FIG. 1

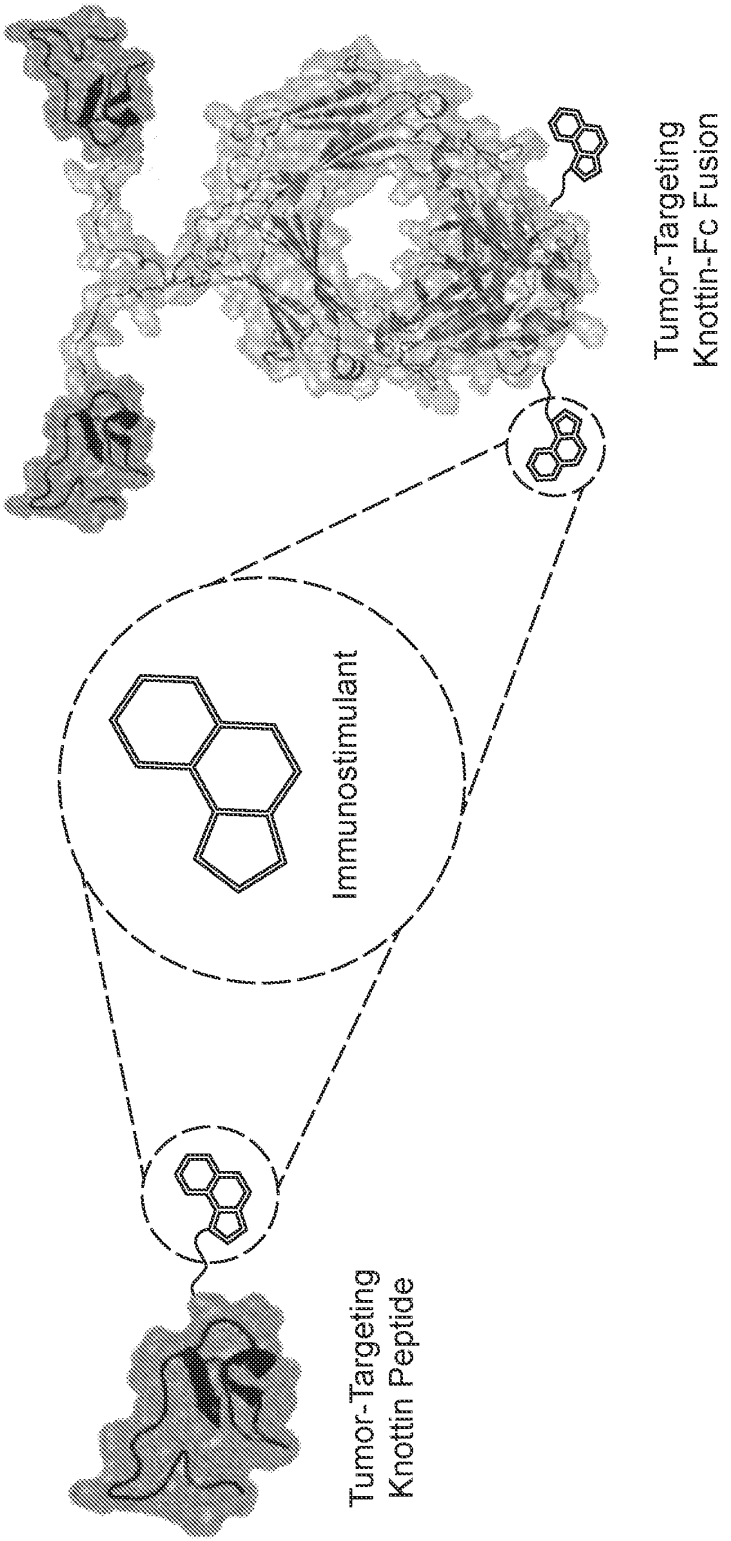

Tumor-Targeting
Knottin-Fc Fusion
Immunostimulant
Tumor-Targeting
Knottin Peptide

FIG. 2

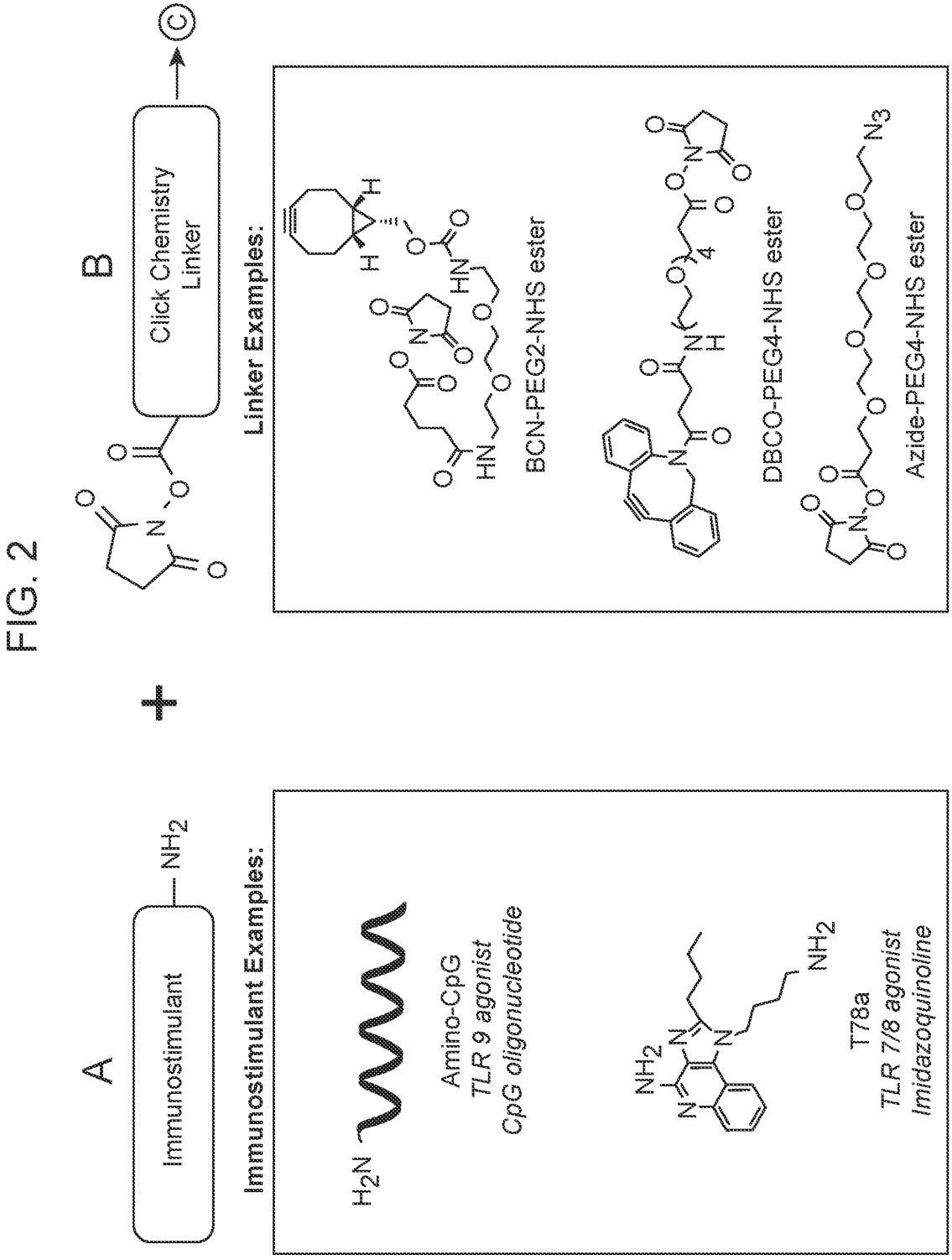

A
Immunostimulant
NH2
+
B
Click Chemistry Linker
C
Immunostimulant Examples:
H2N
Amino-CpG
TLR 9 agonist
CpG oligonucleotide
NH2
NH2
T78a
TLR 7/8 agonist
Imidazoquinoline
Linker Examples:
BCN-PEG2-NHS ester
DBCO-PEG4-NHS ester
N3
Azide-PEG4-NHS ester

FIG. 2 (Cont.)

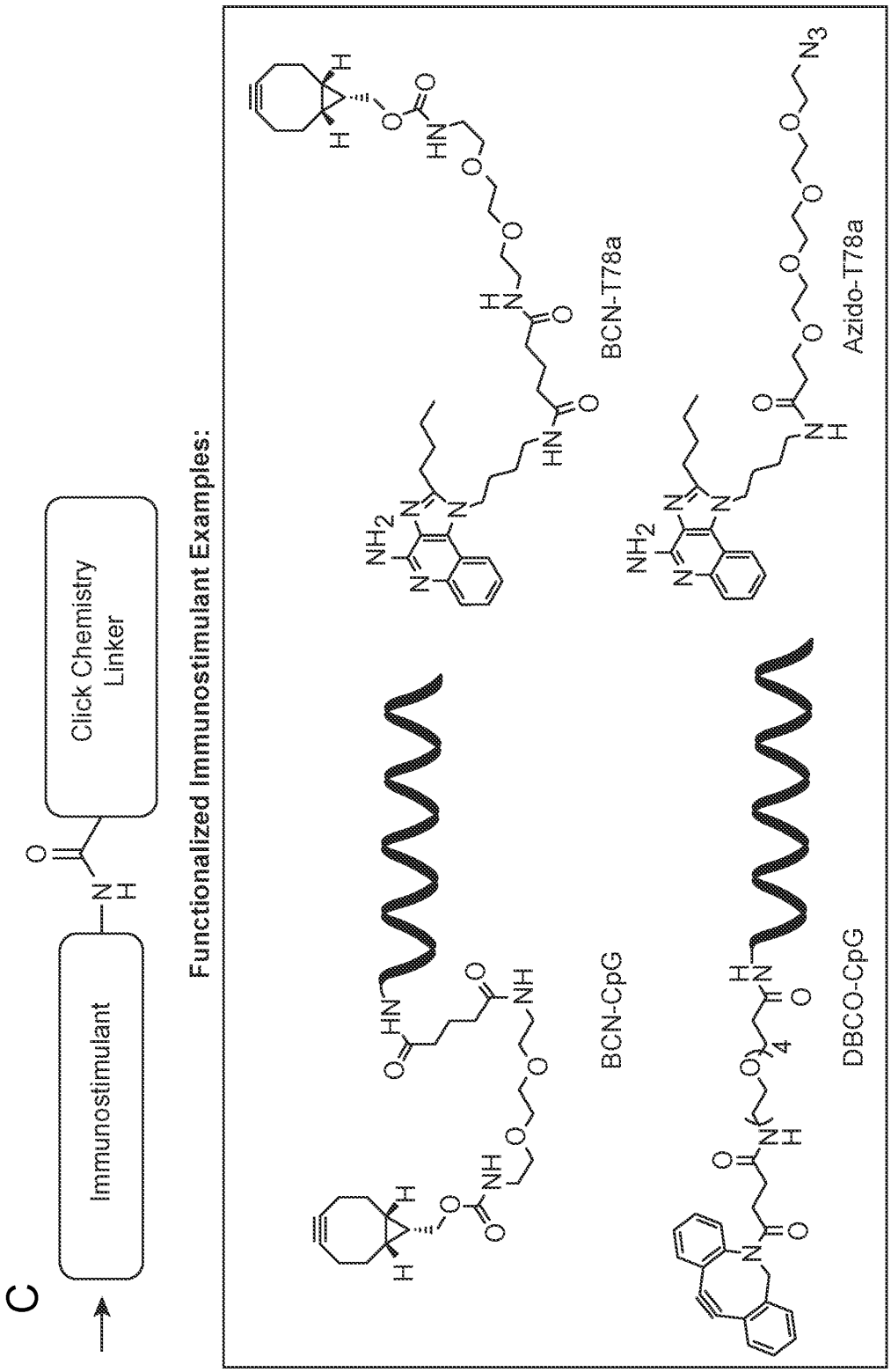
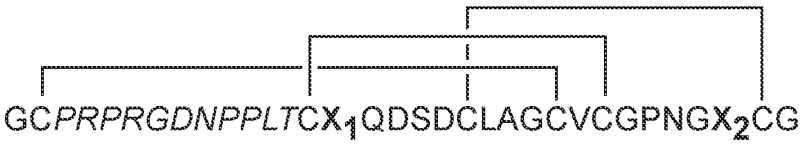

C
Immunostimulant
Click Chemistry Linker
Functionalized Immunostimulant Examples:
BCN-CpG
BCN-T78a
DBCO-CpG
Azido-T78a

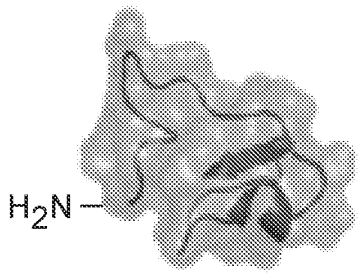
FIG. 3

A

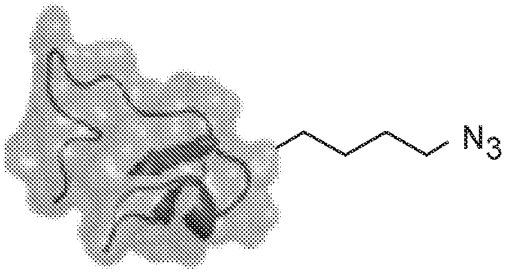


Knottin	X_1	X_2	Conjugation site:
2.5F	Serine	Phenylalanine	N-terminus
3CM	5-azido-L-norvaline	Tyrosine	N-terminus or X_1 Azide

B



2.5F
(N-terminus shown)



3CM
(X_1 azide shown)

FIG. 4

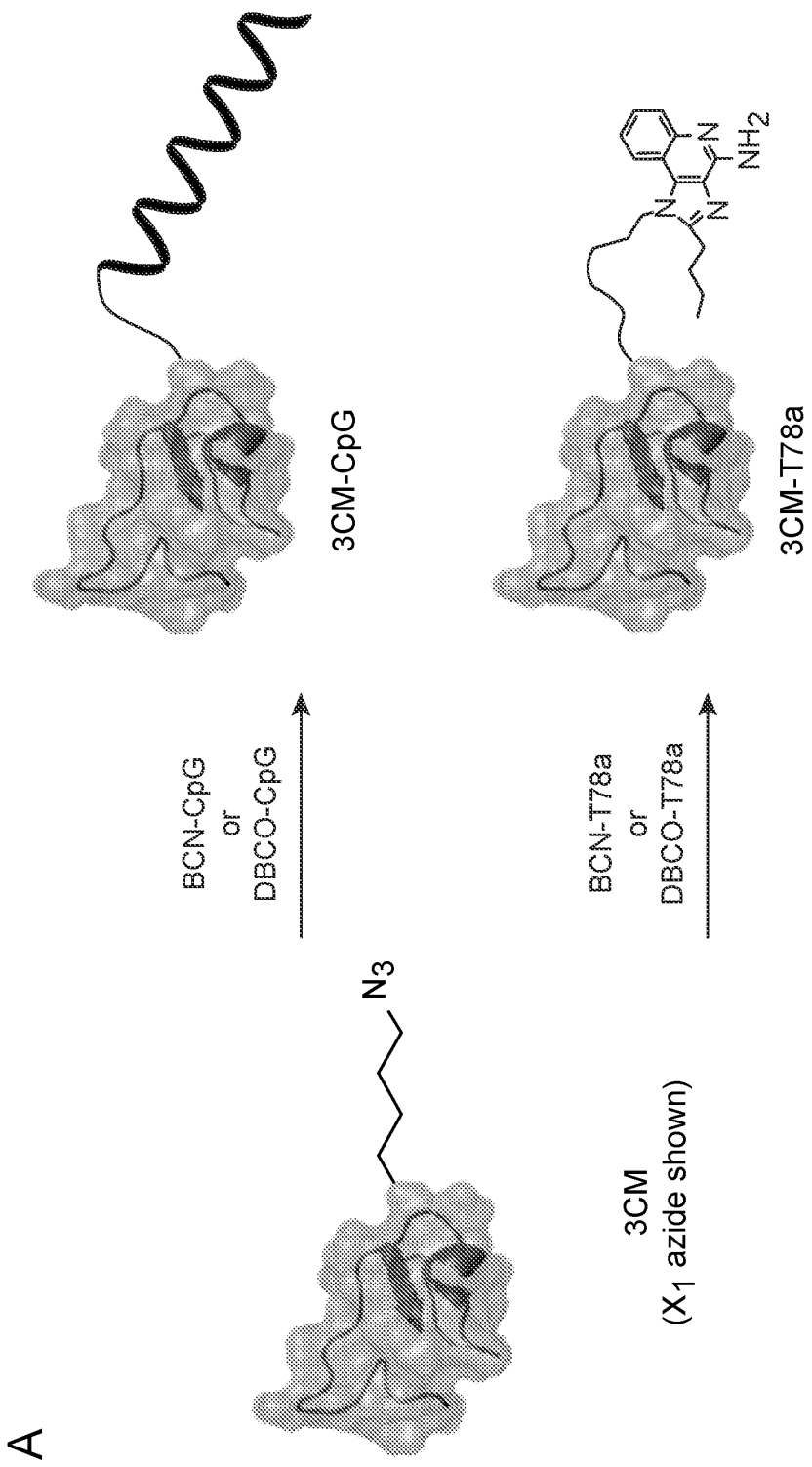

A
3CM
(X1 azide shown)
N3
BCN-CpG
or
DBCO-CpG
3CM-CpG
BCN-T78a
or
DBCO-T78a
3CM-T78a
NH2

FIG. 4 (Cont.)

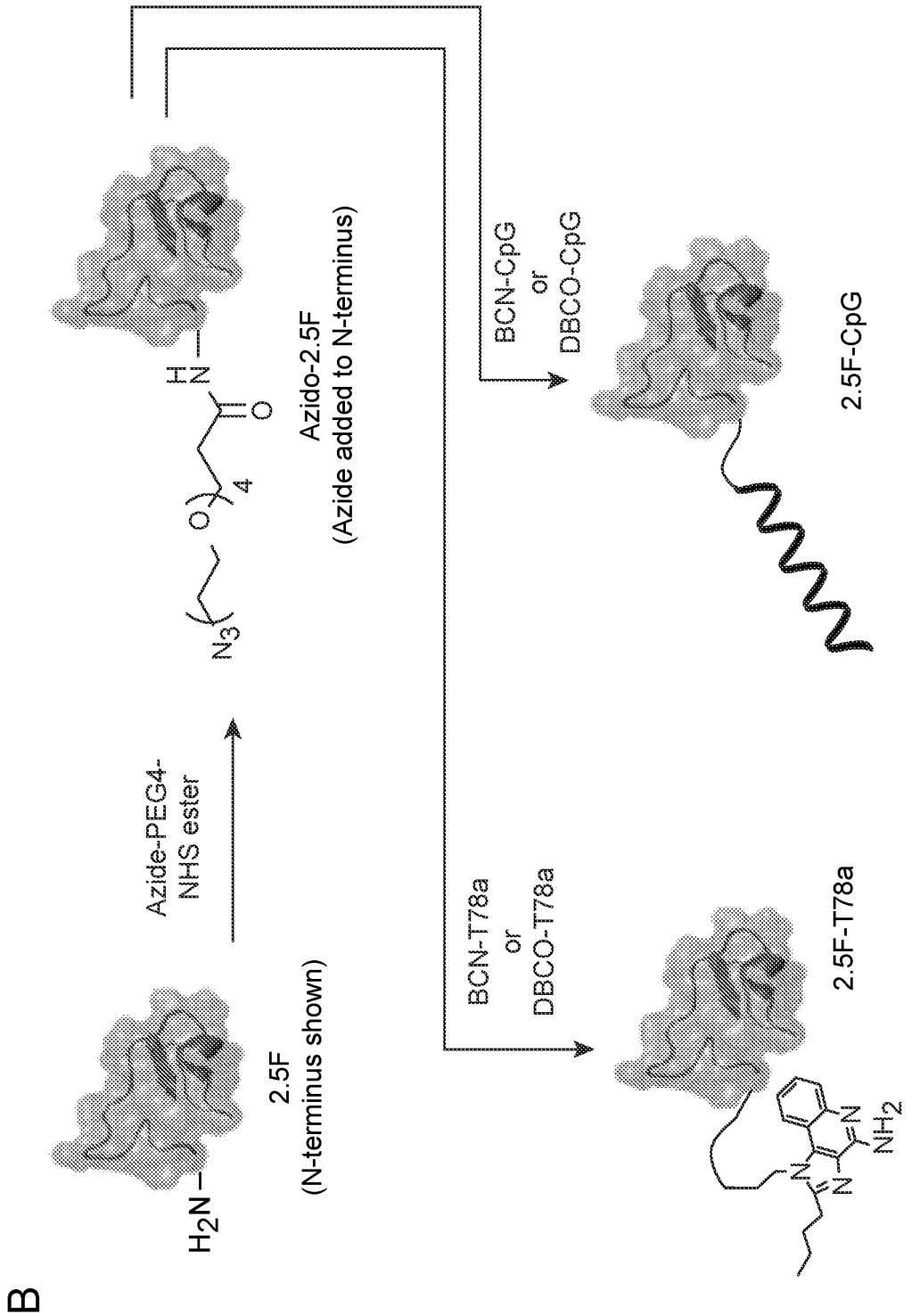

B
H2N—
2.5F
(N-terminus shown)
Azide-PEG4-
NHS ester
Azido-2.5F
(Azide added to N-terminus)
BCN-CpG
or
DBCO-CpG
2.5F-CpG
BCN-T78a
or
DBCO-T78a
2.5F-T78a

FIG. 5

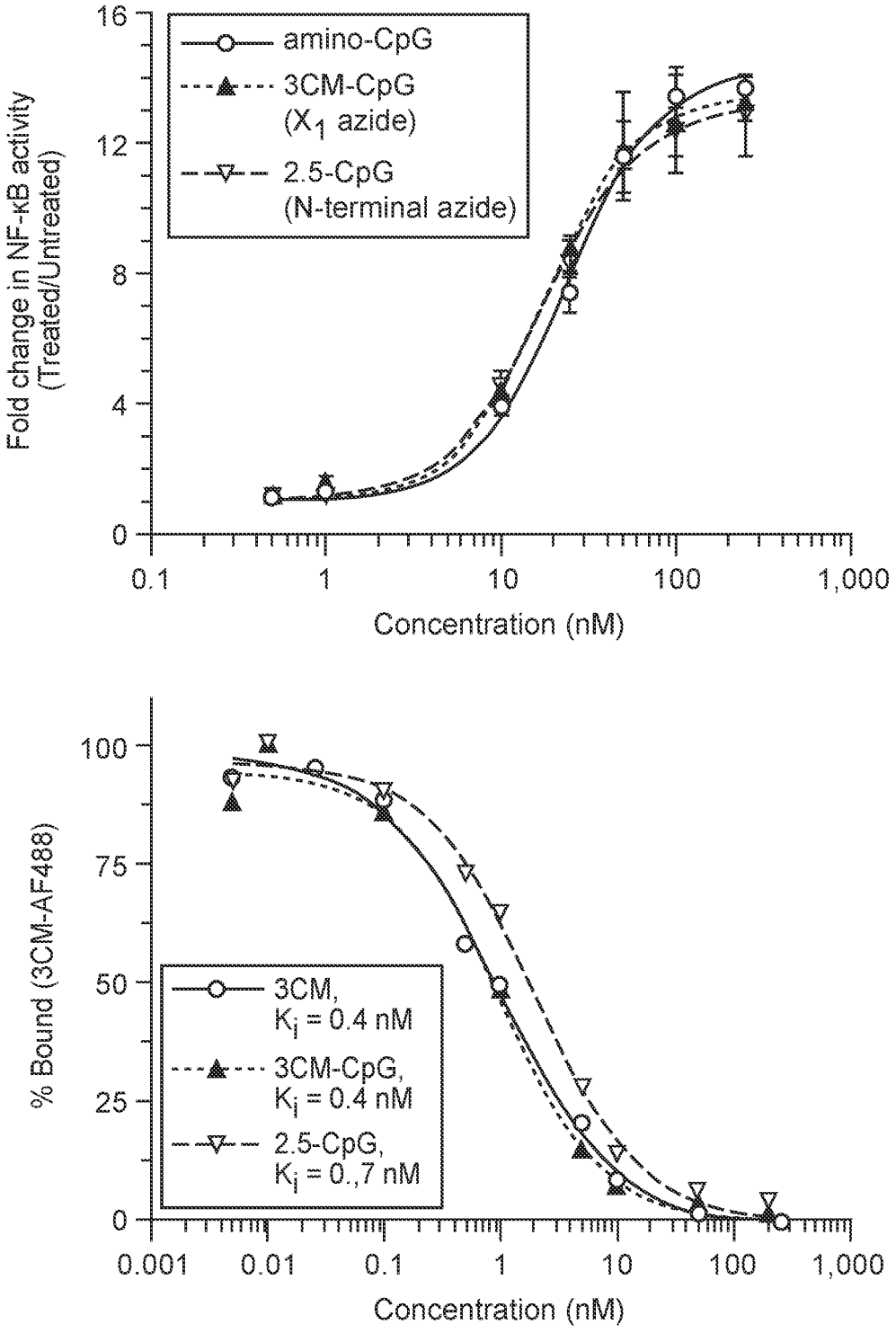

Fold change in NF-κB activity
(Treated/Untreated)
16
12
8
4
0
0.1
1
10
100
1,000
Concentration (nM)
amino-CpG
3CM-CpG
(X1 azide)
2.5-CpG
(N-terminal azide)
% Bound (3CM-AF488)
100
75
50
25
0
0.001
0.01
0.1
1
10
100
1,000
Concentration (nM)
3CM,
Ki = 0.4 nM
3CM-CpG,
Ki = 0.4 nM
2.5-CpG,
Ki = 0.,7 nM

FIG. 6

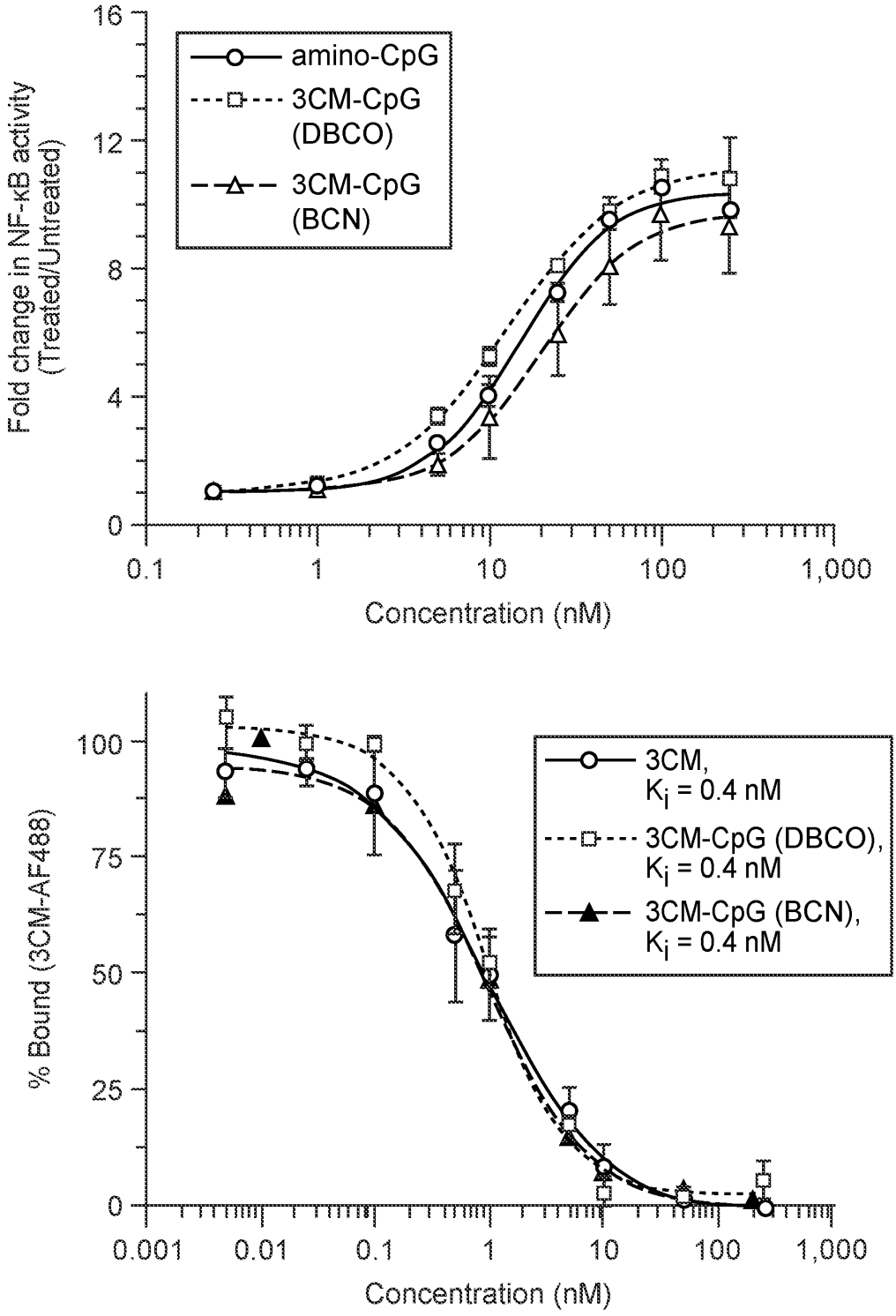
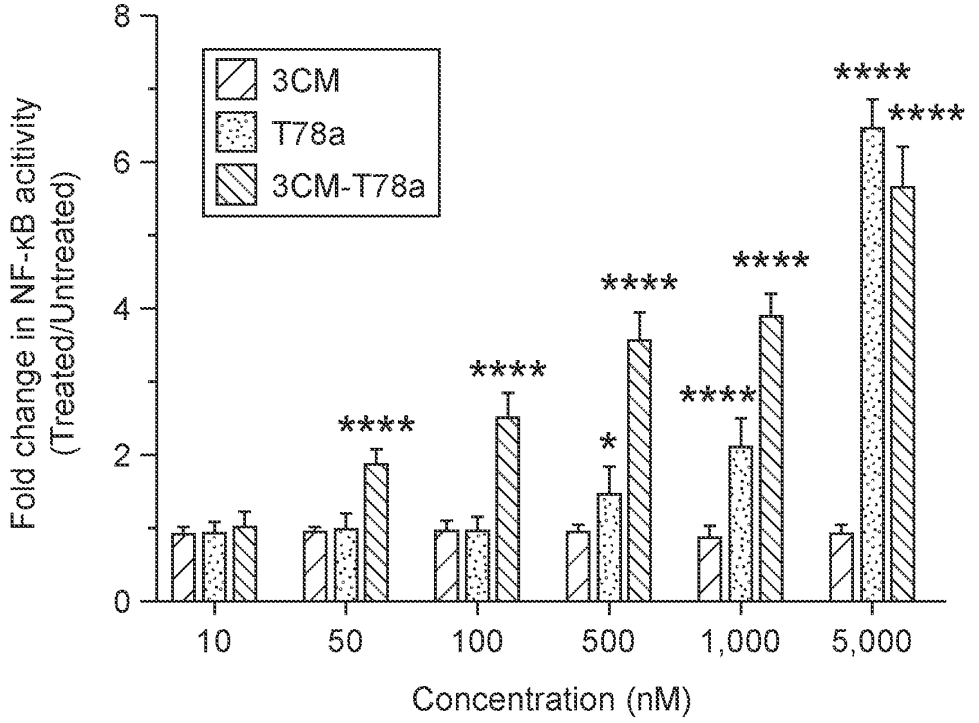

Fold change in NF-κB activity (Treated/Untreated)
16
12
8
4
0
0.1
1
10
100
1,000
Concentration (nM)
amino-CpG
3CM-CpG (DBCO)
3CM-CpG (BCN)
% Bound (3CM-AF488)
100
75
50
25
0
0.001
0.01
0.1
1
10
100
1,000
Concentration (nM)
3CM, Ki = 0.4 nM
3CM-CpG (DBCO), Ki = 0.4 nM
3CM-CpG (BCN), Ki = 0.4 nM

FIG. 7


Fold change in NF-κB acitivity
(Treated/Untreated)
3CM
T78a
3CM-T78a
****
*
10
50
100
500
1,000
5,000
Concentration (nM)

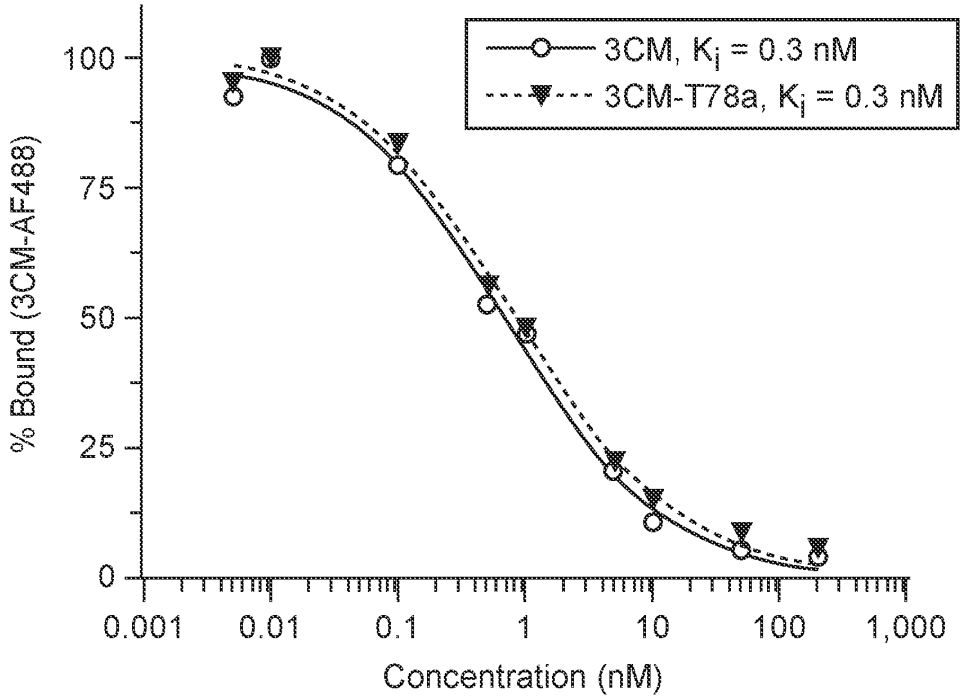

% Bound (3CM-AF488)
3CM, Ki = 0.3 nM
3CM-T78a, Ki = 0.3 nM
0.001
0.01
0.1
1
10
100
1,000
Concentration (nM)

FIG. 8

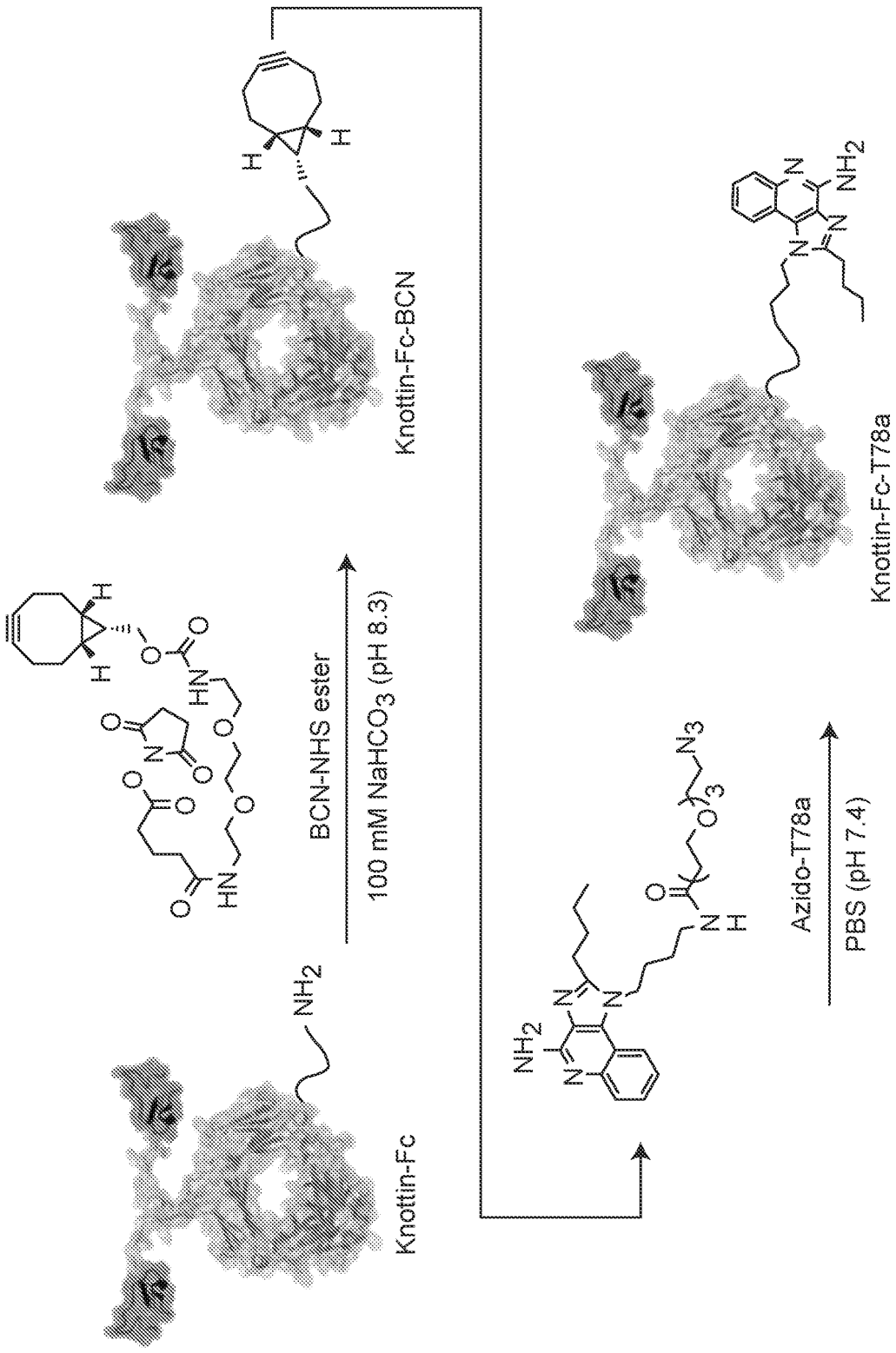

Knottin-Fc
BCN-NHS ester
100 mM NaHCO3 (pH 8.3)
Knottin-Fc-BCN
Azido-T78a
PBS (pH 7.4)
Knottin-Fc-T78a

FIG. 8 (Cont.)

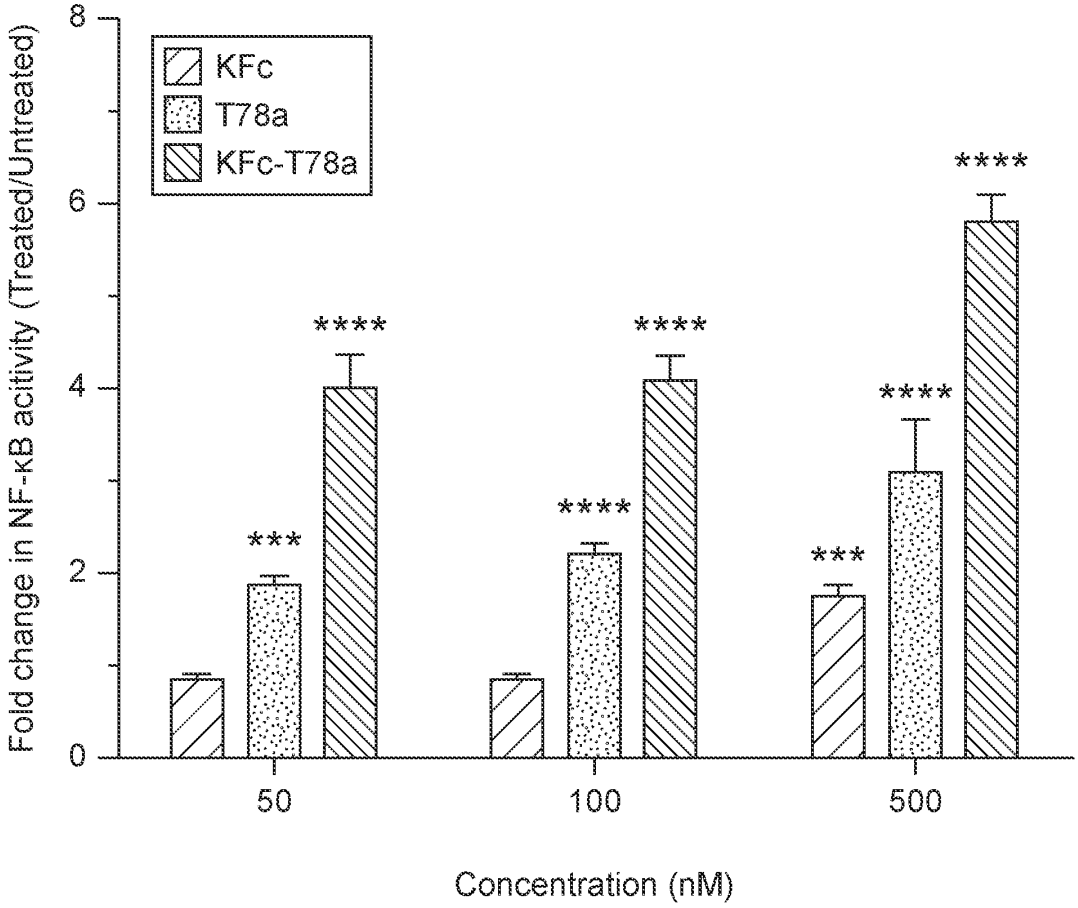

KFc
T78a
KFc-T78a
Fold change in NF-κB acitivity (Treated/Untreated)
0
2
4
6
8
***
****
****
****
***
****
****
50
100
500
Concentration (nM)

FIG. 9

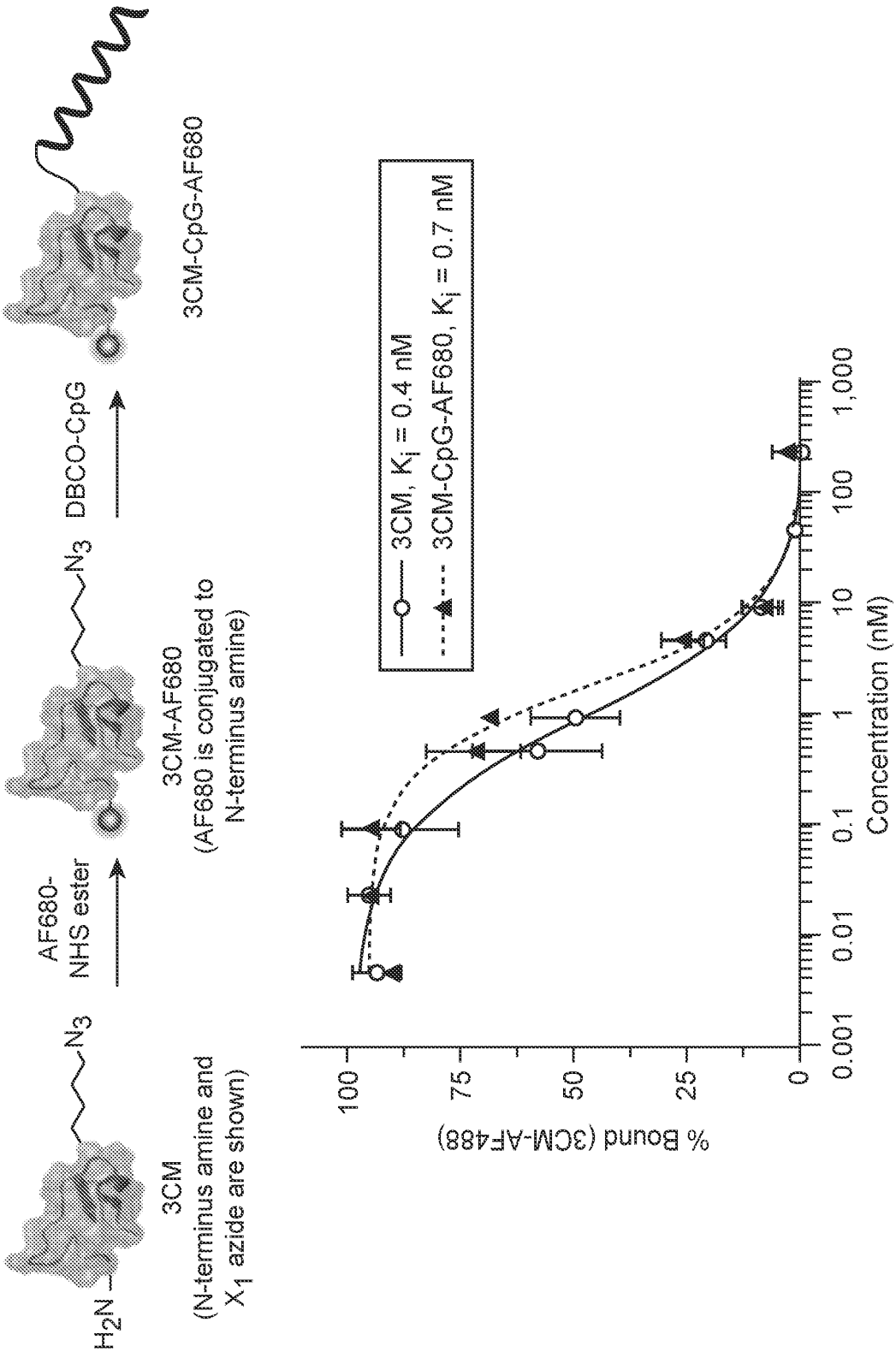

3CM
(N-terminus amine and X1 azide are shown)
AF680-NHS ester
3CM-AF680
(AF680 is conjugated to N-terminus amine)
DBCO-CpG
3CM-CpG-AF680
3CM, Ki = 0.4 nM
3CM-CpG-AF680, Ki = 0.7 nM
% Bound (3CM-AF488)
Concentration (nM)

FIG. 10

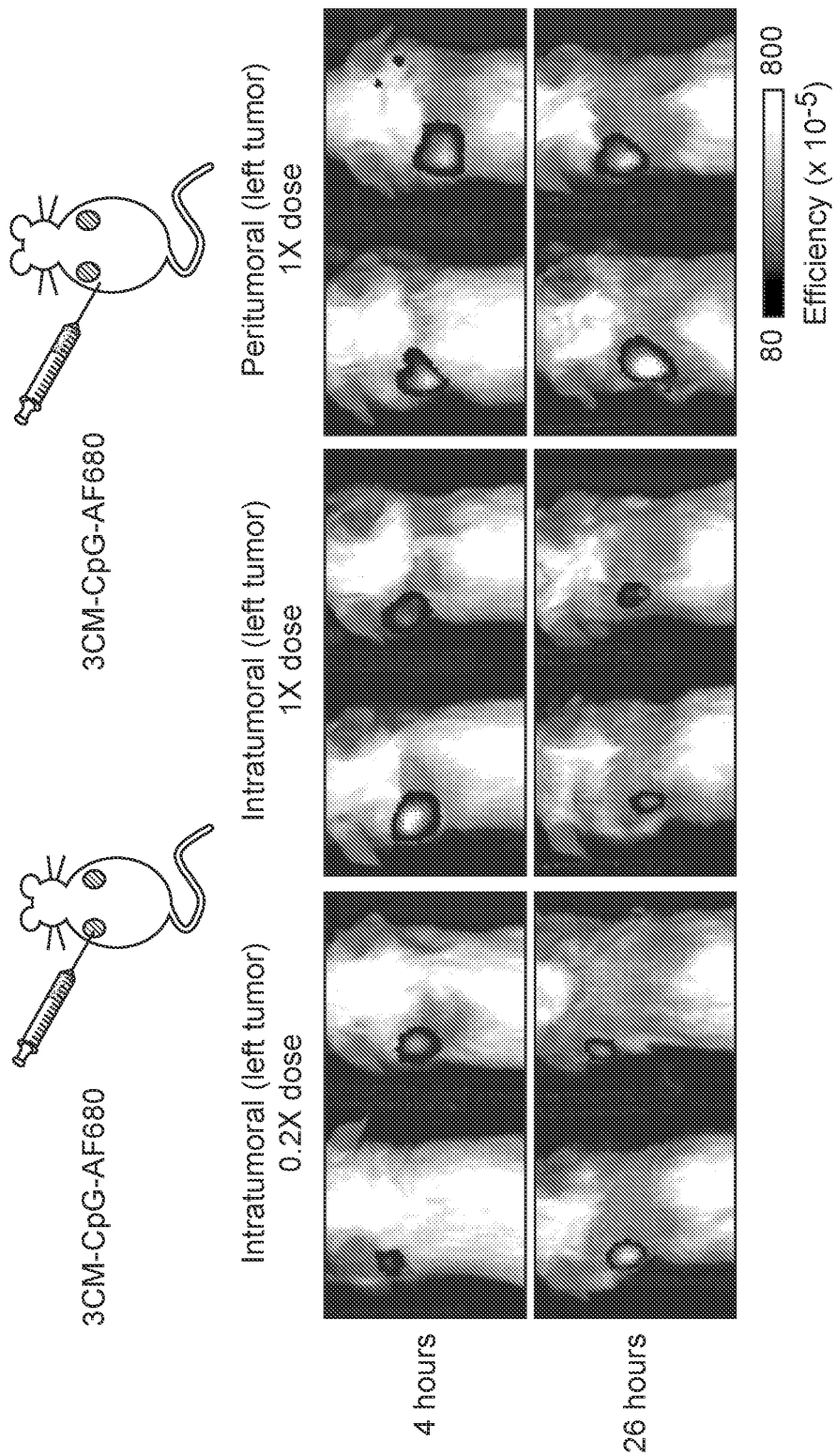

3CM-CpG-AF680
3CM-CpG-AF680
Intratumoral (left tumor)
0.2X dose
Intratumoral (left tumor)
1X dose
Peritumoral (left tumor)
1X dose
4 hours
26 hours
80
800
Efficiency (x 10-5)

FIG. 11

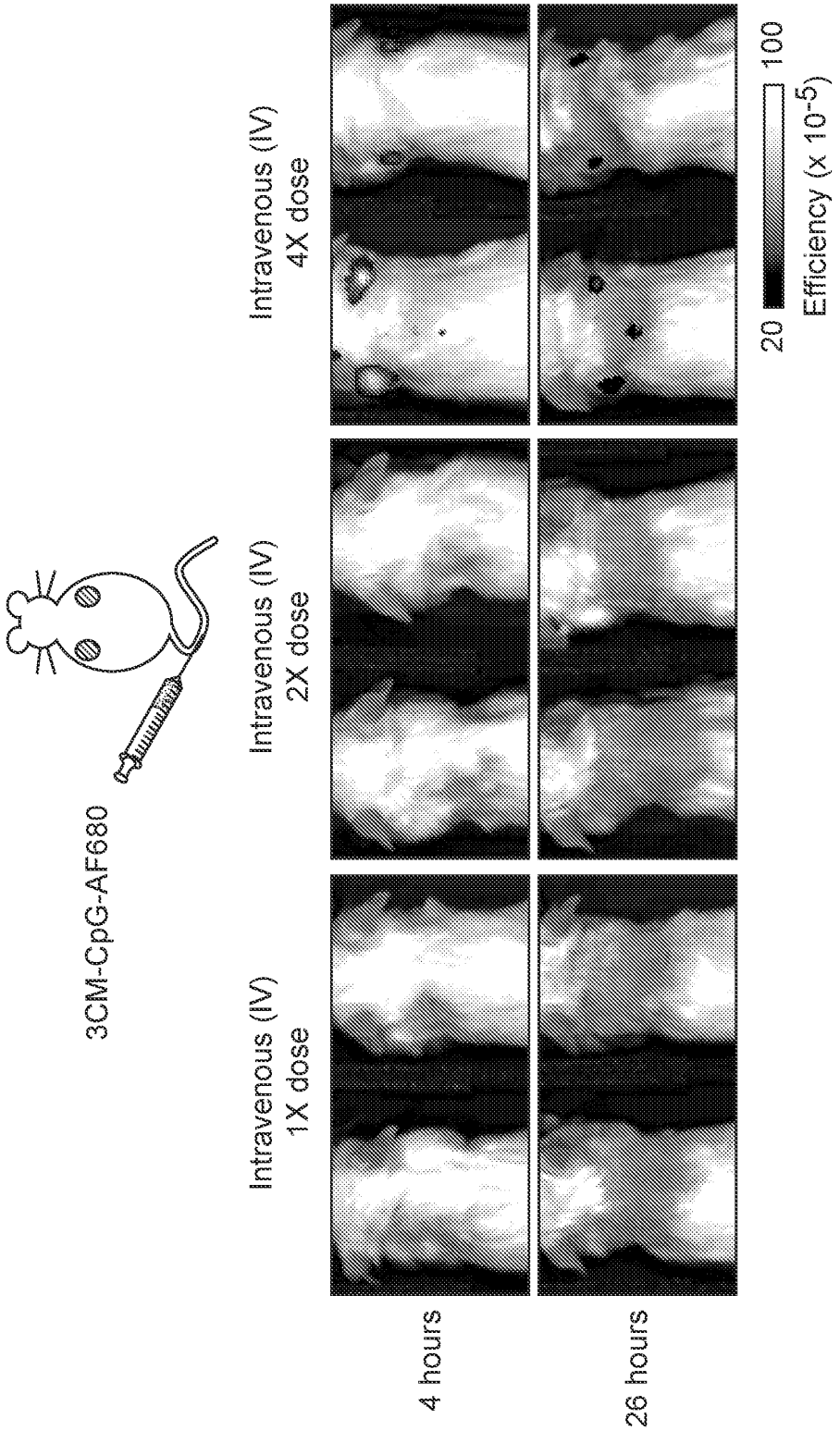

3CM-CpG-AF680
Intravenous (IV)
1X dose
Intravenous (IV)
2X dose
Intravenous (IV)
4X dose
4 hours
26 hours
20
100
Efficiency (x 10-5)

FIG. 12

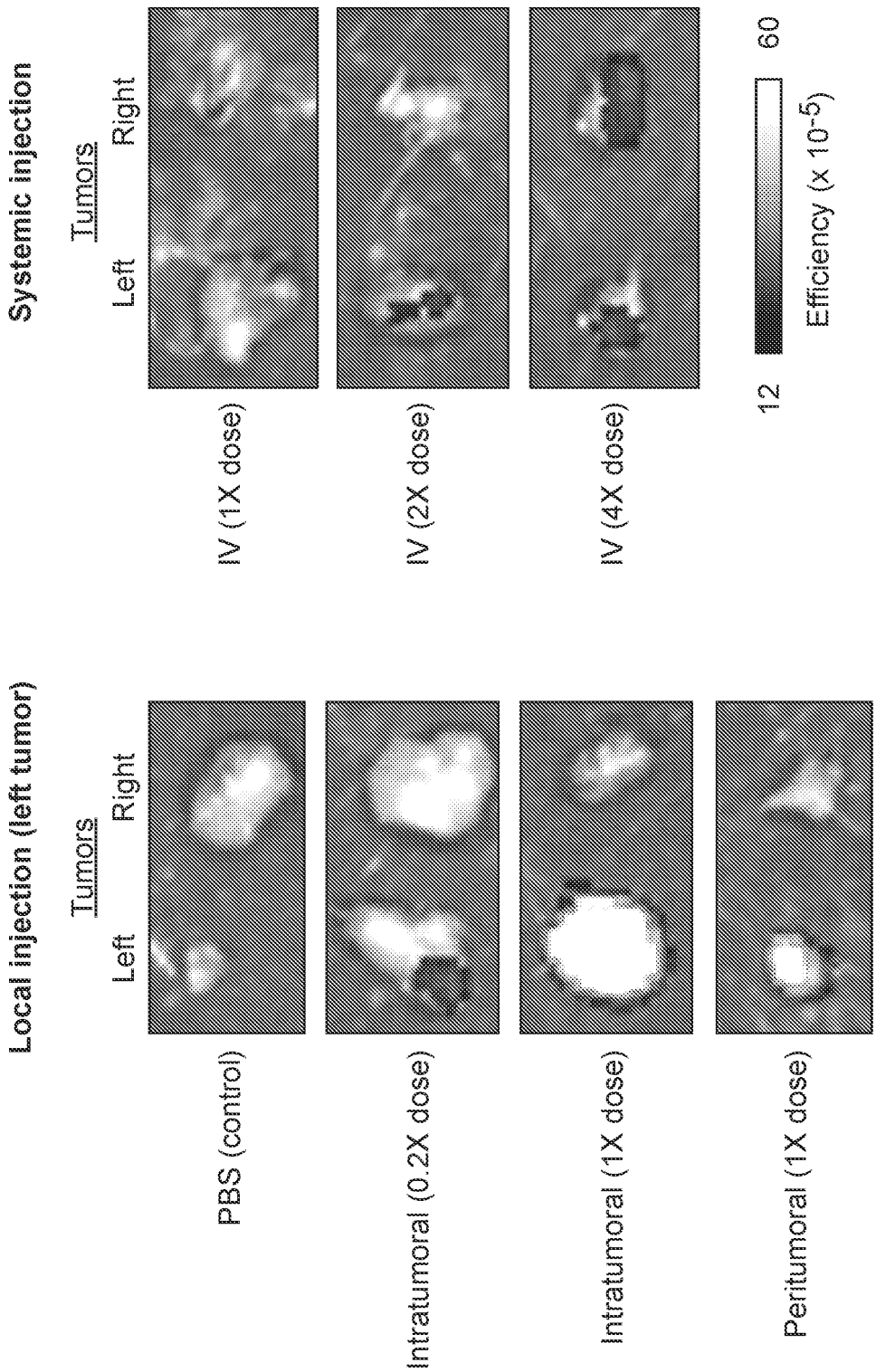

Systemic injection
Tumors
Left
Right
IV (1X dose)
IV (2X dose)
IV (4X dose)
12
60
Efficiency (x 10-5)
Local injection (left tumor)
Tumors
Left
Right
PBS (control)
Intratumoral (0.2X dose)
Intratumoral (1X dose)
Peritumoral (1X dose)

FIG. 13

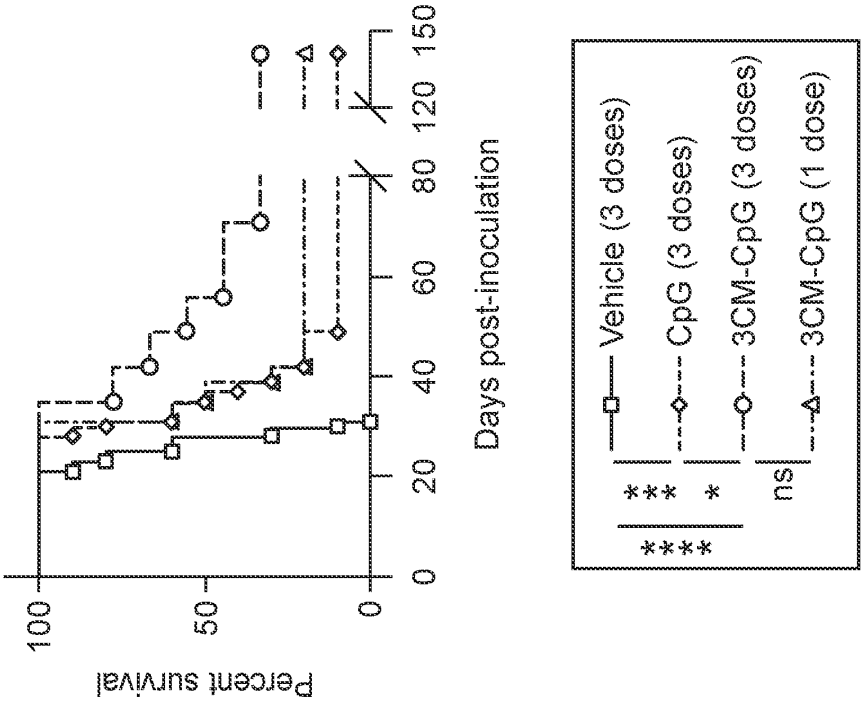

Percent survival
100
50
0
0
20
40
60
80
120
150
Days post-inoculation
Vehicle (3 doses)
CpG (3 doses)
3CM-CpG (3 doses)
3CM-CpG (1 dose)
***
*
****
ns

FIG. 14

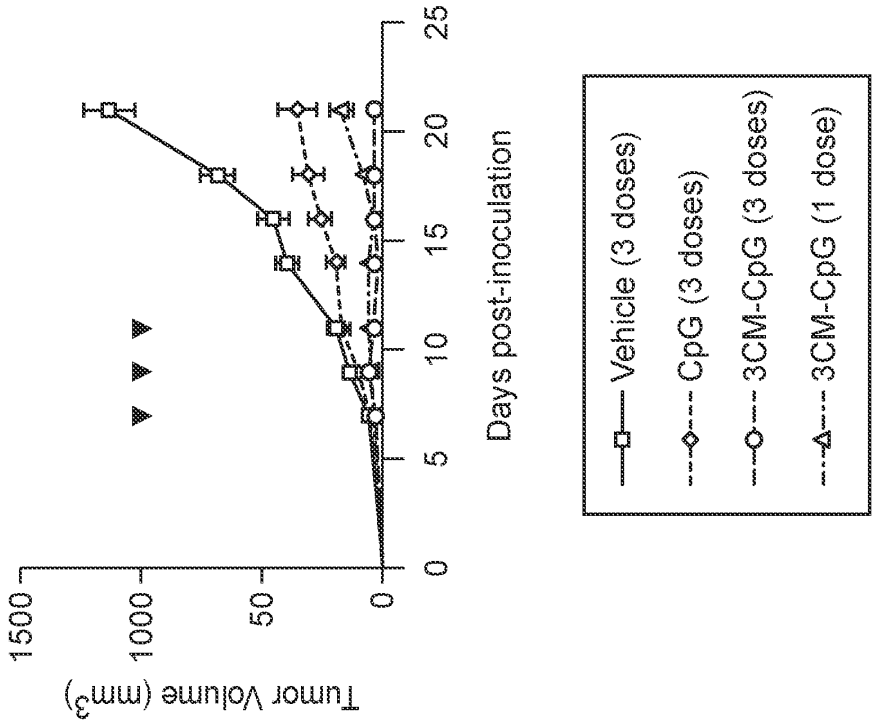

Tumor Volume (mm3)
1500
1000
50
0
0
5
10
15
20
25
Days post-inoculation
Vehicle (3 doses)
CpG (3 doses)
3CM-CpG (3 doses)
3CM-CpG (1 dose)

FIG. 15

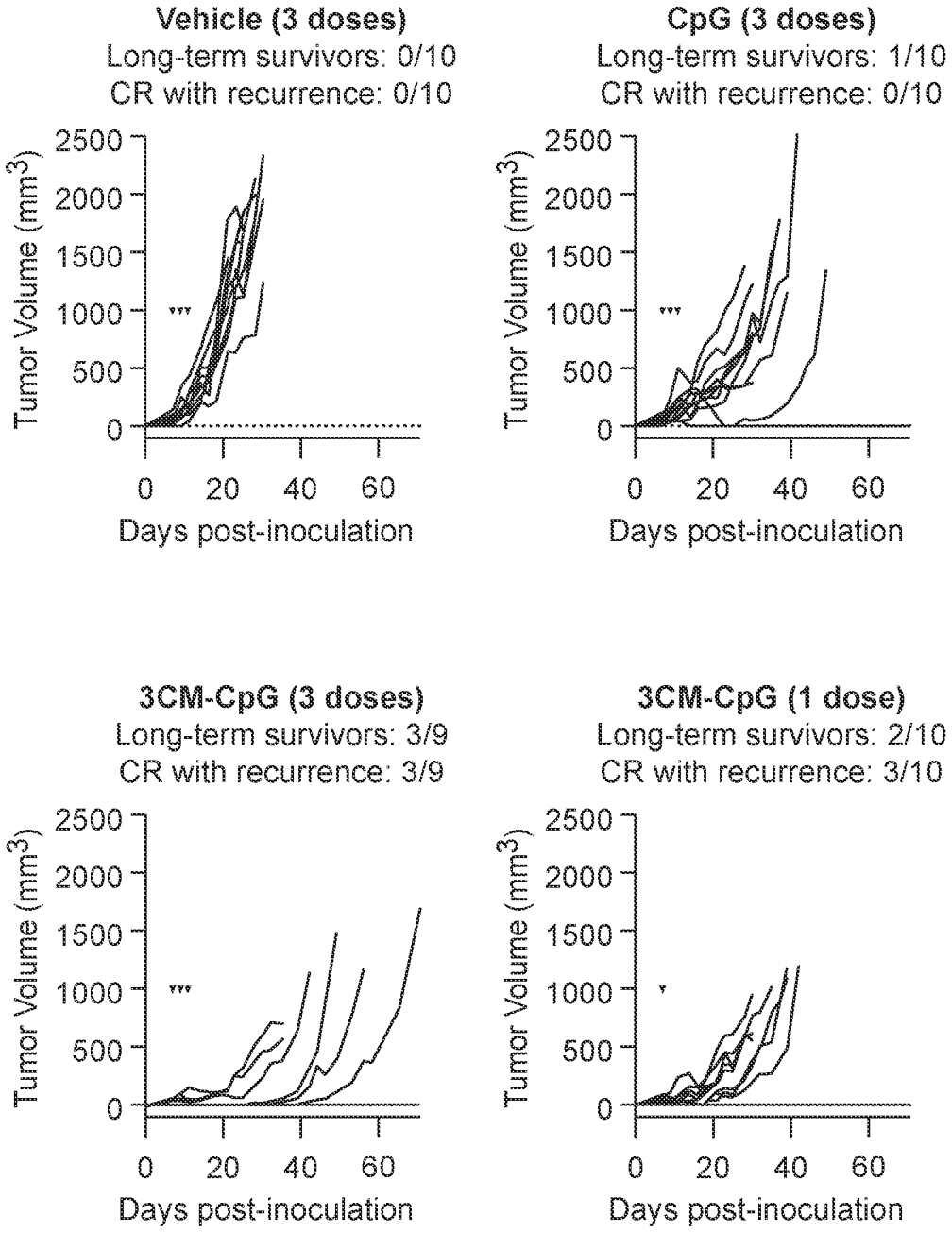

Vehicle (3 doses)
Long-term survivors: 0/10
CR with recurrence: 0/10
CpG (3 doses)
Long-term survivors: 1/10
CR with recurrence: 0/10
3CM-CpG (3 doses)
Long-term survivors: 3/9
CR with recurrence: 3/9
3CM-CpG (1 dose)
Long-term survivors: 2/10
CR with recurrence: 3/10
Tumor Volume (mm3)
2500
2000
1500
1000
500
0
0
20
40
60
Days post-inoculation
***
*

FIG. 16

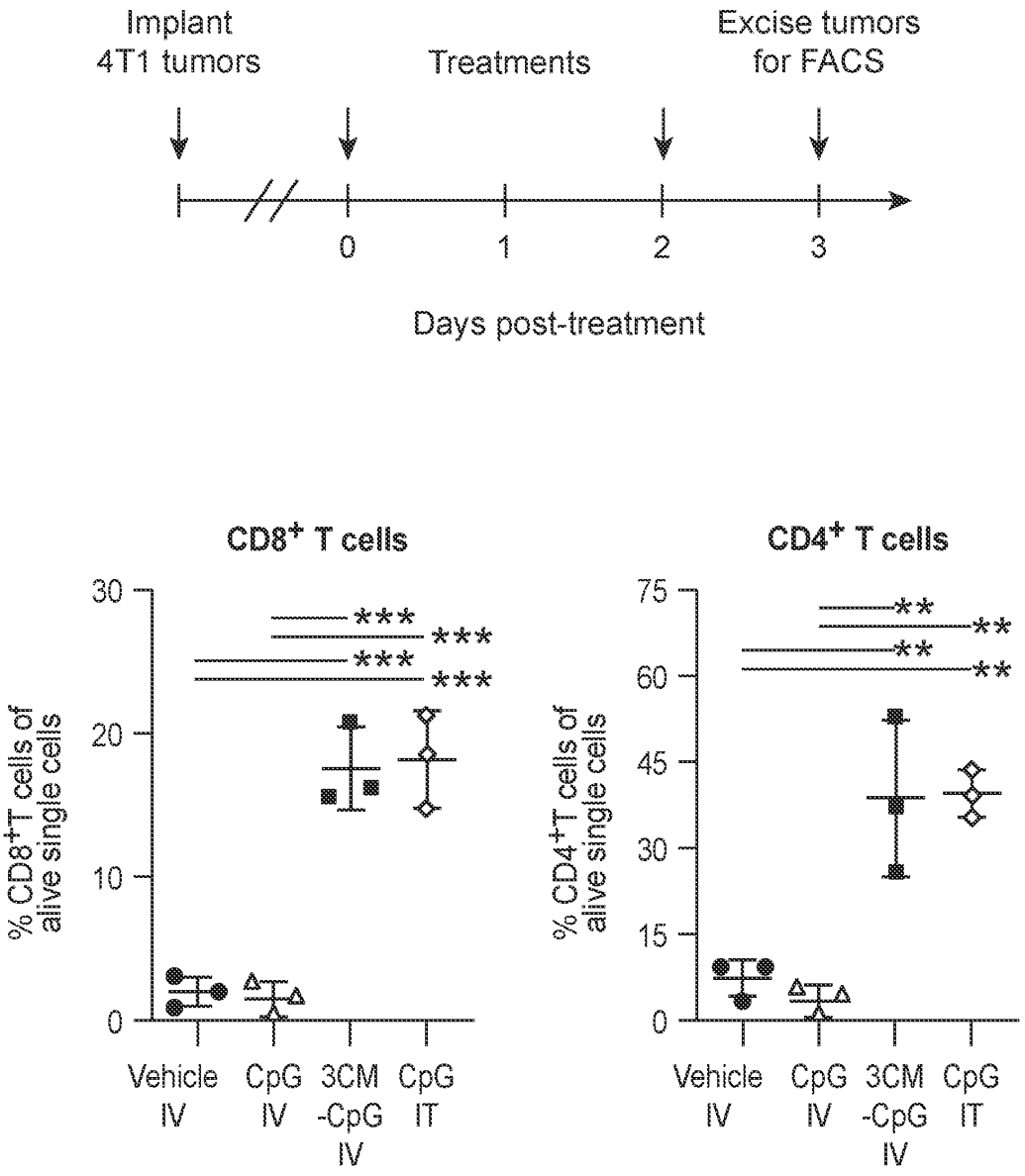

Implant
4T1 tumors
Treatments
Excise tumors
for FACS
0
1
2
3
Days post-treatment
CD8+ T cells
% CD8+T cells of alive single cells
30
20
10
0
***
***
***
***
Vehicle IV
CpG IV
3CM -CpG IV
CpG IT
CD4+ T cells
% CD4+T cells of alive single cells
75
60
45
30
15
0
**
**
**
**
Vehicle IV
CpG IV
3CM -CpG IV
CpG IT

FIG. 16 (Cont.)

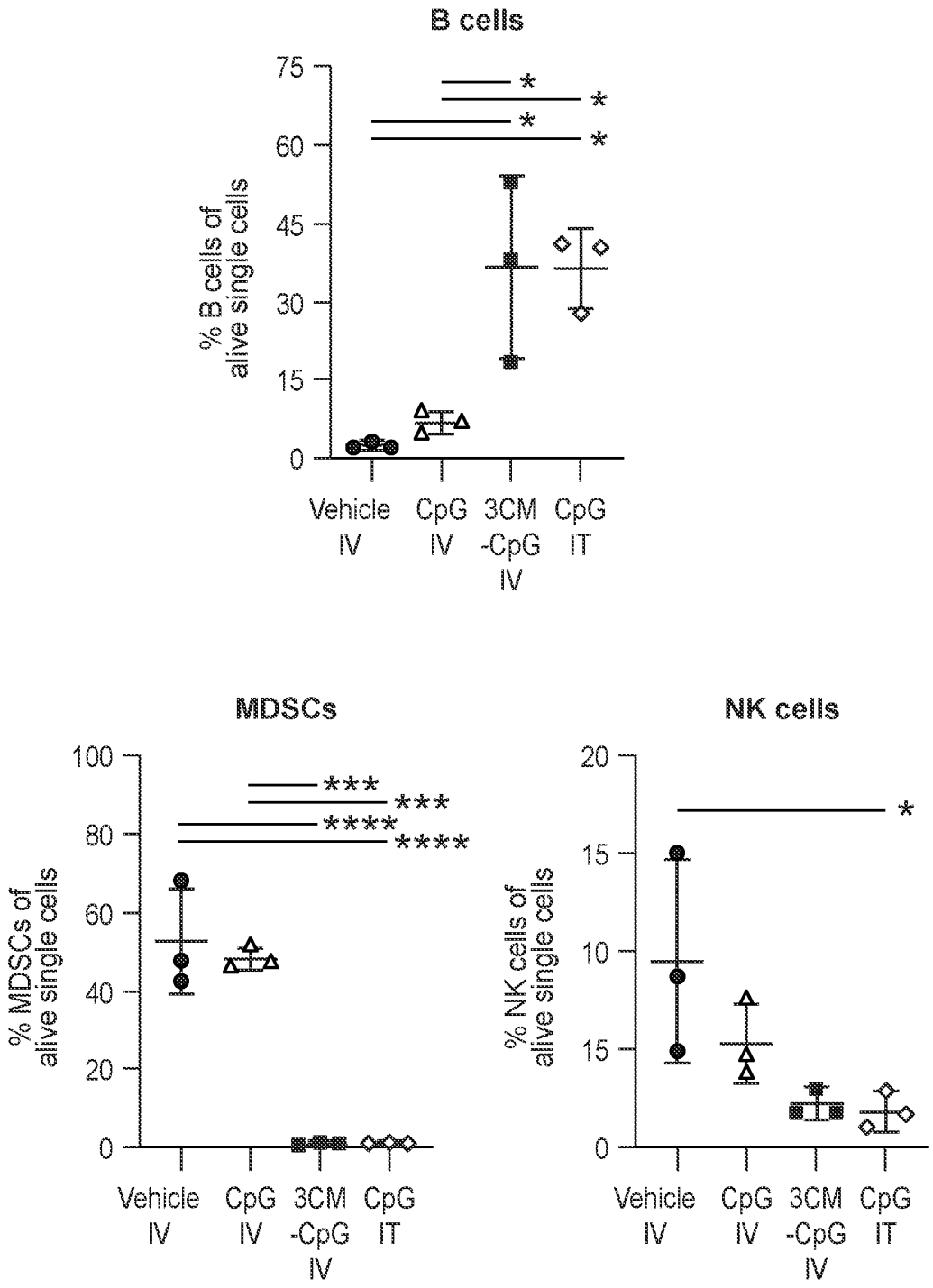
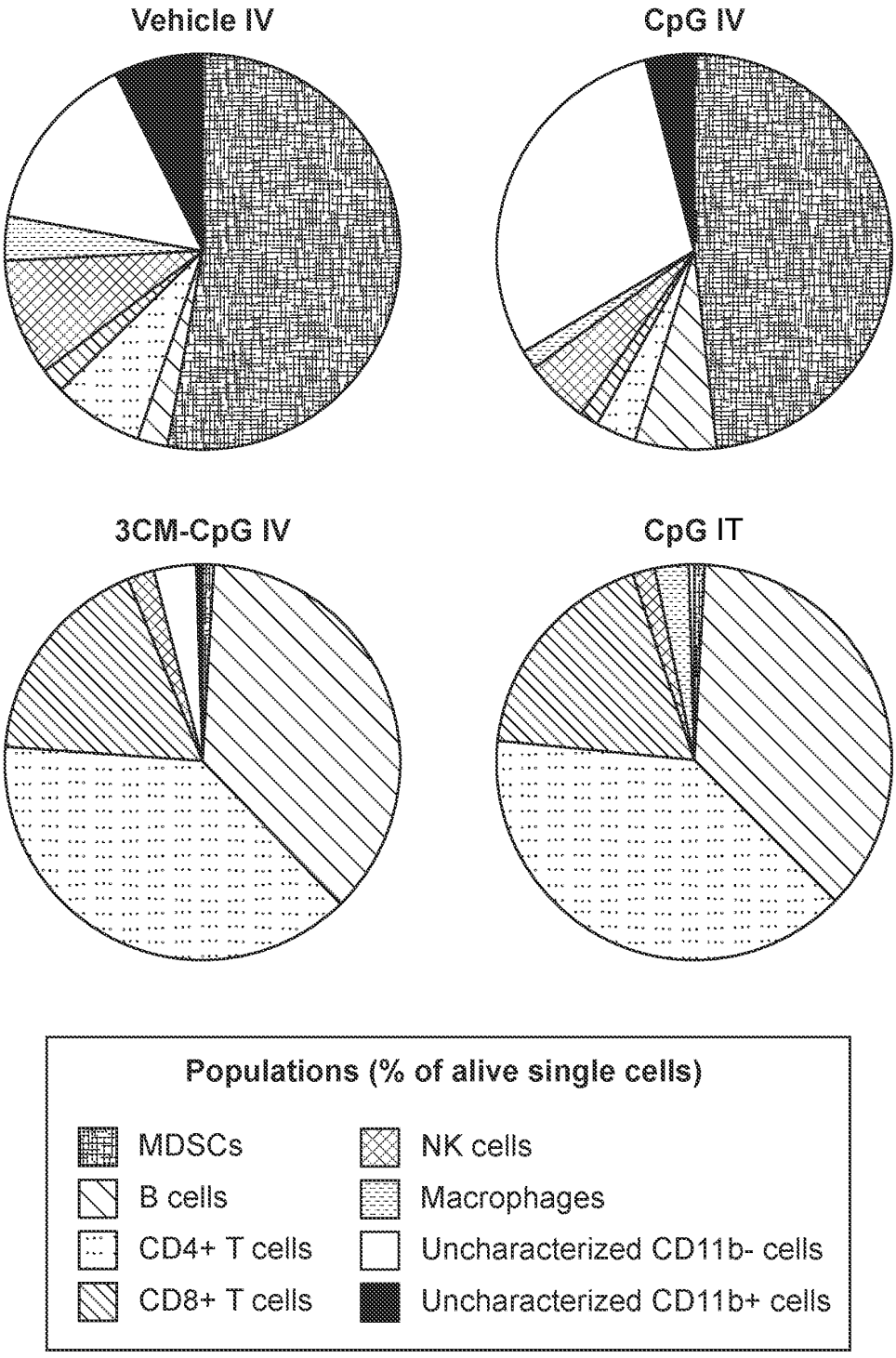

B cells
% B cells of alive single cells
75
60
45
30
15
0
*
*
*
*
Vehicle IV
CpG IV
3CM -CpG IV
CpG IT
MDSCs
% MDSCs of alive single cells
100
80
60
40
20
0
***
***
****
****
Vehicle IV
CpG IV
3CM -CpG IV
CpG IT
NK cells
% NK cells of alive single cells
20
15
10
15
0
*
Vehicle IV
CpG IV
3CM -CpG IV
CpG IT

FIG. 17


Vehicle IV
CpG IV
3CM-CpG IV
CpG IT
Populations (% of alive single cells)
MDSCs
B cells
CD4+ T cells
CD8+ T cells
NK cells
Macrophages
Uncharacterized CD11b- cells
Uncharacterized CD11b+ cells