Figure 1

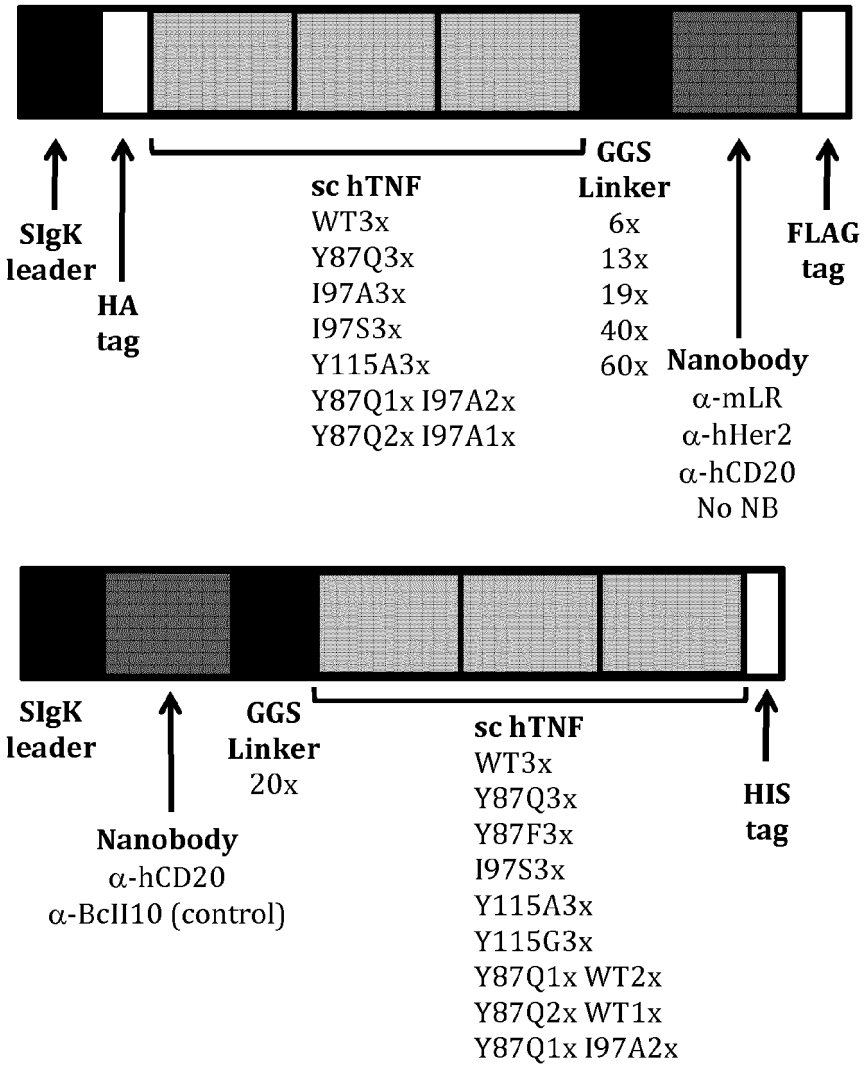
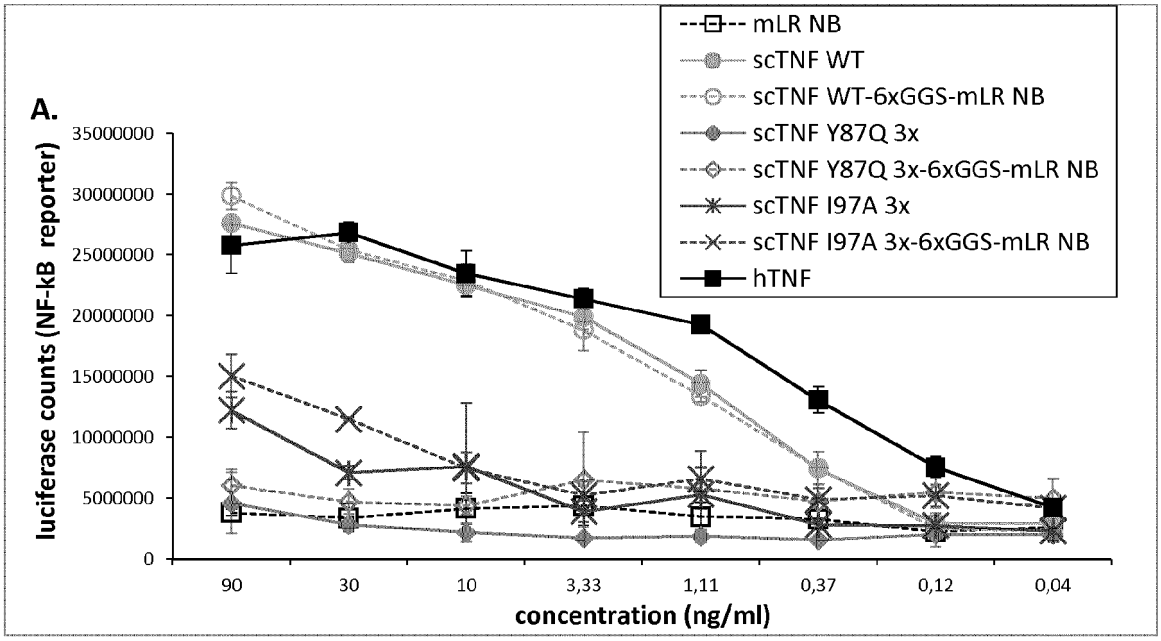

SIgK leader
HA tag
sc hTNF
WT3x
Y87Q3x
I97A3x
I97S3x
Y115A3x
Y87Q1x I97A2x
Y87Q2x I97A1x
GGS Linker
6x
13x
19x
40x
60x
Nanobody
α-mLR
α-hHer2
α-hCD20
No NB
FLAG tag
SIgK leader
Nanobody
α-hCD20
α-BcII10 (control)
GGS Linker
20x
sc hTNF
WT3x
Y87Q3x
Y87F3x
I97S3x
Y115A3x
Y115G3x
Y87Q1x WT2x
Y87Q2x WT1x
Y87Q1x I97A2x
HIS tag

Figure 2


A.
mLR NB
scTNF WT
scTNF WT-6xGGS-mLR NB
scTNF Y87Q 3x
scTNF Y87Q 3x-6xGGS-mLR NB
scTNF I97A 3x
scTNF I97A 3x-6xGGS-mLR NB
hTNF
luciferase counts (NF-kB reporter)
35000000
30000000
25000000
20000000
15000000
10000000
5000000
0
90
30
10
3,33
1,11
0,37
0,12
0,04
concentration (ng/ml)

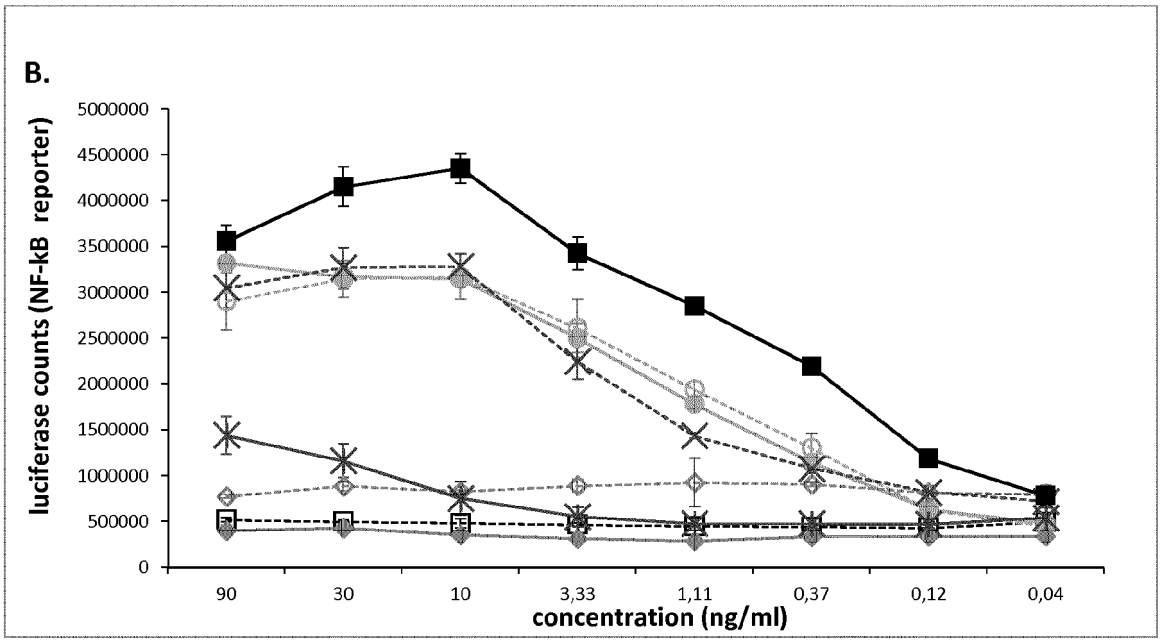
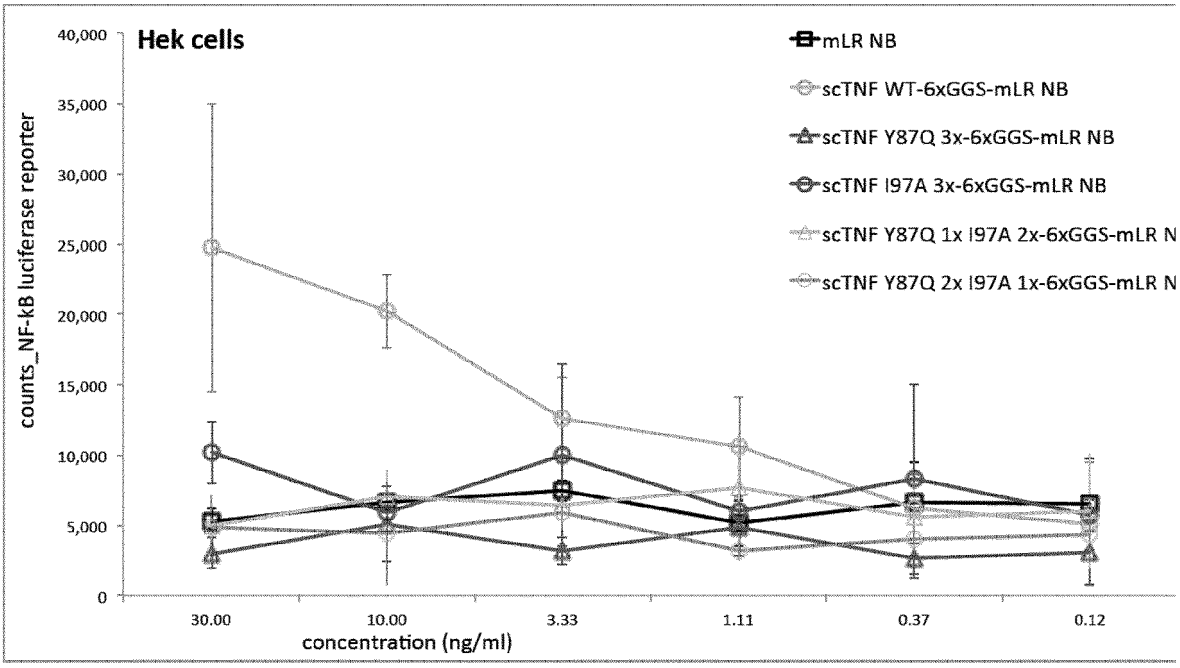

B.
luciferase counts (NF-kB reporter)
5000000
4500000
4000000
3500000
3000000
2500000
2000000
1500000
1000000
500000
0
90
30
10
3,33
1,11
0,37
0,12
0,04
concentration (ng/ml)

Figure 3

A


Hek cells
counts_NF-kB luciferase reporter
40,000
35,000
30,000
25,000
20,000
15,000
10,000
5,000
0
30.00
10.00
3.33
1.11
0.37
0.12
concentration (ng/ml)
mLR NB
scTNF WT-6xGGS-mLR NB
scTNF Y87Q 3x-6xGGS-mLR NB
scTNF I97A 3x-6xGGS-mLR NB
scTNF Y87Q 1x I97A 2x-6xGGS-mLR N
scTNF Y87Q 2x I97A 1x-6xGGS-mLR N

B

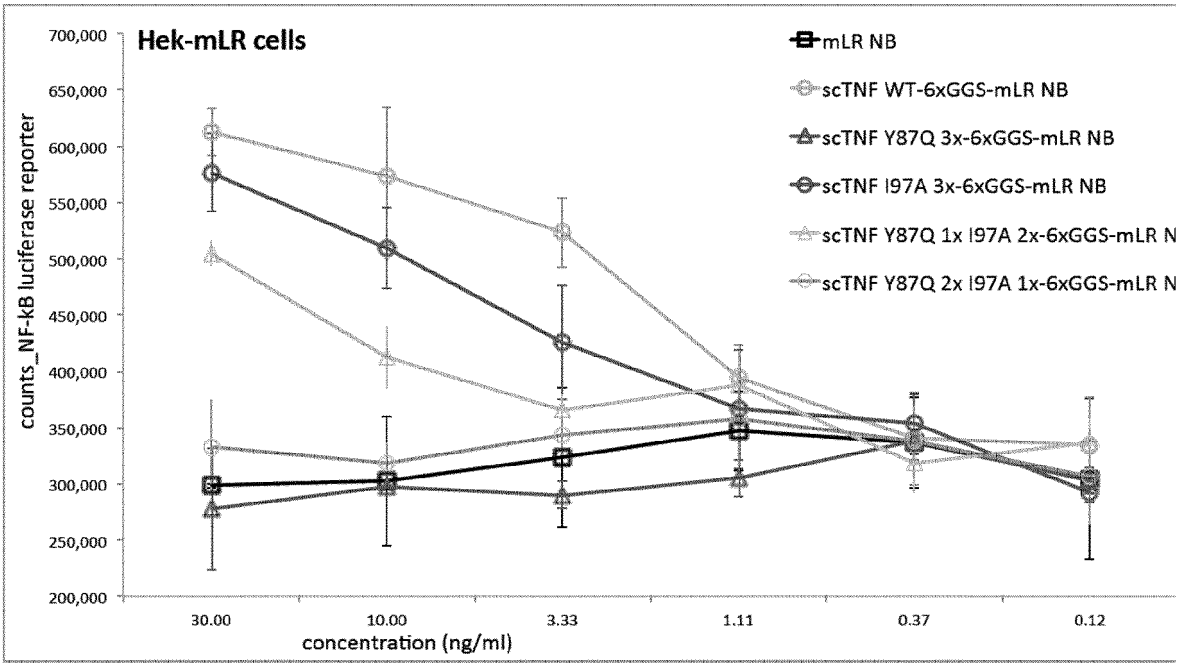
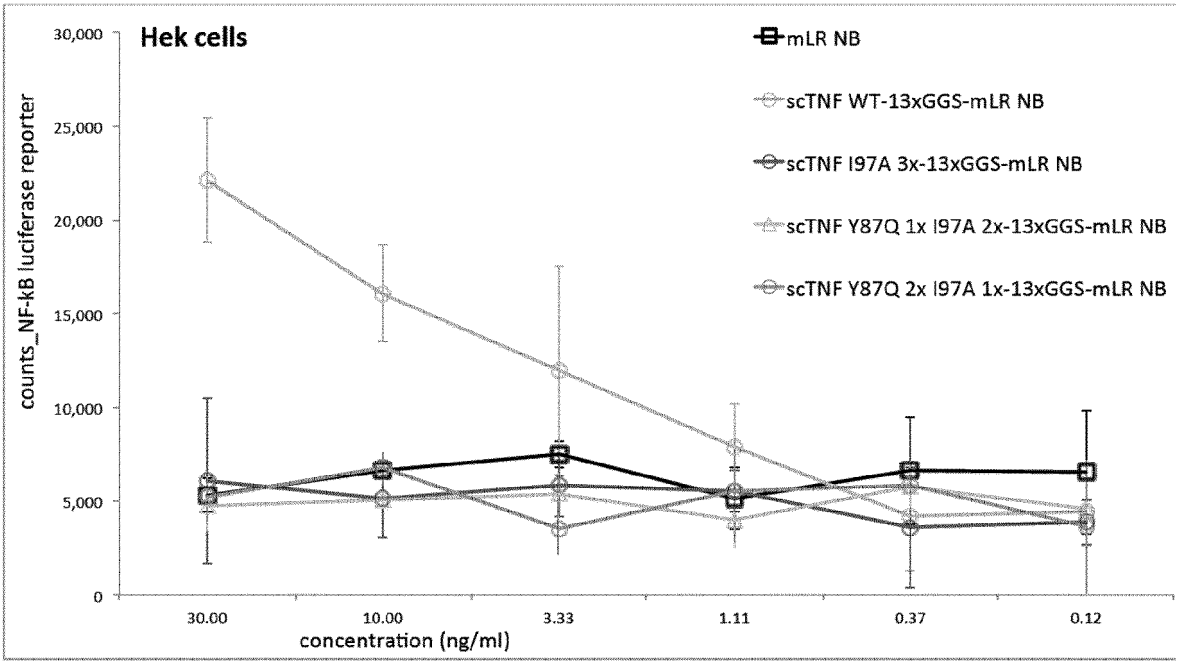

Hek-mLR cells
counts_NF-kB luciferase reporter
700,000
650,000
600,000
550,000
500,000
450,000
400,000
350,000
300,000
250,000
200,000
30.00
10.00
3.33
1.11
0.37
0.12
concentration (ng/ml)
mLR NB
scTNF WT-6xGGS-mLR NB
scTNF Y87Q 3x-6xGGS-mLR NB
scTNF I97A 3x-6xGGS-mLR NB
scTNF Y87Q 1x I97A 2x-6xGGS-mLR N
scTNF Y87Q 2x I97A 1x-6xGGS-mLR N

Figure 4

A


Hek cells
mLR NB
scTNF WT-13xGGS-mLR NB
scTNF I97A 3x-13xGGS-mLR NB
scTNF Y87Q 1x I97A 2x-13xGGS-mLR NB
scTNF Y87Q 2x I97A 1x-13xGGS-mLR NB
counts_NF-kB luciferase reporter
30,000
25,000
20,000
15,000
10,000
5,000
0
30.00
10.00
3.33
1.11
0.37
0.12
concentration (ng/ml)

B

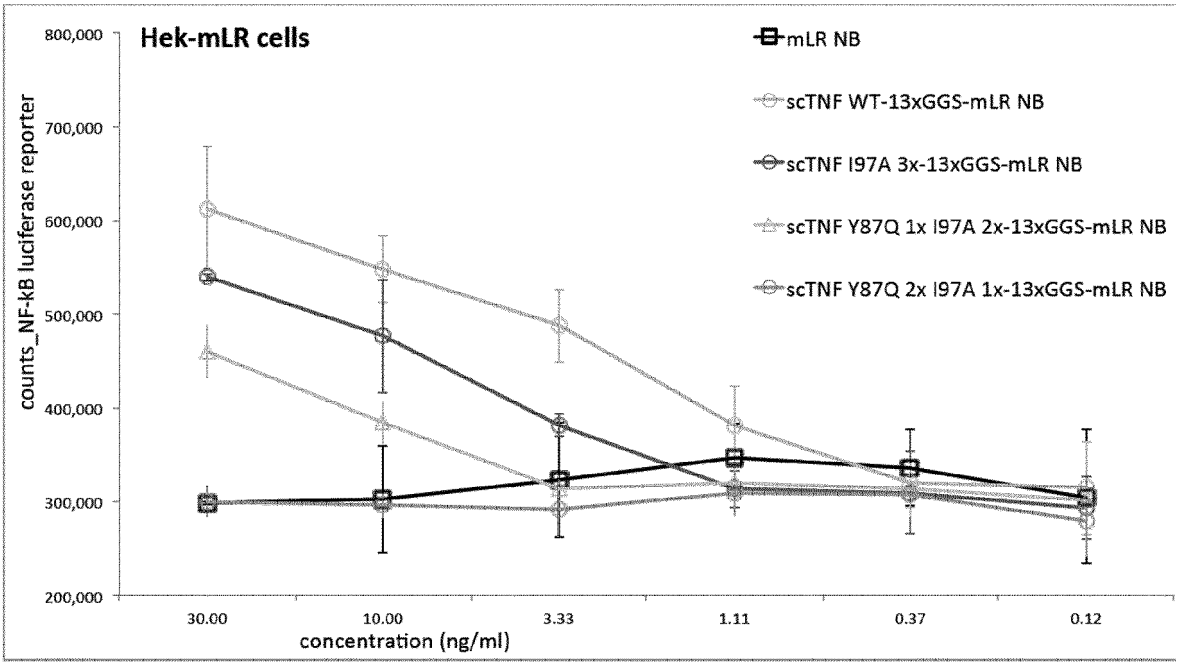
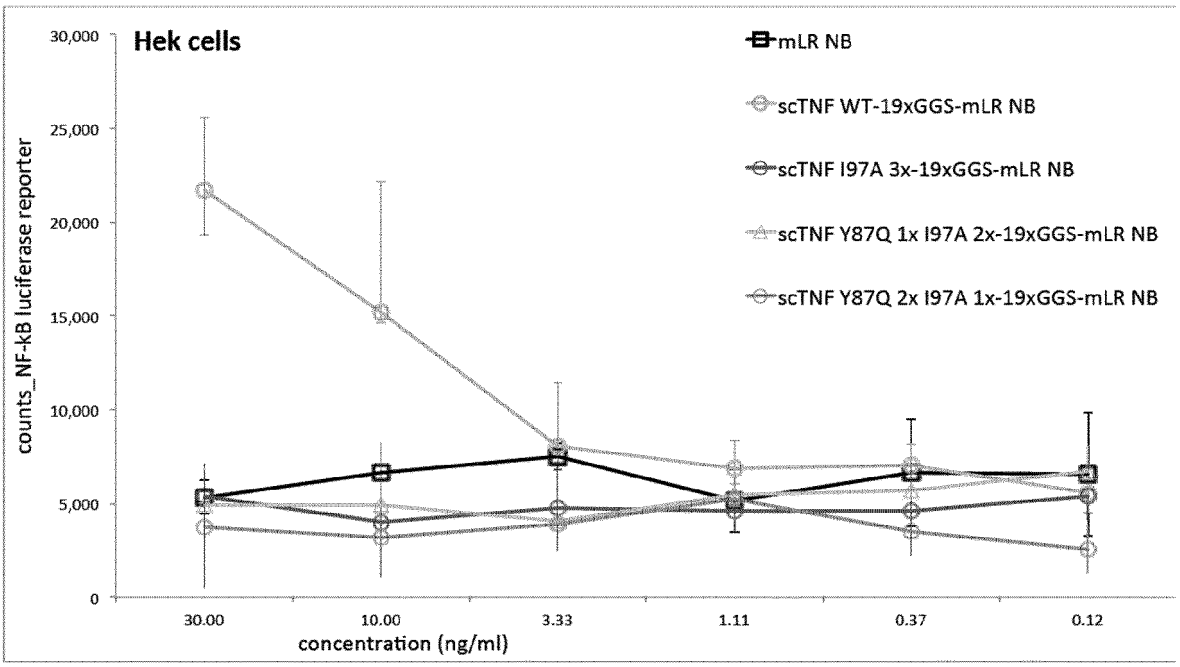

Hek-mLR cells
mLR NB
scTNF WT-13xGGS-mLR NB
scTNF I97A 3x-13xGGS-mLR NB
scTNF Y87Q 1x I97A 2x-13xGGS-mLR NB
scTNF Y87Q 2x I97A 1x-13xGGS-mLR NB
counts_NF-kB luciferase reporter
800,000
700,000
600,000
500,000
400,000
300,000
200,000
30.00
10.00
3.33
1.11
0.37
0.12
concentration (ng/ml)

Figure 5

A


Hek cells
mLR NB
scTNF WT-19xGGS-mLR NB
scTNF I97A 3x-19xGGS-mLR NB
scTNF Y87Q 1x I97A 2x-19xGGS-mLR NB
scTNF Y87Q 2x I97A 1x-19xGGS-mLR NB
counts_NF-kB luciferase reporter
30,000
25,000
20,000
15,000
10,000
5,000
0
30.00
10.00
3.33
1.11
0.37
0.12
concentration (ng/ml)

B

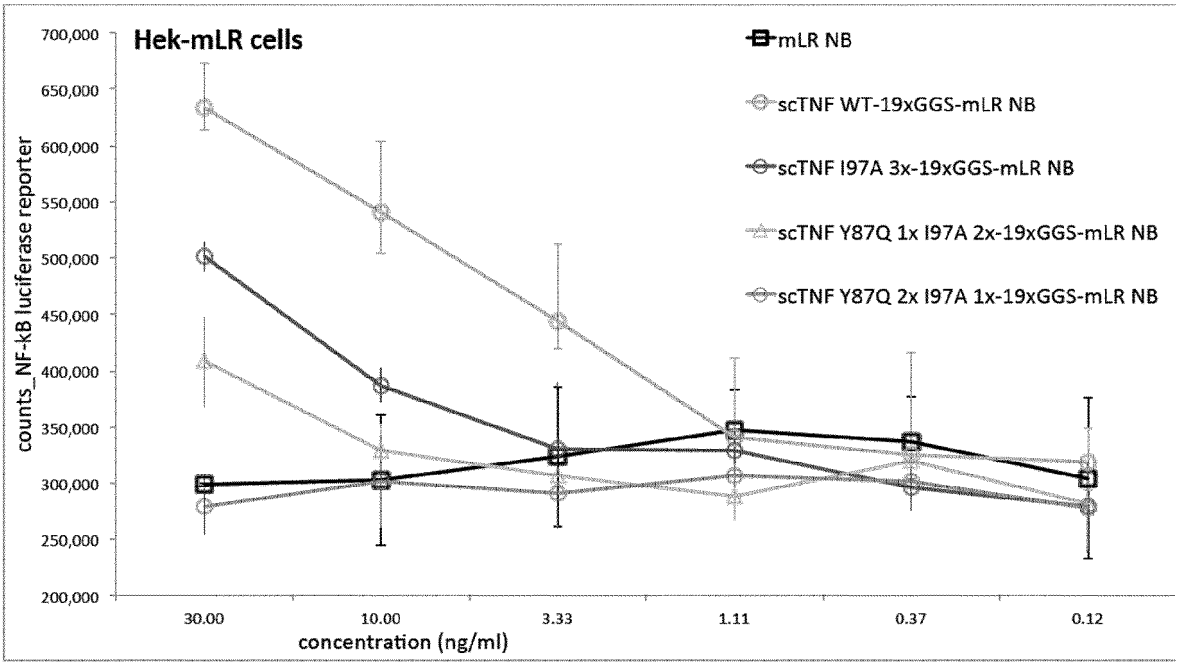

Hek-mLR cells
mLR NB
scTNF WT-19xGGS-mLR NB
scTNF I97A 3x-19xGGS-mLR NB
scTNF Y87Q 1x I97A 2x-19xGGS-mLR NB
scTNF Y87Q 2x I97A 1x-19xGGS-mLR NB
counts_NF-kB luciferase reporter
700,000
650,000
600,000
550,000
500,000
450,000
400,000
350,000
300,000
250,000
200,000
30.00
10.00
3.33
1.11
0.37
0.12
concentration (ng/ml)

Figure 6

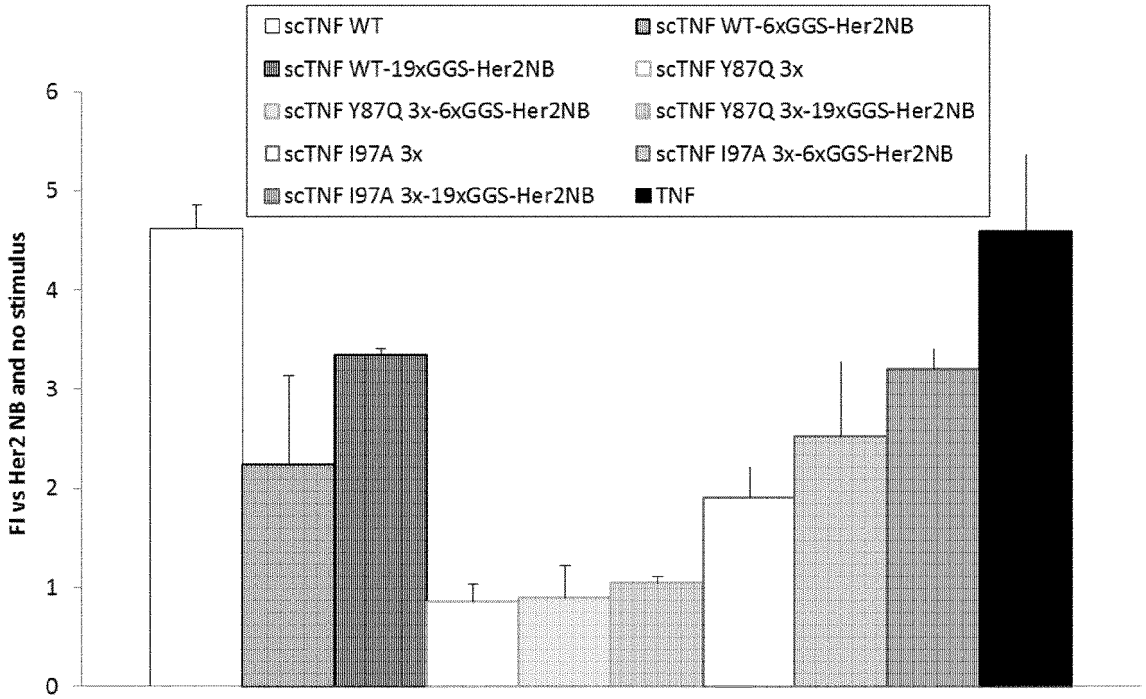


Figure 7

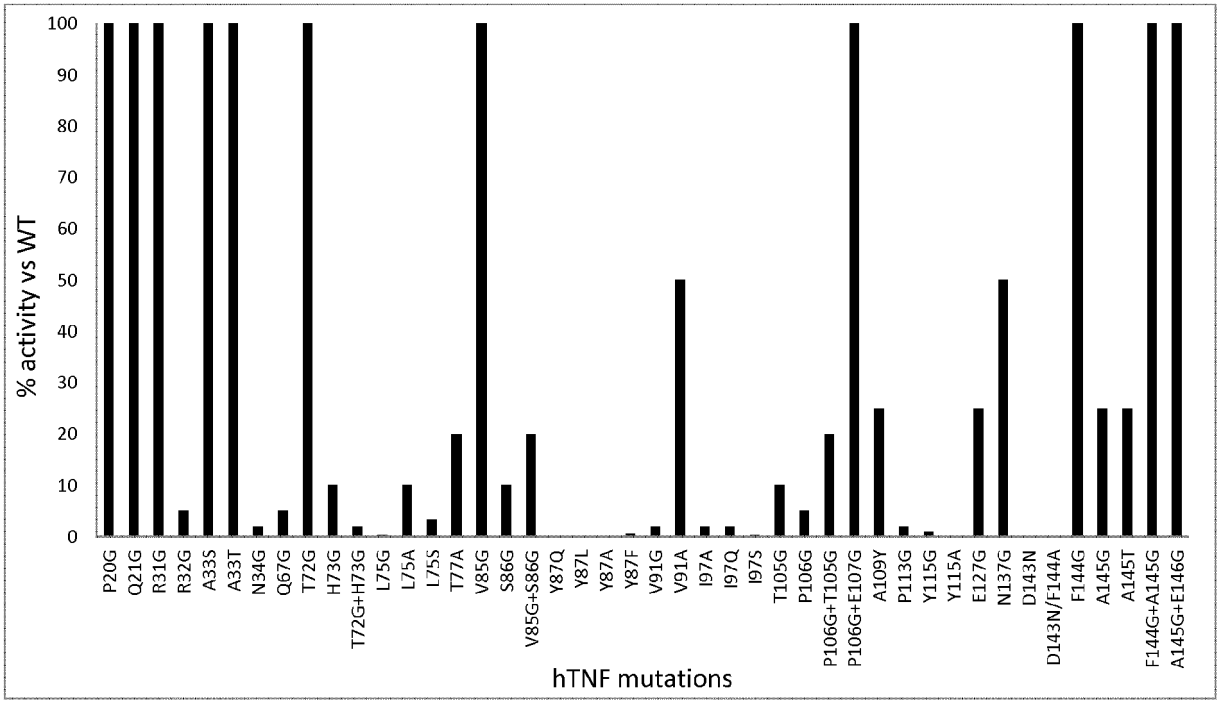
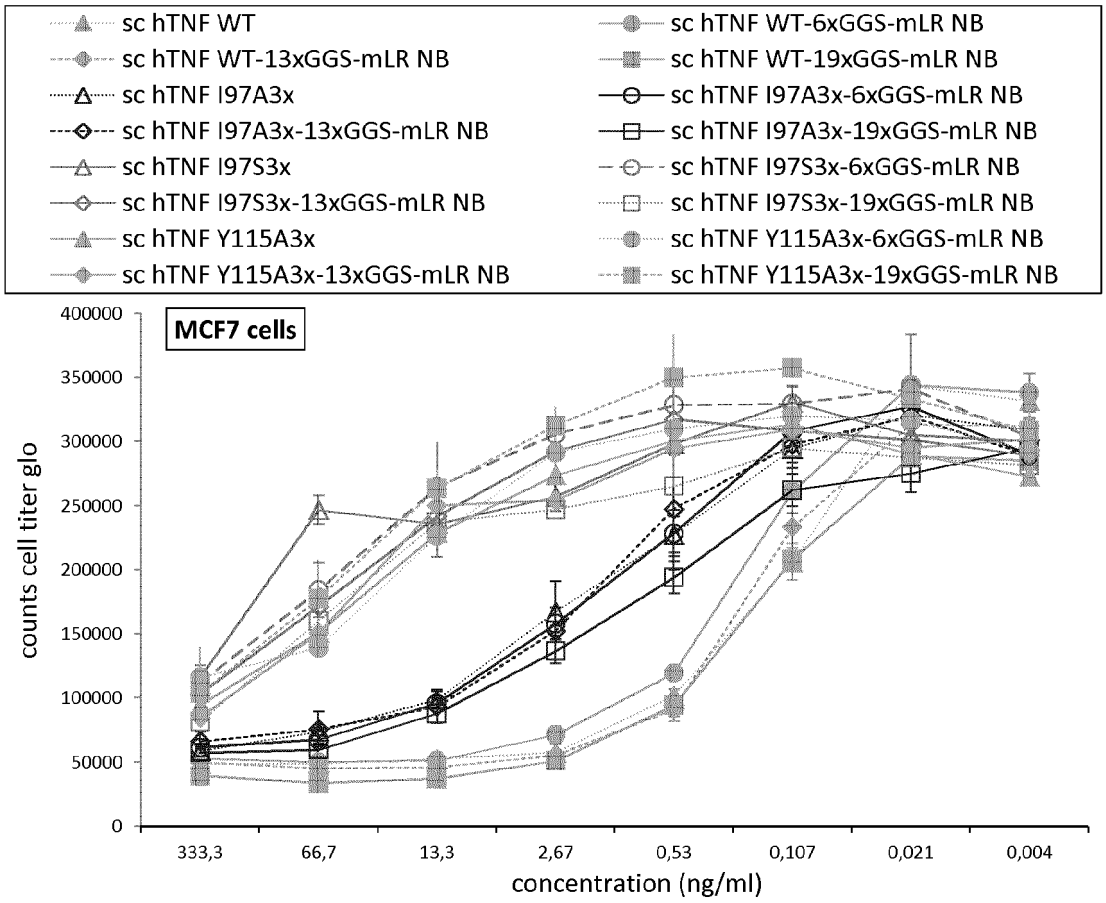


Figure 8

A


sc hTNF WT
sc hTNF WT-6xGGS-mLR NB
sc hTNF WT-13xGGS-mLR NB
sc hTNF WT-19xGGS-mLR NB
sc hTNF I97A3x
sc hTNF I97A3x-6xGGS-mLR NB
sc hTNF I97A3x-13xGGS-mLR NB
sc hTNF I97A3x-19xGGS-mLR NB
sc hTNF I97S3x
sc hTNF I97S3x-6xGGS-mLR NB
sc hTNF I97S3x-13xGGS-mLR NB
sc hTNF I97S3x-19xGGS-mLR NB
sc hTNF Y115A3x
sc hTNF Y115A3x-6xGGS-mLR NB
sc hTNF Y115A3x-13xGGS-mLR NB
sc hTNF Y115A3x-19xGGS-mLR NB
MCF7 cells
counts cell titer glo
400000
350000
300000
250000
200000
150000
100000
50000
0
333,3
66,7
13,3
2,67
0,53
0,107
0,021
0,004
concentration (ng/ml)

B

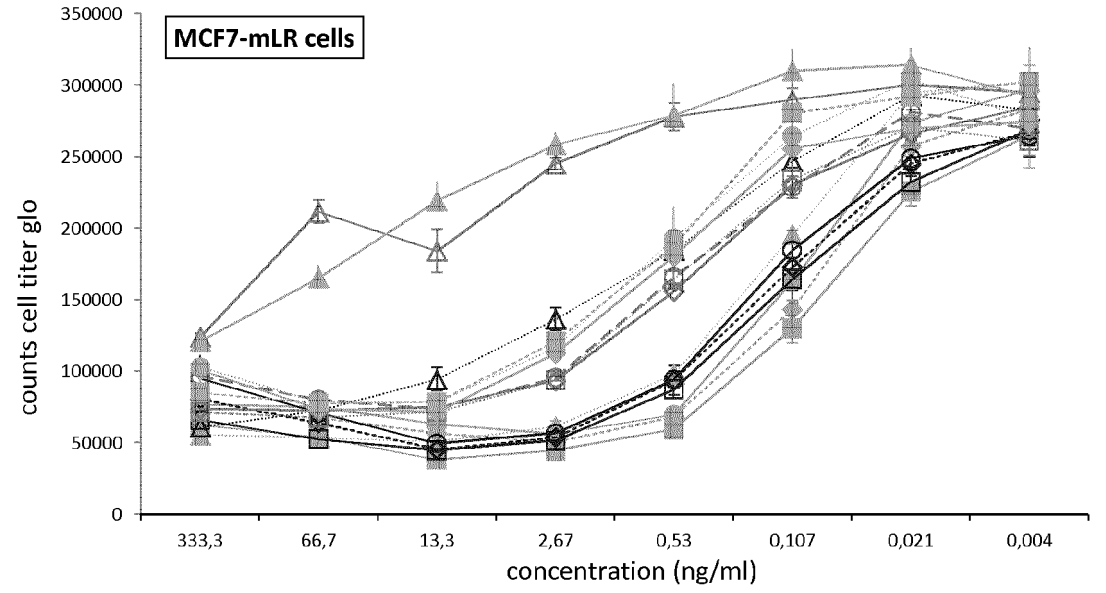
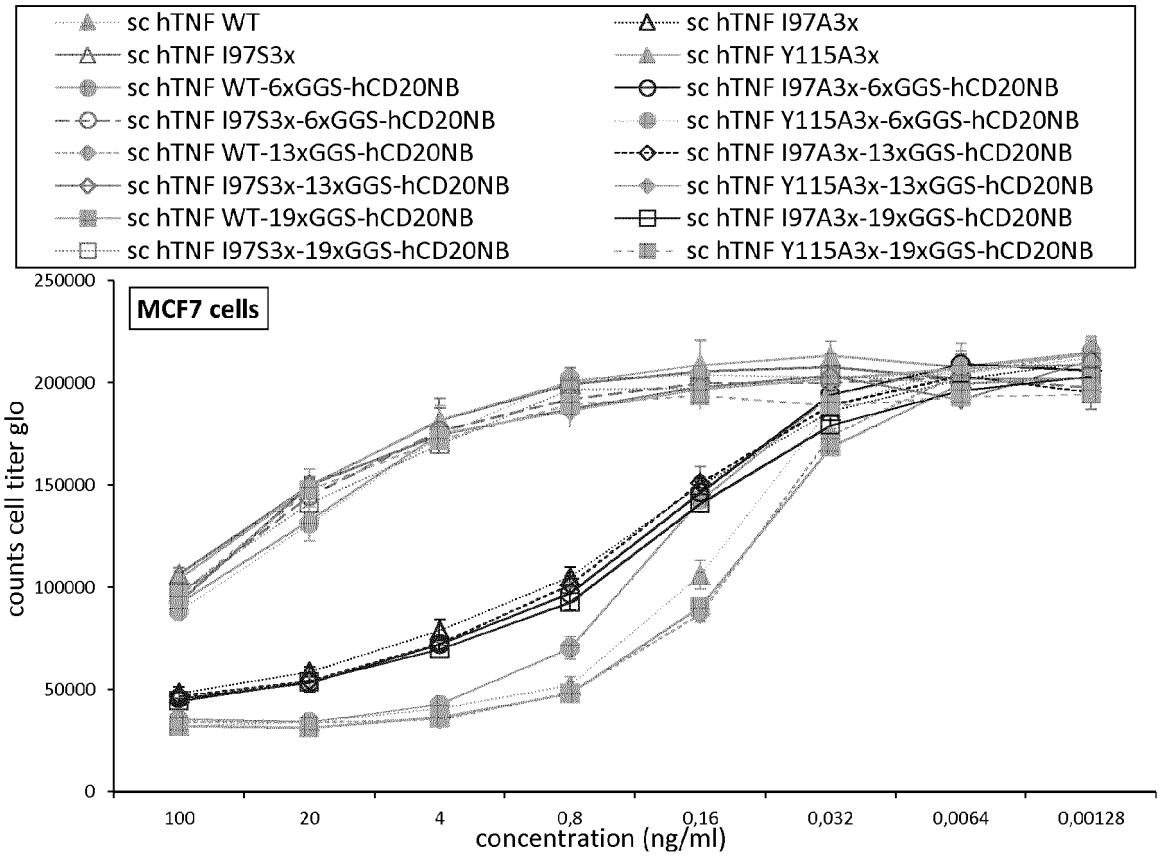

MCF7-mLR cells
counts cell titer glo
350000
300000
250000
200000
150000
100000
50000
0
333,3
66,7
13,3
2,67
0,53
0,107
0,021
0,004
concentration (ng/ml)

Figure 9

A


sc hTNF WT
sc hTNF I97A3x
sc hTNF I97S3x
sc hTNF Y115A3x
sc hTNF WT-6xGGS-hCD20NB
sc hTNF I97A3x-6xGGS-hCD20NB
sc hTNF I97S3x-6xGGS-hCD20NB
sc hTNF Y115A3x-6xGGS-hCD20NB
sc hTNF WT-13xGGS-hCD20NB
sc hTNF I97A3x-13xGGS-hCD20NB
sc hTNF I97S3x-13xGGS-hCD20NB
sc hTNF Y115A3x-13xGGS-hCD20NB
sc hTNF WT-19xGGS-hCD20NB
sc hTNF I97A3x-19xGGS-hCD20NB
sc hTNF I97S3x-19xGGS-hCD20NB
sc hTNF Y115A3x-19xGGS-hCD20NB
MCF7 cells
counts cell titer glo
250000
200000
150000
100000
50000
0
100
20
4
0,8
0,16
0,032
0,0064
0,00128
concentration (ng/ml)

B

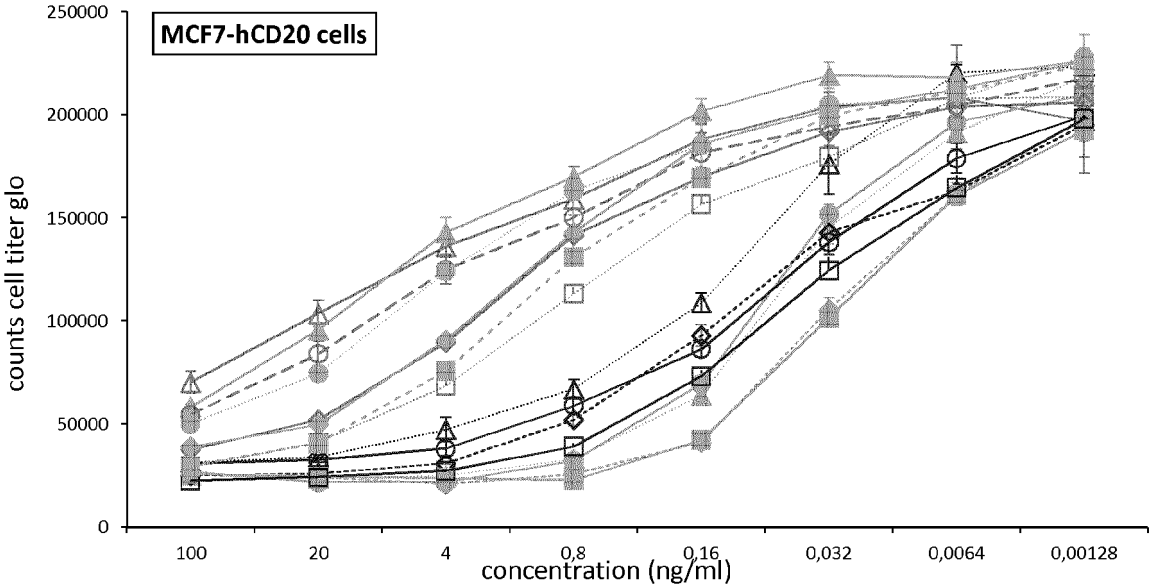
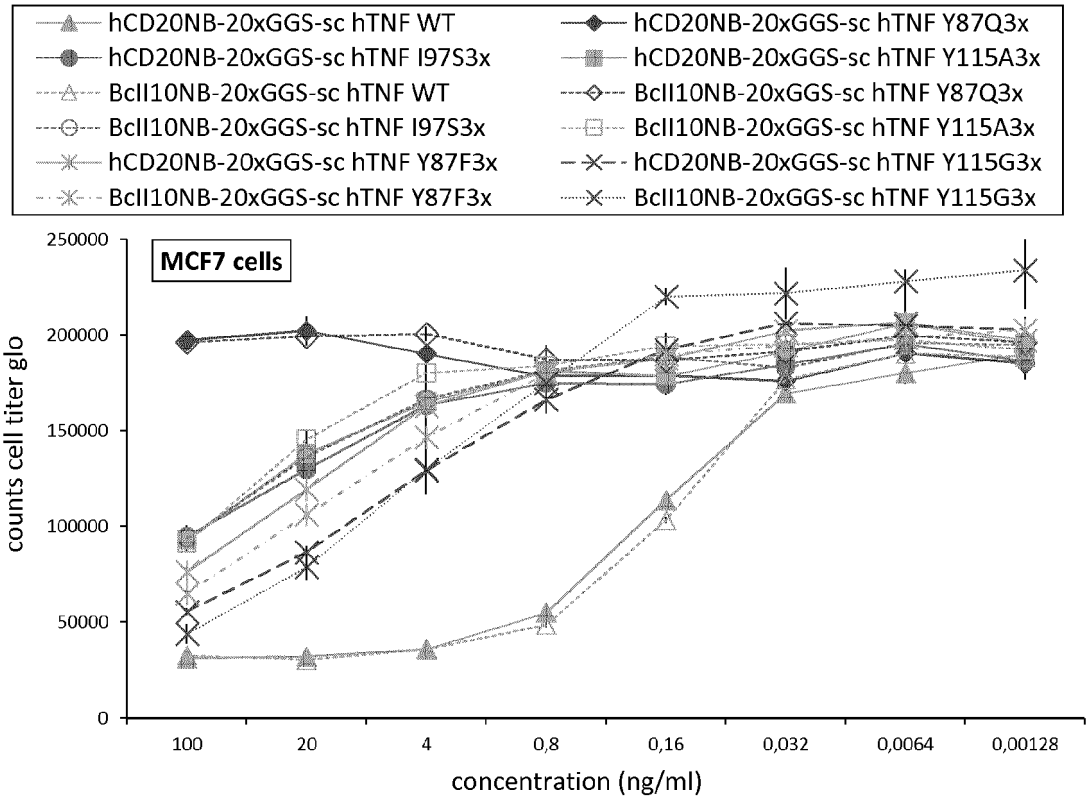

MCF7-hCD20 cells
counts cell titer glo
250000
200000
150000
100000
50000
0
100
20
4
0,8
0,16
0,032
0,0064
0,00128
concentration (ng/ml)

Figure 10

A


hCD20NB-20xGGS-sc hTNF WT
hCD20NB-20xGGS-sc hTNF Y87Q3x
hCD20NB-20xGGS-sc hTNF I97S3x
hCD20NB-20xGGS-sc hTNF Y115A3x
BcII10NB-20xGGS-sc hTNF WT
BcII10NB-20xGGS-sc hTNF Y87Q3x
BcII10NB-20xGGS-sc hTNF I97S3x
BcII10NB-20xGGS-sc hTNF Y115A3x
hCD20NB-20xGGS-sc hTNF Y87F3x
hCD20NB-20xGGS-sc hTNF Y115G3x
BcII10NB-20xGGS-sc hTNF Y87F3x
BcII10NB-20xGGS-sc hTNF Y115G3x
MCF7 cells
counts cell titer glo
250000
200000
150000
100000
50000
0
100
20
4
0,8
0,16
0,032
0,0064
0,00128
concentration (ng/ml)

B

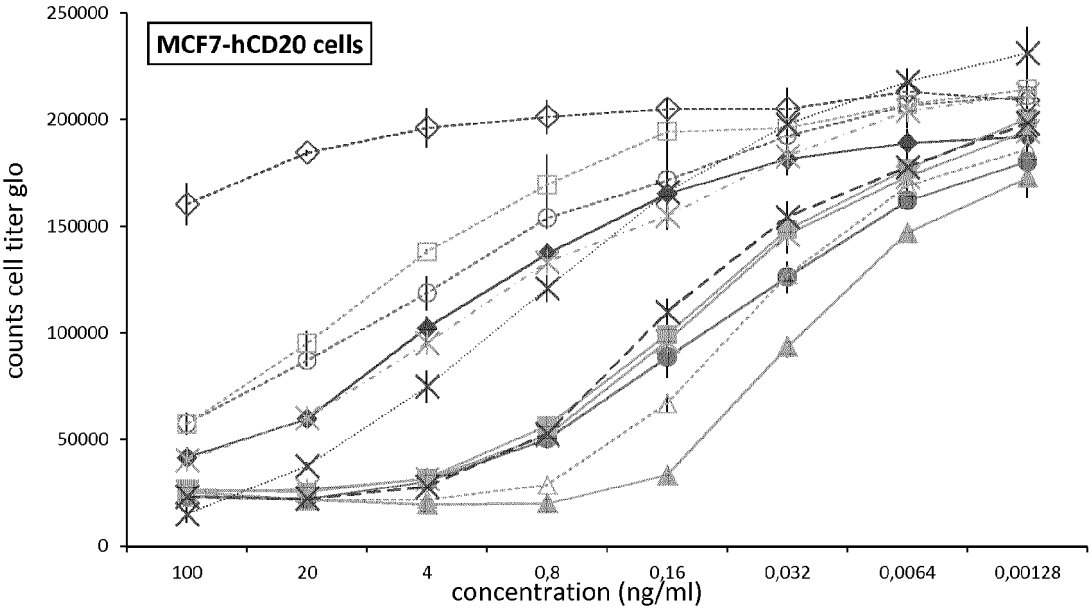
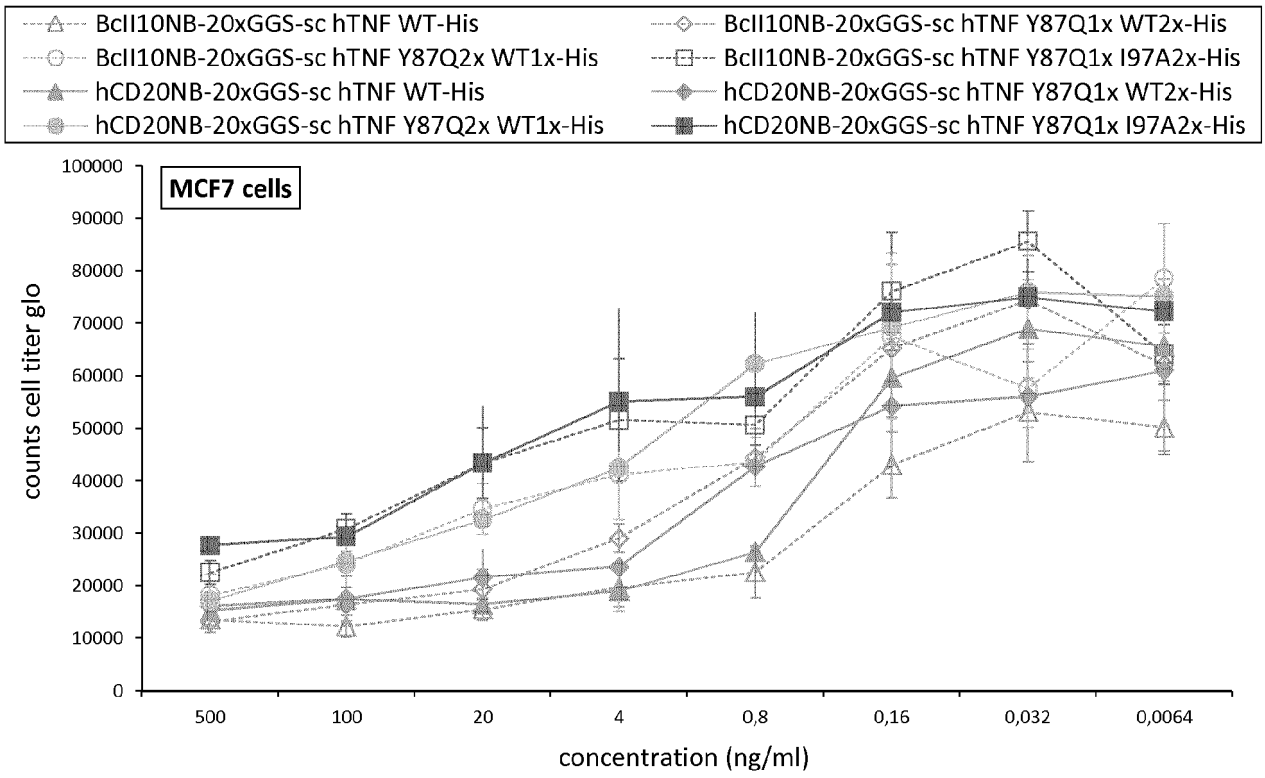

MCF7-hCD20 cells
counts cell titer glo
250000
200000
150000
100000
50000
0
100
20
4
0,8
0,16
0,032
0,0064
0,00128
concentration (ng/ml)

Figure 11

A


BcII10NB-20xGGS-sc hTNF WT-His
BcII10NB-20xGGS-sc hTNF Y87Q1x WT2x-His
BcII10NB-20xGGS-sc hTNF Y87Q2x WT1x-His
BcII10NB-20xGGS-sc hTNF Y87Q1x I97A2x-His
hCD20NB-20xGGS-sc hTNF WT-His
hCD20NB-20xGGS-sc hTNF Y87Q1x WT2x-His
hCD20NB-20xGGS-sc hTNF Y87Q2x WT1x-His
hCD20NB-20xGGS-sc hTNF Y87Q1x I97A2x-His
MCF7 cells
counts cell titer glo
100000
90000
80000
70000
60000
50000
40000
30000
20000
10000
0
500
100
20
4
0,8
0,16
0,032
0,0064
concentration (ng/ml)

B

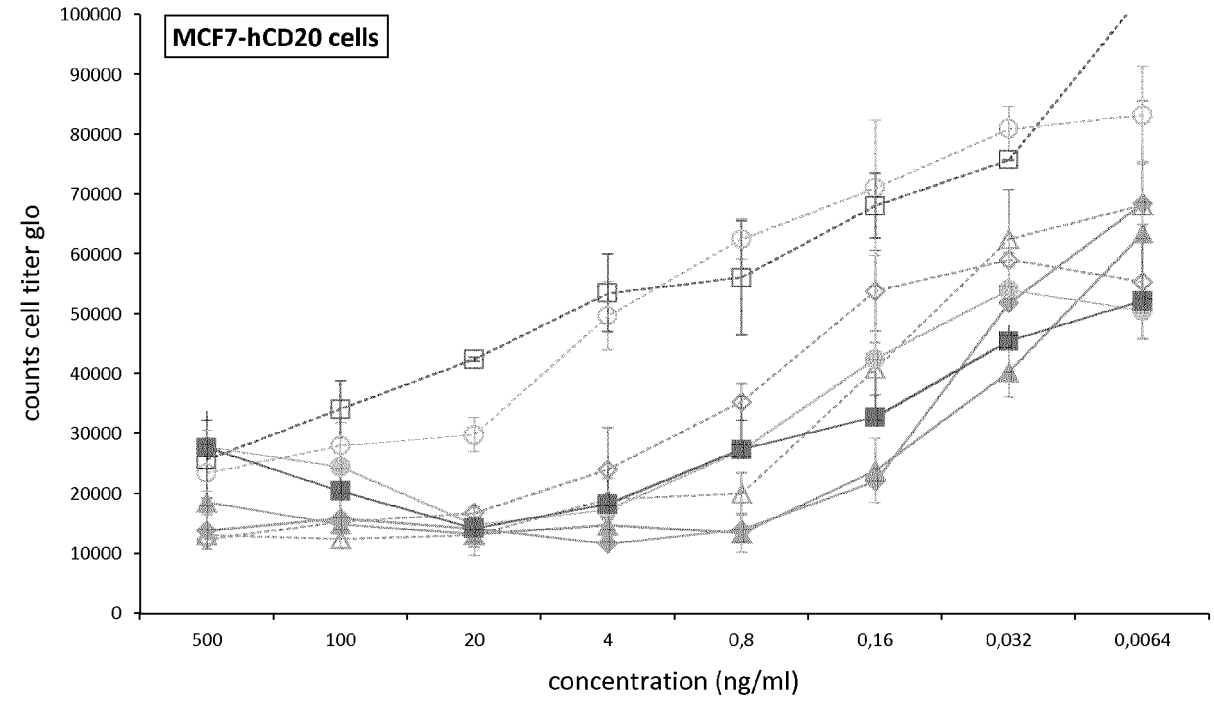
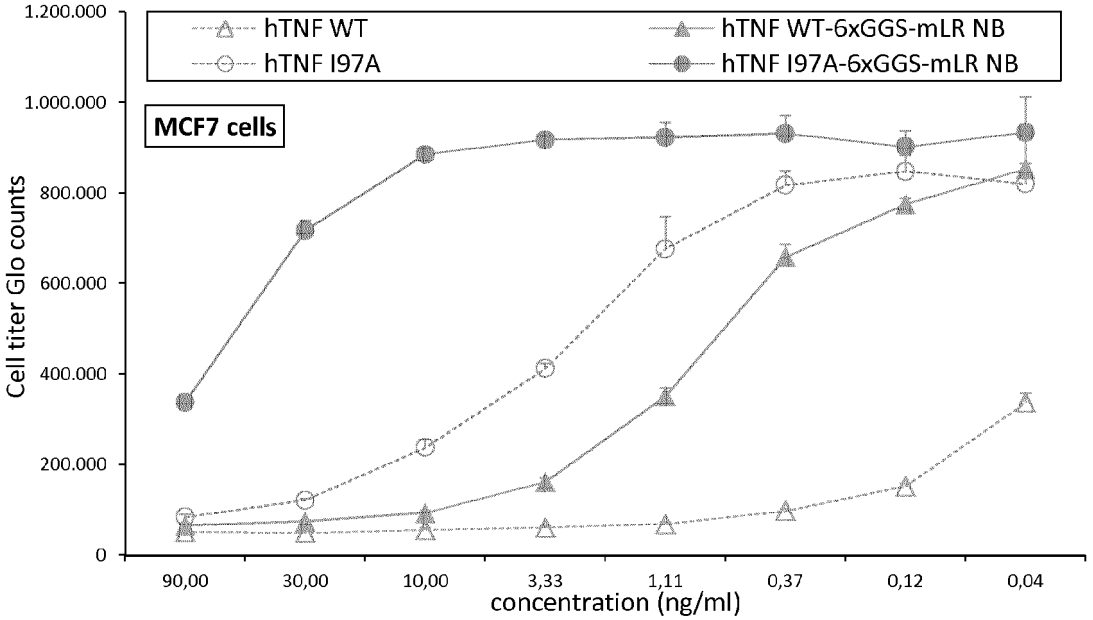

MCF7-hCD20 cells
counts cell titer glo
100000
90000
80000
70000
60000
50000
40000
30000
20000
10000
0
500
100
20
4
0,8
0,16
0,032
0,0064
concentration (ng/ml)

Figure 12

A


hTNF WT
hTNF I97A
hTNF WT-6xGGS-mLR NB
hTNF I97A-6xGGS-mLR NB
MCF7 cells
Cell titer Glo counts
1.200.000
1.000.000
800.000
600.000
400.000
200.000
0
90,00
30,00
10,00
3,33
1,11
0,37
0,12
0,04
concentration (ng/ml)

B

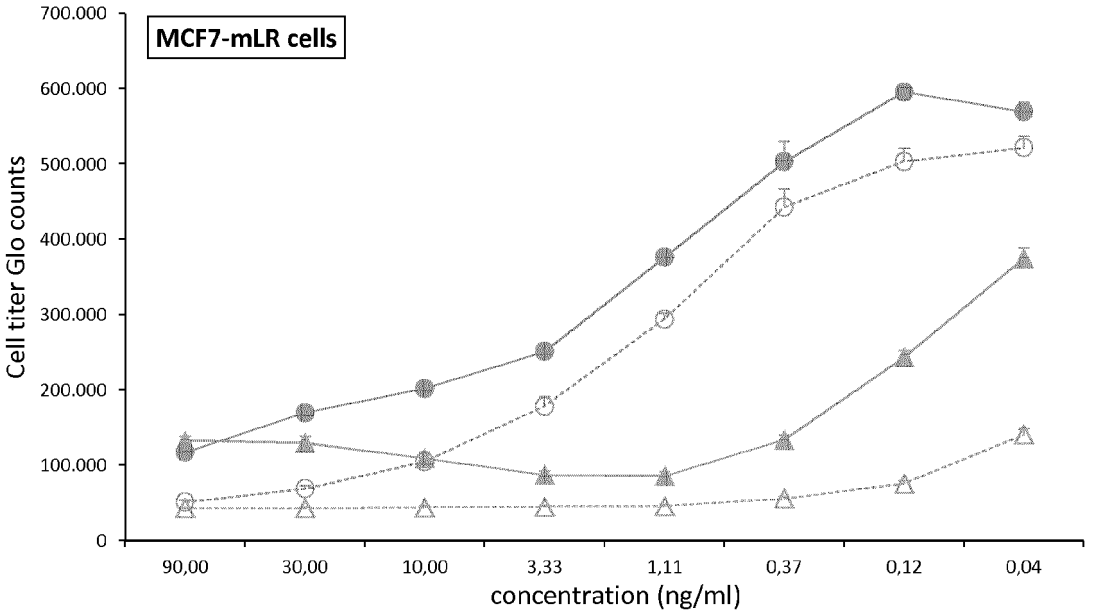
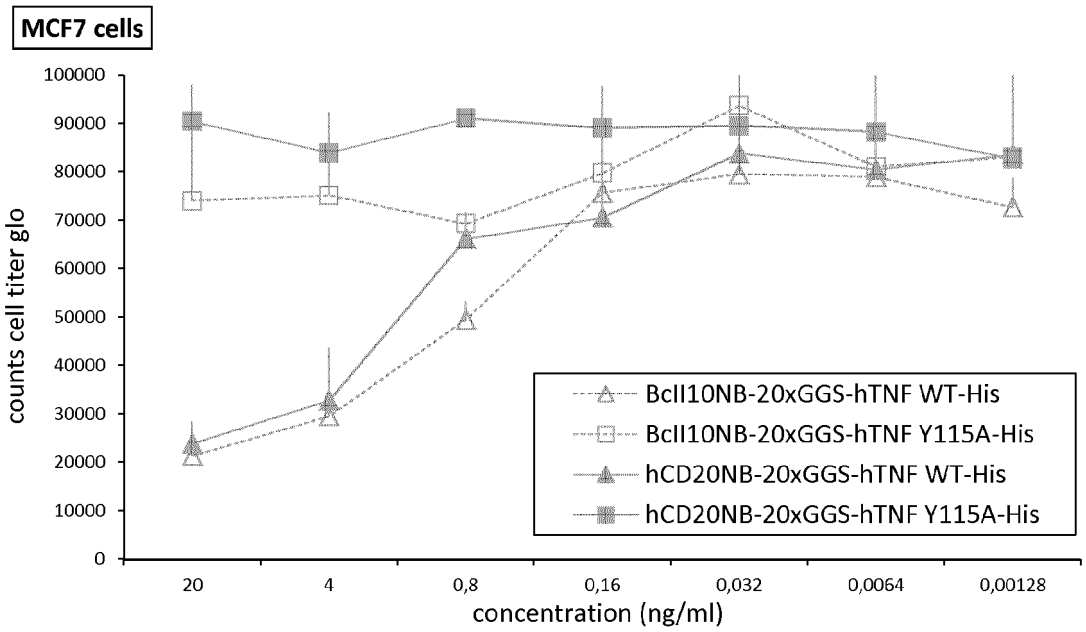

MCF7-mLR cells
Cell titer Glo counts
700.000
600.000
500.000
400.000
300.000
200.000
100.000
0
90,00
30,00
10,00
3,33
1,11
0,37
0,12
0,04
concentration (ng/ml)

Figure 13

A


MCF7 cells
counts cell titer glo
100000
90000
80000
70000
60000
50000
40000
30000
20000
10000
0
20
4
0,8
0,16
0,032
0,0064
0,00128
concentration (ng/ml)
BcII10NB-20xGGS-hTNF WT-His
BcII10NB-20xGGS-hTNF Y115A-His
hCD20NB-20xGGS-hTNF WT-His
hCD20NB-20xGGS-hTNF Y115A-His

B

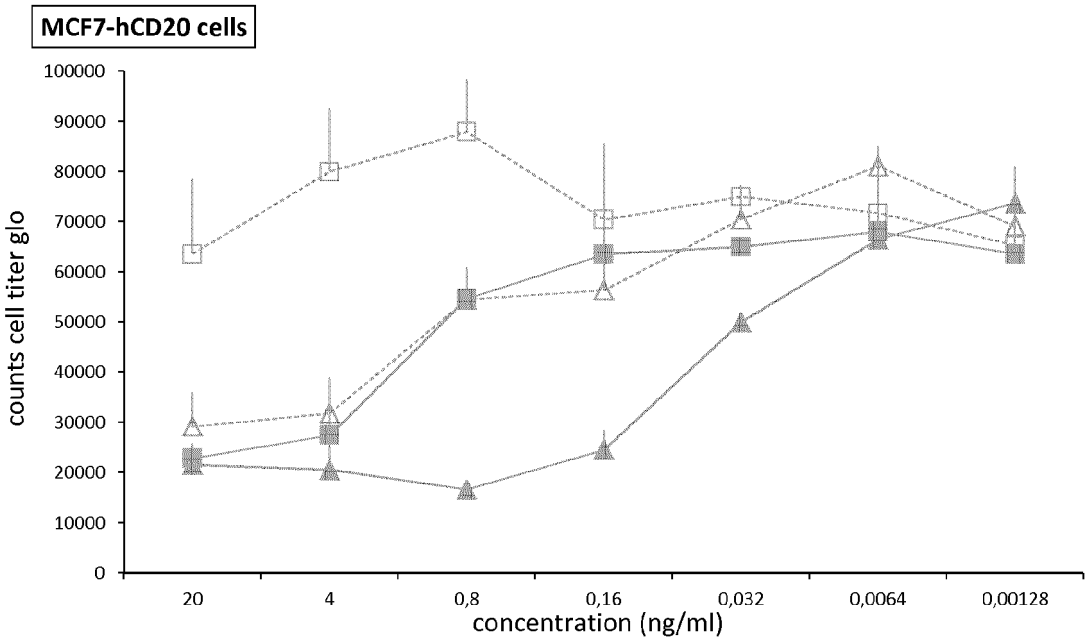
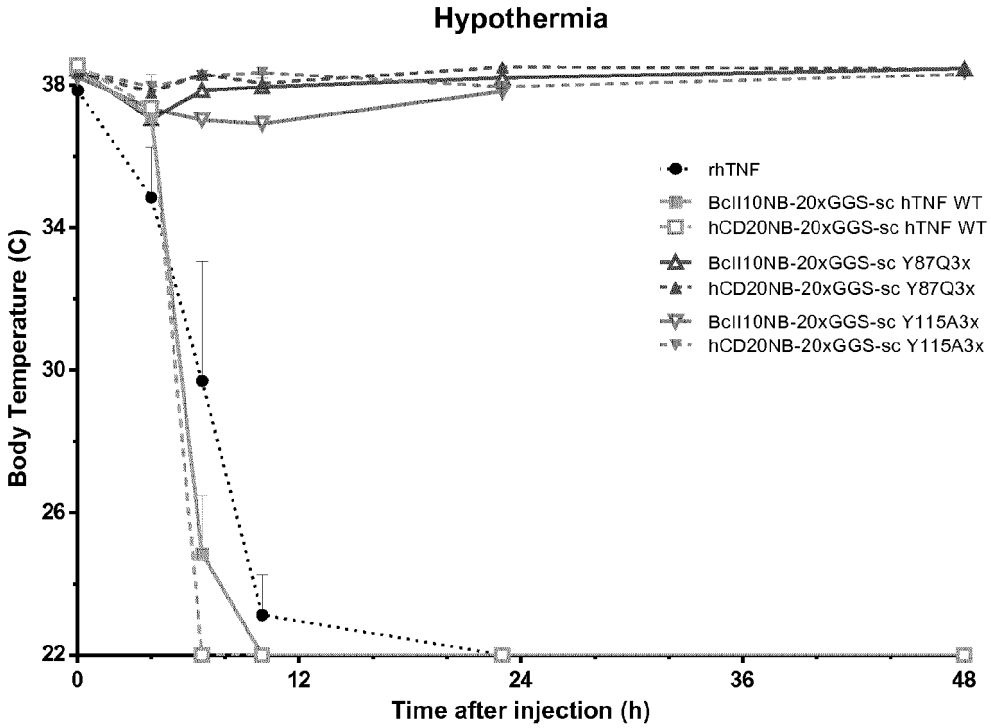

MCF7-hCD20 cells
counts cell titer glo
100000
90000
80000
70000
60000
50000
40000
30000
20000
10000
0
20
4
0,8
0,16
0,032
0,0064
0,00128
concentration (ng/ml)

Figure 14

A


Hypothermia
Body Temperature (C)
38
34
30
26
22
0
12
24
36
48
Time after injection (h)
rhTNF
BclI10NB-20xGGS-sc hTNF WT
hCD20NB-20xGGS-sc hTNF WT
BclI10NB-20xGGS-sc Y87Q3x
hCD20NB-20xGGS-sc Y87Q3x
BclI10NB-20xGGS-sc Y115A3x
hCD20NB-20xGGS-sc Y115A3x

B

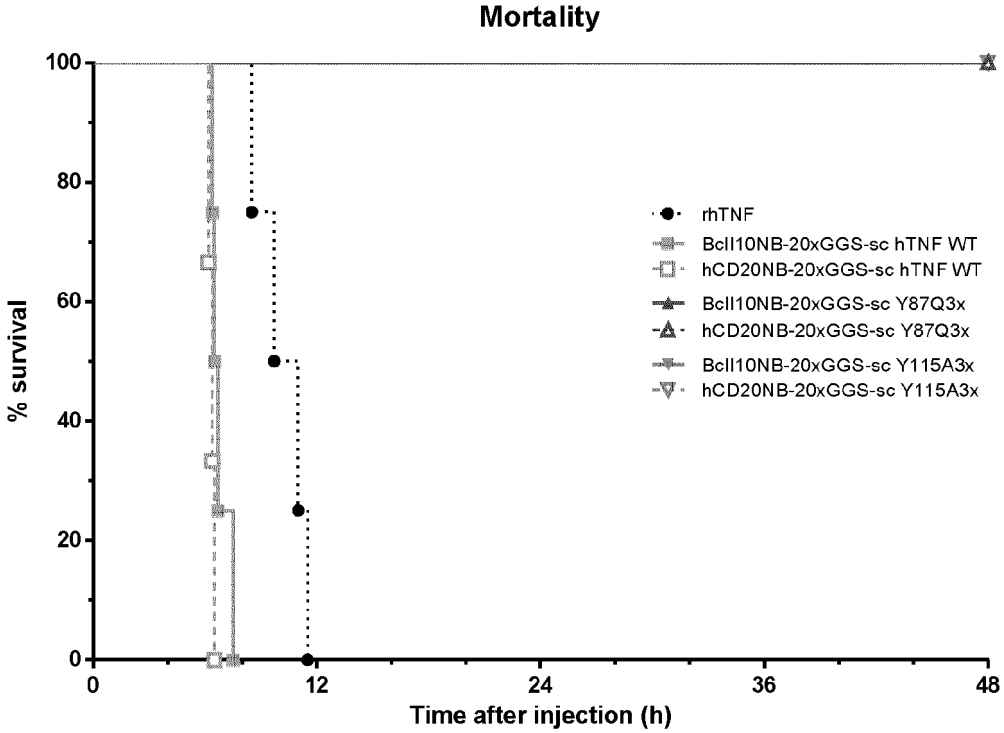
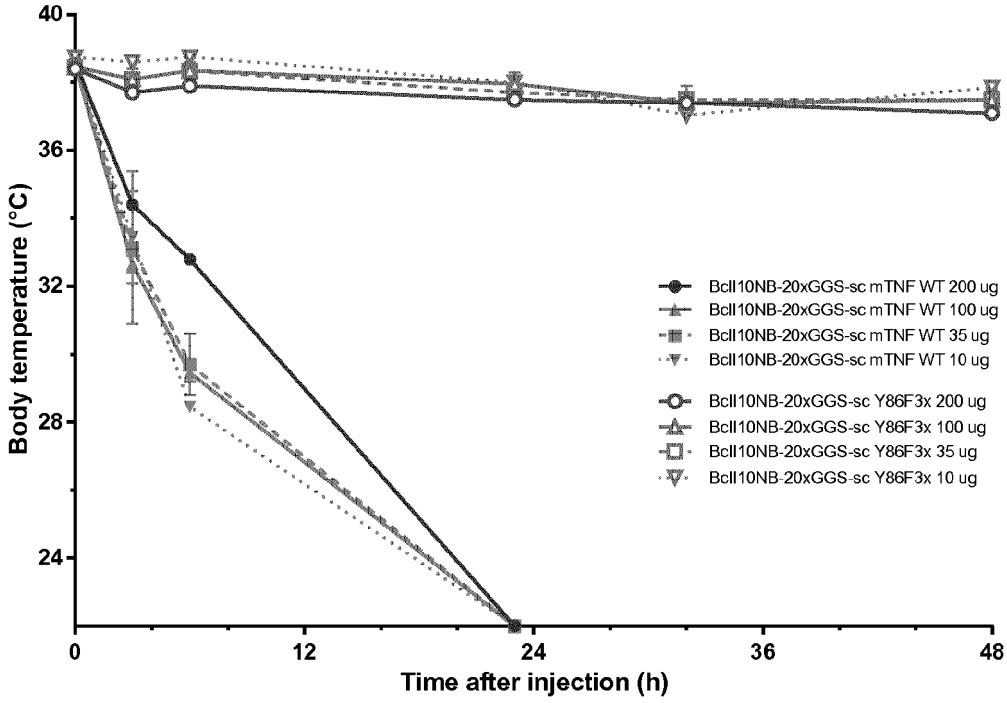

Mortality
% survival
100
80
60
40
20
0
0
12
24
36
48
Time after injection (h)
rhTNF
BclI10NB-20xGGS-sc hTNF WT
hCD20NB-20xGGS-sc hTNF WT
BclI10NB-20xGGS-sc Y87Q3x
hCD20NB-20xGGS-sc Y87Q3x
BclI10NB-20xGGS-sc Y115A3x
hCD20NB-20xGGS-sc Y115A3x

Figure 15

A


Body temperature (°C)
40
36
32
28
24
0
12
24
36
48
Time after injection (h)
BclI10NB-20xGGS-sc mTNF WT 200 ug
BclI10NB-20xGGS-sc mTNF WT 100 ug
BclI10NB-20xGGS-sc mTNF WT 35 ug
BclI10NB-20xGGS-sc mTNF WT 10 ug
BclI10NB-20xGGS-sc Y86F3x 200 ug
BclI10NB-20xGGS-sc Y86F3x 100 ug
BclI10NB-20xGGS-sc Y86F3x 35 ug
BclI10NB-20xGGS-sc Y86F3x 10 ug

B

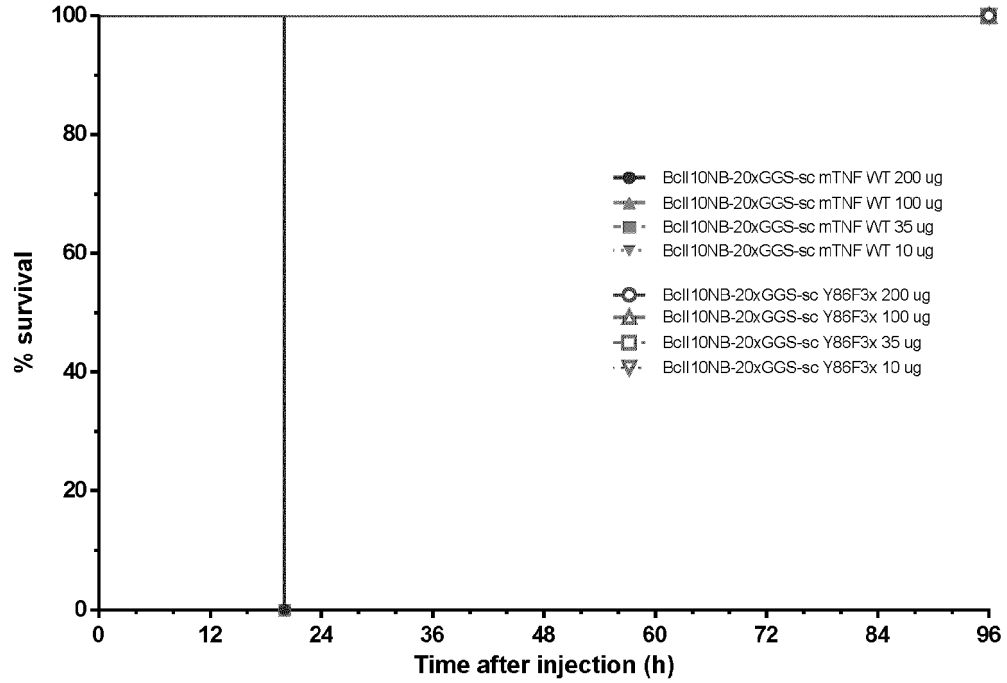
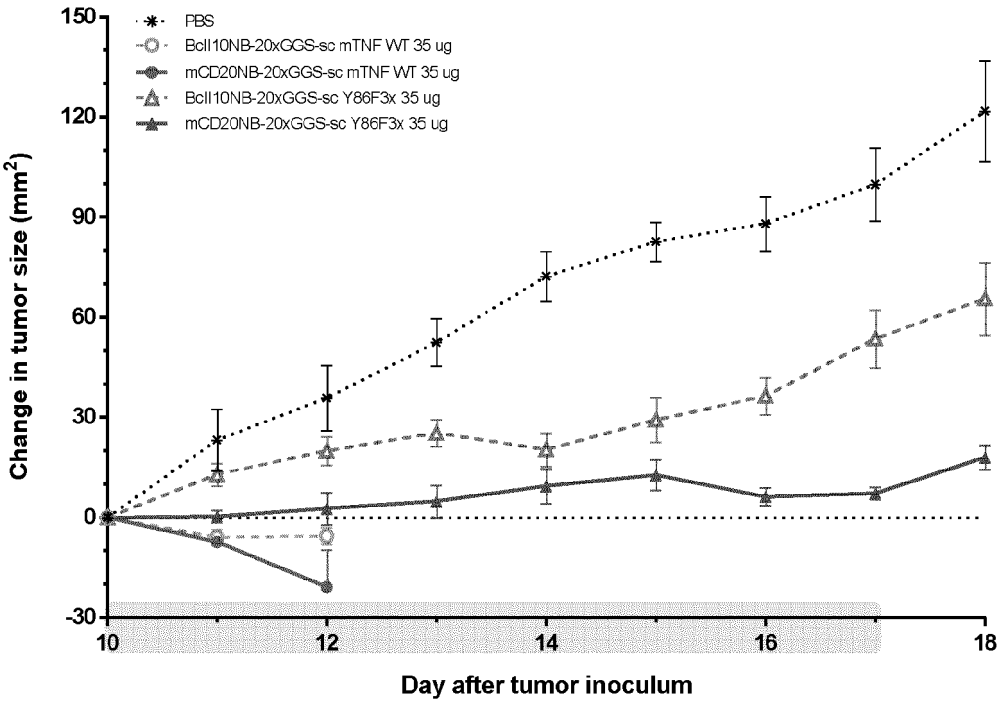
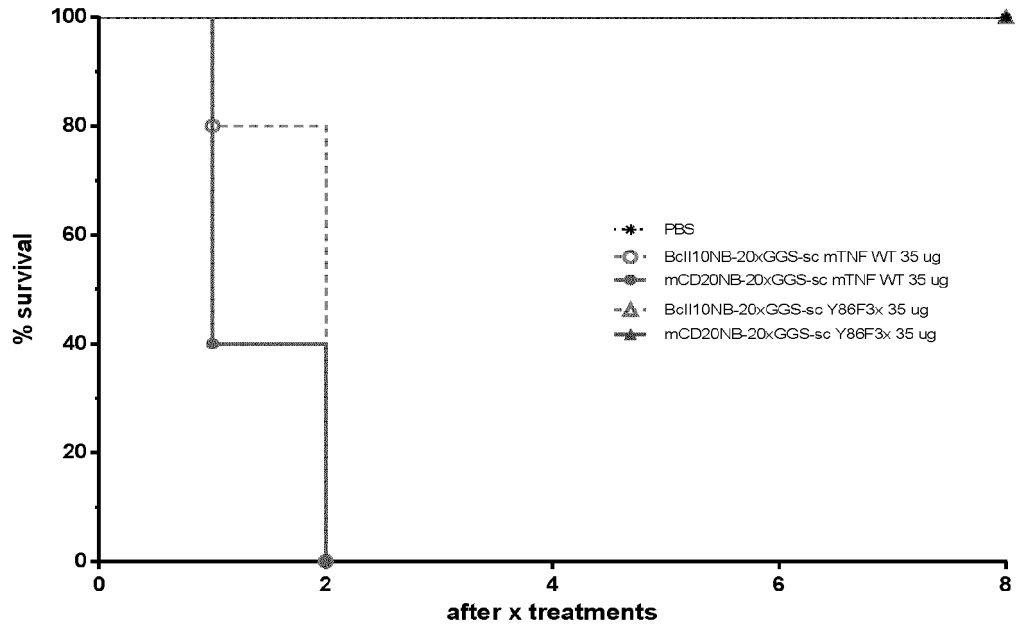

% survival
100
80
60
40
20
0
0
12
24
36
48
60
72
84
96
Time after injection (h)
BclI10NB-20xGGS-sc mTNF WT 200 ug
BclI10NB-20xGGS-sc mTNF WT 100 ug
BclI10NB-20xGGS-sc mTNF WT 35 ug
BclI10NB-20xGGS-sc mTNF WT 10 ug
BclI10NB-20xGGS-sc Y86F3x 200 ug
BclI10NB-20xGGS-sc Y86F3x 100 ug
BclI10NB-20xGGS-sc Y86F3x 35 ug
BclI10NB-20xGGS-sc Y86F3x 10 ug

Figure 16

A


PBS
BclI10NB-20xGGS-sc mTNF WT 35 ug
mCD20NB-20xGGS-sc mTNF WT 35 ug
BclI10NB-20xGGS-sc Y86F3x 35 ug
mCD20NB-20xGGS-sc Y86F3x 35 ug
Change in tumor size (mm2)
150
120
90
60
30
0
-30
10
12
14
16
18
Day after tumor inoculum

B


% survival
100
80
60
40
20
0
0
2
4
6
8
after x treatments
PBS
BclI10NB-20xGGS-sc mTNF WT 35 ug
mCD20NB-20xGGS-sc mTNF WT 35 ug
BclI10NB-20xGGS-sc Y86F3x 35 ug
mCD20NB-20xGGS-sc Y86F3x 35 ug