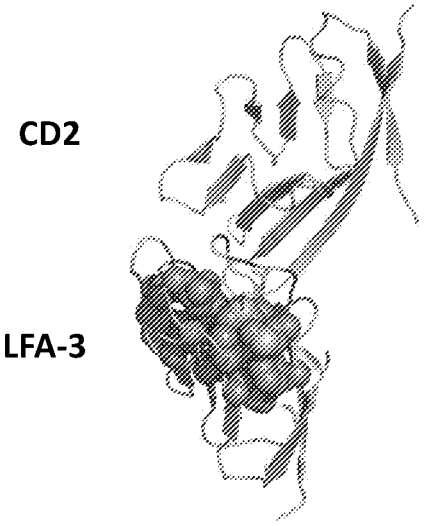
FIG. 1

S library (stability)



AS library (affinity + stability)



Contact Library		Total theoretical diversity = 2.4×10^{10}			
Residue	Position	Mutagenesis	Codon	Diversity	
Glu	25	D, E, N, K, Q, H	VAK	3x1x2	6
Leu	27	F, I, L, V	NTT	4x1x1	4
Lys	29	K, R, M, T	ANG	1X4X1	4
Lys	32	K, R, M, T	ANG	1X4X1	4
Asp	33	D, E, N, K, Q, H	VAM	3x1x2	6
Lys	34	K, R, M, T	ANG	1X4X1	4
Glu	37	D, E, N, K, Q, H	VAK	3x1x2	6
Glu	39	D, E, N, K, Q, H	VAM	3x1x2	6
Glu	42	D, E, N, K, Q, H	VAK	3x1x2	6
Arg	44	K, R, M, T	ANG	1X4X1	4
Phe	46	F, Y, L, H, I, N, V, D	NWT	4x2x1	8
Ser	47	S, T, A, G	RST	2x2x1	4
Pro	80	P, L, H, R	CNT	1x4x1	4
Ile	82	F, I, L, V	NTT	4x1x1	4
Asp	84	D, E, N, K, Q, H	VAM	3x1x2	6

Core Library		Total theoretical diversity = 4.3×10^{9}			
Residue	Position	Mutagenesis	Codon	Diversity	
Phe	15	F, I, L, V	NTT	4x1x1	4
Val	17	F, I, L, V	NTT	4x1x1	4
Leu	23	F, I, L, V	NTT	4x1x1	4
Val	26	F, I, L, V	NTT	4x1x1	4
Trp	28	W, F, L, C	TKS	1x2x2	4
Val	35	F, I, L, V	NTT	4x1x1	4
Ala	36	A, V, S, L	KYA	2x2x1	4
Leu	38	F, I, L, V	NTT	4x1x1	4
Phe	43	F, I, L, V	NTT	4x1x1	4
Ala	45	A, V, S, L	KYA	2x2x1	4
Leu	55	F, I, L, V	NTT	4x1x1	4
Gly	60	S, T, A, G	RST	2x2x1	4
Leu	62	F, I, L, V	NTT	4x1x1	4
Met	77	M, L, I, F	WTS	2x1x2	4
Met	86	M, L, I, F	WTS	2x1x2	4
Phe	88	F, I, L, V	NTT	4x1x1	4

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FIG. 2A

Hotspot residues

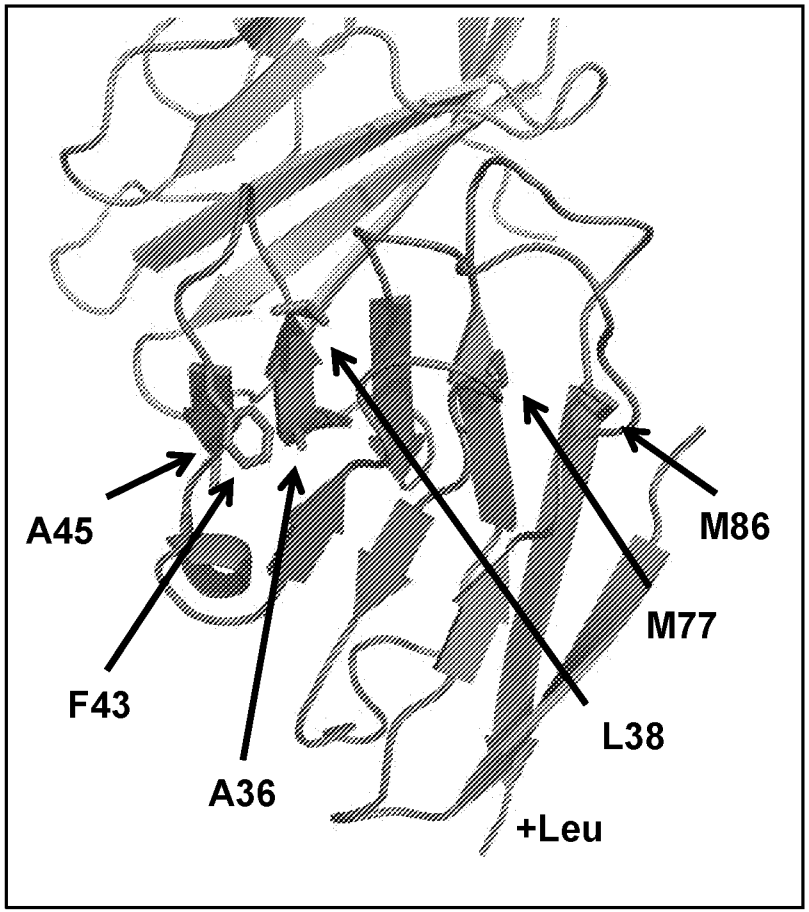

FIG. 2B

LFA3 residues targeted in libraries

FSQQIYGVVYGNVT**F**H**V**PSNVP**L**KE**V**L**W**KKQKDK**V**
AE**L**ENSE**F**R**A**FSSFKNRVY**L**DTVS**G**S**L**TIYNLTS
DEDEYE**M**ESPNITDT**M**K**F**FLYV (SEQ ID NO: 3)

FIG. 2C

Frequency of amino acid variation in hotspot positi

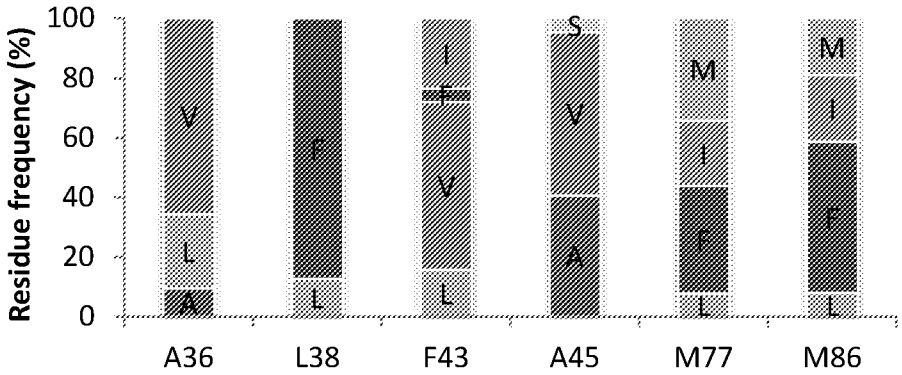

FIG. 2D

Recombinant variants

	36	38	43	45	77	86
M1	V	F	V	A	M	F
M2	V	F	V	A	F	F
M3	V	F	V	V	M	F
M4	V	F	V	V	F	F
M5	V	F	I	V	F	F
M6	L	F	L	V	F	F

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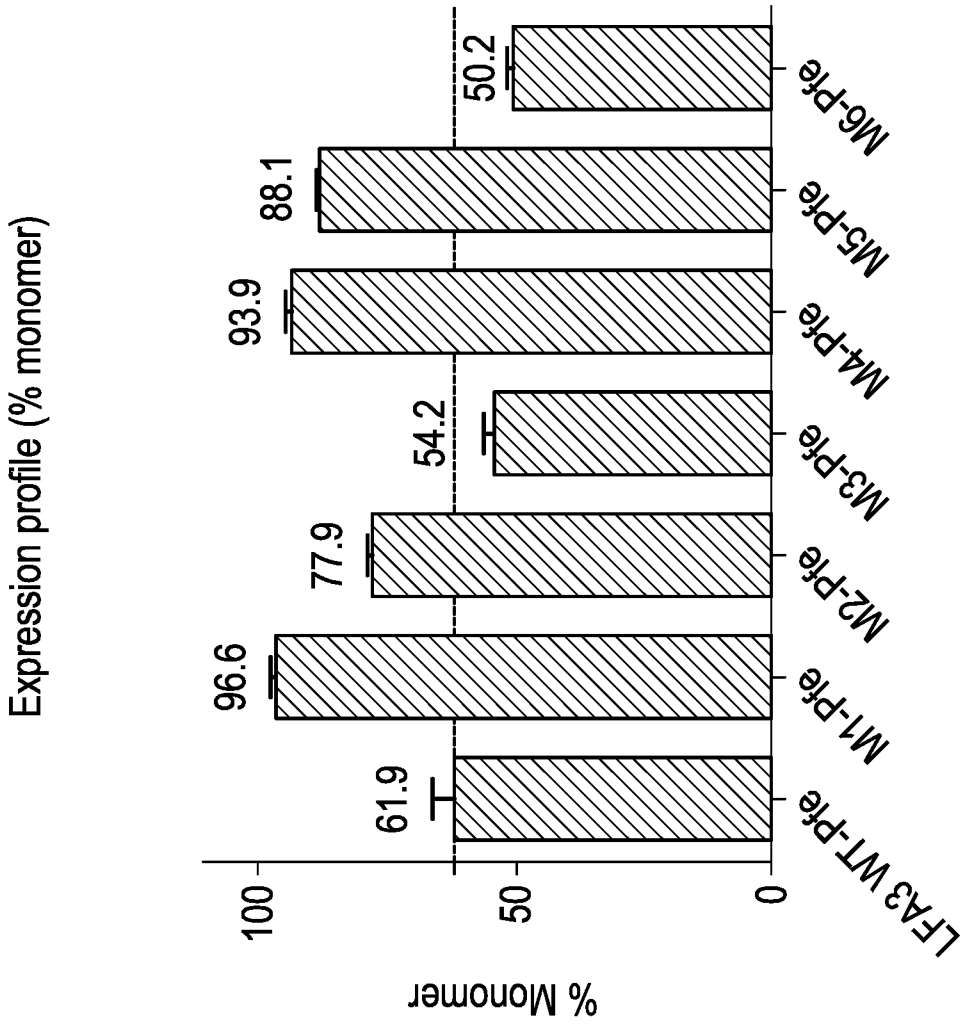
Expression profile (% monomer)
% Monomer
100
50
0
61.9
96.6
77.9
54.2
93.9
88.1
50.2
LFA3 WT-Fle
M1-Fle
M2-Fle
M3-Fle
M4-Fle
M5-Fle
M6-Fle

FIG. 3A

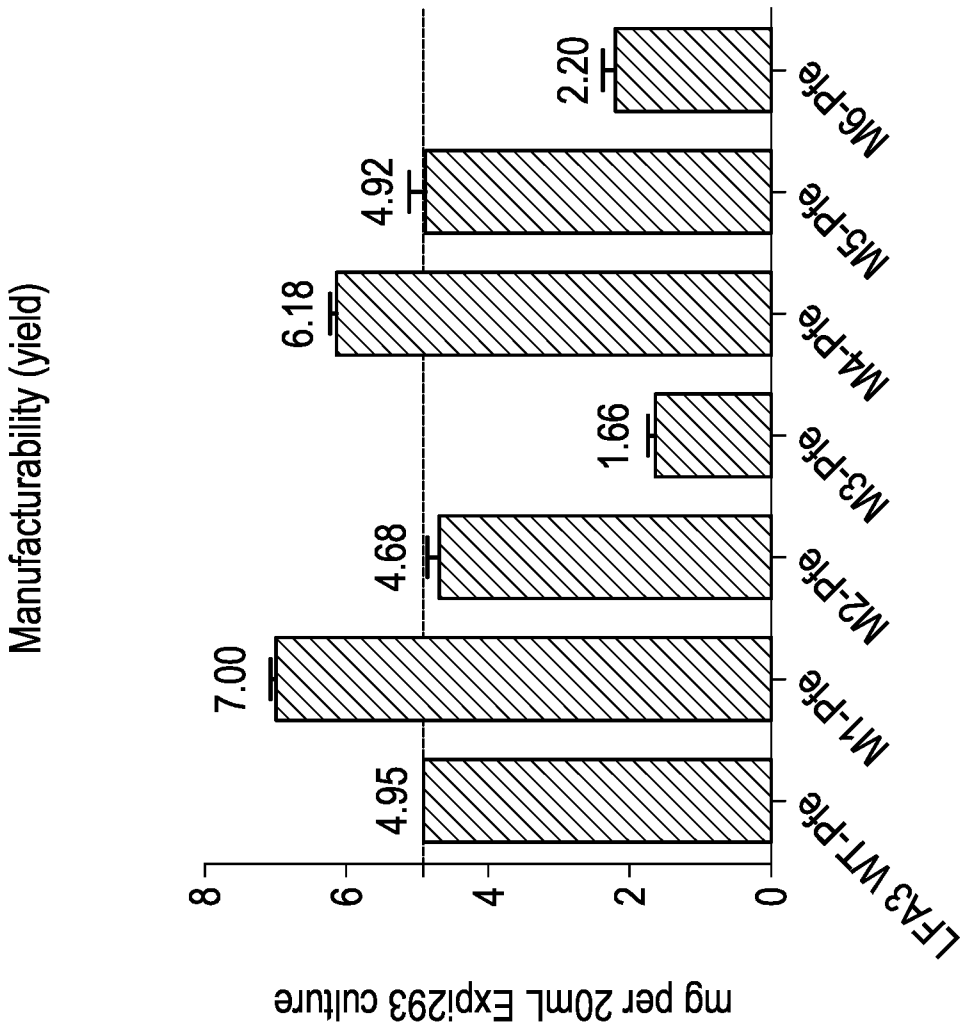
Manufacturability (yield)
mg per 20mL Expi293 culture
8
6
4
2
0
4.95
7.00
4.68
1.66
6.18
4.92
2.20
LFA3 WT-Pfe
M1-Pfe
M2-Pfe
M3-Pfe
M4-Pfe
M5-Pfe
M6-Pfe

FIG. 3B

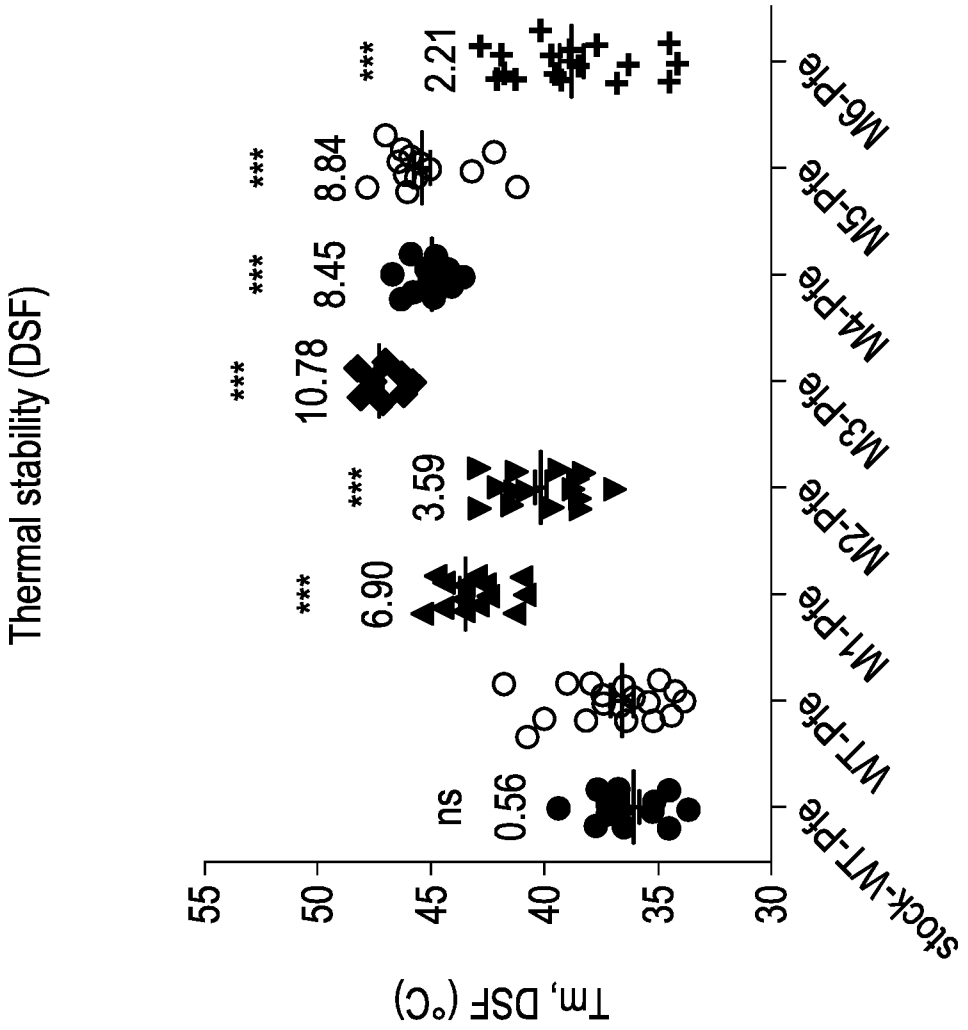
Thermal stability (DSF)
Tm, DSF (°C)
55
50
45
40
35
30
stock-WT-Pfe
WT-Pfe
M1-Pfe
M2-Pfe
M3-Pfe
M4-Pfe
M5-Pfe
M6-Pfe
ns
0.56
***
6.90
***
3.59
***
10.78
***
8.45
***
8.84
***
2.21

FIG. 3C

FIG. 3D

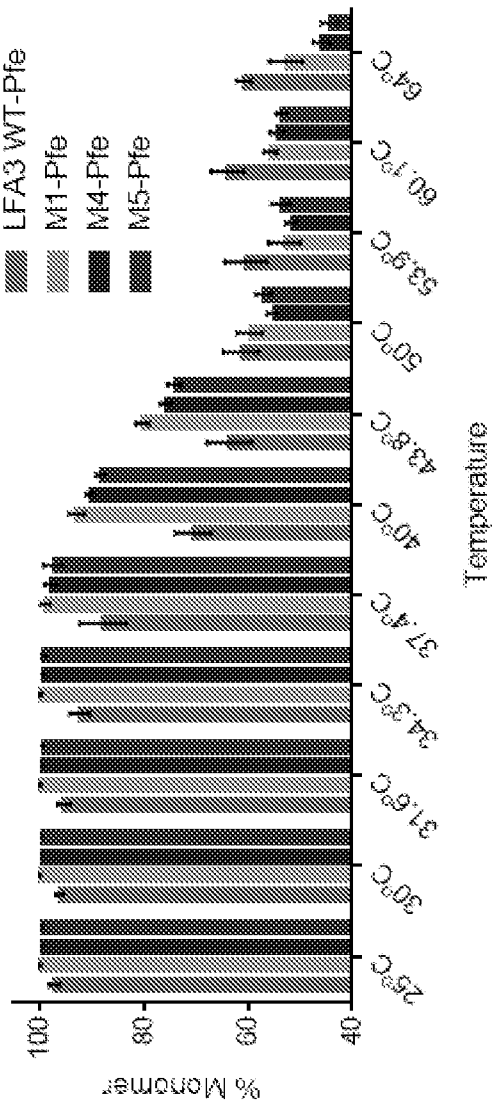
Aggregation Propensity: Heat Forced Aggregation
LFA3 WT-Pfe
M1-Pfe
M4-Pfe
M5-Pfe
% Monomer
100
80
60
40
25°C
30°C
31.6°C
34.3°C
37.4°C
40°C
43.8°C
50°C
53.9°C
60.1°C
64°C
Temperature

FIG. 4

Loop library

Theoretical diversity = 3.4 x10^{10}

Residue	Position	Codon
Ser	79	NNK
Pro	80	NNK
Asn	81	NNK
Ile	82	NNK
Thr	83	NNK
Asp	84	NNK
Ser	85	NNK

- Templates used: WT, M1, M4 and M5
- Library size = 2.9 x 10^{8}

CD2 binding affinity by SPR

Construct	Affinity (μM)
WT LFA3-Fc	1.41
M1d1-Pfe	0.73
M4d1-Pfe	0.75
ML1d1-Pfe	**0.082**
ML3d1-Pfe	**0.052**
CM1d1-Pfe	0.13
CM2d1-Pfe	**0.10**
CM3d1-Pfe	0.088
CM5d1-Pfe	**0.063**
CM6d1-Pfe	0.061

n= 1-3 exp

FIG. 5A

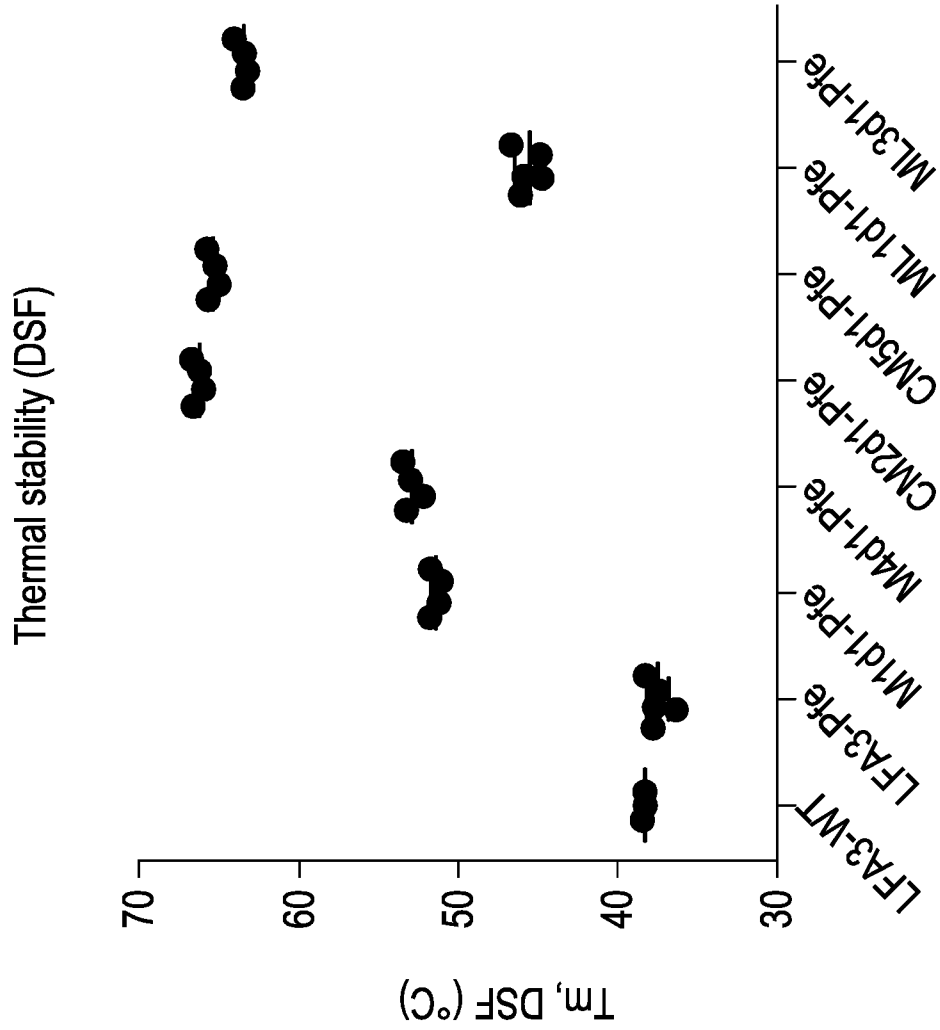
Thermal stability (DSF)
Tm, DSF (°C)
70
60
50
40
30
LFA3-WT
LFA3-Pfe
M1d1-Pfe
M4d1-Pfe
CM2d1-Pfe
CM5d1-Pfe
ML1d1-Pfe
ML3d1-Pfe

FIG. 5B

FIG. 6A

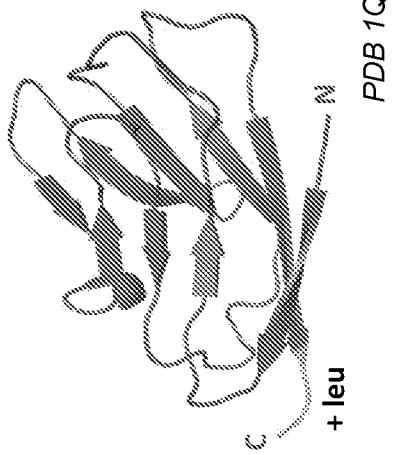

FIG. 6B

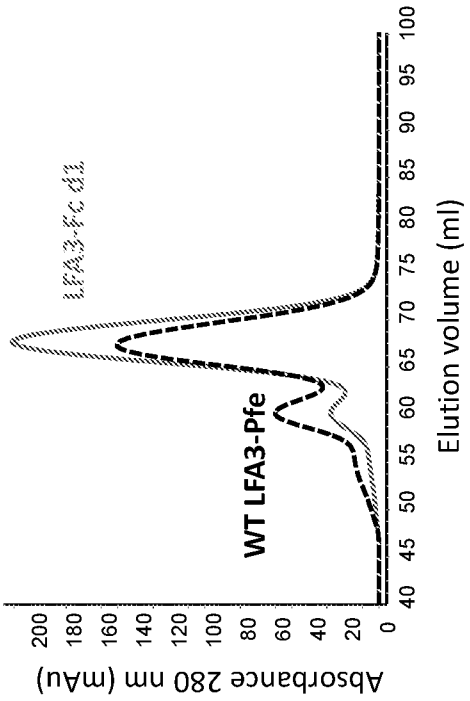

FIG. 6C

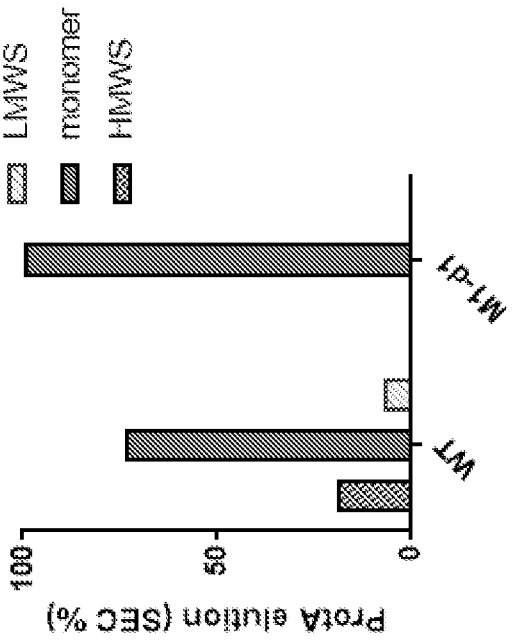

FIG. 6D

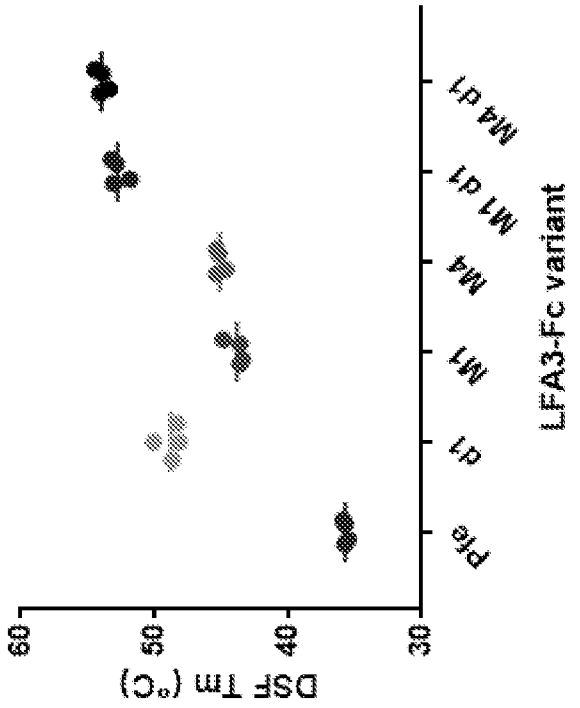

FIG. 7A

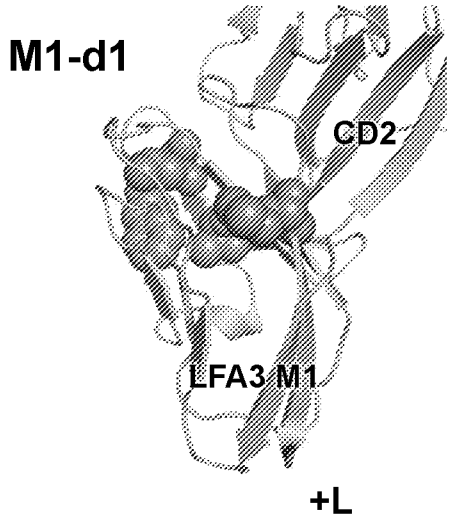

FIG. 7B

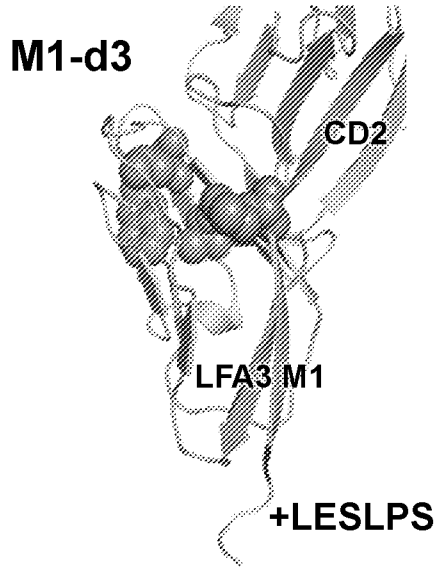

FIG. 7C

Affinity by SPR

construct	human (μM)	cyno (μM)
LFA3-WT	1.47	1.50
M1-d1	1.08	1.06
M1-d3	1.04	1.08

Average of triplicate measurements

FIG. 7D

Thermal stability (DSF)

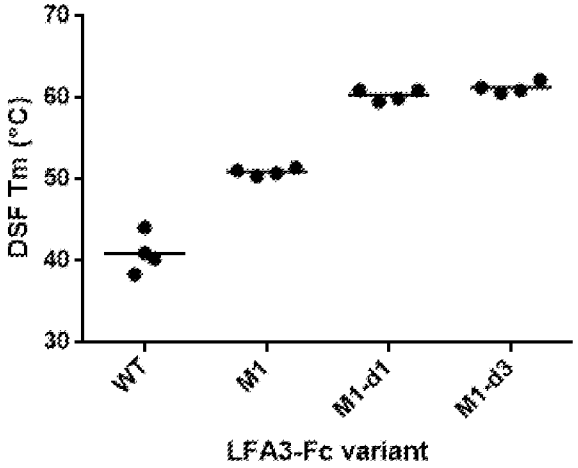

FIG. 8A

Sequence

	36	38	43	45	77	86
WT	**A**	**L**	**F**	**A**	**M**	**M**
M1	V	F	V			F
M4	V	F	V	V	F	F
M7	V	F	V			

FIG. 8B

SEC chromatogram

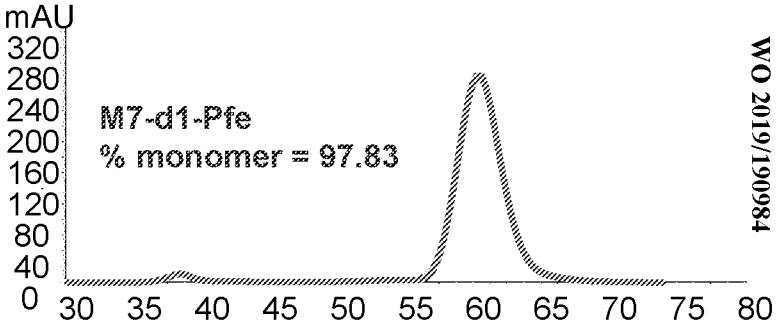

FIG. 8C

Thermal stability by DSF

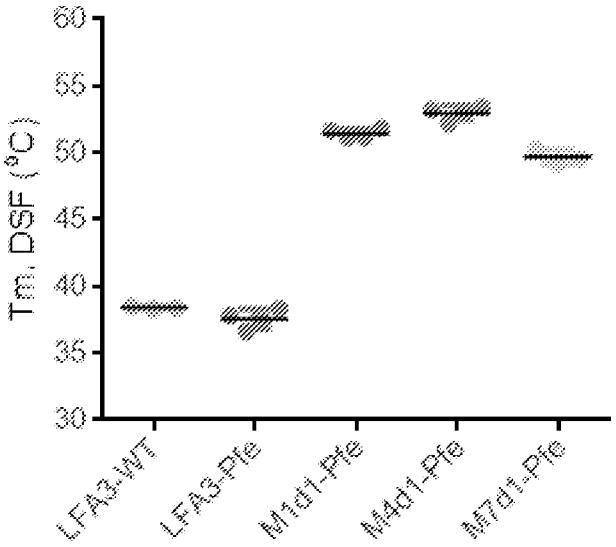

FIG. 8D

Affinity by SPR

Construct	Affinity (μM)
WT	1.41
M1-d1 Pfe	0.73
M4-d1 Pfe	0.75
M7-d1-Pfe	**0.92**

n=4-7

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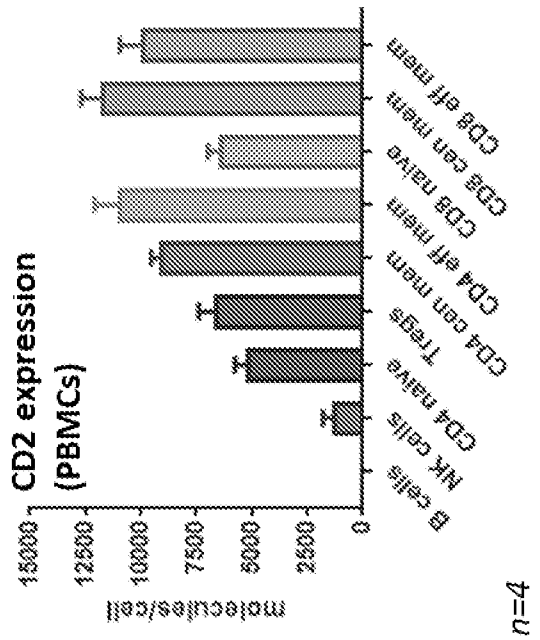
WO 2019/190984 PCT/US2019/023883

FIG. 9


CD2 expression
(PBMCs)
molecules/cell
15000
12500
10000
7500
5000
2500
0
B cells
NK cells
CD4 naive
Tregs
CD4 con mem
CD4 eff mem
CD8 naive
CD8 con mem
CD8 eff mem
n=4

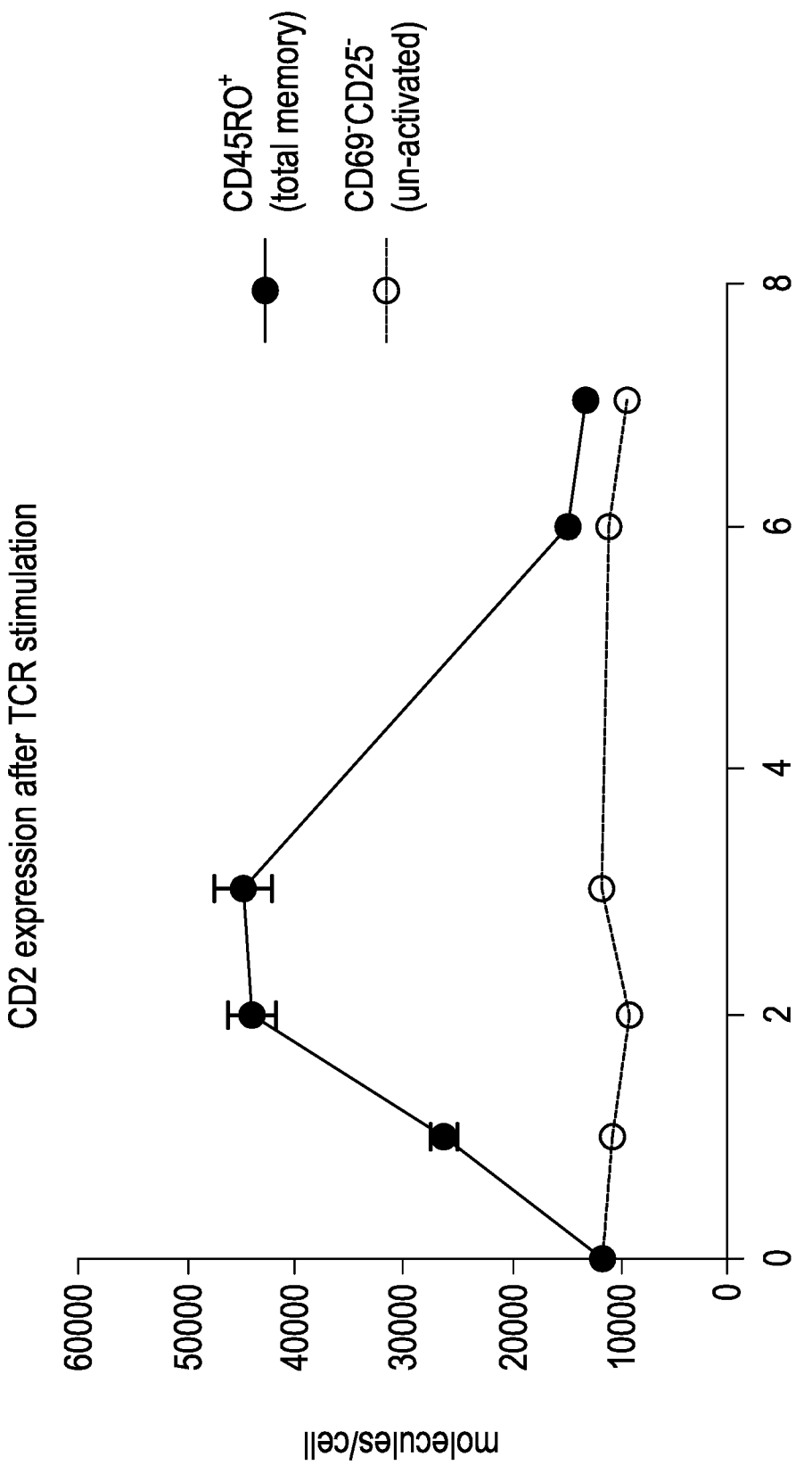

CD2 expression after TCR stimulation
CD45RO+ (total memory)
CD69-CD25- (un-activated)
molecules/cell
60000
50000
40000
30000
20000
10000
0
0
2
4
6
8
Days (αCD3/28)
1 of 4 donors shown

FIG. 10

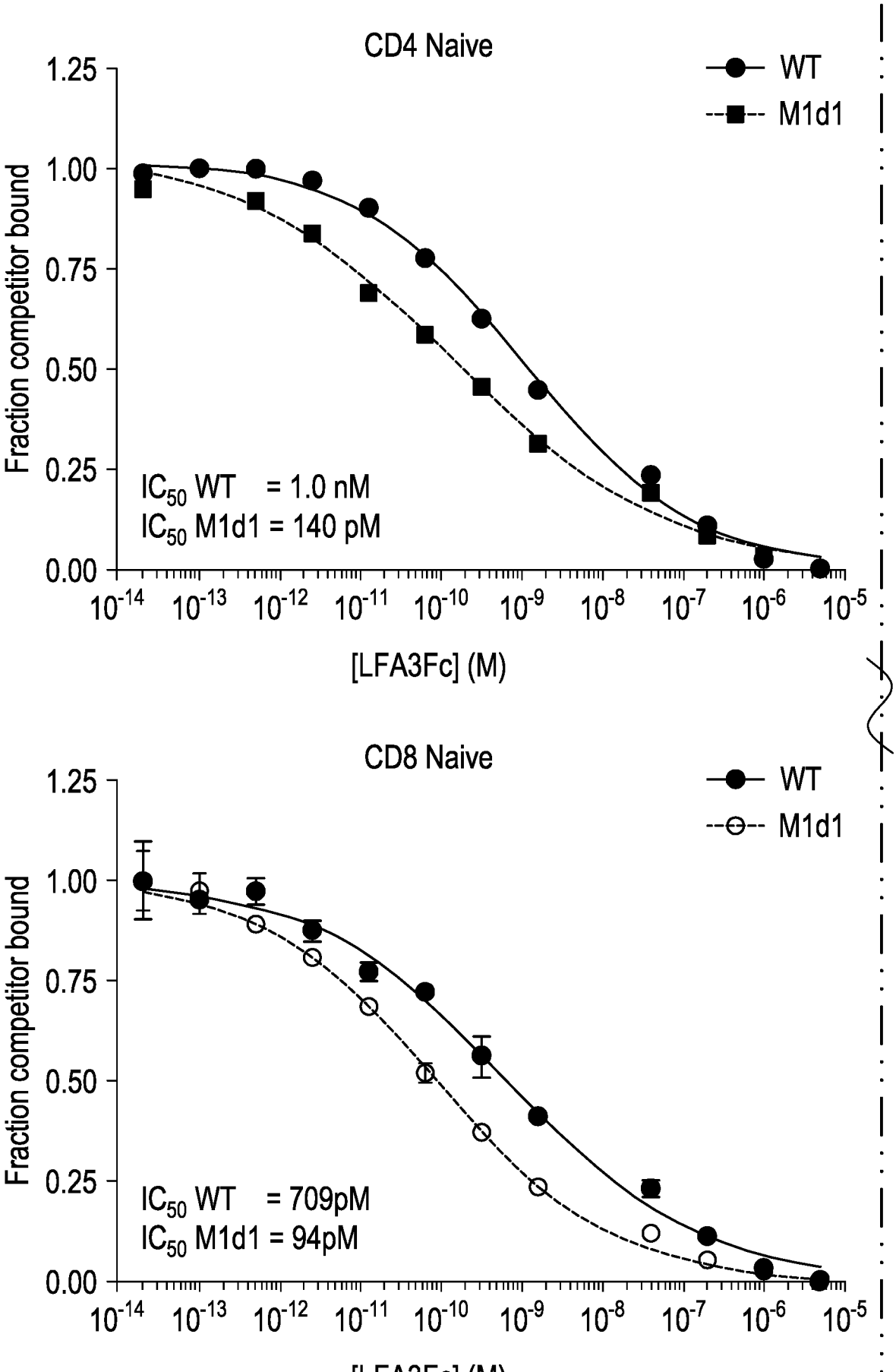

CD4 Naive
WT
M1d1
Fraction competitor bound
1.25
1.00
0.75
0.50
0.25
0.00
IC50 WT = 1.0 nM
IC50 M1d1 = 140 pM
10-14 10-13 10-12 10-11 10-10 10-9 10-8 10-7 10-6 10-5
[LFA3Fc] (M)
CD8 Naive
WT
M1d1
Fraction competitor bound
1.25
1.00
0.75
0.50
0.25
0.00
IC50 WT = 709pM
IC50 M1d1 = 94pM
10-14 10-13 10-12 10-11 10-10 10-9 10-8 10-7 10-6 10-5
[LFA3Fc] (M)

FIG. 11 (part 1)

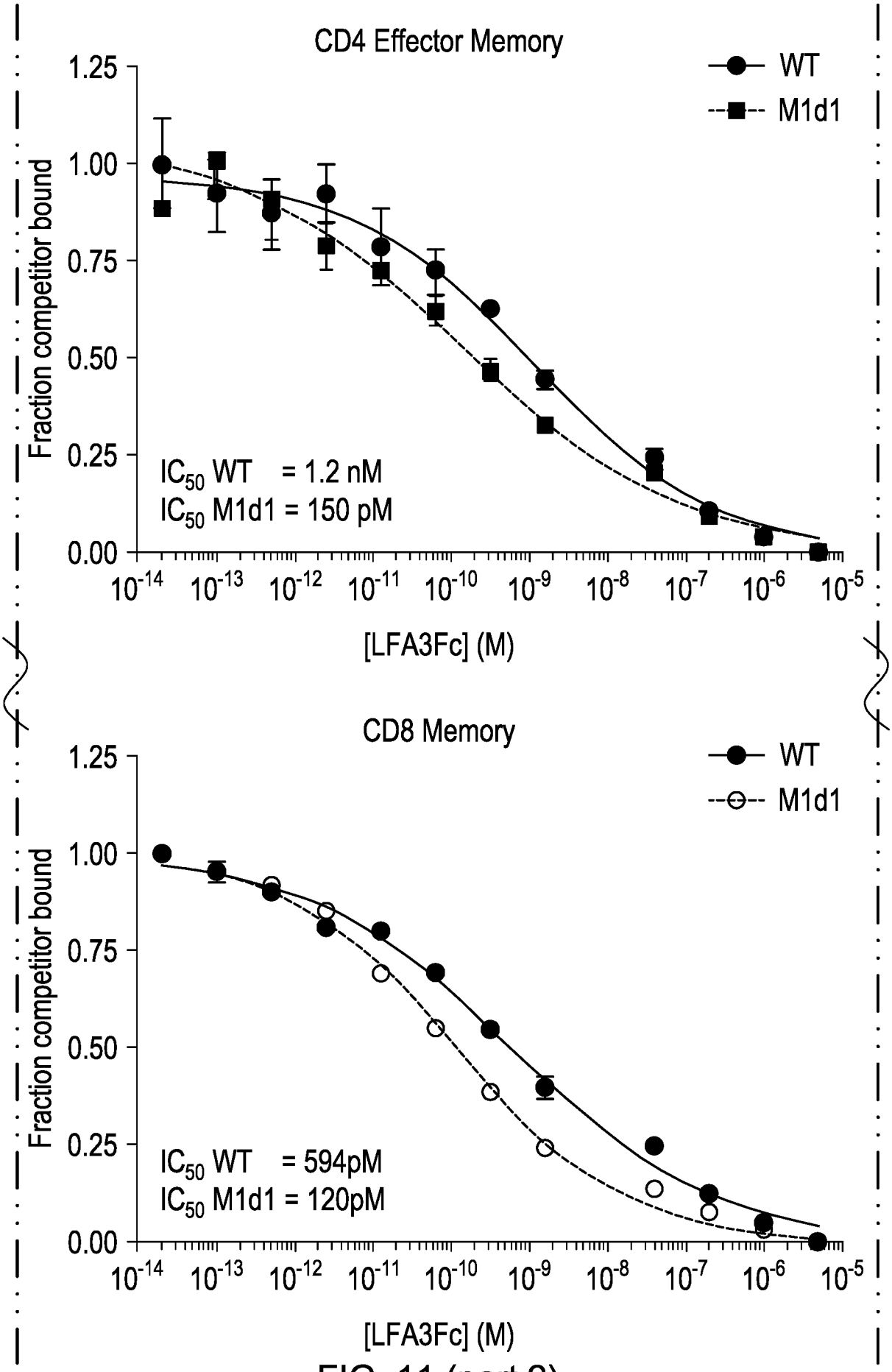

CD4 Effector Memory
WT
M1d1
Fraction competitor bound
1.25
1.00
0.75
0.50
0.25
0.00
IC50 WT = 1.2 nM
IC50 M1d1 = 150 pM
10-14 10-13 10-12 10-11 10-10 10-9 10-8 10-7 10-6 10-5
[LFA3Fc] (M)
CD8 Memory
WT
M1d1
Fraction competitor bound
1.25
1.00
0.75
0.50
0.25
0.00
IC50 WT = 594pM
IC50 M1d1 = 120pM
10-14 10-13 10-12 10-11 10-10 10-9 10-8 10-7 10-6 10-5
[LFA3Fc] (M)

FIG. 11 (part 2)

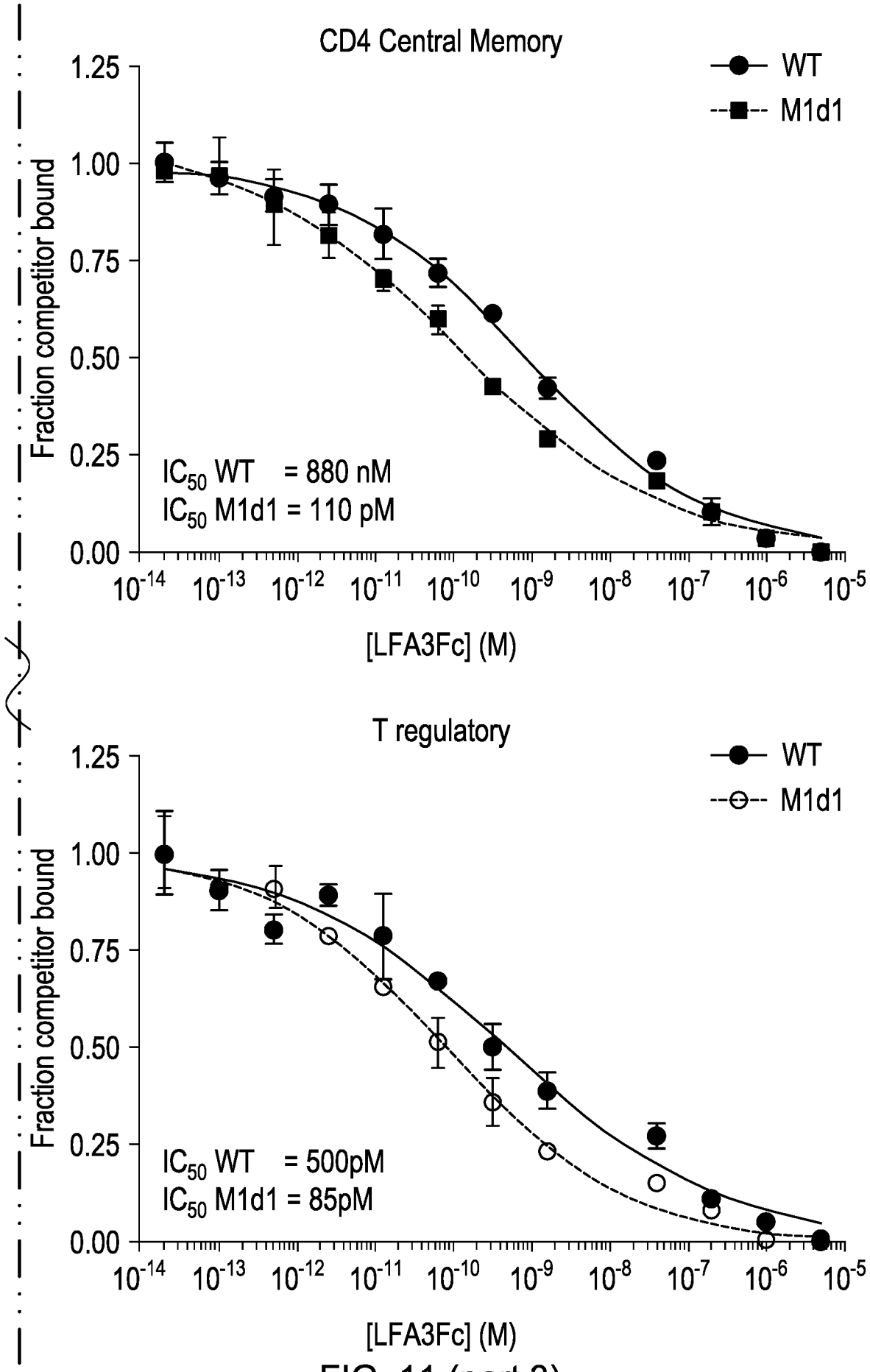

CD4 Central Memory
WT
M1d1
Fraction competitor bound
1.25
1.00
0.75
0.50
0.25
0.00
IC50 WT = 880 nM
IC50 M1d1 = 110 pM
10-14 10-13 10-12 10-11 10-10 10-9 10-8 10-7 10-6 10-5
[LFA3Fc] (M)
T regulatory
WT
M1d1
Fraction competitor bound
1.25
1.00
0.75
0.50
0.25
0.00
IC50 WT = 500pM
IC50 M1d1 = 85pM
10-14 10-13 10-12 10-11 10-10 10-9 10-8 10-7 10-6 10-5
[LFA3Fc] (M)

FIG. 11 (part 3)

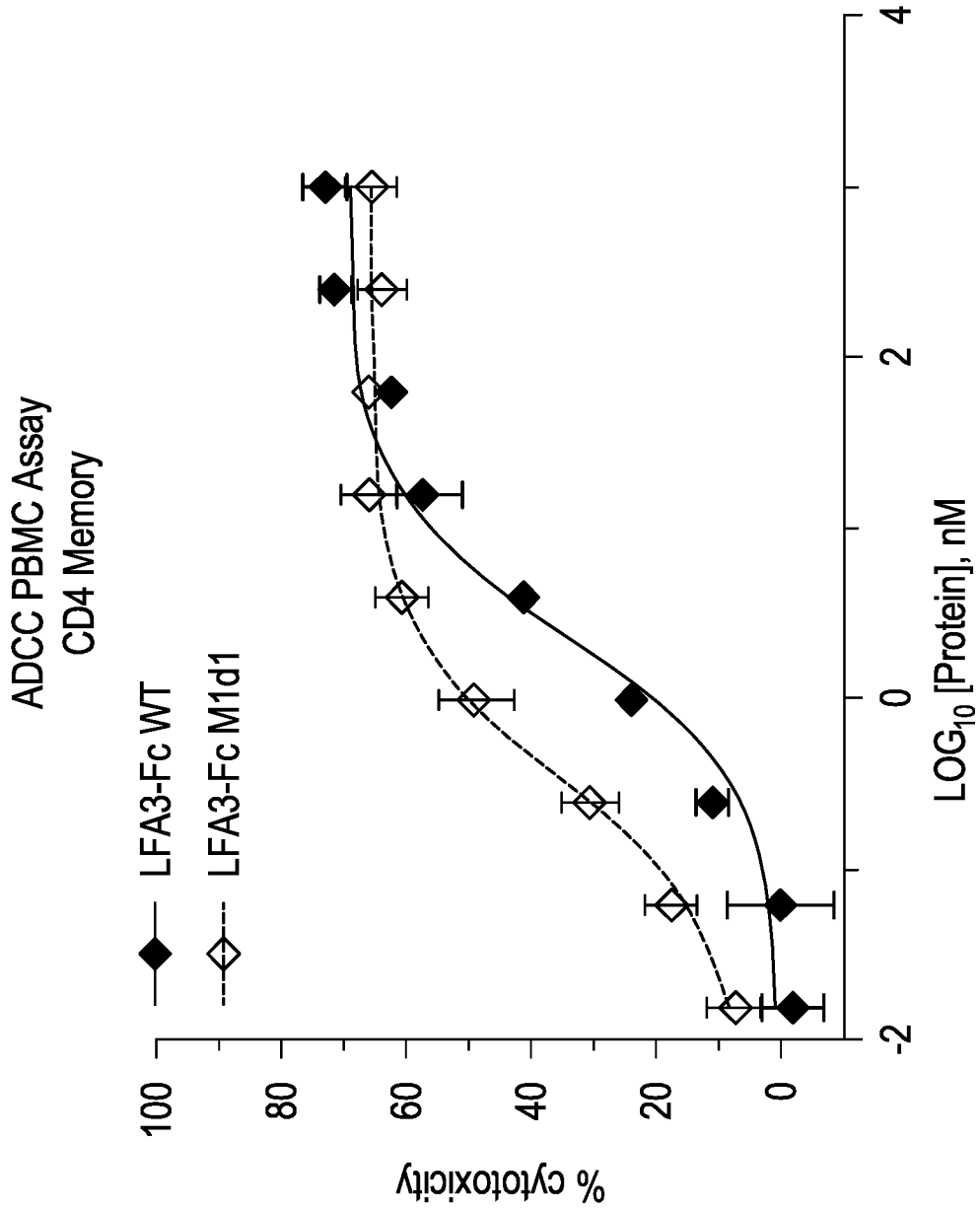

ADCC PBMC Assay
CD4 Memory
LFA3-Fc WT
LFA3-Fc M1d1
% cytotoxicity
100
80
60
40
20
0
-2
0
2
4
LOG10 [Protein], nM

FIG. 12A

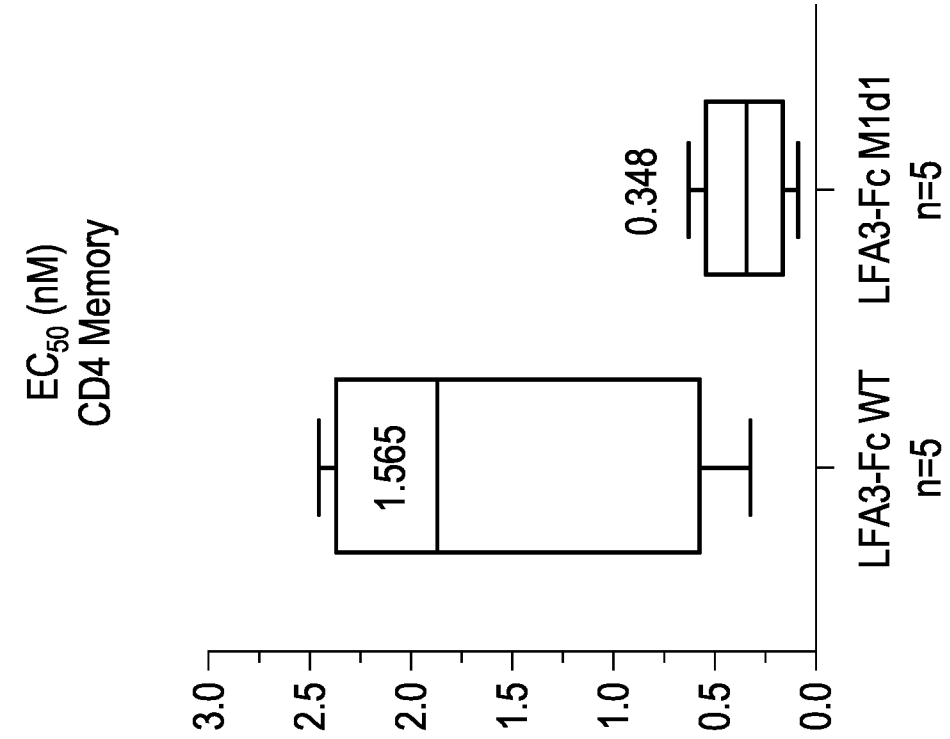

EC50 (nM)
CD4 Memory
3.0
2.5
2.0
1.5
1.0
0.5
0.0
1.565
0.348
LFA3-Fc WT
n=5
LFA3-Fc M1d1
n=5

FIG. 12B

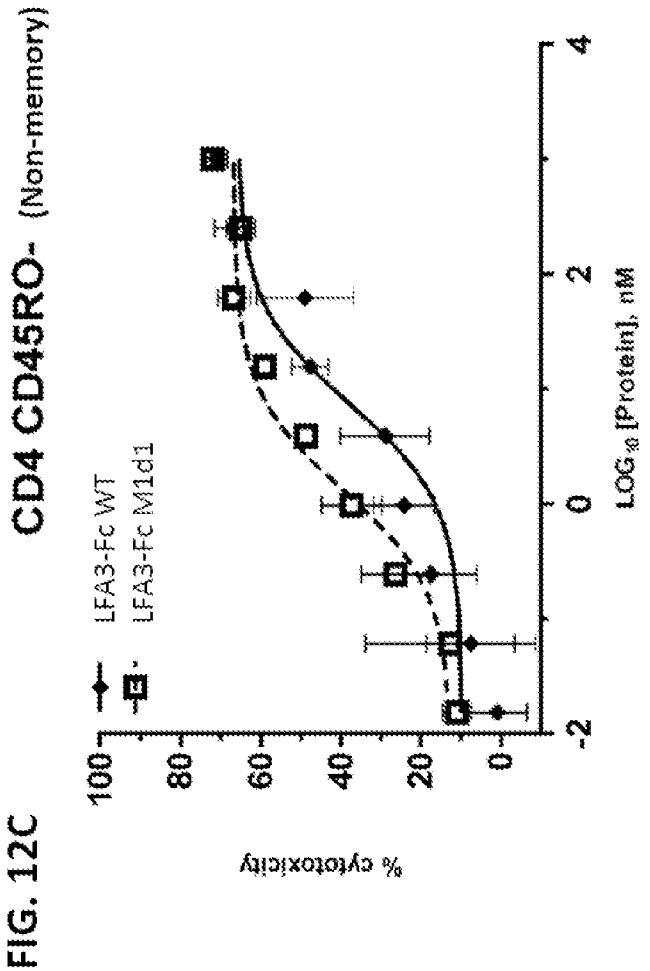

CD4 CD45RO- (Non-memory)
LFA3-Fc WT
LFA3-Fc M1d1
% cytotoxicity
100
80
60
40
20
0
-2
0
2
4
LOG10 [Protein], nM

FIG. 12C

FIG. 12E

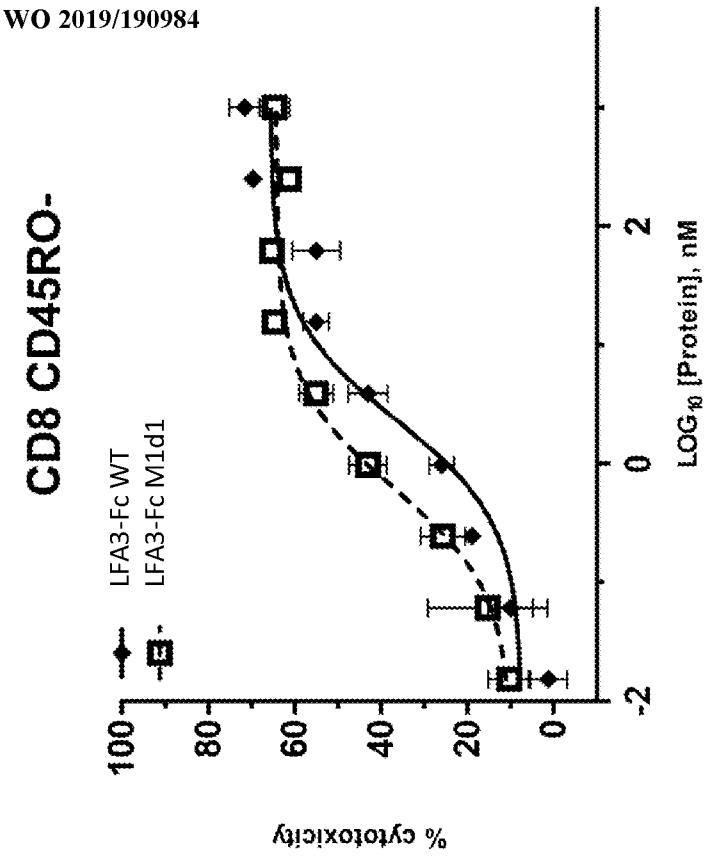


FIG. 12D

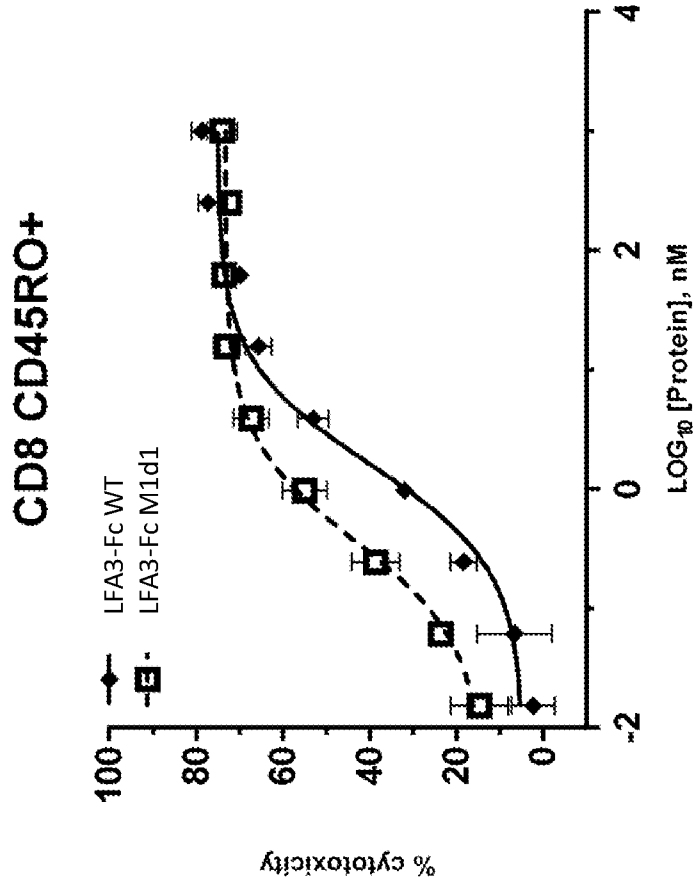


FIG. 13A

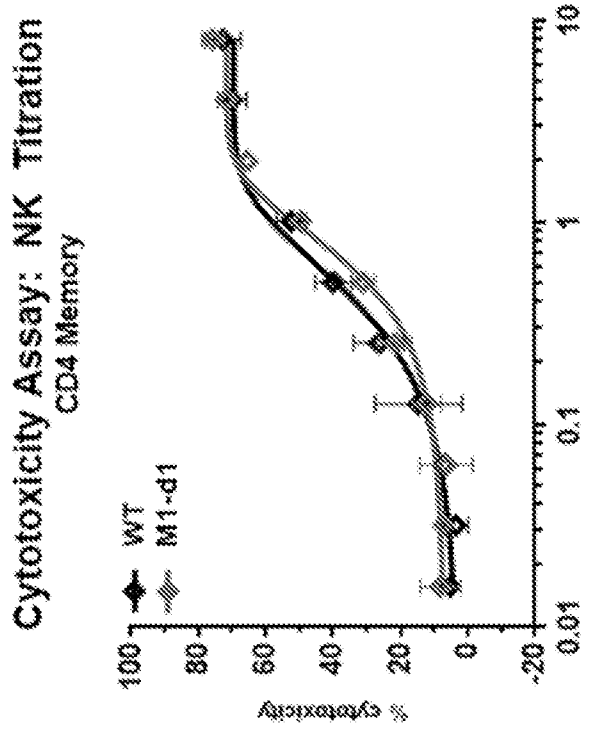


FIG. 13B

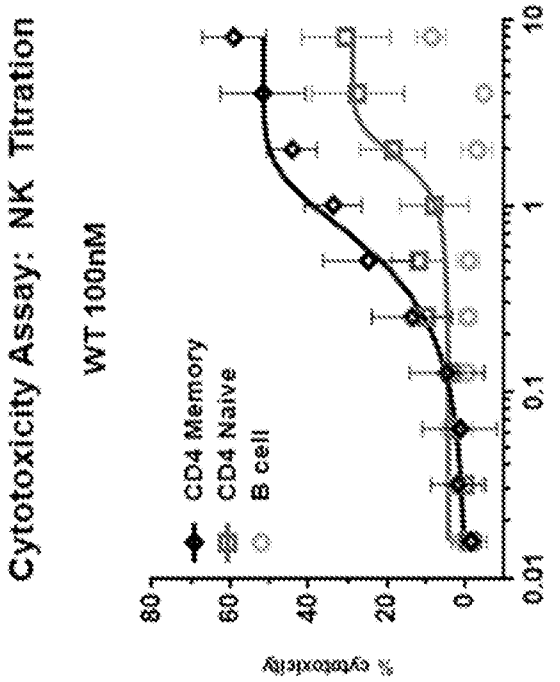
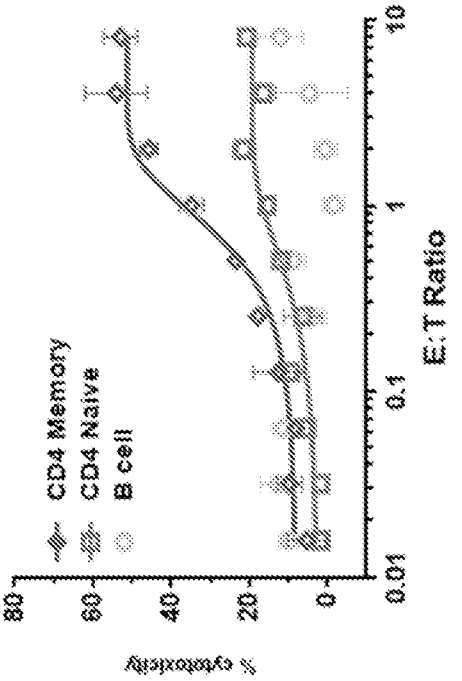


FIG. 13C



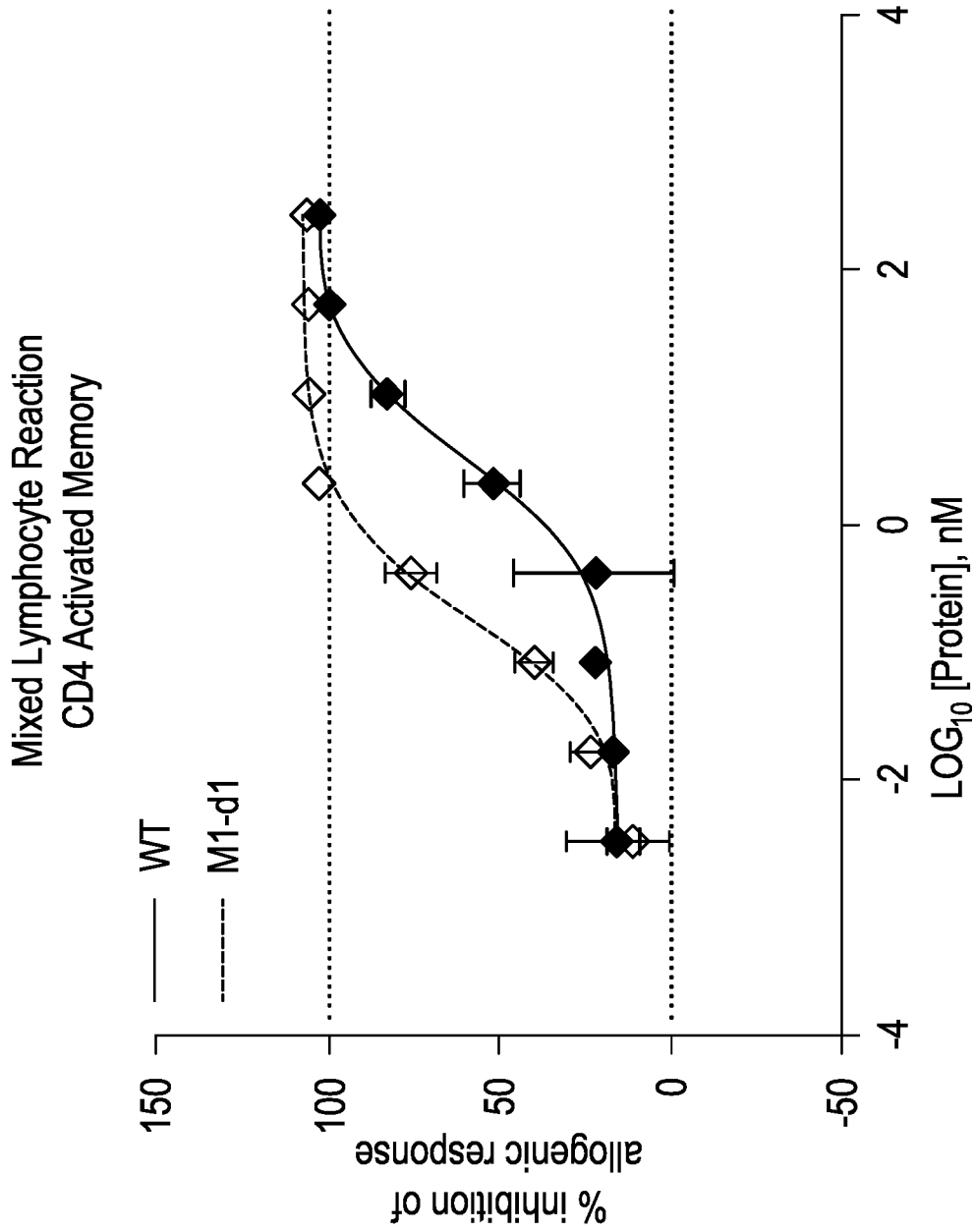

Mixed Lymphocyte Reaction
CD4 Activated Memory
WT
M1-d1
% inhibition of
allogenic response
150
100
50
0
-50
-4
-2
0
2
4
LOG10 [Protein], nM

FIG. 14A

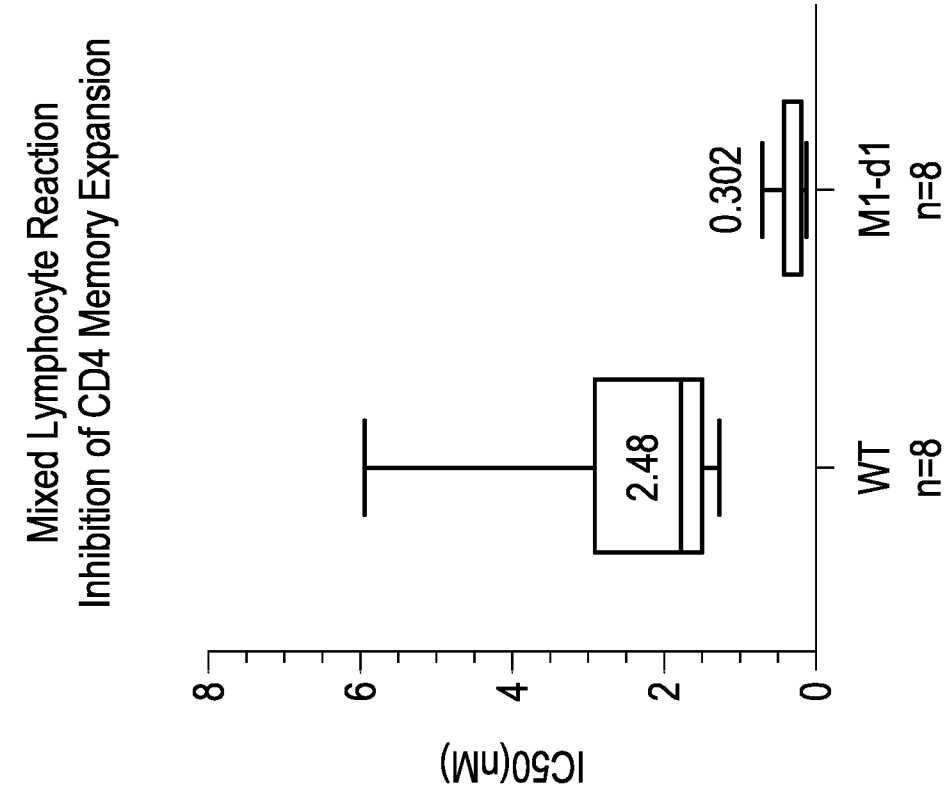

Mixed Lymphocyte Reaction
Inhibition of CD4 Memory Expansion
IC50(nM)
0
2
4
6
8
2.48
0.302
WT
n=8
M1-d1
n=8

FIG. 14B

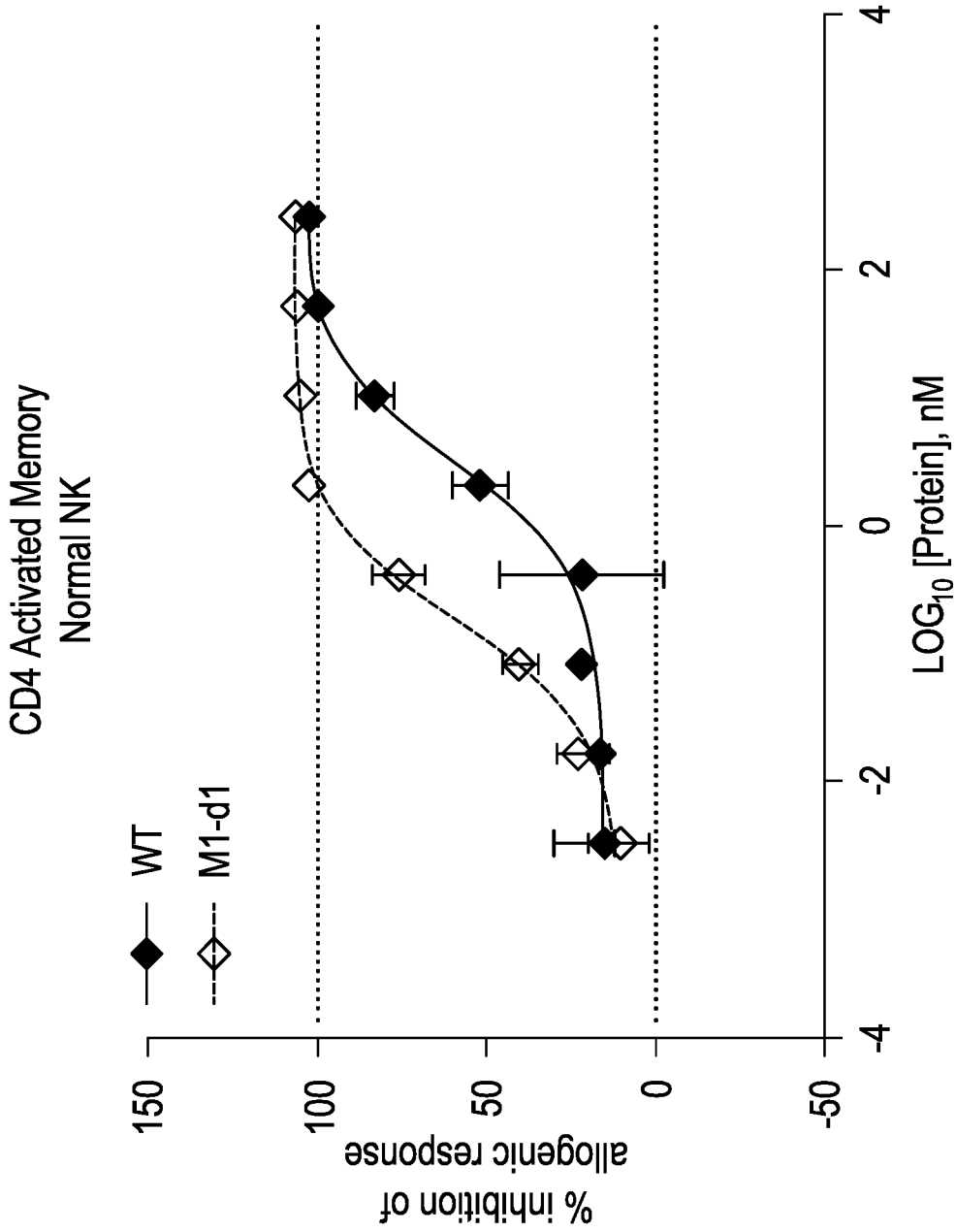

CD4 Activated Memory
Normal NK
WT
M1-d1
% inhibition of
allogenic response
150
100
50
0
-50
-4
-2
0
2
4
LOG10 [Protein], nM

FIG. 15A

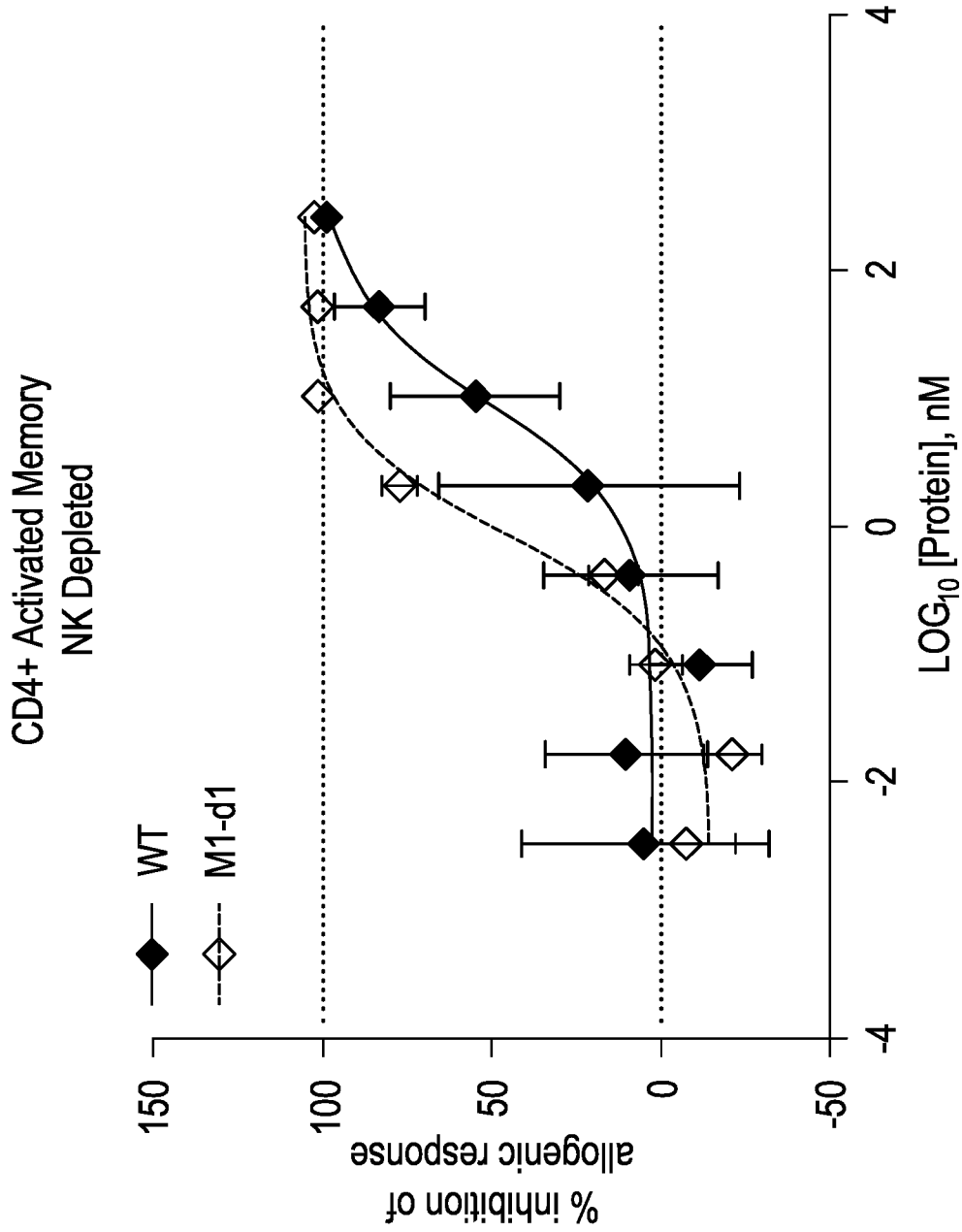

CD4+ Activated Memory
NK Depleted
WT
M1-d1
% inhibition of
allogenic response
150
100
50
0
-50
-4
-2
0
2
4
LOG10 [Protein], nM

FIG. 15B

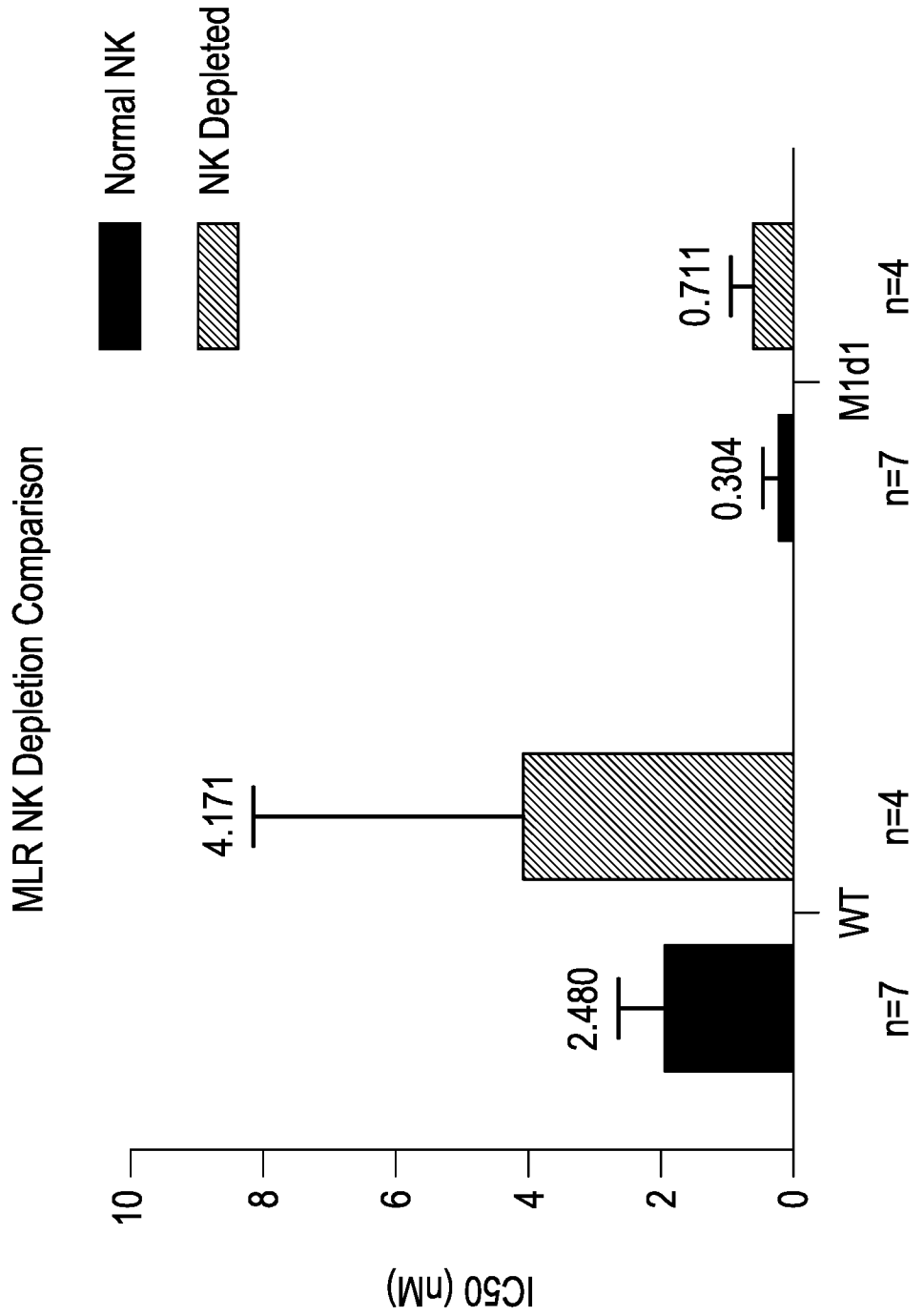

MLR NK Depletion Comparison
Normal NK
NK Depleted
IC50 (nM)
0
2
4
6
8
10
2.480
4.171
0.304
0.711
n=7
n=4
n=7
n=4
WT
M1d1

FIG. 15C

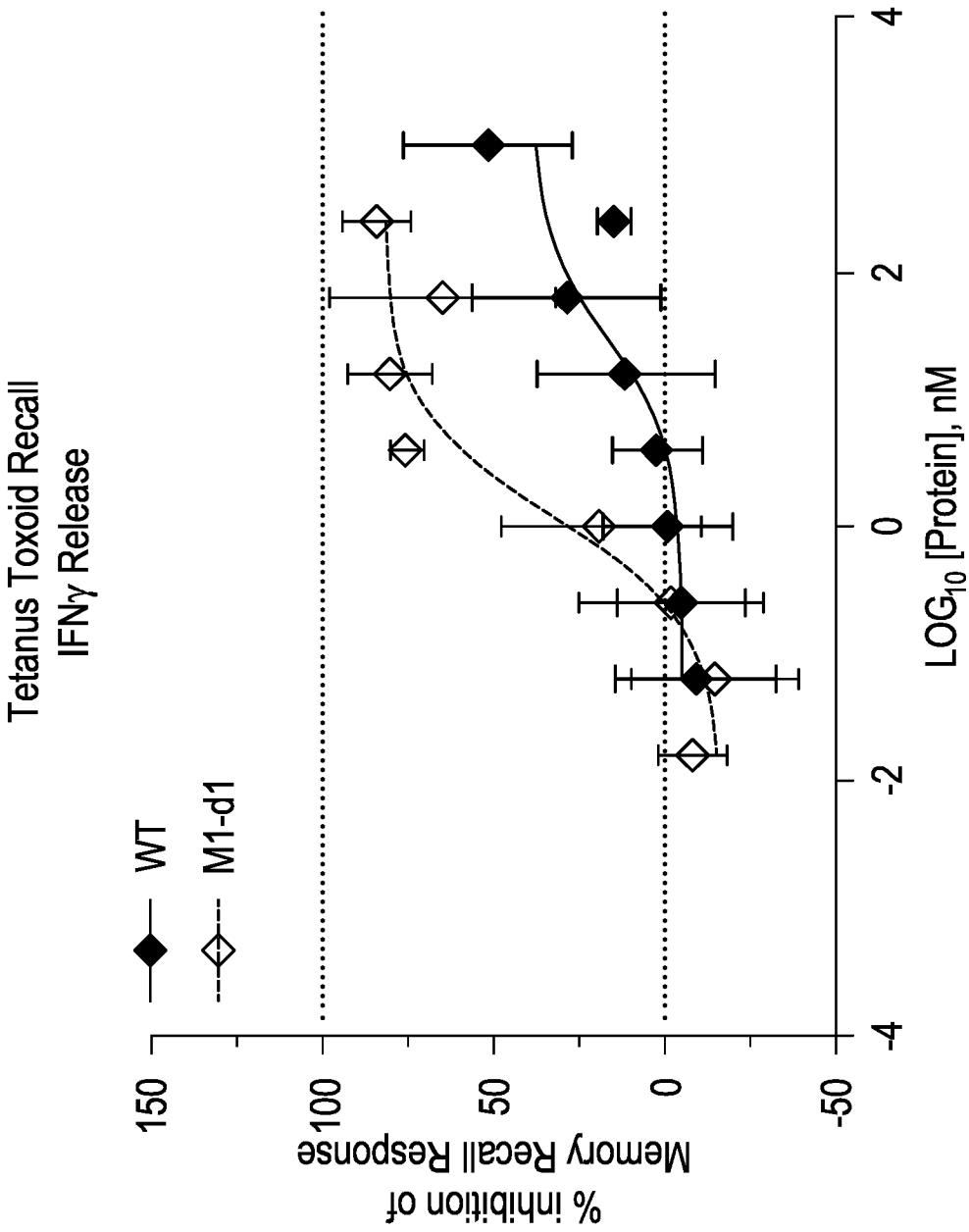

Tetanus Toxoid Recall
IFNγ Release
WT
M1-d1
% inhibition of
Memory Recall Response
150
100
50
0
-50
-4
-2
0
2
4
LOG10 [Protein], nM

FIG. 16A

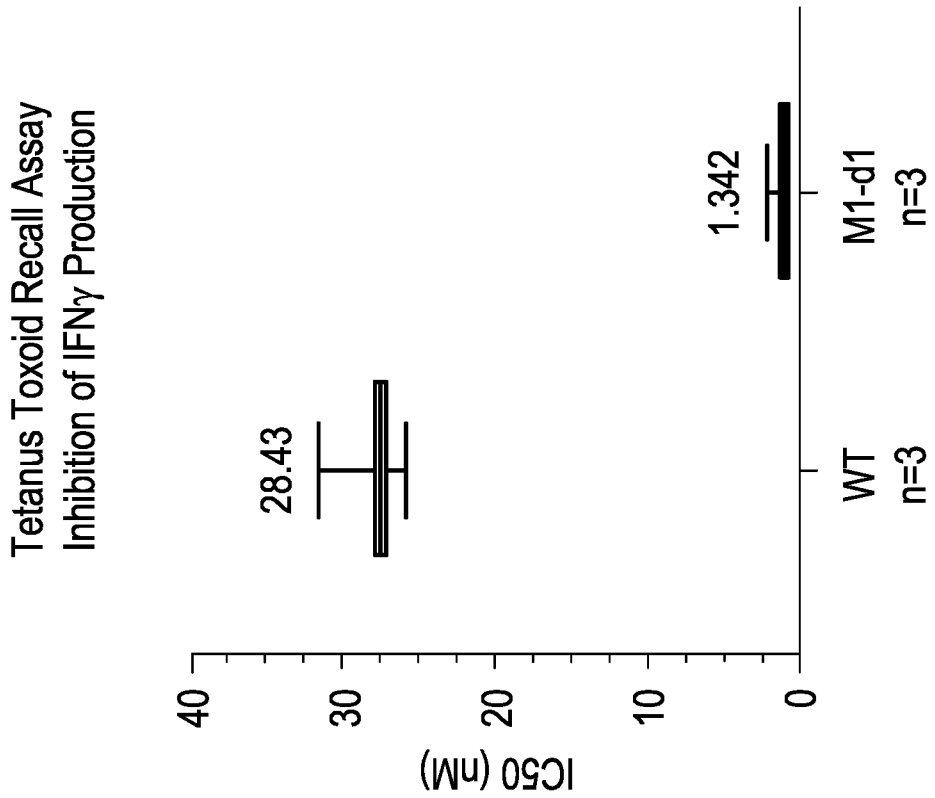

Tetanus Toxoid Recall Assay
Inhibition of IFNγ Production
IC50 (nM)
40
30
20
10
0
28.43
1.342
WT
n=3
M1-d1
n=3

FIG. 16B

FIG. 17

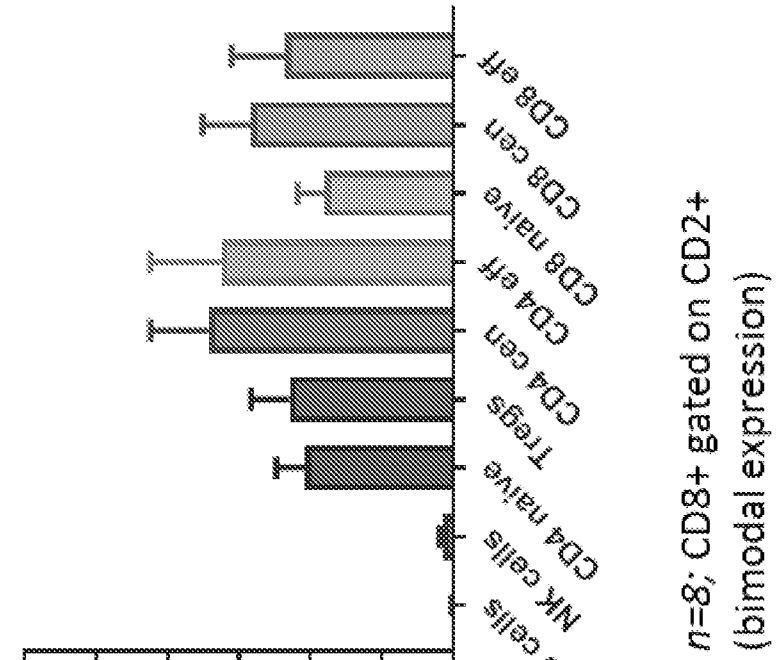

Quantibrite CD2 cyno
molecules/cell
15000
12500
10000
7500
5000
2500
0
B cells
NK cells
CD4 naive
Tregs
CD4 cen
CD4 eff
CD8 naive
CD8 cen
CD8 eff
n=8; CD8+ gated on CD2+
(bimodal expression)

FIG. 18A

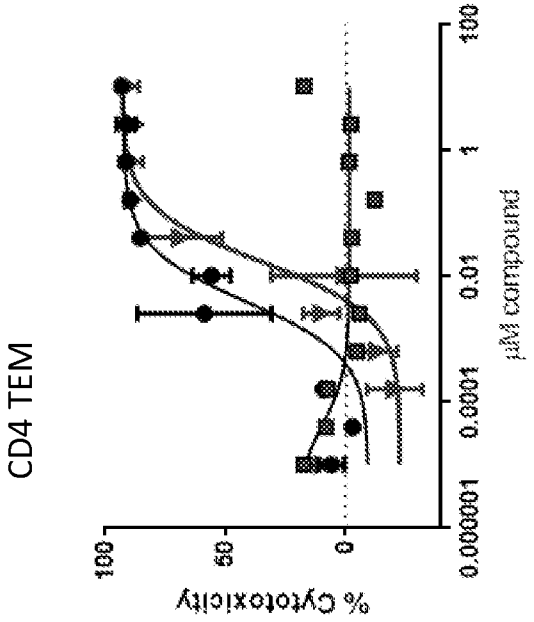
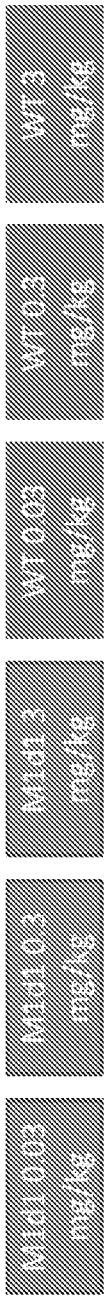


FIG. 18B

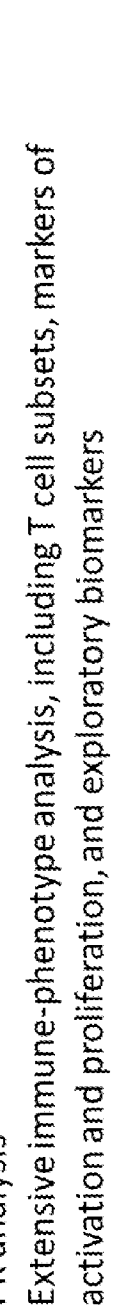
CD4+ TEM	M1d1 EC50 nM	WT EC50 nM
Cyno 1	22.05	33.88
M12817	3.78	16.32
YR669M	1.96	7.71

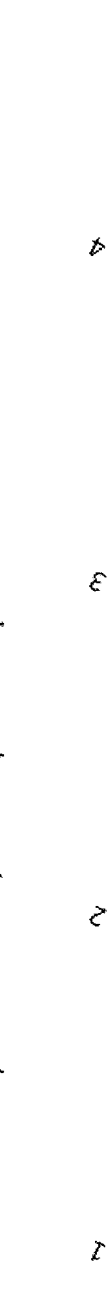
FIG. 19

Goal: Establish in vivo PK/PD repeat dose response relationship for T_{mem} depletion and ratio of Tregs/T_{EM}. Include WT LFA3-Fc to enable comparison to clinical Alefacept dataset.











- PK analysis
- Extensive immune-phenotype analysis, including T cell subsets, markers of activation and proliferation, and exploratory biomarkers

FIG. 20A

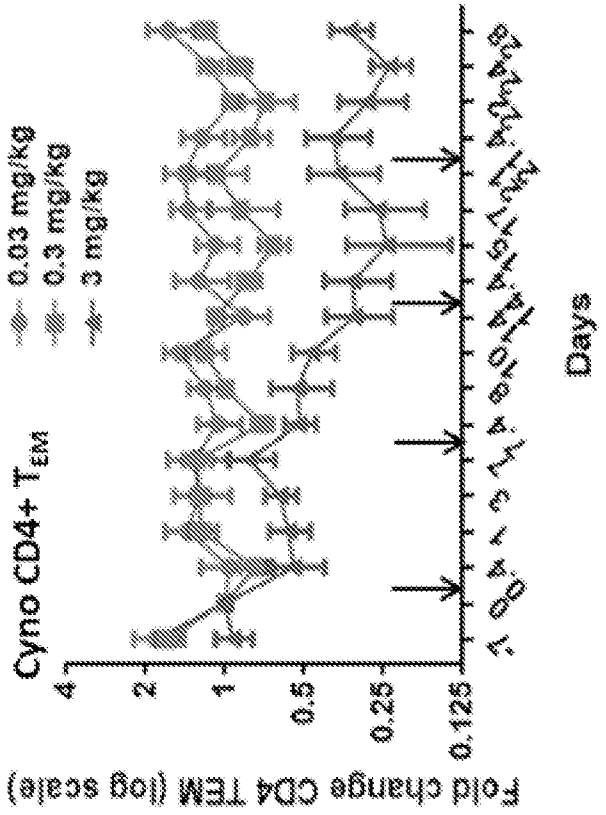
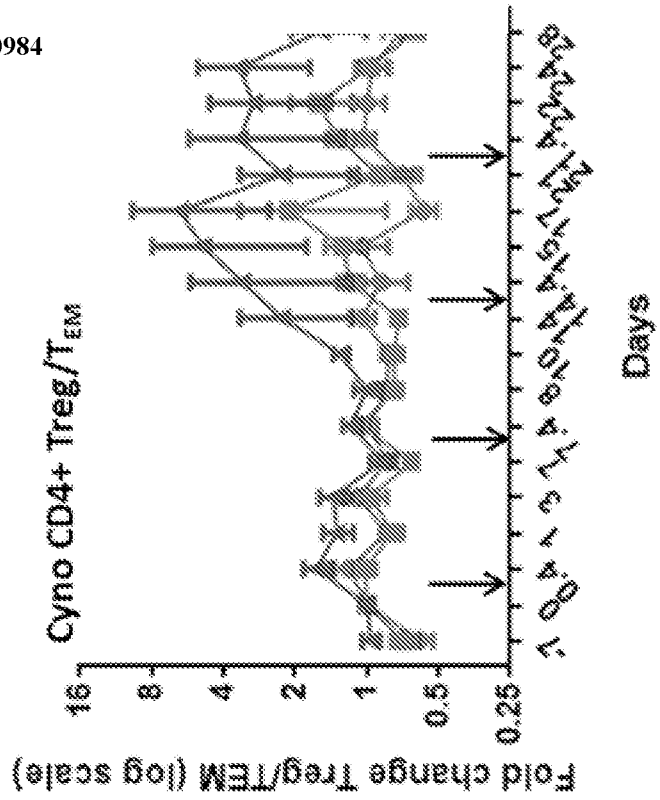
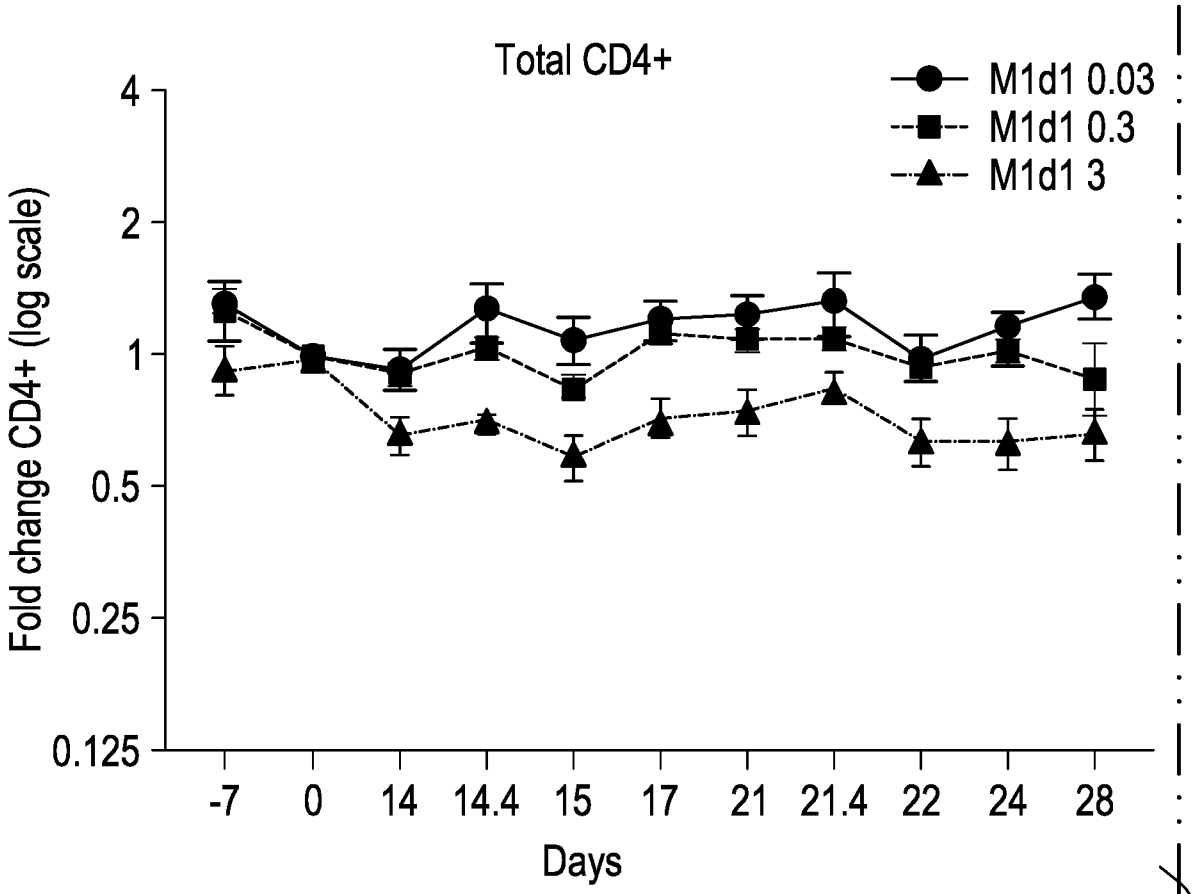


FIG. 20B




Total CD4+
M1d1 0.03
M1d1 0.3
M1d1 3
Fold change CD4+ (log scale)
4
2
1
0.5
0.25
0.125
-7
0
14
14.4
15
17
21
21.4
22
24
28
Days

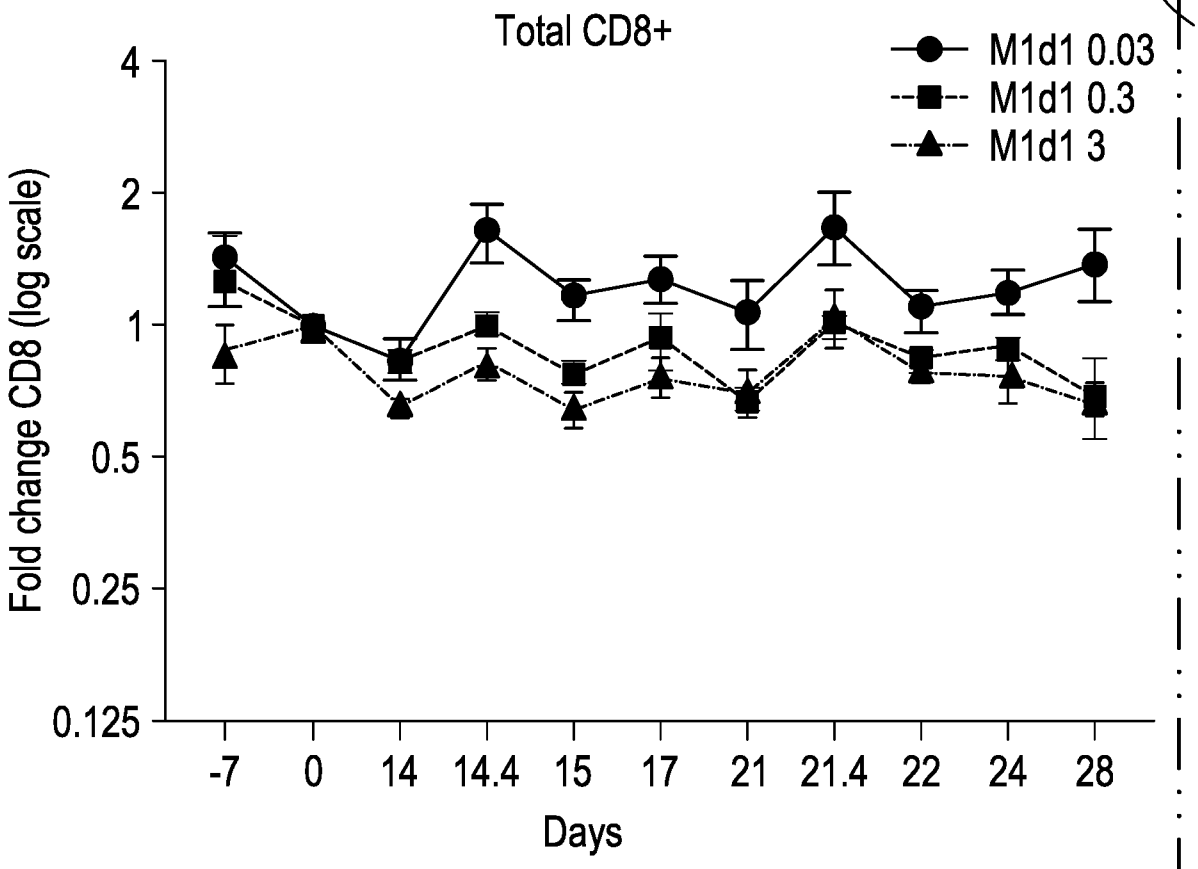

Total CD8+
M1d1 0.03
M1d1 0.3
M1d1 3
Fold change CD8 (log scale)
4
2
1
0.5
0.25
0.125
-7
0
14
14.4
15
17
21
21.4
22
24
28
Days

FIG. 21 (part 1)

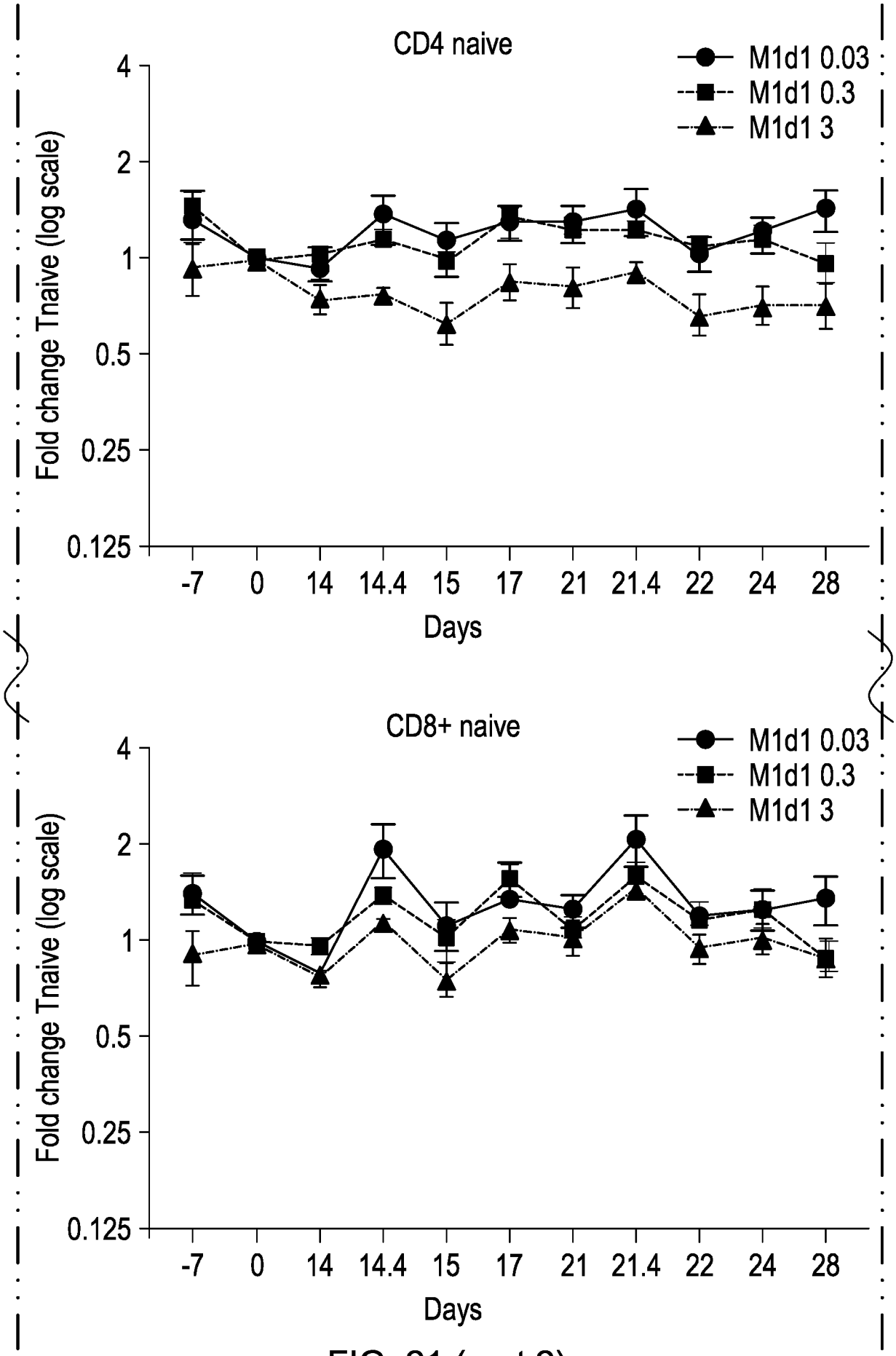
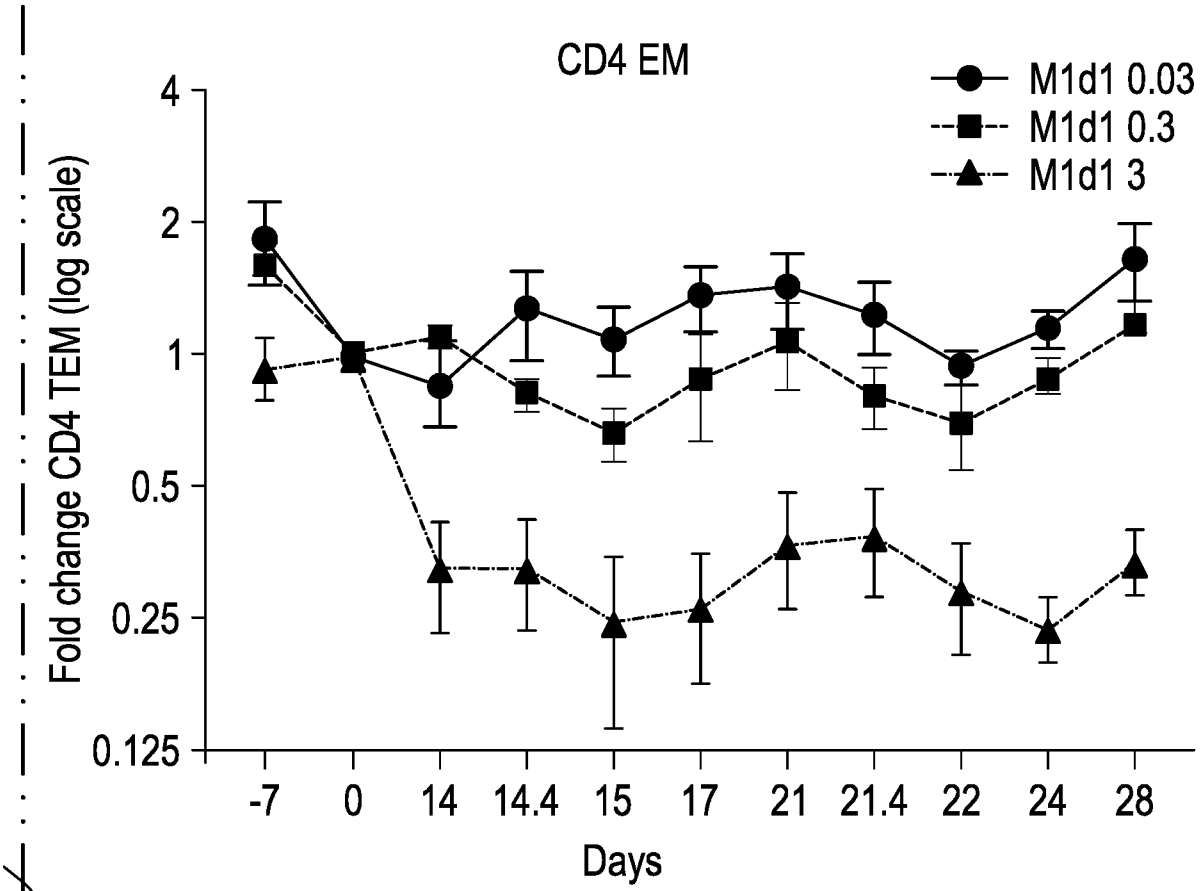

CD4 naive
M1d1 0.03
M1d1 0.3
M1d1 3
Fold change Tnaive (log scale)
4
2
1
0.5
0.25
0.125
-7
0
14
14.4
15
17
21
21.4
22
24
28
Days
CD8+ naive
M1d1 0.03
M1d1 0.3
M1d1 3
Fold change Tnaive (log scale)
4
2
1
0.5
0.25
0.125
-7
0
14
14.4
15
17
21
21.4
22
24
28
Days

FIG. 21 (part 2)


CD4 EM
M1d1 0.03
M1d1 0.3
M1d1 3
Fold change CD4 TEM (log scale)
4
2
1
0.5
0.25
0.125
-7
0
14
14.4
15
17
21
21.4
22
24
28
Days

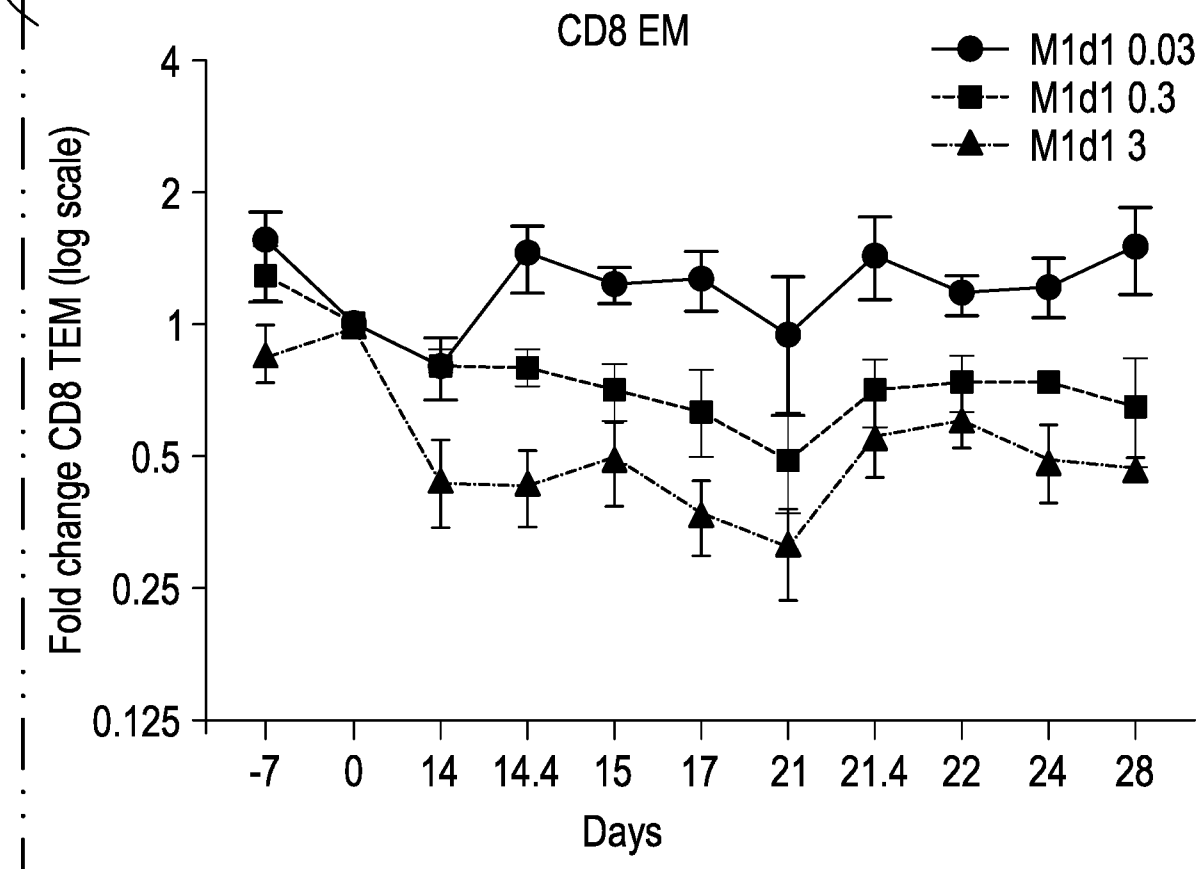

CD8 EM
M1d1 0.03
M1d1 0.3
M1d1 3
Fold change CD8 TEM (log scale)
4
2
1
0.5
0.25
0.125
-7
0
14
14.4
15
17
21
21.4
22
24
28
Days

FIG. 21 (part 3)

FIG. 22B

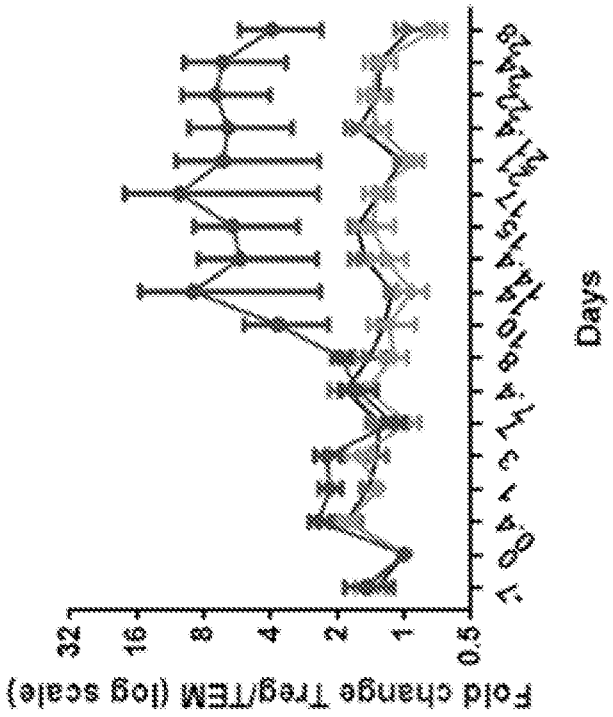


FIG. 22A

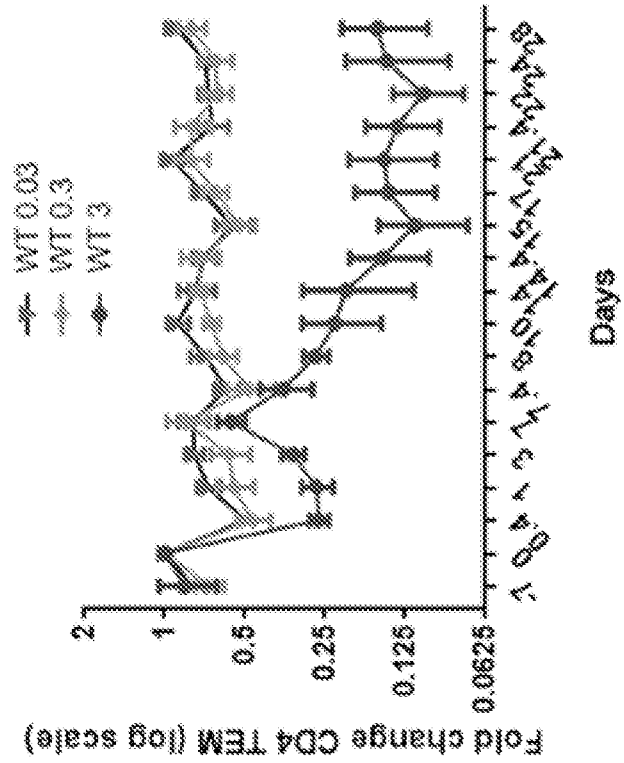


FIG. 23A

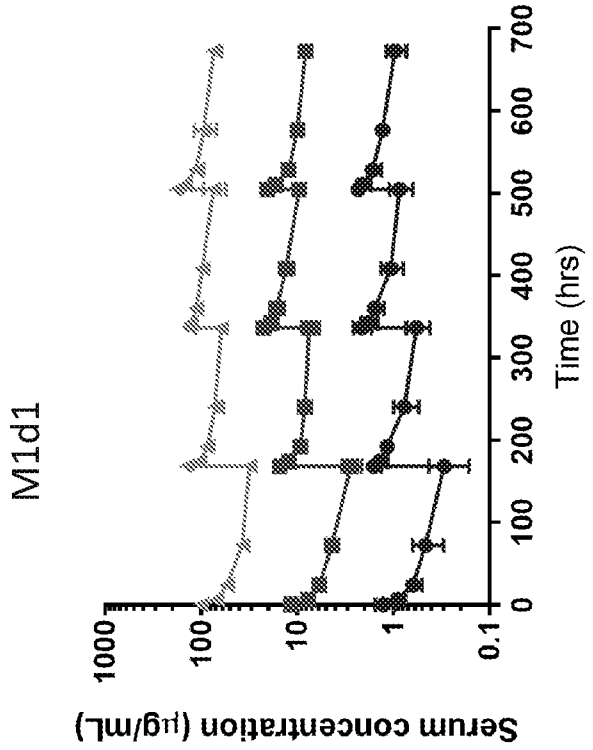


FIG. 23B

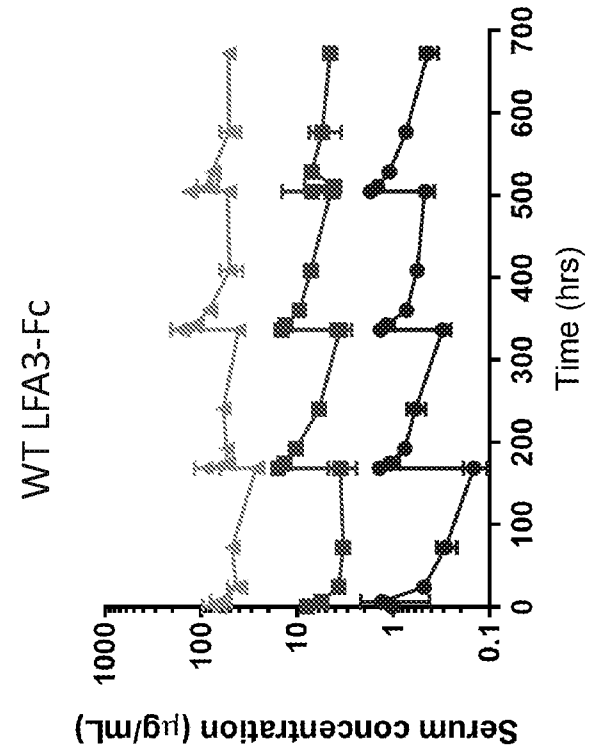
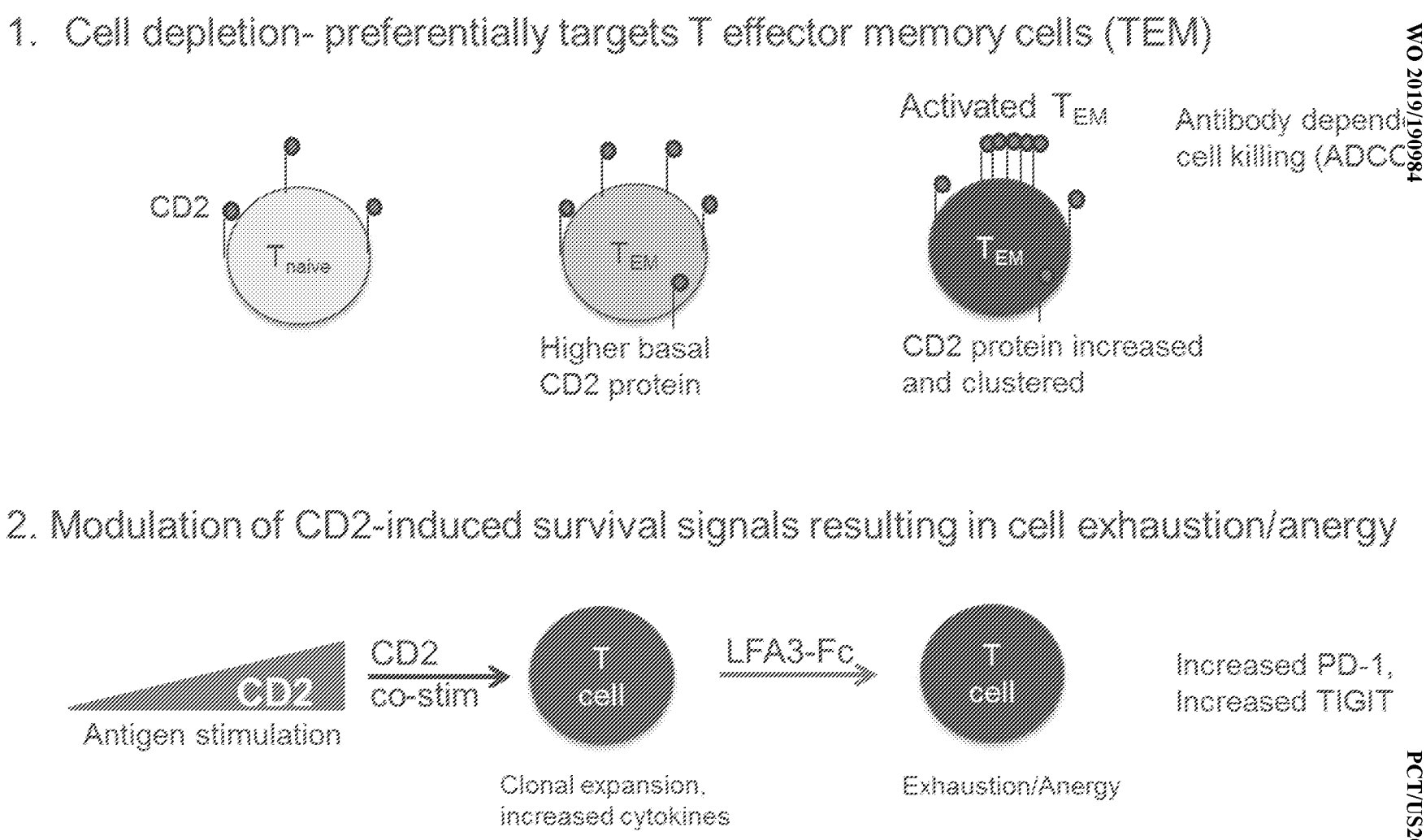


FIG. 24



SUBSTITUTE SHEET (RULE 26)

FIG. 25B

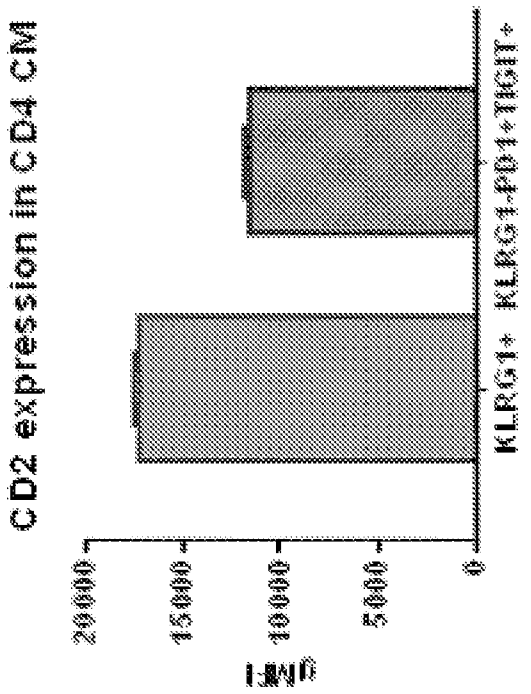


FIG. 25A

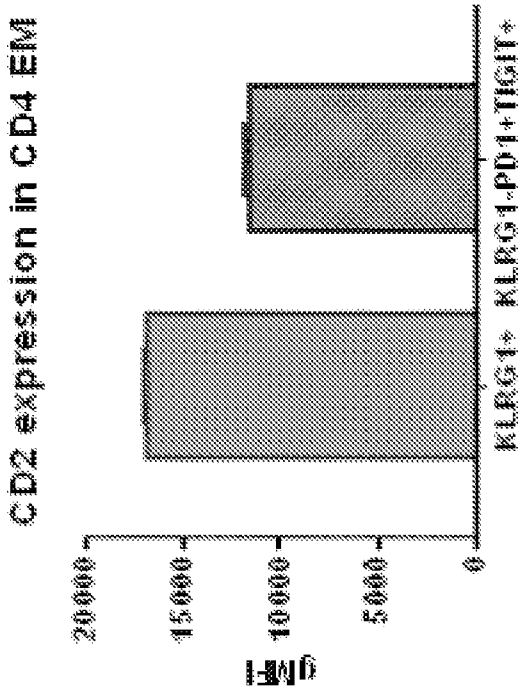


FIG. 26B

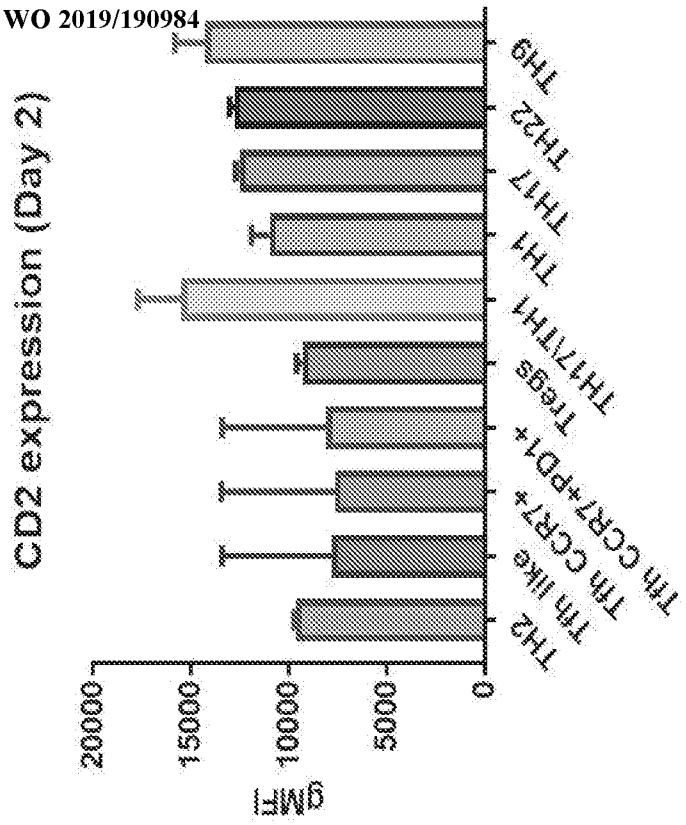


FIG. 26A

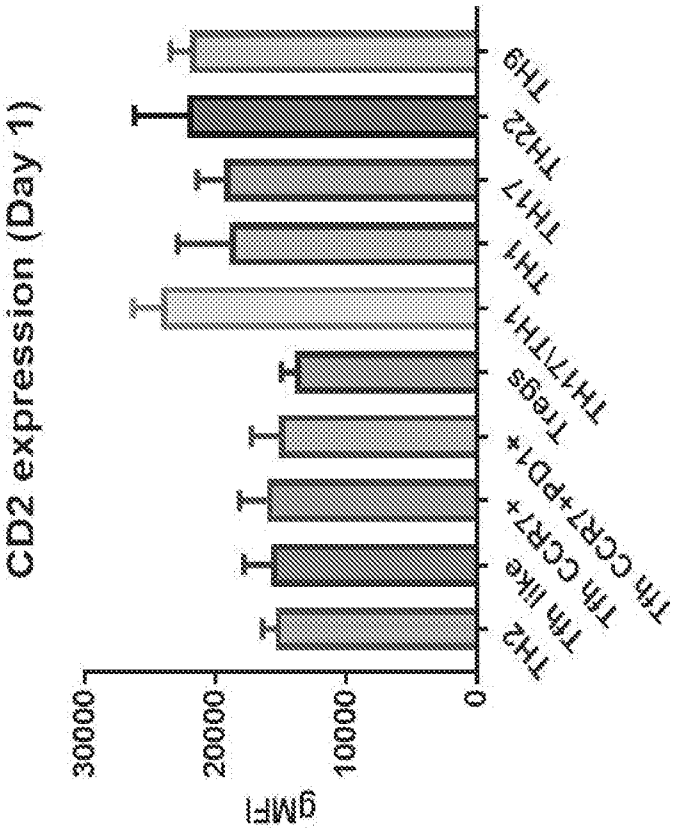


FIG. 28

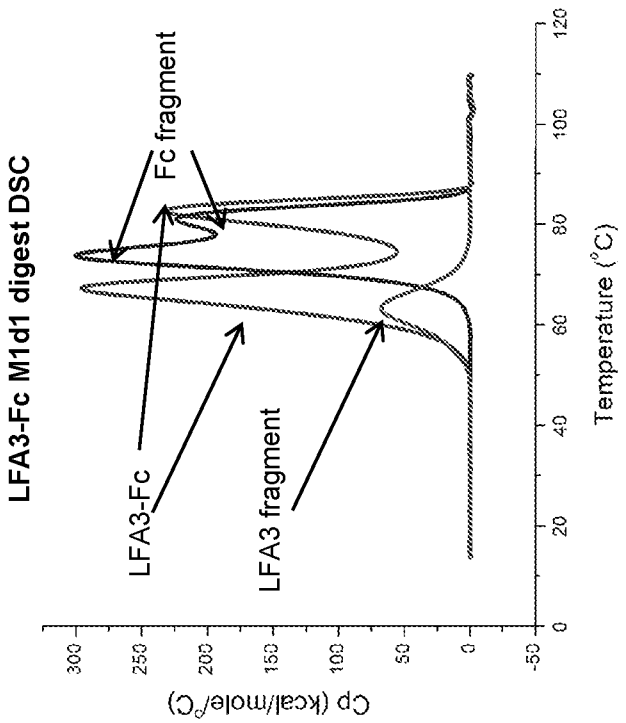
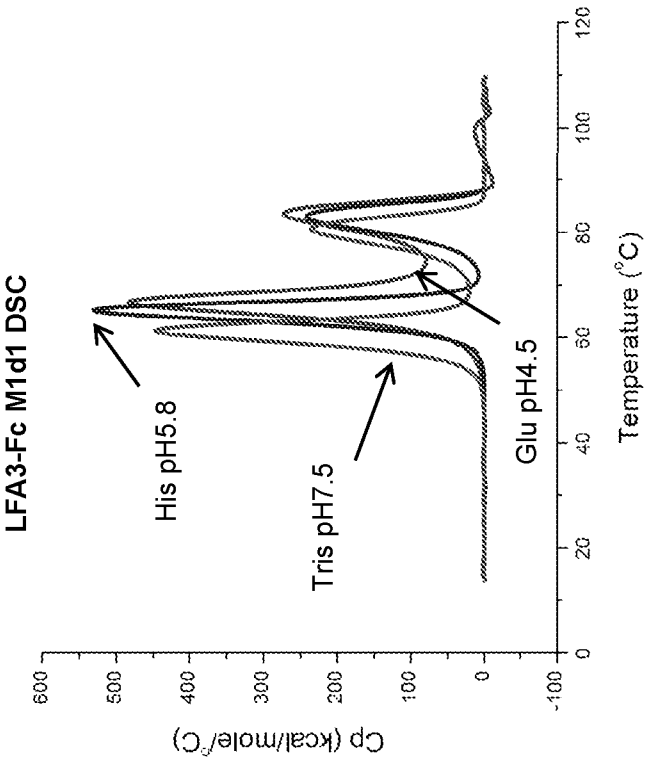

LFA3-Fc M1d1 digest DSC
Fc fragment
LFA3-Fc
LFA3 fragment
Cp (kcal/mole/°C)
Temperature (°C)

FIG. 27


LFA3-Fc M1d1 DSC
His pH5.8
Tris pH7.5
Glu pH4.5
Cp (kcal/mole/°C)
Temperature (°C)

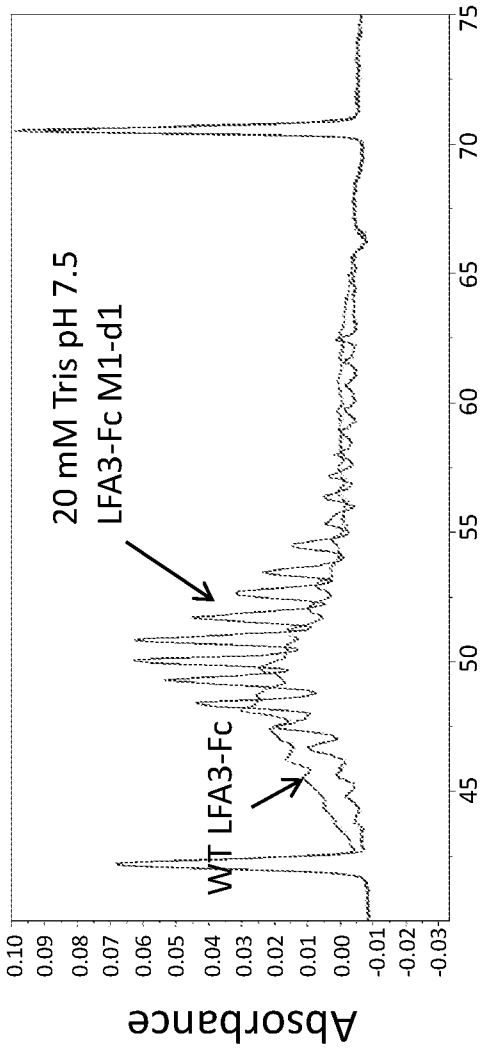


FIG. 29A

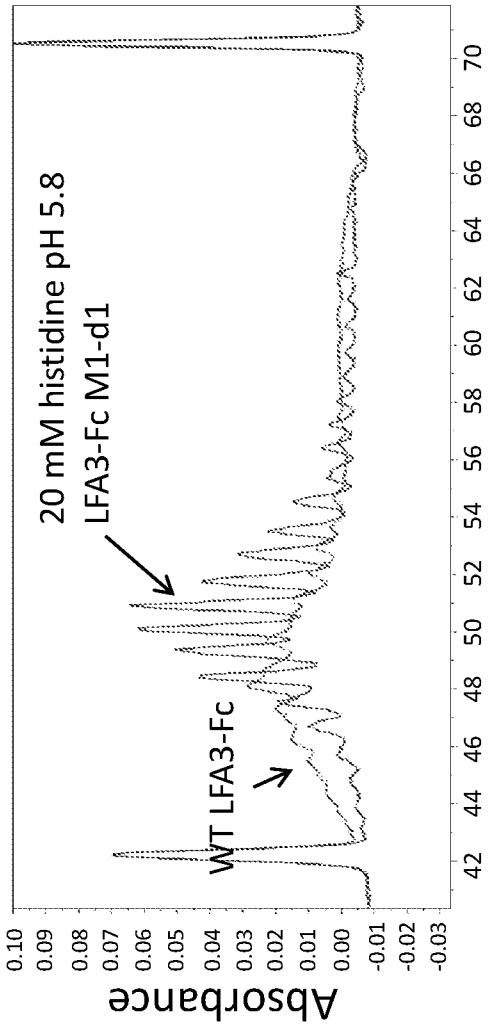


FIG. 29B

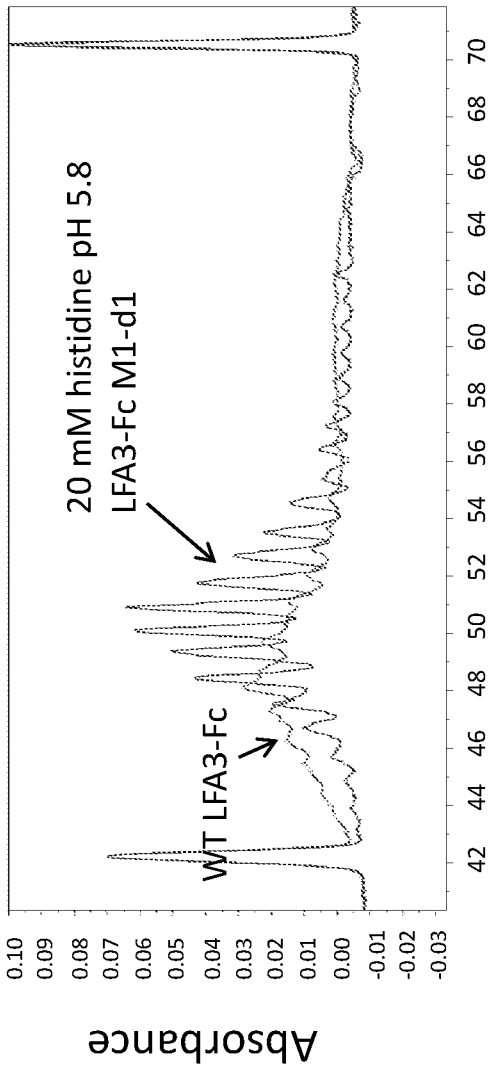

20 mM histidine pH 5.8
LFA3-Fc M1-d1
WT LFA3-Fc
Absorbance
0.10
0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0.00
-0.01
-0.02
-0.03
42
44
46
48
50
52
54
56
58
60
62
64
66
68
70

FIG. 29C

FIG. 30A

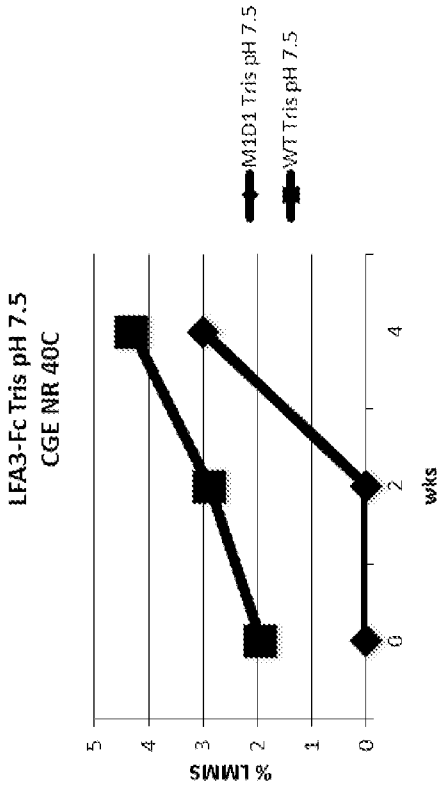


FIG. 30B

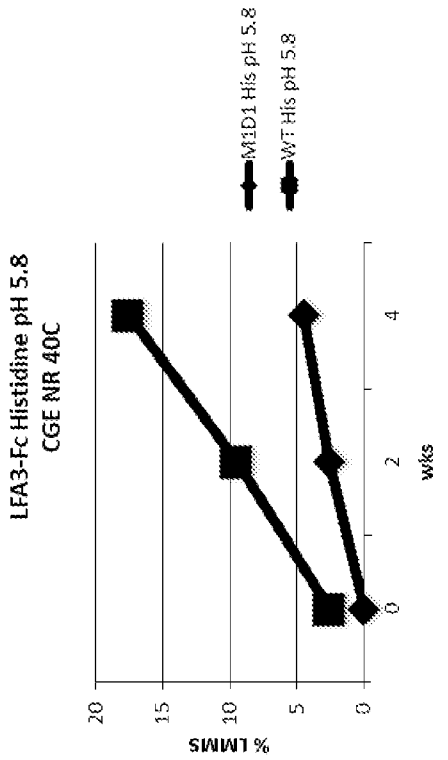


FIG. 30C

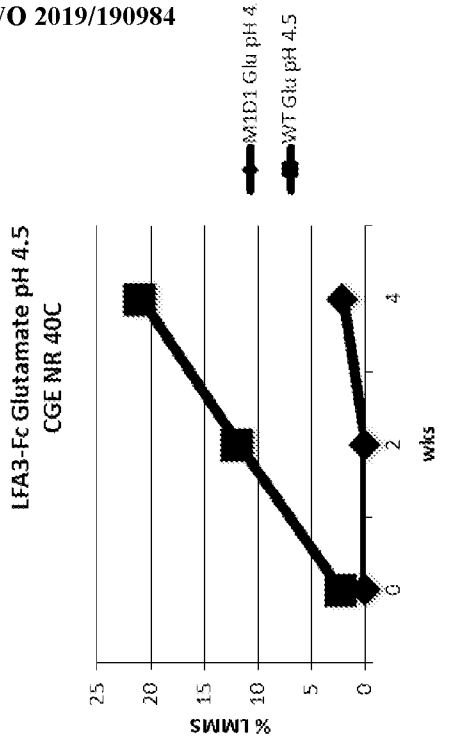


FIG. 31A

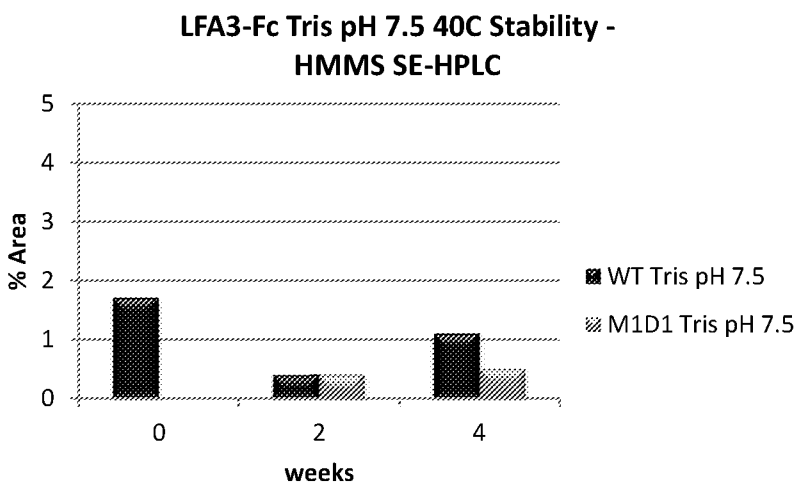


FIG. 31B

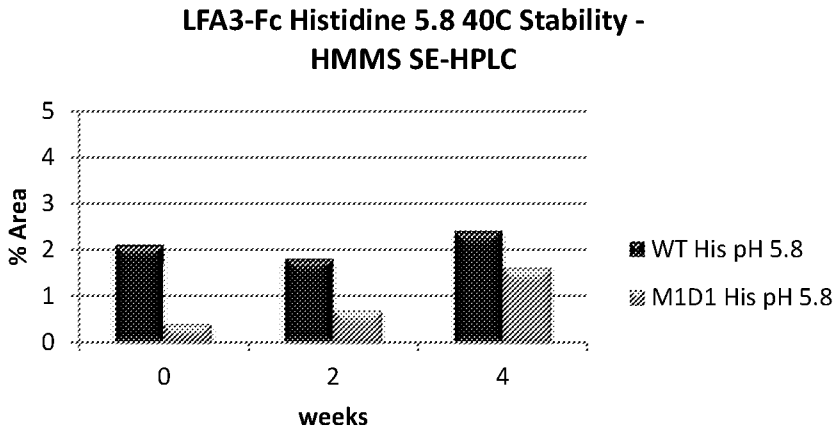


FIG. 31C

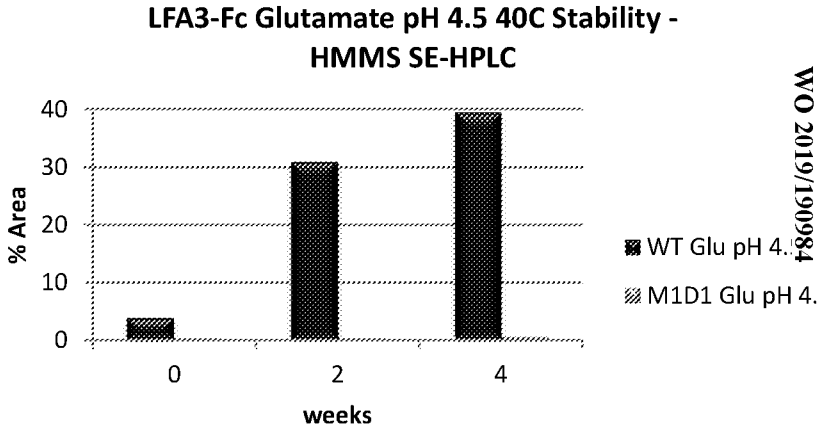
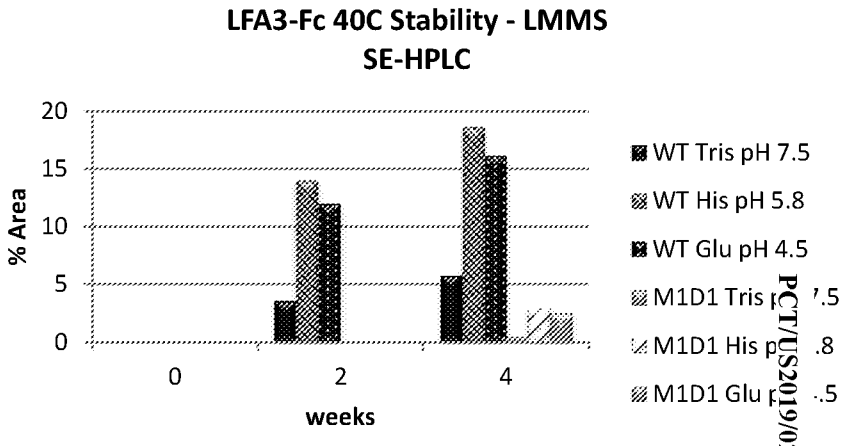


FIG. 31D



SUBSTITUTE SHEET (RULE 26)

FIG. 32A

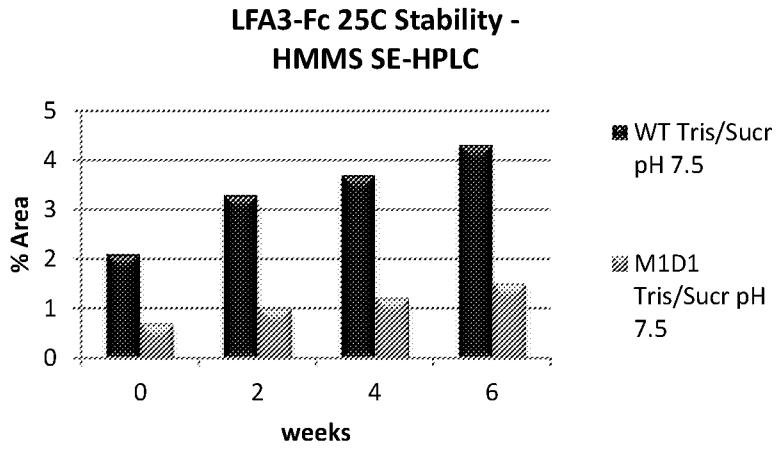


FIG. 32B

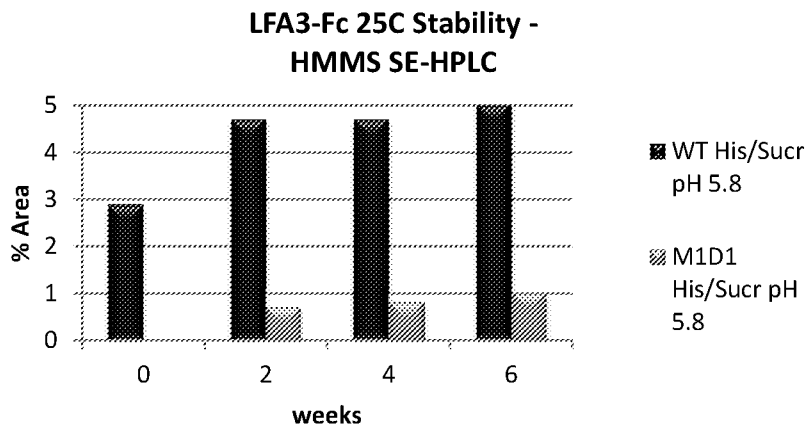


FIG. 32C

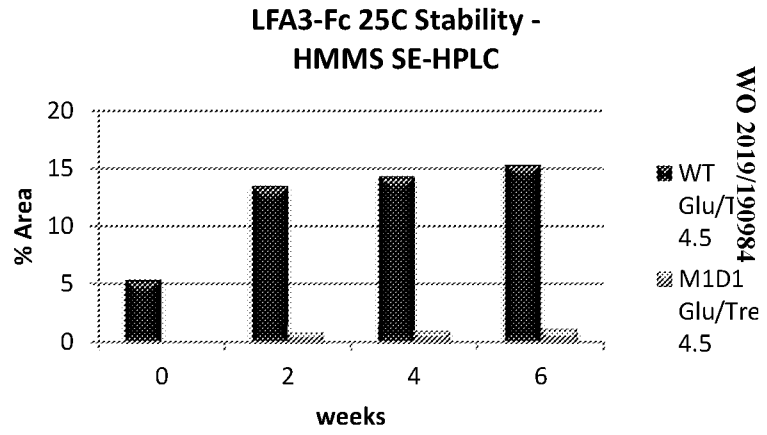
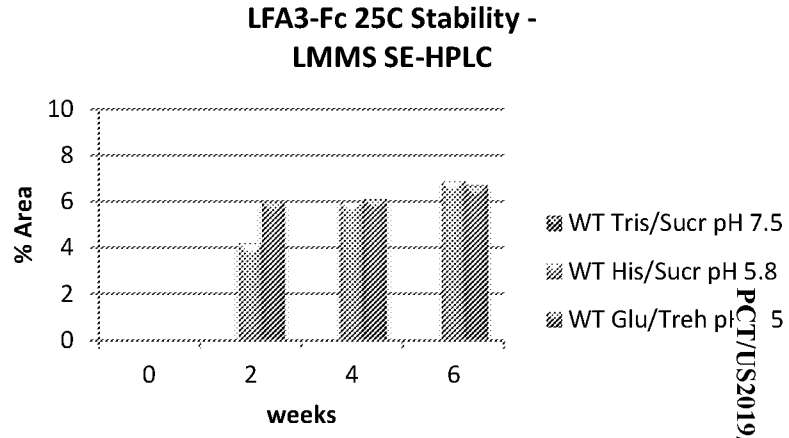


FIG. 32D



SUBSTITUTE SHEET (RULE 26)

FIG. 33A

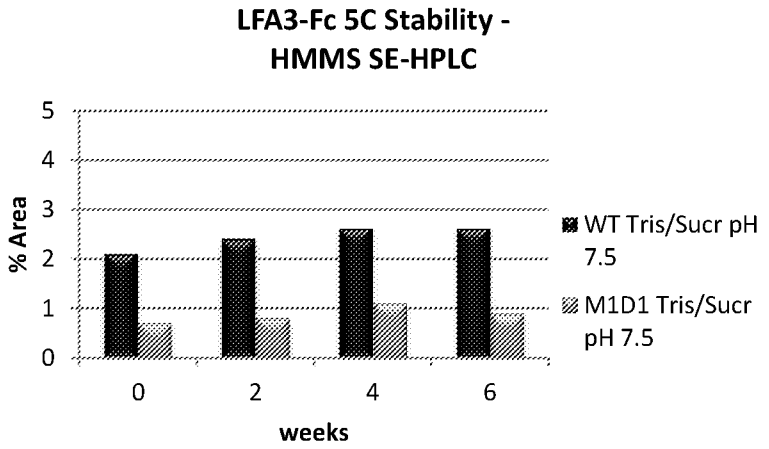


FIG. 33B

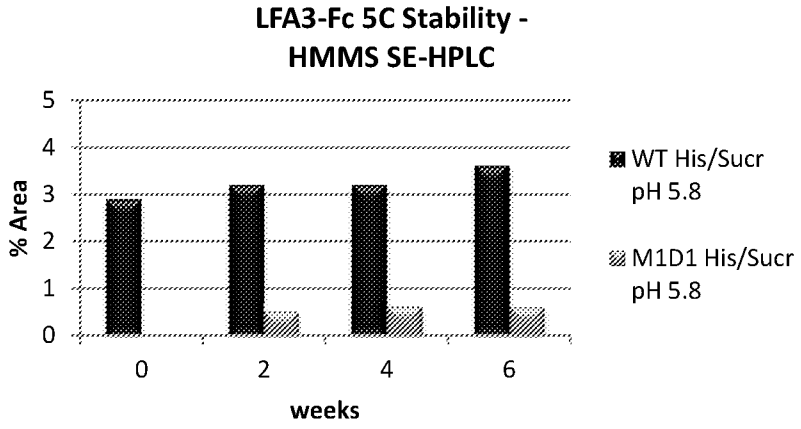


FIG. 33C

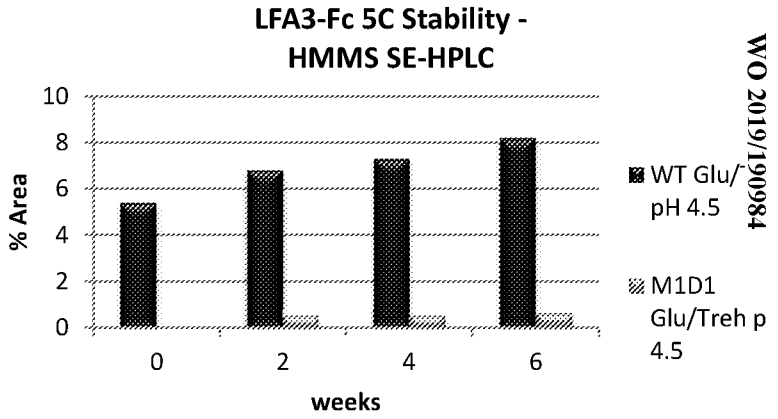
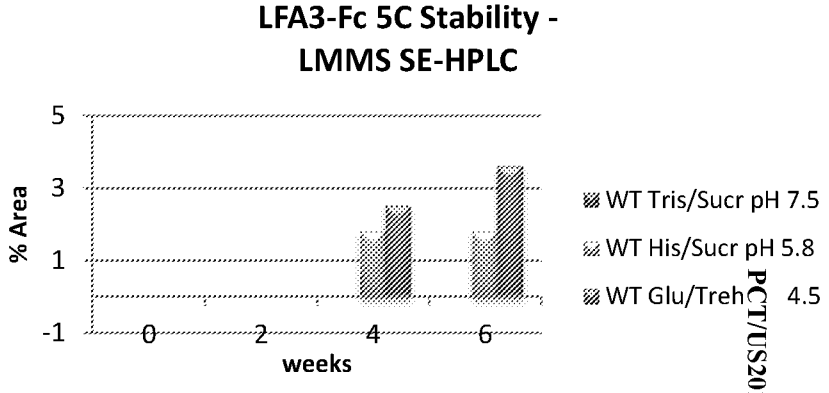


FIG. 33D



SUBSTITUTE SHEET (RULE 26)

FIG. 34B

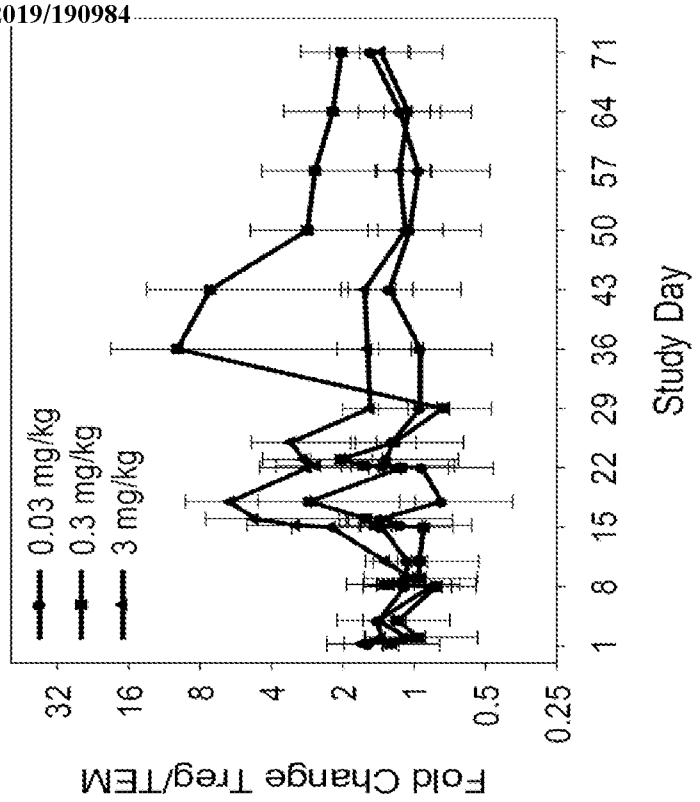


FIG. 34A

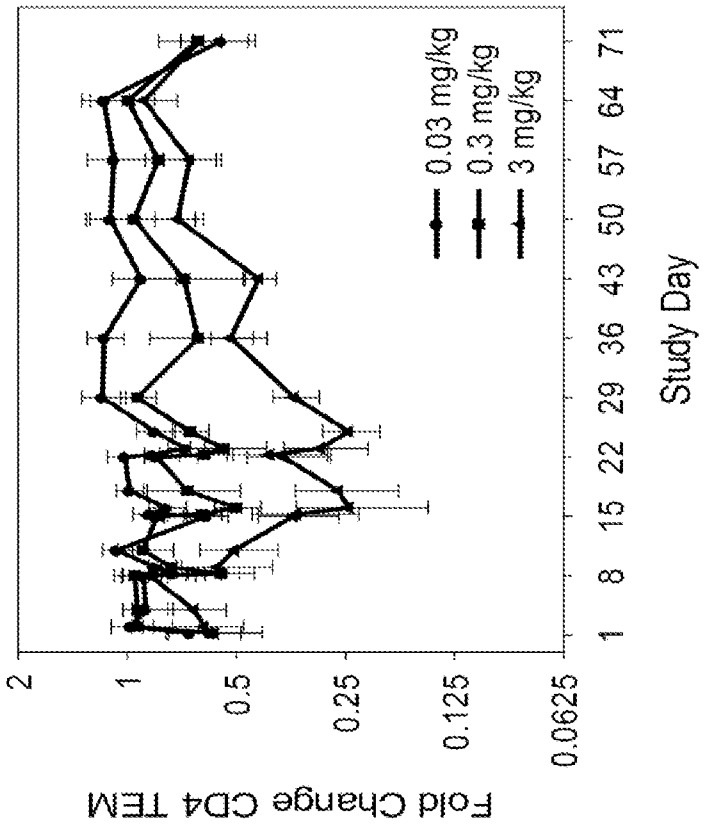


FIG. 35B

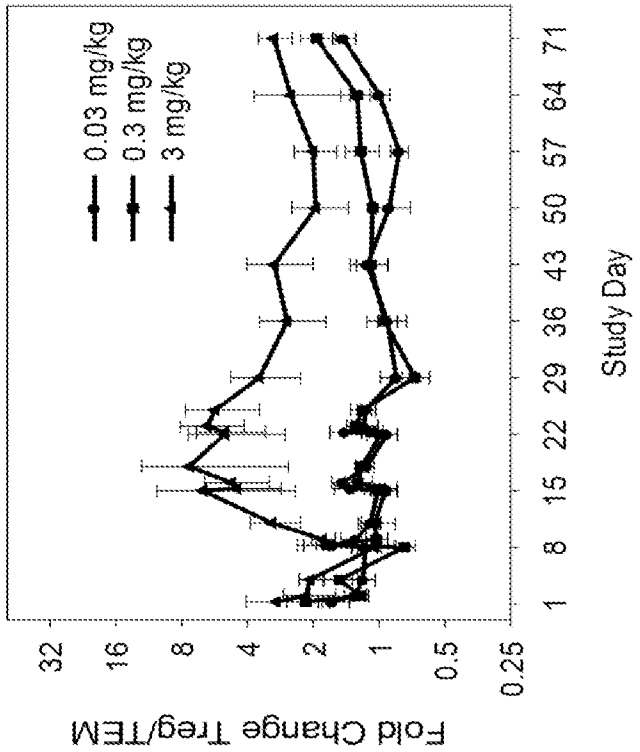


FIG. 35A

