

Figure 1
Schematic of anti-CD33, anti-CD3, and NKG2D binding proteins constructs

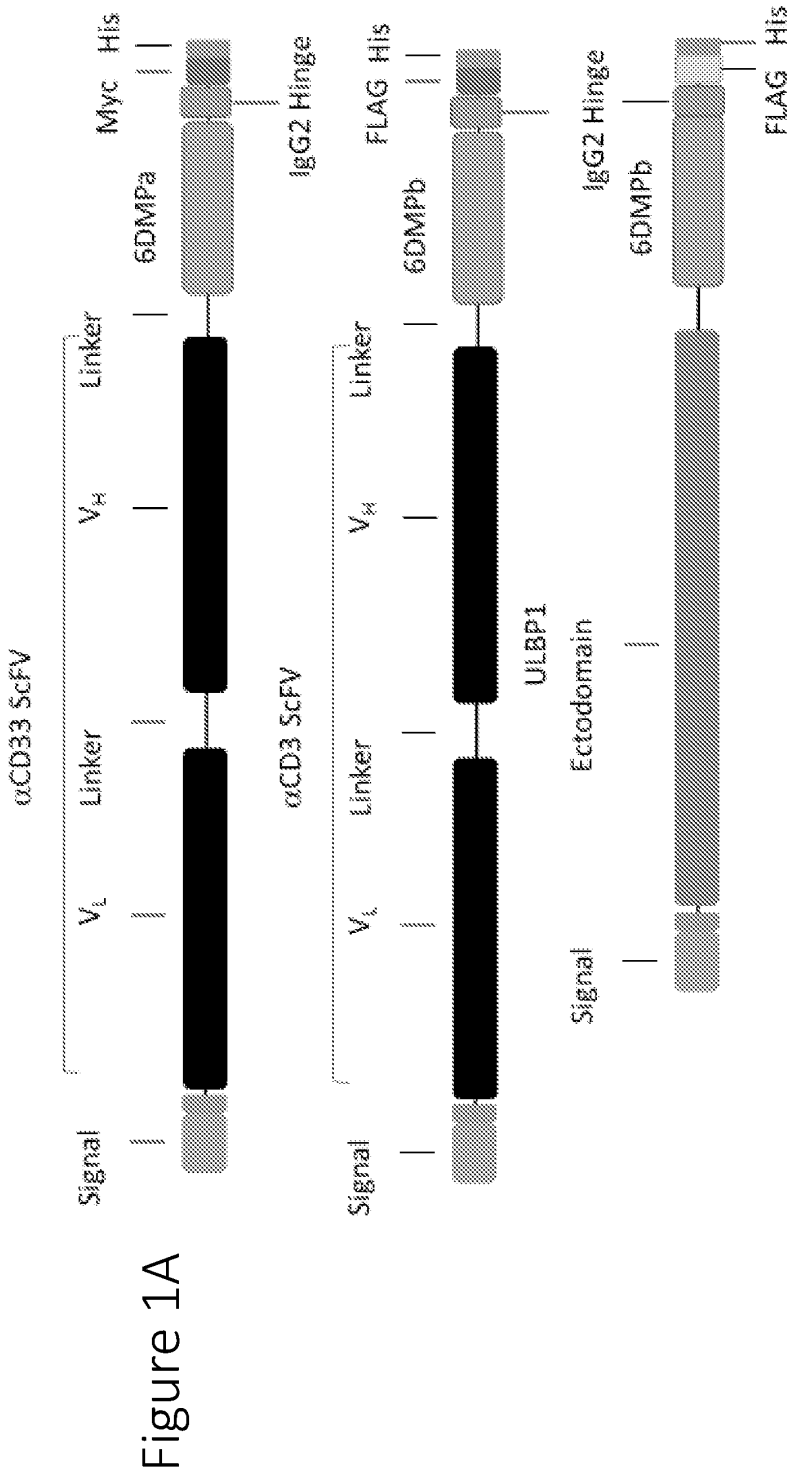


Figure 1B

Schematic of anti-CD33, anti-CD3, and NKG2D binding proteins constructs

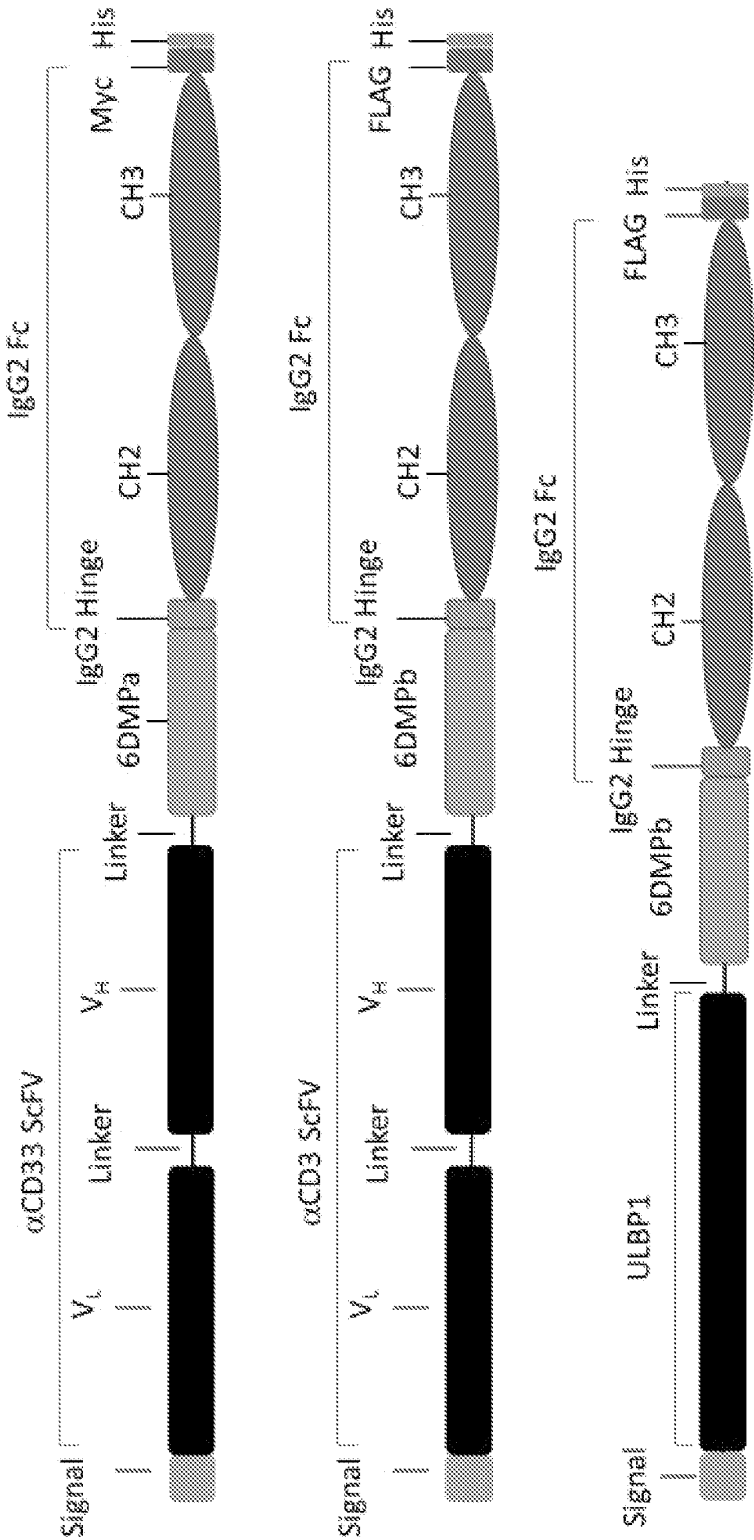


Figure 2

Schematic of bispecific protein dimers engaging an immune cell with cancer cell

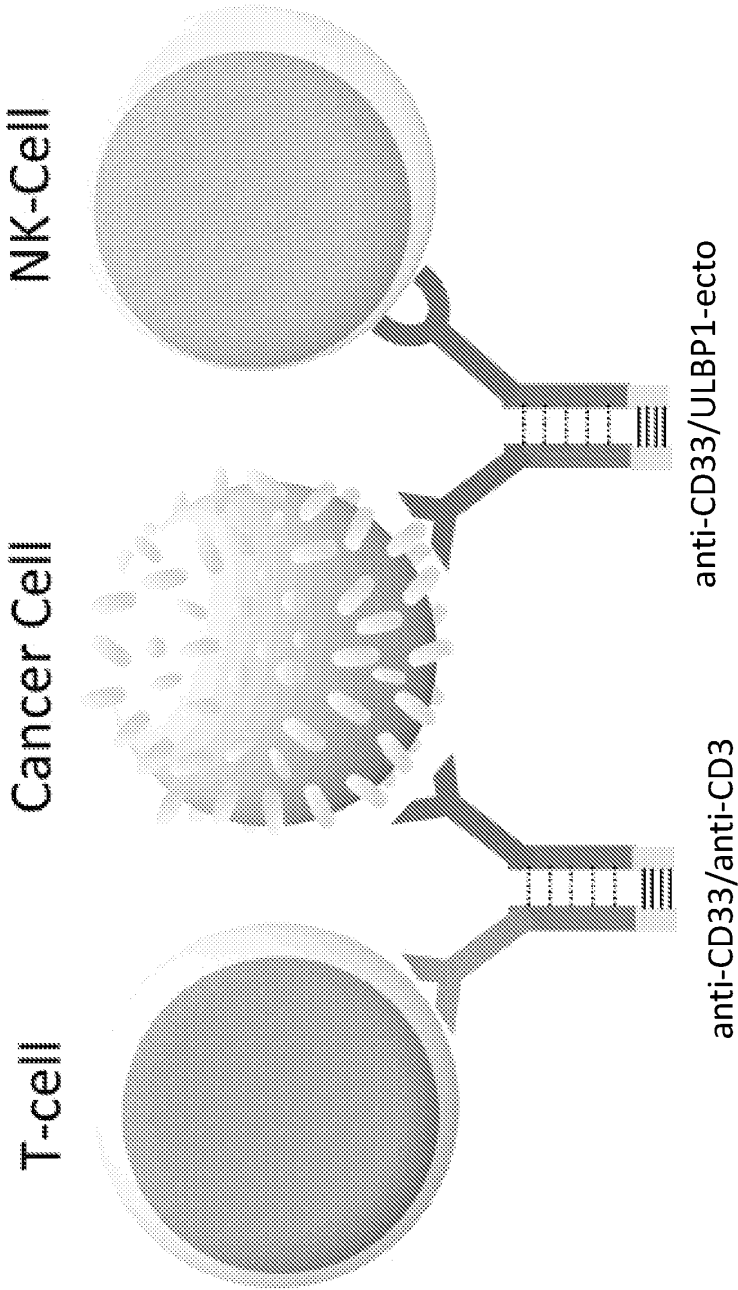


Figure 3
Amino acid sequence of anti-CD33 construct

aCD33_6DMPa_IgG2-Hinge
MYRMQLLSQIALSLALVTNSEIVLTQSPGSLAVSPGERVTMSCKSSQSVFFSSSQKNYLAWYQQIPGQSPRLLIYWASTRE
SGVPDRFTGSGSCTDFTLTISSVQPEDLAIXYCHQYLLSSRTFGQGTKLEIKRGSTSGCKPFGSGEGSTKGQVQLQQPFGAEV
VKPGASVKMSCKASGYTFTSYVHHNFKQTGCGLEWVGVIYFGNDDISYNQKFGQKATLTADKSSSTAYMQLSSLTSEDSA
VYYCAREVRLRYFDVWGQGTVTIVSSSSSAGGGSGGGSGGGSGTKEDILERQKIIERAQEIHRRQQEILEELERIIIR
KPGSSEAMKRMLKLLLEESLRLKELLEISEESAQLLYEQPERKCCVECPCEPKKLISSSDLHHHHHH

Sequence is color coded to identify different regions below:

- IL2 secretory signal
- anti-CD33 scFv
- Linker
- 6DMPa
- IgG2 Hinge
- Myc tag
- 6xHis

Figure 4
Amino acid sequence of ULBP1 construct

ULBP1_6DMPb_IgG2-Hinge
MYRMQLLSCLALSLAVTNSMVDTHCLCYDFIITPKSRPEEQWCEVQGLVDERFFLHYDCVNHKAKAFASLGKKVNVYTKTW
EEQTETLRDVVDFLKGQLLDIQVENLIPIEPLTLQARMSCEHEAHGCHGRGSNQFLFNGQKFLLFDSNNRKWTALHFGAKKM
TEKWEKNROVTMFFCKISLGDCKMNLSEFLMYWEQMLDPTKPPSLAFGGGGSGGGGGSTEKRLLEEAEERAHREQKE
I KKAQELHRRLEEI VRQSGSSEAKKEANKILLEETRELSKRSLELLREILYLSQEQKGSIVPRERKCCVECPCP
DDH HHHHHH

Sequence is color coded to identify different regions below:

- IL2 Signal
- ULBP1
- Linker
- 6DMPb
- IgG2 Hinge
- FLAG tag
- 6xHis

Sequence is color coded to identify different regions below:

IL2 signal
aCD3-VH
VH-VL linker
aCD3-VL
Linker within aCD3-VL
Linker
6DMPb
IgG2 Hinge
FLAG
6xHis

Figure 6
Amino acid sequence of anti-CD33 construct with Fc region

aCD33_6DMPa_IgG2-Hinge&Fc
MYRMQLLSCLALVLTNSSEIVLTQSPGSLAVSPGERVTMSCKSSQSVFFSSQKNYLAWYQQIFGQSPRLIIYWASTRE
SCVPDRFTGSGSGTDFLTITSSVQPEDLAIIYCHQYLSSRTFGQGTKLEIKRGSTSGSGKPGSGEGSTKGQVQLQQPGAEV
VKPGASVKMSCKASGYTFTSYIHHIKQTTPGQGLEWVGVIYPGNDDISYNGKFGKATLTADKSSSTAYMQLSSLTSEDSA
VYYCAREVRLRYFDVWGQGTVTVSSSSAGCGSGGGSGGSGTKEDILERQKII ERAQEI HRRQQEILEELERII R
KPGSSEEAAMKRMKLLEESLRLKELLESESAQLLYEQRRKCCVECPFPAPFPVAGPSVFLFPFKPKDTLMISRTPEV
TCVVVDVSHEDPEVQFNWYVDGVEVHNAKTKPREEQFNSTERVVSVLTVQHQLNGKEYKCKVSNKGLPAPIEKTISKTK
GQPREPQVYTLPPSRREMTKNQVSLTCLVKGFYPSDISVEWESNGQPENNYKTTTPNLDSDGSFFLYSKLLTVDKSRWQQGN
VFSCSVMHREALHNHYTQKSLSLSPGK
IL2 secretory signal
anti-CD33 scFv
Linker
6DMPa
IgG2 Hinge
IgG2 Fc (CH2 and CH3 domains)
Myc tag
6xHis

Sequence is color coded to identify different regions below:

Figure 7 Amino acid sequence of ULBP1 construct with Fc domain

ULBP1_6DMPb_IgG2-Hinge&Fc
MYRMQLLSIALSLALVTNSWVDTHCLCYDFIITPKSRFEFQWCEVQGLVDERPFLHYDCVNHKAKAFASLCKKVNVTKTW
EEQTETLRDVVDFLKGQLLDIQVENLPIEPLTLQARMSCEHAHGHRGSGWQFLENGQKFLLEFDSNNRKWTALHFGAKKM
TEKNEKNRDVTMFFQKISLGDCKMWLEEFMYNEQMLDPTKPPSLAPCGGGGGSGGGGGSTENRLLLEEAEARHREQKE
IINKAQELHRRLEETVRQSGSSEEAKEAKKILEEIRELSKRSLELLREILYLSQEQKGSIVPRERKCCVECPFCPPFVA
GPSVFLFPKPKDTLMISRTPEVTCVVVDVSHEDPEVQFNWYVDGVEVHNNAKTKPREEQFNSTFRVVSULTVYVHQDWLNCK
EYKCKVSNKGLPAPIERTISKTKGQPREPQVYITLPPSREEMTKNQVSLTCLVKGYFIPSDISVENESNGQPENNYKTTTPPML
DSDGSFFLYSKLTVDKSRWQQGNVFSQSVMHREALNNHYTQKSLSPCK

Sequence is color coded to identify different regions below:

- IL2 Signal
- ULBP1
- Linker
- 6DMPb
- IgG2 Hinge
- IgG2 Fc (CH2 and CH3 domains)
- FLAG tag
- 6xHis

Figure 8 Amino acid sequence of anti-CD3 construct with Fc domain

aCD3_6DMPb_IgG2-Hinge&Fc domain

MYRQQLSCIALSLAVTNSDIKIQCSGAELRPGASVKMSCKTSCYTETRYTMHWVKQRFQGGLEWIGYINPSRGY
TNYNQKFKDKATLTDDKSSSTAYMQLSSLTSEDSAVYYCARYDDHYCLDYWGCGTTLTVSSVEGCGSGSGSGGV
DDIQLTQSPAIMASPGOKVYMTORASSSVSYMNNWYQQKSGTSGKRWIYDTSKVASGVYPYRFSGSGSGTSYSLTIGSSME
AEDAATYYCOONSNPITFCAGTKLEIKGGCGSGGGSGGGSTEKRLLEEAERAHREQKEIINKKAQELHRRLEEIVRQ
SGSSEAEAKKEAKKILEEIRELSKRSLELLREILYLSQEQKGSIVPRERKCOVECPFCAPPVAGESVFLERPHKQOTIM
ISRTPEVTCVVVDVSHEDPEVQFNWYVDGVEVHNAKTKPREEQFNSTFRVVSVLTUVHQQDWLNGKEYKCKVSNKGLPAP
IEKTIISKTKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDISVEWESNGQPENNYKTTTPMLEDSDGSFFLYSKL
TVDKSRWQQGNVFPSCSVNHEALHNHYTQKSLSLSPGK HHHHHH

Sequence is color coded to identify different regions below:

- IL2 signal
- aCD3-VH
- VH-VL linker
- aCD3-VL
- IgG2 Fc (CH2 and CH3 domains)
- Linker
- 6DMPb
- IgG2 Hinge
- 6xHis

Figure 9

Figure 9A

Figure 9B

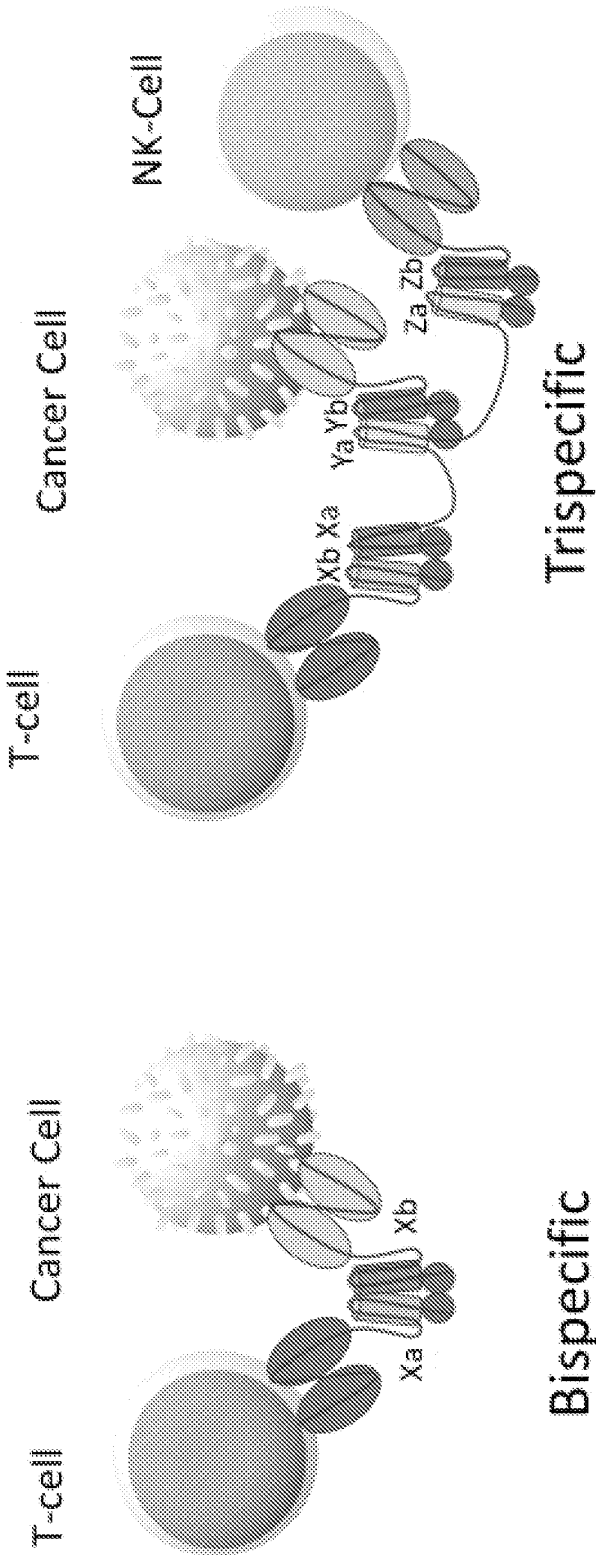


Figure 10

Figure 10A

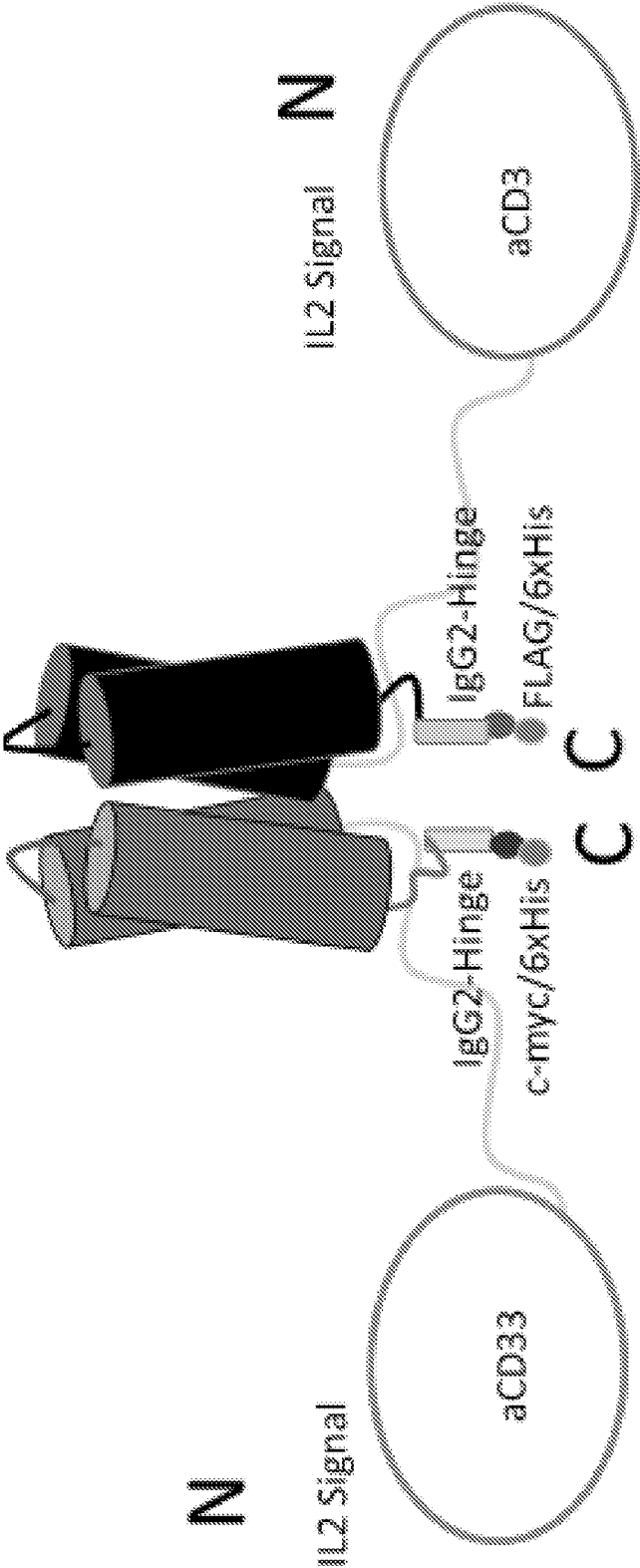


Figure 10B

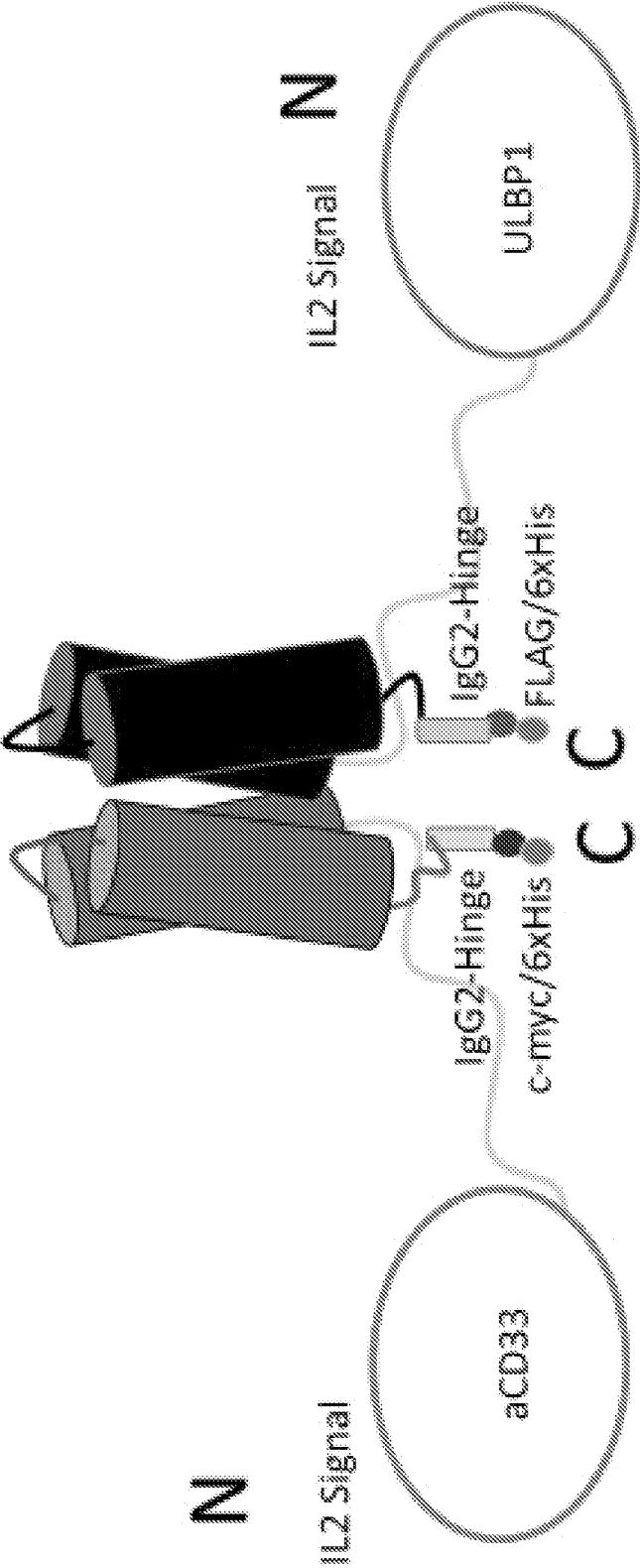
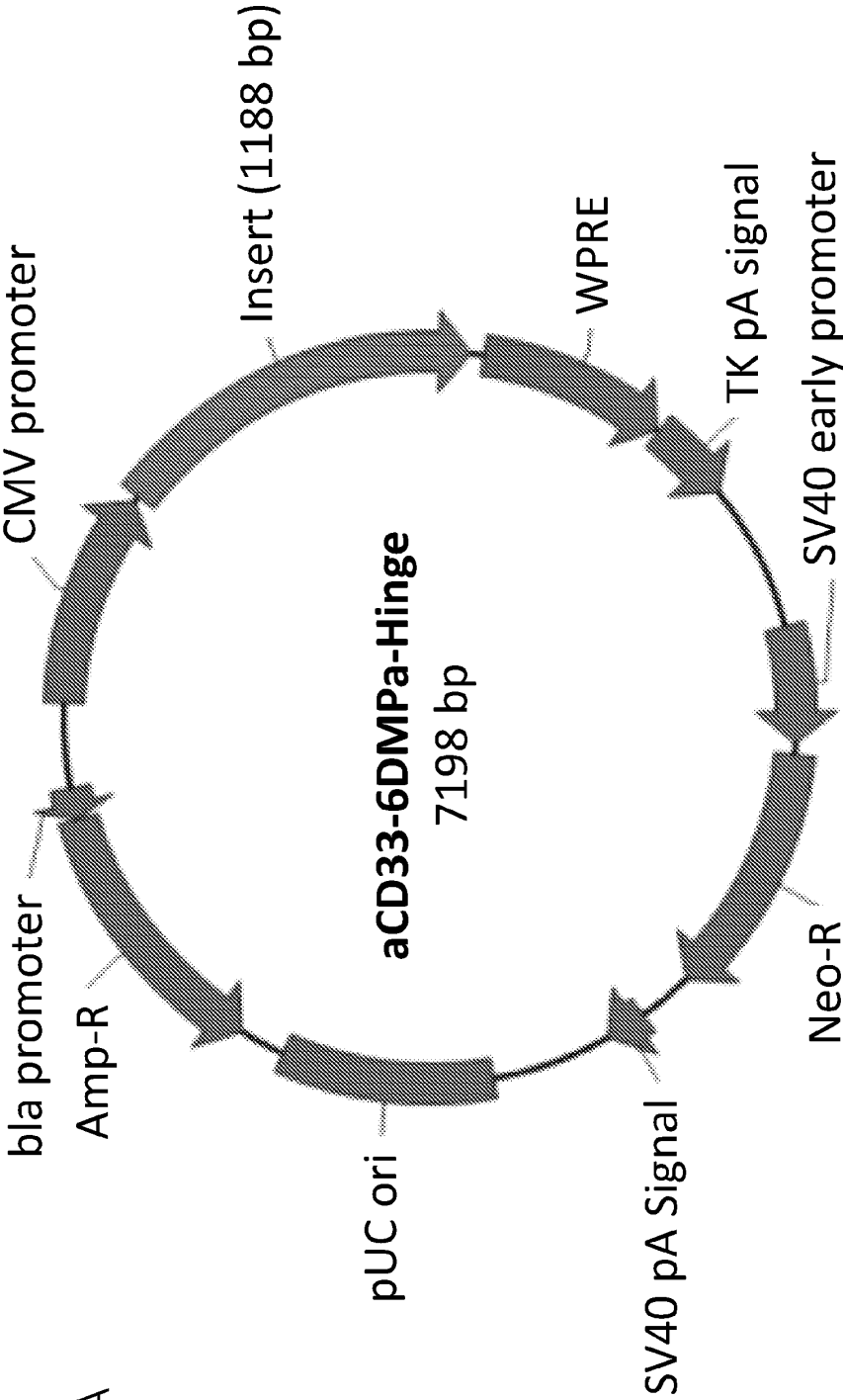


Figure 11

Figure 11A



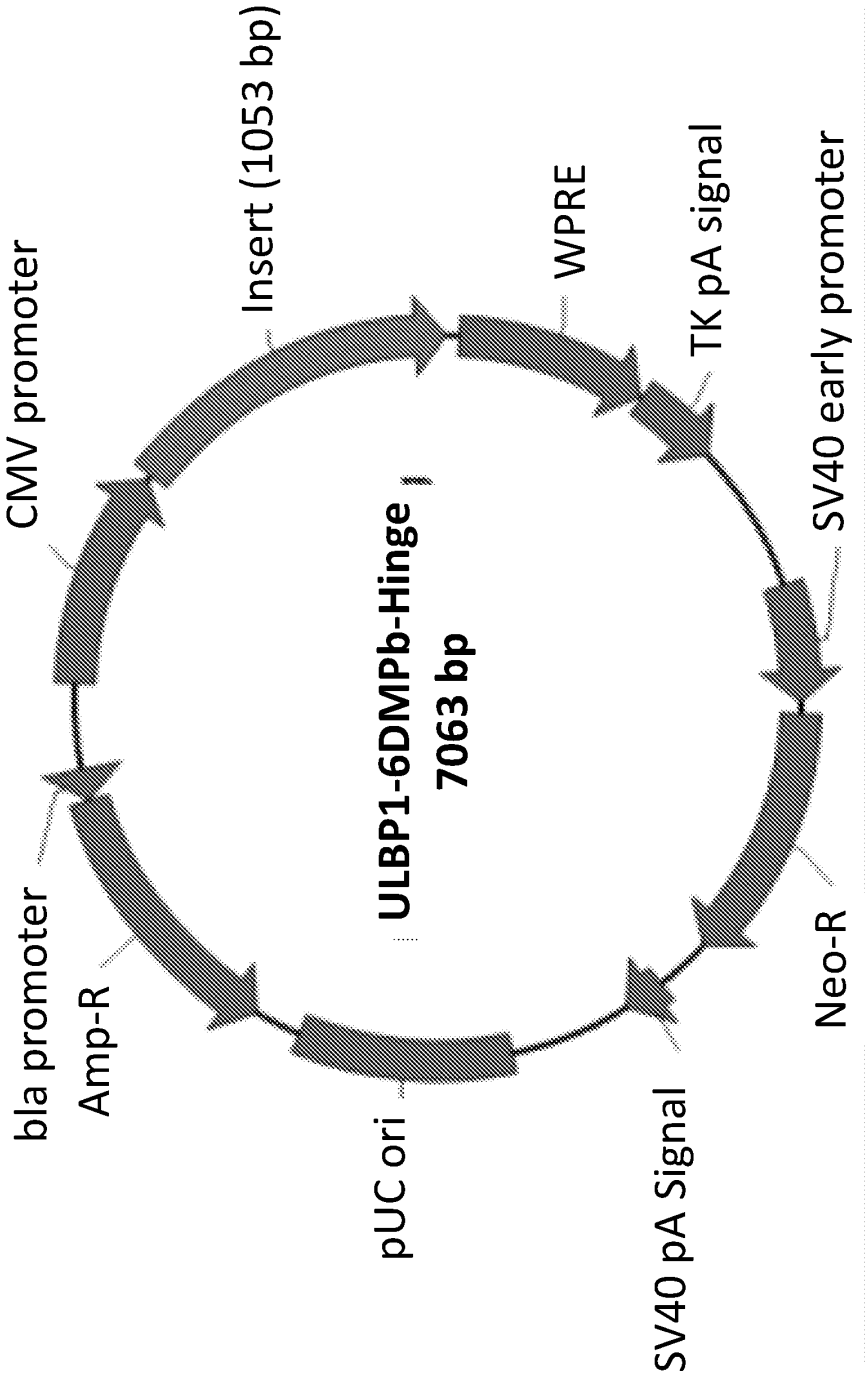


Figure 11B

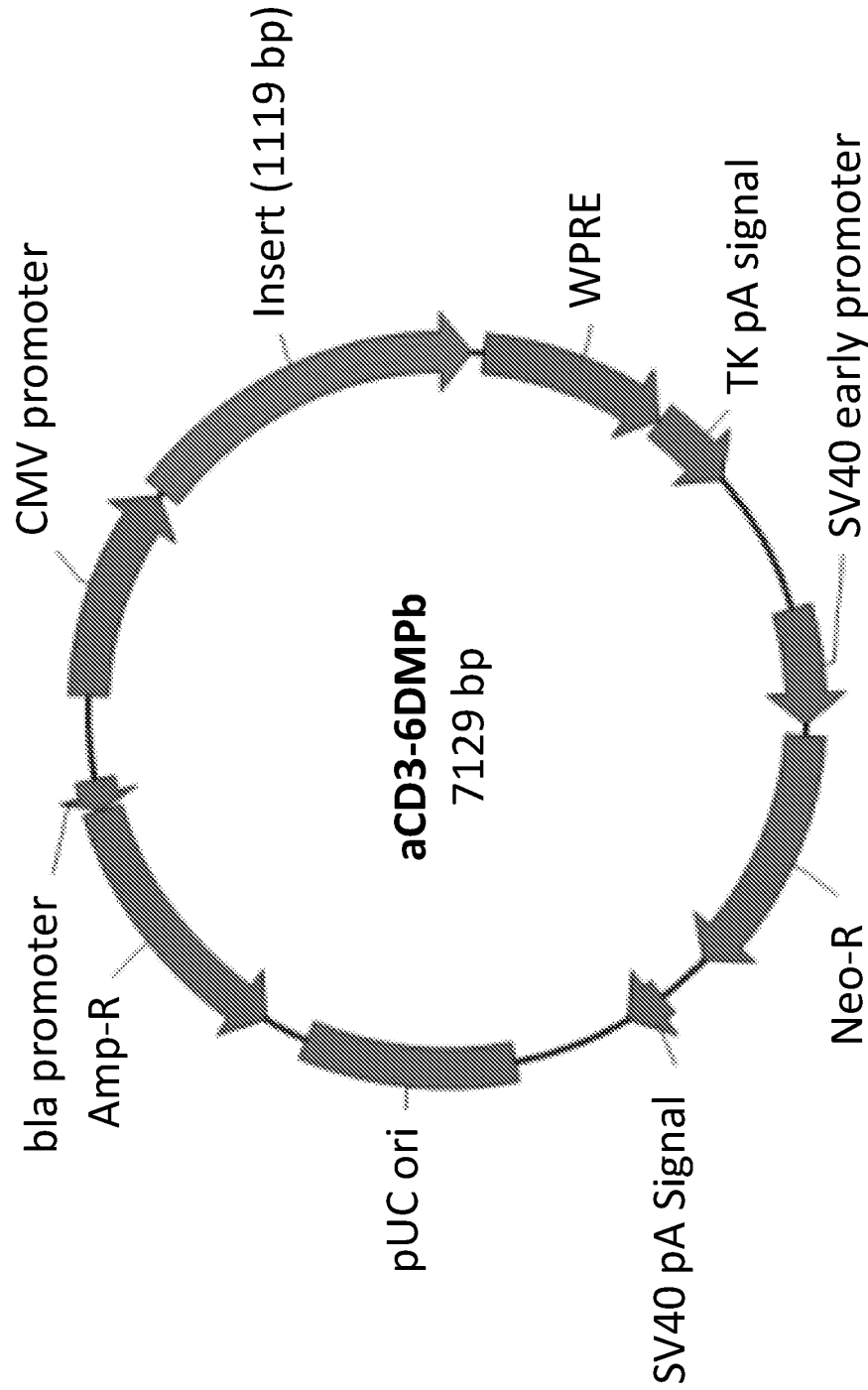


Figure 11C

Expression and purification of CD33-ULBP1 chimera protein

Figure 12

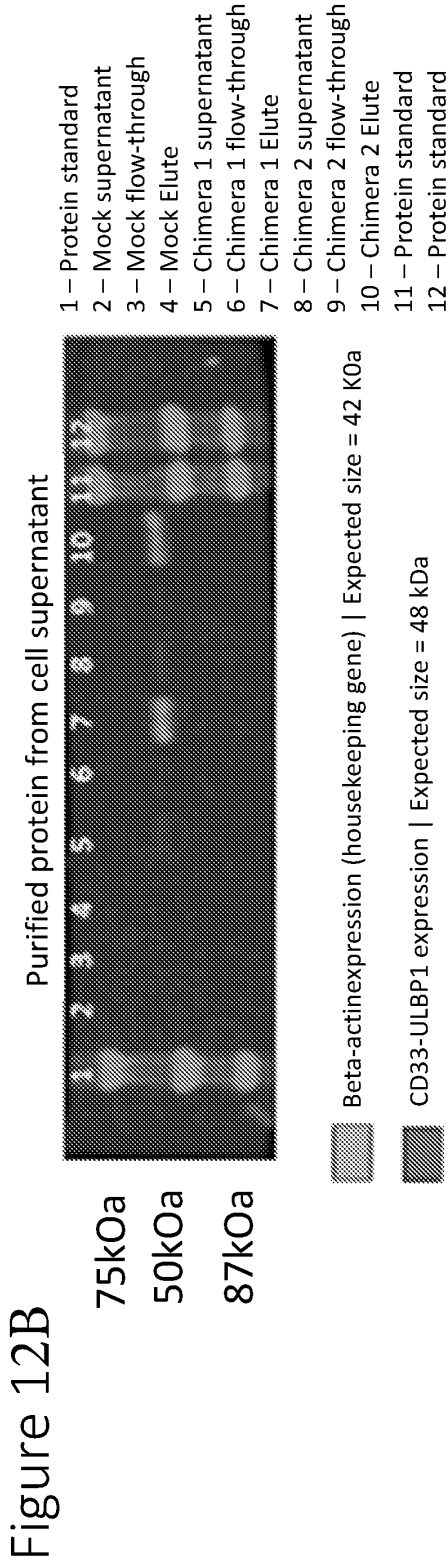
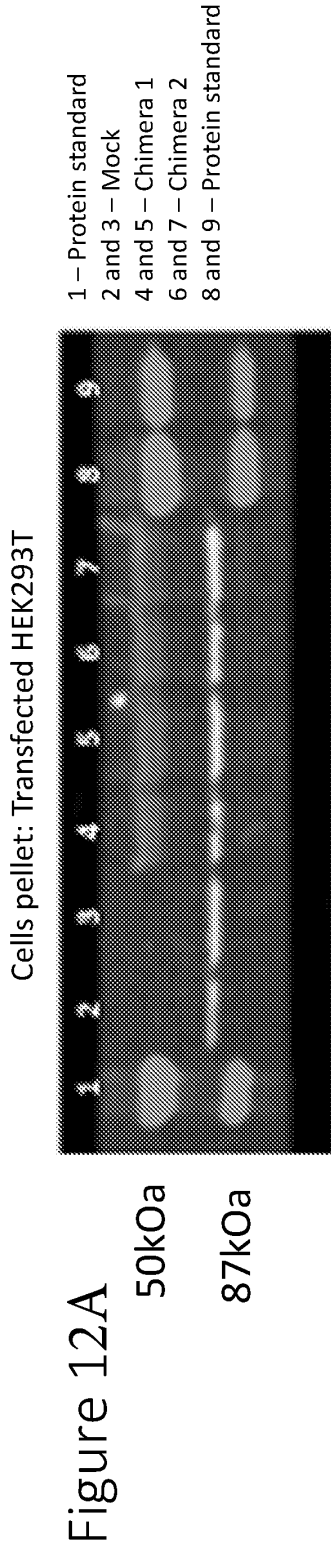


Figure 13

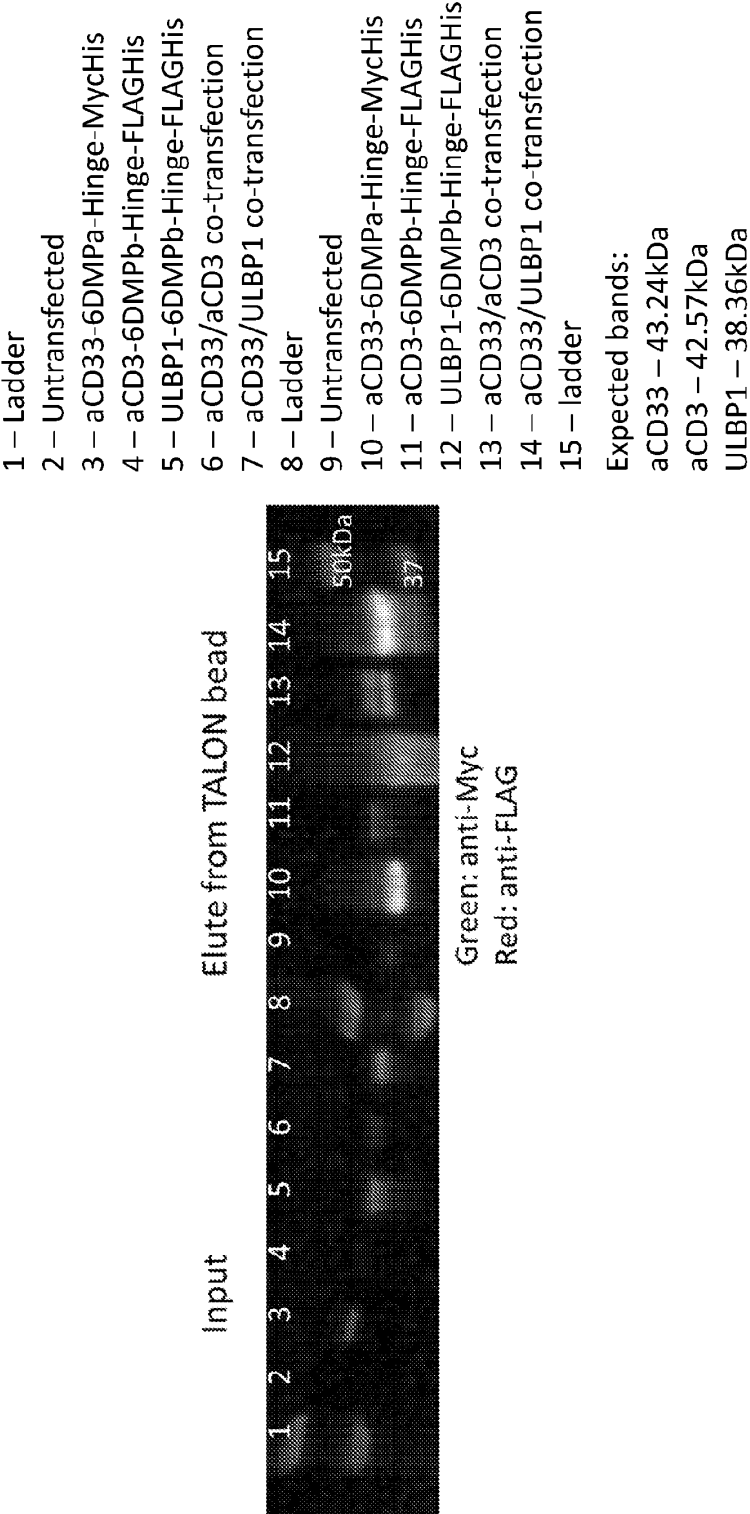
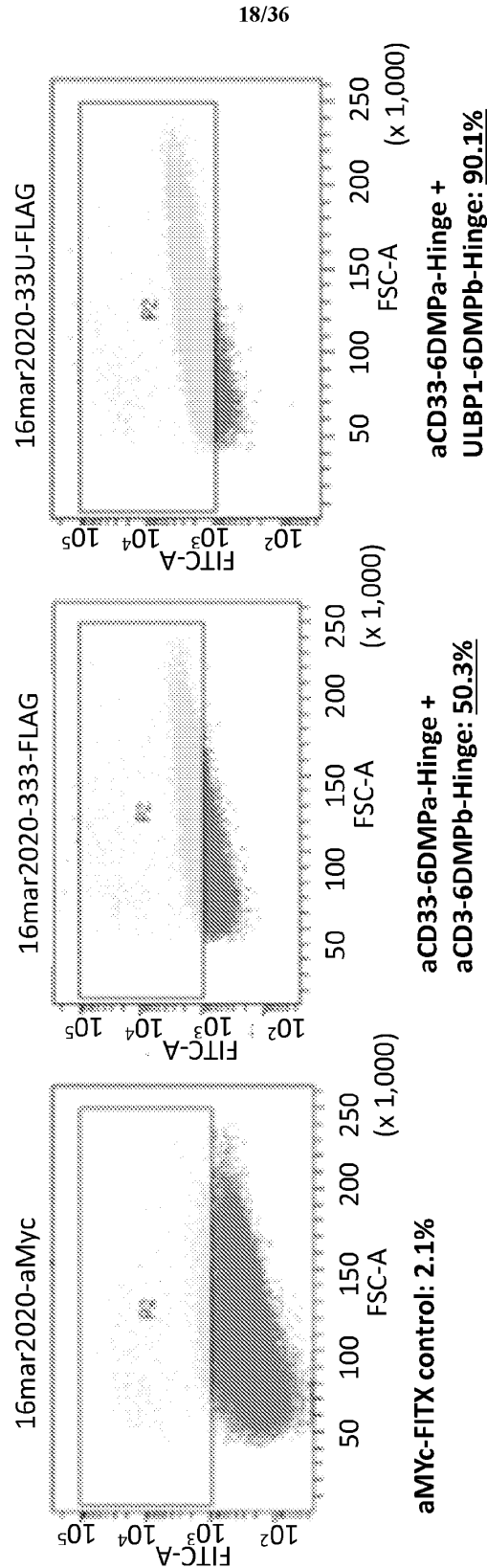


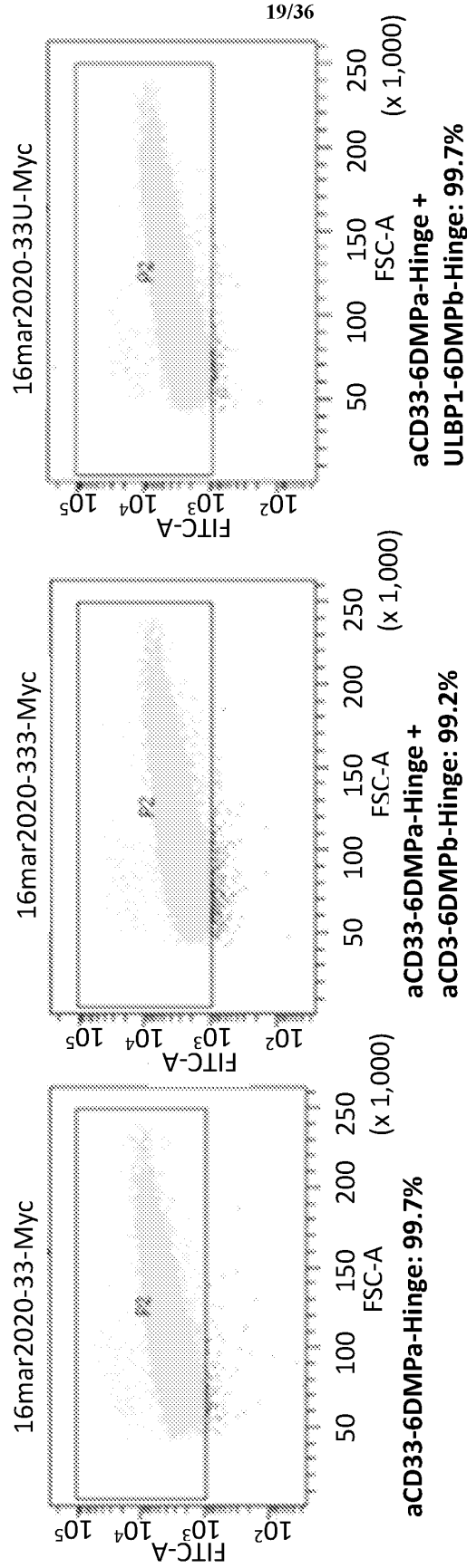
Figure 14 HL60 incubated with purified constructs and
then with antiFLAG-FITC antibodies



FLAG signal suggests presence of aCD33/ULBP1 and of
aCD33/aCD3 dimers; likely some aCD33-monomers

Figure 14A

HL60 (AML cell line) incubated with purified constructs and then with antiMYC-FITC antibodies



Suggests that the aCD33-6DMPa-Hinge construct binds cell-Surface CD33 well, and is not disrupted by 6DMPb-conjugates

Figure 14B

Figure 15

Summary of binding experiments (anti-CD33/antiCD3 and antiCD33/ULBP1) to HL-60

	antibody	Constructs				"Secondary"			
	anti-CD33- FITC	anti-CD33- GDMpA-Myc	anti-CD3- GDMpB-FLAG	ULBP1- GDMpB-FLAG	anti-Myc- FITC	anti-FLAG- FITC	%FITC+	Comments	
1	No	No	No	No	No	No	0.3	Cells only	
2	No	No	No	No	Yes	No	2.1	secondary only control, very low non-specific binding	
3	No	No	No	No	No	Yes	1.7	secondary only control, very low non-specific binding	
4	No	Yes	No	No	Yes	No	99.7	Monomer binding as expected	
5	No	Yes	No	No	No	Yes	2.1	low or no signal as expected	
6	No	No	No	Yes	Yes	No	1.8	low or no signal as expected	
7	No	No	No	Yes	No	Yes	1.9	low or no signal suggest no ULBP1 expression on HL-60	
8	No	No	Yes	No	Yes	No	2	low or no signal as expected	
9	No	No	Yes	No	No	Yes	1.8	low or no signal suggest no CD3 expression on HL-60	
10	No	Yes	Yes	No	Yes	No	99.2	Signal due to both mono and dimer	
11	No	Yes	Yes	No	No	Yes	50.3	Signal due to dimer	
12	No	Yes	No	Yes	Yes	No	99.7	Signal due to both mono and dimer	
13	No	Yes	No	Yes	No	Yes	91	Signal due to dimer	
14	Yes	No	No	No	No	No	99.9	To check CD33 expression using anti-CD33 antibody	

Figure 16

Summary of binding experiments (anti-CD33/antiCD3 and antiCD33/ULBP1) to Jurkat

S/N	Constructs			"Secondary"		%FITC+	%PE+	Comments
	anti-CD33-6DMPa-Myc	anti-CD3-6DMPb-FLAG	ULBP1-6DMPb-FLAG	anti-Myc-FITC	anti-FLAG-PE			
1	No	No	No	No	No	0.1	0	Cells only
2	No	No	No	Yes	No	3.4	0.9	Myc antibody control
3	No	No	No	No	Yes	0	1.2	FLAG antibody control
4	Yes	No	No	Yes	No	4.1	1.4	Low or no CD33 on Jurkat
5	No	Yes	No	Yes	No	5.3	2.2	Low as expected (aCD3-construct lacks myc tag)
6	No	No	Yes	Yes	No	4.8	1.7	Low as expected (ULBP1-construct lacks myc tag)
7	Yes	Yes	No	Yes	No	35.7	2.5	aCD33 construct is on Jurkat surface due to aCD3-half of the dimer; stoichiometry is likely an issue, as well as population shift increasing FITC
8	Yes	No	Yes	Yes	No	7.6	2.5	Low or no CD33 and NKGD2D on Jurkat
9	Yes	No	No	No	Yes	0	2.2	Low as expected (aCD33-construct lacks FLAG)
10	No	Yes	No	No	Yes	0.6	99.8	High binding of aCD3 construct to CD3 on Jurkat
11	No	No	Yes	No	Yes	0.1	2.8	Low or no NKGD2D on Jurkat
12	Yes	Yes	No	No	Yes	0.6	99.8	High binding of aCD3 construct to CD3 on Jurkat; aCD33-6DMPa construct doesn't seem to inhibit binding (stoichiometry issue: compare to row 7)
13	Yes	No	Yes	No	Yes	0.1	3.5	Low or no CD33 and NKGD2D on Jurkat

Figure 17

Summary of binding experiments (anti-CD33/antiCD3 and antiCD33/ULBP1) to PBMC

	Constructs				"Secondary"			
	anti-CD33- 6DMPa-Myc	anti-CD33- 6DMPb- FLAG	ULBP1- 6DMPb- FLAG	anti-Myc- FITC	anti-FLAG- PE	%FITC ⁺	%PE ⁺	
1	No	No	No	No	No	1.5	1.2	Cells only
2	No	No	No	Yes	No	2.1	1.3	Myc secondary control
3	No	No	No	No	Yes	1.3	4.4	FLAG secondary control
4	Yes	No	No	Yes	No	1.3	1	Low or no CD33
5	No	Yes	No	Yes	No	1.1	1	Low as expected (aCD33-constructs lack myc tag)
6	No	No	Yes	Yes	No	1.5	1.1	Low as expected (ULBP1-constructs lack myc tag)
7	Yes	Yes	No	Yes	No	32.6	6	Some aCD33 bound, via the aCD33/aCD3 heterodimer
8	Yes	No	Yes	Yes	No	11.5	2.1	Some aCD33 bound, via the aCD33/ULBP1 heterodimer
9	Yes	No	No	No	Yes	0.6	3.3	Low as expected (aCD33 constructs lack FLAG)
10	No	Yes	No	No	Yes	1.4	97.6	High binding of aCD33-constructs to CD33+ selected PBMC population
11	No	No	Yes	No	Yes	1	98	High binding of ULBP1-constructs to CD33+ selected PBMC population
12	Yes	Yes	No	No	Yes	1.4	96.8	High binding of aCD33-constructs to CD33+ selected PBMC population; not harmed by aCD33-6DMPa
13	Yes	No	Yes	No	Yes	0.9	99.1	High binding of ULBP1-constructs to CD33+ selected PBMC population; not harmed by aCD33-6DMPa

Figure 18

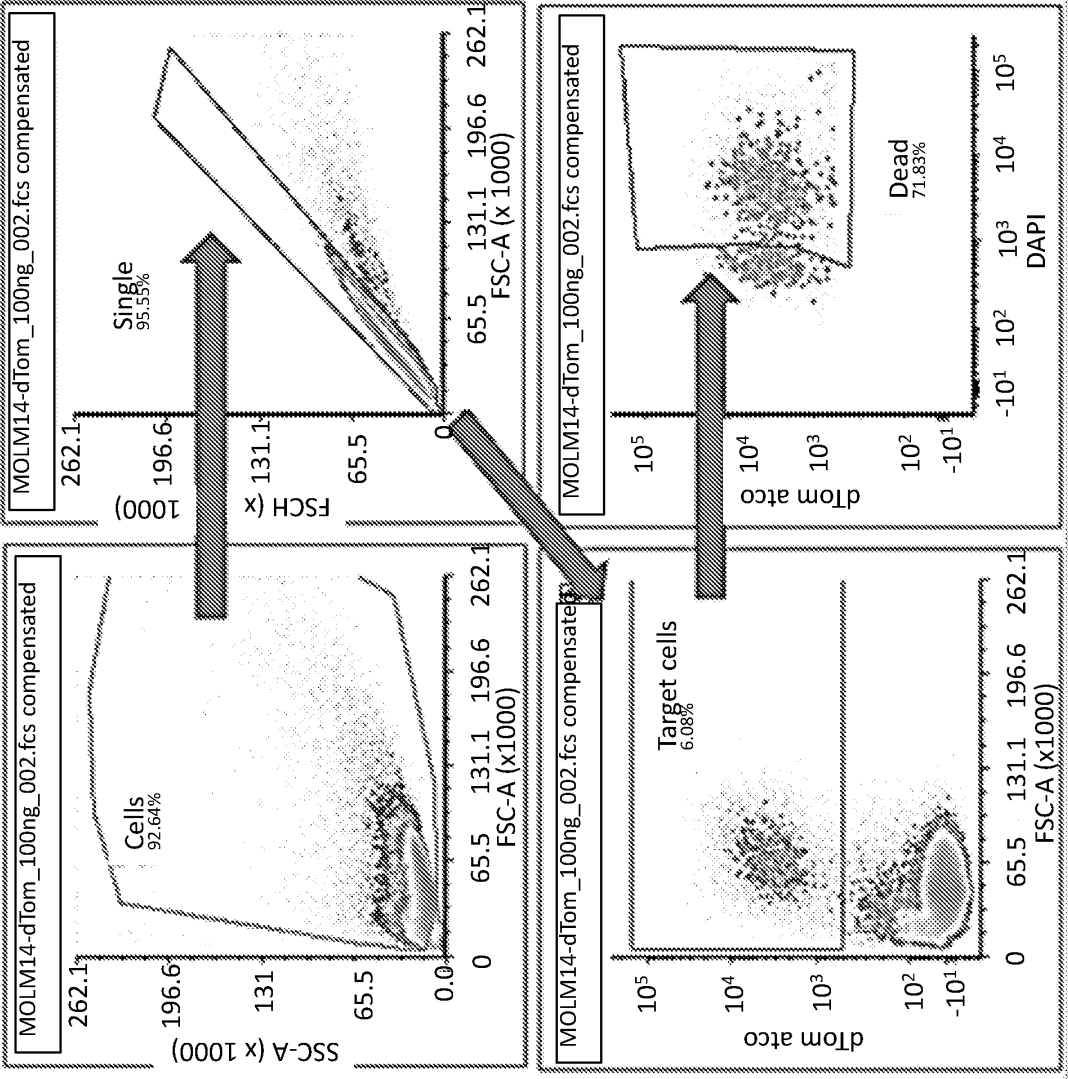


Figure 18A

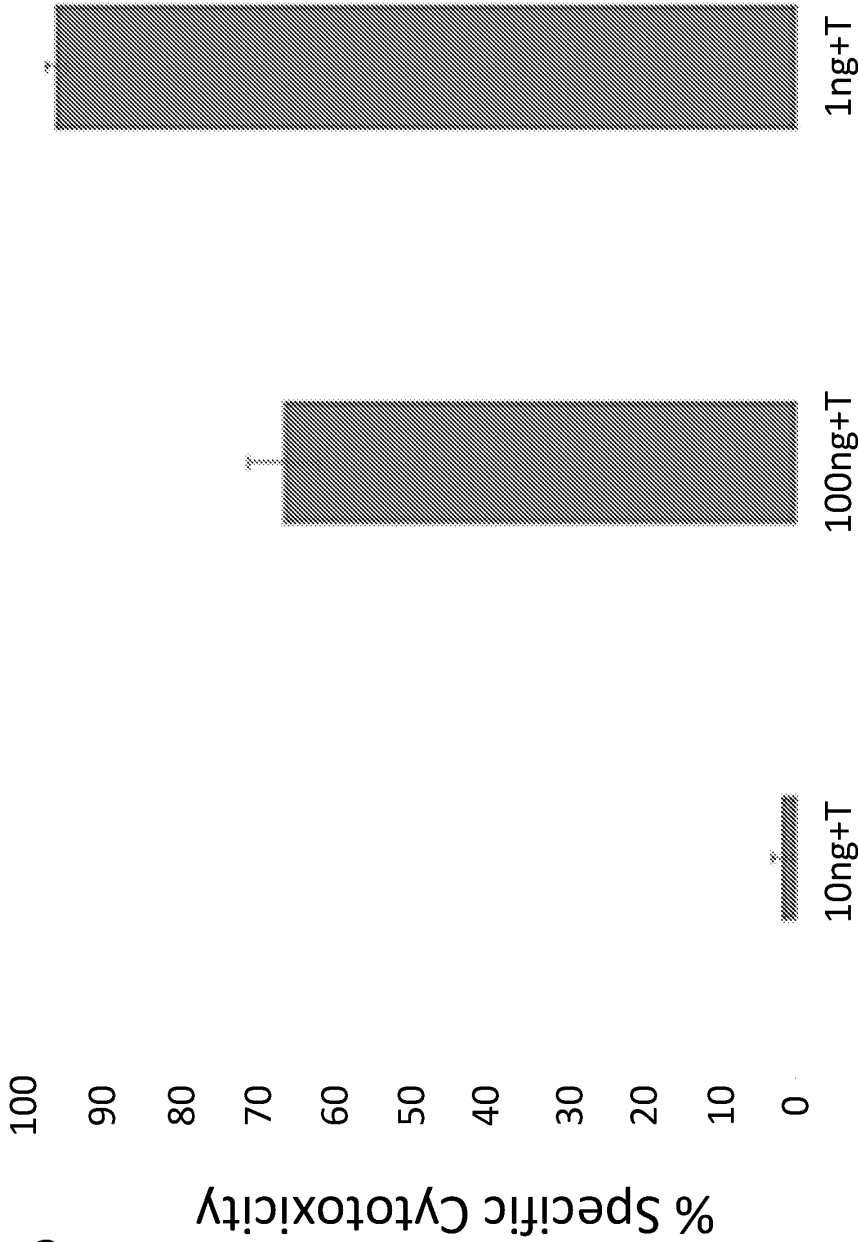


Figure 18B

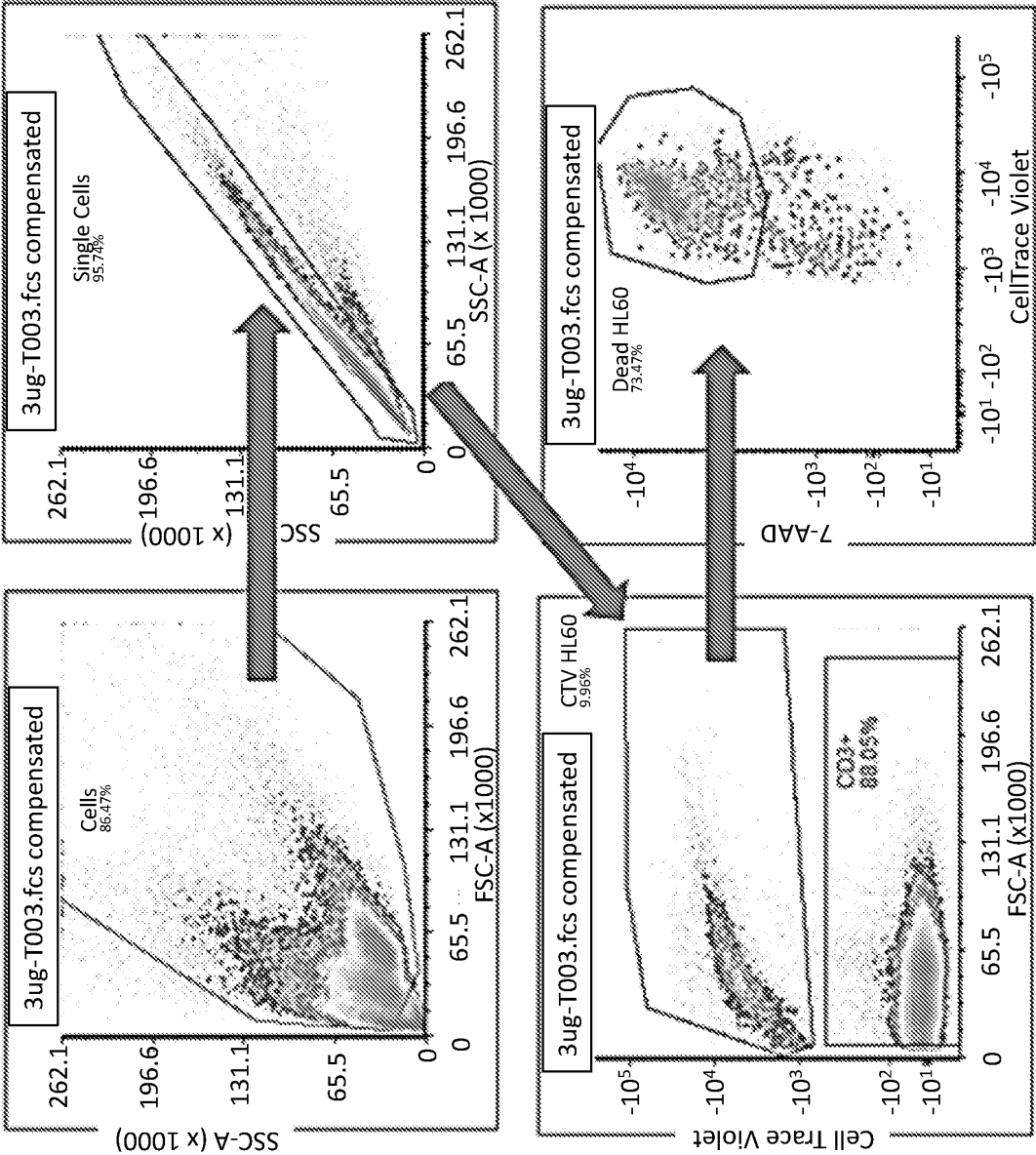
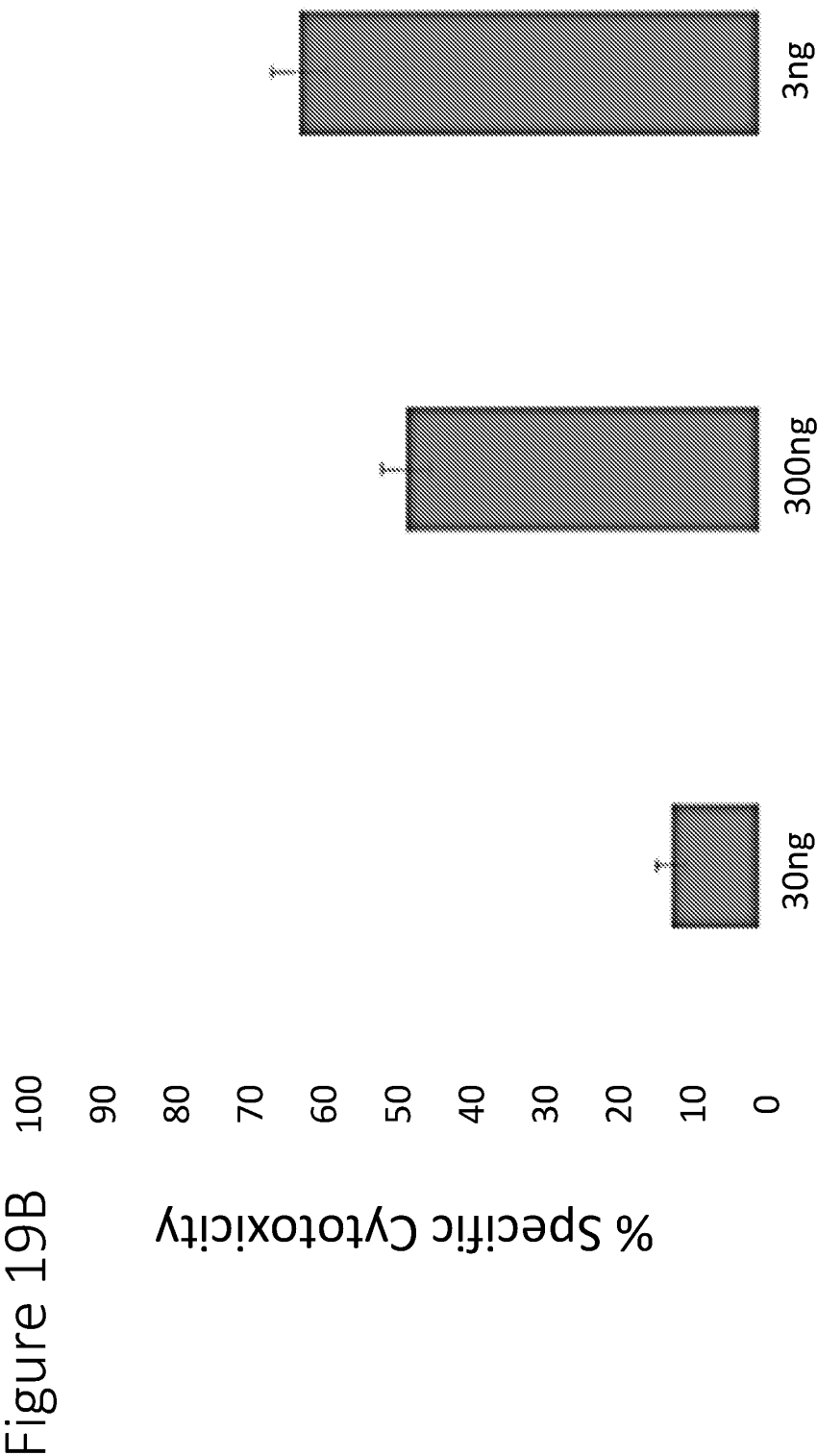


Figure 19

Figure 19A



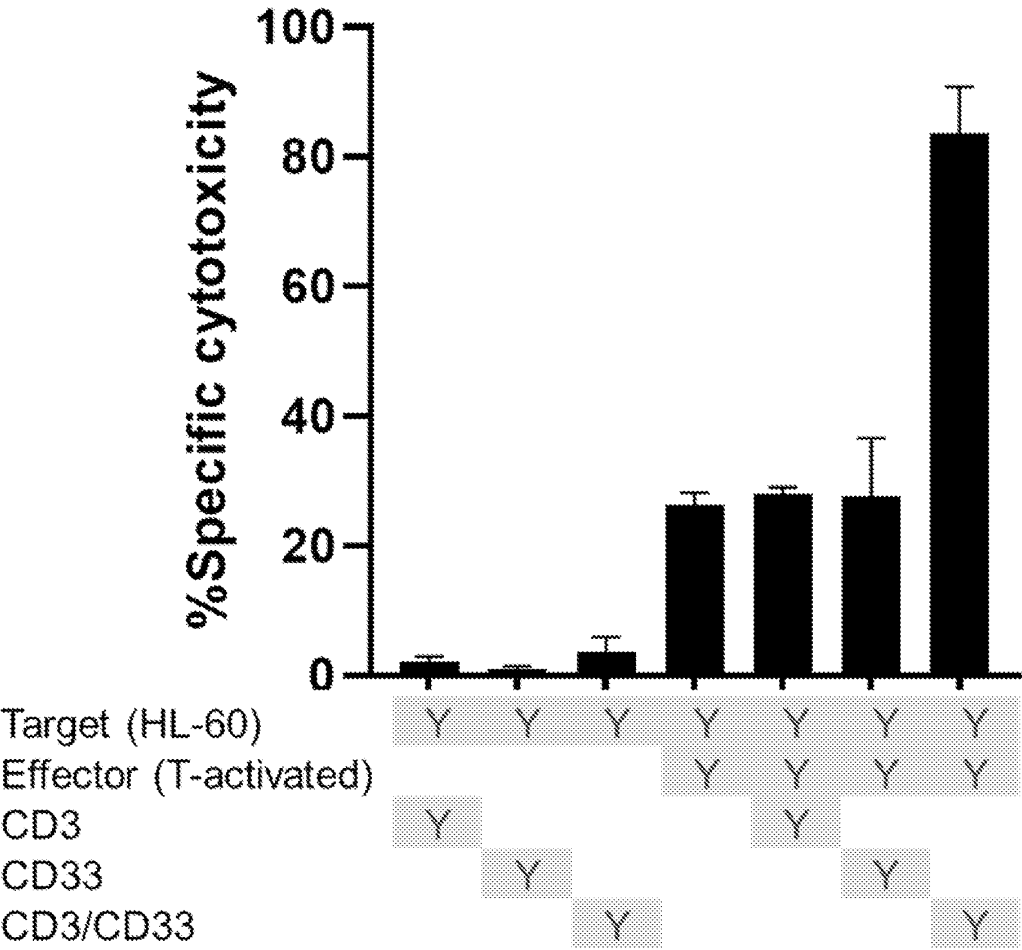


Figure 20

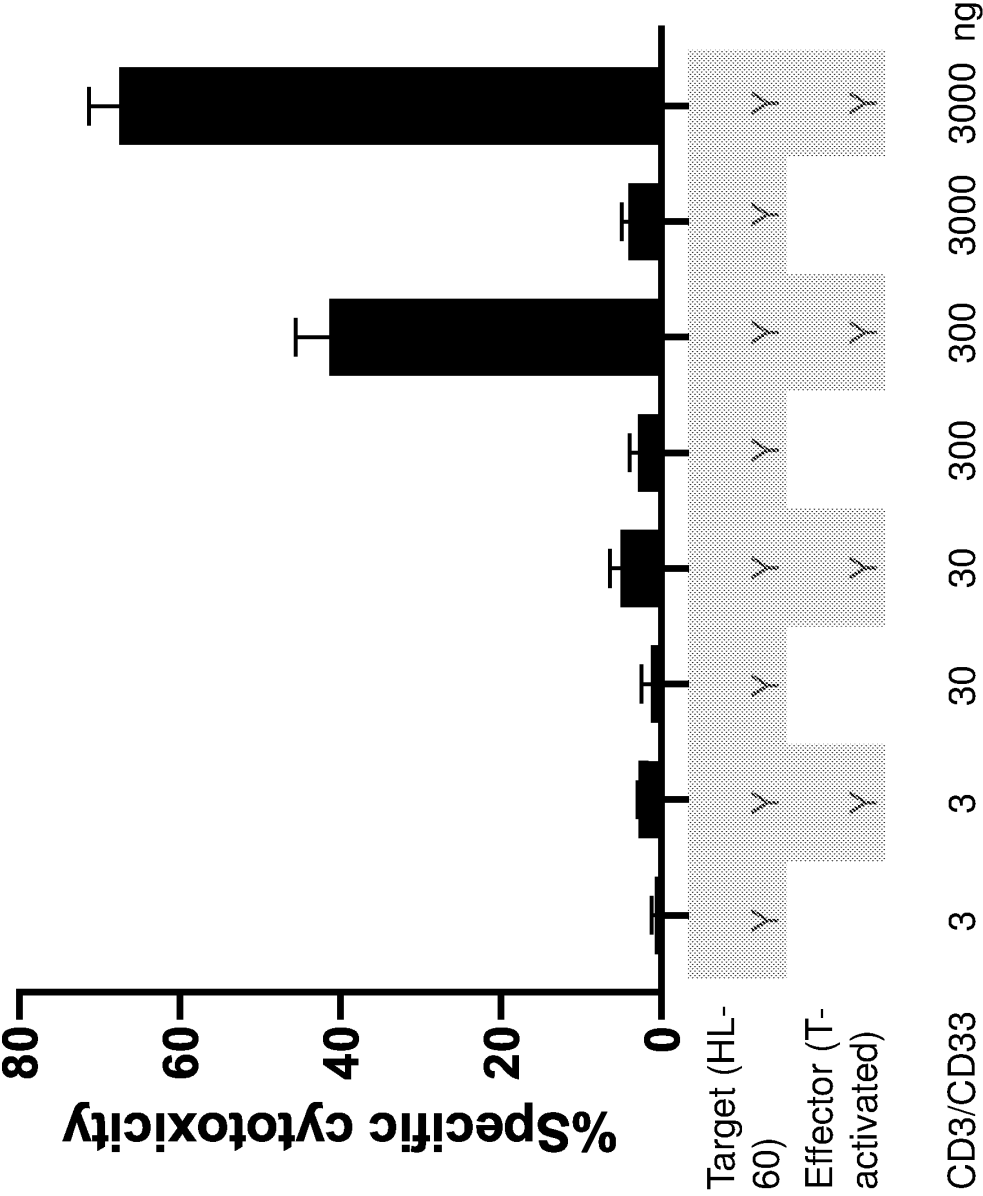


Figure 21

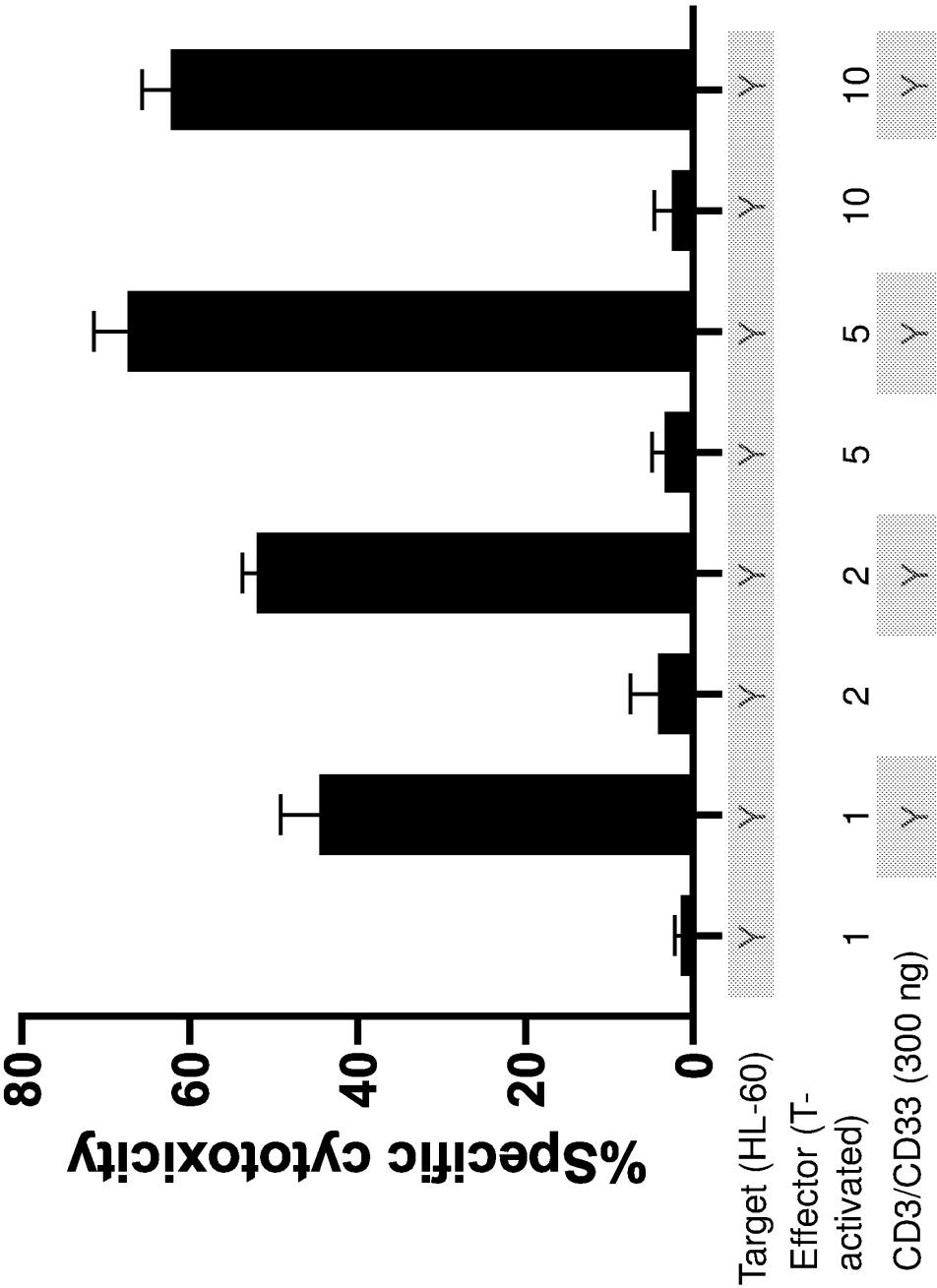


Figure 22

Figure 23

Anti-CD16_6DMPb_IgG2-Hinge

MYRMQLLSICIALSLALVTNSEVQLVESGGGVVRFPGGSLRLSCAASGFTEDDYGMSWVRQAPGKG
LEWVSGINWNGGSTGYADSVKGRFTISRDNAKNSLYLQMNSLRAEDTAVYYCARGSRLLFDYWG
QGTLVTVSRGGGGSGGGSGGGSGGGSSSELTQDPAVSVVALGQTVRITCQGGDSLRSYYASWY
QQKPGQAPVLYYGKNNRPSGIPDRFSGSSSGNTASLTITGAQAKDEADYYCNSRDSNGNHVVF
GGGTKLTVGGGGSGGGSGGGSGGGSTEKRLLEEAEERAHREQKEIKKAQELHRRLEEIVRQSGSS
EEAKKEAKKILEEIRELSKRSLELLREILYLSQEKGSLVPRERKCCVECPFDYKDDDDKHH
HHHH

IL2 signal

aCD16--VH

VH-VL linker

aCD16--VL

linker

6DMPb

IgG2 Hinge

6xHis|

Figure 23A

Figure 23B

67115

Figure 24

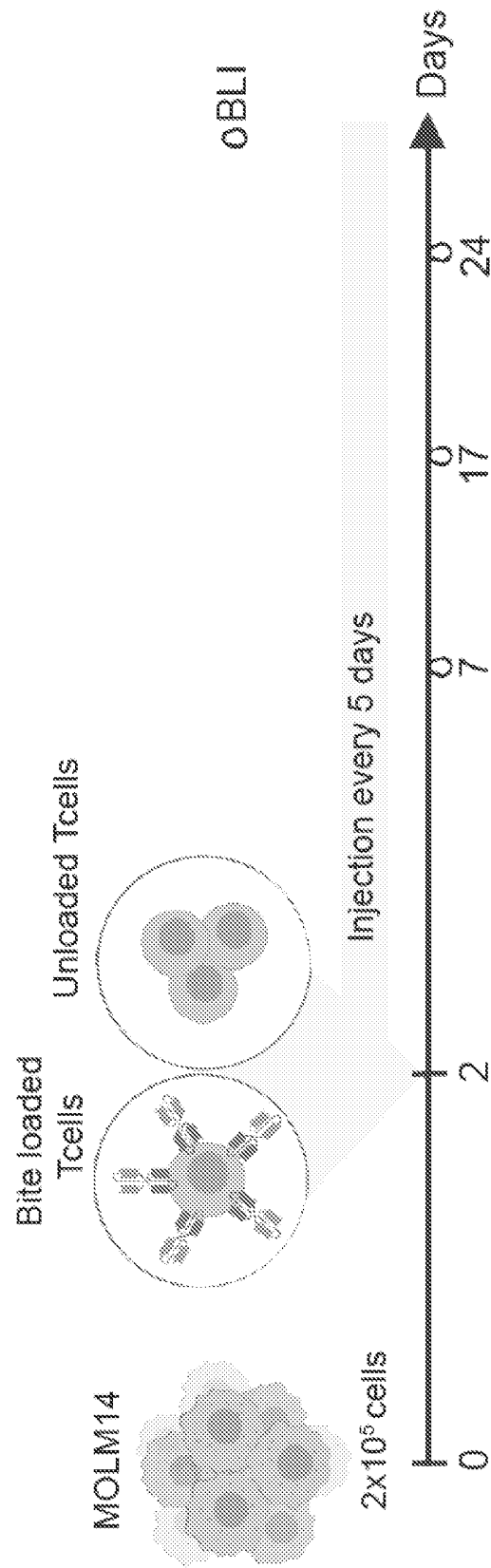
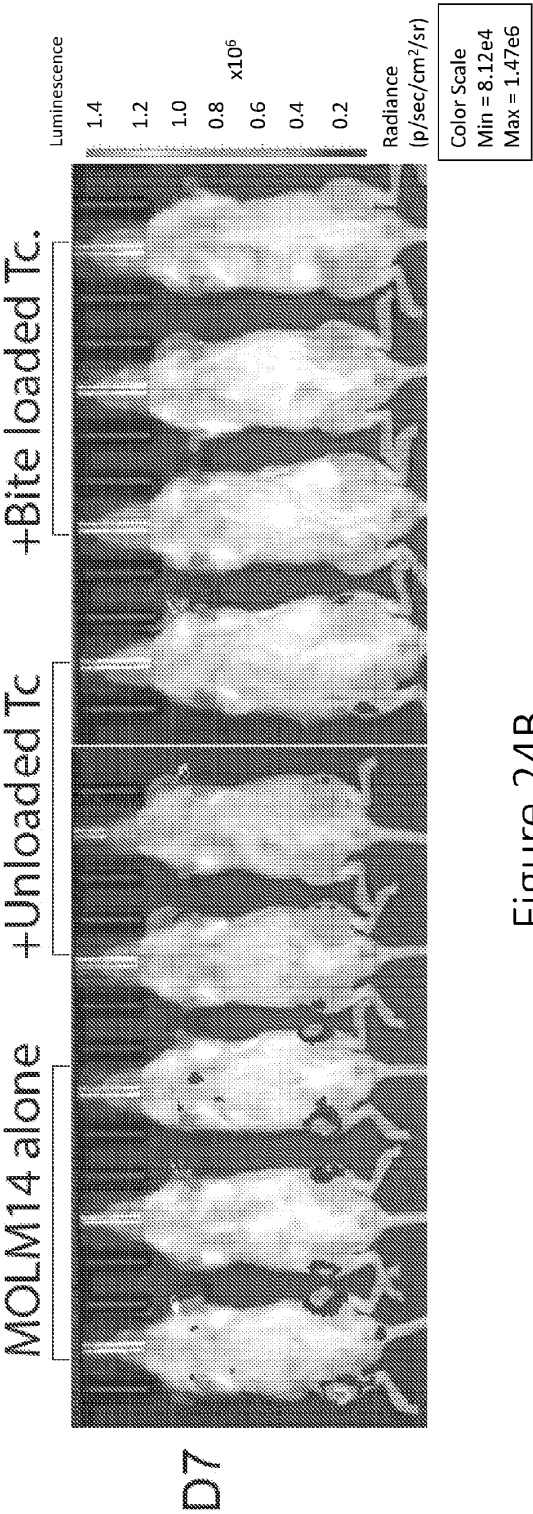


Figure 24A



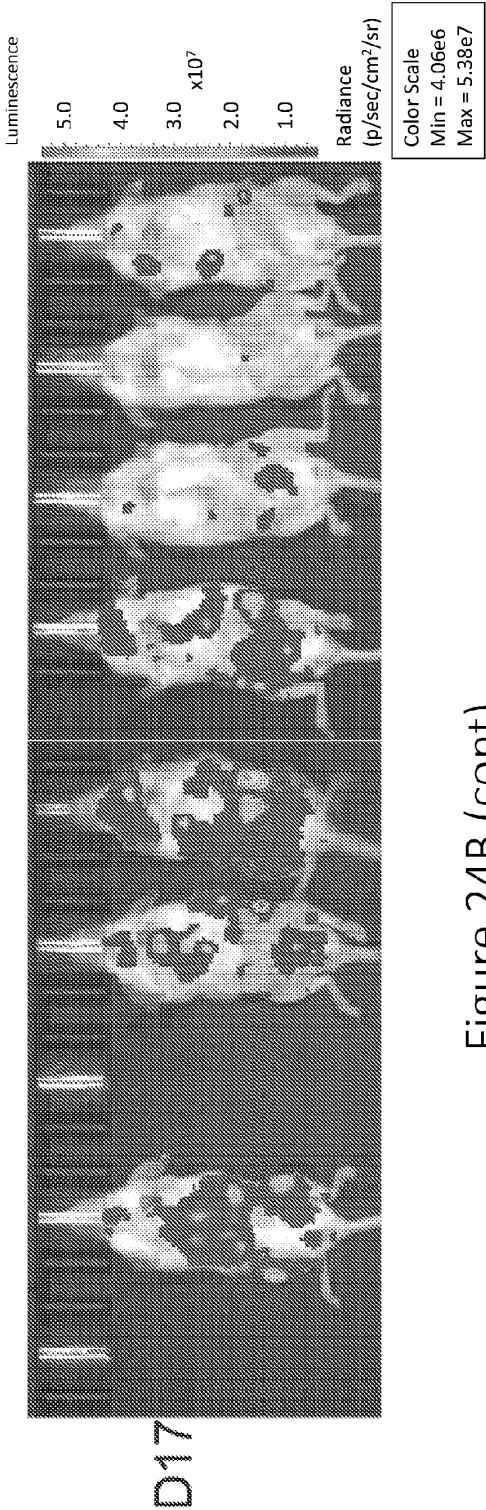


Figure 24B (cont)

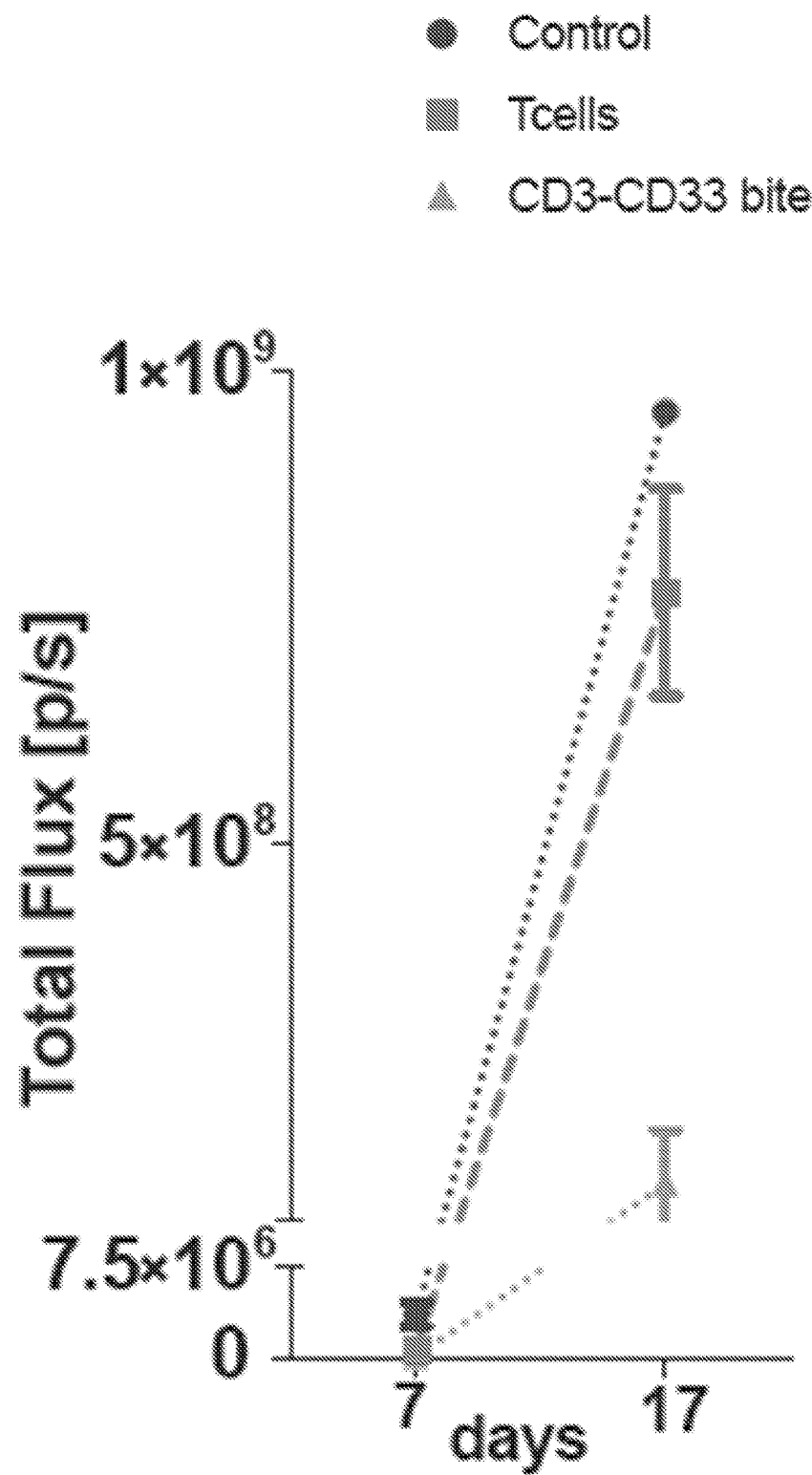


Figure 24C

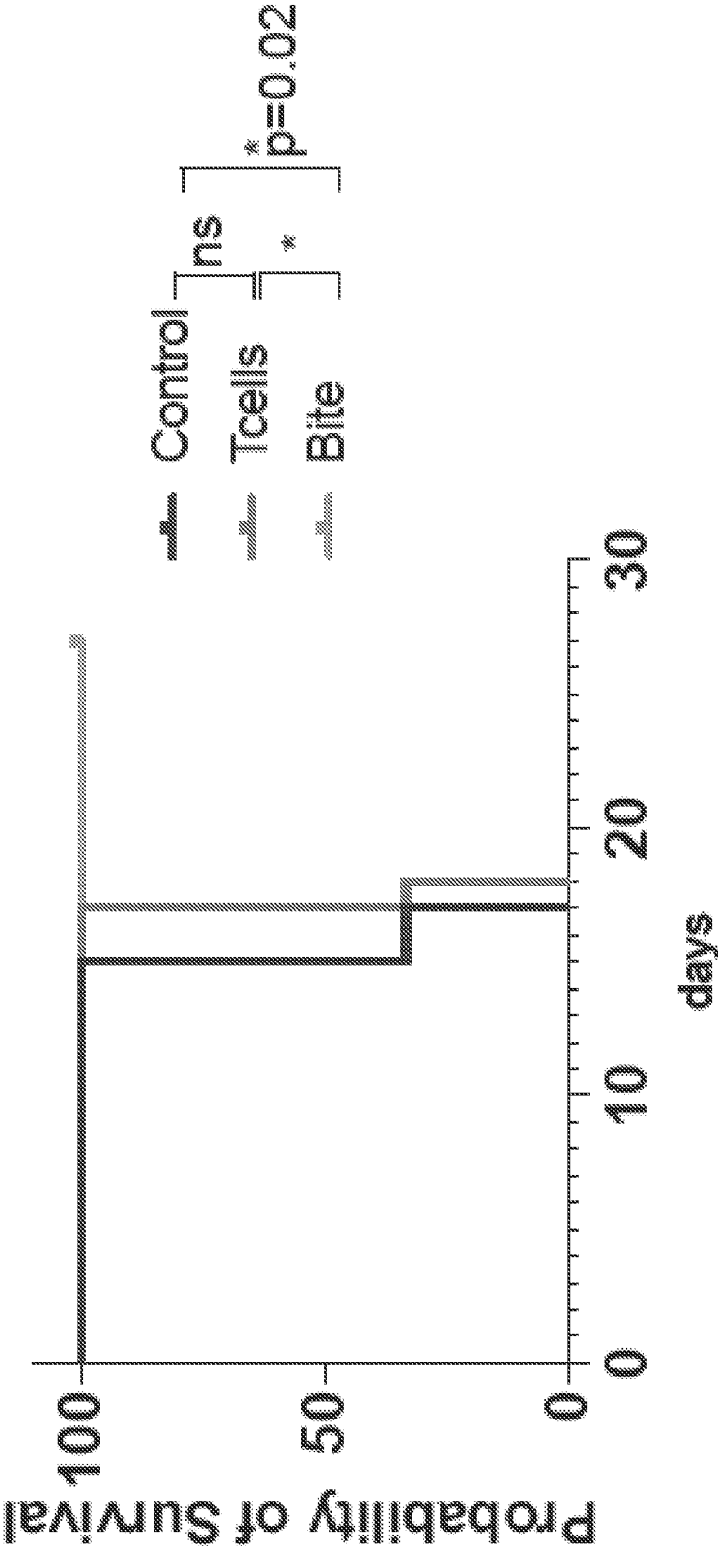


Figure 24D