h2CAR-CD1a
scFv strep-PE-UT
scFv strep-PE-CAR CD1a GFP
scFv strep-PE-CAR CD1a
recombinant protein-UT with protein 200 r
recombinant protein-CAR CD1a GFP with
recombinant protein-CAR CD1a with prot
GFP FITC-A
scFv PE-A
rCD1a APC-A
P3
P4
P5
65%
50%
58%
71%
Count
GFP
scFv (PE)
rCD1a (APC)

Fig. 19 (cont.)

B

	%hCAR-CD1a-(GFP)	%hCAR-CD1a
% GFP	65	-
% scFv	50	58
% rCD1a	65	71

Fig. 20

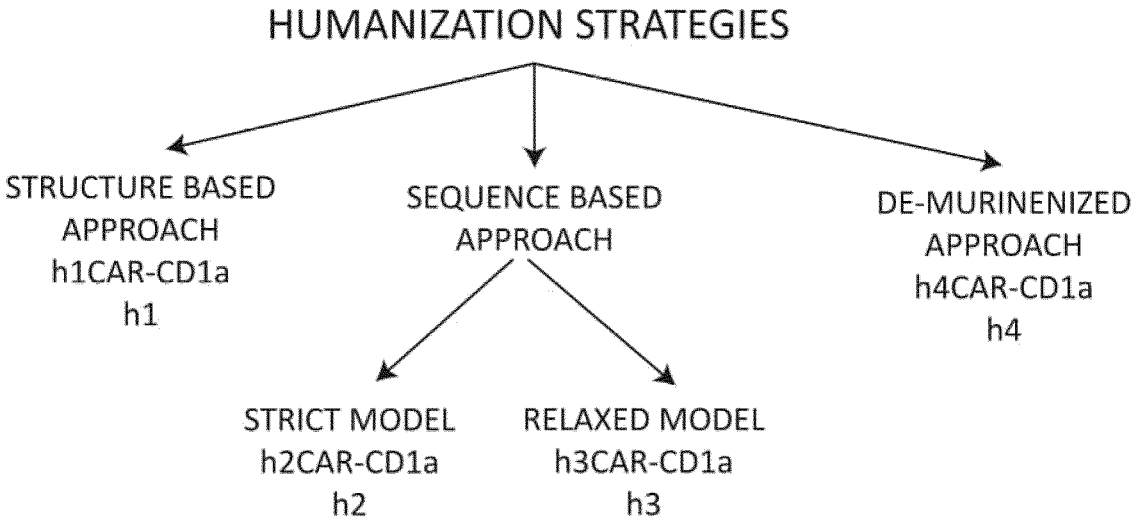

HUMANIZATION STRATEGIES
STRUCTURE BASED APPROACH h1CAR-CD1a h1
SEQUENCE BASED APPROACH
DE-MURINENIZED APPROACH h4CAR-CD1a h4
STRICT MODEL h2CAR-CD1a h2
RELAXED MODEL h3CAR-CD1a h3

Fig. 21

HUMANIZATION STRATEGIES: STRUCTURE-BASED APPROACH
Nt
Light Chain
Nt
Heavy Chain
STRUCTURAL MODEL MURINE
CDRs + stems:
H1: SGYAFSTYTMH H2: YINPNSASTS H3: ARGFYTMDY
L1: QASQDINKYIA L2: IHYTSTL L3: LHYDNLPWT
STRUCTURAL MODEL MURINE-HUMAN 5wn9
Human scFv 5wn9 in grey
Murine scFv
Structurally very similar, RMSD < 2.0
5wn9:https://europepmc.org/article/MED/29523582

Fig. 22

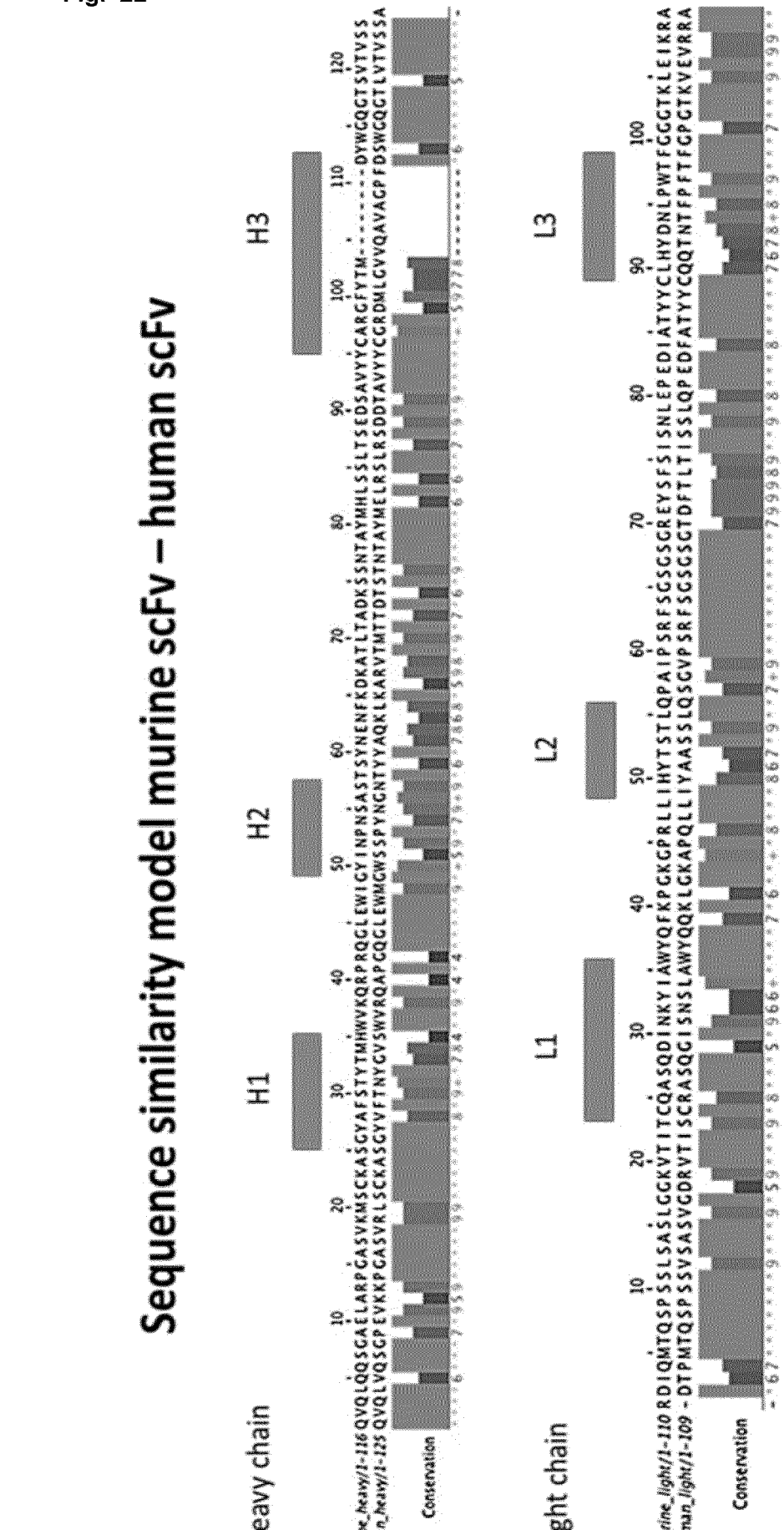

Sequence similarity model murine scFv – human scFv
Heavy chain
H1
H2
H3
murine_heavy/1-116
human_heavy/1-125
Conservation
Light chain
L1
L2
L3
murine_light/1-110
human_light/1-109
Conservation

Fig. 23

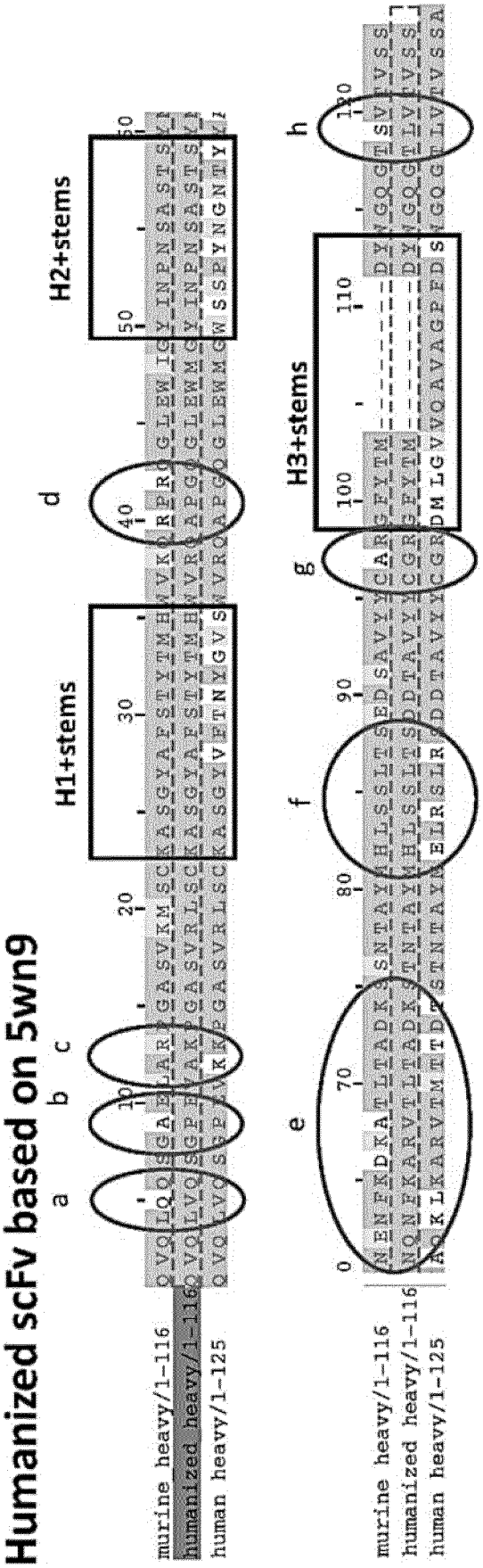

Humanized scFv based on 5wn9
a
b
c
d
e
f
g
h
H1+stems
H2+stems
H3+stems
murine heavy/1-116
humanized heavy/1-116
human heavy/1-125

Fig. 24

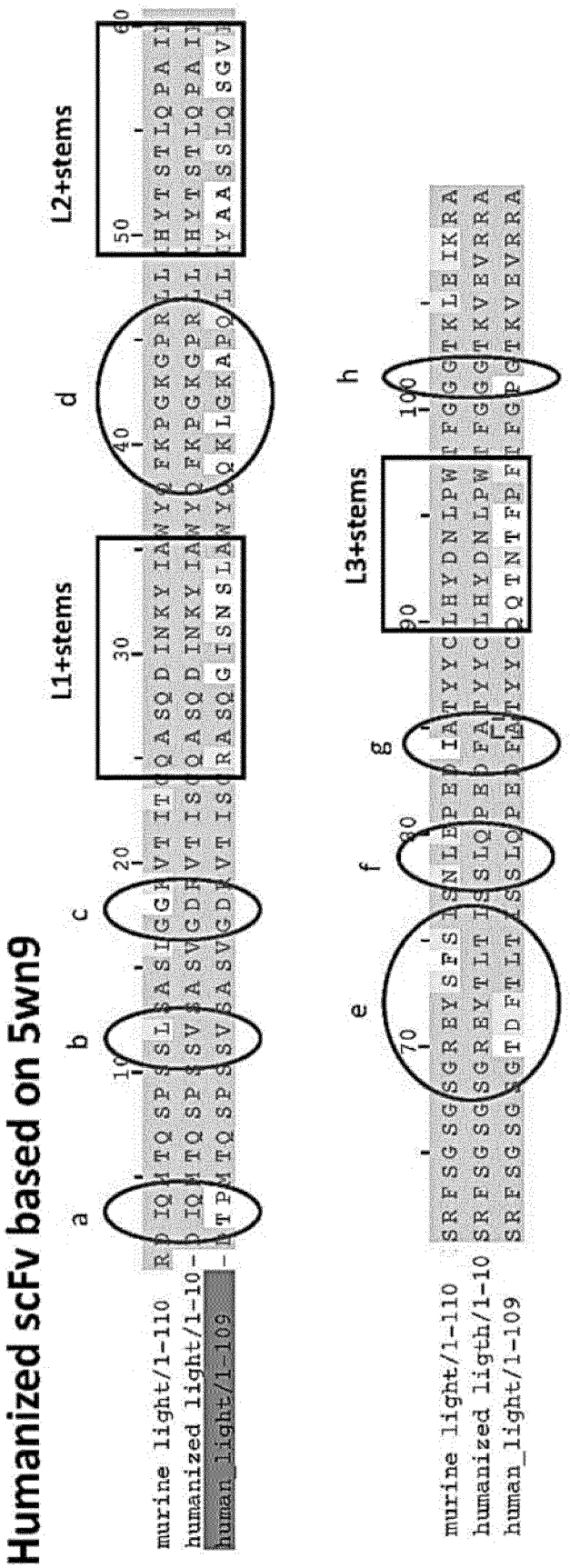

Humanized scFv based on 5wn9
L1+stems
L2+stems
L3+stems
a
b
c
d
e
f
g
h
murine light/1-110
humanized light/1-10-
human_light/1-109

Fig. 25

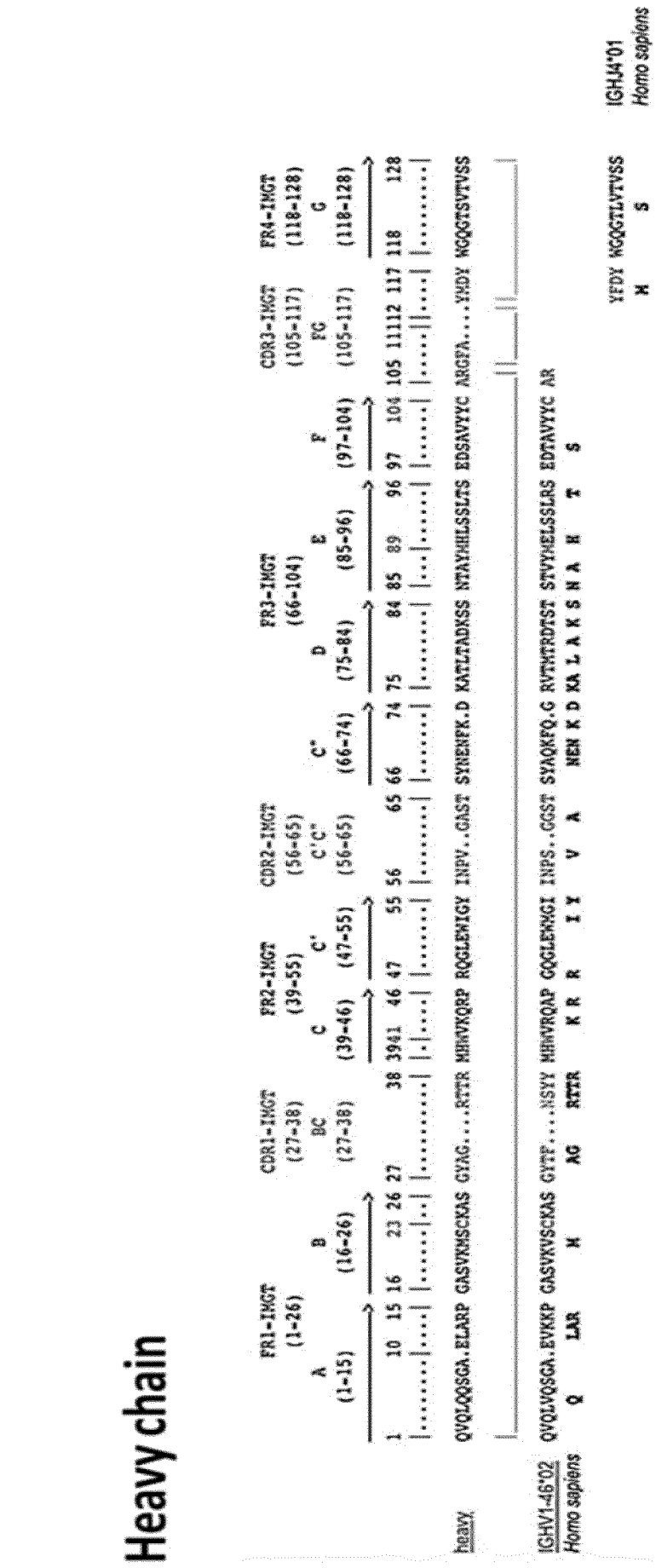

Heavy chain
FR1-IMGT (1-26)
CDR1-IMGT (27-38)
FR2-IMGT (39-55)
CDR2-IMGT (56-65)
FR3-IMGT (66-104)
CDR3-IMGT (105-117)
FR4-IMGT (118-128)
A (1-15)
B (16-26)
BC (27-38)
C (39-46)
C' (47-55)
C'C" (56-65)
C" (66-74)
D (75-84)
E (85-96)
F (97-104)
FG (105-117)
C (118-128)
heavy
QVQLQQSGA.ELARP GASVKMSCKAS GYAG....RTTR MHWVKQRP RQGLEWIGY INPV..GAST SYNEKFK.D KATLTADKSS NTAYMHLSSLTS EDSAVYYC ANGFA....YWDY WGQGTSVTVSS
IGHV1-46*02
Homo sapiens
QVQLVQSGA.EVKKP GASVKVSCKAS GYTF....NSYY MHWVRQAP GQGLEWMGI INPS..GGST SYAQKFQ.G RVTMTRDTST STVYMELSSLRS EDTAVYYC AR
YFDY WGQGTLVTVSS
H S
IGHJ4*01
Homo sapiens

Fig. 26

Light chain
FR1-IMGT (1-26)
A (1-15)
B (16-26)
CDR1-IMGT (27-38)
BC (27-38)
FR2-IMGT (39-55)
C (39-46)
C' (47-55)
CDR2-IMGT (56-65)
C'C" (56-65)
C" (66-74)
FR3-IMGT (66-104)
D (75-84)
E (85-96)
F (97-104)
CDR3-IMGT (105-117)
FG (105-117)
FR4-IMGT (118-128)
G (118-128)
light
DIQMTQSPSSLSASL GGKVTITCQAS QDI.....NKY IAWYQFKP GKGPRLLIH YT.....S TLQPAIP.S RFSGSG..SG REYSFSISNLEP EDIATYYC LHYDN....LPWT FGGGTKLEIK.
IGKV1D-33*01
Homo sapiens
DIQMTQSPSSLSASV GDRVTITCQAS QDI.....SNY LNWYQQKP GKAPKLLIY DA.....S NLETGVP.S RFSGSG..SG TDFTFTISSLQP EDIATYYC QQYDNLP
L GK NK IA F G R H YT T QPAI REYS S N E LH
IGKJ4*01
Homo sapiens
LT FGGGTKVEIK
W L

Fig. 27

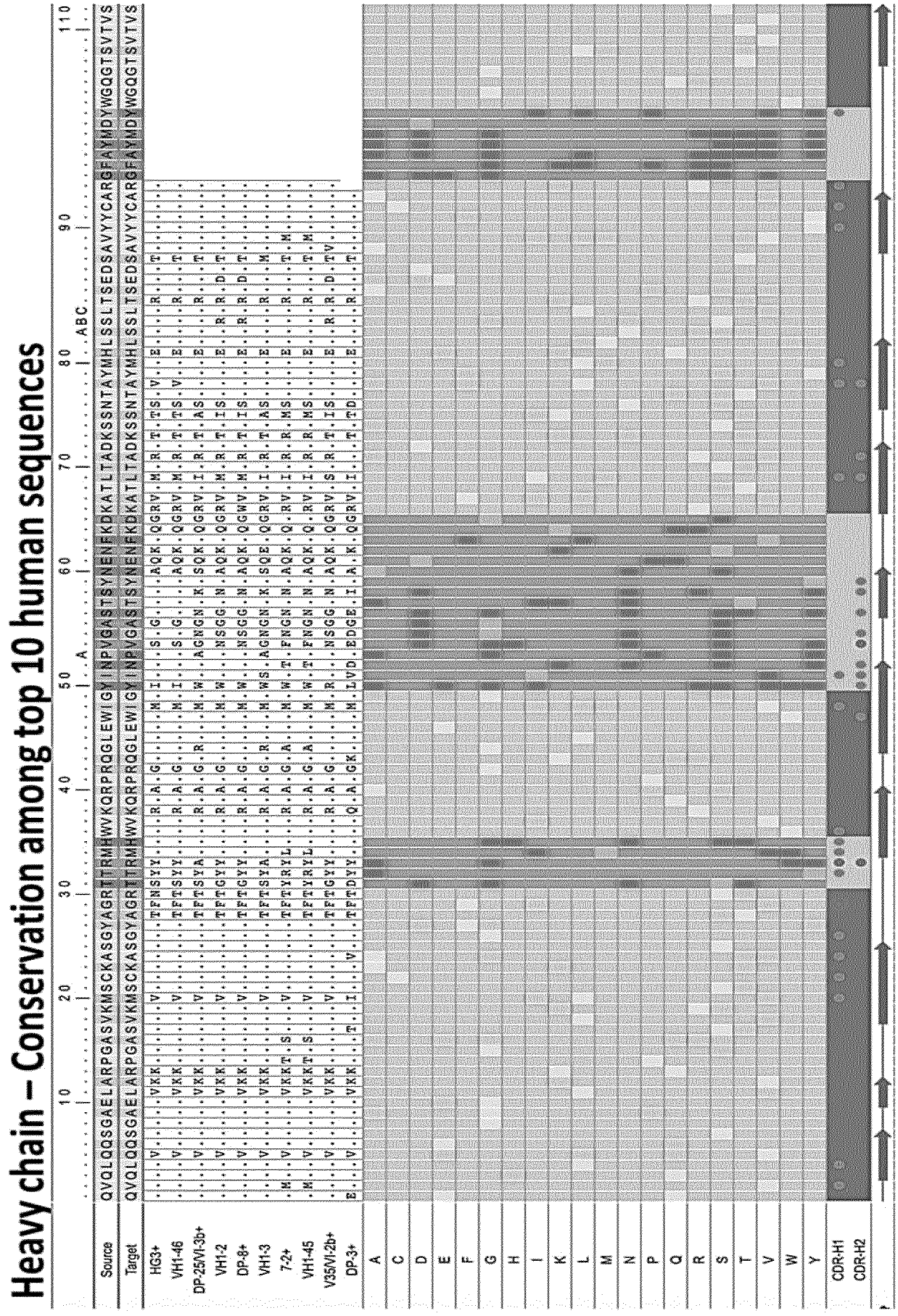

Heavy chain – Conservation among top 10 human sequences
Source
Target
HG3+
VH1-46
DP-25/VI-3b+
VH1-2
DP-8+
VH1-3
7-2+
VH1-45
V35/VI-2b+
DP-3+
A
C
D
E
F
G
H
I
K
L
M
N
P
Q
R
S
T
V
W
Y
CDR-H1
CDR-H2

Fig. 27 (cont.)

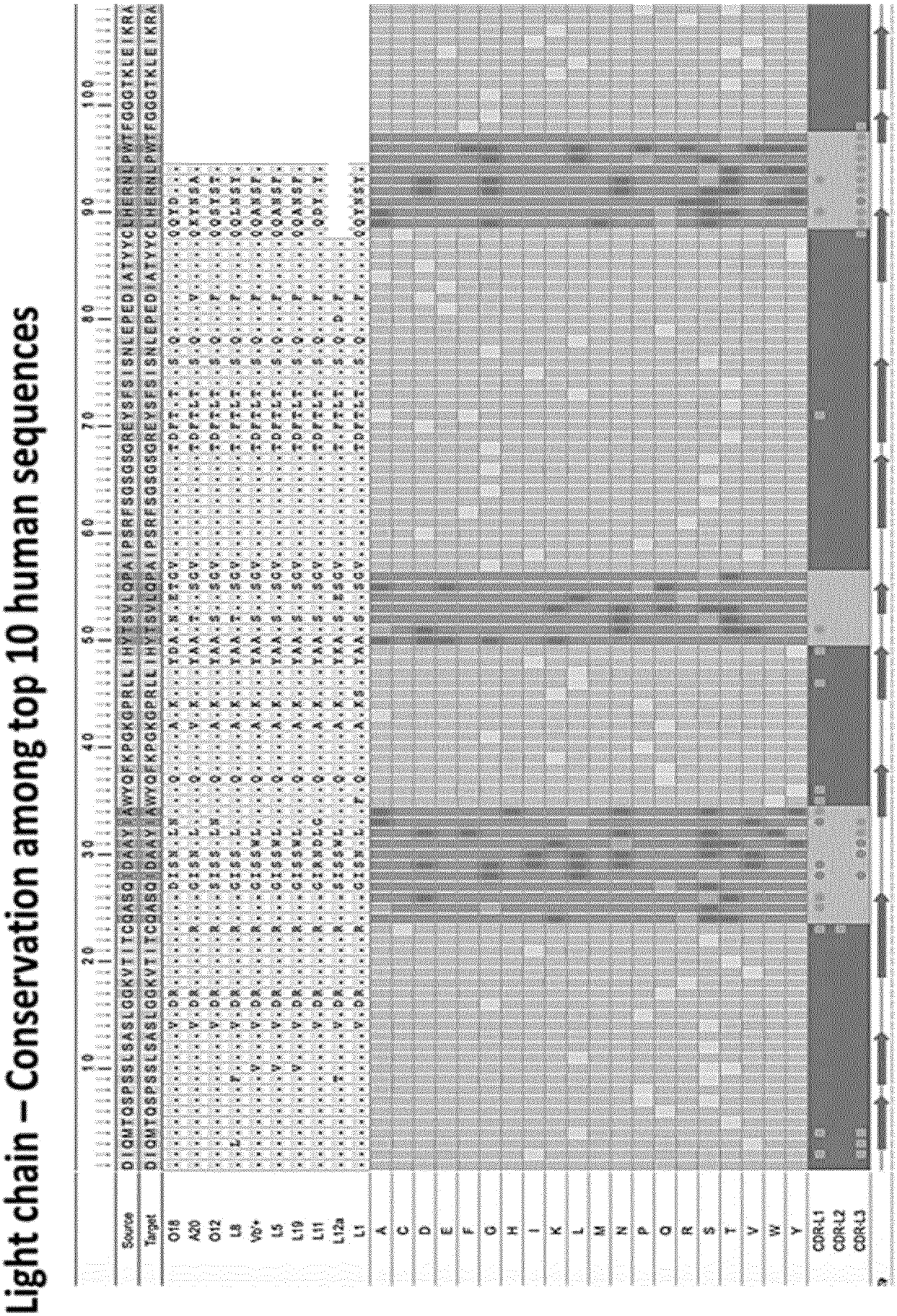

Light chain – Conservation among top 10 human sequences
Source
Target
O18
A20
O12
L8
Vg*
L5
L19
L11
L12a
L1
A
C
D
E
F
G
H
I
K
L
M
N
P
Q
R
S
T
V
W
Y
CDRL1
CDRL2
CDRL3

Fig. 28

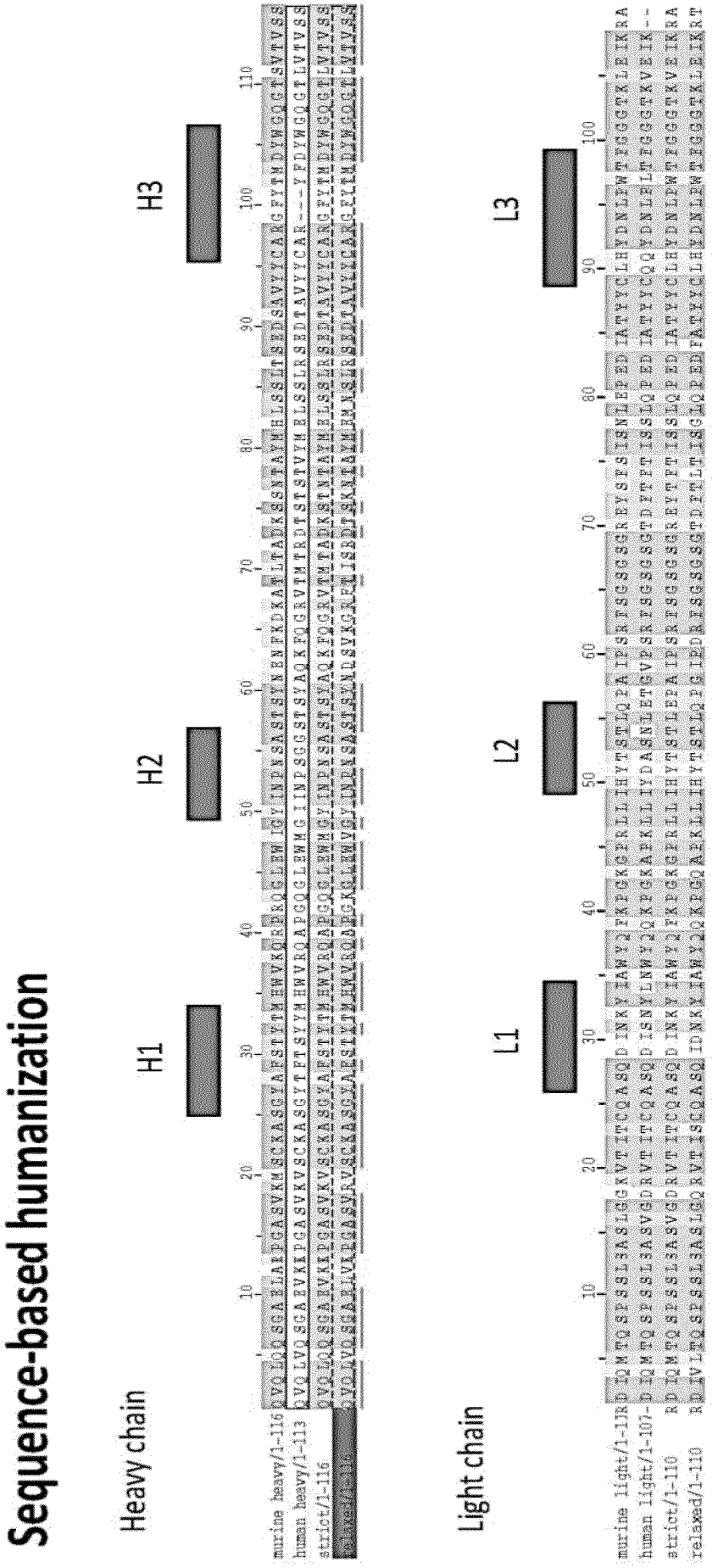

Sequence-based humanization
Heavy chain
H1
H2
H3
murine heavy/1-116
human heavy/1-113
strict/1-116
relaxed/1-116
Light chain
L1
L2
L3
murine light/1-113
human light/1-107
strict/1-110
relaxed/1-110

Fig. 29

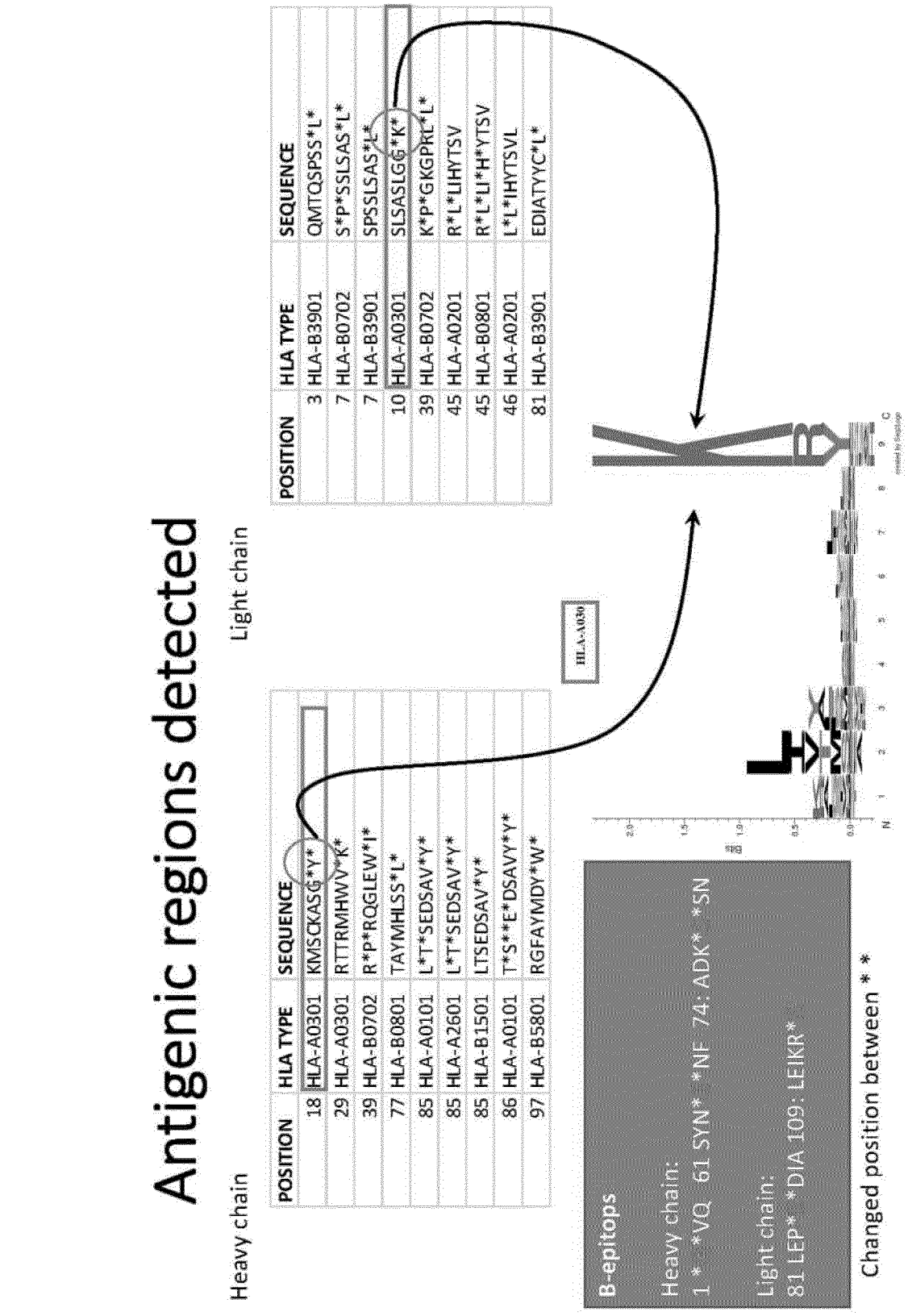

Antigenic regions detected
Heavy chain
POSITION HLA TYPE SEQUENCE
18 HLA-A0301 KMSCKASG*Y*
29 HLA-A0301 RTTRMHWV*K*
39 HLA-B0702 R*P*RQGLEW*I*
77 HLA-B0801 TAYMHLSS*L*
85 HLA-A0101 L*T*SEDSAV*Y*
85 HLA-A2601 L*T*SEDSAV*Y*
85 HLA-B1501 LTSEDSAV*Y*
86 HLA-A0101 T*S**E*DSAVY*Y*
97 HLA-B5801 RGFAYMDY*W*
Light chain
POSITION HLA TYPE SEQUENCE
3 HLA-B3901 QMTQSPSS*L*
7 HLA-B0702 S*P*SSLSAS*L*
7 HLA-B3901 SPSSLSAS*L*
10 HLA-A0301 SLSASLGG*K*
39 HLA-B0702 K*P*GKGPRL*L*
45 HLA-A0201 R*L*LIHYTSV
45 HLA-B0801 R*L*LI*H*YTSV
46 HLA-A0201 L*L*IHYTSVL
81 HLA-B3901 EDIATYYC*L*
HLA-A0301
B-epitops
Heavy chain:
1 * *VQ 61 SYN* *NF 74: ADK* *SN
Light chain:
81 LEP* *DIA 109: LEIKR*
Changed position between * *

Fig. 30

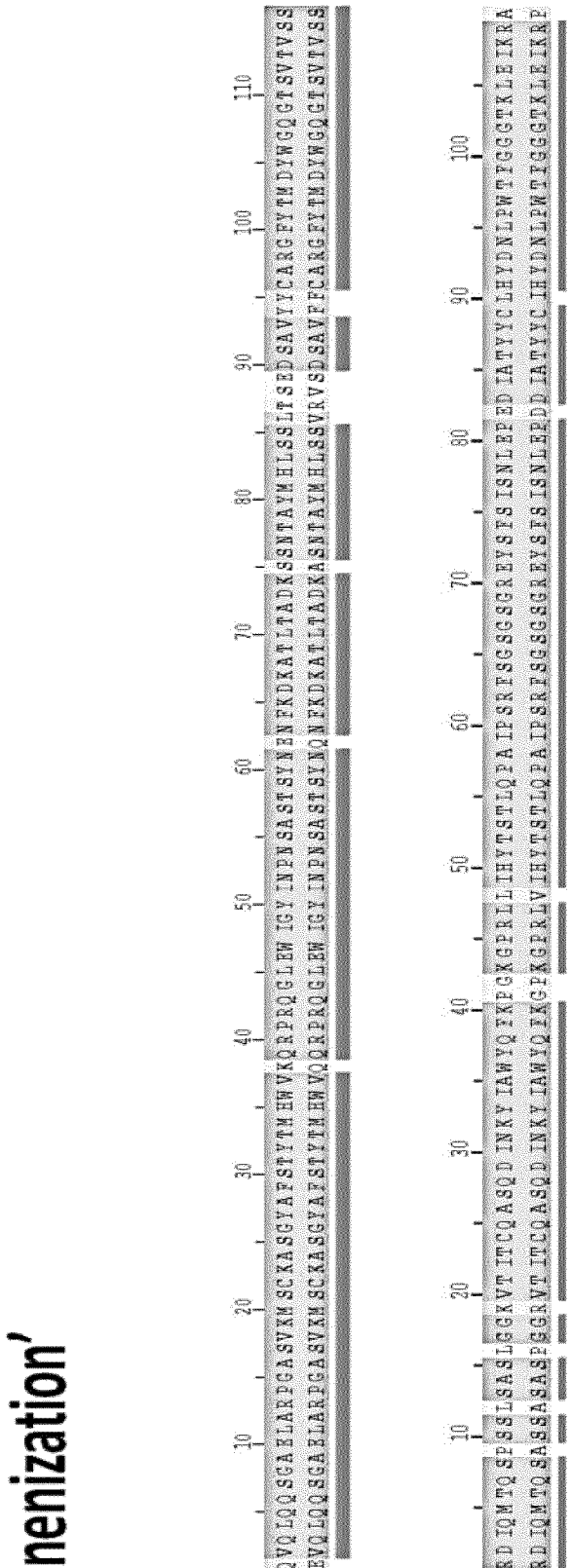

'De-murinenization'
Heavy chain
murine heavy/1-116
murine_heavy_free/1-116
Light chain
murine light/1-110
murine_light_free/1-110

Fig. 31

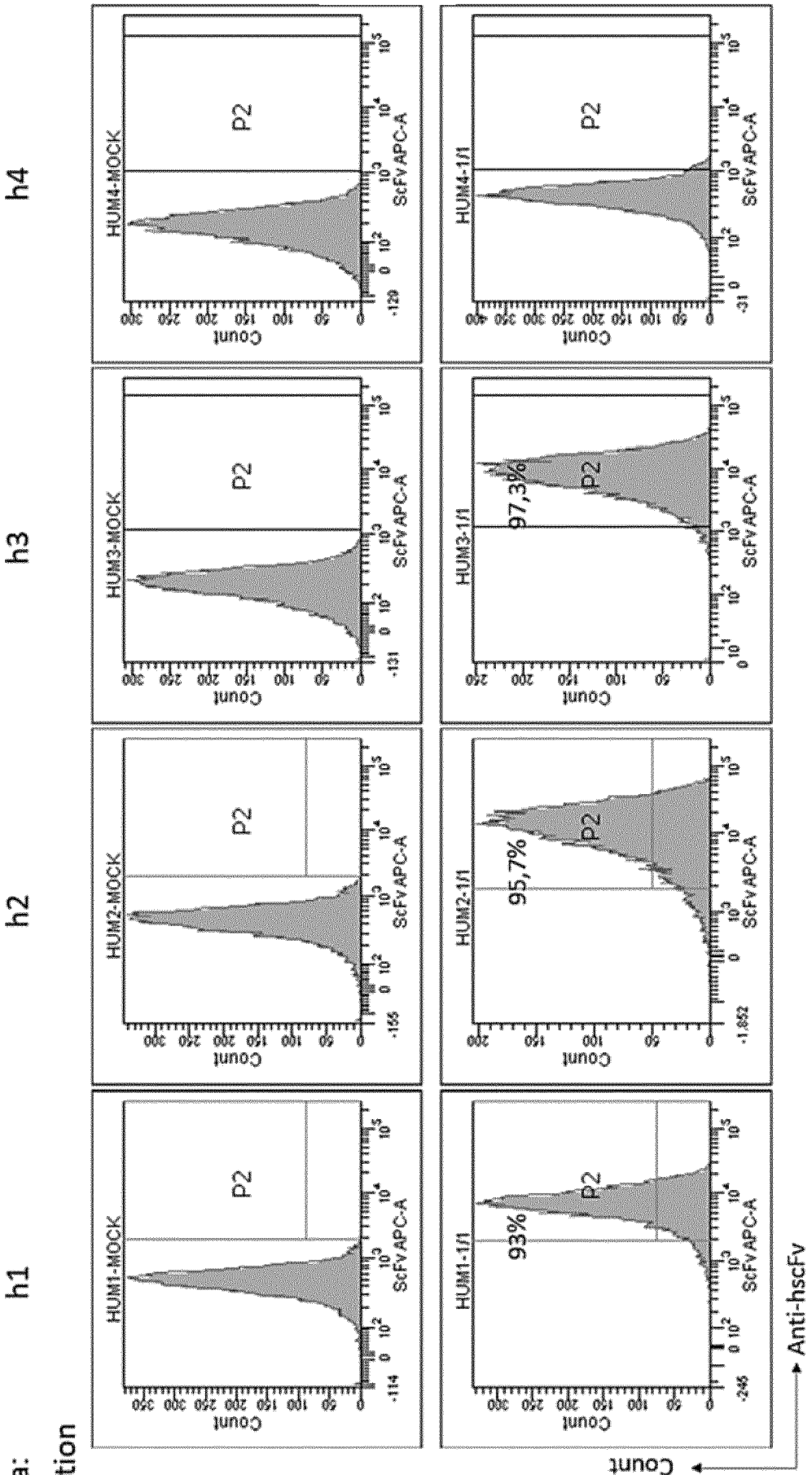

CAR-CD1a:
Virus dilution
h1
h2
h3
h4
No virus
1/1
HUM1-MOCK
HUM2-MOCK
HUM3-MOCK
HUM4-MOCK
HUM1-1/1
HUM2-1/1
HUM3-1/1
HUM4-1/1
P2
93%
95,7%
97,3%
Count
ScFv APC-A
Anti-hscFv

Fig. 31 (cont.)

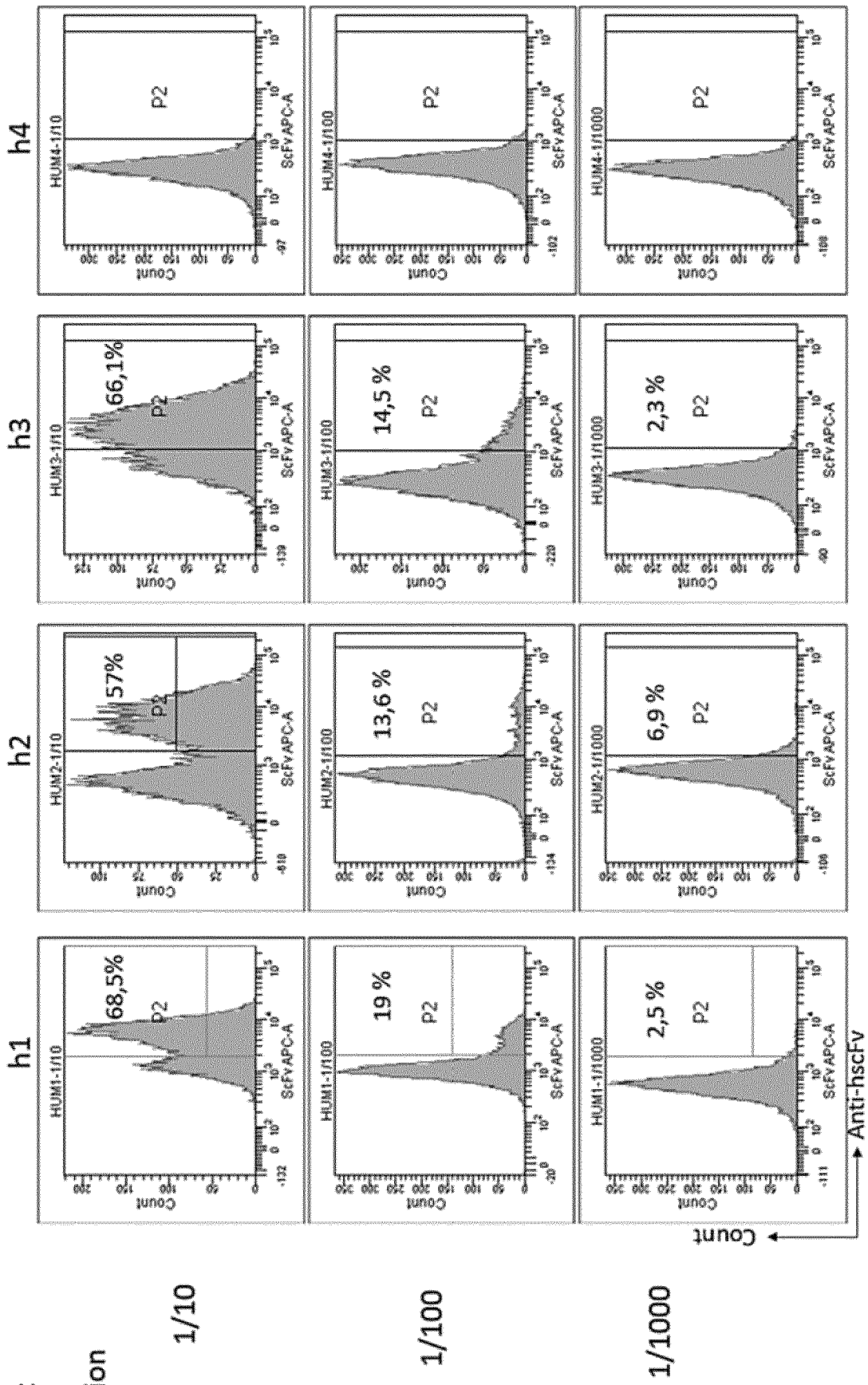

CAR-CD1a:
Virus dilution
1/10
1/100
1/1000
h1
h2
h3
h4
HUM1-1/10
HUM1-1/100
HUM1-1/1000
HUM2-1/10
HUM2-1/100
HUM2-1/1000
HUM3-1/10
HUM3-1/100
HUM3-1/1000
HUM4-1/10
HUM4-1/100
HUM4-1/1000
68,5%
19 %
2,5 %
57%
13,6 %
6,9 %
66,1%
14,5 %
2,3 %
P2
Count
ScFvAPC-A
Anti-hscFv

Fig. 31 (cont.)

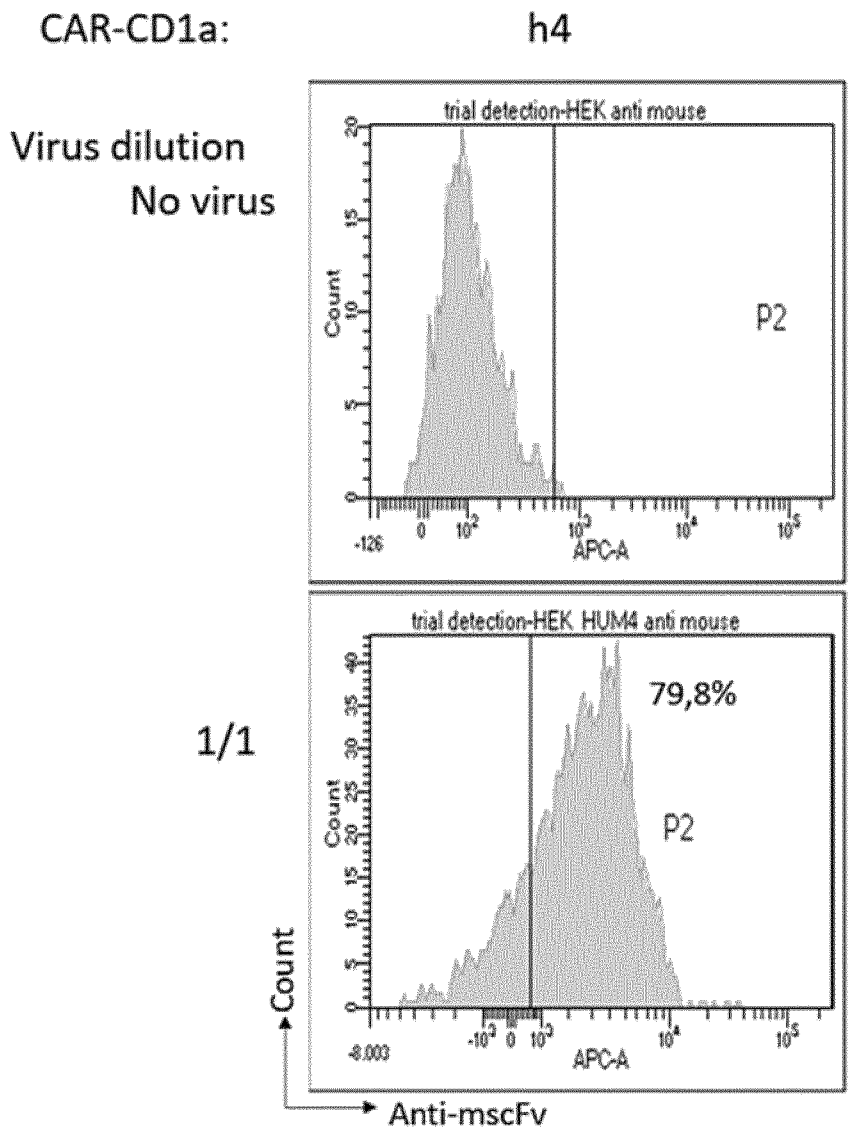

CAR-CD1a:
h4
Virus dilution
No virus
1/1
trial detection-HEK anti mouse
Count
P2
APC-A
trial detection-HEK HUM4 anti mouse
79,8%
P2
Count
APC-A
Anti-mscFv

Fig. 32

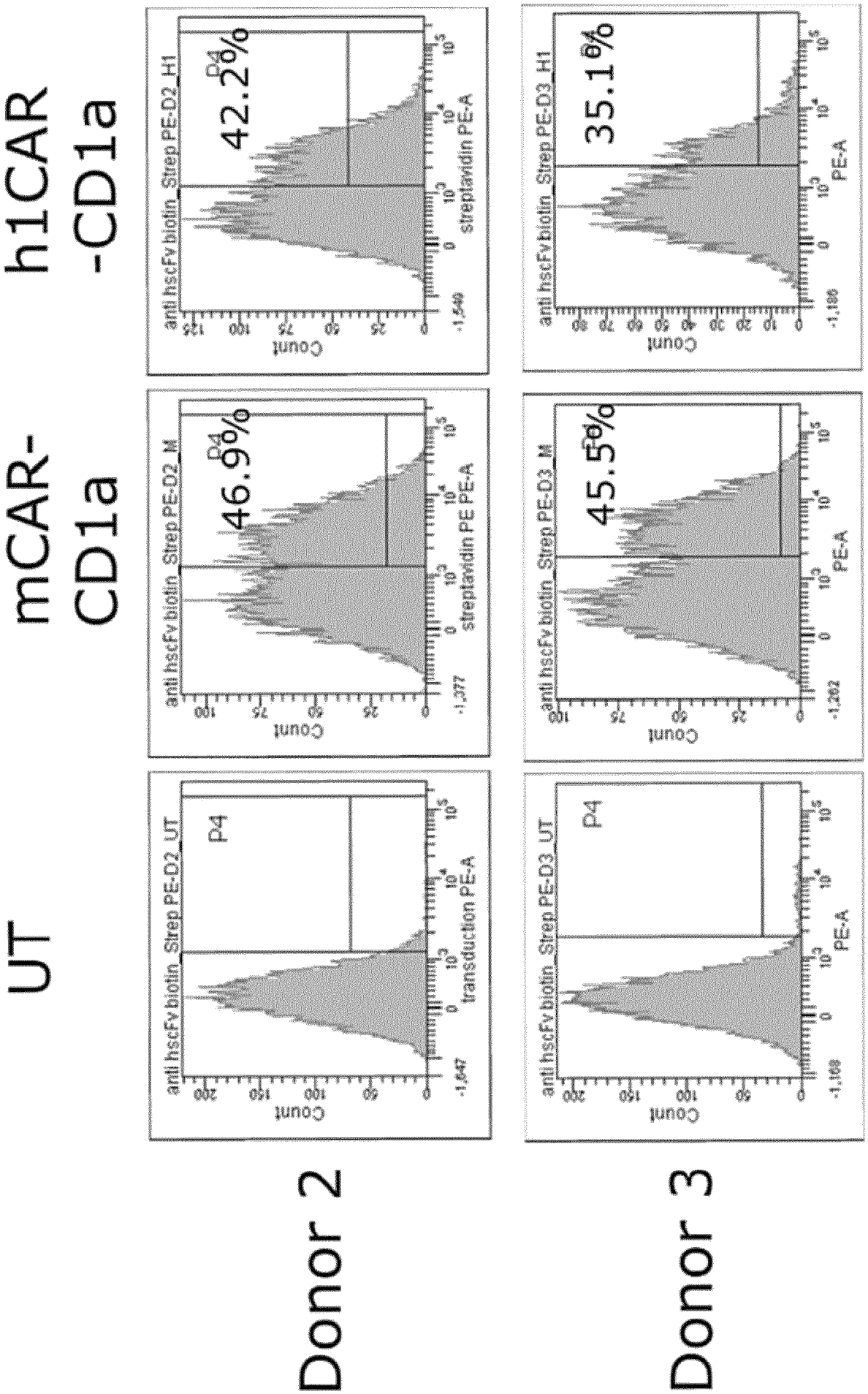

UT
mCAR-CD1a
h1CAR-CD1a
Donor 2
Donor 3
anti hscFv biotin_Strep PE-D2_UT
anti hscFv biotin_Strep PE-D2_M
anti hscFv biotin_Strep PE-D2_H1
anti hscFv biotin_Strep PE-D3_UT
anti hscFv biotin_Strep PE-D3_M
anti hscFv biotin_Strep PE-D3_H1
P4
46.9%
42.2%
45.5%
35.1%
Count
transduction PE-A
streptavidin PE PE-A
streptavidin PE-A
PE-A

Fig. 32 (cont.)

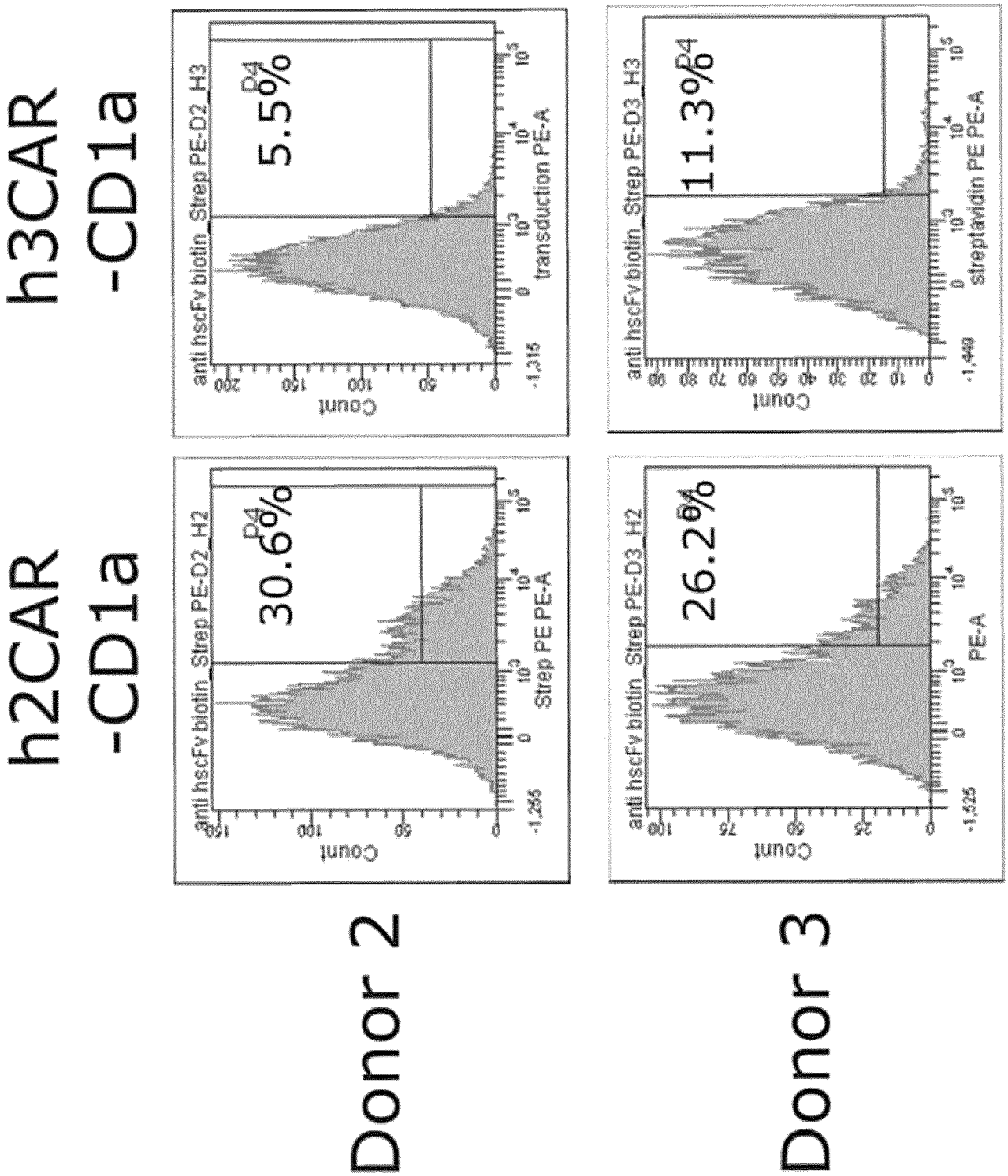

h3CAR -CD1a
h2CAR -CD1a
Donor 2
Donor 3
anti hscFv biotin_Strep PE-D2_H3
anti hscFv biotin_Strep PE-D3_H3
anti hscFv biotin_Strep PE-D2_H2
anti hscFv biotin_Strep PE-D3_H2
5.5%
11.3%
30.6%
26.2%
P4
Count
transduction PE-A
streptavidin PE PE-A
Strep PE PE-A
PE-A

Fig. 33

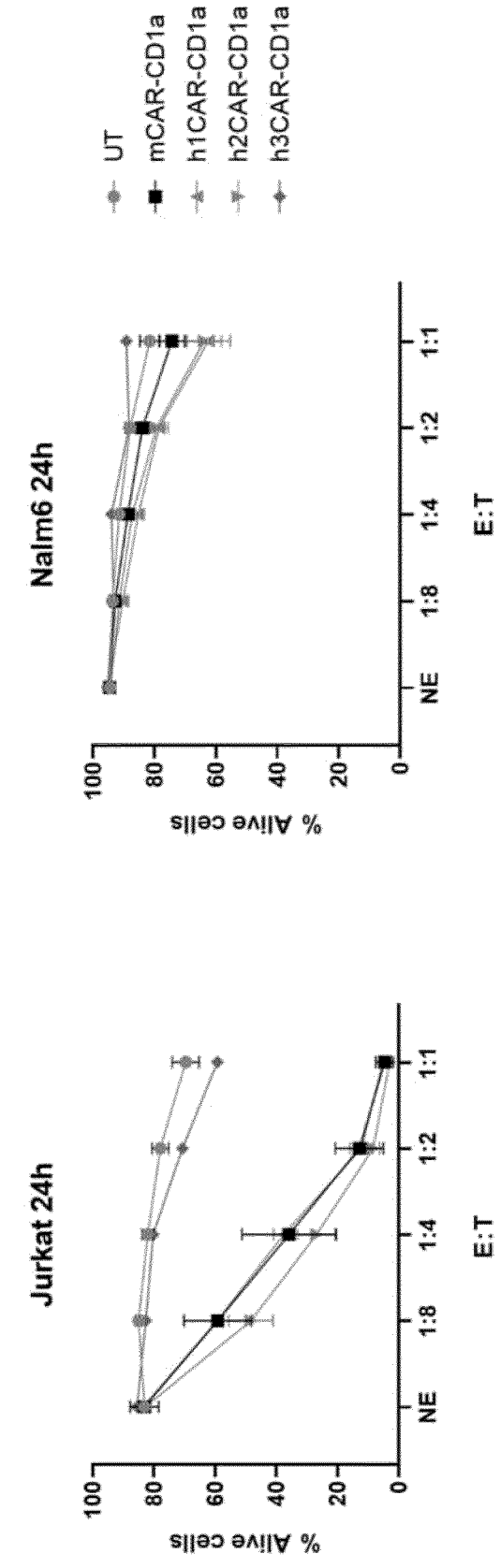

A
Jurkat 24h
Nalm6 24h
% Alive cells
E:T
NE
1:8
1:4
1:2
1:1
0
20
40
60
80
100
UT
mCAR-CD1a
h1CAR-CD1a
h2CAR-CD1a
h3CAR-CD1a

Fig. 33 (cont.)

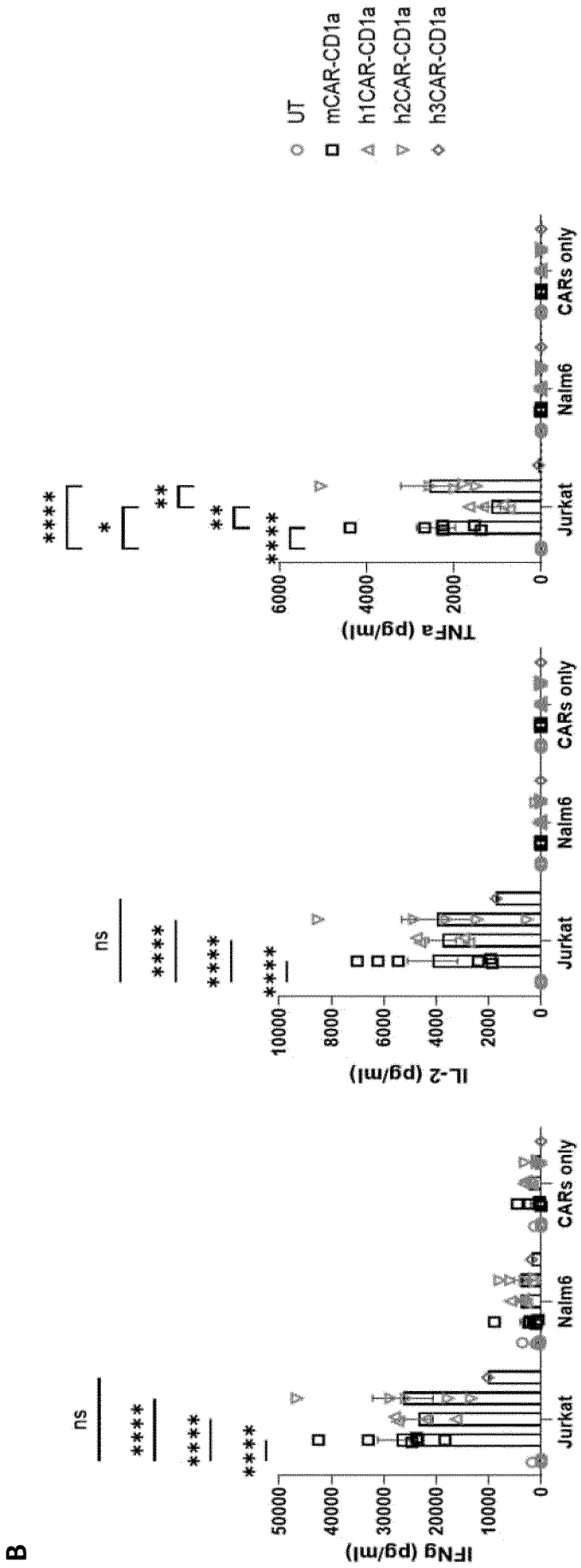

B
IFNg (pg/ml)
50000
40000
30000
20000
10000
0
Jurkat
Nalm6
CARs only
ns
****
****
****
IL-2 (pg/ml)
10000
8000
6000
4000
2000
0
Jurkat
Nalm6
CARs only
ns
****
****
****
TNFa (pg/ml)
6000
4000
2000
0
Jurkat
Nalm6
CARs only
****
*
**
**
UT
mCAR-CD1a
h1CAR-CD1a
h2CAR-CD1a
h3CAR-CD1a

Fig. 34

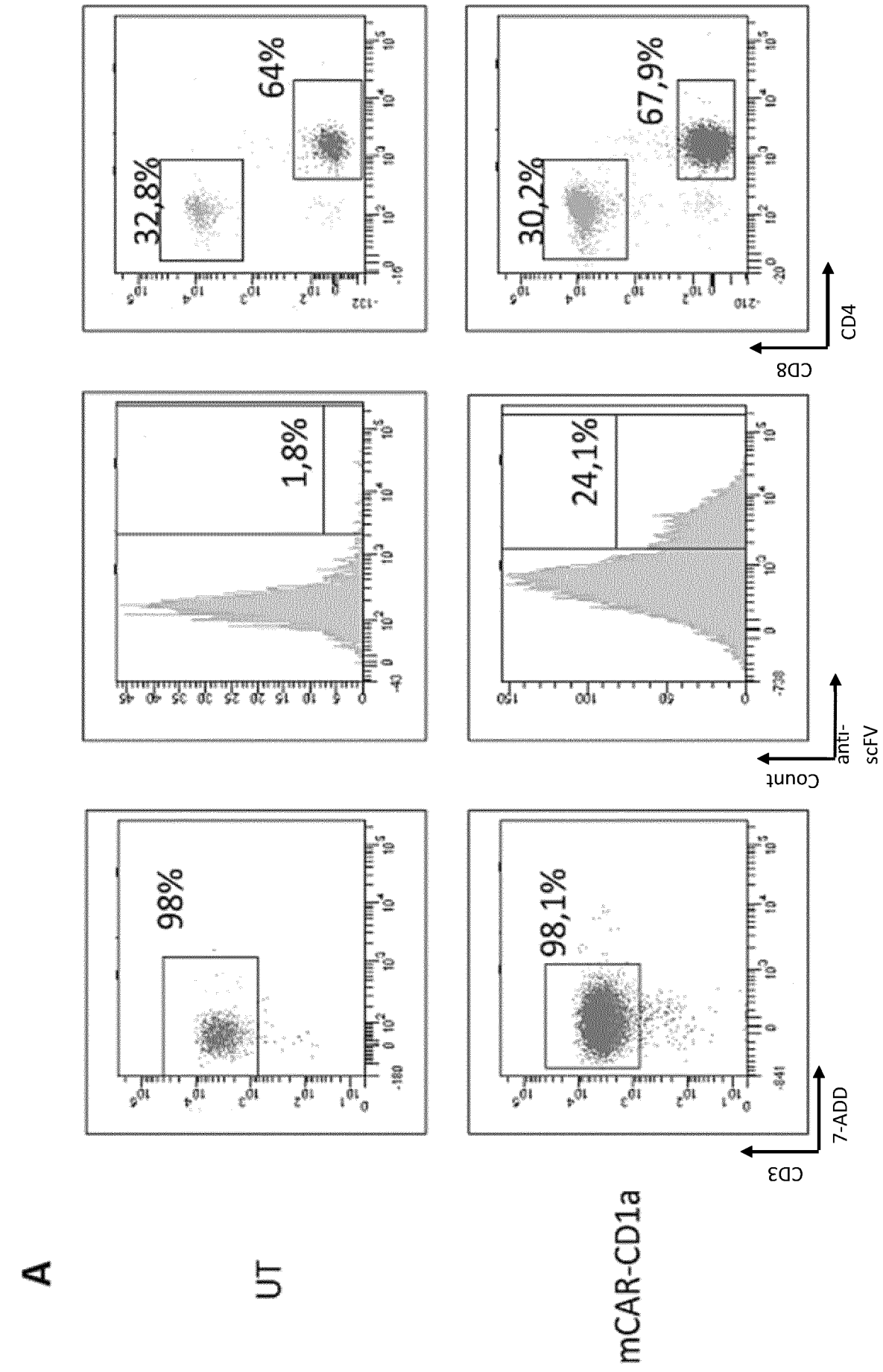

A
UT
mCAR-CD1a
CD3
7-ADD
98%
98,1%
Count
anti-scFV
1,8%
24,1%
CD8
CD4
32,8%
64%
30,2%
67,9%

Fig. 34 (cont.)

A (cont.)

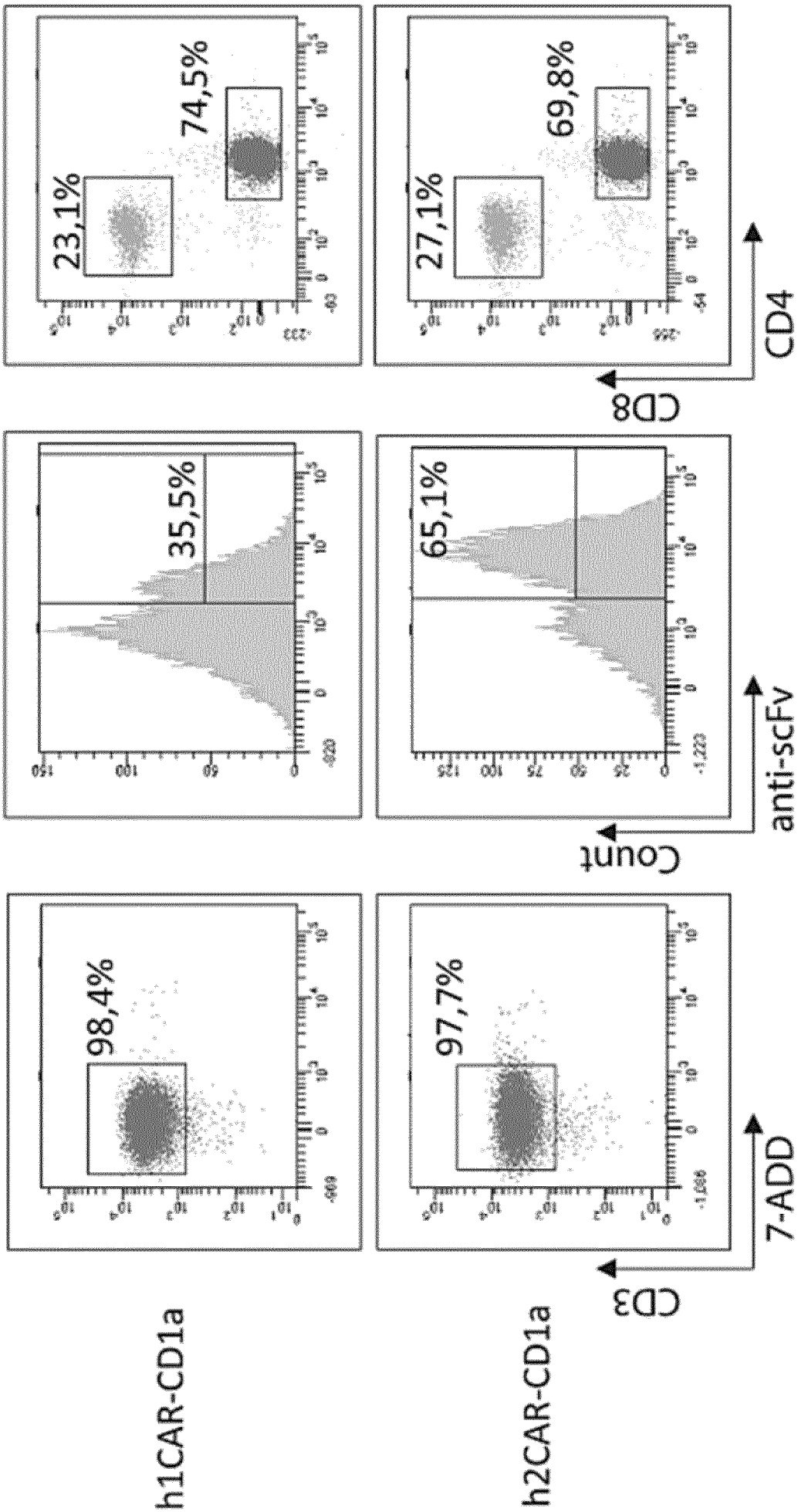


Fig. 34 (cont.)

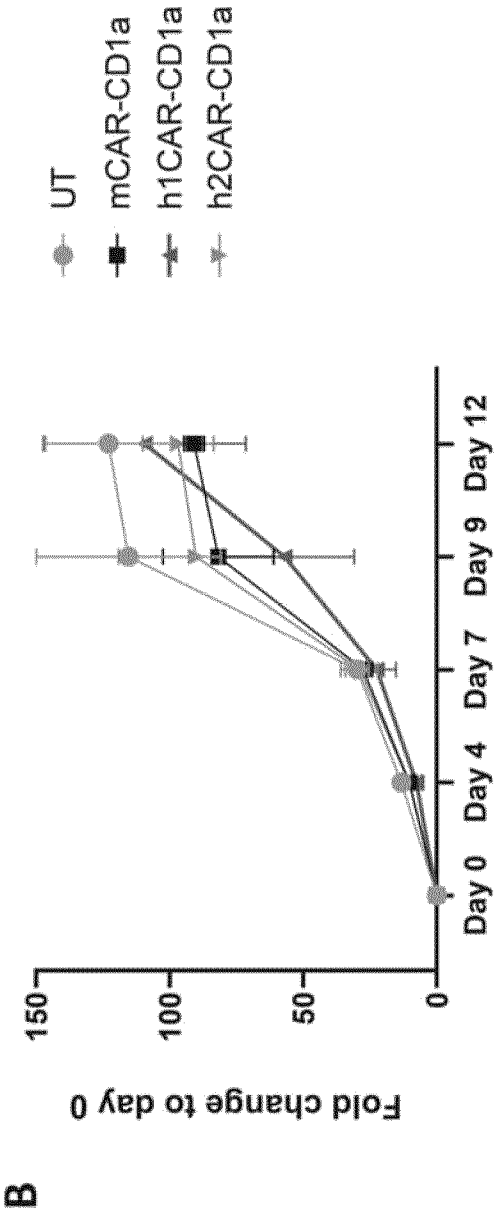

B
Fold change to day 0
150
100
50
0
Day 0
Day 4
Day 7
Day 9
Day 12
UT
mCAR-CD1a
h1CAR-CD1a
h2CAR-CD1a

Fig. 35

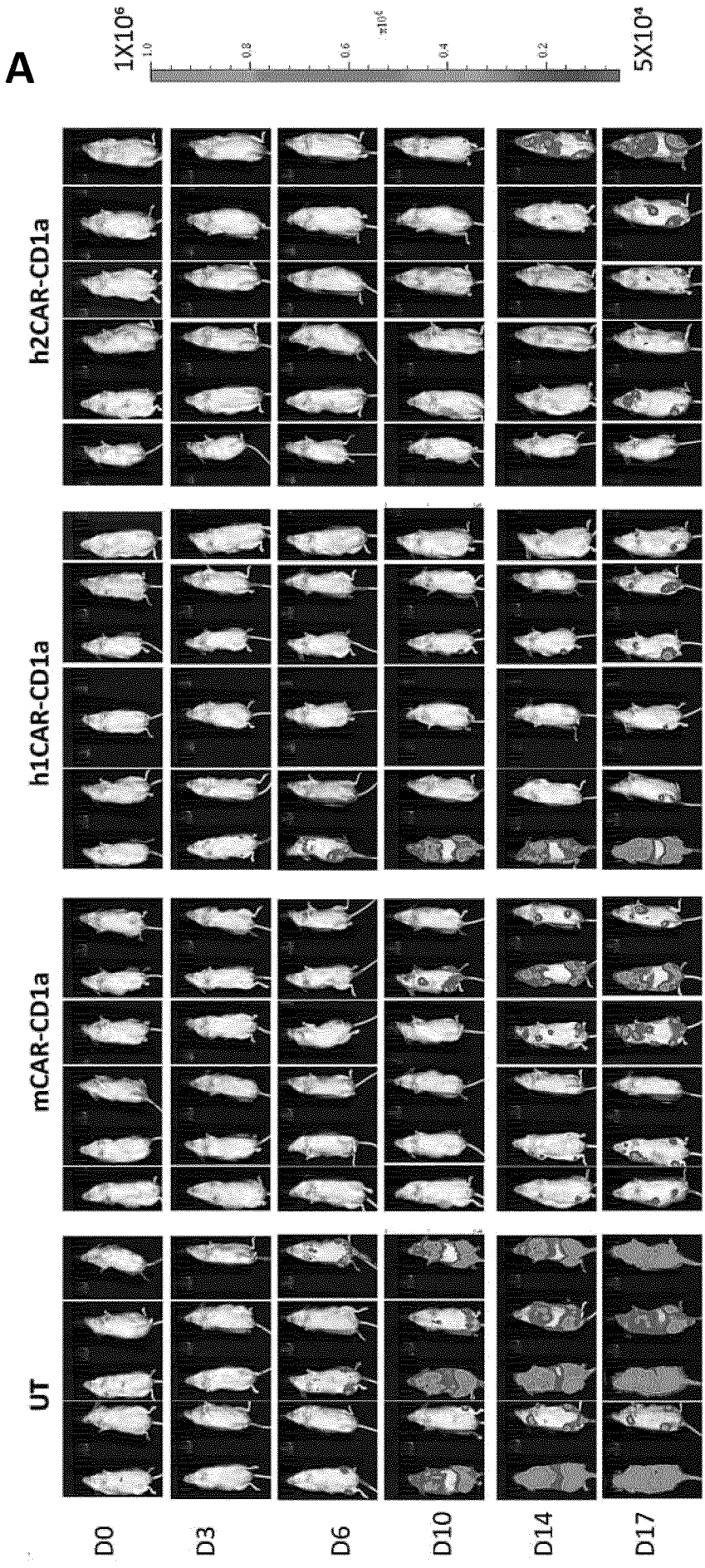

A
1X10⁶
5X10⁴
h2CAR-CD1a
h1CAR-CD1a
mCAR-CD1a
UT
D0
D3
D6
D10
D14
D17

Fig. 35 (cont.)

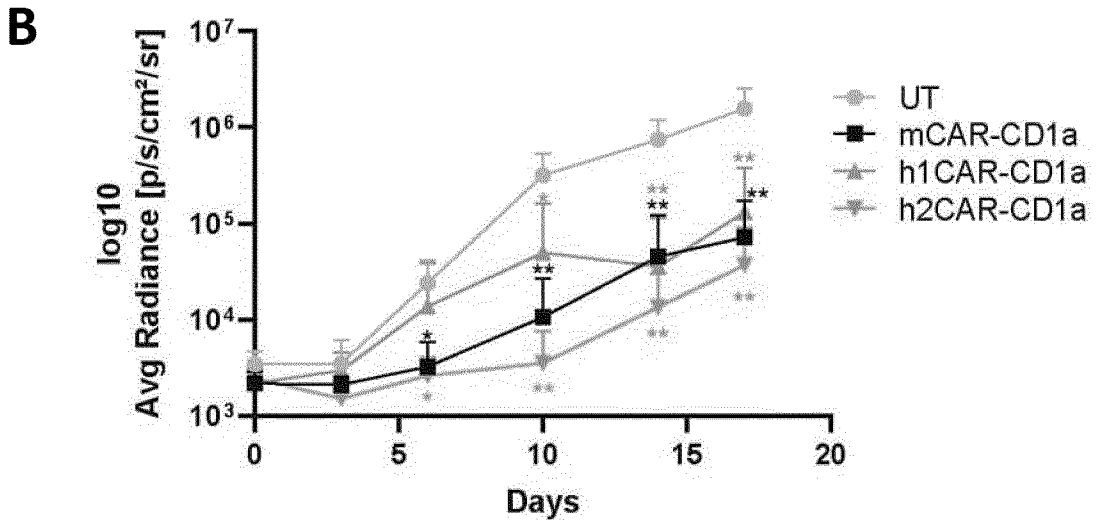

B
log10
Avg Radiance [p/s/cm²/sr]
10⁷
10⁶
10⁵
10⁴
10³
0
5
10
15
20
Days
UT
mCAR-CD1a
h1CAR-CD1a
h2CAR-CD1a

Fig. 35 (Cont.)

C

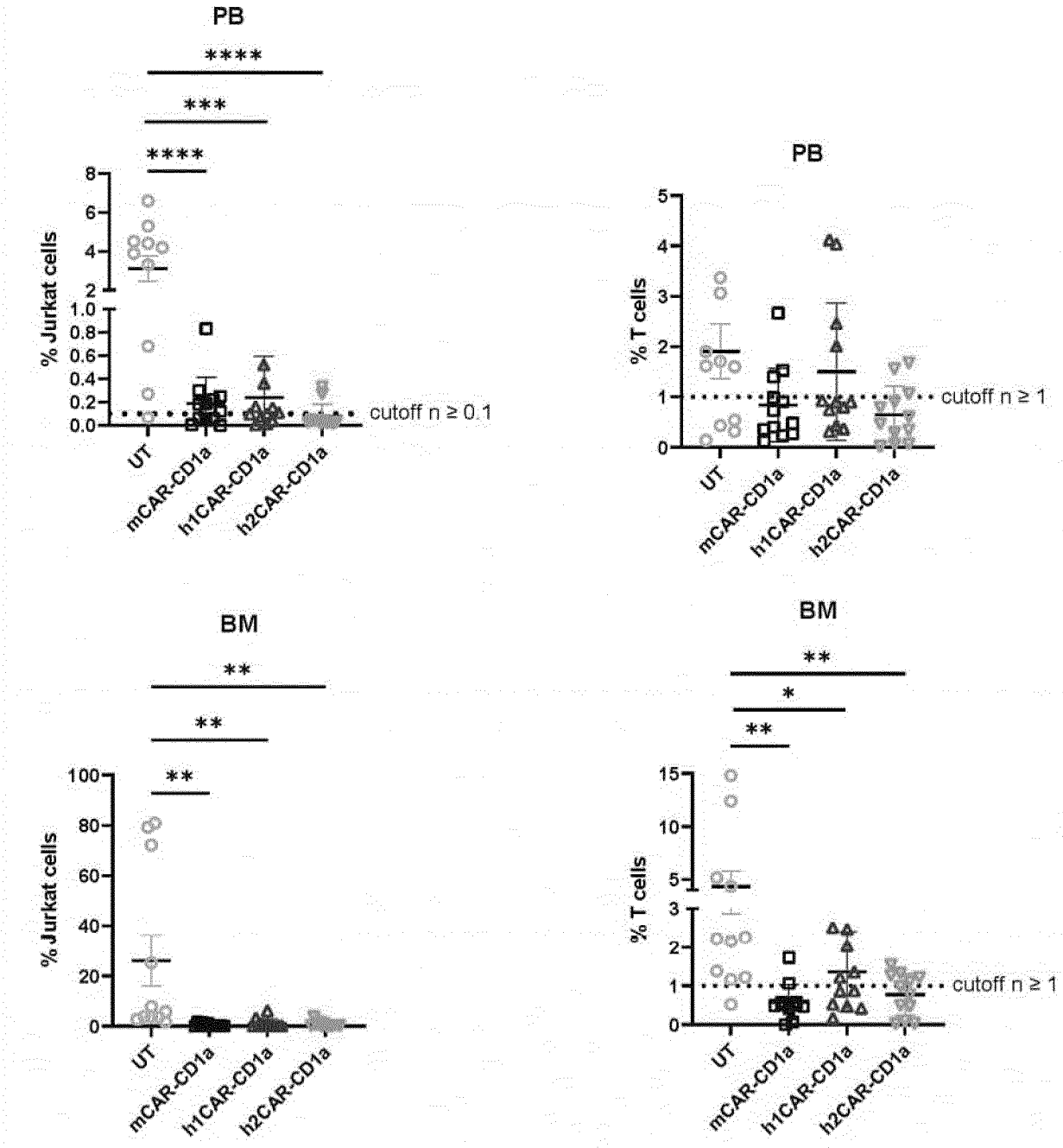
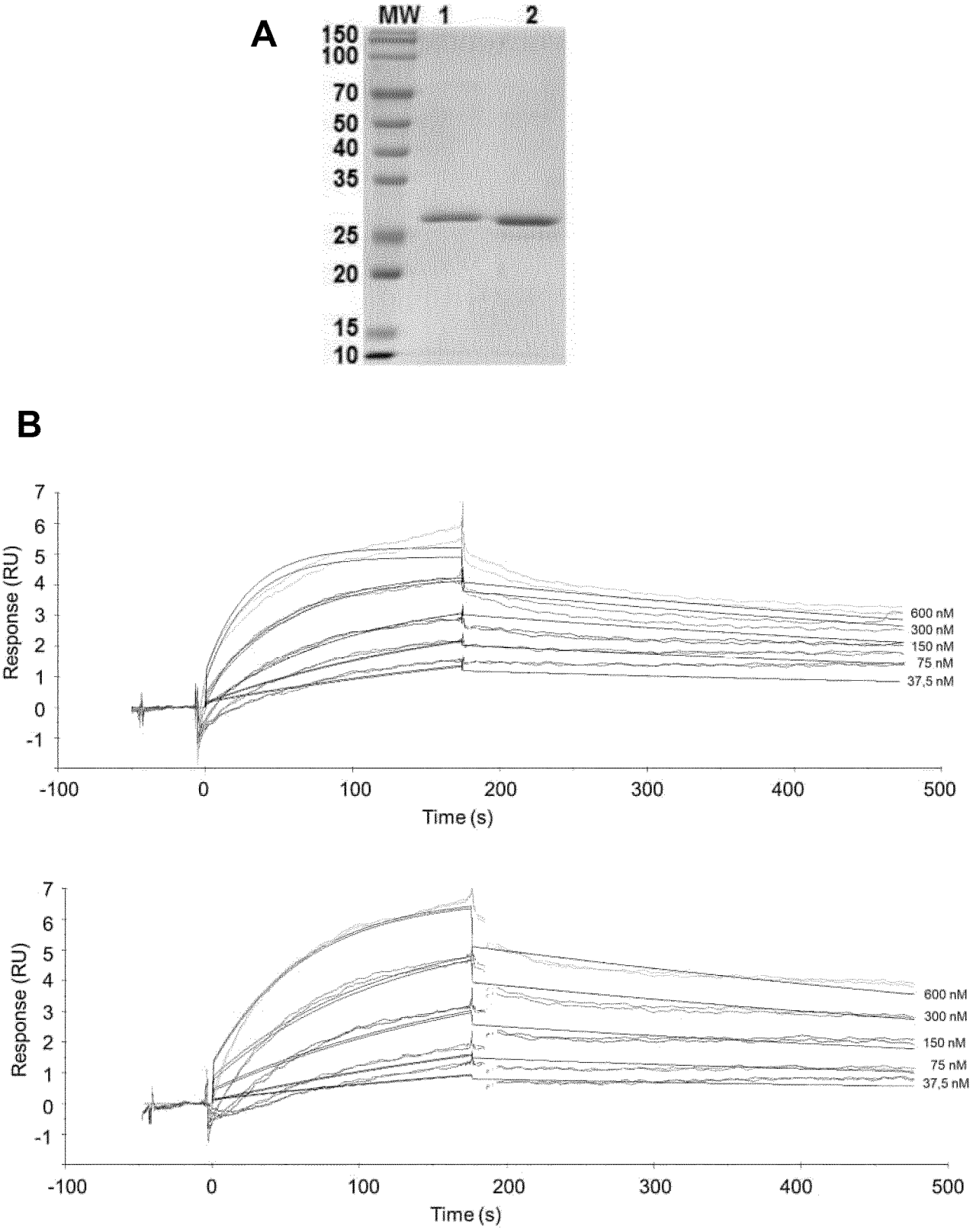

PB
****
***
****
% Jurkat cells
cutoff n ≥ 0.1
UT
mCAR-CD1a
h1CAR-CD1a
h2CAR-CD1a
PB
% T cells
cutoff n ≥ 1
BM
**
**
**
% Jurkat cells
BM
**
*
**
% T cells
cutoff n ≥ 1

Fig. 36


A
MW
1
2
150
100
70
50
40
35
25
20
15
10
B
Response (RU)
Time (s)
600 nM
300 nM
150 nM
75 nM
37,5 nM