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(54) Titre : RECEPTEUR ANTIGENIQUE CHIMERIQUE

(54) Title: CHIMERIC ANTIGEN RECEPTOR

(57) **Abrégé/Abstract:**

The present invention provides a chimeric antigen-receptor (CAR)-forming polypeptide comprising: (i) an anti-gen-binding domain; (ii) a coiled-coil spacer domain; (iii) a transmembrane domain; and (iv) an endodomain. The invention also provides a multimeric CAR formed by association of a plurality of CAR- forming polypeptides by virtue of association of their coiled-coil spacer domains.

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CHIMERIC ANTIGEN RECEPTOR

FIELD OF THE INVENTION

The present invention relates to a chimeric antigen receptor (CAR), comprising a particular spacer domain which causes the formation of multimeric CAR molecules at the cell surface. The multimeric CAR molecule may be “super-sensitive” and capable of inducing T-cell activation in response to binding an antigen which is expressed at low density of a target cell.

BACKGROUND TO THE INVENTION

Chimeric antigen receptors (CARs)

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 a solution to this problem as transgenic expression of Chimeric Antigen Receptor (CAR) allows generation of large numbers of T cells specific to any surface antigen by *ex vivo* viral vector transduction of a bulk population of peripheral blood T-cells.

Chimeric antigen receptors are proteins which graft the specificity of an antigen binder, such as a monoclonal antibody (mAb), to the effector function of a T-cell. Their usual form is that of a type I transmembrane domain protein with an antigen recognizing amino terminus, a spacer, a transmembrane domain all connected to a compound endodomain which transmits T-cell survival and activation signals (see Figure 1A).

The most common forms of these molecules are fusions of single-chain variable fragments (scFv) derived from monoclonal antibodies which recognize 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 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.

CARs often comprise a spacer domain to provide an appropriate distance between the antigen-binding domain and the cell membrane and to allow for suitable orientation, reach and segregation from phosphatases upon ligand engagement.

Common spacers used are the Fc of IgG1, the stalk from CD8 α and CD28 and even just the IgG1 hinge alone or the ectodomain of CD247 can suffice depending on the antigen (Figure 2b).

These common spacers are limited because they either must contain whole domain structures in order to form a functional spacer or they are heavily glycosylated and changes in amino acid length would result in unpredictable changes in spacer size. For example IgG spacers must contain whole numbers of immunoglobulin domains. This requirement for a whole number of structural domains means that the alterations that can be made to the spacers are limited.

In addition, the above listed spacers are typically long primary amino acid sequences which fold to form the required secondary and tertiary structures. As such they are typically encoded by long nucleic acid sequences. This requirement for a long nucleic acid sequence is a problem for the construction of vectors encoding the CARs.

Classical CARs have certain design constraints. As a classical CAR is homodimer, there are commonly two (identical) binding specificities and a 1:1 ratio of antigen binding domains to intracellular T-cell signalling domains. This imposes a certain stoichiometry and limits the flexibility of the system as a whole.

There is thus a need for alternative CARs which offer greater flexibility in terms of design.

Affinity issues

CAR binding domains are usually derived from the variable region of either pre-existing antibody or antibodies selected from a library. As a result, most selected CARs bind cognate ligand with nanomolar affinity. In contrast, the biophysical properties of TCR:peptide:MHC (TCR-pMHC) binding are usually 10-1 μ M (orders of magnitude lower in affinity). Although higher affinity interactions increase specificity for a ligand at a given receptor concentration, there is emerging evidence that the TCR has evolved to have a lower affinity so as to allow T-cells to detect target cells that express low density cognate peptide MHC.

It has been reported that a T-cell can be activated by as few as ten cognate pMHC and that one pMHC can trigger a productive signal in up to 200 TCR molecules. This is thought to be achieved through a process known as serial triggering; where one cognate pMHC present on the target cell can go through a cycle of binding, triggering and then dissociating from a TCR multiple times, effectively amplifying the signal. As a consequence, only a low number of cognate pMHC are needed to transduce a productive signal.

The higher affinity of CARs means that the molecular dissociation of an interaction can take minutes to hours, unlike TCR which is typically in the order of seconds. For this reason it is unlikely that CAR signalling undergoes an effective serial triggering response but relies instead on the ligation of higher numbers of receptors. This may limit CARs to target ligands that are expressed on target cells at high density. It has been estimated that a high affinity CAR requires a target cell to express >10k ligand molecules to elicit an effective killing response. More specifically, using a first generation CAR, James *et al.* have shown a requirement of ~30,000 target molecules/target cell (inducing endocytosis of ~20,000 CAR molecules) to trigger maximum lytic activity (S. James et al., The Journal of Immunology, vol. 184 (8) 4284-4294, 2010). Animal models indicate that target cells that express ligands below the threshold for killing can escape detection and can re-establish disease (U. Anurathapan et al., Molecular Therapy, vol. 22 (3) 623–633, 2014).

One method to increase CAR sensitivity to low density ligands is to use a low affinity binder domain which can then mimic the TCR-pMHC serial triggering response. However there are several limitations to this approach. The use of a low affinity CAR is currently unpredictable, due to the unknown contribution that co-stimulatory, pseudodimer formation and adhesion molecules play in TCR-pMHC serial triggering responses. In CAR therapy, these molecular interactions vary depending on the target cells and it is therefore difficult to obtain a robust serial triggering response. Furthermore, the methods to reduce the affinity of CARs to be on par with TCR usually involve starting with a high affinity CAR and then mutating the CDRs. This is a lengthy and often unsuccessful approach which can cause unpredictable results and increase the risk of off target binding.

Engineering a CAR that is able to trigger in the presence of low density ligand would allow the therapy to target many more cancers and also reduce the chance of cancer escape.

There is thus a need for CARs which are not associated with the problems outlined above.

SUMMARY OF ASPECTS OF THE INVENTION

In a first aspect the present invention provides a chimeric antigen receptor (CAR)-forming polypeptide comprising:

- (i) an antigen-binding domain;
- (ii) a coiled-coil spacer domain;
- (iii) a transmembrane domain; and
- (iv) an endodomain.

The present invention also provides an accessory polypeptide comprising:

- (i) a coiled-coil spacer domain;
- (iii) a transmembrane domain; and
- (iv) an endodomain.

The coiled-coil domain enables the multimerization of a plurality of CAR-forming polypeptides and/or accessory polypeptides, such as at least three CAR-forming polypeptides/accessory polypeptides, to form a multimeric CAR.

The coiled-coil domain may be derived from any of the following: cartilage-oligomeric matrix protein (COMP), mannose-binding protein A, coiled-coil serine-rich protein 1, polypeptide release factor 2, SNAP-25, SNARE, Lac repressor or apolipoprotein E.

The coiled-coil domain may comprise the sequence shown as SEQ ID No. 1 or a fragment thereof, or a variant thereof which has at least 80% sequence identity.

The endodomain may comprise at least one of CD3 zeta endodomain, CD28 endodomain, 41BB endodomain and OX40 endodomain.

The endodomain may comprise the sequence shown as SEQ ID No. 7 or a variant thereof which has at least 80% sequence identity.

The antigen-binding domain may bind an antigen which is expressed at a low density on a target cell. For example, the antigen-binding domain may bind to ROR-1, Typr-1 or BCMA.

The CAR-forming polypeptide may comprise an element capable of forming a bridge with another CAR.

For example, the element may be capable of forming a di-sulphide bridge with another CAR which contains such an element.

The second aspect of the invention relates to multimeric CARs which form due to interactions between the coiled-coil spacer domains or CAR-forming polypeptide(s) and/or accessory polypeptide(s).

In a first embodiment of the second aspect the present invention provides a multimeric chimeric antigen receptor (CAR) comprising a plurality of CAR-forming polypeptides as defined above.

In a second embodiment of the second aspect of the invention there is provided a multimeric chimeric antigen receptor (CAR) comprising one or more CAR-forming polypeptides and one or more accessory polypeptides as defined above.

The CAR-forming polypeptide(s) and/or accessory polypeptide(s) in a multimeric CAR may comprise different endodomains.

If the multimeric CAR comprises two or more CAR-forming polypeptides, they may have different antigen-binding domains, for example antigen-binding domains with different binding specificities.

One of the endodomains of the CAR-forming polypeptide and the accessory polypeptide may comprise a CD3 zeta endodomain and the other endodomain of the CAR-forming polypeptide and the accessory polypeptide may comprise a 41BB endodomain. Where there are two accessory polypeptides, one may comprise the 41BB endodomain and the other may comprise the CD28 endodomain.

The multimeric CAR may, for example, be dimeric, trimeric, tetrameric, pentameric, hexameric or heptameric.

A pentameric CAR may comprise any of the following combinations of CAR-forming polypeptide and accessory polypeptide chains:

CAR-forming polypeptide	Accessory polypeptide
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5	0
4	1
3	2
2	3
1	4
0	5

Where a multimeric CAR comprises first and second CAR-forming polypeptides according to the first aspect of the invention, the antigen-binding domain of the first CAR may bind to a different epitope than the antigen-binding domain of the second CAR.

In this embodiment, the antigen-binding domain of the first CAR may bind to a different antigen than the antigen-binding domain of the second CAR.

The present invention also provides an engaged complex which comprises at least two multimeric CARs according to the second aspect of the invention, wherein a first CAR on a first multimeric CAR forms a bridge with a second CAR on a second multimeric CAR, such that the first and second multimeric CARs engage to form a complex.

The bridge may be a disulphide bridge or an additional coiled coil structure.

In a third aspect the present invention provides a chimeric antigen receptor (CAR) signalling system, which comprises:

- (i) a multimeric CAR comprising a CAR-forming polypeptide or accessory polypeptide as defined above which comprises a first heterodimerization domain; and
- (ii) an intracellular signalling component comprising a signalling domain and a second heterodimerization domain;

wherein heterodimerization between the first and second heterodimerization domains causes the multimeric CAR and signalling component to form a functional CAR complex.

The or each CAR-forming polypeptide(s) or accessory polypeptide(s) may comprise a plurality of heterodimerisation domains, such that a single CAR-forming polypeptide or accessory polypeptide is capable of heterodimerising with a plurality of signalling components.

The signalling component of a CAR signalling system may comprise a plurality of signalling domains.

In a fourth aspect the present invention provides a nucleic acid which encodes a CAR-forming polypeptide according to the first aspect of the invention and/or an accessory polypeptide as defined above.

The fifth aspect of the invention relates to nucleic acid constructs which comprise two or more nucleic acid sequences.

In a first embodiment of the fifth aspect of the invention there is provided a nucleic acid construct which encodes two or more CAR forming polypeptides according to the first aspect of the invention.

In a second embodiment of the fifth aspect of the invention there is provided a nucleic acid construct which encodes at least one CAR-forming polypeptide and at least one accessory polypeptide as defined above.

In a first embodiment of the fifth aspect of the invention there is provided a nucleic acid construct which encodes:

- (i) at least one CAR-forming polypeptide according to the first aspect of the invention, which forms a multimeric CAR according to the second aspect of the invention; and
- (ii) an intracellular signalling component as defined in relation to the fifth aspect of the invention.

In a sixth aspect the present invention provides a vector which comprises a nucleic acid sequence according to the fourth aspect of the invention or a nucleic acid construct according to the fifth aspect of the invention.

The vector may be, for example, a retroviral vector or a lentiviral vector or a transposon.

In a seventh aspect the present invention provides a cell which expresses a CAR-forming polypeptide or accessory polypeptide according to the first aspect of the invention, a multimeric CAR according the second aspect of the present invention, a CAR signalling system according to the third aspect of the invention; or an engaged complex as defined above.

The cell may be a T cell or NK cell.

In an eighth aspect the present invention provides a pharmaceutical composition which comprises a cell according to the seventh aspect of the invention.

In a ninth aspect the present invention relates to a cell according to the seventh aspect of the invention for use in treating a disease.

In a tenth aspect the present invention relates to the use of a cell according to the seventh aspect of the invention in the manufacture of a medicament for treating a disease.

In an eleventh aspect the present invention relates to a method for treating a disease which comprises the step of administering a cell according to the seventh aspect of the invention to a subject.

The disease may be cancer, for example Chronic lymphocytic leukaemia (CLL), melanoma or myeloma.

In a twelfth aspect the present invention relates to a kit which comprises a nucleic acid according to fourth aspect of the invention, a nucleic acid construct according to the fifth aspect of the invention or a vector according to the sixth aspect of the present invention.

In a thirteenth aspect the present invention provides a kit which comprises a cell according to the seventh aspect of the invention.

In a fourteenth aspect the present invention relates to a method for making a cell according to the seventh aspect of the invention, which comprises the step of introducing a nucleic acid according to fourth aspect of the invention, a nucleic acid construct according to the fifth aspect of the invention or a vector according to the sixth aspect of the present invention.

The cell may be from a sample isolated from a subject.

The use of a coiled coil domain as a spacer in a CAR provides a number of advantages over spacers which have been used previously, such as Fc domains derived from IgG.

For example, the use of a coiled coil domain enables the spacer dimensions to be altered in 0.15nm increments. The addition or subtraction of individual amino acids or a number of

amino acids means that the size of the coiled coil spacer can be incrementally altered. In contrast, the use of IgG spacers only allows the addition or removal of whole immunoglobulin domains. This means that the lowest increment of change is ~4nm (i.e. the size of a folded immunoglobulin domain).

Coiled coil domains are coded by a smaller DNA fragment (e.g ~100 nucleotides) compared to the Fc domain derived from IgG (~700 nucleotides). This allows for a smaller DNA vector which is important for improving viral titre and transduction efficiency.

The use of a coiled coil spacer allows a selection from a large number of coiled coil spacers that will not cross-hybridize with other coiled coil domains. This is in contrast with other spacers where there are a more limited numbers of spacer options.

The use of a coiled coil spacer also much greater flexibility in terms of CAR design than a classical CAR. For example, it allows the formation of homo- or hetero-oligomeric CAR complexes. Hetero-oligomeric CAR complexes are useful when engineering multi-chain CARs with CD28/OX40/41BB and TCRz endodomains in order to ensure that each of the endodomains is located with optimal proximity to the membrane and present at the desired ratios.

The present inventors have engineered a hyper-sensitive CAR without changing the biophysical properties of the binder domain. This is desirable because methods to reduce the affinity of CAR binders are unpredictable and often have uncharacterised specificity.

The hyper-sensitive CAR is provided by increasing the valency of the CAR. In particular, the use of a coiled coil spacer domain which is capable of interacting to form a multimer comprising more than two CARs increases the sensitivity to targets expressing low density ligands due to the increase in ITAMs and avidity to the oligomeric CAR complex.

Sensitivity may be increased by increasing the ratio of coil-signal to scFv-coil, so each scFv is attached to many signaling elements (see Figure 10c). Sensitivity may also be increased via the formation of complexes of multimeric CARs (Figure 10f).

In a multimeric CAR of the invention, the signaling endodomains are provided in trans in a membrane proximal location, enabling fine tuning of the T-cell signalling domain combinations (Figure 10 a and b); and the incorporation of more than three distinct

intracellular signalling domains (Figure 10d) so that the structure includes more endodomain signals than a third generation CAR (Figure 1d).

The use of a separate intracellular signalling component molecule which heterodimerizes with the CAR intracellularly enables the further amplification on the number of endodomains per antigen binding domain, producing a “superCAR” (Figure 15).

A multimeric CAR of the invention may comprise more than one antigen-binding specificity, enabling a plurality of epitopes or antigens to be targeted (Figure 10e).

A multimeric CAR having a plurality of binding domains will have much greater avidity than a classical homodimeric CAR. This can be important, for example for binding domains with low affinity, as the accumulated strength of multiple affinities provides high specificity binding. A multimeric CAR may bind antigen in a fashion analogous to IgM, which comprises multiple immunoglobulins covalently linked to form a pentameric or hexameric structure,

DESCRIPTION OF THE FIGURES

Figure 1 - a) Schematic diagram illustrating a classical CAR. (b) to (d): Different generations and permutations of CAR endodomains: (b) initial designs transmitted ITAM signals alone through FcεR1-γ or CD3ζ endodomain, while later designs transmitted additional (c) one or (d) two co-stimulatory signals in the same compound endodomain.

Figure 2 - a) Schematic diagram illustrating a classical CAR. b) Schematic diagram illustrating common CAR spacers. “S” denotes disulfide bonds.

Figure 3 - Naturally occurring dimeric, trimeric and tetrameric coiled coil structures (modified from Andrei N. Lupas and Markus Gruber; Adv Protein Chem. 2005;70:37-78)

Figure 4 - Crystal structure of the pentameric coiled coil motif from collagen oligomeric matrix protein (COMP) and human IgG1. Individual chains are depicted with different colours. The coiled coil COMP structure is displayed from the N-terminus with the C-terminus extending into the page and also displayed from the profile with the C-terminus left to the N-terminus right. The human IgG1 is displayed from the profile with the N-terminus (top) to C-terminus (bottom).

Figure 5 - Coiled coil spacer CAR. a) Schematic diagram illustrating a CAR with a pentameric coiled coil spacer derived from COMP. b) Construct map displaying the ORF of the COMP CARs and control CARs. c) Amino acid sequence of the ORF of the anti-CD33 COMP CAR and anti-ROR-1 COMP CAR. d) DNA sequence of the ORF of the anti-CD33 COMP CAR and anti-ROR-1 COMP CAR.

Figure 6 - Coiled coil CAR surface expression levels. a) A murine T-cell line was transduced with the anti-CD33 COMP CAR or anti-CD33 IgG1 CAR. These cells were then stained with chimeric soluble CD33 fused to murine Fc IgG2a before a secondary stain with anti-mouse IgG PE. b) A murine T-cell line was transduced with the anti-ROR-1 COMP CAR or anti-ROR-1 IgG1 CAR. These cells were then stained with soluble His tagged ROR-1 followed by a secondary stain with anti-His-biotin and then a third stain with streptavidin-APC.

Figure 7 - Stimulation of anti-ROR-1 COMP CAR T-cells with immobilised ligand. Transduced murine T-cells were co-cultured with anti-His beads that were pre-coated with different concentrations of soluble His tagged ROR-1 supernatant. The amount of IL-2 in the co-culture supernatant was analysed after 16-24 hours via ELISA.

Figure 8 - Expression levels of ROR-1 on target cells. The SKW cell line naturally expresses low levels of ROR-1. These cells were transduced with ROR-1 to increase the expression levels. These cells were stained with anti-ROR-1 APC and compared to non-stained cells.

Figure 9 - Stimulation of anti-ROR-1 COMP CAR T-cells with ROR-1 positive SKW cells. Transduced murine T-cells were co-cultured with SKW target cells that express the ROR-1 ligand at low or high density. T-cells were maintained at a constant number and the number of target cells was varied. The amount of IL-2 in the co-culture supernatant was analysed after 16-24 hours via ELISA. The grey shaded region denotes the standard curve range for that experiment. The dotted blue line is the average IL-2 secretion from PMA and Ionomycin stimulation. The red dotted line is the average IL-2 detected from cultures of just T-cells (non-stimulated).

Figure 10 – Coiled coil CAR designs. a) Schematic diagram illustrating a CAR made up of a CAR-forming polypeptide and an accessory polypeptide. The CAR-forming polypeptide provides signal one to the T-cell and consists of a scFv binder on the N-terminus followed by a COMP spacer, transmembrane and TCRz. The accessory polypeptide provides signal three to the T-cell and consists of no N-terminal ligand binder but begins with the COMP spacer followed by a transmembrane and the signalling motif of 41BB; b) Schematic diagram

illustrating another coiled coil CAR system which includes a CAR-forming polypeptide and two accessory polypeptides. The CAR-forming polypeptide provides signal one to the T-cell and consists of a scFv binder on the N-terminus followed by a COMP spacer, transmembrane and TCRz. The first accessory polypeptide provides signal two to the T-cell and consists of no N-terminal ligand binder but begins with the COMP spacer followed by a transmembrane and the signalling motif of CD28. The second accessory polypeptide provides signal three to the T-cell and consists of no N-terminal ligand binder but begins with the COMP spacer followed by a transmembrane and the signalling motif of 41BB. "S" denotes disulfide bonds. This arrangement allows the signalling endodomains to be in *trans* in a membrane-proximal location, leading to better signalling; c) Schematic diagram illustrating another coiled coil CAR system in which the ratio of scFv:coil is much less than that of coil:signal, so each scFv is attached to many signalling elements. This is an amplification system: by limiting the scFv to one per complex, each engaged ligand will signal through 5 TCRz chains (two ligands through 10 TCRz). This is as opposed to the traditional dimeric CARs where two ligands signal through 2 TCRz chains. This system will essentially increase triggering power by 5 fold; d) Schematic diagram illustrating another coiled coil CAR system which comprises more endodomains than a third generation CAR. The coiled coil spacer enables the introduction of two additional signalling domains ("EXTRA") over and above a traditional third generation CAR; e) Schematic diagram illustrating another coiled coil CAR signalling system which comprises multiple targets (here two target-binding specificities are shown: one which binds ligand A and one which binds ligand B). This arrangement is an alternative architecture for a TanCAR; f) Schematic diagram illustrating another coiled coil CAR system which comprises an element which forms a link such as a bridging di-sulphide with another coiled-coil spacer CAR giving an engaged complex which further increases the valency of the scFv:signalling domain. Like the arrangement shown in Figure 10c), this is an amplification system. Each engaged complex will signal through 10 TCRz chains. As opposed to the traditional dimeric CARs that signal through 2 TCRz chains. This system will essentially increase triggering power by 5 fold. Replacing the IgG hinge with a trimeric coiled coil structure would increase this to just short of 8 fold, whereas a tetrameric coiled coil would increase it by 10 fold.

Figure 11 – Truncation of the COMP spacer

- a) schematic diagram showing the anti-ROR-1 COMP CAR, the COMP spacer was truncated from the N-terminus from 45 amino acids to "x" amino acids
- b) 293T cells were transfected with the truncated constructs and analysed by FACS.

Figure 12 – Schematic diagram showing the multimeric and classical CARs tested in Example 6

A) a heteromultimeric CAR which comprises: a polypeptide having an anti-CD19 antigen binding domain; a coiled-coil spacer domain and a CD3zeta endodomain; and an accessory polypeptide having a coiled-coil spacer domain and a 41BB endodomain. The CAR is encoded by a bicistronic construct having the structure: aCD19fmc63-COMP-CD28tmZ-2A-COMP-CD28tm-41BB. In this CAR structure, the 41BB and TCRzeta signalling motifs are in parallel.

B) a homomultimeric CAR made up of polypeptides comprising an anti-CD19 antigen binding domain; a coiled-coil spacer domain and a combined 41BB/CD3zeta endodomain. The CAR is encoded by a construct having the structure: aCD19fmc63-COMP-CD8TM-41BB-Z. In this CAR structure, the 41BB and TCRzeta signalling motifs are in sequential order.

C) a classical second generation homodimeric CAR which comprises two polypeptides having an anti-CD19 antigen-binding domain, a CD8 stalk spacer domain and a combined 41BB/CD3zeta endodomain. The CAR is encoded by a bicistronic construct which also encodes the suicide gene RQR8. The construct has the structure: RQR8-2A-aCD19fmc63-CD8STK-41BBZ.

Figure 13 – Killing of CD19+ SupT1 target cells by the CARs shown in Figure 12 at day 2.

Figure 14 - Killing of CD19+ SupT1 target cells by the CARs shown in Figure 12 at day 5.

Figure 15 – Schematic diagram of the Coiled-coil SuperCAR constructs tested in Example 7:

aCD19-IgGFc-Z – a classical homodimeric CAR comprising 2 TCRz molecules per molecule, having the fmc63 aCD19 binder.

A coiled coil SuperCAR made up of five polypeptides each comprising four separate AD1 domains. The coiled-coil SuperCAR therefore comprises 20 AD1 domains.

COMP_x4AD1 – the coiled-coil SuperCAR was tested in combination with a signalling component having 0 copies of the TCR zeta signalling domain. This was used as a negative control.

COMP_x4AD1 + Z-DDD1-Z - the coiled-coil SuperCAR was tested in combination with a signalling component having 2 copies of the TCR zeta signalling domain. As DDD1 binds AD1 in a 2:1 stoichiometry, this signalling domains gives 80 copies of the TCR zeta domain for each 5-polypeptide coiled-coil CAR targeting component.

Figure 16 – IL2 release following challenge with target cells expressing the cognate antigen (CD19) at different concentrations: low, mid and high.

DETAILED DESCRIPTION

CHIMERIC ANTIGEN RECEPTORS (CARs)

Classical CARs, which are shown schematically in Figure 1, are chimeric type I trans-membrane proteins which connect an extracellular antigen-recognizing domain (binder) to an intracellular signalling domain (endodomain). The binder is typically a single-chain variable fragment (scFv) derived from a monoclonal antibody (mAb), but it can be based on other formats which comprise an antibody-like or ligand-based antigen binding site. A trans-membrane domain anchors the protein in the cell membrane and connects the spacer to the endodomain.

Early CAR designs had endodomains derived from the intracellular parts of either the γ chain of the Fc ϵ R1 or CD3 ζ . 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 have been constructed: fusion of the intracellular part of a T-cell co-stimulatory molecule to that of CD3 ζ results in second generation receptors which can transmit an activating and co-stimulatory signal simultaneously after antigen recognition. The co-stimulatory domain most commonly used is that of CD28. This supplies the most potent co-stimulatory signal - namely immunological signal 2, which triggers T-cell proliferation. Some receptors have also been described which include TNF receptor family endodomains, such as the closely related OX40 and 41BB which transmit survival signals. Even more potent third generation CARs have now been described which have endodomains capable of transmitting activation, proliferation and survival signals.

CAR-encoding nucleic acids may be transferred to T cells using, for example, retroviral vectors. In this way, a large number of antigen-specific T cells can be generated for adoptive cell transfer. When the CAR binds the target-antigen, this results in the transmission of an activating signal to the T-cell it is expressed on. Thus the CAR directs the specificity and cytotoxicity of the T cell towards cells expressing the targeted antigen.

The present CAR comprises an antigen-binding domain, a coiled-coil spacer domain, a transmembrane domain and an endodomain. The coiled-coil spacer domain provides numerous advantages over the spacers previously described in the art.

COILED COIL DOMAIN

CARs typically comprise a spacer sequence to connect the antigen-binding domain with the transmembrane domain. The spacer allows the antigen-binding domain to have a suitable orientation and reach. The spacer also provides segregation from phosphatases upon ligand engagement.

The CAR of the present invention comprises a coiled coil spacer domain.

A coiled coil is a structural motif in which two to seven alpha-helices are wrapped together like the strands of a rope (Figure 3). Many endogenous proteins incorporate coiled coil domains. The coiled coil domain may be involved in protein folding (e.g. it interacts with several alpha helical motifs within the same protein chain) or responsible for protein-protein interaction. In the latter case, the coiled coil can initiate homo or hetero oligomer structures.

As used herein, the terms 'multimer' and 'multimerization' are synonymous and interchangeable with 'oligomer' and 'oligomerization'.

The structure of coiled coil domains is well known in the art. For example as described by Lupas & Gruber (Advances in Protein Chemistry; 2007; 70; 37-38).

Coiled coils usually contain a repeated pattern, hxxhcx, of hydrophobic (h) and charged (c) amino-acid residues, referred to as a heptad repeat. The positions in the heptad repeat are usually labeled abcdefg, where a and d are the hydrophobic positions, often being occupied by isoleucine, leucine, or valine. Folding a sequence with this repeating pattern into an alpha-helical secondary structure causes the hydrophobic residues to be presented as a 'stripe' that coils gently around the helix in left-handed fashion, forming an amphipathic structure. The most favourable way for two such helices to arrange themselves in the cytoplasm is to wrap the hydrophobic strands against each other sandwiched between the hydrophilic amino acids. Thus, it is the burial of hydrophobic surfaces that provides the thermodynamic driving force for the oligomerization. The packing in a coiled-coil interface is exceptionally tight, with almost complete van der Waals contact between the side-chains of the a and d residues.

The α -helices may be parallel or anti-parallel, and usually adopt a left-handed super-coil. Although disfavoured, a few right-handed coiled coils have also been observed in nature and in designed proteins.

The coiled coil domain may be any coiled coil domain which is capable of forming a coiled coil multimer such that a complex of CARs or accessory polypeptides comprising the coiled coil domain is formed.

The relationship between the sequence and the final folded structure of a coiled coil domain are well understood in the art (Mahrenholz *et al*; Molecular & Cellular Proteomics; 2011; 10(5):M110.004994). As such the coiled coil domain may be a synthetically generated coiled coil domain.

Examples of proteins which contain a coiled coil domain include, but are not limited to, kinesin motor protein, hepatitis D delta antigen, archaeal box C/D sRNP core protein, cartilage-oligomeric matrix protein (COMP), mannose-binding protein A, coiled-coil serine-rich protein 1, polypeptide release factor 2, SNAP-25, SNARE, Lac repressor or apolipoprotein E.

The sequence of various coiled coil domains is shown below:

Kinesin motor protein: parallel homodimer (SEQ ID No. 30)

MHAALSTEVVHLRQRTEELLRCNEQQAAELETCKEQLFQSNMERKELHNTVMDLRGN

Hepatitis D delta antigen: parallel homodimer (SEQ ID No. 31)

GREILEQWVSGRKKLEELERDLRKLKKKIKKLEEDNPWLGNIIGIIGKY

Archaeal box C/D sRNP core protein: anti-parallel heterodimer (SEQ ID No. 32)

RYVVALVKALEEIDESINMLNEKLEDIRAVKESEITEKFEEKIRELRELRRDVEREIEEVM

Mannose-binding protein A: parallel homotrimer (SEQ ID No. 33)

AIEVKLANMEAEINTLKSLELTNKLHAFSM

Coiled-coil serine-rich protein 1: parallel homotrimer (SEQ ID No. 34)

EWEALEKKLAALLESKLQALEKKLEALEHG

Polypeptide release factor 2: anti-parallel heterotrimer

Chain A: INPVNNRIQDLTERSDVLRGYLDY (SEQ ID No. 35)

Chain B: VVDTLDQMKQGLEDVSGLLLELAVEADDEETFNEAVAELDALEEKLAQLEFR (SEQ ID No. 36)

SNAP-25 and SNARE: parallel heterotetramer

Chain A: IETRHSEIIKLENSIRELHDMFMDMAMLVESQGEMIDRIEYNVEHAVDYVE (SEQ ID No. 37)

Chain B: ALSEIETRHSEIIKLENSIRELHDMFMDMAMLVESQGEMIDRIEYNVEHAVDYVERA VSDTKKAVKY (SEQ ID No. 38)

Chain C: ELEEMQRRADQLADESLESTRMLQLVEESKDAGIRTLVMLDEQGEQLERIEE GMDQINKDMKEAEKNL (SEQ ID No. 39)

Chain D: IETRHSEIIKLENSIRELHDMFMDMAMLVESQGEMIDRIEYNVEHAVDYVE (SEQ ID No. 40)

Lac repressor: parallel homotetramer

SPRALADSLMQLARQVSRLE (SEQ ID No. 41)

Apolipoprotein E: anti-parallel heterotetramer

SGQRWELALGRFWDYLRWVQTLSEQVQEELLSSQVTQELRALMDETMKELKAYKSELEE QLTARLSKELQAAQARLGADMEDVCGRLVQYRGEVQAMLGQSTEELRVRLASHLRKLRKR LLRDADDLQKRLAVYQA (SEQ ID No. 42)

The coiled coil domain is capable of oligomerization. In certain embodiments, the coiled coil domain may be capable of forming a trimer, a tetramer, a pentamer, a hexamer or a heptamer.

A coiled-coil domain is different from a leucine zipper. Leucine zippers are super-secondary structures that function as a dimerization domains. Their presence generates adhesion forces in parallel alpha helices. A single leucine zipper consists of multiple leucine residues at approximately 7-residue intervals, which forms an amphipathic alpha helix with a hydrophobic region running along one side. This hydrophobic region provides an area for dimerization, allowing the motifs to "zip" together. Leucine zippers are typically 20 to 40 amino acids in length, for example approximately 30 amino acids.

Leucine zippers are typically formed by two different sequences, for example an acidic leucine zipper heterodimerizes with a basic leucine zipper. An example of a leucine zipper is

the docking domain (DDD1) and anchoring domain (AD1) which are described in more detail below.

Leucine zippers form dimers, whereas the coiled-coiled spacers of the present invention form multimers (trimers and above). Leucine zippers heterodimerise in the dimerization portion of the sequence, whereas coiled-coil domains homodimerise.

In one embodiment, the present invention provides a hyper-sensitive CAR.

The hyper-sensitive CAR is provided by increasing the valency of the CAR. In particular, the use of a coiled coil spacer domain which is capable of interacting to form a multimer comprising more than two coiled coil domains, and therefore more than two CARs, increases the sensitivity to targets expressing low density ligands due to increasing the number of ITAMs present and avidity of the oligomeric CAR complex.

Thus in one embodiment the present CAR-forming polypeptide comprises a coiled coil spacer domain which enables the multimerization of at least three CAR-forming polypeptides. In other words, the CAR comprises a coiled coil domain which is capable of forming a trimer, a tetramer, a pentamer, a hexamer or a heptamer of coiled coil domains.

Examples of coiled coil domains which are capable of forming multimers comprising more than two coiled coil domains include, but are not limited to, those from cartilage-oligomeric matrix protein (COMP), mannose-binding protein A, coiled-coil serine-rich protein 1, polypeptide release factor 2, SNAP-25, SNARE, Lac repressor or apolipoprotein E (see SEQ ID Nos. 30-42 above).

The coiled coil domain may be the COMP coiled coil domain.

COMP is one of the most stable protein complexes in nature (stable from 0°C-100°C and a wide range of pH) and can only be denatured with 4-6M guanidine hydrochloride. The COMP coiled coil domain is capable of forming a pentamer. COMP is also an endogenously expressed protein that is naturally expressed in the extracellular space. This reduces the risk of immunogenicity compared to synthetic spacers. Furthermore, the crystal structure of the COMP coiled coil motif has been solved which gives an accurate estimation on the spacer length (Figure 4). The COMP structure is ~5.6nm in length (compared to the hinge and CH2CH3 domains from human IgG which is ~8.1nm).

The coiled coil domain may consist of or comprise the sequence shown as SEQ ID No. 1 or a fragment thereof.

SEQ ID No. 1

DLGPQMLRELQETNAALQDVRELLRQQVREITFLKNTVMECDACG

As shown in Figure 11, it is possible to truncate the COMP coiled-coil domain at the N-terminus and retain surface expression. The coiled-coil domain may therefore comprise or consist of a truncated version of SEQ ID No. 1, which is truncated at the N-terminus. The truncated COMP may comprise the 5 C-terminal amino acids of SEQ ID No. 1, i.e. the sequence CDACG. The truncated COMP may comprise 5 to 44 amino acids, for example, at least 5, 10, 15, 20, 25, 30, 35 or 40 amino acids. The truncated COMP may correspond to the C-terminus of SEQ ID No. 1. For example a truncated COMP comprising 20 amino acids may comprise the sequences QQVREITFLKNTVMECDACG. Truncated COMP may retain the cysteine residue(s) involved in multimerisation. Truncated COMP may retain the capacity to form multimers.

Various coiled coil domains are known which form hexamers such as gp41 derived from HIV, and an artificial protein designed hexamer coiled coil described by N. Zaccai et al. (2011) Nature Chem. Bio., (7) 935-941). A mutant form of the GCN4-p1 leucine zipper forms a heptameric coiled-coil structure (J. Liu. et al., (2006) PNAS (103) 15457–15462).

The coiled coil domain may comprise a variant of one of the coiled coil domains described above, providing that the variant sequence retains the capacity to form a coiled coil oligomer. For example, the coiled coil domain may comprise a variant of the sequence shown as SEQ ID No. 1 or 30 to 42 having at least 80, 85, 90, 95, 98 or 99% sequence identity, providing that the variant sequence retains the capacity to form a coiled coil oligomer.

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>.

ANTIGEN BINDING DOMAIN

The antigen-binding domain is the portion of a classical CAR which recognizes antigen.

Numerous antigen-binding domains are known in the art, including those based on the antigen binding site of an antibody, antibody mimetics, and T-cell receptors. For example,

the antigen-binding domain may comprise: a single-chain variable fragment (scFv) derived from a monoclonal antibody; a natural ligand of the target antigen; a peptide with sufficient affinity for the target; a single domain binder such as a camelid; an artificial binder single as a Darpin; or a single-chain derived from a T-cell receptor.

Various tumour associated antigens (TAA) are known, as shown in the following Table 1. The antigen-binding domain used in the present invention may be a domain which is capable of binding a TAA as indicated therein.

Table 1

Cancer type	TAA
Diffuse Large B-cell Lymphoma	CD19, CD20, CD22
Breast cancer	ErbB2, MUC1
AML	CD13, CD33
Neuroblastoma	GD2, NCAM, ALK, GD2
B-CLL	CD19, CD52, CD160
Colorectal cancer	Folate binding protein, CA-125
Chronic Lymphocytic Leukaemia	CD5, CD19
Glioma	EGFR, Vimentin
Multiple myeloma	BCMA, CD138
Renal Cell Carcinoma	Carbonic anhydrase IX, G250
Prostate cancer	PSMA
Bowel cancer	A33

In certain embodiments, the present invention provides a hyper-sensitive CAR which is capable of stimulating cell activation in response to antigen which is expressed on a target cell at a low density.

The antigen binding domain may bind a TAA which is expressed on a cell, for example a cancer cell, at a low density. A TAA expressed at low density may refer, for example, to a TAA expressed at a level of 10s to 1000s molecules per cell.

Examples of TAAs which are known to be expressed at low densities in certain cancers include, but are not limited to, ROR1 in CLL, Typr-1 in melanoma and BCMA in myeloma.

Antigen-binding domains (such as scFvs or mAbs) which bind these TAAs have previously been described, for example as shown in the following table:

Tumour-associated antigen	Antigen-binding domain	Reference
ROR-1	2A2, 2D11	S. Baskar et al., Landes Bioscience, vol. 4, (3) 349–361), R12, R11, Y31 (J. Yang et al., PLOSONe, vol. 6, (6), e21018, 2011
Tyrb-1	TA99	P. Boross et al., Immunology Letters, vol. 160, (2), 151-157, 2014
BCMA	C12A3.2 and C11D5.3	R. Carpenter et al., Clin Cancer Res., vol. 19, (8) 2048–2060, 2013), J6M0 (Y. Tai et al., Blood, vol 123, (20), 3128-3138, 2014

TRANSMEMBRANE DOMAIN

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

SIGNAL PEPTIDE

The CAR-forming polypeptides and/or accessory polypeptides of the present invention may comprise a signal peptide so that when it is expressed in 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 signal peptide may comprise the sequence shown as SEQ ID No. 2, 3 or 4 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. 2: MGTSLLCWMALCLLGADHADG

The signal peptide of SEQ ID No. 2 is compact and highly efficient and is derived from TCR beta chain. It is predicted to give about 95% cleavage after the terminal glycine, giving efficient removal by signal peptidase.

SEQ ID No. 3: MSLPVTALLPLALLHAARP

The signal peptide of SEQ ID No. 3 is derived from IgG1.

SEQ ID No. 4: MAVPTQVLGLLLLWLTDARC

The signal peptide of SEQ ID No. 4 is derived from CD8a.

ENDODOMAIN

The endodomain is the portion of a classical CAR which is located on the intracellular side of the membrane.

The endodomain is the signal-transmission portion of a classical CAR. After antigen recognition by the antigen binding domain, individual CAR molecules cluster, native CD45 and CD148 are excluded from the synapse and a signal is transmitted to the cell.

The endodomain of a coiled-coil spacer CAR may be or comprise an intracellular signalling domain. In an alternative embodiment, the endodomain of the present CAR may be capable of interacting with an intracellular signalling molecule which is present in the cytoplasm, leading to signalling.

The intracellular signalling domain or separate intracellular signalling molecule may be or comprise a T cell signalling domain.

The most commonly used signalling domain is that of CD3-zeta endodomain, 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 signalling may be needed. For example, chimeric CD28 and OX40 can be used with CD3-Zeta to transmit a proliferative / survival signal, or all three can be used together (illustrated in Figure 1B).

The present CAR may comprise the CD3-Zeta endodomain alone, the CD3-Zeta endodomain with that of either CD28 or OX40 or the CD28 endodomain and OX40 and CD3-Zeta endodomain (Figure 1).

The endodomain may comprise one or more of the following: an ICOS endodomain, a CD27 endodomain, a BTLA endodomain, a CD30 endodomain, a GITR endodomain and an HVEM endodomain.

The endomain may comprise the sequence shown as SEQ ID No. 5 to 13 or a variant thereof having at least 80% sequence identity.

SEQ ID No. 5 - CD3 Z endodomain

RVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGGKPRRKNPQEGL
YNELQKDKMAEAYSEIGMKGERRRGKGGHDGLYQGLSTATKDTYDALHMQALPPR

SEQ ID No. 6 - CD28 and CD3 Zeta endodomains

SKRSRLLHSDYMNMTPRRPGPTRKHYPYAPPRDFAAYRSRVKFSRSADAPAYQQGQNQ
LYNELNLGRREEYDVLDKRRGRDPEMGGKPRRKNPQEGLYNELQKDKMAEAYSEIGMKG
ERRRGKGGHDGLYQGLSTATKDTYDALHMQALPPR

SEQ ID No. 7 - CD28, OX40 and CD3 Zeta endodomains

SKRSRLLHSDYMNMTPRRPGPTRKHYPYAPPRDFAAYRSRDQRLPPDAHKKPPGGGSFR
TPIQEEQADAHSTLAKIRVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGRDP
EMGGKPRRKNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGGHDGLYQGLSTATKDTYD
ALHMQALPPR

SEQ ID No. 8 - ICOS endodomain

CWLTKKKYSSSVHDPNGEYMFMRAVNTAKKSRLTDVTL

SEQ ID No. 9 - CD27 endodomain

QRRKYRSNKGESPVEPAEPCHYSCPREEEGSTIPIQEDYRKPEPACSP

SEQ ID No. 10 - BTLA endodomain

RRHQGKQNELSDTAGREINLVDAHLKSEQTEASTRQNSQVLLSETGIYDNDPDLCFRMQE
GSEVYSNPCLEENKPGIVYASLNHHSVIGPNSRLARNVKEAPTEYASICVRS

SEQ ID No. 11 - CD30 endodomain

HRRACRKRIKRLHLCYPVQTSQPKLELVDSRPRRSSTQLRSGASVTEPVAEERGLMSQP
LMETCHSVGAAYLESPLQDASPAGGPSSPRDLPEPRVSTEHTNNKIEKIYIMKADTVIVGT
VKAELPEGRGLAGPAEPELEEELEADHTPHYPEQETEPPLGSCSDVMLSVEEEGKEDPLPT
AASGK

SEQ ID No. 12 - GITR endodomain

QLGLHIWQLRSQCMWPRETQLLLEVPPSTEDARSCQFPEEERGERSAEEKGRLGDLWW

SEQ ID No. 13 - HVEM endodomain

CVKRRKPRGDVVKVIVSVQRKRQEAEGEATVIEALQAPPDVTTVAVEETIPSFTGRSPNH

A variant sequence may have at least 80%, 85%, 90%, 95%, 98% or 99% sequence identity to SEQ ID No. 5 to 13, provided that the sequence provides an effective intracellular signalling domain.

CHIMERIC ANTIGEN RECEPTOR (CAR)

In one aspect the present invention provides a CAR comprising a CAR-forming polypeptide according to the first aspect of the invention and an accessory polypeptide which comprises (i) a coiled-coil spacer domain; (ii) a transmembrane domain; and (iii) an endodomain, wherein the coiled-coil spacer domain of the accessory polypeptide is capable of interacting with the coiled-coil domain of the CAR-forming polypeptide.

The CAR-forming polypeptide provides the antigen-binding domain and hence the antigen specificity.

The accessory polypeptide provides an additional endodomain which may be used for generating a desired signalling response. This is advantageous over a compound signalling domain since each signalling domain remains unencumbered from other signalling domains.

In addition, it allows each signalling domain to be localised at an optimal proximity to the membrane for signalling.

The endodomain of the CAR-forming polypeptide may comprise at least a first intracellular signalling domain; and the endodomain of the accessory polypeptide may comprise at least a second intracellular signalling domain. For example, one of the endodomain of the CAR-forming polypeptide and the accessory polypeptide may comprise a CD3 zeta endodomain and the other endodomain of the CAR and the accessory polypeptide may comprise a 41BB endodomain.

In another embodiment, the present invention provides a CAR according to the second aspect of the present invention, further comprising a second accessory polypeptide comprising: (i) a coiled-coil domain; (ii) a transmembrane domain; and (iii) an endodomain; wherein the coiled-coil domain of the second accessory polypeptide is capable of interacting with the coiled-coil domains of the CAR-forming polypeptide and the first accessory polypeptide.

The endodomain of the CAR-forming polypeptide may comprise at least a first intracellular signalling domain, the endodomain of the first accessory polypeptide may comprise at least a second intracellular signalling domain and the endodomain of the second accessory polypeptide may comprise at least a third intracellular signalling domain.

For example, the endodomains of the CAR, the first accessory polypeptide and the second accessory polypeptide may comprise between them a CD3 zeta endodomain; a 41BB endodomain; and a CD28 endodomain.

The present invention also provides an accessory polypeptide suitable for use in a CAR as described herein.

MULTIMERIC CAR

The present invention provides a multimeric CAR which comprises a plurality of CAR-forming polypeptides according to the present invention and optionally accessory polypeptide(s) which form a complex due to interactions between the coiled coil spacer domains.

The multimeric CAR may be, for example, trimeric, tetrameric, pentameric, hexameric or heptameric.

The number of CAR-forming polypeptides vs accessory proteins in each type of CAR is summarised in the Tables below:

Trimeric CAR:

Number of CAR-forming polypeptides	Number of accessory polypeptides
1	2
2	1
3	0

Tetrameric CAR:

Number of CAR-forming polypeptides	Number of accessory polypeptides
1	3
2	2
3	1
4	0

Pentameric CAR:

Number of CAR-forming polypeptides	Number of accessory polypeptides
1	4
2	3
3	2
4	1
5	0

The association of CAR-forming polypeptides and accessory polypeptides within a cell will be random, so the options given in the tables above may refer to a single multimeric CAR, in which the number of CAR-forming polypeptides and accessory polypeptides can be

precisely defined, or the average number of CAR-forming polypeptides and accessory polypeptides in multiplexed CARs expressed on a cell. In systems where there is a high accessory polypeptide:CAR-forming polypeptide ratio, it is possible that some multiplexes of accessory polypeptide alone will be expressed on the cell surface. This is not a problem, as long as at least some of the multiplexes expressed at the cell surface comprise a CAR-forming polypeptide.

The plurality of CAR-forming polypeptides and optionally accessory polypeptide(s) may comprise the same endodomain.

Alternatively, the plurality of CAR-forming polypeptides and optionally accessory polypeptide(s) may comprise different endodomains. In this way, multiple different endodomains can be activated simultaneously. This is advantageous over a compound signalling domain since each signalling domain remains unencumbered from other signalling domains. In addition, it allows each signalling domain to be localised at an optimal proximity to the membrane for signalling.

Where a multimeric CAR comprises a plurality of antigen binding domains, this will increase the avidity of antigen binding. The multimeric CAR may mimic antigen binding by IgM, which comprises a pentameric or hexameric arrangement of immunoglobulins.

CAR SIGNALLING SYSTEM

The present invention also provides a chimeric antigen receptor (CAR) signalling system, which comprises:

- (i) a multimeric CAR comprising a CAR-forming polypeptide or accessory polypeptide as defined above which comprises a first heterodimerization domain; and
- (ii) an intracellular signalling component comprising a signalling domain and a second heterodimerization domain;

wherein heterodimerization between the first and second heterodimerization domains causes the multimeric CAR and signalling component to form a functional CAR complex.

Each CAR-forming polypeptide(s) or accessory polypeptide(s) may comprise a plurality of heterodimerisation domains, such that a single CAR-forming polypeptide or accessory polypeptide is capable of heterodimerising with a plurality of signalling components. An example of such a system is illustrated in Figure 15.

In order to increase the signalling domain: antigen-binding domain even further, each signalling component may comprise a plurality of signalling domains.

Heterodimerisation may occur only in the presence of a small molecule, for example using a system such as the one described in WO2016/030691.

Alternatively heterodimerization may occur spontaneously. The first and second heterodimerization domains are capable of spontaneous dimerization with each other. Heterodimerization occurs with the first and second heterodimerization domains alone, without the need for any separate molecule acting as an “inducer” of dimerization.

The signalling system of the present invention is not limited by the arrangement of a specific pair of heterodimerization domains. The targeting component (i.e. the multimeric CAR) may comprise either domain from a pair of heterodimerizing domains so long as the signalling component comprises the corresponding, complementary domain which enables the targeting component and the signalling component to co-localize at the cell membrane.

The heterodimerization domains for use in the present CAR system are not limited to those which interact at a 1:1 ratio. For example, heterodimerization domains may interact to form multimers (e.g. trimers or tetramers). The domains may interact in a manner which co-localises a single first heterodimerization domain with multiple (e.g. 2 or 3) second heterodimerization domains. Herein it may be advantageous to have a signalling domain which comprises the second heterodimerization domain, such that multiple signalling components can co-localise with a single multimeric CAR. This may be advantageous, for example, when a high level of signalling is required upon binding of antigen to the multimeric CAR.

The multimeric CAR may comprise a plurality of heterodimerization domains, so that it interacts with a plurality of signalling components. For example, the multimeric CAR may comprise more than two heterodimerization domains, such as 3 to 10 heterodimerization domains. Figure 15 shows a multimeric CAR which comprises 20 heterodimerization domains, four per CAR-forming polypeptide/accessory polypeptide.

For convenience, the term heterodimerization domain is used herein for all domains which mediate co-localization of the multimeric CAR and signalling components.

A large variety of appropriate heterodimerization domains are known in the art, examples of which are provided herein.

The first and second heterodimerization domains may be leucine zippers.

Leucine zippers are well known in the art (see Hakoshima; Encyclopedia of Life Sciences; 2005, for example). The leucine zipper is a super-secondary structure that functions as a dimerization domain. Its presence generates adhesion forces in parallel alpha helices. A single leucine zipper consists of multiple leucine residues at approximately 7-residue intervals, which forms an amphipathic alpha helix with a hydrophobic region running along one side. This hydrophobic region provides an area for dimerization, allowing the motifs to "zip" together. Leucine zippers are typically 20 to 40 amino acids in length, for example approximately 30 amino acids.

The first and/or second heterodimerization domain may comprise the sequence shown as SEQ ID NO: 43 or 44. The first heterodimerization domain may comprise the sequence shown as SEQ ID NO: 43 and the second heterodimerization domain may comprise the sequence shown as SEQ ID NO: 44, or vice versa.

SEQ ID NO: 43: QLEKELQALEKENAQLEWELQALEKELAQ

SEQ ID NO: 44: QLEKKLQALKKKNAQLKWKLQALKKKLAQ

In certain embodiments, the first and second heterodimerization domains may be acidic (e.g. SEQ ID NO: 43) or basic (e.g. SEQ ID NO: 44) leucine zippers. In particular, where the first heterodimerization domain is an acidic leucine zipper, the second heterodimerization is a basic leucine zipper and vice versa.

The first and second heterodimerization domains may be dimerization and docking domain (DDD1) and anchoring domain (AD1). These domains and the interaction between them is known in the art (Rossi et al.; PNAS; 2006; 103(18); 6841-6846).

DDD1 is a short alpha helical structure derived from Protein Kinase A (PKA). AD1 is a short alpha helical structure derived from A-kinase anchor proteins (AKAPs).

The DDD1 domain may comprise the sequence shown as SEQ ID NO: 45.

SEQ ID NO: 45: SHIQIPPGLTELLQGYTVEVLRQQPPDLVEFAVEYFTRLREARA

The AD1 domain may comprise the sequence shown as SEQ ID NO: 46

SEQ ID NO: 46: VQIEYLAKQIVDNAIQQA

Since the DDD1/AD1 interaction is trimeric, an AD1 domain present on the CAR endodomain will recruit three signalling domains comprising a DDD1 domain. Thus in a particular embodiment, the CAR endodomain comprises an AD1 domain and the intracellular signalling component comprises a DDD1 domain.

The heterodimerization domains may be derived from the Bacterial Ribonuclease (Barnase) and Barnstar peptides.

Barnase is the *Bacillus amyloliquefaciens* ribonuclease protein. It is composed on 110 amino acids. Barnstar functions to inhibit the nuclease activity of Barnase and therefore binds Barnstar with a very high affinity (an on-rate of $10^8 \text{s}^{-1} \text{M}^{-1}$).

The heterodimerization domains may be derived from Human Pancreatic RNases and S-peptide.

Human Pancreatic RNase are pyrimidine-specific endonucleases. S-peptide is the enzymatically inactive proteolytic fragment of RNase A, which lacks the RNA binding site.

The present invention also encompasses variants of the heterodimerization sequences described herein which retain the ability to dimerize with the corresponding heterodimerization domain. The heterodimerization domain may be a variant having 5, 4, 3, 2 or 1 amino acid mutations (insertions, substitutions or additions) or at least 80%, 85%, 90%, 95%, 98% or 99% sequence identity compared to the sequence shown as SEQ ID No. 43, 44, 45 or 46 provided that they still cause heterodimerization between the CAR and the signalling component.

NUCLEIC ACID

The present invention further provides a nucleic acid encoding the CAR-forming polypeptide according to the first aspect of the present invention and/or an accessory polypeptide as defined in the first aspect of the invention.

As used herein, the terms “polynucleotide”, “nucleotide”, and “nucleic acid” are intended to be synonymous with each other.

It will be understood by a skilled person that numerous different polynucleotides and nucleic acids can encode the same polypeptide as a result of the degeneracy of the genetic code. In addition, it is to be understood that skilled persons may, using routine techniques, make nucleotide substitutions that do not affect the polypeptide sequence encoded by the polynucleotides described here to reflect the codon usage of any particular host organism in which the polypeptides are to be expressed.

Nucleic acids according to the invention may comprise DNA or RNA. They may be single-stranded or double-stranded. They may also be polynucleotides which include within them synthetic or modified nucleotides. A number of different types of modification to oligonucleotides are known in the art. These include methylphosphonate and phosphorothioate backbones, addition of acridine or polylysine chains at the 3' and/or 5' ends of the molecule. For the purposes of the use as described herein, it is to be understood that the polynucleotides may be modified by any method available in the art. Such modifications may be carried out in order to enhance the in vivo activity or life span of polynucleotides of interest.

The terms “variant”, “homologue” or “derivative” in relation to a nucleotide sequence include any substitution of, variation of, modification of, replacement of, deletion of or addition of one (or more) nucleic acid from or to the sequence.

The present invention also provides a nucleic acid sequence encoding an accessory polypeptide suitable for use in the CAR according to the second aspect of the present invention.

NUCLEIC ACID CONSTRUCT

The present invention also provides a nucleic acid construct which encodes a plurality of nucleic acid sequences.

For example the nucleic acid construct may encode two or more CAR forming polypeptides as defined in the first aspect of the invention.

In this embodiment, the nucleic acid construct may comprise at least two nucleic acid sequences:

- (i) a first nucleic acid sequence which encodes a first CAR-forming polypeptide; and
- (ii) a second nucleic acid sequence which encodes a second CAR-forming polypeptide.

The nucleic acid construct may encode at least one CAR-forming polypeptide as defined in the first aspect of the invention and at least one accessory polypeptide as defined above.

In this embodiment, the nucleic acid construct may comprise at least two nucleic acid sequences:

- (i) a first nucleic acid sequence which encodes a CAR-forming polypeptide; and
- (ii) a second nucleic acid sequence which encodes an accessory polypeptide.

The nucleic acid construct may encode:

- (i) at least one CAR-forming polypeptide, which forms a multimeric CAR as defined in the second aspect of the invention; and
- (ii) an intracellular signalling component as defined above.

In this embodiment, the nucleic acid construct may comprise at least two nucleic acid sequences:

- (i) a first nucleic acid sequence which encodes a CAR-forming polypeptide; and
- (ii) a second nucleic acid sequence which encodes an intracellular signalling component.

The nucleic acid construct may comprise a third nucleic acid sequence which encodes an accessory polypeptide.

The nucleic acid construct may therefore produce two or more polypeptide(s) joined by a cleavage site(s). The cleavage site may be self-cleaving, such that when the nascent translation product is produced, it is immediately cleaved into individual polypeptides without the need for any external cleavage activity.

The cleavage site may be any sequence which enables the polypeptide comprising multiple CARs and/or accessory polypeptides to become separated.

The term “cleavage” is used herein for convenience, but the cleavage site may cause the peptides to separate into individual entities by a mechanism other than classical cleavage. For example, for the Foot-and-Mouth disease virus (FMDV) 2A self-cleaving peptide (see below), various models have been proposed for to account for the “cleavage” activity: proteolysis by a host-cell proteinase, autoproteolysis or a translational effect (Donnelly et al (2001) J. Gen. Virol. 82:1027-1041). The exact mechanism of such “cleavage” is not important for the purposes of the present invention, as long as the cleavage site, when positioned between nucleic acid sequences which encode proteins, causes the proteins to be expressed as separate entities.

The cleavage site may be a furin cleavage site.

Furin is an enzyme which belongs to the subtilisin-like proprotein convertase family. The members of this family are proprotein convertases that process latent precursor proteins into their biologically active products. Furin is a calcium-dependent serine endoprotease that can efficiently cleave precursor proteins at their paired basic amino acid processing sites. Examples of furin substrates include proparathyroid hormone, transforming growth factor beta 1 precursor, proalbumin, pro-beta-secretase, membrane type-1 matrix metalloproteinase, beta subunit of pro-nerve growth factor and von Willebrand factor. Furin cleaves proteins just downstream of a basic amino acid target sequence (canonically, Arg-X-(Arg/Lys)-Arg') and is enriched in the Golgi apparatus.

The cleavage site may be a Tobacco Etch Virus (TEV) cleavage site.

TEV protease is a highly sequence-specific cysteine protease which is chymotrypsin-like proteases. It is very specific for its target cleavage site and is therefore frequently used for the controlled cleavage of fusion proteins both *in vitro* and *in vivo*. The consensus TEV cleavage site is ENLYFQ\S (where ‘\’ denotes the cleaved peptide bond). Mammalian cells, such as human cells, do not express TEV protease. Thus in embodiments in which the present nucleic acid construct comprises a TEV cleavage site and is expressed in a mammalian cell – exogenous TEV protease must also expressed in the mammalian cell.

The cleavage site may encode a self-cleaving peptide.

A ‘self-cleaving peptide’ refers to a peptide which functions such that when the nascent product comprising the polypeptides and the self-cleaving peptide is produced, it is

immediately “cleaved” or separated into distinct and discrete first and second polypeptides without the need for any external cleavage activity.

The self-cleaving peptide may be a 2A self-cleaving peptide from an aphtho- or a cardiovirus. The primary 2A/2B cleavage of the aphtho- and cardioviruses is mediated by 2A “cleaving” at its own C-terminus. In aphthoviruses, such as foot-and-mouth disease viruses (FMDV) and equine rhinitis A virus, the 2A region is a short section of about 18 amino acids, which, together with the N-terminal residue of protein 2B (a conserved proline residue) represents an autonomous element capable of mediating “cleavage” at its own C-terminus (Donnelly et al (2001) as above).

“2A-like” sequences have been found in picornaviruses other than aphtho- or cardioviruses, ‘picornavirus-like’ insect viruses, type C rotaviruses and repeated sequences within Trypanosoma spp and a bacterial sequence (Donnelly et al (2001) as above). The cleavage site may comprise one of these 2A-like sequences, such as:

YHADYYKQRLIHDVEMNPGP (SEQ ID No. 14)
 HYAGYFADLLIHDIETNPGP (SEQ ID No. 15)
 QCTNYALLKLAGDVESNPGP (SEQ ID No. 16)
 ATNFSLLKQAGDVEENPGP (SEQ ID No. 17)
 AARQMLLLLSGDVETNPGP (SEQ ID No. 18)
 RAEGRGSLTTCGDVEENPGP (SEQ ID No. 19)
 TRAEIEDELIRAGIESNPGP (SEQ ID No. 20)
 TRAEIEDELIRADIESNPGP (SEQ ID No. 21)
 AKFQIDKILISGDVELNPGP (SEQ ID No. 22)
 SSIIRTKMLVSGDVEENPGP (SEQ ID No. 23)
 CDAQRRQKLLLSGDIEQNPGP (SEQ ID No. 24)
 YPIDFGGFLVKADSEFNPGP (SEQ ID No. 25)

The cleavage site may comprise the 2A-like sequence shown as SEQ ID No. 19 (RAEGRGSLTTCGDVEENPGP).

The present invention also provides a kit comprising one or more nucleic acid sequence(s) encoding a CAR-forming polypeptide according to the first aspect of the present invention and/or an accessory polypeptide suitable for producing a CAR according to the second aspect of the invention.

VECTOR

The present invention also provides a vector, or kit of vectors, which comprises one or more nucleic acid sequence(s) or nucleic acid construct as defined above. Such a vector may be used to introduce the nucleic acid sequence(s) into a host cell so that it expresses a CAR-forming polypeptide or an accessory peptide according to the first aspect of the invention and/or a CAR according to the second 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, or a transposon based vector or synthetic mRNA.

The vector may be capable of transfecting or transducing an immune cell such as a T cell or a NK cell.

CELL

The present invention also relates to a cell, such as an immune cell, comprising a CAR-forming polypeptide, CAR, or CAR signalling system as described above.

The cell may comprise a nucleic acid, nucleic acid construct or a vector of the present invention.

The cell may be an immune cell, in particular a cytolytic immune cell, such as a T cell or an NK cell.

T cells or T lymphocytes are a type of lymphocyte that play a central role in cell-mediated immunity. They can be distinguished from other lymphocytes, such as B cells and natural killer cells (NK cells), by the presence of a T-cell receptor (TCR) on the cell surface. There are various types of T cell, as summarised below.

Helper T helper cells (TH cells) assist other white blood cells in immunologic processes, including maturation of B cells into plasma cells and memory B cells, and activation of cytotoxic T cells and macrophages. TH cells express CD4 on their surface. TH cells become activated when they are presented with peptide antigens by MHC class II molecules on the surface of antigen presenting cells (APCs). These cells can differentiate into one of several subtypes, including TH1, TH2, TH3, TH17, Th9, or TFH, which secrete different cytokines to facilitate different types of immune responses.

Cytolytic T cells (TC cells, or CTLs) destroy virally infected cells and tumor cells, and are also implicated in transplant rejection. CTLs express the CD8 at their surface. These cells recognize their targets by binding to antigen associated with MHC class I, which is present on the surface of all nucleated cells. Through IL-10, adenosine and other molecules secreted by regulatory T cells, the CD8+ cells can be inactivated to an anergic state, which prevent autoimmune diseases such as experimental autoimmune encephalomyelitis.

Memory T cells are a subset of antigen-specific T cells that persist long-term after an infection has resolved. They quickly expand to large numbers of effector T cells upon re-exposure to their cognate antigen, thus providing the immune system with "memory" against past infections. Memory T cells comprise three subtypes: central memory T cells (TCM cells) and two types of effector memory T cells (TEM cells and TEMRA cells). Memory cells may be either CD4+ or CD8+. Memory T cells typically express the cell surface protein CD45RO.

Regulatory T cells (Treg cells), formerly known as suppressor T cells, are crucial for the maintenance of immunological tolerance. Their major role is to shut down T cell-mediated immunity toward the end of an immune reaction and to suppress auto-reactive T cells that escaped the process of negative selection in the thymus.

Two major classes of CD4+ Treg cells have been described — naturally occurring Treg cells and adaptive Treg cells.

Naturally occurring Treg cells (also known as CD4+CD25+FoxP3+ Treg cells) arise in the thymus and have been linked to interactions between developing T cells with both myeloid (CD11c+) and plasmacytoid (CD123+) dendritic cells that have been activated with TSLP. Naturally occurring Treg cells can be distinguished from other T cells by the presence of an intracellular molecule called FoxP3. Mutations of the FOXP3 gene can prevent regulatory T cell development, causing the fatal autoimmune disease IPEX.

Adaptive Treg cells (also known as Tr1 cells or Th3 cells) may originate during a normal immune response.

The cell may be a Natural Killer cell (or NK cell). NK cells form part of the innate immune system. NK cells provide rapid responses to innate signals from virally infected cells in an MHC independent manner

NK cells (belonging to the group of innate lymphoid cells) are defined as large granular lymphocytes (LGL) and constitute the third kind of cells differentiated from the common lymphoid progenitor generating B and T lymphocytes. NK cells are known to differentiate and mature in the bone marrow, lymph node, spleen, tonsils and thymus where they then enter into the circulation.

The CAR cells of the invention may be any of the cell types mentioned above.

T or NK cells expressing a CAR according to the invention or components thereof, 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, T or NK cells expressing a CAR according to the the invention or components thereof may be derived from *ex vivo* differentiation of inducible progenitor cells or embryonic progenitor cells to T or NK cells. Alternatively, an immortalized T-cell line which retains its lytic function and could act as a therapeutic may be used.

In all these embodiments, CAR cells are generated by introducing DNA or RNA coding for the CAR of the invention or a component(s) or a component thereof by one of many means including transduction with a viral vector, transfection with DNA or RNA.

The CAR cell of the invention may be an *ex vivo* T or NK cell from a subject. The T or NK cell may be from a peripheral blood mononuclear cell (PBMC) sample. T or NK cells may be activated and/or expanded prior to being transduced with nucleic acid encoding the molecules providing the CAR of the invention or a component(s) of the CAR, for example by treatment with an anti-CD3 monoclonal antibody.

The T or NK cell of the invention may be made by:

- (i) isolation of a T or NK cell-containing sample from a subject or other sources listed above; and
- (ii) transduction or transfection of the T or NK cells with one or more a nucleic acid sequence(s) or nucleic acid construct(s) as described above.

The T or NK cells may then be purified, for example, selected on the basis of expression of the antigen-binding domain of the antigen-binding polypeptide.

PHARMACEUTICAL COMPOSITION

The present invention also relates to a pharmaceutical composition containing a plurality of cells expressing the CAR according to the invention or the components thereof.

The pharmaceutical composition may additionally comprise a pharmaceutically acceptable carrier, diluent or excipient. The pharmaceutical composition may optionally comprise 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

The present invention provides a method for treating and/or preventing a disease which comprises the step of administering the cells of the present invention (for example in a pharmaceutical composition as described above) to a subject.

A method for treating a disease relates to the therapeutic use of the cells of the present invention. Herein 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 for preventing a disease relates to the prophylactic use of the cells of the present invention. Herein such cells may be administered to a subject who has not yet contracted the disease and/or who is not showing any symptoms of the disease to prevent or impair the cause of the disease or to reduce or prevent development of at least one symptom associated with the disease. The subject may have a predisposition for, or be thought to be at risk of developing, the disease.

The method may involve the steps of:

- (i) isolating a T or NK cell-containing sample;
- (ii) transducing or transfecting such cells with a nucleic acid sequence, nucleic acid construct or vector of the invention;
- (iii) administering the cells from (ii) to a subject.

The T or NK cell-containing sample may be isolated from a subject or from other sources, for example as described above. The T or NK cells may be isolated from a subject'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).

The present invention provides a CAR cell of the present invention for use in treating and/or preventing a disease.

The invention also relates to the use of a CAR cell of the present invention in the manufacture of a medicament for the treatment and/or prevention of a disease.

The disease to be treated and/or prevented by the methods of the present invention may be a cancerous disease, such as bladder cancer, breast cancer, colon cancer, endometrial cancer, kidney cancer (renal cell), leukaemia, lung cancer, melanoma, non-Hodgkin lymphoma, pancreatic cancer, prostate cancer and thyroid cancer.

The CAR cells of the present invention may be capable of killing target cells, such as cancer cells. The target cell may be recognisable by expression of a TAA, for example the expression of a TAA provided above in Table 1.

The CAR cells of the present invention may be capable of killing target cells, such as cancer cells, which express a low density of the TAA. Examples of TAAs which are known to be expressed at low densities in certain cancers include, but are not limited to, ROR1 in CLL, Typr-1 in melanoma and BCMA in myeloma.

The CAR cells and pharmaceutical compositions of present invention may be for use in the treatment and/or prevention of the diseases described above.

The CAR cells and pharmaceutical compositions of present invention may be for use in any of the methods described above.

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 – Expression of COMP CARs at the cell surface

A murine T-cell line was transduced with the anti-CD33 COMP CAR (amino acid sequence shown in Figure 5c and nucleic acid sequence shown in Figure 5d) or anti-CD33 IgG1 CAR.

These cells were then stained with chimeric soluble CD33 fused to murine Fc IgG2a before a secondary stain with anti-mouse IgG PE (Figure 6a)

A murine T-cell line was transduced with the anti-ROR-1 COMP CAR CAR (amino acid sequence shown in Figure 5c and nucleic acid sequence shown in Figure 5d) or anti-ROR-1 IgG1 CAR.

These cells were then stained with soluble His tagged ROR-1 followed by a secondary stain with anti-His-biotin and then a third stain with streptavidin-APC (Figure 6b).

All four CARs were successfully expressed on the cell surface. These data also demonstrate that the CAR binding domain is orientated in a way that does not impede ligand binding when linked to a COMP spacer.

Example 2 – Stimulation of COMP CAR T-cells with immobilised ligand

T-cells with beads coated with immobilised ligand were used to stimulate COMP ROR-1 CAR T-cells. To achieve this, soluble His-tag ROR-1 was constructed and expressed. Supernatants containing these soluble ligands were then incubated at various concentrations with a set number of anti-His beads. The beads were then washed to remove unbound ligand and these beads were used to stimulate T-cells transduced with either the COMP CAR platforms or an equivalent CAR with an IgG spacer.

Transduced murine T-cells were co-cultured with anti-His beads that were pre-coated with different concentrations of soluble His tagged ROR-1 supernatant. The amount of IL-2 in the co-culture supernatant was analysed after 16-24hours via ELISA (Figure 7).

Example 3 – Expression levels of ROR-1 target cells

The SKW cell line naturally expresses low levels of ROR-1. These cells were transduced with ROR-1 to increase the expression levels. These cells were stained with anti-ROR-1 APC and compared to non-stained cells (Figure 8).

Example 4 – Stimulation of anti-ROR-1 COMP CAR T-cells with ROR-1 positive SKW cells

Transduced murine T-cells (described in Examples 1 and 2) were co-cultured with SKW target cells that express the ROR-1 ligand at either a low or a high density. T-cells were maintained at a constant number and the target cells were varied. The amount of IL-2 in the co-culture supernatant was analysed after 16-24 hours via ELISA (Figure 9).

Higher levels of IL-2 were detected when the anti-ROR-1 COMP CAR T cells were co-cultured with SKW target cells expressing a low density of ROR-1 ligand compared to the anti-ROR-1 IgG1 CAR.

Both CARs were able to initiate an activation response with SKW-high target cells.

Example 5 – Truncation of the COMP spacer

The aROR-1 CAR with a COMP spacer was truncated from its original length of 45 amino acids. These COMP truncated constructs were transfected into 293T cells and then stained for CAR surface expression with sROR-1 His followed by anti-His-Biotin followed by a streptavidin-PE.Cy7. These cells were also stained for the RQR8 marker with an anti-CD34-FITC antibody. These FACS plots show stable surface expression of various truncated forms of the COMP spacer, displaying the ability to vary the length of the coiled coil spacer by one to a few amino acids at a time (Figure 11).

Example 6 – Comparison of multimeric coiled-coil spacer CARs with a classical dimeric CAR

In order to compare the function of coiled-coil spacers CARs of the invention with a conventional CAR, a series of constructs were made with the same antigen-binding domains and equivalent endodomains, but with different spacers, leading to a completely different CAR structure. The different CARs are shown in Figure 12.

All CARs comprised an anti-CD19 antigen-binding domain based on fmc63 and a “second generation” endodomain comprising 41BB and CD3zeta endodomains. The following formats were tested:

a) a heteromultimeric CAR which comprises: a polypeptide having an anti-CD19 antigen binding domain; a coiled-coil spacer domain and a CD3zeta endodomain; and an accessory polypeptide having a coiled-coil spacer domain and a 41BB endodomain (Figure 12A);

b) a homomultimeric CAR made up of polypeptides comprising an anti-CD19 antigen binding domain; a coiled-coil spacer domain and a combined 41BB/CD3zeta endodomain (Figure 12B); and

c) a classical second generation homodimeric CAR which comprises two polypeptides having an anti-CD19 antigen-binding domain, a CD8 stalk spacer domain and a combined 41BB/CD3zeta endodomain (Figure 12C).

Vectors encoding the CAR illustrated in Figure 12 were

RD114-pseudotyped retrovirus encoding the various CAR structures was produced.

T cells were depleted of CD56-expressing cells and co-cultured with an equal number of SupT1 to achieve an effector:target ratio of 1:1. Prior to analysis by flow cytometry, an equal number of fluorescent counting beads was added to each co-culture to allow normalization of cell numbers and to account for any differences in uptake volumes. CAR-mediated cytotoxicity was assessed by flow cytometry as follows: T cells were differentiated from tumour cells by staining for CD3 expression vs FCS and tumour cells identified by their lack of CD3 and higher FCS signal. Viability was assessed by staining with the dead cell exclusion dye 7-AAD and viable cells defined as those which did not uptake the dye. Viable tumour cells were enumerated for each co-culture condition and percentage cytotoxicity was calculated by normalizing the number of viable tumour cells to that recovered from co-cultures carried out with non-transduced PBMCs (100%). Killing of targets cells was assessed at day 2 and 5.

The results are shown in Figure 13 (day 2) and Figure 14 (day 5). After two days, all three CAR structures showed killing of CD19+ SupT1 target cells. The two multimeric CARs showed superior killing to the equivalent classical homodimeric CAR. The homomultimeric CAR (Fmc63-COMP-41BBz) showed the most killing. At day 5, some residual target cells remained with the classical homodimeric CAR (Fmc63-CD8STK-41BBz) but viable target cells were virtually undetectable for both the multimeric CARs.

Example 7 – Production and testing of coiled-coil SuperCARs

A major challenge for CAR technologies is the detection of antigens which are present at low densities on target cells. In order to address this issue, the present inventors have designed “SuperCARs” based on the coiled-coil spacer format which recruit multiple TCRzeta chains for each antigen interaction.

The intracellular part of the polypeptide making up the coiled-coil CAR structure comprises a plurality of heterodimerization domains, each of which is capable of interacting with one or more intracellular signalling components which comprises one or more intracellular signalling domains.

In the constructs illustrated in Figure 15, intracellular dimerization is between dimerization and docking domain (DDD1) and anchoring domain (AD1). Each polypeptide making up the coiled-coil spacer CAR comprises four separate AD1 domains. A coiled-coil CAR comprising 5 polypeptides will therefore comprise 20 AD1 domains

The coiled coil SuperCAR was tested in combination with different signalling components having 0 or 2 copies of the TCR zeta signalling domain. As DDD1 binds AD1 in a 2:1 stoichiometry, these signalling domains give 0 and 80 copies of the TCR zeta domain respectively for each 5-polypeptide coiled-coil CAR targeting component.

As a control, a classical homodimeric anti-CD19 CAR was used (Figure 15: aCD19-IgGFc-Z) with the same antigen-binding domain.

The murine T-cell line BW5 was transduced with each CAR and challenged with SupT1 cells expressing the cognate antigen (CD19) at different concentrations: low, mid and high. These SupT1 cells were engineered to express CD19 at different levels by the use of suboptimal signal peptides and/or the introduction of cytoplasmic retention motifs derived from Tyrp-1 (inserted proximal to the membrane) or glycoprotein E3-19k from adenovirus (inserted on the C-terminus). IL-2 release was measured after antigen challenge.

The results are shown in Figure 16. It was found that coiled-coil the superCAR comprising 80 copies of TCR zeta per 5-mer coiled-coil CAR gave a much greater response to antigen than the equivalent classical CAR comprising two copies of TCR zeta per molecule.

All publications mentioned in the above specification are herein incorporated by reference. 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 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, cell biology or related fields are intended to be within the scope of the following claims.

CLAIMS

1. A chimeric antigen receptor (CAR)-forming polypeptide comprising:
 - (i) an antigen-binding domain;
 - (ii) a coiled-coil spacer domain;
 - (iii) a transmembrane domain; and
 - (iv) an endodomain.
2. A CAR-forming polypeptide according to claim 1, wherein the antigen-binding domain binds an antigen which is expressed at a low density on a target cell.
3. A CAR-forming polypeptide according to claim 2 wherein the antigen-binding domain binds to ROR-1, Typr-1 or BCMA.
4. An accessory polypeptide comprising:
 - (i) a coiled-coil spacer domain;
 - (iii) a transmembrane domain; and
 - (iv) an endodomain.
5. A CAR-forming polypeptide according to any of claims 1 to 3 or an accessory polypeptide according to claim 4, wherein the coiled-coil spacer domain enables the multimerization of at least three CAR-forming polypeptides/accessory polypeptides.
6. A CAR-forming polypeptide or accessory polypeptide according to claim 5 wherein the coiled-coil spacer domain is from: cartilage-oligomeric matrix protein (COMP), mannose-binding protein A, coiled-coil serine-rich protein 1, polypeptide release factor 2, SNAP-25, SNARE, Lac repressor or apolipoprotein E.
7. A CAR-forming polypeptide or accessory polypeptide according to claim 6 wherein the coiled-coil spacer domain comprises one of the sequences shown as SEQ ID No. 1 or 30 to 42 or a fragment thereof or a variant thereof which has at least 80% sequence identity.
8. A CAR-forming polypeptide or accessory polypeptide according to any preceding claim wherein the endodomain comprises at least one of CD3 zeta endodomain, CD28 endodomain, 41BB endodomain and OX40 endodomain.

9. A CAR-forming polypeptide or accessory polypeptide according to any preceding claim wherein the endodomain comprises the sequence shown as SEQ ID No. 7 or a variant thereof which has at least 80% sequence identity.
10. A CAR-forming polypeptide or accessory polypeptide according to any preceding claim, wherein the endodomain comprises a heterodimerisation domain, capable of heterodimerising with an intracellular signalling component.
11. A CAR-forming polypeptide or accessory polypeptide according to claim 10, wherein the heterodimerization domain comprises one of the following: a leucine zipper domain, an DDD1 or AD1 domain; a Barnase or Barnstar domains; a human pancreatic RNase or S-peptide domains.
12. A CAR-forming polypeptide or accessory polypeptide according to any preceding claim, which comprises an element capable of forming a bridge with another CAR.
13. A CAR-forming polypeptide or accessory polypeptide according to claim 10, wherein the element is capable of forming a di-sulphide bridge with another CAR-forming polypeptide or accessory polypeptide which contains such an element.
14. A multimeric chimeric antigen receptor (CAR) comprising a plurality of CAR-forming polypeptides as defined in any preceding claim.
15. A multimeric chimeric antigen receptor (CAR) comprising one or more CAR-forming polypeptides as defined in any of claims 1 to 11 and one or more accessory polypeptides as defined in any of claims 1 to 11.
.
16. A multimeric CAR according claim 12 or 13 wherein the CAR-forming polypeptide(s) and/or accessory polypeptide(s) comprise different endodomains.
17. A multimeric CAR according to any of claims 12 to 14 comprising at least two CAR-forming polypeptides having different antigen-binding domains..

18. An engaged complex which comprises first and second multimeric CARs according to any of claims 12 to 15, wherein the first multimeric CAR forms a bridge with the second multimeric CAR, such that the first and second multimeric CARs engage to form a complex.
19. An engaged complex according to claim 16, wherein the bridge is a disulphide bridge.
20. A chimeric antigen receptor (CAR) signalling system, which comprises:
(i) a multimeric CAR comprising a CAR-forming polypeptide or accessory polypeptide as defined in claim 10 or 11 which comprises a first heterodimerization domain; and
(ii) an intracellular signalling component comprising a signalling domain and a second heterodimerization domain;
wherein heterodimerization between the first and second heterodimerization domains causes the multimeric CAR and signalling component to form a functional CAR complex.
21. A CAR signalling system according to claim 20, wherein the or each CAR-forming polypeptide(s) or accessory polypeptide(s) comprises a plurality of heterodimerisation domains, such that a single CAR-forming polypeptide or accessory polypeptide is capable of heterodimerising with a plurality of signalling components.
22. A CAR signalling system according to claim 20 or 21, wherein each signalling component comprises a plurality of signalling domains.
23. A nucleic acid which encodes a CAR-forming polypeptide or accessory polypeptide according to any of claims 1 to 13.
24. A nucleic acid construct which encodes two or more CAR forming polypeptides as defined in any of claims 1 to 3 and 5 to 13.
25. A nucleic acid construct which encodes at least one CAR-forming polypeptide as defined in any of claims 1 to 3 and 5 to 13 and at least one accessory polypeptide as defined in any of claims 4 to 13.
26. A nucleic acid construct which encodes:
(i) at least one CAR-forming polypeptide as defined in any of claims 1 to 3 and 5 to 13, which forms a multimeric CAR as defined in any of claims 20 to 22; and
(ii) an intracellular signalling component as defined in any of claims 20 to 22.

27. A vector which comprises a nucleic acid sequence according to claim 23 or a nucleic acid construct according to any of claims 24 to 26..
28. A vector according to claim 27 which is a retroviral vector or a lentiviral vector or a transposon.
29. A cell which expresses a CAR-forming polypeptide or accessory polypeptide according to any of claims 1 to 13; a CAR signalling system according any of claims 20 to 22; a multimeric CAR according to any of claims 14 to 17; or an engaged complex according claim 18 or 19.
30. A cell according to claim 29 which is a T cell or NK cell.
31. A pharmaceutical composition which comprises a cell according to claim 29 or 30.
32. A cell according to claim 30 or 31 for use in treating a disease.
33. The use of a cell according to claim 29 or 30 in the manufacture of a medicament for treating a disease.
34. A method for treating a disease which comprises the step of administering a cell according to claim 29 or 30 to a subject.
35. A cell for use according to claim 32, a use according to claim 33, or a method according to claim 34 wherein the disease is cancer.
36. A cell, use or method according to claim 35 wherein the cancer is Chronic lymphocytic leukaemia (CLL), melanoma or myeloma.
37. A method for making a cell according to claim 29 or 30, which comprises the step of introducing a nucleic acid according to claim 23, a nucleic acid construct according to any of claims 24 to 26 or a vector according to claim 27 or 28 into the cell.
39. A method according to claim 37 wherein the cell is from a sample isolated from a subject.

FIGURE 1

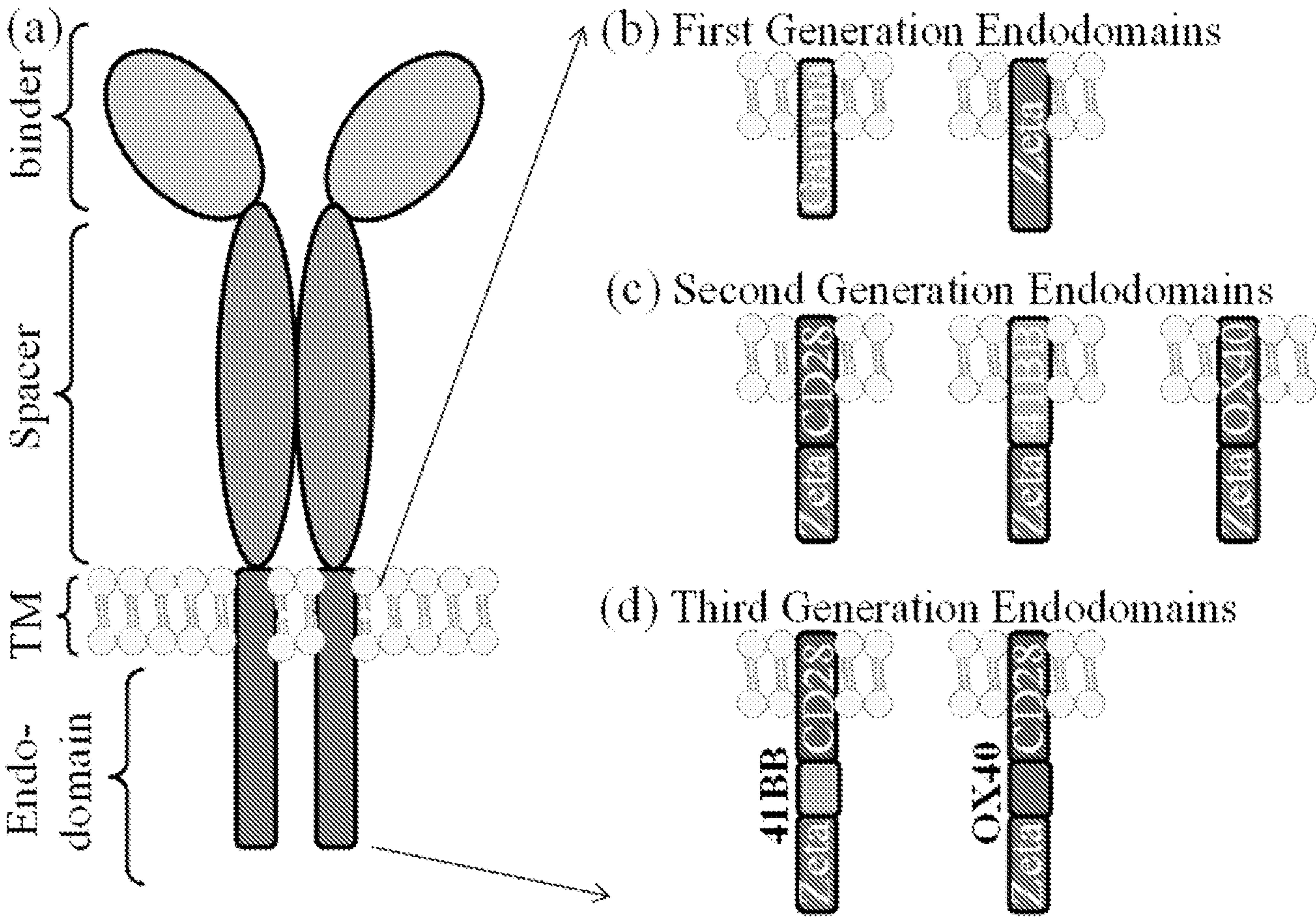
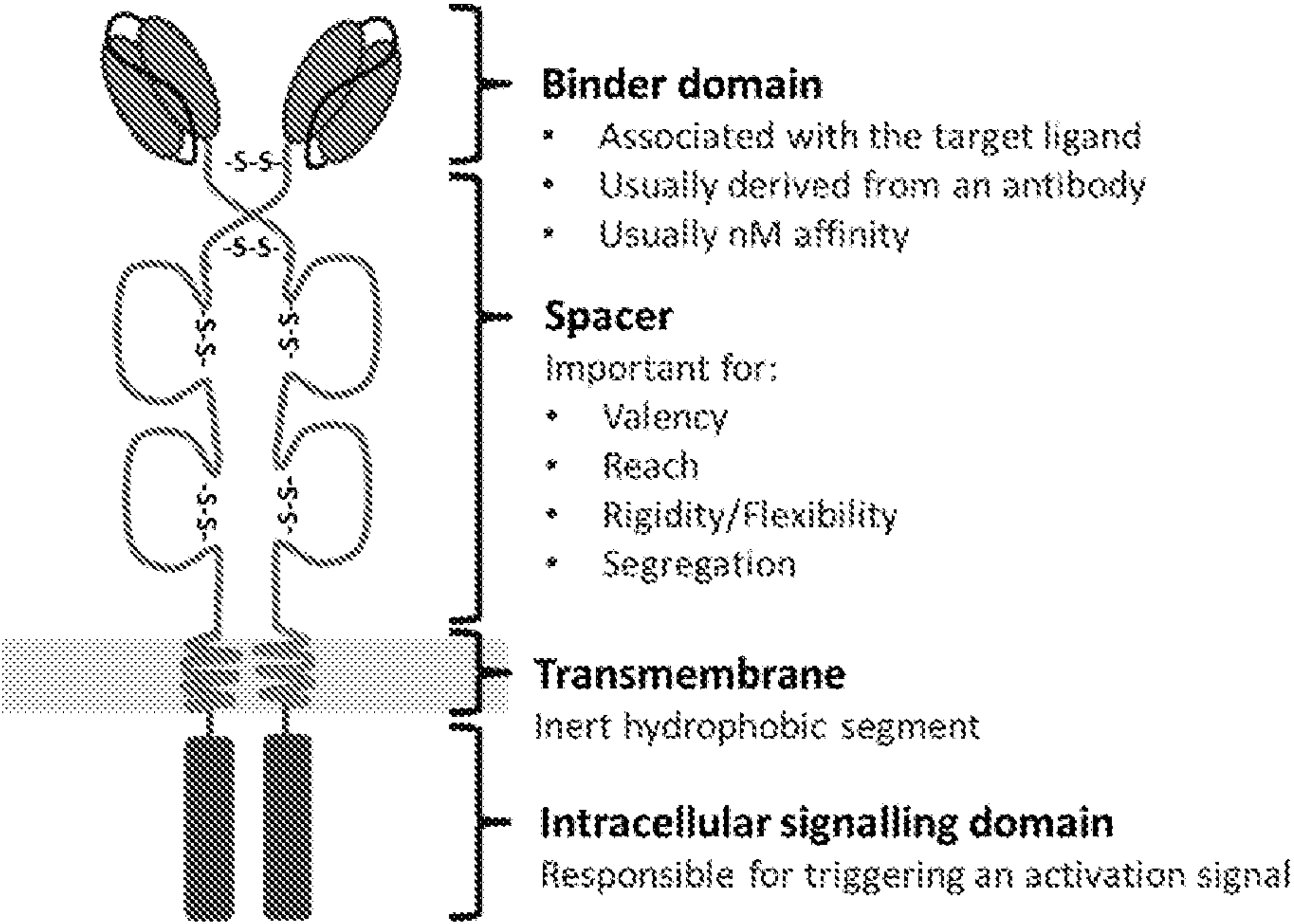


FIGURE 2

A)



B)

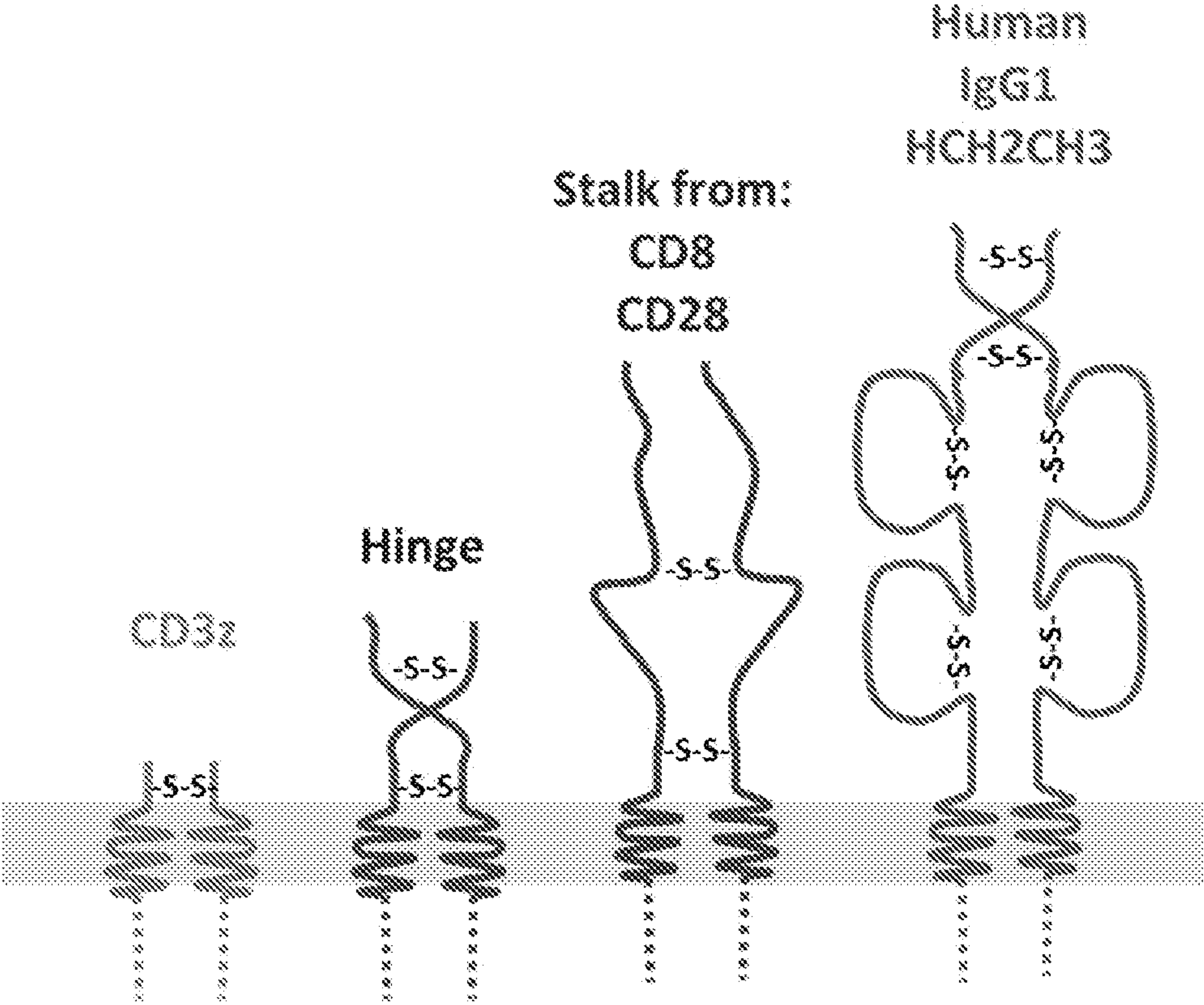


FIGURE 3

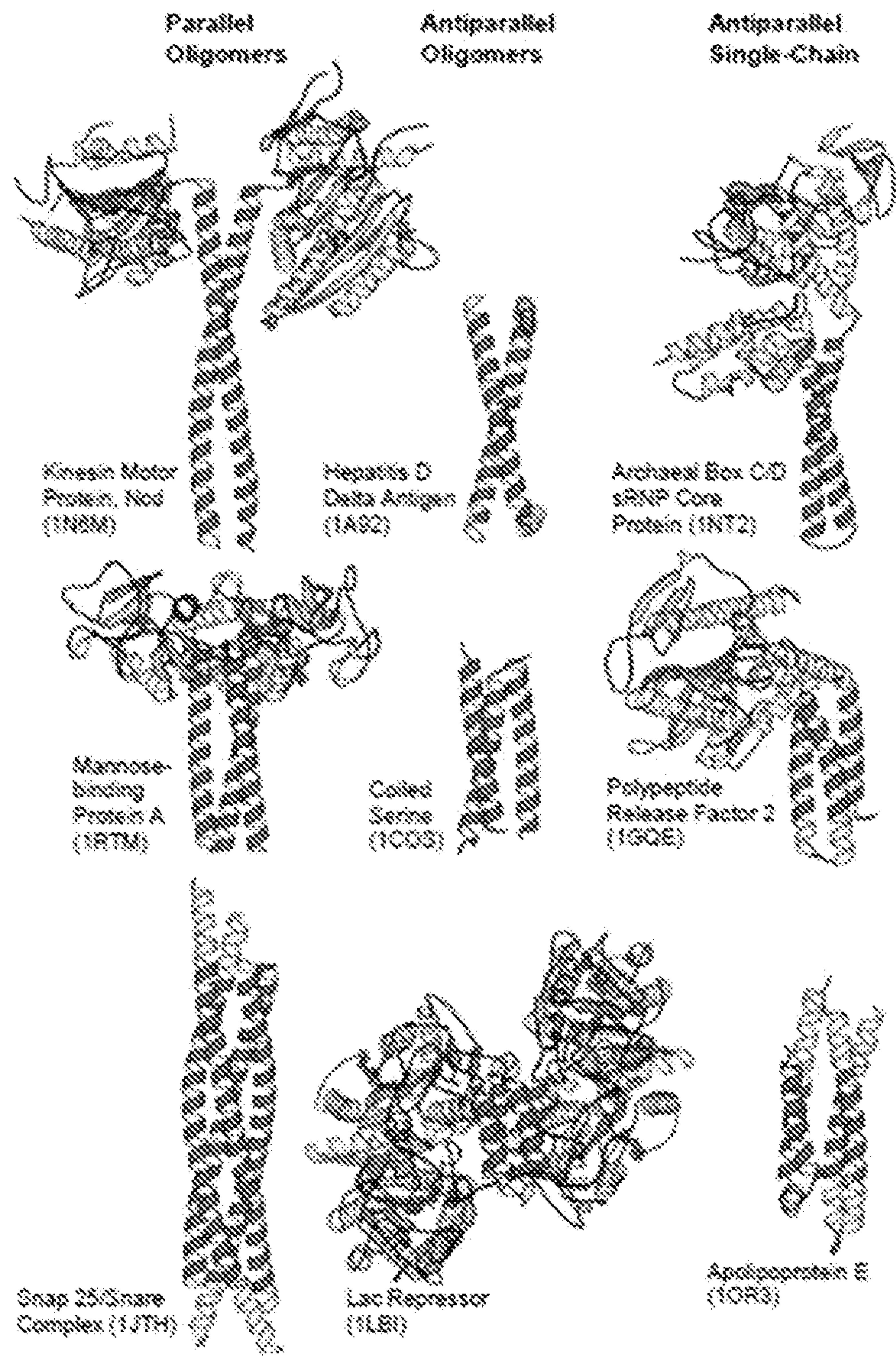


FIGURE 4

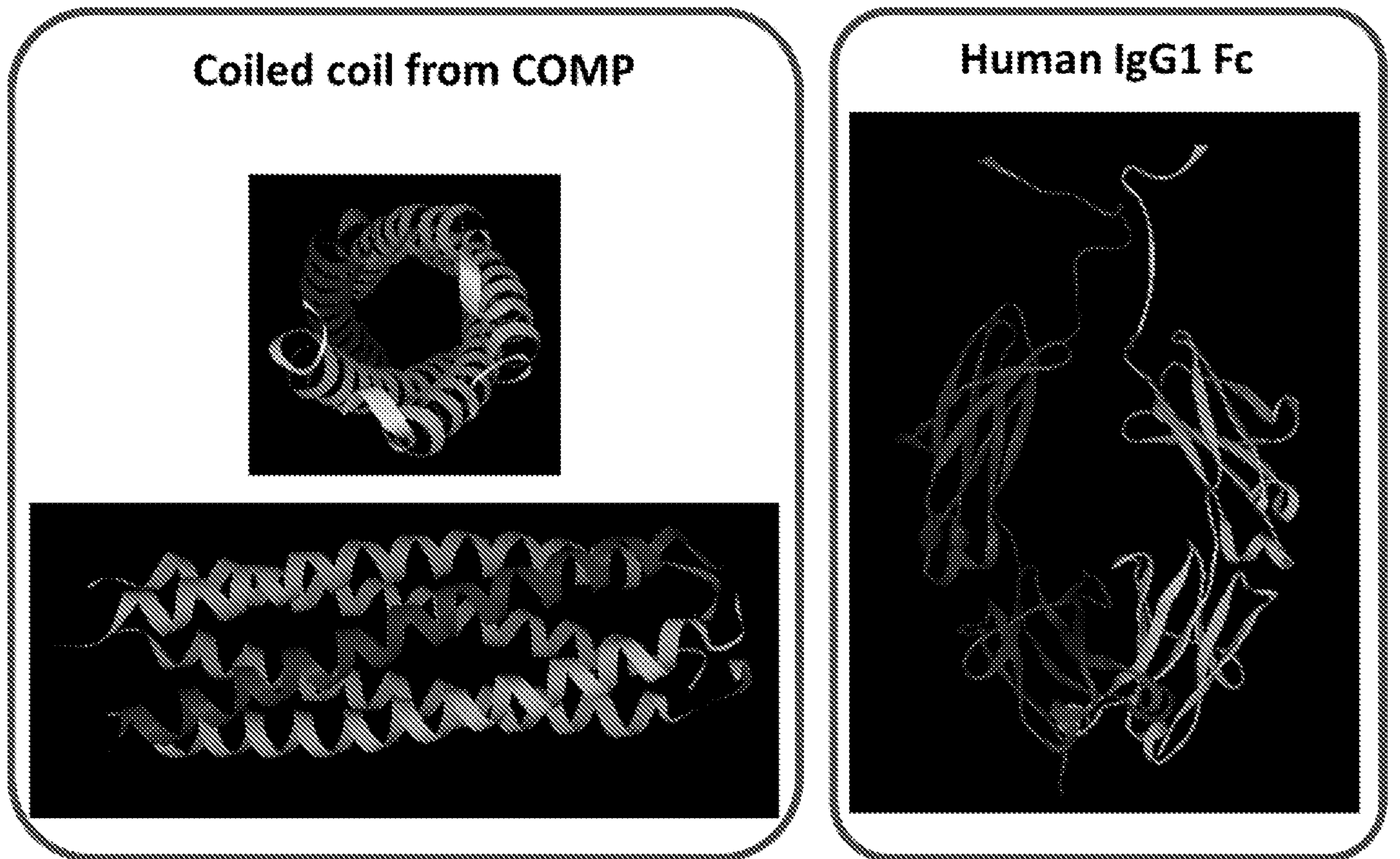
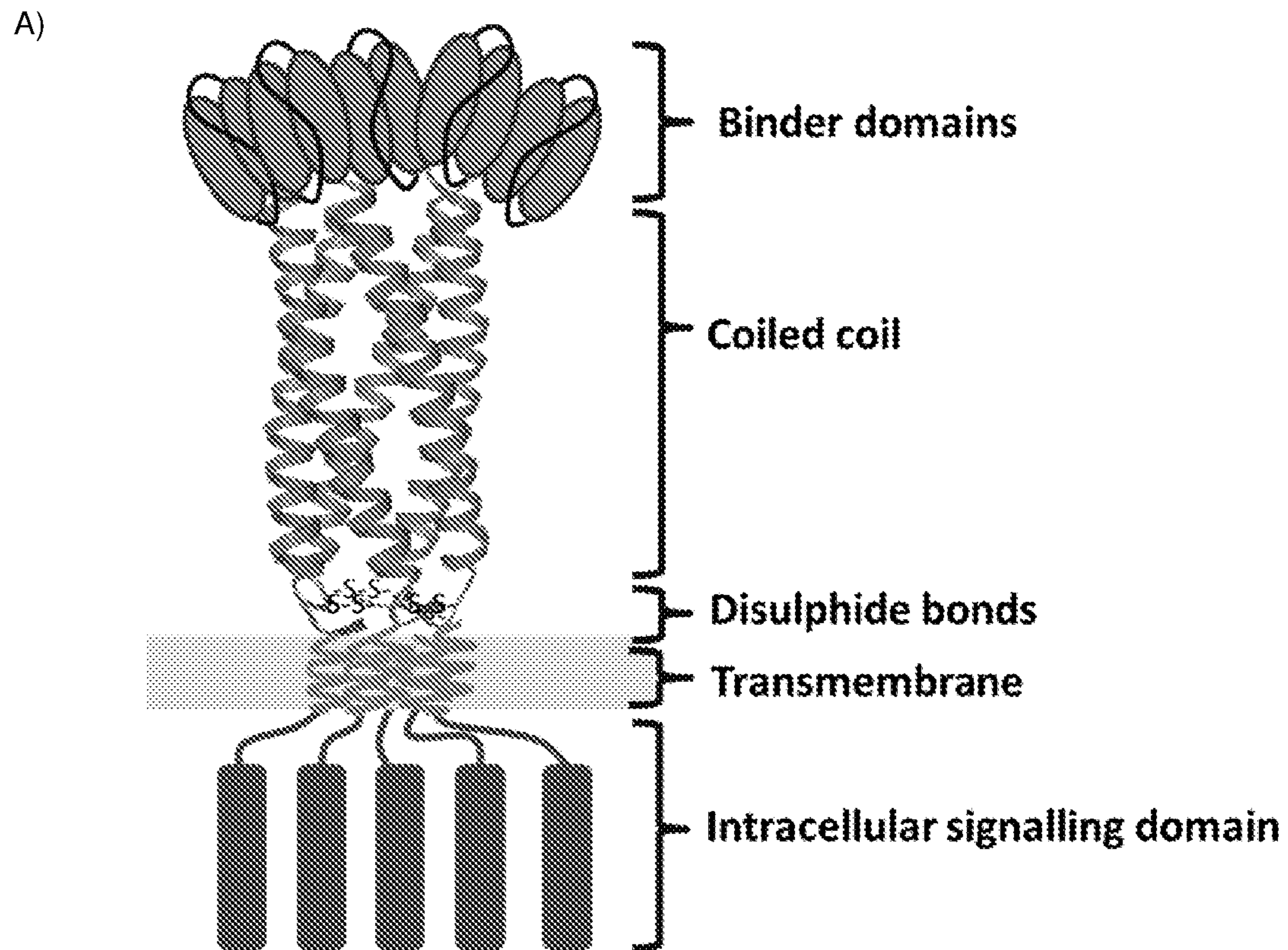


FIGURE 5



B)

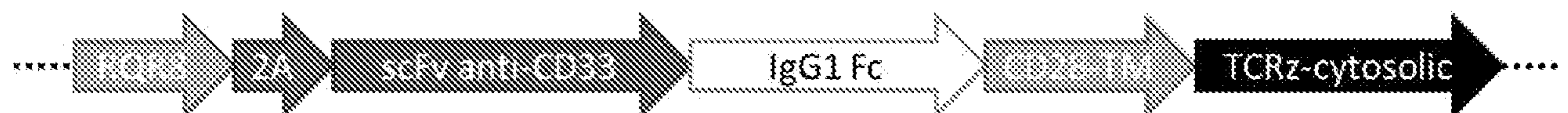
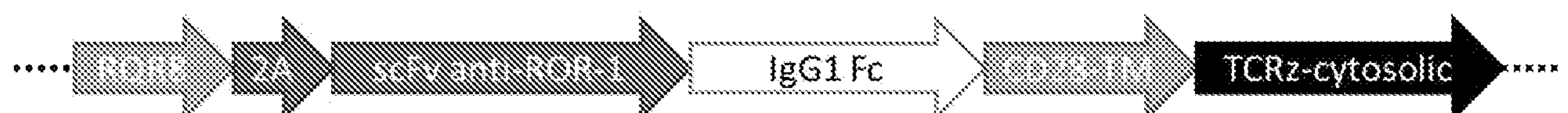
anti-CD33 COMP CAR:**Control anti-CD33 IgG1 CAR:****anti-ROR-1 COMP CAR:****Control anti-ROR-1 IgG1 CAR:**

FIGURE 5 – CONT.

C)

Amino acid sequence of the coiled coil anti-CD33 COMP CAR (SEQ ID No. 26):

MGTSLLCWMALCLLGADHADACPYSNPSCSGGGGSELPTQGTFENVSTNVSPAKPTTTACPYSNPSCSGGGGSPAPRPPTPAPTIASQ
PLSLRPEACRPAAGGAVHTRGLDFACDIYIWAPLAGTCGVLLLSLVITLYCNHRNRRRVCKCPRPVVRAEGRGSLTCGDVEENPGPMAVP
TQVLGLLLLWLTDARCDIQMTQSPSSLSASVGDRVTITCRASEDIYFNLVWYQQKPGKAPKLLIYDTNRLADGVPSRFSGSGSGTQYTLTISS
LQPEDFATYYCQHYKNYPLTFGQGTKLEIKRSGGGGSGGGGSGGGGSGGGGSRSEVQLVESGGGLVQPGGSLRLSCAASGFTLSNYGMH
WIRQAPGKGLEWVSSISLNGGSTYYRDSVKGRFTISRDNASTLYLQMNSLRAEDTAVYYCAAQDAYTGGYFDYWGQGTLVTVSSMDPA
GSDLGPQMLRELQETNAALQDVRELLRQQVREITFLKNTVMECDACGSGKKDPKFWVLVVGGVLACYSLLVTVAFIIFWVRSRVKFSRS
ADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPGEMGGKPRRKNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGL
STATKDTYDALHMQALPPR

Amino acid sequence of the coiled coil anti-ROR-1 COMP CAR(SEQ ID No. 27):

MGTSLLCWMALCLLGADHADACPYSNPSCSGGGGSELPTQGTFENVSTNVSPAKPTTTACPYSNPSCSGGGGSPAPRPPTPAPTIASQPLS
LRPEACRPAAGGAVHTRGLDFACDIYIWAPLAGTCGVLLLSLVITLYCNHRNRRRVCKCPRPVVRAEGRGSLTCGDVEENPGPMETDTLLLW
VLLLWVPGSTGQSVKESEGLVTPAGNLTCTASGSDINDYPISWVRQAPGKGLEWIGFINSGGSTWYASWVKGRFTISRTSTTVDLKMTSL
TTDDTATYFCARGYSTYYGDFNIWGPGLTVTISSGGGGSGGGGSGGGGSELVMTQTPSSTSGAVGGTITINCQASQSIDSNLAWFQQKPGQ
PPTLLIYRASNLASGVPSRFSGSRSGTEYTLISGVQREDAATYYCLGGVGNVSYRTSFGGGTEVVVKRSDPAGSDLGPQMLRELQETNAALQD
VRELLRQQVREITFLKNTVMECDACGSGKKDPKFWVLVVGGVLACYSLLVTVAFIIFWVRSRVKFSRSADAPAYQQGQNQLYNELNLGRREE
YDVLDKRRGRDPGEMGGKPRRKNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTATKDTYDALHMQALPPR

FIGURE 5 – CONT.

D) **DNA sequence of the coiled coil anti-CD33 COMP CAR (SEQ ID No. 28):**

ATGGGCACCAGCCTGCTGTGCTGGATGGCCCTGTGCCTGCTGGGCGCCGACCACGCCGATGCCTGCCCCTACAGCAACCCCAGCC
TGTGCAGCGGAGGCGGCGGCAGCGAGCTGCCACCCAGGGCACCTTCTCCAACGTGTCCACCAACGTGAGCCCAGCCAAGCCCA
CCACCACCGCCTGTCTTATTCCAATCCTTCCCTGTGTAGCGGAGGGGGAGGCAGCCCAGCCCCAGACCTCCCACCCCAGCCCCC
ACCATCGCCAGCCAGCCTCTGAGCCTGAGACCCGAGGCCTGCCGCCAGCCGCCGCGCGCCGTGCACACCAGAGGCCTGGAT
TTCGCCTGCGATATCTACATCTGGGCCCCACTGGCCGGCACCTGTGGCGTGCTGCTGCTGAGCCTGGTGATCACCTGTACTGCAA
CCACCGCAACCGCAGGCGCGTGTGCAAGTGCCCCAGGCCCGTGGTGAGAGCCGAGGGCAGAGGCAGCCTGCTGACCTGCGGCG
ACGTGGAGGAGAACCCAGGCCCCATGGCCGTGCCCACTCAGGTCCTGGGGTTGTTGCTACTGTGGCTTACAGATGCCAGATGTG
ACATCCAGATGACACAGTCTCCATCTTCCCTGTCTGCATCTGTCGGAGATCGCGTCACCATCACCTGTGAGCAAGTGAGGACATT
TATTTTAATTTAGTGTGGTATCAGCAGAAACCAGGAAAGGCCCTAAGCTCCTGATCTATGATACAAATCGCTTGGCAGATGGGG
TCCCATCACGTTTCACTGGCTCTGGATCTGGCACACAGTATACTCTAACCATAAGTAGCCTGCAACCCGAAGATTTGCAACCTAT
TATTGTCAACACTATAAGAATTATCCGCTCACGTTCCGTCAGGGGACCAAGCTGGAAATCAAAGATCTGGTGGCGGAGGGTCA
GGAGGCGGAGGCAGCGGAGGCGGTGGCTCGGGAGGCGGAGGCTCGAGATCTGAGGTGCAGTTGGTGGAGTCTGGGGGCGGC
TTGGTGCAGCCTGGAGGGTCCCTGAGGCTCTCCTGTGCAGCCTCAGGATTAAGTCTCAGTAATTATGGCATGCACTGGATCAGGC
AGGCTCCAGGGAAGGGTCTGGAGTGGGTCTCGTCTATTAGTCTTAATGGTGGTAGCACTTACTATCGAGACTCCGTGAAGGGCC
GATTAAGTCTCAGGACAAATGCAAAAAGCACCTCTACCTTCAAATGAATAGTCTGAGGGCCGAGGACACGGCCGTCTATTA
CTGTGCAGCACAGGACGCTTATACGGGAGGTTACTTTGATTACTGGGGCCAAGGAACGCTGGTCAAGTCTCGTCTATGGATCCC
GCCGGGAGCGACCTGGGCCCTCAGATGCTGCGGGAGCTGCAGGAGACAAATGCCGCCCTGCAGGACGTGCGCGAGCTGCTGAG
ACAGCAGGTGCGGGAGATTACATTCCTGAAGAACACCGTGATGGAGTGCGATGCCTGCGGATCTGGGAAGAAGGACCCCAAGT
TCTGGGTCTGGTGGTGGTGGGAGGCGTGCTGGCCTGTTACTCTCTCCTGGTGACCGTGGCCTTCATCATCTTTTGGGTGCGCTCC
CGGGTGAAGTTTCTCGCTCTGCCGATGCCCCAGCCTATCAGCAGGGCCAGAATCAGCTGTACAATGAACTGAACCTGGGCAGGC
GGGAGGAGTACGACGTGCTGGATAAGCGGAGAGGCAGAGACCCCGAGATGGGCGGCAAACACGGCGCAAAAATCCCCAGGA
GGGACTCTATAACGAGCTGCAGAAGGACAAAATGGCCGAGGCCTATTCCGAGATCGGCATGAAGGGAGAGAGAAGACGCGGA
AAGGGCCACGACGGCCTGTATCAGGGATTGTCCACCGCTACAAAAGATACATATGATGCCCTGCACATGCAGGCCCTGCCACCCA
GATGA

DNA sequence of the coiled coil anti-ROR-1 COMP CAR (SEQ ID No. 29):

ATGGGCACCAGCCTGCTGTGCTGGATGGCCCTGTGCCTGCTGGGCGCCGACCACGCCGATGCCTGCCCCTACAGCAACCCCAGCCTGT
GCAGCGGAGGCGGCGGCAGCGAGCTGCCACCCAGGGCACCTTCTCCAACGTGTCCACCAACGTGAGCCCAGCCAAGCCCACCACCA
CCGCTGTCTTATTCCAATCCTTCCCTGTGTAGCGGAGGGGGAGGCAGCCCAGCCCCAGACCTCCCACCCCAGCCCCCACCATCGCC
AGCCAGCCTCTGAGCCTGAGACCCGAGGCCTGCCGCCAGCCGCCGCGCGCCGTGCACACCAGAGGCCTGGATTTGCGCTGCGAT
ATCTACATCTGGGCCCCACTGGCCGGCACCTGTGGCGTGCTGCTGCTGAGCCTGGTGATCACCTGTACTGCAACCACCGCAACCGCA
GGCGCGTGTGCAAGTGCCCCAGGCCCGTGGTGAGAGCCGAGGGCAGAGGCAGCCTGCTGACCTGCGGCGACGTGGAGGAGAACCC
AGGCCCCATGGAGACCGACACCTGCTGCTGTGGGTGCTGCTGCTGTGGGTGCCCGCAGCACCAGGCGCAGAGCGTGAAGGAGAGCG
AGGGCGACCTGGTGACCCAGCCGGCAACCTGACCTGACCTGCACCGCCAGCGGCAGCGACATCAACGACTACCCCATCAGCTGGG
TGCGGCAGGCCCCAGGCAAGGGCCTGGAGTGGATCGGCTTCATCAACAGCGGCGGCAGCACCTGGTACGCCAGCTGGGTGAAGGGC
CGGTTACCATCAGCCGACCAGCACCACCGTGGACCTGAAGATGACCAGCCTGACCACCGACGACACCGCCACCTACTTCTGCGCCA
GAGGCTACAGCACCTACTACGGCGACTTCAACATCTGGGGACCCGGCACCTGGTGACCATCAGCAGCGGAGGCGGAGGGTCTGGG
GGCGGCGGTAGCGGCGGAGGAGGGAGCGAGCTGGTGATGACCCAGACCCCAAGCAGCACCAGCGGCGCCGTGGGCGGCACCGTGA
CCATCAACTGCCAGGCCAGCCAGAGCATCGACAGCAACCTGGCCTGGTTCCAGCAGAAGCCTGGCCAGCCACCCACCTGCTGATCTA
CCGGGCCAGCAACCTGGCCAGCGGCGTGCCAGCCGTTTACGCGGCAGCCGAGCGGCACCGAGTACACCTGACCATCAGCGGCG
TGCAGCGGGAGGACGCGGCCACCTACTACTGCCTGGGCGGAGTGGGCAACGTGAGCTACCGGACCAGCTTCGGCGGAGGCACCGAG
GTGGTGGTGAAGCGGTCGGATCCCGCCGGGAGCGACCTGGGCCCTCAGATGCTGCGGGAGCTGCAGGAGACAAATGCCGCCCTGCA
GGACGTGCGCGAGCTGCTGAGACAGCAGGTGCGGGAGATTACATTCCTGAAGAACACCGTGATGGAGTGCGATGCCTGCGGATCTG
GGAAGAAGGACCCCAAGTTCTGGGTCTGGTGGTGGGAGGCGTGCTGGCCTGTTACTCTCTCCTGGTGACCGTGGCCTTCATCAT
CTTTTGGGTGCGCTCCCGGGTGAAGTTTTCTCGCTCTGCCGATGCCCCAGCCTATCAGCAGGGCCAGAATCAGCTGTACAATGAACTG
AACCTGGGCAGGCGGGAGGAGTACGACGTGCTGGATAAGCGGAGAGGCAGAGACCCCGAGATGGGCGGCAAACACGGCGCAAAA
ATCCCCAGGAGGGACTCTATAACGAGCTGCAGAAGGACAAAATGGCCGAGGCCTATTCCGAGATCGGCATGAAGGGAGAGAGAAGA
CGCGGAAAGGGCCACGACGGCCTGTATCAGGGATTGTCCACCGCTACAAAAGATACATATGATGCCCTGCACATGCAGGCCCTGCCA
CCCAGA

FIGURE 6

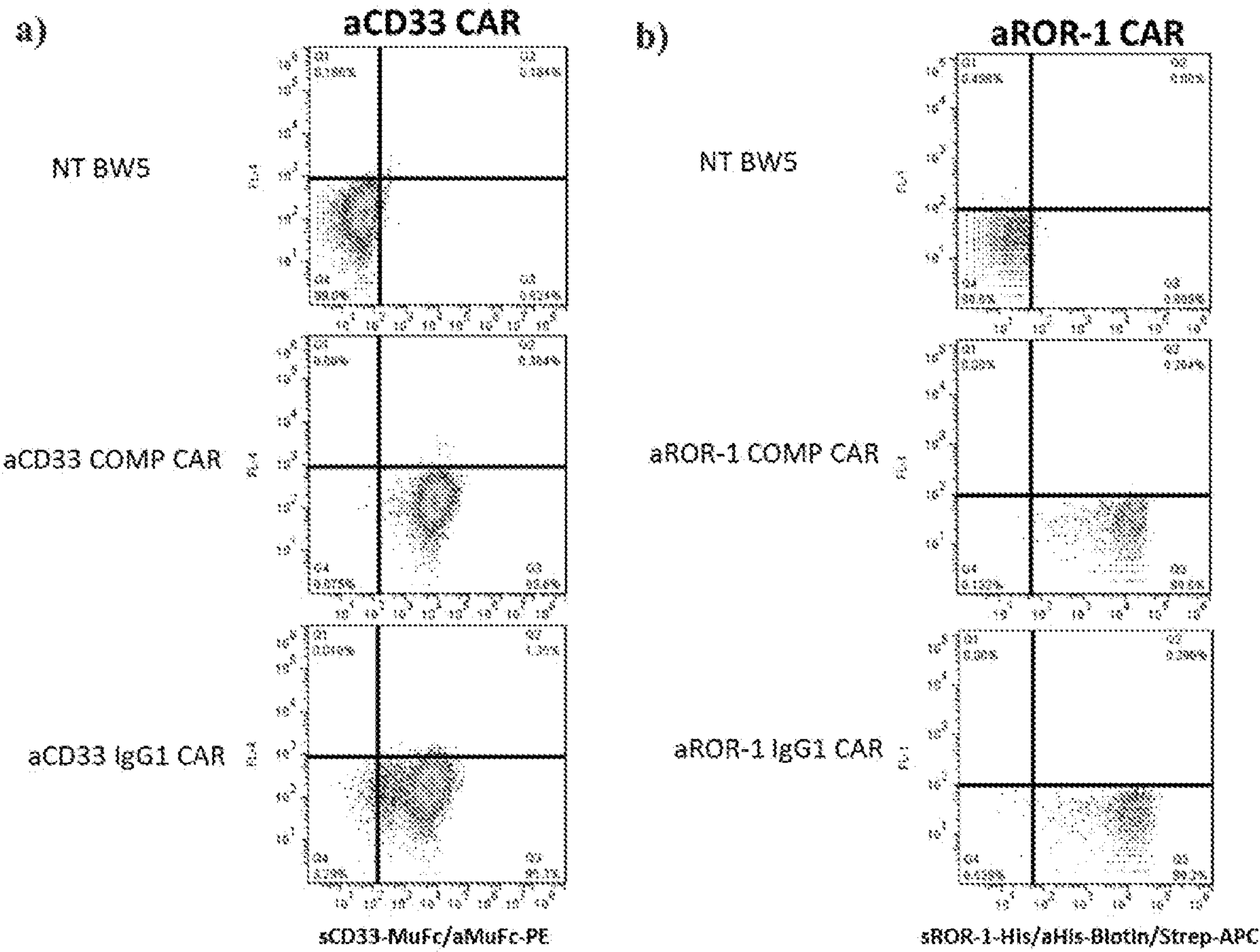


FIGURE 7



FIGURE 8

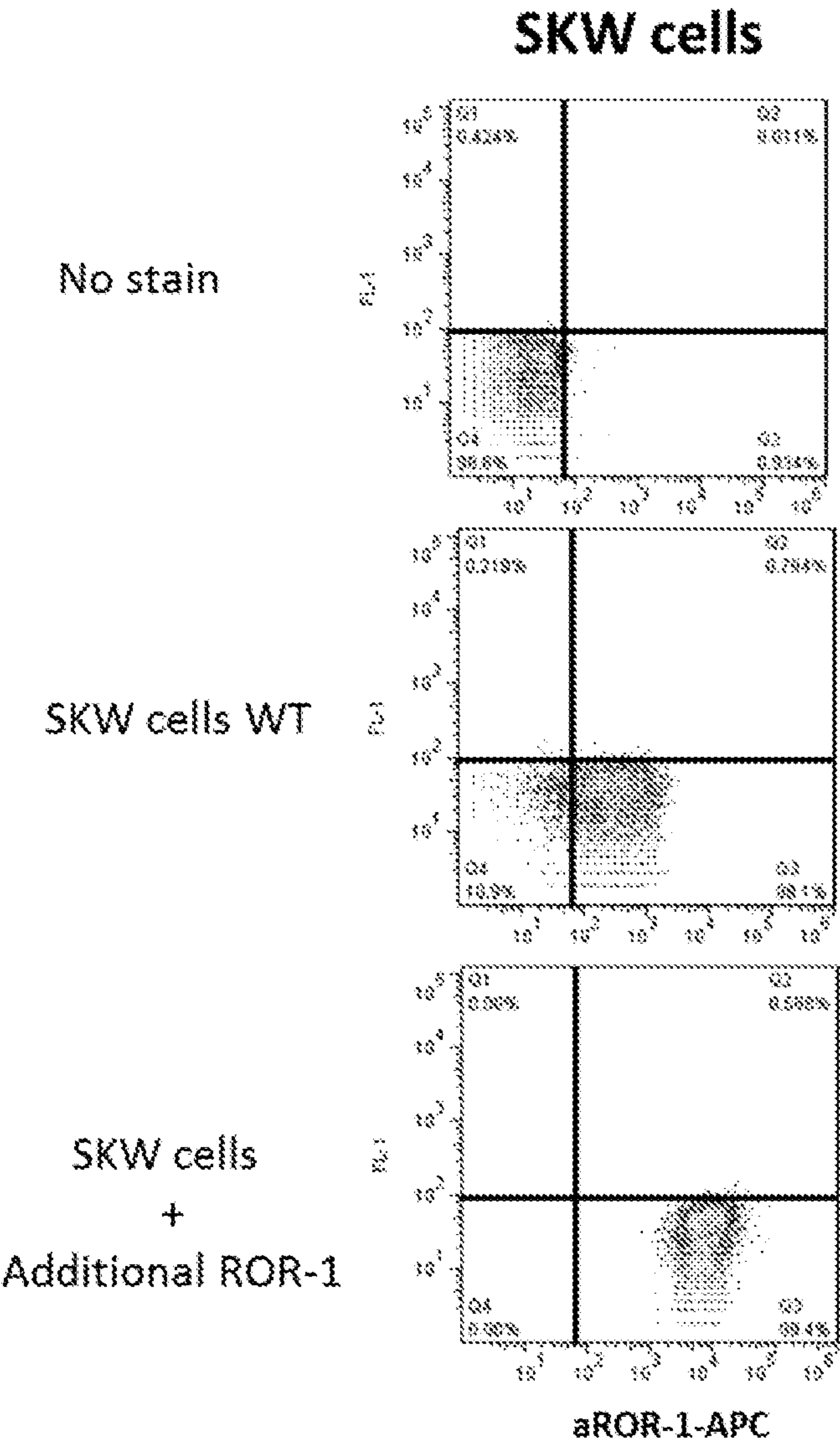


FIGURE 9

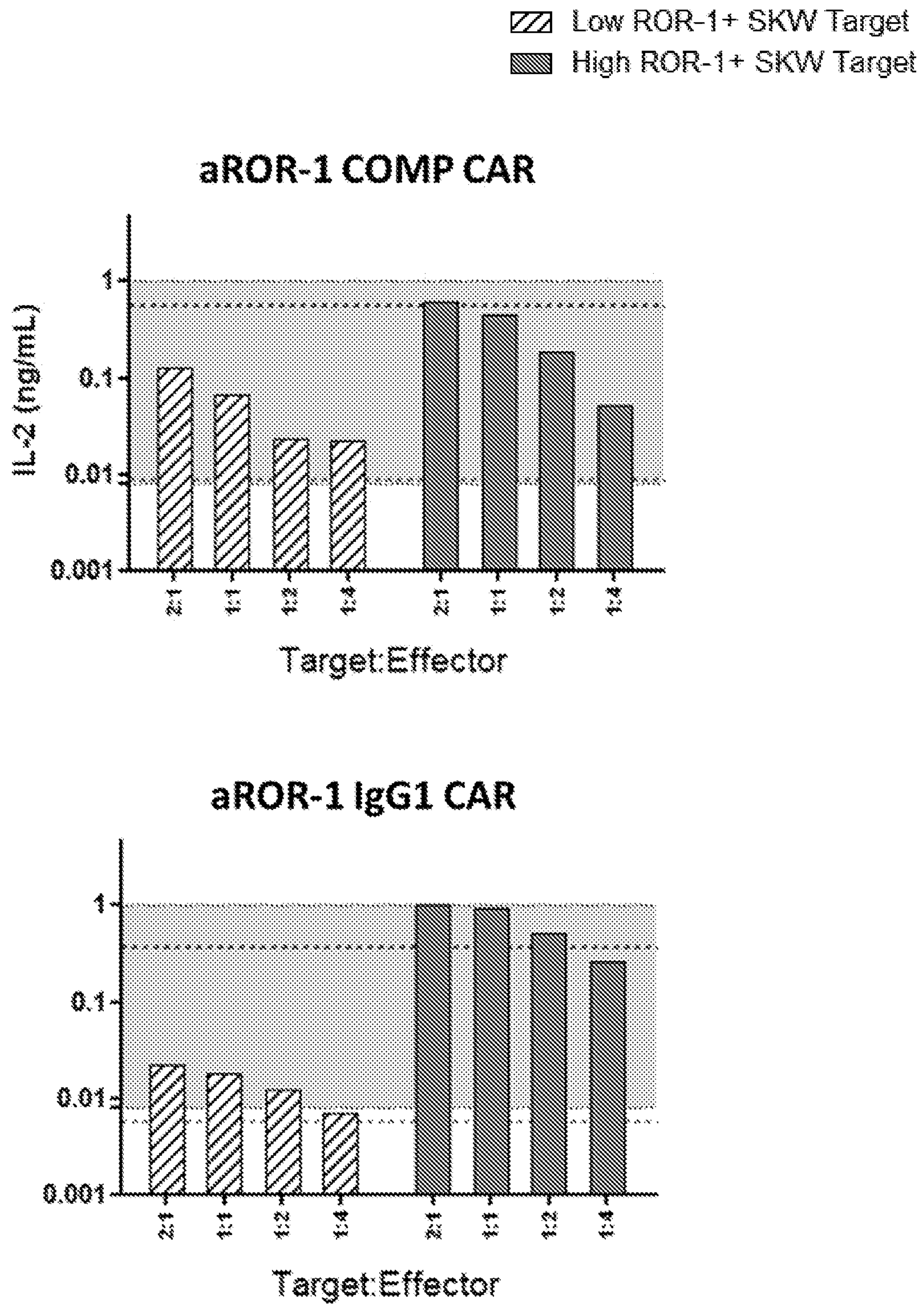


FIGURE 10

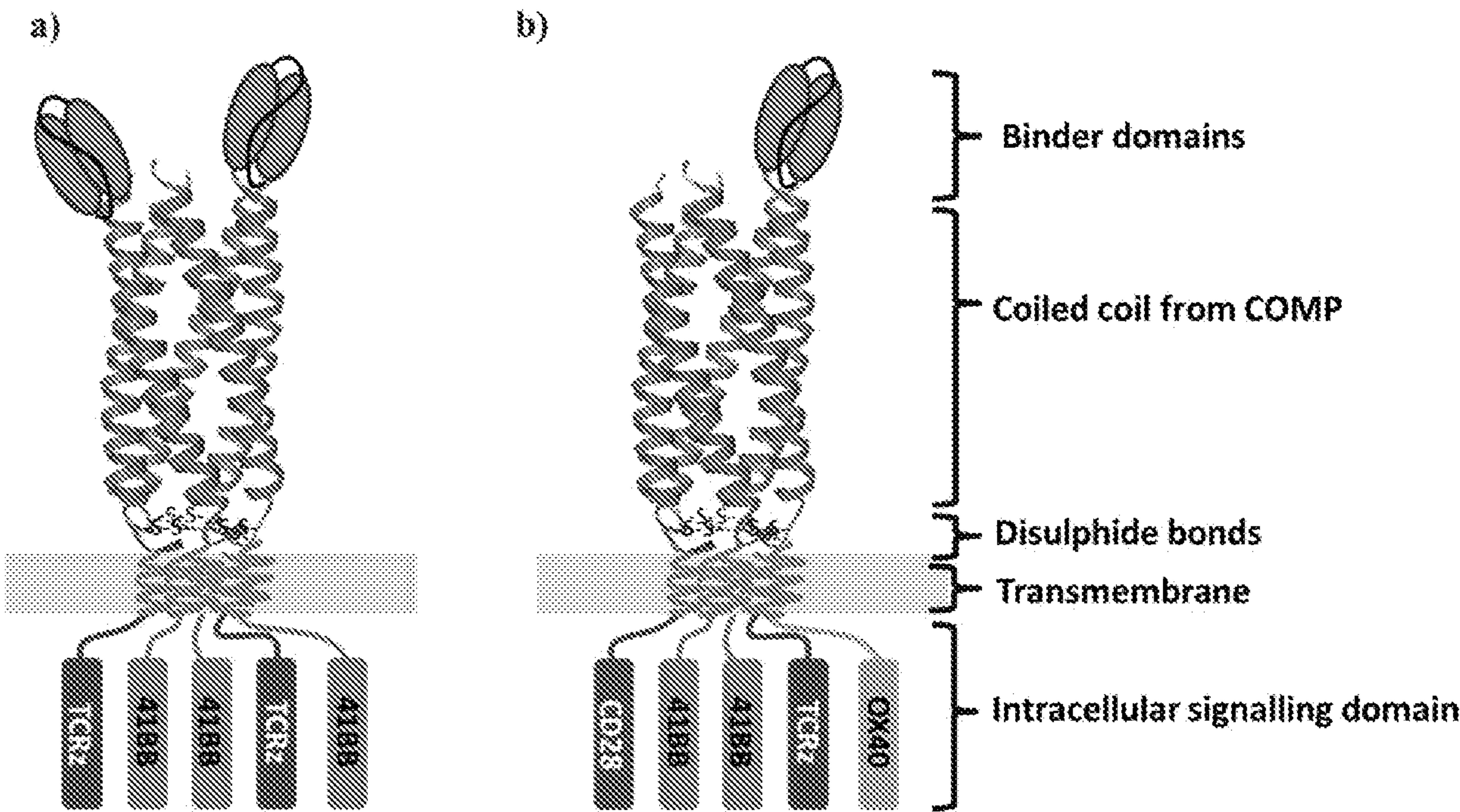


FIGURE 10c

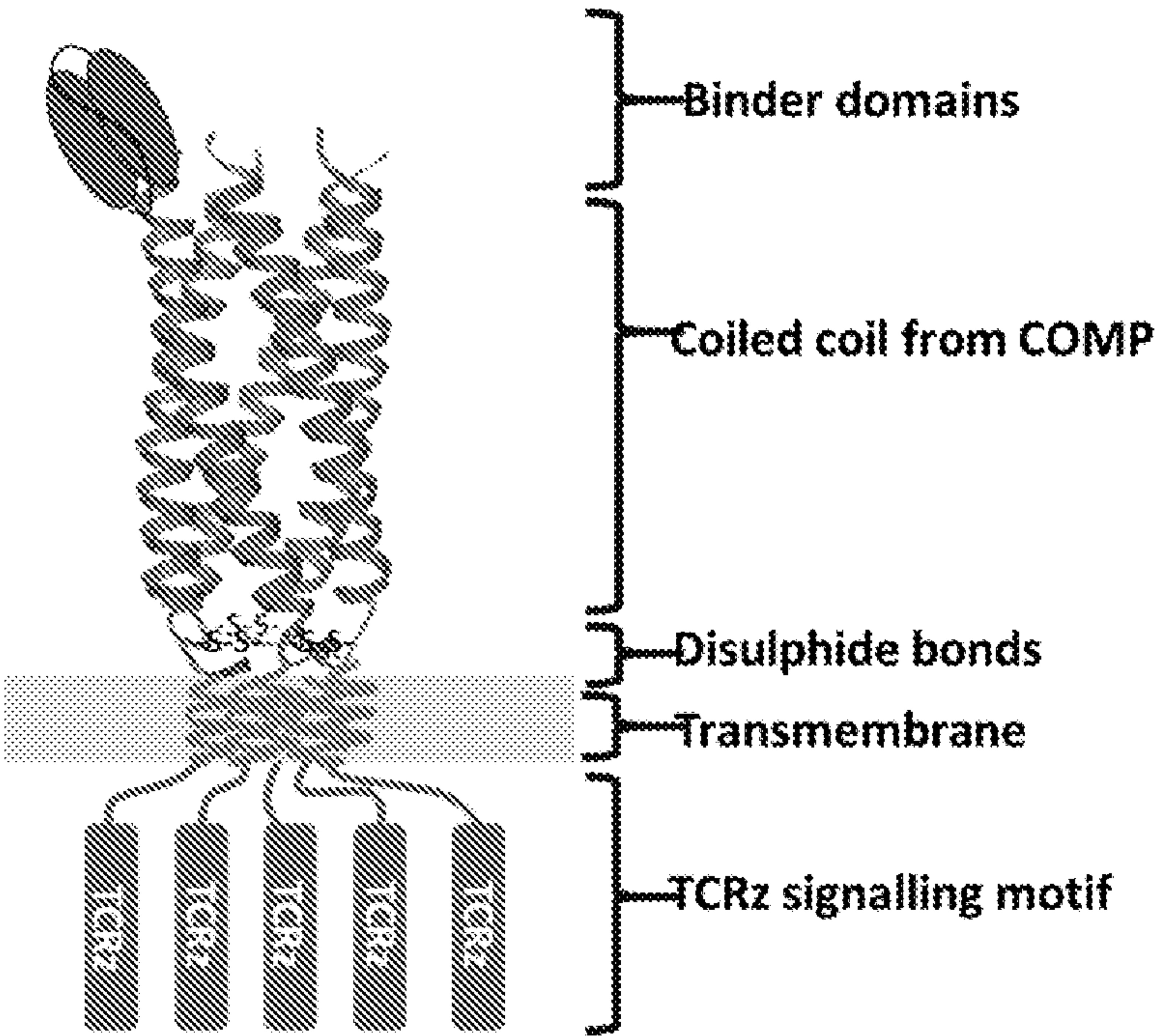


FIGURE 10d

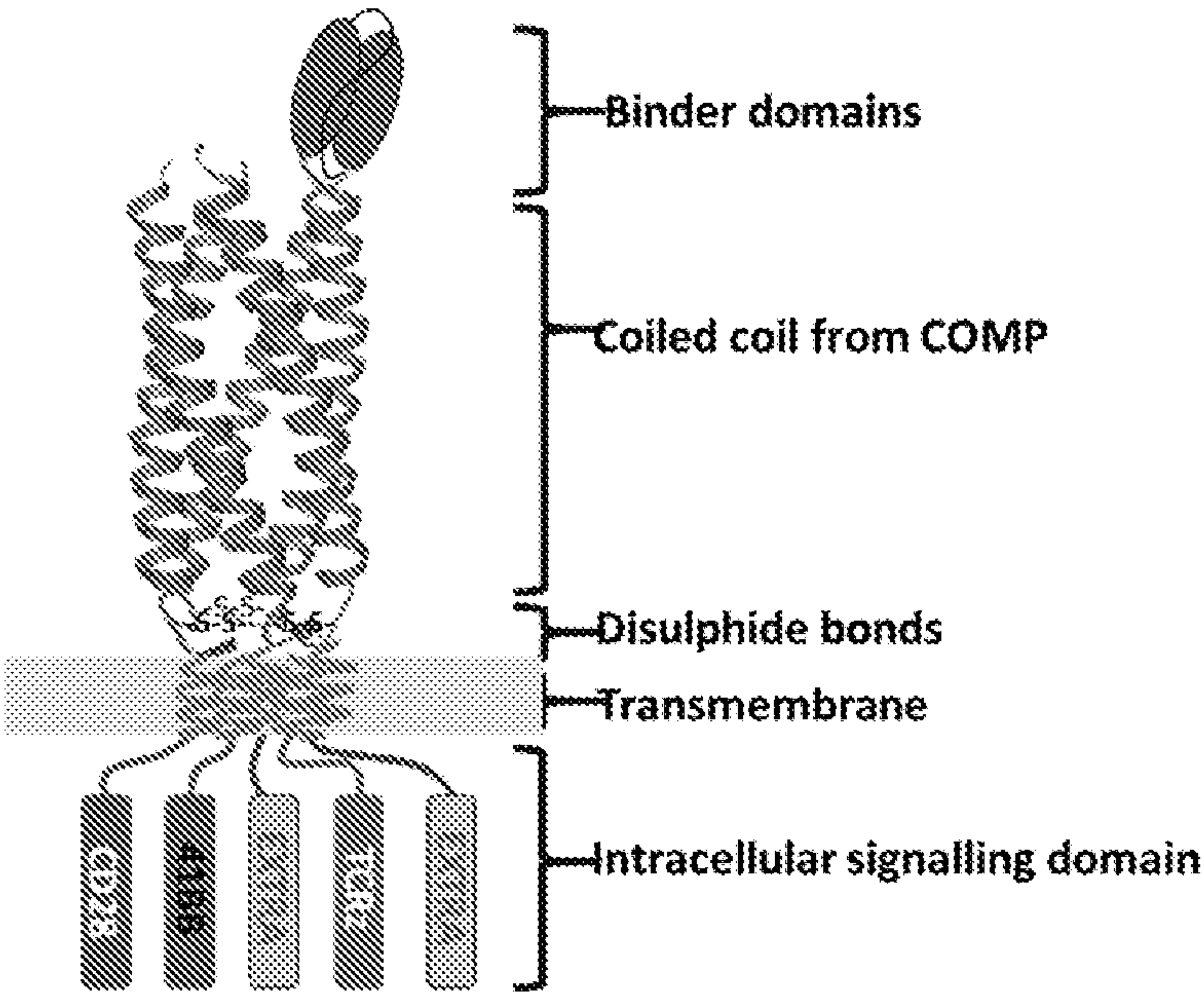


FIGURE 10e

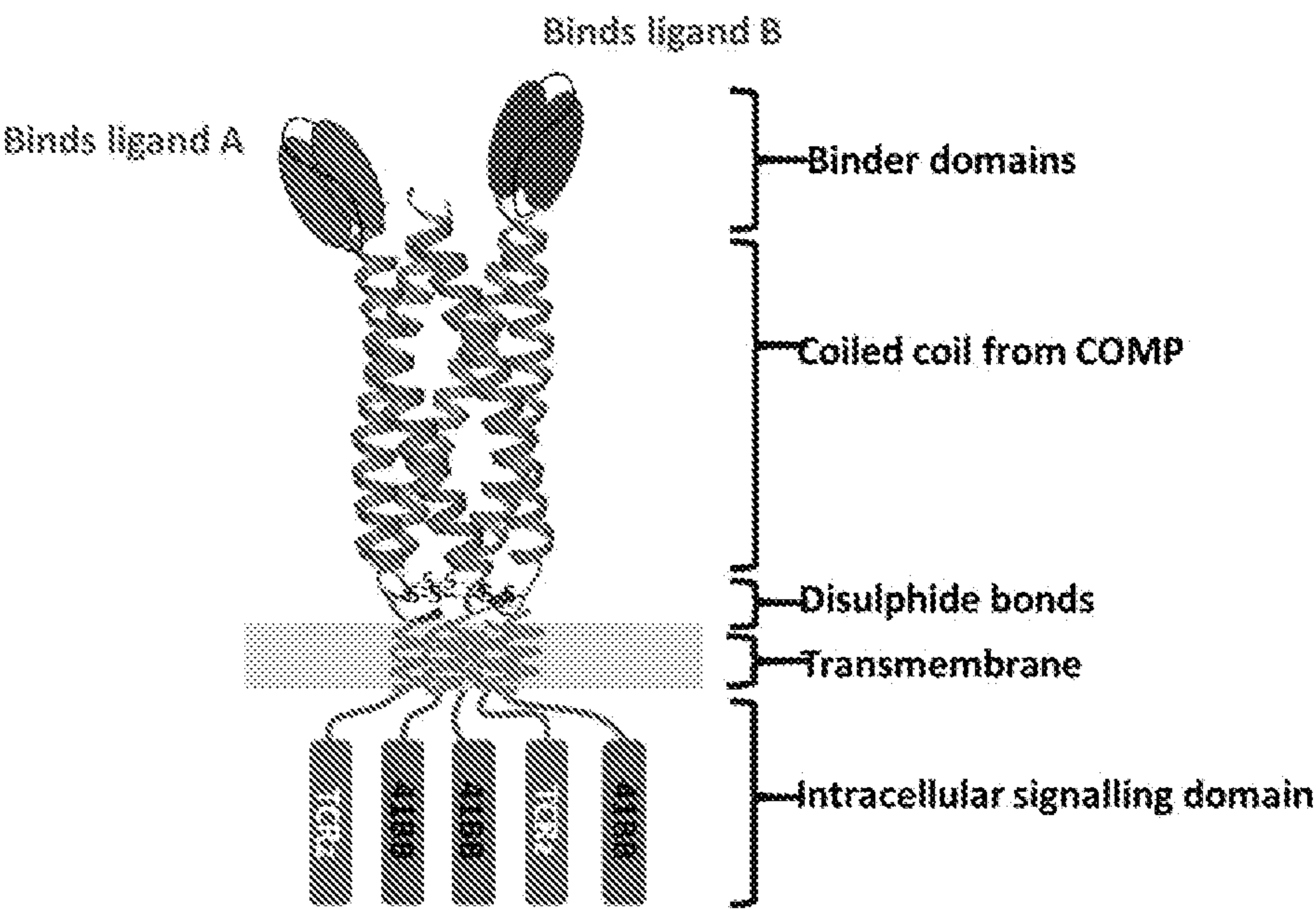


FIGURE 10f

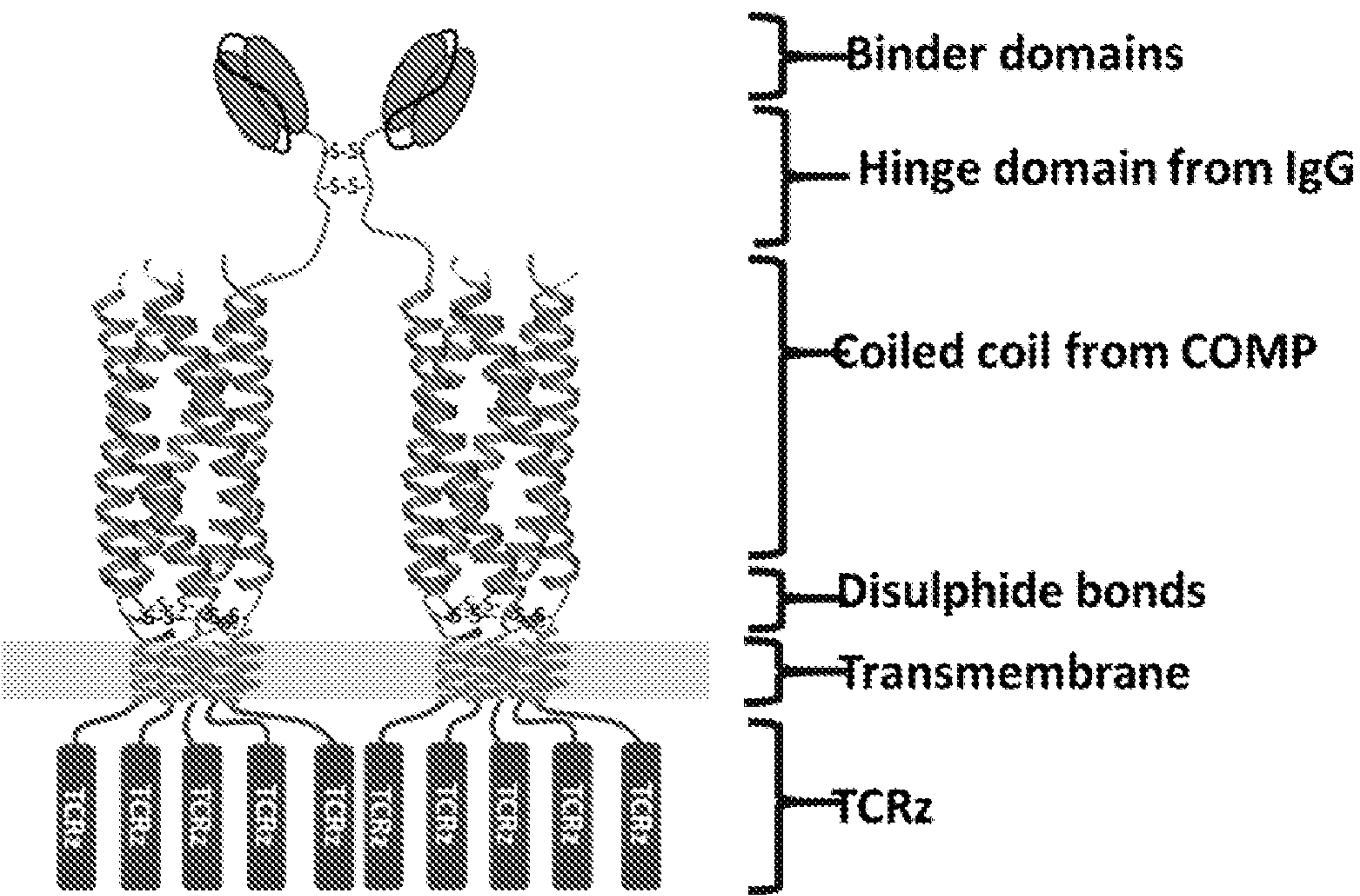


FIGURE 11

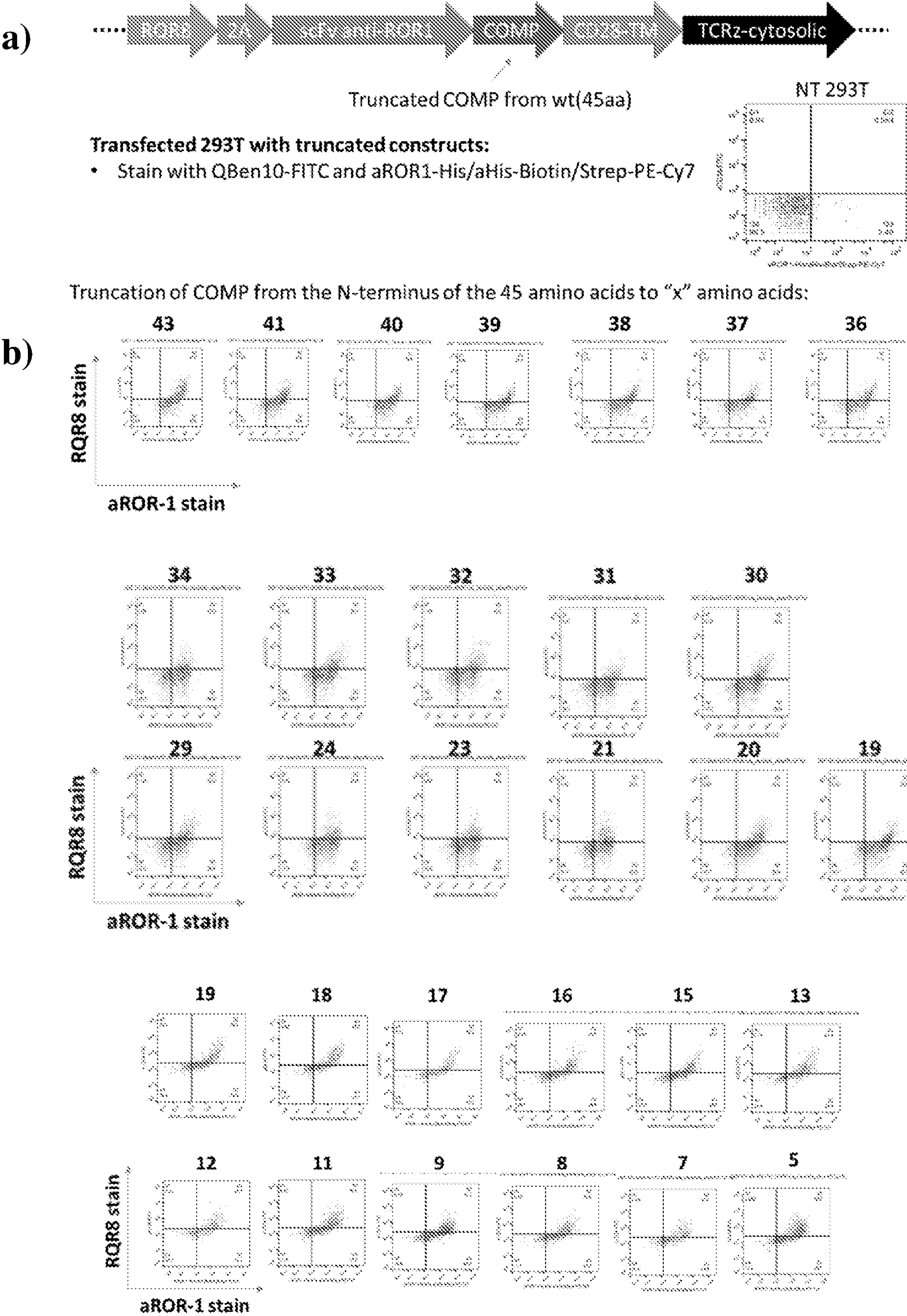


FIGURE 12

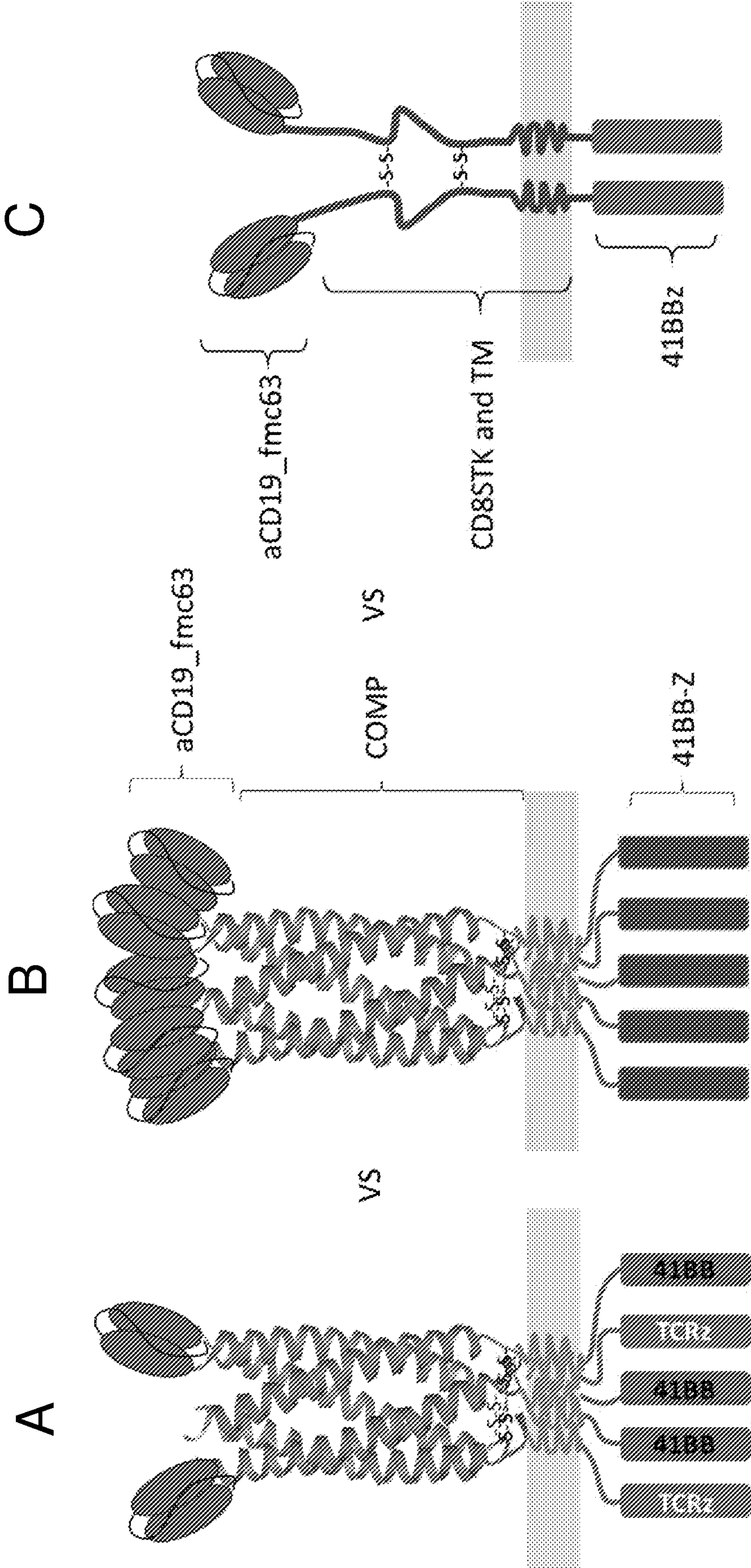


FIGURE 13

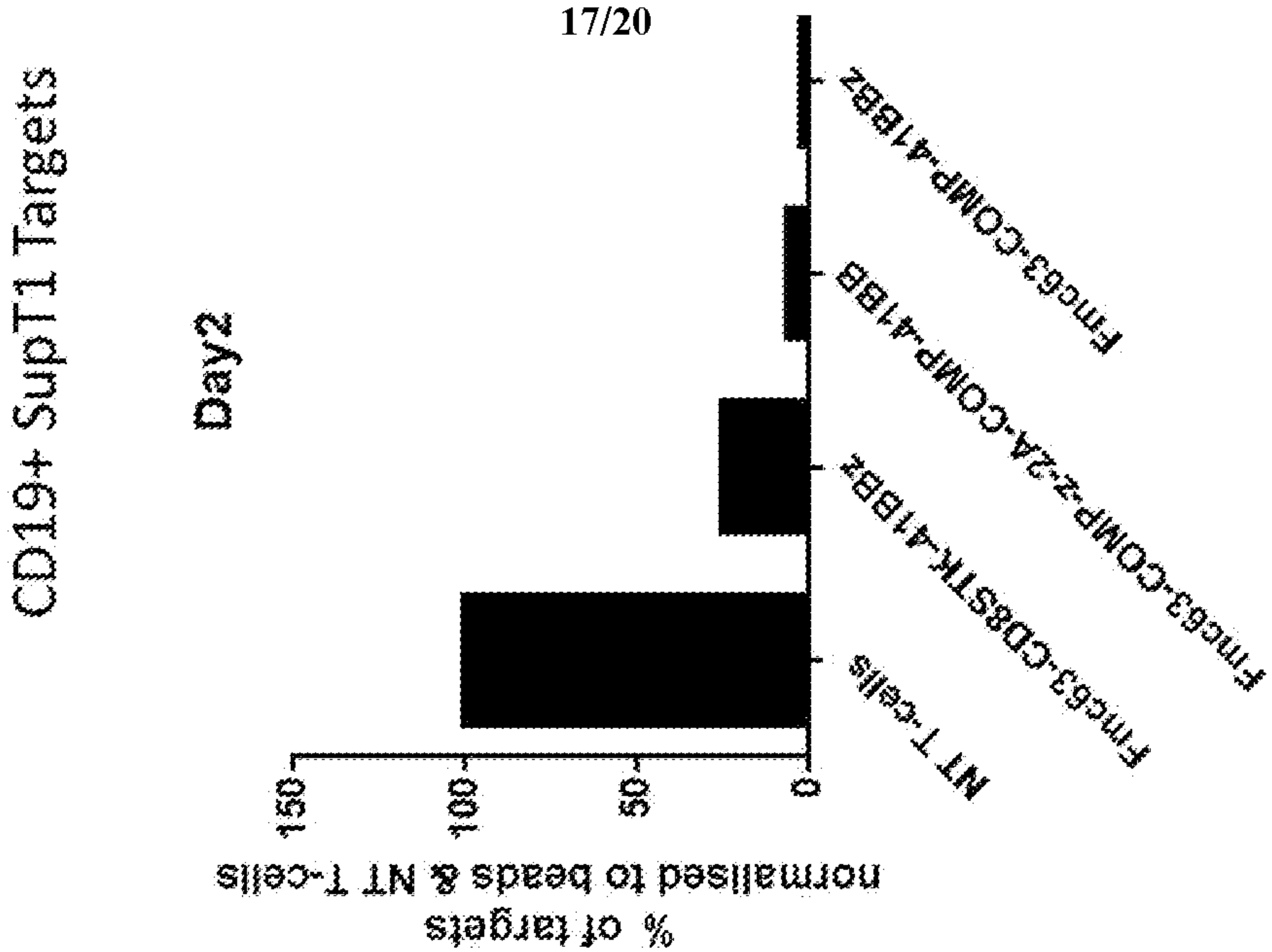
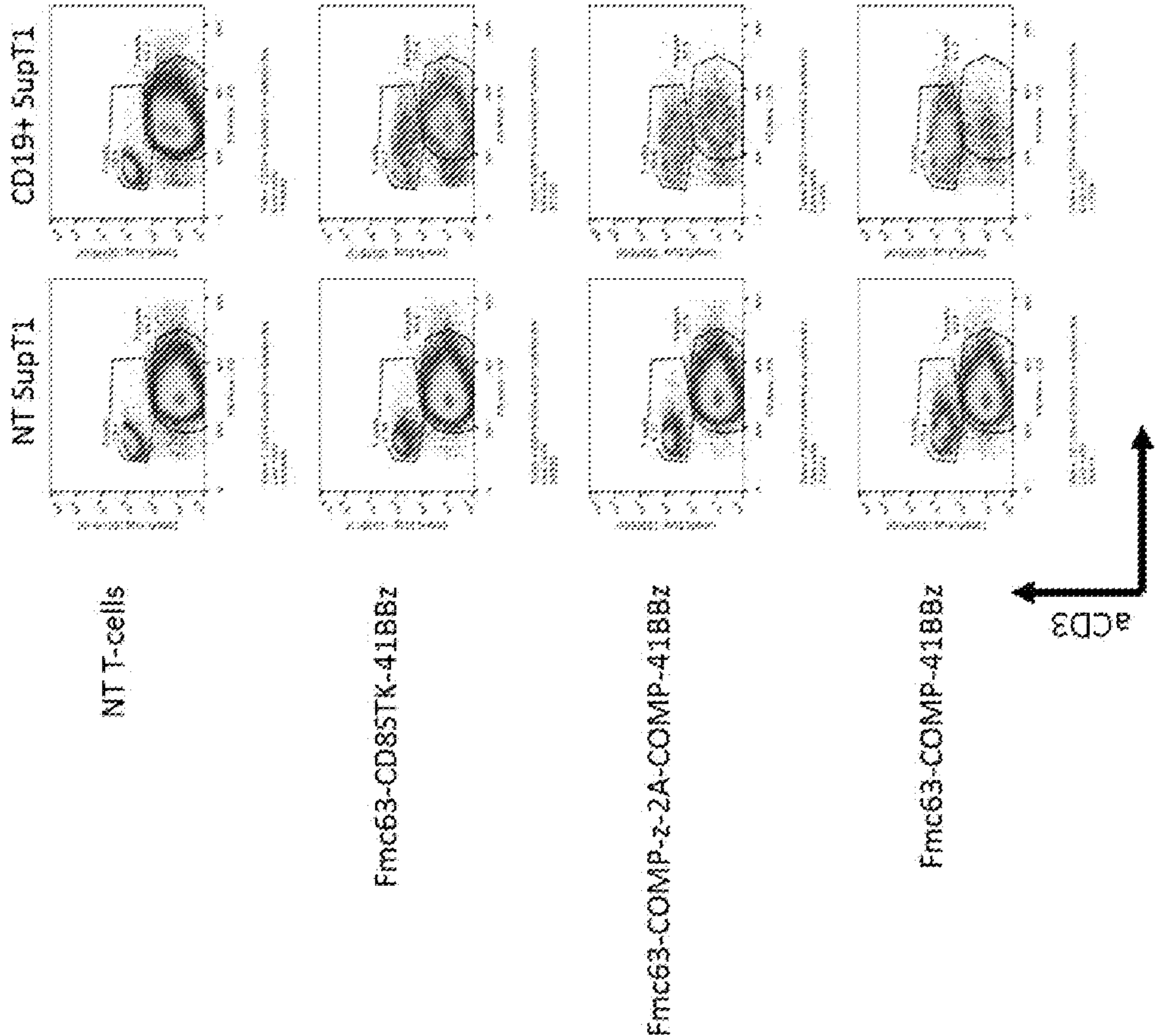


FIGURE 14

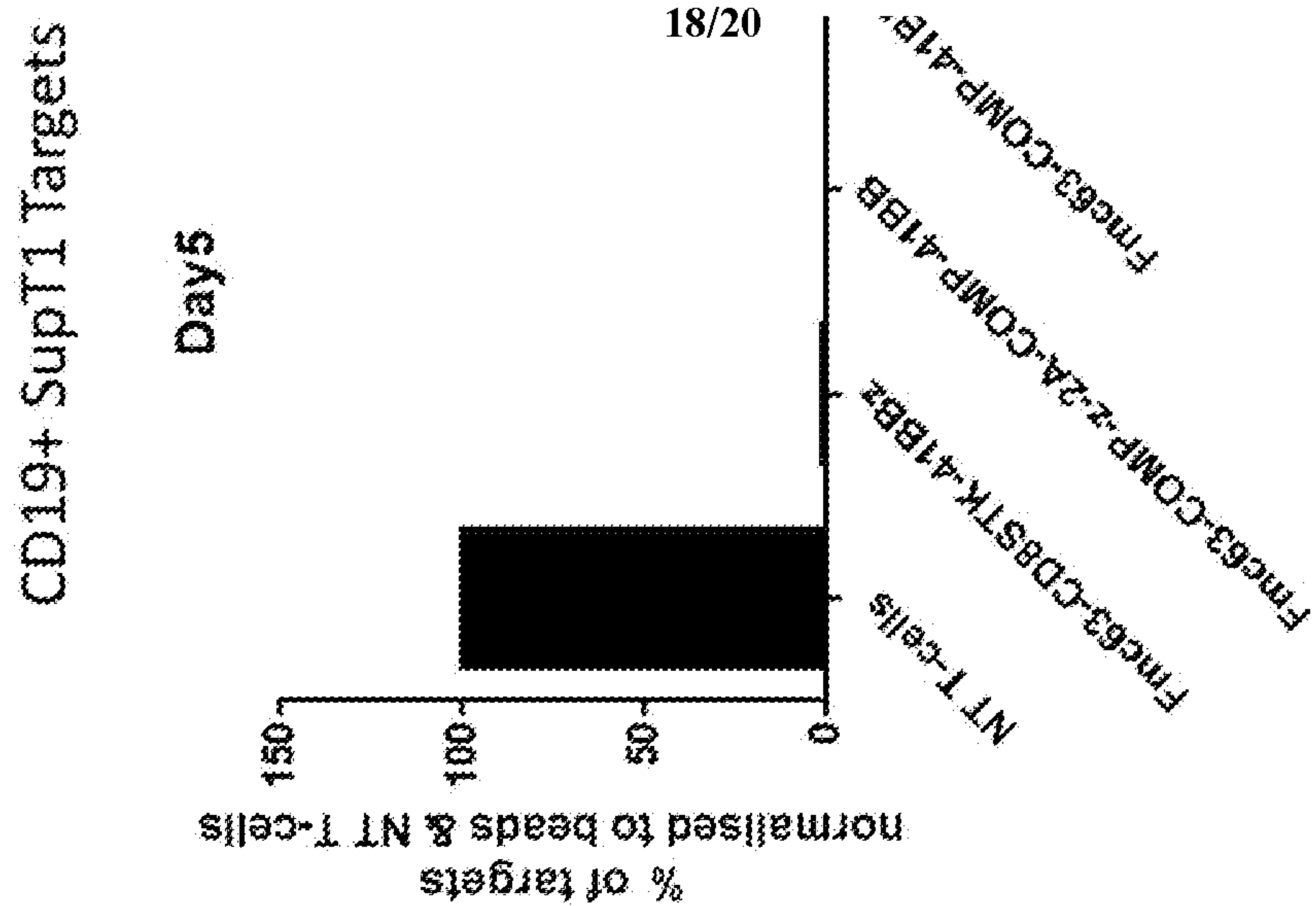
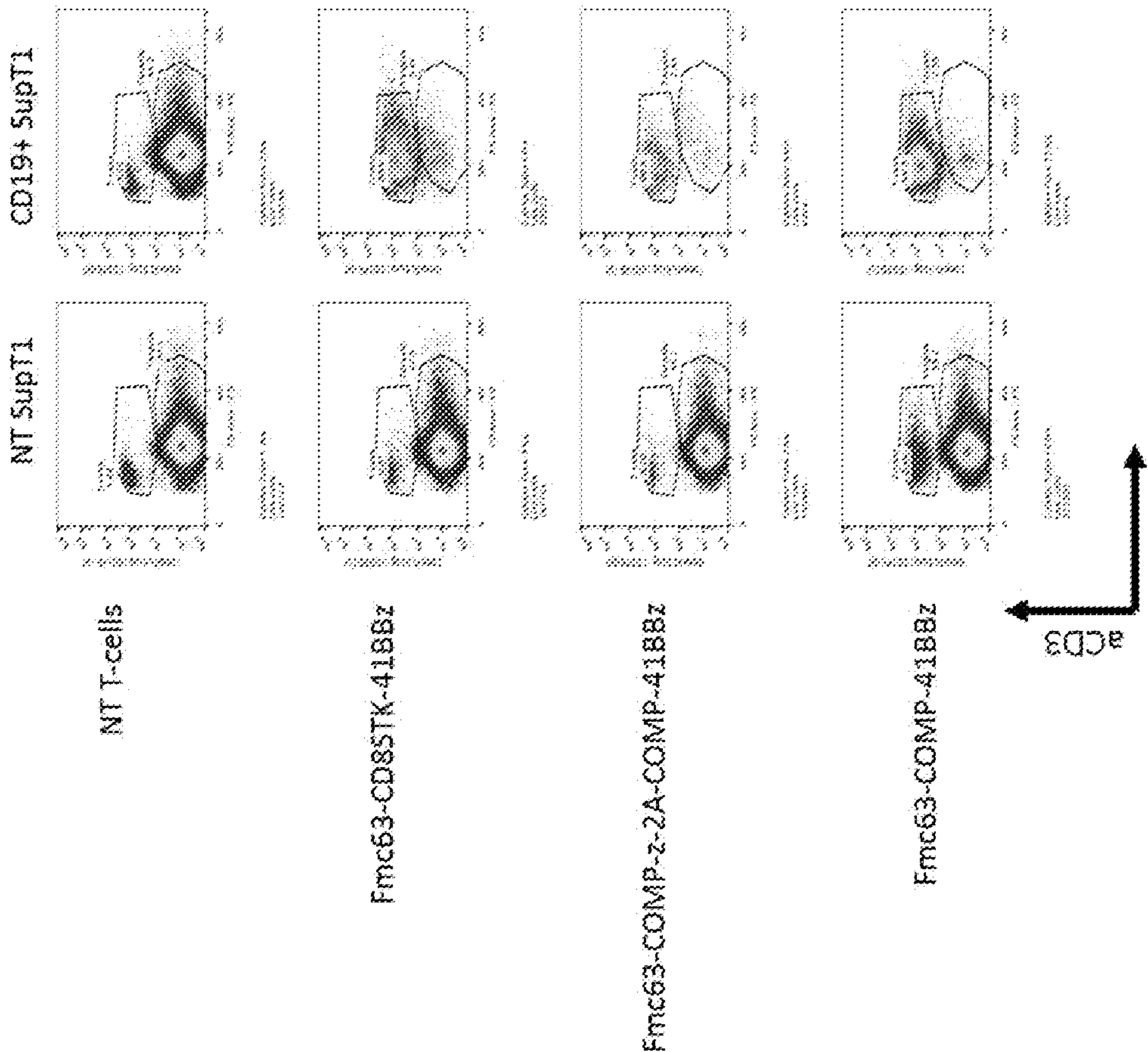


FIGURE 15

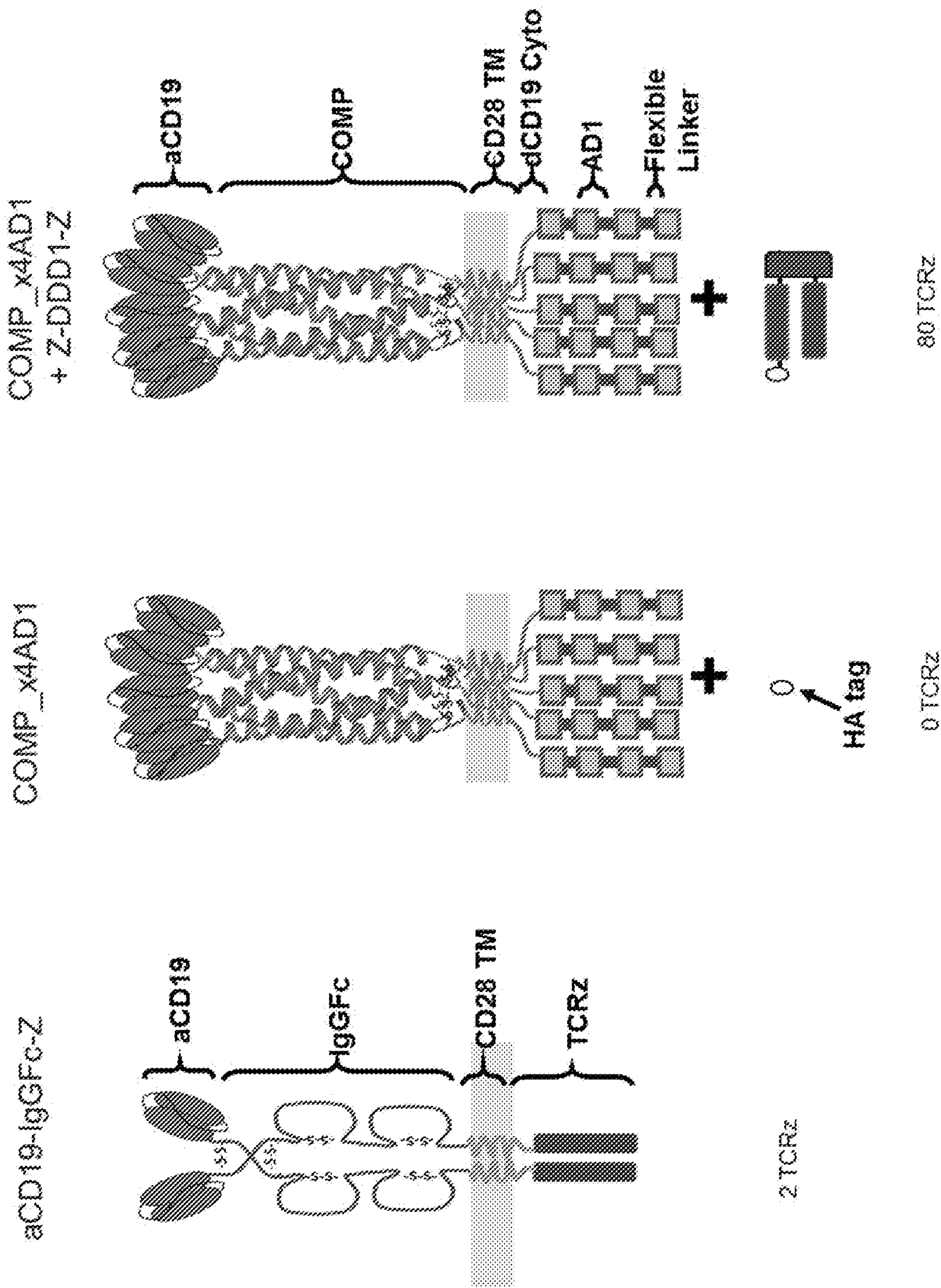
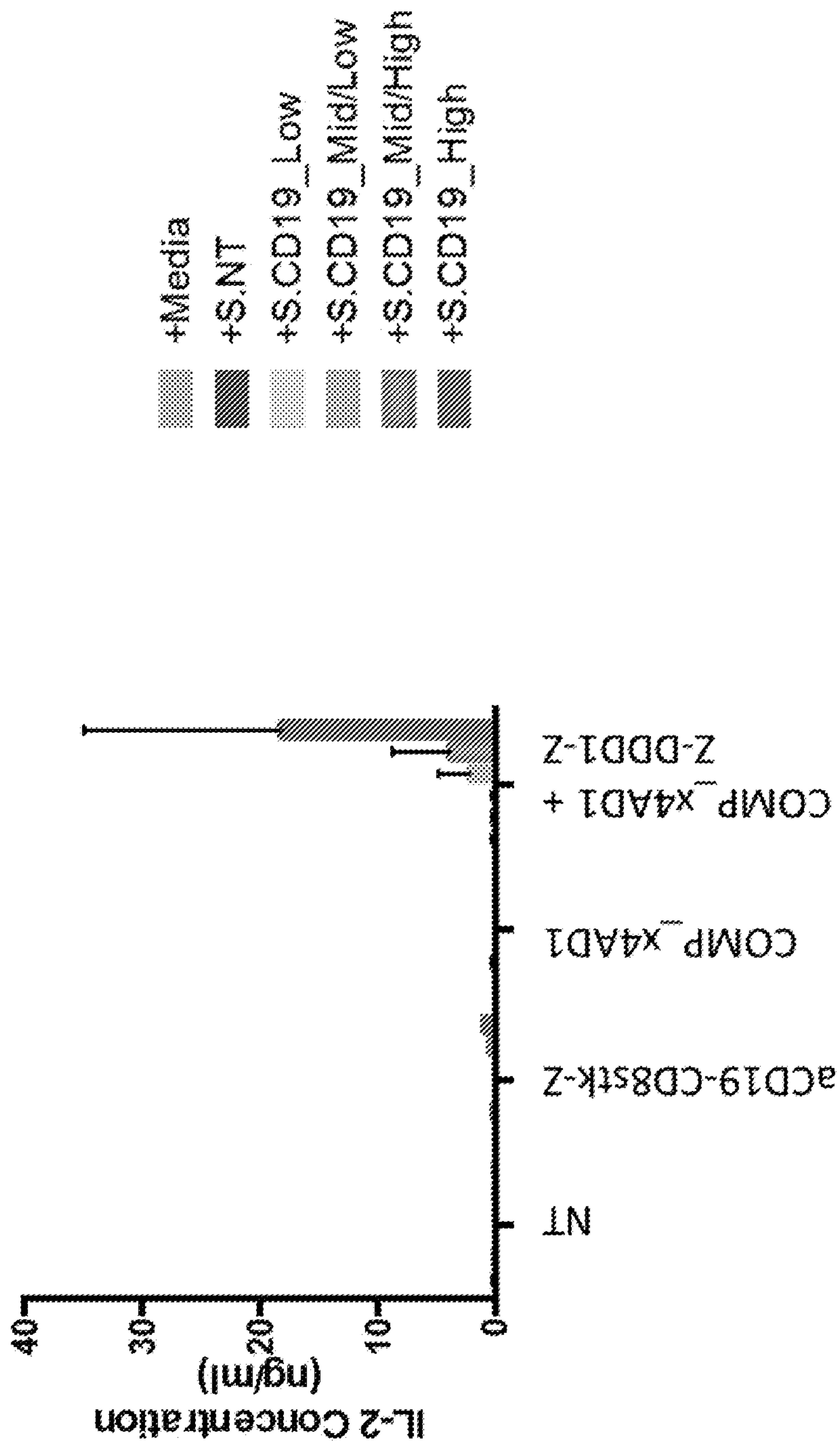


FIGURE 16



+Media
+S.NT
+S.CD19_Low
+S.CD19_Mid/Low
+S.CD19_Mid/High
+S.CD19_High