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(54) Titre : RECEPTEUR D'ANTIGENE CHIMERE (CAR) COMPRENANT UN DOMAINE DE LIAISON A CD19
(54) Title: CHIMERIC ANTIGEN RECEPTOR (CAR) COMPRISING A CD19-BINDING DOMAIN

(57) Abrégé/Abstract:
There is provided a chimeric antigen receptor (CAR) comprising a CD19-binding domain which comprises a) a heavy chain variable region (VH) having complementarity determining regions (CDRs) with the following sequences: CDR1 → GYAFSSS (SEQ ID No. 1); CDR2 → YPGDED (SEQ ID No. 2) CDR3 → SLLYGDYLDY (SEQ ID No. 3); and b) a light chain variable region (VL) having CDRs with the following sequences: CDR1 → SASSSVSYMH (SEQ ID No. 4); CDR2 → DTSKLAS (SEQ ID No. 5) CDR3 → QQWNINPLT (SEQ ID No. 6). There is also provided a cell comprising such a CAR, and the use of such a cell in the treatment of cancer, in particular a B cell malignancy.

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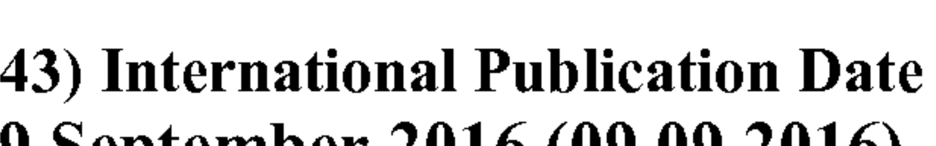

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(54) Title: CHIMERIC ANTIGEN RECEPTOR (CAR) COMPRISING A CD19-BINDING DOMAIN

(57) Abstract: There is provided a chimeric antigen receptor (CAR) comprising a CD19-binding domain which comprises a) a heavy chain variable region (VH) having complementarity determining regions (CDRs) with the following sequences: CDR1 – GYAFSSS (SEQ ID No. 1); CDR2 – YPGDED (SEQ ID No. 2) CDR3 – SLLYGDYLDY (SEQ ID No. 3); and b) a light chain variable region (VL) having CDRs with the following sequences: CDR1 – SASSSVSYMH (SEQ ID No. 4); CDR2 – DTSKLAS (SEQ ID No. 5) CDR3 – QQWNINPLT (SEQ ID No. 6). There is also provided a cell comprising such a CAR, and the use of such a cell in the treatment of cancer, in particular a B cell malignancy.

CHIMERIC ANTIGEN RECEPTOR (CAR) COMPRISING A CD19-BINDING DOMAIN

FIELD OF THE INVENTION

The present invention relates to a chimeric antigen receptor (CAR) which binds the B-lymphocyte antigen CD19 (Cluster of Differentiation 19). T cells expressing such a CAR are useful in the treatment of cancerous diseases such as B-cell leukemias and lymphomas.

BACKGROUND TO THE INVENTION

Chimeric Antigen Receptors

Traditionally, antigen-specific T-cells have been generated by selective expansion of peripheral blood T-cells natively specific for the target antigen. However, it is difficult and quite often impossible to select and expand large numbers of T-cells specific for most cancer antigens. Gene-therapy with integrating vectors affords us a solution to this problem: transgenic expression of Chimeric Antigen Receptor (CAR) allows large numbers of T-cells specific to any surface antigen to be easily generated by *ex vivo* viral vector transduction of a bulk population of peripheral blood T-cells.

The most common forms of these molecules are fusions of single-chain variable fragments (scFv) derived from monoclonal antibodies which recognise a target antigen, fused via a spacer and a transmembrane domain to a signalling endodomain. Such molecules result in activation of the T-cell in response to recognition by the scFv of its cognate target. When T cells express such a CAR, they recognize and kill target cells that express the target antigen. Several CARs have been developed against tumour associated antigens, and adoptive transfer approaches using such CAR-expressing T cells are currently in clinical trial for the treatment of various cancers. To-date however, the main clinical exploration and potential application of CAR therapy is as treatment for B-cell malignancies.

CARs directed against CD19

CD19 is a B-cell antigen which is expressed very early in B-cell differentiation and is only lost at terminal B-cell differentiation into plasma cells. Hence, CD19 is

expressed on all B-cell malignancies apart from multiple myeloma. It is not expressed on other haematopoietic populations or non-haematopoietic cells and therefore targeting this antigen should not lead to toxicity to the bone marrow or non-haematopoietic organs. Loss of the normal B-cell compartment is considered an
5 acceptable toxicity when treating lymphoid malignancies, because although effective CD19 CAR T cell therapy will result in B cell aplasia, the consequent hypogammaglobulinaemia can be treated with pooled immunoglobulin.

CD19 is therefore an attractive CAR target. To date, the main clinical focus of the
10 CAR field has been studies targeting CD19 on refractory B-cell cancers, as summarised in Table 1.

Different designs of CARs have been tested against CD19 in different centres, as outlined in Table 1:

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Centre	***Binder***	***Endodomain***	***Comment***
University College London	Fmc63	CD3-Zeta	Low-level brief persistence
Memorial Sloane Kettering	SJ25C1	CD28-Zeta	Short-term persistence
NCI/KITE	Fmc63	CD28-Zeta	Long-term low-level persistence
Baylor, Centre for Cell and Gene Therapy	Fmc63	CD3-Zeta/ CD28-Zeta	Short-term low-level persistence
UPENN/Novartis	Fmc63	41BB-Zeta	Long-term high-level persistence

Table 1. Summary of CAR experience targeting CD19

Most of the studies have tested CD19 CARs based on a scFv derived from the hybridoma fmc63. The most promising have been in the treatment of Acute
20 Lymphoblastic Leukaemia (ALL).

Clinical Experience with CARs against CD19

CD19 directed CAR therapy appears most effective in ALL. The first studies in ALL
25 were published in Spring 2013, by groups from Memorial Sloane Kettering (Brentjens,

et al. (2013) Leukemia. Sci. Transl. Med. 5, 177ra38) and the University of Pennsylvania. An update report of the latter study has recently been made (Maude et al. (2014) N. Engl. J. Med. 371, 1507–1517). Here, 25 patients under the age of 25 years and 5 over this age were treated. 90% achieved a complete response at one month, 22 of 28 evaluable cases achieved an MRD negative status and the 6 month event free survival rate was 67%. 15 patients received no further therapy after the study.

Brentjens et al., (as above) in the adult setting, treated 5 ALL patients (2 with refractory relapse, 2 with MRD positive disease and 1 who was MRD negative) with autologous T cells retrovirally transduced to express a CD19 CAR incorporating an scFv derived from the SJ25C1 hybridoma and a CD28 co-stimulatory domain. All of these achieved a deep molecular remission, enabling 4 of these patients to receive an allogeneic SCT. This precluded assessment of the durability of responses but CAR T cells were only detectable in the blood or bone marrow for 3-8 weeks after infusion. The patient who was not transplanted relapsed at 90 days with CD19+ disease. Subsequently, Davila et al. ((2014). Sci. Transl. Med. 6, 224ra25) have updated this cohort. 14 of 16 adult patients had detectable disease at the point of CAR T cell infusion, despite salvage chemotherapy and cyclophosphamide conditioning. 14 of 16 achieved a complete remission with or without count recovery including 7 of 9 patients with morphologic evidence of residual disease detectable after salvage chemotherapy. 12 of 16 patients achieved MRD negativity and this allowed 7 to undergo allogeneic transplantation by the time of publication. Responses were durable in some patients with 4 of 8 non-transplanted patients continuing in morphological remission at up to 24 months follow-up although the survival curves for this cohort are not yet stable.

A recently published study in a cohort of paediatric and young adult patients predominantly with ALL provides the first intention-to-treat analysis of its outcomes. This may help remove the bias inherent in excluding patients who do not receive the anticipated dose of CAR T cells (Lee et al. (2014) Lancet. doi:10.1016/S0140-6736(14)61403-3). 21 patients were treated with a CD28 domain-containing second generation CAR. All but 2 patients received the anticipated T cell dose, highlighting the feasibility of delivering this treatment to those with refractory or multiply-relapsed ALL. This study shows the following efficacy: 67% achieving a complete remission and 60% of those with ALL achieving MRD negative status.

Immune toxicity of CD19 CAR therapy

Cytokine release syndrome (CRS) encompasses a range of inflammatory symptoms ranging from mild to multi-organ failure with hypotension and respiratory failure. Some degree of CRS occurs commonly in patients treated with CD19 CAR T cells. Approximately 30% (21/73) patients treated in recent cohorts showed some degree of CRS (Davilia et al (2014) as above; Lee et al (2014) as above; Kochenderfer (2014) J. Clin. Oncol. Off. J. Am. Soc. Clin. Oncol.. doi:10.1200/JCO.2014.56.2025). CRS has also been seen in patients treated with blinatumomab, a bi-specific recombinant single-chain antibody recognising both CD19 and CD3. CRS typically occurs 5-21 days after CAR T cell infusion.

CRS can be life threatening and requires treatment in an intensive care setting. CRS is associated with elevated serum cytokine levels. The cytokines most significantly elevated are IL-6, IL-10 and interferon gamma (IFNγ). Clinical manifestations of severe CRS (fever, hepatosplenomegaly, coagulopathy and hyperferritinaemia) resemble macrophage activation syndrome (MAS) found for instance in patients with congenital defects in T-cells. This suggests that common immunopathological processes are involved. At present it is not clear which cell type (CAR T cells, dying tumour cells, or locally-activated macrophages) are responsible for production of the key cytokines, particularly IL-6. However, a key initiating factor in MAS is release of copious Interferon-gamma (López-Alvarez et al. (2009). Clin. Vaccine Immunol. CVI 16, 142–145).

Neurotoxicity

A number of patients in CD19 CAR studies across institutions have developed transient neurotoxicity with a spectrum of severity from aphasia to obtundation, delirium and seizures (Davilia et al (2014) as above). This appears to be restricted to patients with ALL and a similar syndrome has been documented after blinatumomab therapy. Brain imaging appears normal. Neurotoxicity may reflect high levels of systemic cytokines crossing the blood-brain barrier.

Persistence, relapse and T-cell exhaustion

Durable responses appeared to correlate with higher peak levels of circulating CAR transduced T cells, as well as with the duration of B cell aplasia. With exception of

patients relapsing with CD19- disease, relapse was generally associated with loss of circulating CAR T cells and recovery of normal B cells.

T cell exhaustion is a state of T cell dysfunction that arises during many chronic infections and cancer. It is defined by poor effector function, sustained expression of inhibitory receptors and a transcriptional state distinct from that of functional effector or memory T cells. Exhaustion prevents optimal control of infection and tumors. Recently, a clearer picture of the functional and phenotypic profile of exhausted T cells has emerged with expression of inhibitory receptor programmed death 1 (PD-1; also known as PDCD1), a negative regulator of activated T cells, being a key feature (Day et al. (2006) Nature 443, 350–354).

Responses in CD19 CAR studies suggest that persistence of T-cells for a protracted period at high levels seems to be important in effecting durable responses. A CD19 CAR which reduces T-cell exhaustion may result in improved clinical responses.

There is thus a need for an alternative CAR directed against CD19 which is not associated with the above disadvantages.

DESCRIPTION OF THE FIGURES

Figure 1. Annotated and numbered (a) CAT19 VH sequences; (b) CAT19 VL
Sequences of the VH and VL are numbered using Chothia numbering. The framework and CDR regions are shown. Insertions are also shown.

Figure 2. Staining of CD19 positive cells with recombinant CAT19
SupT1 cells do not normally express CD19 but were engineered to do so in this study. CAT19 VH and VL sequences were cloned into mouse IgG2a heavy chain format and mouse kappa light chain format, both in mammalian expression plasmids. 293T cells were transfected simultaneously with both heavy and light chain and the resultant antibody purified with protein A. SupT1 cells and SupT1.CD19 cells were stained with this recombinant antibody (or plain 293T supernatant) and further stained with a fluorescently conjugated anti-mouse secondary. Binding of recombinant CAT19 antibody could readily be detected by flow-cytometry.

Figure 3. Staining of CD19 positive cells with CAT19 scFv

The VH and VL of CAT19 were cloned such that they form a scFv whereby the VH and VL are separated by a $(SGGGGS)_3$ linker. Two scFvs were generated with the CAT scFv in both VH-VL and VL-VH orientations. In addition, scFvs were generated, also in either orientation, from the anti-CD19 antibodies fmc63 and 4g7. (a) scFv display format: this is a retroviral vector whereby the scFv is cloned onto a human IgG1 Fc spacer which has the CD8 transmembrane domain and the first 12 residues of the CD8 endodomain. This in turn is in frame with the FMD-2A peptide TaV and truncated human CD34. In this way, the scFv is displayed on the surface of a cell, and the transgene expression can be controlled for by detecting CD34 separately. SupT1 cells were generated which express either of the 6 different scFv formats and these cells were stained with recombinant human truncated CD19 – mouse IgG2a Fc fusion and anti-CD34; (b) Staining with fmc63 VH-VL and VL-VH format; (c) Staining with 4g7 VH-VL and VL-VH format; and (d) Staining with CAT19 VH-VL and VL-VH format. Surprisingly, the CAT19 VH-VL scFv bound well, while the VL-VH scFv gave significantly less detectable binding.

Figure 4. Different generations of CARs and initial CARs tested

(a) A typical CAR format comprising of an antigen binding domain (which most usually is a scFv), a spacer domain, a transmembrane domain and one or several signalling domains. (b) First generation CARs transmit an activation signal; their endodomain is derived from either the FcGamma receptor endodomain or the CD3 Zeta endodomain; (c) Second generation receptors transmit two signals: their endodomains comprise a co-stimulatory domain connected to the endodomain of CD3-Zeta. The co-stimulatory domain is usually either the endodomain of CD28, the endodomain of OX40 or the endodomain of 41BB. (d) Third generation receptors transmit three signals: their endodomains comprise a fusion of the CD28 endodomain with the 41BB endodomain and with the CD3-Zeta endodomain, or the CD28 endodomain with the OX40 endodomain and with the CD3-Zeta endodomain. (e) The CAT19 based CAR initially tested which comprises a scFv in the VH-VL orientation, a CD8 stalk spacer and 2nd generation endodomain comprising of 41BB-Zeta (Campana CAR format).

Figure 5. In vitro comparison of CAT19 CAR function against fmc36 CAR

Primary human T-cells from 5 different donors were transduced with lentiviral vectors coding for CAT19 CAR in Campana format, or the Campana CAR itself. These T-cells were then used in various assays. (a) Chromium release assay was performed against SupT1 cells. These cells are CD19 negative. Neither CAR T-cells responded

against this cell line (dotted lines). Chromium release assay was performed against SupT1.CD9. Both CARs performed equally against this cell line (unbroken lines). Next a degranulation assay was performed using either NT T-cells, fmc63 CAR T-cells, or CAT19 CAR T-cells against either SupT1 or SupT1.CD19. (b) data gated on CD4+ T-cells, and (c) CD8+ T-cells is shown. Degranulation was increased with CAR19 CAR T-cells. (d) Proliferation was estimated using tritiated thymidilation incorporation. NT, fmc63 CAR T-cells, CAT19 CAR T-cells were tested against SupT1. CD19. In this experiment, an irrelevant CAR targeting GD2 was also tested. There was a trend to increased proliferation with CAR19 CAR T-cells. (e) Interferon-gamma release from either NT T-cells ,fmc63 CAR T-cells, CAT19 CAR T-cells or GD2 CAR T-cells 24 hours after challenge against SupT1 or SupT1.CD19 cells. CAT19 CAR T-cells produced significantly less IF-G than fmc63 CAR T-cells when challenged with CD19+ targets.

Figure 6. In vivo model of CAT19 efficacy.

(a) Outline of experimental set-up for in vivo model. NSG mice were injected with 2.5x10^5 Raji.FLuc cells via tail vein injection. 24 hours later 4x10^6 of either NT primary human T-cells, or T-cells transduced with fmc63 CAR, or T-cells transduced with CAT19 CAR were administered via tail-vein. Tumour response was measured sequentially by bioluminescence imaging. Tail-vein blood was sampled at day 4 for engraftment and serum cytokine. The animals were culled at day 11 and tissues studied for persistence of CAR T-cells and tumour burden. (b) Bioluminescence imaging of the different mouse cohorts at day 10. Extensive disease is seen in the pelvis, spine, ribs, skull and spleen of mice treated with NT T-cells, while minimal signal is evident in mice who received either CAT19 CAR T-cells, or fmc63 CAR T-cells. (c) Quantitative bioluminescent signal averaged from different mouse cohorts over time. Y-axis is a log-scale; A clear difference is seen between signal accumulation in mice who received NT T-cells, and mice who received CAR T-cells. No difference in signal accumulation is seen in mice who received fmc63 CAR T-cells or CAT19 CAR T-cells. (d) Flow-cytometric determined tumour burden in bone-marrow from mice at the end of the experiment. Practically no Raji cells could be detected in marrow of mice who received either fmc63 or CAT19 CAR T-cells.

Figure 7. Characterization of in vivo persisting CAR T-cells

(a) Absolute numbers of CAR T-cells in spleens of mice from animals treated with fmc63 CAR T-cells or CAT19 CAR T-cells in the model outlined above. This shows the same numbers are present in both; (b) Absolute numbers of CAR T-cells in bone-

marrow of mice treated with fmc63 CAR T-cells or CAT19 CAR T-cells. This shows the same numbers of cells are present in both; (c) Absolute numbers of PD1-expressing CAR T-cells in spleen and (d) bone-marrow of mice treated with either fmc63 CAR T-cells or CAT19 CAR T-cells. Fewer of the CAT19 T-cells are PD1+ in both compartments.

SUMMARY OF ASPECTS OF THE INVENTION

The present inventors have developed a new CD19-specific CAR with CDRs that have not previously been described. It has equivalent potency to the fmc63-based CAR used in the UPENN studies, but results in reduced toxicity and reduced T-cell exhaustion.

Thus, in a first aspect the present invention provides a chimeric antigen receptor (CAR) comprising a CD19-binding domain which comprises a) a heavy chain variable region (VH) having complementarity determining regions (CDRs) with the following sequences:
CDR1 – GYAFSSS (SEQ ID No. 1);
CDR2 – YPGDED (SEQ ID No. 2)
CDR3 – SLLYGDYLDY (SEQ ID No. 3); and
b) a light chain variable region (VL) having CDRs with the following sequences:
CDR1 – SASSSVSYMH (SEQ ID No. 4);
CDR2 – DTSKLAS (SEQ ID No. 5)
CDR3 – QQWNINPLT (SEQ ID No. 6).

The CD19 binding domain may comprise a VH domain having the sequence shown as SEQ ID No. 7 and/or or a VL domain having the sequence shown as SEQ ID No 8 or a variant thereof having at least 95% sequence identity.

The CD19 binding domain may comprise an scFv in the orientation VH-VL.

The CD19 binding domain may comprise the sequence shown as SEQ ID No 9 or a variant thereof having at least 90% sequence identity.

The CD19 binding domain may comprise the 6 CDRs defined in claim 1 grafted on to a human antibody framework.

The CD19-binding domain and the transmembrane domain may be connected by a spacer, which may comprise one of the following: a human an IgG1 Fc domain; an IgG1 hinge; or a CD8 stalk. The spacer may comprise a CD8 stalk.

5 The CAR may comprise or associate with an intracellular T cell signalling domain.

The intracellular T cell signalling domain may comprise one or more of the following endodomains: CD28 endodomain; 41BB endodomain, OX40 endodomain and the CD3-Zeta endodomain.

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In particular the CAR may comprise a CD8 stalk spacer and an intracellular T-cell signalling domain which comprises the 41BB endodomain and the CD3-Zeta endodomain.

15 In particular the CAR may comprise a CD8 stalk spacer and an intracellular T-cell signalling domain which comprises the OX40 endodomain and the CD3-Zeta endodomain.

In an alternative embodiment, the intracellular T cell signalling domain may comprise
20 all of the following endodomains: CD28 endodomain; OX40 and CD3-Zeta endodomain.

The CAR may comprise the sequence shown as any of SEQ ID No. 10 to 15 or a variant thereof which has at least 80% sequence identity but retains the capacity to i)
25 bind CD19 and ii) induce T cell signalling.The CAR may have advantageous properties compared to the fmc63-based CAR used in the UPENN studies. For example, the CAR, when expressed by a T-cell and used to target a CD19 expressing cell, may cause lower IFNγ release by the CD19-expressing target cell than that caused by a T-cell expressing a CAR comprising a CD19-binding domain
30 which comprises: a) a heavy chain variable region (VH) having complementarity determining regions (CDRs) with the following sequences:CDR1 – GVSLPDY (SEQ ID No. 16); CDR2 – WGSET (SEQ ID No. 17); CDR3 – HYYYGGSYAMDY (SEQ ID No. 18); and b) a light chain variable region (VL) having CDRs with the following sequences:CDR1 – RASQDISKYLN (SEQ ID No. 19); CDR2 – HTSRLHS (SEQ ID
35 No. 20) CDR3 – QQGNTLPYT (SEQ ID No. 21). The CDRs may be grafted on to a human or humanised framework.

In a second aspect, the present invention provides a nucleic acid sequence which encodes a CAR according to the first aspect of the invention.

In a third aspect, there is provided a vector which comprises a nucleic acid sequence according to the second aspect of the invention.

In a third aspect there is provided a cell which comprises a CAR according to the first aspect of the invention.

The cell may be a cytolytic immune cell, such as a T cell or a natural killer (NK) cell.

In a fourth aspect there is provided a cell composition which comprises a plurality of cells according to the third aspect of the invention.

In a fifth aspect, there is provided a method for making a cell according to the third aspect of the invention, which comprises the step of transducing or transfecting a cell with a vector according to the third aspect of the invention.

In a sixth aspect there is provided a method for making a cell composition according to the fourth aspect of the invention which comprises the step of transducing or transfecting a sample of cells from a subject *ex vivo* with a vector according to the third aspect of the invention.

The sample of cells may, for example, be a blood sample or a derivative thereof, such as a peripheral blood mononuclear cell (PBMC) sample.

In a seventh aspect, there is provided a pharmaceutical composition which comprises a cell according to the first aspect of the invention, or a cell composition according to the fourth aspect of the invention, together with a pharmaceutically acceptable carrier, diluent or excipient.

In an eighth aspect, there is provided a method for treating cancer which comprises the step of administering a cell according to the first aspect of the invention, a cell composition according to the fourth aspect of the invention or a pharmaceutical composition according to the seventh aspect of the invention to a subject.

The method may comprise the step of transducing or transfecting cells from the subject *ex vivo* with a vector according to the third aspect of the invention, then administering the, or some of the, transfected cells back to the subject.

There is also provided a pharmaceutical composition according to the seventh aspect of the invention for use in treating cancer.

There is also provided the use of a cell according to the third aspect of the invention in the manufacture of a pharmaceutical composition for treating cancer.

The cancer may, for example, be a B cell malignancy.

DETAILED DESCRIPTION

CHIMERIC ANTIGEN RECEPTORS (CARS)

Chimeric antigen receptors (CARs), also known as chimeric T cell receptors, artificial T cell receptors and chimeric immunoreceptors, are engineered receptors, which graft an arbitrary specificity onto an immune effector cell. In a classical CAR, the specificity of a monoclonal antibody is grafted on to a T cell. CAR-encoding nucleic acids may be transferred to T cells using, for example, retroviral vectors. In this way, a large number of cancer-specific T cells can be generated for adoptive cell transfer. Phase I clinical studies of this approach show efficacy.

The target-antigen binding domain of a CAR is commonly fused via a spacer and transmembrane domain to an endodomain. The endodomain may comprise or associate with an intracellular T-cell signalling domain. When the CAR binds the target-antigen, this results in the transmission of an activating signal to the T-cell it is expressed on.

The CAR of the present invention comprises a CD19 binding domain which is based on a mouse anti-CD19 monoclonal antibody.

The CAR of the present invention comprises a CD19-binding domain which comprises

a) a heavy chain variable region (VH) having complementarity determining regions (CDRs) with the following sequences:

CDR1 – GYAFSSS (SEQ ID No. 1);

CDR2 – YPGDED (SEQ ID No. 2)

5 CDR3 – SLLYGDYLDY (SEQ ID No. 3); and

b) a light chain variable region (VL) having CDRs with the following sequences:

CDR1 – SASSSVSYMH (SEQ ID No. 4);

CDR2 – DTSKLAS (SEQ ID No. 5)

10 CDR3 – QQWNINPLT (SEQ ID No. 6).

It may be possible to introduce one or more mutations (substitutions, additions or deletions) into each CDR without negatively affecting CD19-binding activity. Each CDR may, for example, have one, two or three amino acid mutations.

15

The CDRs may be in the format of a single-chain variable fragment (scFv), which is a fusion protein of the heavy variable region (VH) and light chain variable region (VL) of an antibody, connected with a short linker peptide of ten to about 25 amino acids. The scFv may be in the orientation VH-VL, i.e. the VH is at the amino-terminus of the CAR
20 molecule and the VL domain is linked to the spacer and, in turn the transmembrane domain and endodomain.

The CDRs may be grafted on to the framework of a human antibody or scFv. For example, the CAR of the present invention may comprise a CD19-binding domain
25 consisting or comprising one of the following sequences

The CAR of the present invention may comprise the following VH sequence:

SEQ ID No. 7 – VH sequence from murine monoclonal antibody

30 QVQLQQSGPELVKPGASVKISCKASGYAFSSSWMNWVKQRPGKGLEWIGRIYPGDEDTNYSGK
FKDKATLTADKSSTTAYMQLSSLTSEDSAVYFCARSLLYGDYLDYWGQGTTLTVSS

The CAR of the present invention may comprise the following VL sequence:

35 SEQ ID No 8 – VL sequence from murine monoclonal antibody

QIVLTQSPAIMSASPGEKVTMTCSASSSVSYMHWYQQKSGTSPKRWIYDTSKLASGVPDRFSG
SGSGTSYFLTINNMEAEDAATYYCQQWNINPLTFGAGTKLELKR

The CAR of the invention may comprise the following scFv sequence:

SEQ ID No 9 – VH-VL scFv sequence from murine monoclonal antibody

QVQLQQSGPELVKPGASVKISCKASGYAFSSSWMNWVKQRPGKGLEWIGRIYPGDEDTNYSGK
FKDKATLTADKSSTTAYMQLSSLTSEDSAVYFCARSLLYGDYLDYWGQGTTLTVSSGGGGSGG
5 GGSGGGGSQIVLTQSPAIMSASPGEKVTMTCSASSSVSYMHWYQQKSGTSPKRWIYDTSKLAS
GVPDRFSGSGSGTSYFLTINNMEAEDAATYYCQQWNINPLTFGAGTKLELKR

The CAR may consist of or comprise one of the following sequences:

10 SEQ ID No. 10 – CAT19 CAR using “Campana” architecture (see Examples)

MGTSLLCWMALCLLGADHADAQVQLQQSGPELVKPGASVKISCKASGYAFSSSWMNWVKQRPG
KGLEWIGRIYPGDEDTNYSGKFKDKATLTADKSSTTAYMQLSSLTSEDSAVYFCARSLLYGDY
LDYWGQGTTLTVSSGGGGSGGGGSGGGGSQIVLTQSPAIMSASPGEKVTMTCSASSSVSYMHW
YQQKSGTSPKRWIYDTSKLASGVPDRFSGSGSGTSYFLTINNMEAEDAATYYCQQWNINPLTF
15 GAGTKLELKRSDPTTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDFACDIYIWA
PLAGTCGVLLLSLVITLYCKRGRKKLLYIFKQPFMRPVQTTQEEDGCSCRFPEEEEGGCELRV
KFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGGKPRRKNPQEGLYNELQKD
KMAEAYSEIGMKGERRRGKGHDGLYQGLSTATKDTYDALHMQALPPR

20 SEQ ID No. 11 – CAT19 CAR with an OX40-Zeta endodomain

MGTSLLCWMALCLLGADHADAQVQLQQSGPELVKPGASVKISCKASGYAFSSSWMNWVKQRPG
KGLEWIGRIYPGDEDTNYSGKFKDKATLTADKSSTTAYMQLSSLTSEDSAVYFCARSLLYGDY
LDYWGQGTTLTVSSGGGGSGGGGSGGGGSQIVLTQSPAIMSASPGEKVTMTCSASSSVSYMHW
YQQKSGTSPKRWIYDTSKLASGVPDRFSGSGSGTSYFLTINNMEAEDAATYYCQQWNINPLTF
25 GAGTKLELKRSDPTTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDFACDIYIWA
PLAGTCGVLLLSLVITLYCRRDQRLPPDAHKPPGGGSFRTPIQEEQADAHSTLAKIRVKFSRS
ADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGGKPRRKNPQEGLYNELQKDKMAEA
YSEIGMKGERRRGKGHDGLYQGLSTATKDTYDALHMQALPPR

30 SEQ ID No. 12 – CAT19 CAR with a CD28-Zeta endodomain

MGTSLLCWMALCLLGADHADAQVQLQQSGPELVKPGASVKISCKASGYAFSSSWMNWVKQRPG
KGLEWIGRIYPGDEDTNYSGKFKDKATLTADKSSTTAYMQLSSLTSEDSAVYFCARSLLYGDY
LDYWGQGTTLTVSSGGGGSGGGGSGGGGSQIVLTQSPAIMSASPGEKVTMTCSASSSVSYMHW
YQQKSGTSPKRWIYDTSKLASGVPDRFSGSGSGTSYFLTINNMEAEDAATYYCQQWNINPLTF
35 GAGTKLELKRSDPTTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDFACDIYIWA
PLAGTCGVLLLSLVITLYCRSKRSRLLHSDYMNMTPRRPGPTRKHYQPYAPPRDFAAYRSRVK
FSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGGKPRRKNPQEGLYNELQKDK
MAEAYSEIGMKGERRRGKGHDGLYQGLSTATKDTYDALHMQALPPR

40 SEQ ID No. 13 – Third generation CD19 CAR

MGTSLLCWMALCLLGADHADAQVQLQQSGPELVKPGASVKISCKASGYAFSSSWMNWVKQRPG
KGLEWIGRIYPGDEDTNYSGKFKDKATLTADKSSTTAYMQLSSLTSEDSAVYFCARSLLYGDY
LDYWGQGTTLTVSSGGGGSGGGGSGGGGSQIVLTQSPAIMSASPGEKVTMTCSASSSVSYMHW
YQQKSGTSPKRWIYDTSKLASGVPDRFSGSGSGTSYFLTINNMEAEDAATYYCQQWNINPLTF
45 GAGTKLELKRSDPTTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDFACDIFWVL
VVVGGVLACYSLLVTVAFIIFWVRSKRSRLLHSDYMNMTPRRPGPTRKHYQPYAPPRDFAAYR
SRDQRLPPDAHKPPGGGSFRTPIQEEQADAHSTLAKIRVKFSRSADAPAYQQGQNQLYNELNL
GRREEYDVLDKRRGRDPEMGGKPRRKNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGL
YQGLSTATKDTYDALHMQALPPR

50

SEQ ID No. 14 - CD19 CAR with IgG1 hinge spacer

MGTSLLCWMALCLLGADHADAQVQLQQSGPELVKPGASVKISCKASGYAFSSSWMNWVKQRPG
KGLEWIGRIYPGDEDTNYSGKFKDKATLTADKSSTTAYMQLSSLTSEDSAVYFCARSLLYGDY
LDYWGQGTTLTVSSGGGGSGGGGSGGGGSQIVLTQSPAIMSASPGEKVTMTCSASSSVSYMHW
5 YQQKSGTSPKRWIYDTSKLASGVPDRFSGSGSGTSYFLTINNMEAEDAATYYCQQWNINPLTF
GAGTKLELKRSDPAEPKSPDKTHTCPPCPKDPKFWVLVVVGGVLACYSLLVTVAFIIFWVRSK
RSRLLHSDYMNMTPRRPGPTRKHYQPYAPPRDFAAYRSRVKFSRSADAPAYQQGQNQLYNELN
LGRREEYDVLDKRRGRDPEMGGKPRRKNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDG
LYQGLSTATKDTYDALHMQALPPR

10

SEQ ID No. 15 - CD19 CAR with hinge-CH2-CH3 of human IgG1 with FcR binding sites mutated out

MGTSLLCWMALCLLGADHADAQVQLQQSGPELVKPGASVKISCKASGYAFSSSWMNWVKQRPG
KGLEWIGRIYPGDEDTNYSGKFKDKATLTADKSSTTAYMQLSSLTSEDSAVYFCARSLLYGDY
15 LDYWGQGTTLTVSSGGGGSGGGGSGGGGSQIVLTQSPAIMSASPGEKVTMTCSASSSVSYMHW
YQQKSGTSPKRWIYDTSKLASGVPDRFSGSGSGTSYFLTINNMEAEDAATYYCQQWNINPLTF
GAGTKLELKRSDPAEPKSPDKTHTCPPCPAPPVAGPSVFLFPPKPKDTLMIARTPEVTCVVVD
VSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKAL
PAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNY
20 KTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGKKDPKFWV
LVVVGGVLACYSLLVTVAFIIFWVRSKRSRLLHSDYMNMTPRRPGPTRKHYQPYAPPRDFAAY
RSRVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGGKPRRKNPQEGLYNE
LQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTATKDTYDALHMQALPPR

25 The CAR of the invention may comprise a variant of the sequence shown as SEQ ID No. 7, 8, 9, 10, 11, 12, 13, 14 or 15 having at least 80, 85, 90, 95, 98 or 99% sequence identity, provided that the variant sequence retain the capacity to bind CD19 (when in conjunction with a complementary VL or VH domain, if appropriate).

30 The percentage identity between two polypeptide sequences may be readily determined by programs such as BLAST which is freely available at http://blast.ncbi.nlm.nih.gov.

TRANSMEMBRANE DOMAIN

35

The CAR of the invention may also comprise a transmembrane domain which spans the membrane. It may comprise a hydrophobic alpha helix. The transmembrane domain may be derived from CD28, which gives good receptor stability.

40 The transmembrane domain may comprise the sequence shown as SEQ ID No. 22.

SEQ ID No. 22

FWVLVVVGGVLACYSLLVTVAFIIFWV

INTRACELLULAR T CELL SIGNALING DOMAIN (ENDODOMAIN)

The endodomain is the signal-transmission portion of the CAR. After antigen recognition, receptors cluster and a signal is transmitted to the cell. The most commonly used endodomain component is that of CD3-zeta which contains 3 ITAMs. This transmits an activation signal to the T cell after antigen is bound. CD3-zeta may not provide a fully competent activation signal and additional co-stimulatory signaling may be needed. For example, endodomains from CD28, or OX40 or 41BB can be used with CD3-Zeta to transmit a proliferative / survival signal, or all three can be used together.

Early CAR designs had endodomains derived from the intracellular parts of either the γ chain of the FcεR1 or CD3ζ. Consequently, these first generation receptors transmitted immunological signal 1, which was sufficient to trigger T-cell killing of cognate target cells but failed to fully activate the T-cell to proliferate and survive. To overcome this limitation, compound endodomains were constructed. Fusion of the intracellular part of a T-cell co-stimulatory molecule to that of CD3ζ resulted in second generation receptors which could transmit an activating and co-stimulatory signal simultaneously after antigen recognition. The co-stimulatory domain most commonly used was that of CD28. This supplies the most potent co-stimulatory signal, namely immunological signal 2, which triggers T-cell proliferation. Some receptors were also described which included TNF receptor family endodomains such as OX40 and 41BB which transmit survival signals. Finally, even more potent third generation CARs were described which had endodomains capable of transmitting activation, proliferation and survival signals. CARs and their different generations are summarized in Figure 4.

The endodomain of the CAR of the present invention may comprise combinations of one or more of the CD3-Zeta endodomain, the 41BB endodomain, the OX40 endodomain or the CD28 endodomain.

The intracellular T-cell signalling domain (endodomain) of the CAR of the present invention may comprise the sequence shown as SEQ ID No. 23, 24, 25, 26, 27, 28, 29 or 30 or a variant thereof having at least 80% sequence identity.

SEQ ID No. 23 (CD3 zeta endodomain)

RSRVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGGKPRRKNPQEGLYNE
LQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTATKDTYDALHMQALPPR

SEQ ID No. 24 (41BB endodomain)
KRGRKKLLYIFKQPFMRPVQTTQEEDGCSCRFPEEEEGGCEL

5 SEQ ID No. 25 (OX40 endodomain)
RRDQRLPPDAHKPPGGGSFRTPIQEEQADAHSTLAKI

SEQ ID No. 26 (CD28 endodomain)
KRSRLLHSDYMNMTPRRPGPTRKHYQPYAPPRDFAAY

10

Examples of combinations of such endodomains include 41BB-Z, OX40-Z, CD28-Z and CD28-OX40-Zeta.

SEQ ID No. 27 (41BB-Z endodomain fusion)
15 KRGRKKLLYIFKQPFMRPVQTTQEEDGCSCRFPEEEEGGCELRVKFSRSADAPAYQQGQNQLY
NELNLGRREEYDVLDKRRGRDPEMGGKPRRKNPQEGLYNELQKDKMAEAYSEIGMKGERRRGK
GHDGLYQGLSTATKDTYDALHMQALPPR

SEQ ID No. 28 (OX40-Z endodomain fusion)
20 RRDQRLPPDAHKPPGGGSFRTPIQEEQADAHSTLAKIRVKFSRSADAPAYQQGQNQLYNELNL
GRREEYDVLDKRRGRDPEMGGKPRRKNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGL
YQGLSTATKDTYDALHMQALPPR

SEQ ID No. 29 (CD28Z endodomain fusion)
25 KRSRLLHSDYMNMTPRRPGPTRKHYQPYAPPRDFAAYRSRVKFSRSADAPAYQQGQNQLYNEL
NLGRREEYDVLDKRRGRDPEMGGKPRRKNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHD
GLYQGLSTATKDTYDALHMQALPPR

SEQ ID No. 30 (CD28OXZ)
30 KRSRLLHSDYMNMTPRRPGPTRKHYQPYAPPRDFAAYRSRDQRLPPDAHKPPGGGSFRTPIQE
EQADAHSTLAKIRVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGGKPRR
KNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTATKDTYDALHMQALPPR

A variant sequence may have at least 80%, 85%, 90%, 95%, 98% or 99% sequence
35 identity to SEQ ID No. 22, 23, 24, 25, 26, 27, 28, 29 or 30 provided that the sequence provides an effective transmembrane domain/intracellular T cell signaling domain.

SIGNAL PEPTIDE

40 The CAR of the present invention may comprise a signal peptide so that when the CAR is expressed inside a cell, such as a T-cell, the nascent protein is directed to the endoplasmic reticulum and subsequently to the cell surface, where it is expressed.

The core of the signal peptide may contain a long stretch of hydrophobic amino acids that has a tendency to form a single alpha-helix. The signal peptide may begin with a short positively charged stretch of amino acids, which helps to enforce proper topology of the polypeptide during translocation. At the end of the signal peptide there is typically a stretch of amino acids that is recognized and cleaved by signal peptidase. Signal peptidase may cleave either during or after completion of translocation to generate a free signal peptide and a mature protein. The free signal peptides are then digested by specific proteases.

The signal peptide may be at the amino terminus of the molecule.

The CAR of the invention may have the general formula:

Signal peptide – CD19-binding domain – spacer domain - transmembrane domain/intracellular T cell signaling domain.

The signal peptide may comprise the SEQ ID No. 31 or a variant thereof having 5, 4, 3, 2 or 1 amino acid mutations (insertions, substitutions or additions) provided that the signal peptide still functions to cause cell surface expression of the CAR.

SEQ ID No. 31: METDTLLLWVLLLWVPGSTG

The signal peptide of SEQ ID No. 31 is compact and highly efficient. It is predicted to give about 95% cleavage after the terminal glycine, giving efficient removal by signal peptidase.

SPACER

The CAR of the present invention may comprise a spacer sequence to connect the CD19-binding domain with the transmembrane domain and spatially separate the CD19-binding domain from the endodomain. A flexible spacer allows to the CD19-binding domain to orient in different directions to enable CD19 binding.

The spacer sequence may, for example, comprise an IgG1 Fc region, an IgG1 hinge or a CD8 stalk, or a combination thereof. The spacer may alternatively comprise an alternative sequence which has similar length and/or domain spacing properties as an IgG1 Fc region, an IgG1 hinge or a CD8 stalk.

A human IgG1 spacer may be altered to remove Fc binding motifs.

Examples of amino acid sequences for these spacers are given below:

SEQ ID No. 32 (hinge-CH2CH3 of human IgG1)
AEPKSPDKTHTCPPCPAPPVAGPSVFLFPPKPKDTLMIARTPEVTCVVVDVSHEDPE
VKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKA
LPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESN
GQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQK
SLSLSPGKKD

SEQ ID No. 33 (human CD8 stalk):
TTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDFACDI

SEQ ID No. 34 (human IgG1 hinge):
AEPKSPDKTHTCPPCPKDPK

SEQ ID No. 35 (IgG1 Hinge-Fc)
AEPKSPDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDP
EVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNK
ALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESN
GQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQK
SLSLSPGKKDPK

SEQ ID No. 36 (IgG1 Hinge – Fc modified to remove Fc receptor recognition motifs)
AEPKSPDKTHTCPPCPAPPVA*GPSVFLFPPKPKDTLMIARTPEVTCVVVDVSHEDP
EVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNK
ALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESN
GQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQK
SLSLSPGKKDPK

Modified residues are underlined; * denotes a deletion.

INTERFERON RELEASE AND CAR T-CELL EXHAUSTION

The present inventors have found that a CD19 CAR based on the CAT19 scFv has properties which may result in lower toxicity and better efficacy.

Given that the main experience with CD19 CAR therapy has been with CARs based on the fmc63 scFv, and that the oldest, largest and perhaps most significant clinical data set is with the fmc63 based Campana CAR, the present inventors took this Campana CAR as the "gold-standard". A comparison was hence made between the fmc63-Campana CAR and a similar CAR but with CAT19 scFv instead of fmc63. Surprisingly, the present inventors found that while CAT19 CAR T-cells effected killing of target cell expressing CD19, and proliferated in response to CD19 expressing targets, Interferon-gamma release was less. Further, a small animal model of an aggressive B-cell lymphoma showed equal efficacy and equal engraftment between the fmc63 and CAT19 based CARs, but surprisingly, less of the CAT19 CAR T-cells were exhausted than fmc63 CAR T-cells.

The CAR of the invention may cause 25, 50, 70 or 90% lower IFNγ release in a comparative assay involving bringing CAR T cells into contact with target cells.

The CAR of the invention may result in a smaller proportion of CAR T cells becoming exhausted than fmc63 CAR T cells. T cell exhaustion may be assessed using methods known in the art, such as analysis of PD-1 expression. The CAR of the present invention may cause 20, 30, 40, 50, 60 of 70% fewer CAR T cells to express PD-1 that fmc63 CAR T cells in a comparative assay involving bringing CAR T cells into contact with target cells.

NUCLEIC ACID SEQUENCE

The second aspect of the invention relates to a nucleic acid sequence which codes for a CAR of the first aspect of the invention.

The nucleic acid sequence may be capable of encoding a CAR having the amino acid sequence shown as any of SEQ ID No. 10-15.

VECTOR

The present invention also provides a vector which comprises a nucleic acid sequence according to the present invention. Such a vector may be used to

introduce the nucleic acid sequence into a host cell so that it expresses and produces a molecule according to the first aspect of the invention.

The vector may, for example, be a plasmid or a viral vector, such as a retroviral vector or a lentiviral vector.

The vector may be capable of transfecting or transducing a cell, such as a T cell.

CELL

The invention also provides a cell which comprises a nucleic acid according to the invention. The invention provides a cell which expresses a CAR according to the first aspect of the invention at the cell surface.

The cell may be a cytolytic immune cell, such as a T-cell or natural killer (NK) cell.

A cell capable of expressing a CAR according to the invention may be made by transducing or transfecting a cell with CAR-encoding nucleic acid.

The CAR-expressing cell of the invention may be generated *ex vivo*. The cell may be from a cell sample, such as a peripheral blood mononuclear cell (PBMC) sample from the patient or a donor. Cells may be activated and/or expanded prior to being transduced with CAR-encoding nucleic acid, for example by treatment with an anti-CD3 monoclonal antibody.

PHARMACEUTICAL COMPOSITION

The present invention also relates to a pharmaceutical composition containing a CAR-expressing cell, or plurality of cells, of the invention together with a pharmaceutically acceptable carrier, diluent or excipient, and optionally one or more further pharmaceutically active polypeptides and/or compounds. Such a formulation may, for example, be in a form suitable for intravenous infusion.

METHOD OF TREATMENT

CAR-expressing cells of the present invention may be capable of killing cancer cells, such as B-cell lymphoma cells. CAR-expressing cells, such as T-cells or NK cells,

may either be created *ex vivo* either from a patient's own peripheral blood (1st party), or in the setting of a haematopoietic stem cell transplant from donor peripheral blood (2nd party), or peripheral blood from an unconnected donor (3rd party). Alternatively, CAR-expressing cells may be derived from *ex vivo* differentiation of inducible progenitor cells or embryonic progenitor cells to cells such as T-cells. In these instances, CAR cells are generated by introducing DNA or RNA coding for the CAR by one of many means including transduction with a viral vector, transfection with DNA or RNA.

T or NK cells expressing a CAR molecule of the present invention may be used for the treatment of a cancerous disease, in particular a cancerous disease associated with CD19 expression.

A method for the treatment of disease relates to the therapeutic use of a cell or population of cells of the invention. In this respect, the cells may be administered to a subject having an existing disease or condition in order to lessen, reduce or improve at least one symptom associated with the disease and/or to slow down, reduce or block the progression of the disease. The method of the invention may cause or promote cell mediated killing of CD19-expressing cells, such as B cells.

The invention will now be further described by way of Examples, which are meant to serve to assist one of ordinary skill in the art in carrying out the invention and are not intended in any way to limit the scope of the invention.

EXAMPLES

Example 1 – Cloning of VH and VL and demonstration of CD19 binding

The VH and VL were cloned from a mouse anti-CD19 monoclonal antibody and fused in frame with the human kappa constant region and the human IgG1 constant region. These chimeric heavy and light chains were cloned into an expression vector and used to transfect 293T cells. The subsequent produced antibody was used to stain SupT1 cells (a T-cell line which is CD19 negative), and SupT1 cells which have been engineered to be CD19 positive. This staining shows specific binding of the CD19 (Figure 2).

Example 2 - Demonstration that the VH/VL can form an scFv which binds CD19

It was then investigated whether the cloned VH and VL could bind CD19 in a scFv format. The VH and VL were cloned as an scFv in two orientations: VH-VL and VL-VH, where the two variable regions were separated by a linker comprising of (SGGGG)4. These scFv were cloned into a non-signalling CAR co-expressed with truncated CD34 as shown in Figure 3a. Briefly, this comprises of a signal peptide, scFv, hinge-CH2-CH3 of human IgG1, the CD8 transmembrane domain, the first 12 residues of the CD8 endodomain, a FMD-2A peptide TeV, truncated human CD34. To allow comparison, scFv from fmc63, and scFv from another anti-CD19 hybridoma 4g7, were cloned in the same format in both VH-VL and VL-VH orientations.

In this way, several parameters can be studied: (1) the binding of target antigen to the CAR by use of recombinant cognate target antigen fused to murine Fc, unencumbered by internalization of the receptor due to signalling; (2) The stability of the receptor can be determined using polyclonal anti-Fc; (3) the expression levels of the cassette can be controlled for by co-staining for CD34.

These constructs were transduced into SupT1 cells. Recombinant CD19-mouse IgG2aFc fusion was generated. SupT1 cells were stained for mouse-Fc, human-Fc and anti-CD34 with antibodies conjugated to different fluorophores and stability / binding interrogated by flow-cytometery.

The sequences of the different scFvs used are detailed below:

>scFv_fmc63_VH-VL (SEQ ID No. 37)

EVKLQESGPGLVAPSQSLSVTCTVSGVSLPDYGVSWIRQPPRKGLEWLGVIWGSETTYYNSAL
KSRLTIIKDNSKSQVFLKMNSLQTDDTAIYYCAKHYYYGGSYAMDYWGQGTSVTVSSGGGGSG
GGGSGGGGSDIQMTQTTSSLSASLGDRVTISCRASQDISKYLNWYQQKPDGTVKLLIYHTSRL
HSGVPSRFSGSGSGTDYSLTISNLEQEDIATYFCQQGNTLPYTFGGGTKLEITKA

>scFv_fmc63_VL-VH (SEQ ID No. 38)

DIQMTQTTSSLSASLGDRVTISCRASQDISKYLNWYQQKPDGTVKLLIYHTSRLHSGVPSRFS
GSGSGTDYSLTISNLEQEDIATYFCQQGNTLPYTFGGGTKLEITKAGGGGSGGGGSGGGGSEV
KLQESGPGLVAPSQSLSVTCTVSGVSLPDYGVSWIRQPPRKGLEWLGVIWGSETTYYNSALKS
RLTIIKDNSKSQVFLKMNSLQTDDTAIYYCAKHYYYGGSYAMDYWGQGTSVTVSS

>scFv_4g7_VH-VL (SEQ ID No. 39)

EVQLQQSGPELIKPGASVKMSCKASGYTFTSYVMHWVKQKPGQGLEWIGYINPYNDGTKYNEK
FKGKATLTSDKSSSTAYMELSSLTSEDSAVYYCARGTYYYGSRVFDYWGQGTTLTVSSGGGGS
GGGGSGGGGSDIVMTQAAPSIPVTPGESVSISCRSSKSLLNSNGNTYLYWFLQRPGQSPQLLI
YRMSNLASGVPDRFSGSGSGTAFTLRISRVEAEDVGVYYCMQHLEYPFTFGAGTKLELKR

>scFv_4g7_VL-VH (SEQ ID No. 40)

DIVMTQAAPSIPVTPGESVSISCRSSKSLLNSNGNTYLYWFLQRPGQSPQLLIYRMSNLASGV
PDRFSGSGSGTAFTLRISRVEAEDVGVYYCMQHLEYPFTFGAGTKLELKRSGGGGSGGGGSGG
GGSEVQLQQSGPELIKPGASVKMSCKASGYTFTSYVMHWVKQKPGQGLEWIGYINPYNDGTKY
5 NEKFKGKATLTSDKSSSTAYMELSSLTSEDSAVYYCARGTYYYGSRVFDYWGQGTTLTVSS

>scFv_CAT_VH-VL (SEQ ID No. 9)

QVQLQQSGPELVKPGASVKISCKASGYAFSSSWMNWVKQRPGKGLEWIGRIYPGDEDTNYSGK
FKDKATLTADKSSTTAYMQLSSLTSEDSAVYFCARSLLYGDYLDYWGQGTTLTVSSGGGGSGG
10 GGSGGGGSQIVLTQSPAIMSASPGEKVTMTCSASSSVSYMHWYQQKSGTSPKRWIYDTSKLAS
GVPDRFSGSGSGTSYFLTINNMEAEDAATYYCQQWNINPLTFGAGTKLELKR

>scFv_CAT_VL-VH (SEQ ID No. 41)

QIVLTQSPAIMSASPGEKVTMTCSASSSVSYMHWYQQKSGTSPKRWIYDTSKLASGVPDRFSG
15 SGSGTSYFLTINNMEAEDAATYYCQQWNINPLTFGAGTKLELKRSGGGGSGGGGSGGGGSQVQ
LQQSGPELVKPGASVKISCKASGYAFSSSWMNWVKQRPGKGLEWIGRIYPGDEDTNYSGKFKD
KATLTADKSSTTAYMQLSSLTSEDSAVYFCARSLLYGDYLDYWGQGTTLTVSS

The construct used and the staining results are summarized in Figure 3. Surprisingly,
20 the CAT CAR with scFv in VH-VL orientation binds CD19, while the CAT19 CAR with
scFv in the VL-VH orientation gave minimal CD19 binding. This was in contrast to the
fmc63 CARs and 4g7 CARs which bound CD19 in both the HL and LH orientations.
Binding and stability of the HL CAT CAR appeared equal to that with fmc63.

25 **Example 3** - *In vitro* comparison of CAT19 CAR function against fmc36 CAR

The CAT scFv in HL orientation was cloned into a CAR scaffold designed by
Campana (Imai et al (2004) Leuk. Off. J. Leuk. Soc, Am. Leuk, Res. Fund. UK
18:676-684). Effectively the fmc63 scFv was replaced with a CAT scFv, and
30 compared with the original fmc63 based CAR. This CAR comprises a signal peptide,
the scFv, a CD8 stalk spacer and transmembrane and 41BB and Zeta endodomains.
The amino acid sequences of the CAT CAR and fmc63 CAR are given below:

>CAT19_CAR (SEQ ID No. 10)

35 MGTSLLCWMALCLLGADHADAQVQLQQSGPELVKPGASVKISCKASGYAFSSSWM
NWVKQRPGKGLEWIGRIYPGDEDTNYSGKFKDKATLTADKSSTTAYMQLSSLTSED
SAVYFCARSLLYGDYLDYWGQGTTLTVSSGGGGSGGGGSGGGGSQIVLTQSPAIM
SASPGEKVTMTCSASSSVSYMHWYQQKSGTSPKRWIYDTSKLASGVPDRFSGSGS
GTSYFLTINNMEAEDAATYYCQQWNINPLTFGAGTKLELKRSDPTTTPAPRPPTPAP
40 TIASQPLSLRPEACRPAAGGAVHTRGLDFACDIYIWAPLAGTCGVLLLSLVITLYCKR
GRKKLLYIFKQPFMRPVQTTQEEDGCSCRFPEEEEGGCELRVKFSRSADAPAYQQ

GQNQLYNELNLGRREEYDVLDKRRGRDPEMGGKPRRKNPQEGLYNELQKDKMAE
AYSEIGMKGERRRGKGHDGLYQGLSTATKDTYDALHMQALPPR

>Fmc63_CAR, as described by Imai et al (2004) as above (SEQ ID No. 42)
5 METDTLLLWVLLLWVPGSTGDIQMTQTTSSLSASLGDRVTISCRASQDISKYLNWYQ
QKPDGTVKLLIYHTSRLHSGVPSRFSGSGSGTDYSLTISNLEQEDIATYFCQQGNTL
PYTFGGGTKLEITKAGGGGSGGGGSGGGGSGGGGSEVKLQESGPGLVAPSQSLS
VTCTVSGVSLPDYGVSWIRQPPRKGLEWLGVIWGSETTYYNSALKSRLTIIKDNSKS
QVFLKMNSLQTDDTAIYYCAKHYYYGGSYAMDYWGQGTSVTVSSDPTTTPAPRPP
10 TPAPTIASQPLSLRPEACRPAAGGAVHTRGLDFACDIYIWAPLAGTCGVLLLSLVITLY
CKRGRKKLLYIFKQPFMRPVQTTQEEDGCSCRFPEEEEGGCELRVKFSRSADAPAY
QQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGGKPRRKNPQEGLYNELQKDKM
AEAYSEIGMKGERRRGKGHDGLYQGLSTATKDTYDALHMQALPPR

15 Primary human T-cells from 5 different donors were transduced with lentiviral vectors coding for CAT19 CAR in Campana format, or the fmc63 Campana CAR itself. These T-cells were then used in various assays. Chromium release assay was performed against SupT1 cells. These cells are CD19 negative. Neither CAR T-cells responded against this cell line demonstrating that CAR19 CAR has no non-specific killing
20 activity against CD19 negative cells [Figure 5(a)]. (b) Chromium release assay was also performed against SupT1 cells engineering to express CD19. Both CARs performed equally against this cell line in this assay with high-levels of killing [Figure 5(b)]. Next a degranulation assay was performed by staining for CD107 on the surface of effector cells after co-culture with target cells. Here either NT T-cells,
25 fmc63 CAR T-cells, or CAT19 CAR T-cells were used as effectors and either SupT1 or SupT1.CD19 cells were used as targets. Surface CD107 was detected by flow-cytometry which allowed differential measurement of degranulation of CD4+ and CD8+ cells. [Figure 5(c) and (d) respectively]. Degranulation was increased with CAT19 CAR T-cells in comparison with fmc63 CAR T-cells. Proliferation was
30 estimated using tritiated thymidilate incorporation. Here, NT, fmc63 CAR T-cells, CAT19 CAR T-cells were co-cultured with SupT1 cells engineered to express CD19. Incorporation of thymidiln this experiment, an irrelevant CAR targeting GD2 was also tested. There was a trend to increased proliferation with CAR19 CAR T-cells [Figure 4(e)]. Next, interferon-gamma release from either NT T-cells ,fmc63 CAR T-cells,
35 CAT19 CAR T-cells or GD2 CAR T-cells 24 hours after challenge against SupT1 or SupT1.CD19 cells was measured by ELISA. CAT19 CAR T-cells produced

significantly less interferon-gamma than fmc63 CAR T-cells when challenged with CD19+ targets.

Example 4 - Demonstration of in vivo efficacy of CAT19 CAR therapy.

5 An outline of experimental set-up for this in vivo model is present in figure 6(a). Briefly NSG (NOD scid gamma, NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ) mice are sufficiently immunocompromised that they are permissive for engraftment of human cell lines and primary human T-cells. Raji cells are a B-cell line derived from Burkitt's lymphoma. These cells readily engraft within the bone-marrow of NSG mice causing 10 an aggressive leukaemia-like syndrome. Raji cells were engineered to express fire-fly Luciferase to allow non-invasive tracking using bioluminescence imaging (BLI). Mice were injected with 2.5x10^5 Raji.FLuc cells via tail vein injection. 24 hours later 4x10^6 of either NT primary human T-cells, or T-cells transduced with fmc63 CAR, or T-cells transduced with CAT19 CAR were administered via tail-vein. Tumour 15 response was measured sequentially by BLI. Tail-vein blood was sampled at day 4 for engraftment and serum cytokine. The animals were culled at day 11 and tissues studied for persistence of CAR T-cells and tumour burden. BLI imaging of the different mouse cohorts at day 10 is shown in figure 6(b). Extensive disease is seen in the pelvis, spine, ribs, skull and spleen of mice treated with NT T-cells, while 20 minimal signal is evident in mice who received either CAT19 CAR T-cells, or fmc63 CAR T-cells. Quantitative bioluminescent signal averaged from different mouse cohorts over time is shown on a log-scale in figure 6(c). A clear difference is seen between signal accumulation in mice who received NT T-cells, and mice who received CAR T-cells. No difference in signal accumulation is seen in mice who 25 received fmc63 CAR T-cells or CAT19 CAR T-cells. Finally, after sacrifice, flow-cytometric analysis of bone-marrow from each mouse was performed to directly determine tumour burden. Raji cells are easily distinguishable from mouse haematopoietic cells and from adoptively transferred T-cells, since they express human B-cell markers. Minimal Raji cells could be detected in marrow of mice who 30 received either fmc63 or CAT19 CAR T-cells.

Example 5 - Characterization of *in vivo* persisting CAR T-cells

From the above animal models, the present inventors sought to determine if both types of CAR T-cells engrafted within the bone-marrow and spleen of these NSG 35 mice. Flow-cytometric analysis of bone-marrow and spleen with counting beads allowed determination of absolute numbers of CAR T-cells. This data is shown in figures 7(a) and (b). The absolute numbers of CAR T-cells in spleens of mice from

animals treated with fmc63 CAR T-cells or CAT19 CAR T-cells was similar. Next, the present inventors proceeded to determine if there was any difference in the numbers of exhausted T-cells in these different tissues. By co-staining for PD1 expression in the above samples the numbers of exhausted T-cells could be determined. This data is
5 shown in figures 7(c) and (d). Surprisingly, fewer exhausted T-cells were present in both tissue compartments with the CAT19 CAR than the fmc63 CAR.

Various modifications and variations of the described methods and system of the invention will be apparent to those skilled in the art without departing from the scope
10 and spirit of the invention. Although the invention has been described in connection with specific preferred embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes for carrying out the invention which are obvious to those skilled in molecular biology, CAR technology or related fields are intended to
15 be within the scope of the following claims.

CLAIMS

1. A chimeric antigen receptor (CAR) comprising a CD19-binding domain which comprises

5 a) a heavy chain variable region (VH) having complementarity determining regions (CDRs) with the following sequences:

CDR1 – GYAFSSS (SEQ ID No. 1);

CDR2 – YPGDED (SEQ ID No. 2)

CDR3 – SLLYGDYLDY (SEQ ID No. 3); and

10 b) a light chain variable region (VL) having CDRs with the following sequences:

CDR1 – SASSSVSYMH (SEQ ID No. 4);

CDR2 – DTSKLAS (SEQ ID No. 5)

CDR3 – QQWNINPLT (SEQ ID No. 6).

15 2. The CAR according to claim 1, wherein the CD19 binding domain comprises a VH domain having the sequence shown as SEQ ID No. 7 and/or or a VL domain having the sequence shown as SEQ ID No. 8 or a variant of SEQ ID No. 7 or 8 having at least 95% sequence identity thereof.

20 3. The CAR according to claim 1, wherein the CD19 binding domain comprises an scFv in the orientation VH-VL

4. The CAR according to claim 3, wherein the CD19 binding domain comprises the sequence shown as SEQ ID No. 9 or a variant thereof having at least 90%
25 sequence identity.

5. The CAR according to any one of claims 1 to 4, wherein CD19-binding domain and the transmembrane domain are connected by a spacer.

30 6. The CAR according to claim 5, wherein the spacer comprises one of the following: a human an IgG1 Fc domain; an IgG1 hinge; or a CD8 stalk.

7. The CAR according to any one of claims 1 to 6, which also comprises an intracellular T cell signalling domain; optionally wherein the intracellular T cell signalling
35 domain comprises one or more of the following endodomains: CD28 endodomain; 41BB endodomain, OX40 endodomain and the CD3-Zeta endodomain.

8. The CAR according to claim 7, wherein the intracellular T-cell signalling domain comprises:

(i) the 41BB endodomain and the CD3-Zeta endodomain;

(ii) the OX40 endodomain and the CD3-Zeta endodomain; or

5 (iii) all of the following endodomains: CD28 endodomain; OX40 endodomain and CD3-Zeta endodomain.

9. The CAR according to any one of claims 1 to 8, which comprises the sequence shown as any of SEQ ID No. 10 to 15 or a variant thereof which has at least 80%
10 sequence identity but retains the capacity to i) bind CD19 and ii) induce T cell signalling.

10. The CAR according to any one of claims 1 to 9, which, when expressed by a T-cell and used to target a CD19 expressing cell, causes lower IFNγ release by the CD19-expressing target cell than that caused by a T-cell expressing a CAR comprising
15 a CD19-binding domain which comprises:

a) a heavy chain variable region (VH) having complementarity determining regions (CDRs) with the following sequences:

CDR1 – GVSLPDY (SEQ ID No. 16);

CDR2 – WGSET (SEQ ID No. 17);

20 CDR3 – HYYYGGSYAMDY (SEQ ID No. 18); and

b) a light chain variable region (VL) having CDRs with the following sequences:

CDR1 – RASQDISKYLN (SEQ ID No. 19);

CDR2 – HTSRLHS (SEQ ID No. 20)

CDR3 – QQGNTLPYT (SEQ ID No. 21).

25

11. The CAR according to any one of claims 1 to 10, wherein the CDRs are grafted on to a human or humanised framework.

12. A nucleic acid sequence which encodes the CAR according to any any one of
30 claims 1 to 11.

13. A vector which comprises a nucleic acid sequence according to claim 12.

14. A cell which comprises the CAR according to any one of claims 1 to 11.

35

15. The cell according to claim 14, wherein the cell is a T cell or a natural killer (NK) cell.

16. A cell composition which comprises a plurality of cells according to claim 14 or 15.

5 17. A method for making (i) a cell according to claim 14 or 15, which comprises the step of transducing or transfecting a cell with a vector according to claim 13; or (ii) a cell composition according to claim 16 which comprises the step of transducing or transfecting a sample of cells from a subject ex vivo with a vector according to claim 13.

10

18. A pharmaceutical composition which comprises a cell according to claim 14 or 15, or a cell composition according to claim 16, together with a pharmaceutically acceptable carrier, diluent or excipient.

15 19. The cell according to claim 14 or 15 for use in treating cancer.

20. The cell according to claim 19, wherein the cancer is a B cell malignancy.

21. The cell composition according to claim 16 for use in treating cancer.

20

22. The cell composition according to claim 21, wherein the cancer is a B cell malignancy.

23. The pharmaceutical composition according to claim 18 for use in treating
25 cancer.

24. The pharmaceutical composition according to claim 23, wherein the cancer is a B cell malignancy.

30

(a)

Query protein sequence	Q	V	Q	L	Q	Q	S	G	P	E	L	V	K	P	G	A	S	V	K	I
Chothia numbering	H1	H2	H3	H4	H5	H6	H7	H8	H9	H10	H11	H12	H13	H14	H15	H16	H17	H18	H19	H20
CHOTHIA REGIONS	HFR1																			

S	C	K	A	S	G	Y	A	F	S	S	S	W	M	N	W	V	K	Q	R	P	G	K
H21	H22	H23	H24	H25	H26	H27	H28	H29	H30	H31	H32	H33	H34	H35	H36	H37	H38	H39	H40	H41	H42	H43
					CDR-H1							HFR2										

G	L	E	W	I	G	R	I	Y	P	G	D	E	D	T	N	Y	S	G	K	F	K	D
H44	H45	H46	H47	H48	H49	H50	H51	H52	H52A	H53	H54	H55	H56	H57	H58	H59	H60	H61	H62	H63	H64	H65
								CDR-H2						HFR3								
												⇧			☆							

K	A	T	L	T	A	D	K	S	S	T	T	A	Y	M	Q	L	S	S	L	T	S	E
H66	H67	H68	H69	H70	H71	H72	H73	H74	H75	H76	H77	H78	H79	H80	H81	H82	H82A	H82B	H82C	H83	H84	H85

D	S	A	V	Y	F	C	A	R	S	L	L	Y	G	D	Y	L	D	Y	W	G	Q	G
H86	H87	H88	H89	H90	H91	H92	H93	H94	H95	H96	H97	H98	H99	H100	H100A	H100B	H101	H102	H103	H104	H105	H106
									CDR-H3										HFR4			

T	T	L	T	V	S	S
H107	H108	H109	H110	H111	H112	H113

▨ Insertion
☆ Predicted N-Linked Glycosylation Site
⇧ Unusual residue (<1% of sequences)

FIG. 1

(b)

Query protein sequence	Q	I	V	L	T	Q	S	P	A	I	M	S	A	S	P	G	E	K	V	T
Chothia numbering	L1	L2	L3	L4	L5	L6	L7	L8	L9	L10	L11	L12	L13	L14	L15	L16	L17	L18	L19	L20
CHOTHIA REGIONS	LFR1																			

M	T	C	S	A	S	S	S	V	S	Y	M	H	W	Y	Q	Q	K	S	G	T	S	P
L21	L22	L23	L24	L25	L26	L27	L28	L29	L30	L32	L33	L34	L35	L36	L37	L38	L39	L40	L41	L42	L43	L44
			CDR-L1										LFR2									

K	R	W	I	Y	D	T	S	K	L	A	S	G	V	P	D	R	F	S	G	S	G	S
L45	L46	L47	L48	L49	L50	L51	L52	L53	L54	L55	L56	L57	L58	L59	L60	L61	L62	L63	L64	L65	L66	L67
					CDR-L2							LFR3										

G	T	S	Y	F	L	T	I	N	N	M	E	A	E	D	A	A	T	Y	Y	C	Q	Q
L68	L69	L70	L71	L72	L73	L74	L75	L76	L77	L78	L79	L80	L81	L82	L83	L84	L85	L86	L87	L88	L89	L90
				⇧																	CDR-L3	

W	N	I	N	P	L	T	F	G	A	G	T	K	L	E	L	K	R
L91	L92	L93	L94	L95	L96	L97	L98	L99	L100	L101	L102	L103	L104	L105	L106	L107	L108
⇧							LFR4										

⇧ Unusual residue (<1% of sequences)

FIG. 1 (Continued)

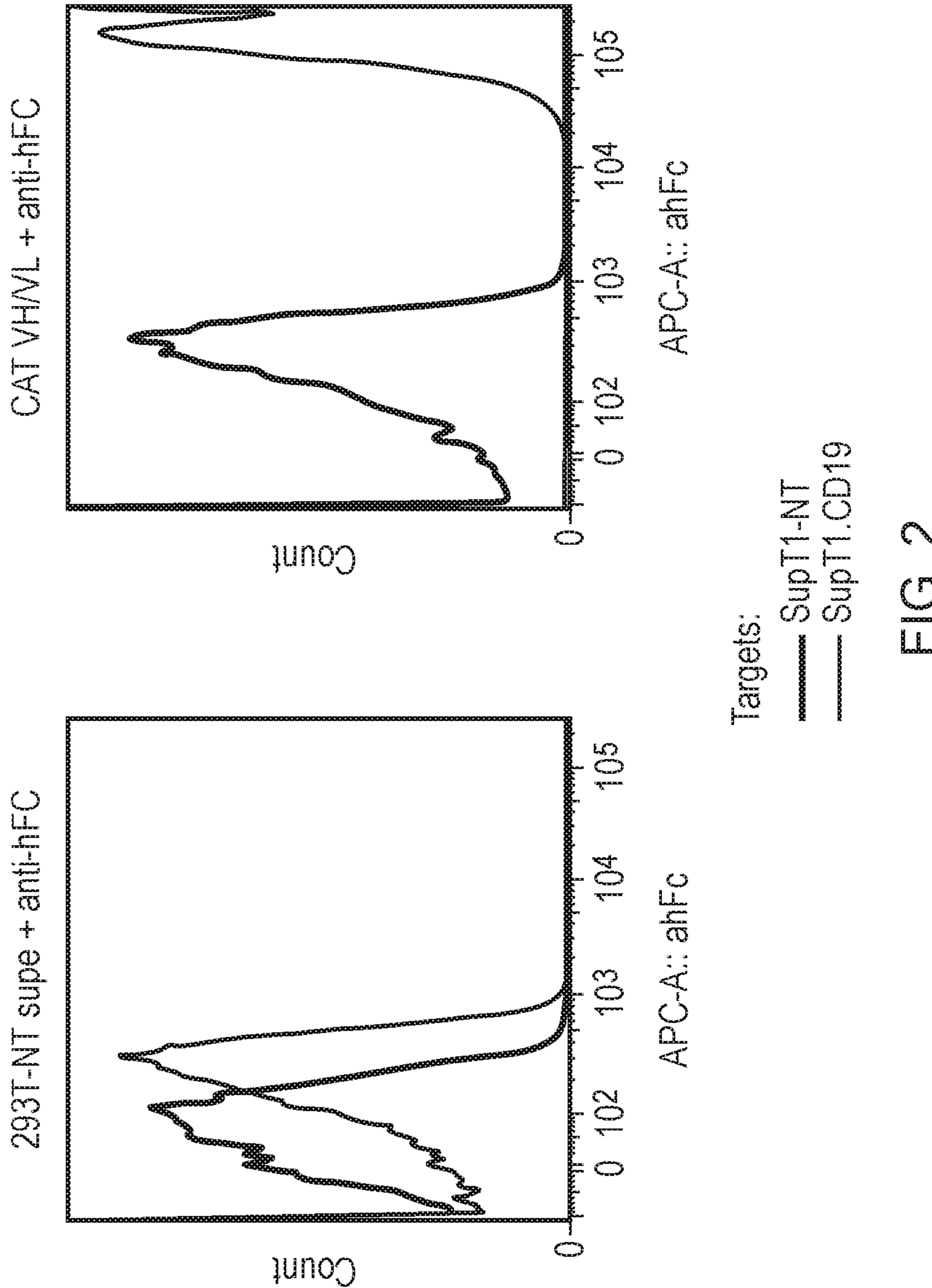
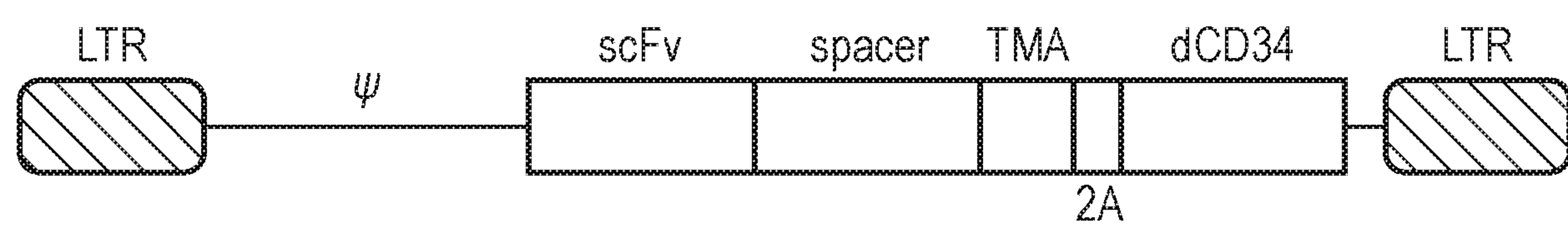


FIG. 2

(a)



(b)

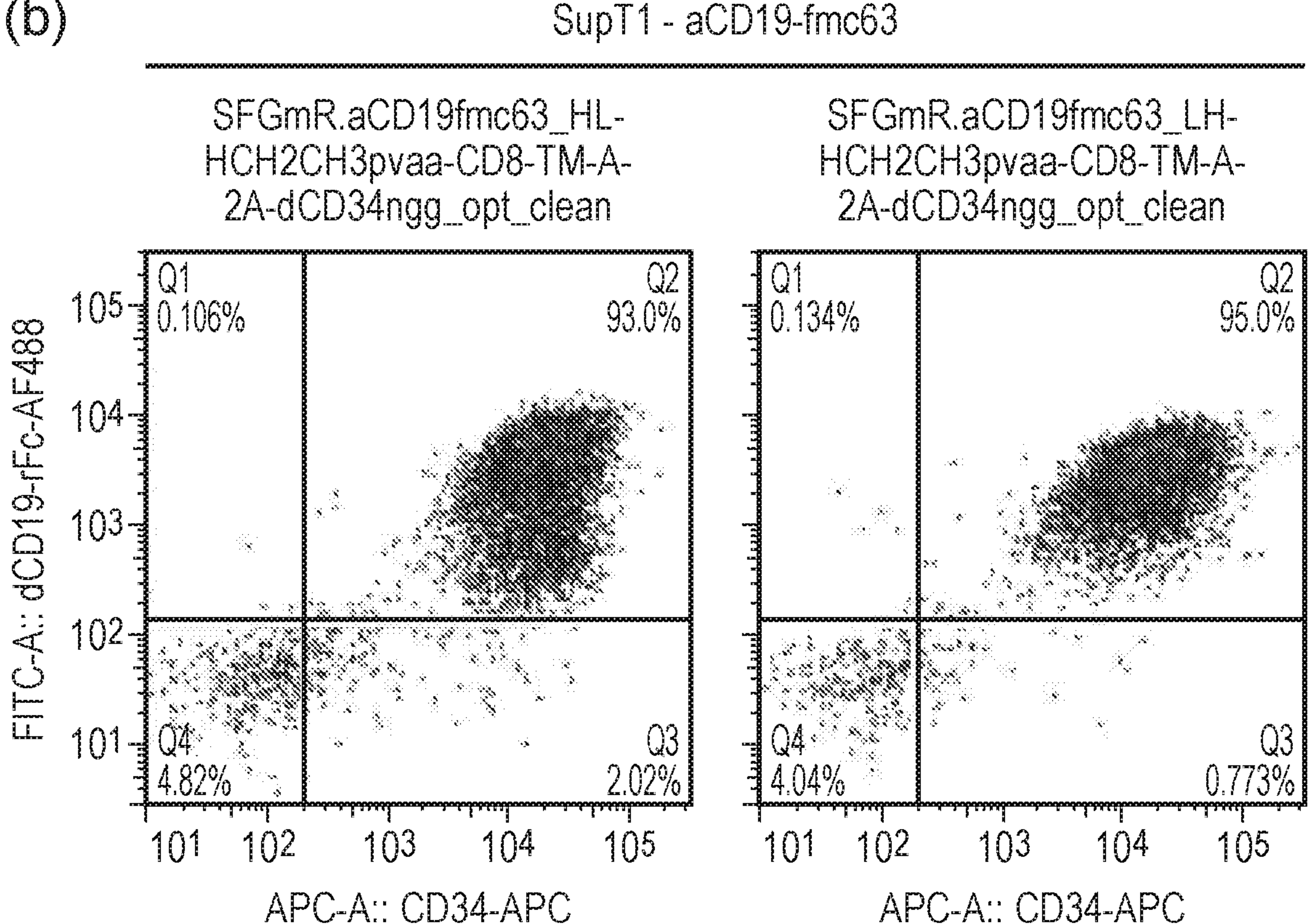


FIG. 3

(c) SupT1 - aCD19-4g7

SFGmR.aCD19_4g7_HL-HCH2CH3pvaa-CD8-TM-A-2A-dCD34ngg_opt_clean

Q1 0.00% | Q2 90.2% | Q3 0.872% | Q4 8.90%

SFGmR.aCD19_4g7_LH-HCH2CH3pvaa-CD8-TM-A-2A-dCD34ngg_opt_clean

Q1 0.00% | Q2 92.4% | Q3 0.951% | Q4 6.67%

Y axis: FITC-A:: dCD19-rFc-AF488 (10^1–10^5)

X axis: APC-A:: CD34-APC (10^1–10^5)

(d) SupT1 - aCD19-CAT-13 1E10

SFGmR.aCD19cat_HL-HCH2CH3pvaa-CD8-TM-A-2A-dCD34ngg_opt_clean

Q1 0.003% | Q2 94.8% | Q3 1.25% | Q4 3.85%

SFGmR.aCD19cat_LH-HCH2CH3pvaa-CD8-TM-A-2A-dCD34ngg_opt_clean

Q1 0.00% | Q2 0.072% | Q3 99.5% | Q4 0.467%

Y axis: FITC-A:: dCD19-rFc-AF488 (10^1–10^5)

X axis: APC-A:: CD34-APC (10^1–10^5)

aCD19-rFc + arFc-488

aCD34-APC

FIG. 3 (Continued)

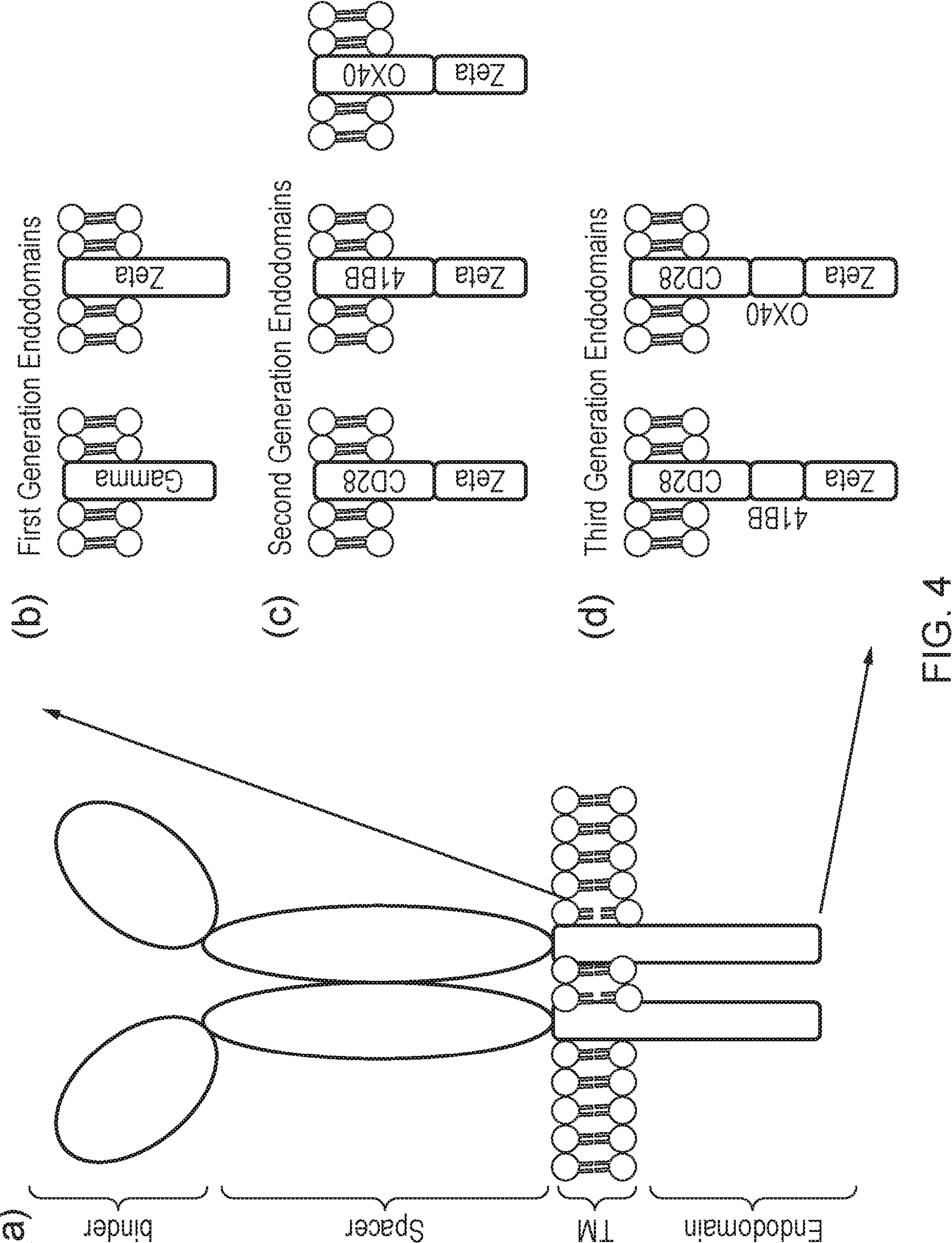


FIG. 4

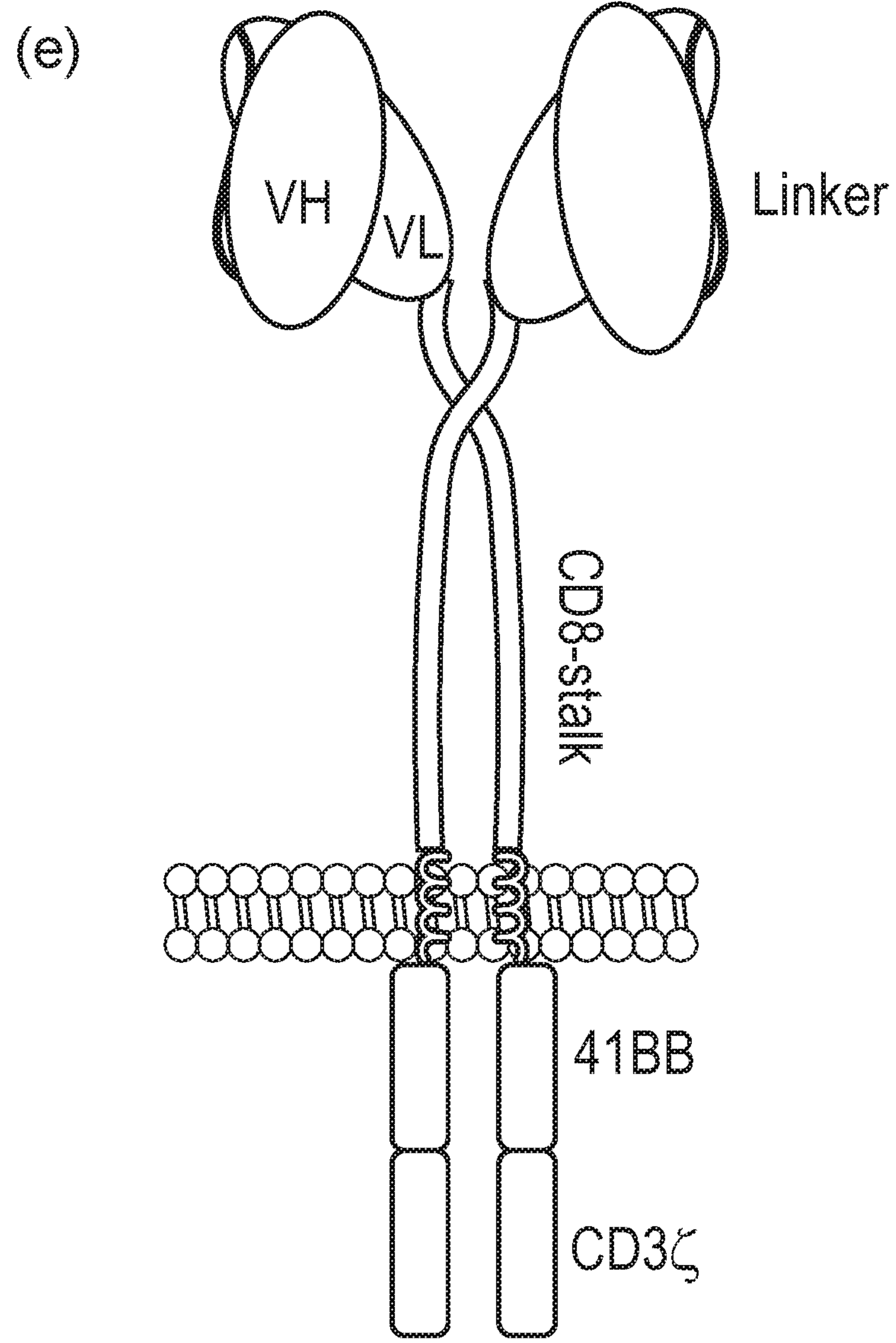


FIG. 4 (Continued)

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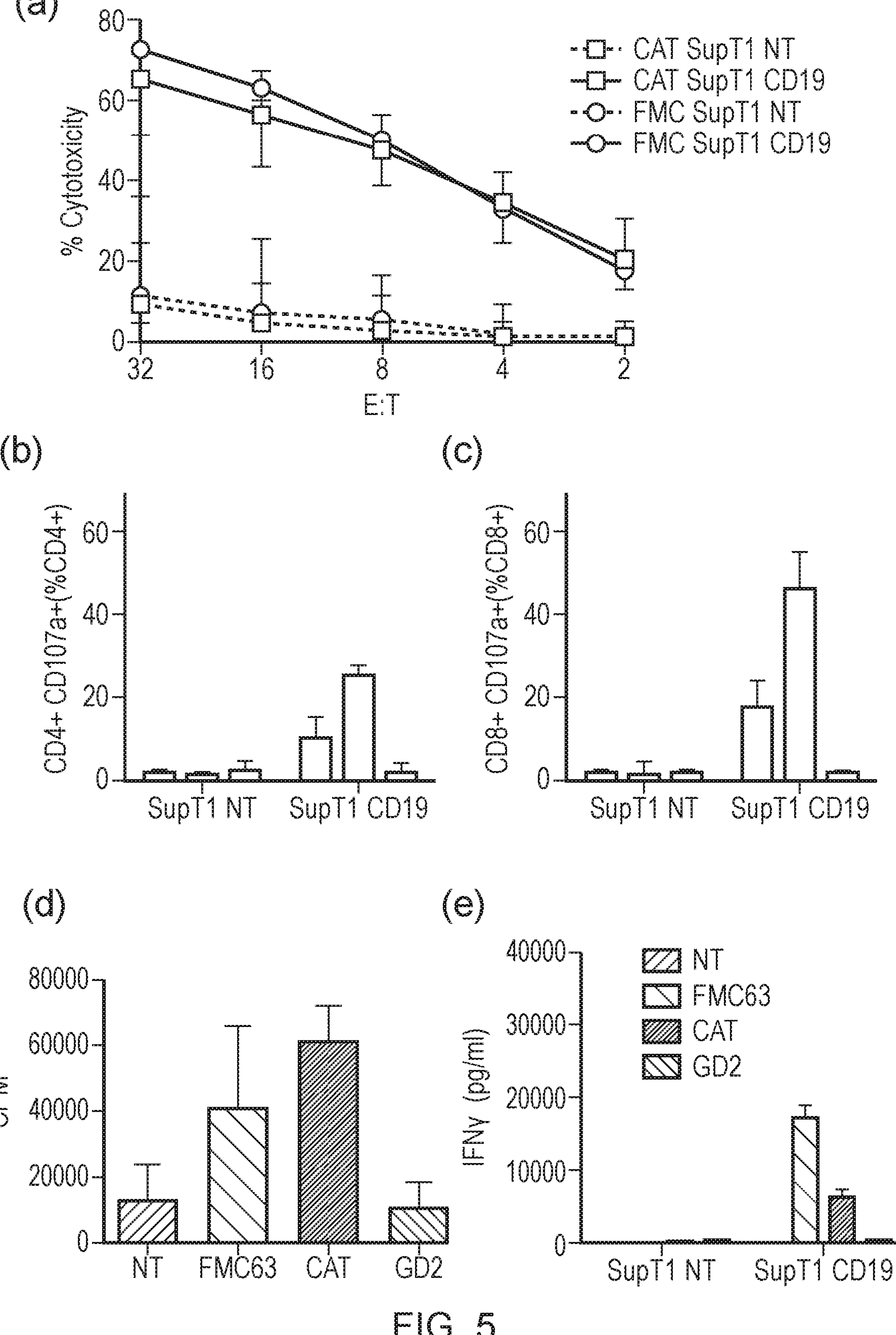
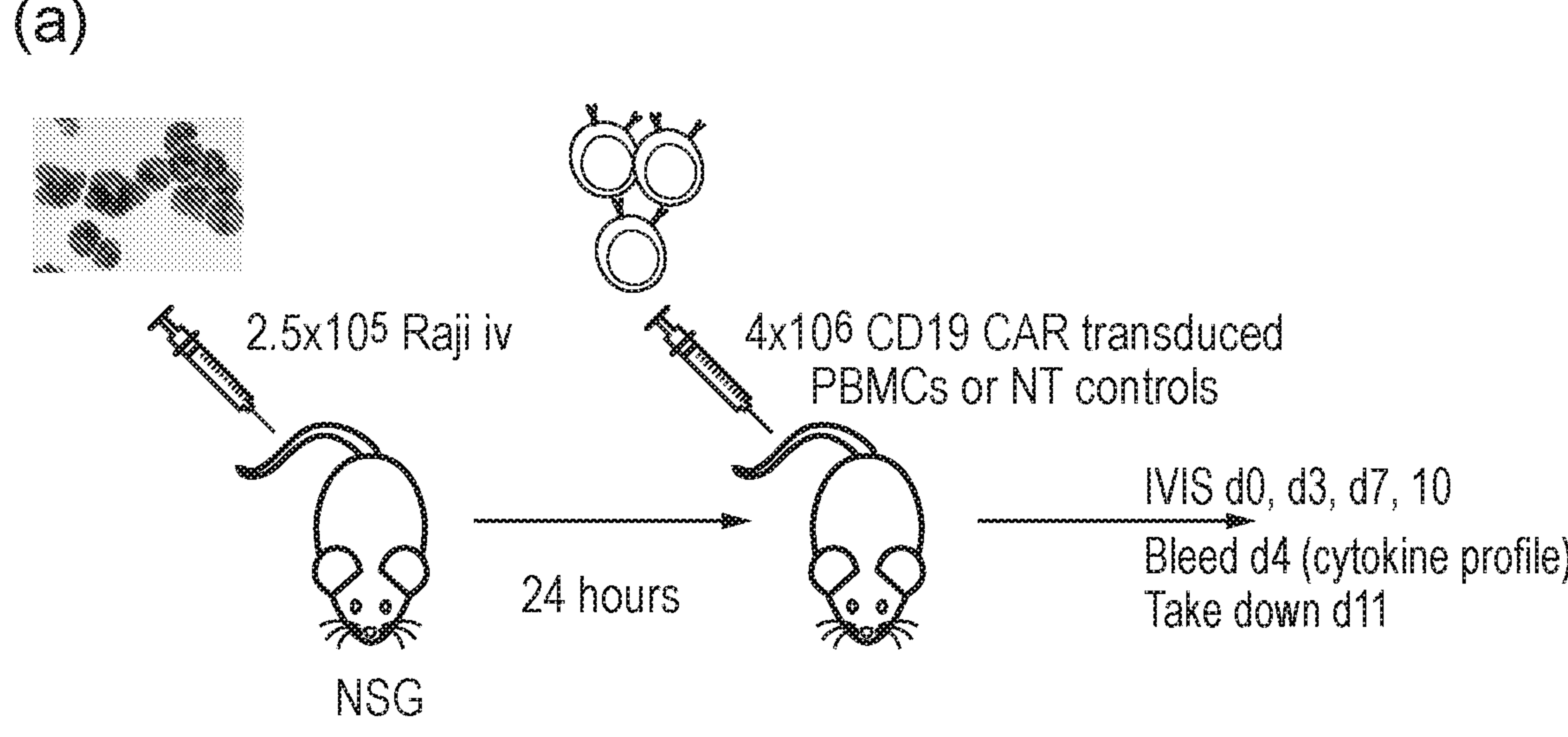
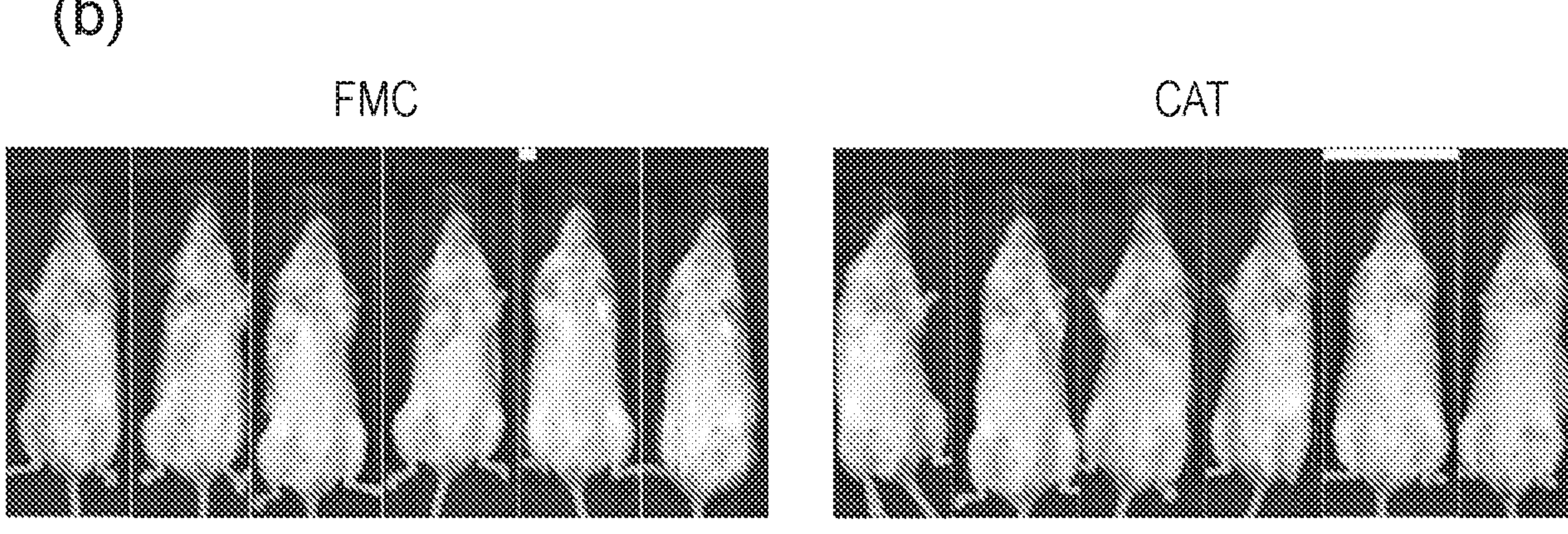


FIG. 5

(a)



(b)



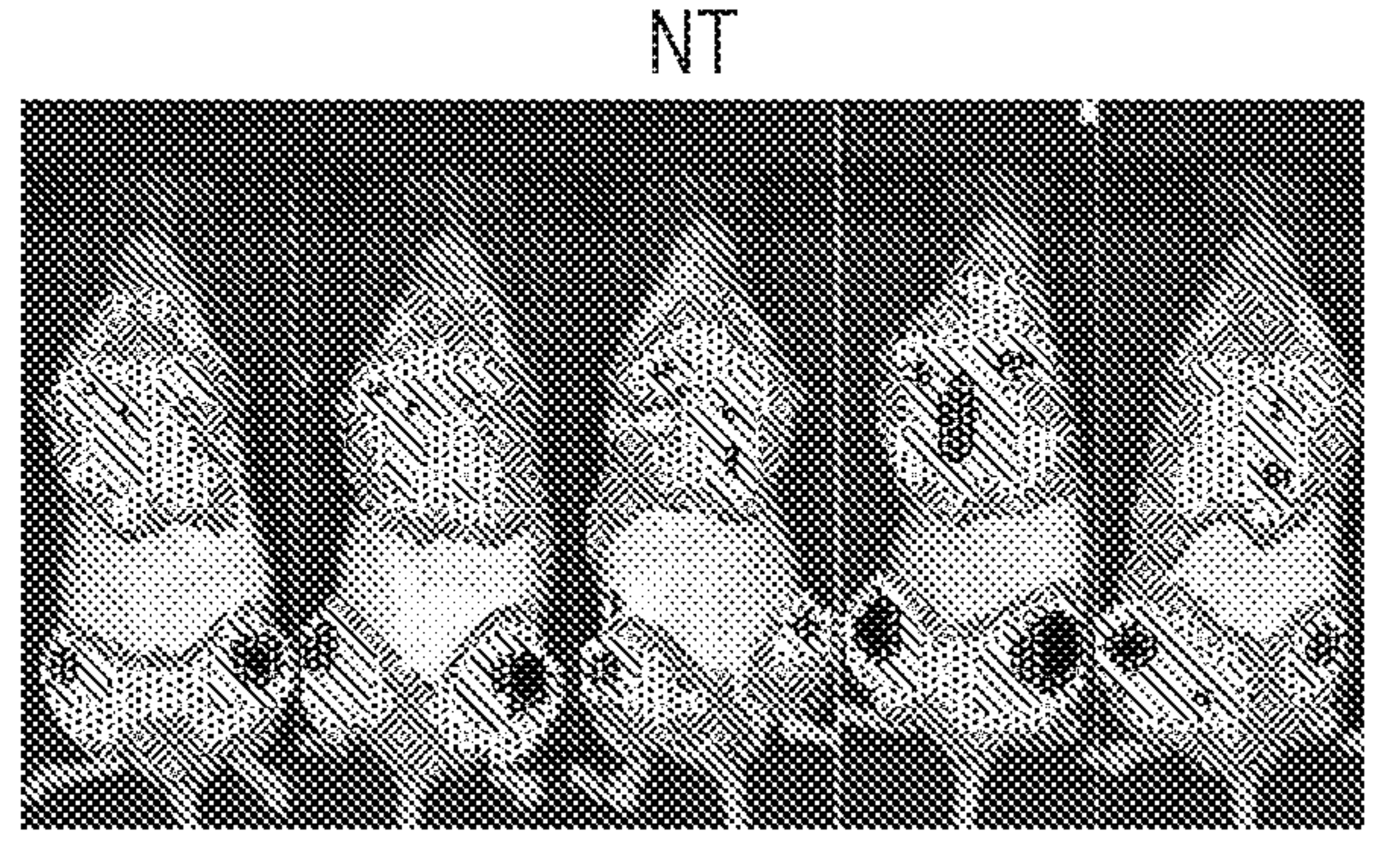


FIG. 6

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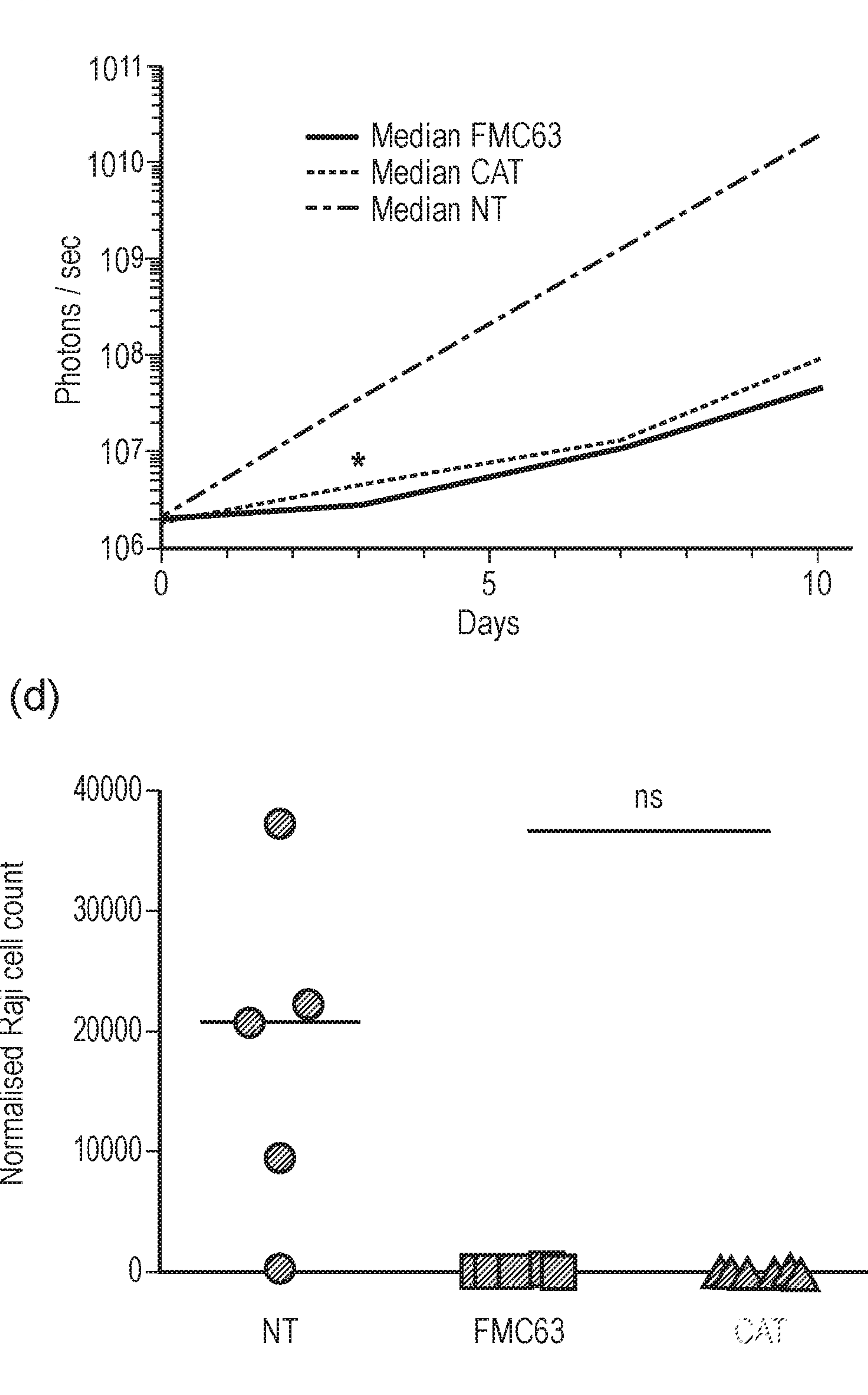

FIG. 6 (Continued)

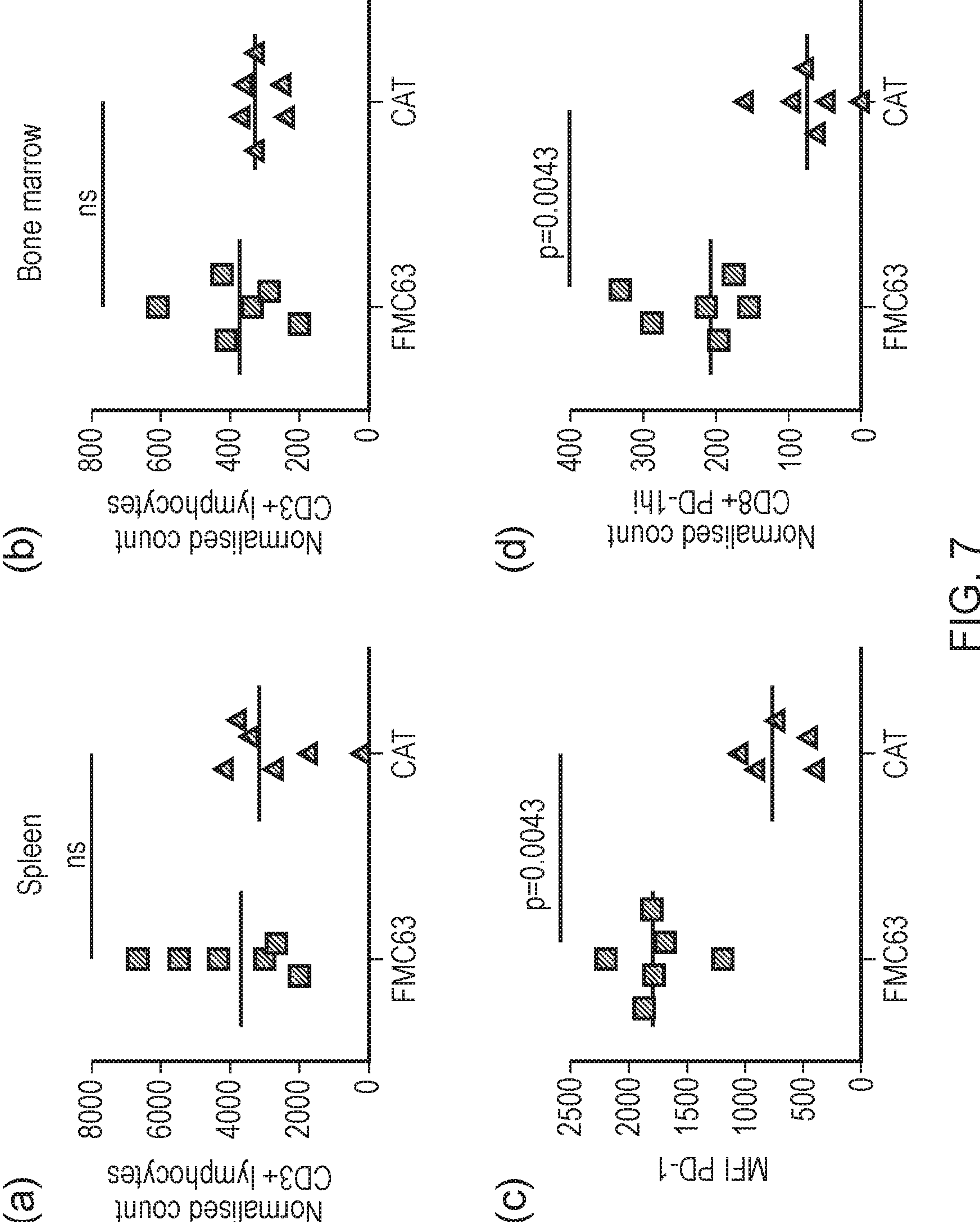

FIG. 7