(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
06 September 2019 (06.09.2019)

WIPO | PCT

(10) International Publication Number
WO 2019/166453 A1

(51) International Patent Classification:
G01N 33/566 (2006.01) *C07K 16/46* (2006.01)
C07K 14/725 (2006.01) *G01N 33/577* (2006.01)

(21) International Application Number:
PCT/EP2019/054786

(22) International Filing Date:
27 February 2019 (27.02.2019)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
18159401.1 01 March 2018 (01.03.2018) EP

(71) Applicant *(for all designated States except US)*: **F. HOFFMANN-LA ROCHE AG** [CH/CH]; Grenzacherstrasse 124, 4070 Basel (CH).

(71) Applicant *(for US only)*: **HOFFMANN-LA ROCHE INC.** [US/US]; Overlook at Great Notch, 150 Clove Road 8th Floor, Suite 8, Legal Department, Little Falls, New Jersey 07424 (US).

(72) Inventors: **BRINKMANN, Ulrich**; c/o Roche Diagnostics GmbH, Nonnenwald 2, 82377 Penzberg (DE). **DAROWSKI, Diana**; c/o Roche Glycart AG, Wagistrasse 10, 8952 Schlieren (CH). **DICKOPF, Steffen**; c/o Roche Diagnostics GmbH, Nonnenwald 2, 82377 Penzberg (DE). **JOST, Christian**; c/o Roche Glycart AG, Wagistrasse 10, 8952 Schlieren (CH). **KLEIN, Christian**; c/o Roche Glycart AG, Wagistrasse 10, 8952 Schlieren (CH).

(74) Agent: **BRODBECK, Michel**; F. Hoffmann-La Roche AG, Grenzacherstrasse 124, 4070 Basel (CH).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JO, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Declarations under Rule 4.17:
— *of inventorship (Rule 4.17(iv))*

Published:
— *with international search report (Art. 21(3))*
— *with sequence listing part of description (Rule 5.2(a))*

WO 2019/166453 A1

(54) Title: SPECIFICITY ASSAY FOR NOVEL TARGET ANTIGEN BINDING MOIETIES

(57) Abstract: The present invention generally relates to specificity assays using cell cultures, in particular to chimeric antigen receptor (CAR) expressing reporter T (CAR-T) cell assays to test novel target antigen binding moieties in different formats. Furthermore, the present invention relates to the use of reporter CAR-T cells, transfected/transduced with an engineered CAR capable of specific binding to a recognition domain comprising a tag.

Specificity assay for novel target antigen binding moieties

FIELD OF THE INVENTION

The present invention generally relates to specificity assays using cell cultures, in particular to chimeric antigen receptor (CAR) expressing reporter T (CAR-T) cell assays to test novel target antigen binding moieties in different formats. Furthermore, the present invention relates to the use of reporter CAR-T cells, transfected/transduced with an engineered CAR capable of specific binding to a recognition domain comprising a tag.

BACKGROUND

Over the last 15 years, antibody based therapies have evolved and represent now a valuable combination or alternative to chemotherapeutic approaches in the treatment of hematological malignancies and solid tumors. Unlike chemotherapy, antibody therapies target specific antigens on cancer cells thus allowing a more site-directed treatment thereby reducing the side effects on healthy tissue. In the process of developing an antibody-based therapeutic reagent, various assays are required to identify the best candidates to bring into clinical trials and eventually to the market. In a first early preclinical phase, the antibodies have to be generated and analyzed for their target-specificity, as well as their affinity to the target.

Binding properties can be analyzed using various protein-protein interaction assays, such as FRET-based methods, Surface Plasmon Resonance (SPR) or fluorescence-activated cell sorting (FACS). However, available assay formats might not always reproduce the *in vivo* situation comprehensively and integrative. For example targeting of cancer cells with therapeutic antibodies binding to cell surface receptors can have impacts on multiple levels, e.g., intracellular signaling via the binding and cross-linking of surface molecules as well as marking the tumor cells to engage immune cells. Furthermore, the recognition cascade from antigen binding to establishing of an effector function, e.g., T cell cytotoxicity, requires a well-orchestrated sequence of cell surface interactions, wherein binding affinity of an antigen binding moiety is one among several factors. Plain protein-protein affinity interaction assays may therefore not provide the complete picture, although these assays are a very valuable tool for early candidate development.

Still, there remains a need to develop binding assays which do provide meaningful predictions for the *in vivo* interactions in a more comprehensive setup minimizing non-specific effects on target-antibody binding as far as possible.

The inventors of the present invention developed a novel assay which is applicable to a wide variety of different cancer cell types to assess binding of antibodies to their target. The innovative assay includes modified T-cells as reporter cells combining straight-forward readout with a comprehensive and inclusive result.

Furthermore, the present invention provides assays which combine the assessment of binding and functionality of antibodies and antibody-like constructs (e.g., ligands). The novel assay is useful for example for screening or characterization purposes of therapeutic antibody drug candidates, i.e., in high-throughput formats.

This new assay represents a valuable tool for early and late stage screening and characterization of antibody binding to the native target and assessing functionality which will allow identifying the best binders in the development of the drug candidate.

SUMMARY OF THE INVENTION

The present invention generally relates to a method for assessing and selecting novel antigen binding moieties, particularly in the drug development process, and combines the determination of binding to a target antigen, e.g., on a tumor cell, with the activation of T cells in response to the antibody-target binding. Herein provided is a method for assessing the specificity of a target antigen binding moiety capable of specific binding to a target antigen, the method comprising the steps of:

a) providing an antigen binding molecule comprising an antigen binding domain and a recognition domain, wherein the antigen binding domain comprises the target antigen binding moiety, and wherein the recognition domain comprises a tag;
b) contacting the antigen binding molecule with a target cell comprising the target antigen on the surface, particularly wherein the target cell is a cancer cell;
c) contacting the antigen binding molecule with a chimeric antigen receptor (CAR) expressing reporter T (CAR-T) cell wherein the reporter CAR-T cell comprises:
    i. a CAR capable of specific binding to the recognition domain comprising the tag, wherein the CAR is operationally coupled to a response element;
    ii. a reporter gene under the control of the response element; and
d) determining T cell activation by measuring the expression of the reporter gene to establish the specificity of the target antigen binding moiety.

In one embodiment, the antigen binding molecule is an IgG class antibody, particularly an IgG1 or IgG4 isotype antibody, or a fragment thereof.

In one embodiment, the antigen binding domain is a Fab fragment and the recognition domain is an Fc domain.

In one embodiment, the antigen binding domain and the recognition domain are the same domain, in particular a Fab fragment.

In one embodiment, the tag is a hapten molecule.

In one embodiment, the hapten molecule is Digoxigenin (DIG).

In one embodiment, the tag is a polypeptide tag.

In one embodiment, the polypeptide tag is selected from the group consisting of myc-tag, HA-tag, AviTag, FLAG-tag, His-tag, GCN4-tag and NE-tag.

In one embodiment, the target antigen binding moiety is a Fab fragment, in particular a Fab fragment deriving from a phage display library screening.

In one embodiment, binding of the target antigen binding moiety to the target antigen and binding of the reporter CAR-T cell to the antigen binding molecule comprising the target antigen binding moiety leads to expression of the reporter gene.

In one embodiment, the target antigen is a cell surface antigen or receptor.

In one embodiment, the target antigen is a peptide bound to a molecule of the human major histocompatibility complex (MHC).

In one embodiment, the target antigen binding moiety is a T cell receptor like (TCRL) antigen binding moiety.

In one embodiment, provided is a chimeric antigen receptor (CAR) comprising an anchoring transmembrane domain and an extracellular domain comprising an antigen binding moiety, wherein the antigen binding moiety is capable of specific binding to a recognition domain comprising a tag but not capable of specific binding to the recognition domain not comprising the tag.

In one embodiment, the antigen binding moiety is a scFv, a Fab, a crossFab or a scFab, in particular a Fab or a crossFab.

In one embodiment, the tag is a hapten.

In one embodiment, the hapten molecule is Digoxigenin (DIG).

In one embodiment, the tag is a polypeptide tag.

In one embodiment, the polypeptide tag is selected from the group consisting of myc-tag, HA-tag, AviTag, FLAG-tag, His-tag, GCN4-tag and NE-tag.

SHORT DESCRIPTION OF THE FIGURES

Figure 1 depicts the architecture of exemplary antigen binding receptors (CARs) used according to the invention. Figure 1A shows the architecture of the scFv format. The antigen binding moiety capable of specific binding to the recognition domain consists of a variable heavy (VH) and a variable light (VL) chain. Attached to the VL chain, a Gly4Ser linker connects the antigen recognition domain with the CD28 transmembrane domain (TM) which is fused to the intracellular costimulatory signaling domain (CSD) of CD28 which in turn is fused to the stimulatory signaling domain (SSD) of CD3z. Figures 1B and 1C show the architecture of the Fab (Figure 1B) and crossFab (Figure 1C) formats. The antigen binding moiety consists of an Ig heavy chain and an Ig light chain. Attached to the heavy chain, a Gly4Ser linker connects the antigen recognition domain with the CD28 transmembrane domain which is fused to the intracellular co-stimulatory signaling domain of CD28 which in turn is fused to the stimulatory signaling domain of CD3z.

Figure 2 depicts a schematic representation illustrating the modular composition of exemplary expression constructs CARs used according to the invention. Figure 2A depicts the scFv format. Figure 2B depicts the Fab format. Figure 2C depicts a crossFab format.

Figure 3 depicts the structural formula of the Digoxigenin (DIG) molecule.

Figure 4 depicts an exemplary digoxigeninylated IgG1 molecule which can be specifically recognized by an anti-Digoxigenin CAR.

Figure 5 depicts alternative digoxigenylated antigen binding molecules which are recognized by an anti-Digoxigenin CAR. In this embodiment, the target antigen binding domain and the recognition domain are the same domain, i.e., the Digoxigenin hapten tag is coupled to the antigen binding domain wherein the antigen binding domain exerts also the function of the recognition domain. Figure 5A depicts an digoxigeninylated Fab molecule which can be recognized by an anti-Digoxigenin CAR. Figure 5B depicts an digoxigenylated scFv molecule which can be recognized by an anti-Digoxigenin CAR.

Figure 6 depicts a Western Blot confirming successful digoxigenylation of the anti-CD20 targeting antibody GA101. Digoxigeninylation was detected by anti-Digoxigenin-AP Fab fragments by Western Blot analysis.

Figure 7 depicts surface detection of anti-Digoxigenin-ds-scFv on Jurkat NFAT reporter cells.

Figure 8 depicts a schematic representation of a Jurkat NFAT reporter CAR-T cell assay. The target antigen bound IgG which is digoxigeninylated at the Fc (recognition domain) can be recognized by the anti-Digoxigenin CAR expressing Jurkat NFAT reporter T cell. This

recognition leads to the activation of the cell which can be detected by measuring luciferase luminescence (CPS/RLU).

Figure 9 depicts the Jurkat NFAT reporter CAR-T cell assay using CD20 expressing SUDHDL4 tumor cells as target cells and an anti-CD20 IgG antibody (GA101) digoxigeninylated with a ten times molar excess of Digoxigenin-3-O-methylcarbonyl-e-aminocaproic acid-N-hydroxysuccinimide ester. The antibody recognizes on the one hand the tumor associated antigen and on the other hand is recognized by Jurkat NFAT reporter CAR-T cells. A sorted pool of anti-Digoxigenin-ds-scFv-CD28ATDCD28CSD-CD3zSSD expressing Jurkat NFAT reporter CAR-T cells was used as effector cells.

Figure 10 depicts activation of anti-Digoxigenin-ds-scFv-CD28ATDCD28CSD-CD3zSSD expressing Jurkat NFAT reporter CAR-T cells. Activation is dependent on an anti-CD20 IgG antibody (GA101), coupled with different amounts of digoxigeninylated molecules.

Figure 11 depicts the Jurkat NFAT reporter CAR-T cell assay using CD20 expressing SUDHDL4 tumor cells as target cells. An anti-CD20 IgG antibody (GA101) digoxygeninylated at the Fc with approximately one Digoxigenin (equimolar Dig-NHS:antibody ratio) molecule on average was used. The antibody recognizes on the one hand the tumor associated antigen and on the other hand is recognized by Jurkat NFAT reporter CAR-T cells. A sorted pool of anti-Digoxigenin-ds-scFv-CD28ATDCD28CSD-CD3zSSD expressing Jurkat NFAT reporter CAR-T cells was used as effector cells.

Figure 12 depicts a schematic representation of an alternative Jurkat NFAT reporter CAR-T cell assay using a bridging Biotin-Digoxigenin adapter. The Biotin-Digoxigenin adapter bound to the Fc domain via a Biotin binding moiety forms the recognition domain in this setup. The Digoxigenin moiety can be recognized by the anti-Digoxigenin CAR expressing Jurkat NFAT reporter CAR-T cell.

Figure 13 depicts the Jurkat NFAT reporter CAR-T cell assay using MCF7 cells as target cells. An anti-LeY/Biotin antibody and a bridging Biotin-Digoxigenin adapter was used. The antibody recognizes on one hand the tumor associated antigen (LeY) and on the other hand the Biotin of the adapter molecule. The adapter-bound Digoxigenin is recognized by the Jurkat NFAT reporter CAR-T cells. A sorted pool of anti-Digoxigenin-ds-scFv-CD28ATDCD28CSD-CD3zSSD expressing Jurkat NFAT reporter CAR-T cells was used as effector cells. As negative control a non-targeting Biotin-coupled anti-CD33 antibody was used.

DETAILED DESCRIPTION

Definitions

"Affinity" refers to the strength of the sum total of non-covalent interactions between a single binding site of a molecule (e.g., an antibody or a CAR) and its binding partner (e.g., a ligand). Unless indicated otherwise, as used herein, "binding affinity" refers to intrinsic binding affinity which reflects a 1:1 interaction between members of a binding pair (e.g., an antigen binding moiety and an antigen and/or a receptor and its ligand). The affinity of a molecule X for its partner Y can generally be represented by the dissociation constant (K_D), which is the ratio of dissociation and association rate constants (k_{off} and k_{on}, respectively). Thus, equivalent affinities may comprise different rate constants, as long as the ratio of the rate constants remains the same. Affinity can be measured by well-established methods known in the art, including those described herein. A preferred method for measuring affinity is Surface Plasmon Resonance (SPR) and a preferred temperature for the measurement is 25°C.

The term "amino acid" ("aa") refers to naturally occurring and synthetic amino acids, as well as amino acid analogs and amino acid mimetics that function in a manner similar to the naturally occurring amino acids. Naturally occurring amino acids are those encoded by the genetic code, as well as those amino acids that are later modified, e.g., hydroxyproline, γ-carboxyglutamate, and O-phosphoserine. Amino acid analogs refer to compounds that have the same basic chemical structure as a naturally occurring amino acid, i.e., an α carbon that is bound to a hydrogen, a carboxyl group, an amino group, and an R group, e.g., homoserine, norleucine, methionine sulfoxide, methionine methyl sulfonium. Such analogs have modified R groups (e.g., norleucine) or modified peptide backbones, but retain the same basic chemical structure as a naturally occurring amino acid. Amino acid mimetics refers to chemical compounds that have a structure that is different from the general chemical structure of an amino acid, but that function in a manner similar to a naturally occurring amino acid. Amino acids may be referred to herein by either their commonly known three letter symbols or by the one-letter symbols recommended by the IUPAC-IUB Biochemical Nomenclature Commission.

The term "amino acid mutation" as used herein is meant to encompass amino acid substitutions, deletions, insertions, and modifications. Any combination of substitution, deletion, insertion, and modification can be made to arrive at the final construct, provided that the final construct possesses the desired characteristics. Amino acid sequence deletions and insertions include amino- and/or carboxy-terminal deletions and insertions of amino acids. Particular amino acid mutations are amino acid substitutions. Amino acid substitutions

include replacement by non-naturally occurring amino acids or by naturally occurring amino acid derivatives of the twenty standard amino acids (e.g., 4-hydroxyproline, 3-methylhistidine, ornithine, homoserine, 5-hydroxylysine). Amino acid mutations can be generated using genetic or chemical methods well known in the art. Genetic methods may include site-directed mutagenesis, PCR, gene synthesis and the like. It is contemplated that methods of altering the side chain group of an amino acid by methods other than genetic engineering, such as chemical modification, may also be useful.

The term "antibody" herein is used in the broadest sense and encompasses various antibody structures, including but not limited to monoclonal antibodies, polyclonal antibodies, and antibody fragments so long as they exhibit the desired antigen-binding activity. Accordingly, in the context of the present invention, the term antibody relates to full immunoglobulin molecules as well as to parts of such immunoglobulin molecules. Furthermore, the term relates, as discussed herein, to modified and/or altered antibody molecules, in particular to modified antibody molecules. The term also relates to recombinantly or synthetically generated/synthesized antibodies. In the context of the present invention the term antibody is used interchangeably with the term immunoglobulin.

An "antibody fragment" refers to a molecule other than an intact antibody that comprises a portion of an intact antibody that binds the antigen to which the intact antibody binds. Examples of antibody fragments include but are not limited to Fv, Fab, crossover Fab, Fab', Fab'-SH, $F(ab')_2$, diabodies, linear antibodies, single-domain antibodies, single-chain antibody molecules (e.g., scFv, scFab), and single-domain antibodies. For a review of certain antibody fragments, see Hudson et al., Nat Med 9, 129-134 (2003). For a review of scFv fragments, see e.g., Plückthun, in The Pharmacology of Monoclonal Antibodies, vol. 113, Rosenburg and Moore eds., Springer-Verlag, New York, pp. 269-315 (1994); see also WO 93/16185; and U.S. Patent Nos. 5,571,894 and 5,587,458. Diabodies are antibody fragments with two antigen-binding sites that may be bivalent or bispecific. See, for example, EP 404,097; WO 1993/01161; Hudson et al., Nat Med 9, 129-134 (2003); and Hollinger et al., Proc Natl Acad Sci USA 90, 6444-6448 (1993). Triabodies and tetrabodies are also described in Hudson et al., Nat Med 9, 129-134 (2003). Single-domain antibodies are antibody fragments comprising all or a portion of the heavy chain variable domain or all or a portion of the light chain variable domain of an antibody (Domantis, Inc., Waltham, MA; see e.g., U.S. Patent No. 6,248,516 B1). Antibody fragments can be made by various techniques, including but not limited to proteolytic digestion of an intact antibody as well as production by recombinant host cells (e.g., E. coli or phage), as described herein.

As used herein, the term "antigen binding molecule" refers in its broadest sense to a molecule that specifically binds an antigenic determinant. Examples of antigen binding molecules are antibodies/immunoglobulins and derivatives, e.g., fragments, thereof. Furthermore, the term relates, as discussed herein, to modified and/or altered antigen binding molecules, in particular to modified antibody molecules. The term also relates to recombinantly or synthetically generated/synthesized antibodies. In the context of the present invention the antigen binding molecule is preferably an antibody or fragment thereof.

As used herein, the term "antigen binding moiety" refers to a polypeptide molecule that specifically binds to an antigenic determinant. In one embodiment, an antigen binding moiety is able to direct the entity to which it is attached (e.g., an immunoglobulin or a CAR) to a target site, for example to a specific type of tumor cell or tumor stroma bearing the antigenic determinant or to an immunoglobulin binding to the antigenic determinant on a tumor cell. In another embodiment an antigen binding moiety is able to activate signaling through its target antigen, for example signaling is activated upon binding of an antigenic determinant to a CAR on a T cell. In the context of the present invention, antigen binding moieties may be included in antibodies and fragments thereof as well as in antigen binding receptors (e.g., CARs) and fragments thereof as further defined herein. Antigen binding moieties include an antigen binding domain, e.g., comprising an immunoglobulin heavy chain variable region and an immunoglobulin light chain variable region.

In the context of the present invention the term "antigen binding receptor" relates to an molecule comprising an anchoring transmembrane domain and an extracellular domain comprising at least one antigen binding moiety. An antigen binding receptor (e.g., a CAR) can be made of polypeptide parts from different sources. Accordingly, it may be also understood as a "fusion protein" and/or a "chimeric protein". Usually, fusion proteins are proteins created through the joining of two or more genes (or preferably cDNAs) that originally coded for separate proteins. Translation of this fusion gene (or fusion cDNA) results in a single polypeptide, preferably with functional properties derived from each of the original proteins. Recombinant fusion proteins are created artificially by recombinant DNA technology for use in biological research or therapeutics. In the context of the present invention a CAR (chimeric antigen receptor) is understood to be an antigen binding receptor comprising an extracellular portion comprising an antigen binding moiety fused by a spacer sequence to an anchoring transmembrane domain which is itself fused to the intracellular signaling domains of e.g., CD3z and CD28.

An "antigen binding site" refers to the site, i.e., one or more amino acid residues, of an antigen binding molecule which provides interaction with the antigen. A native immunoglobulin molecule typically has two antigen binding sites, a Fab or a scFv molecule typically has a single antigen binding site.

The term "antigen binding domain" refers to the part of an antibody or an antigen binding receptor (e.g., a CAR) that comprises the area which specifically binds to and is complementary to part or all of an antigen. An antigen binding domain may be provided by, for example, one or more immunoglobulin variable domains (also called variable regions). Particularly, an antigen binding domain comprises an immunoglobulin light chain variable region (VL) and an immunoglobulin heavy chain variable region (VH).

The term "variable region" or "variable domain" refers to the domain of an immunoglobulin heavy or light chain that is involved in binding the antigen. The variable domains of the heavy chain and light chain (VH and VL, respectively) of a native antibody generally have similar structures, with each domain comprising four conserved framework regions (FRs) and three hypervariable regions (HVRs). See, e.g., Kindt et al., Kuby Immunology, 6th ed., W.H. Freeman and Co, page 91 (2007). A single VH or VL domain is usually sufficient to confer antigen-binding specificity.

The term "ATD" as used herein refers to "anchoring transmembrane domain" which defines a polypeptide stretch capable of integrating in (the) cellular membrane(s) of a cell. The ATD can be fused to further extracellular and/or intracellular polypeptide domains wherein these extracellular and/or intracellular polypeptide domains will be confined to the cell membrane as well. In the context of the antigen binding receptors as used in the present invention the ATD confers membrane attachment and confinement of the antigen binding receptor, e.g., a CAR used according to the present invention.

The term "binding to" as used in the context of the antigen binding receptors (e.g., CARs) used according to the present invention defines a binding (interaction) of an "antigen-interaction-site" and an antigen with each other. The term "antigen-interaction-site" defines a motif of a polypeptide which shows the capacity of specific interaction with a specific antigen or a specific group of antigens. Said binding/interaction is also understood to define a "specific recognition". The term "specifically recognizing" means in accordance with this invention that the antigen binding receptor is capable of specifically interacting with and/or binding to the recognition domain, i.e., a modified molecule as defined herein whereas the non-modified molecule is not recognized. The antigen binding moiety of an antigen binding receptor (e.g., a CAR) can recognize, interact and/or bind to different epitopes on the same

molecule. This term relates to the specificity of the antigen binding receptor, i.e., to its ability to discriminate between the specific regions of a modified molecule, i.e., a modified Fc domain, as defined herein. The specific interaction of the antigen-interaction-site with its specific antigen may result in an initiation of a signal, e.g., due to the induction of a change of the conformation of the polypeptide comprising the antigen, an oligomerization of the polypeptide comprising the antigen, an oligomerization of the antigen binding receptor, etc. Thus, a specific motif in the amino acid sequence of the antigen-interaction-site and the antigen bind to each other as a result of their primary, secondary or tertiary structure as well as the result of secondary modifications of said structure. The term binding to does not only relate to a linear epitope but may also relate to a conformational epitope, a structural epitope or a discontinuous epitope consisting of two regions of the target molecules or parts thereof. In the context of this invention, a conformational epitope is defined by two or more discrete amino acid sequences separated in the primary sequence which comes together on the surface of the molecule when the polypeptide folds to the native protein (Sela, Science 166 (1969), 1365 and Laver, Cell 61 (1990), 553-536). Moreover, the term "binding to" is interchangeably used in the context of the present invention with the term "interacting with". The ability of the antigen binding moiety (e.g., a Fab or scFv domain) of a CAR or an antibody to bind to a specific target antigenic determinant can be measured either through an enzyme-linked immunosorbent assay (ELISA) or other techniques familiar to one of skill in the art, e.g., surface plasmon resonance (SPR) technique (analyzed on a BIAcore instrument) (Liljeblad et al., Glyco J 17, 323-329 (2000)), and traditional binding assays (Heeley, Endocr Res 28, 217-229 (2002)). In one embodiment, the extent of binding of an antigen binding moiety to an unrelated protein is less than about 10% of the binding of the antigen binding moiety to the target antigen as measured, in particular by SPR. In certain embodiments, an antigen binding moiety that binds to the target antigen, has a dissociation constant (K_D) of $\leq$ 1 μM, $\leq$ 100 nM, $\leq$ 10 nM, $\leq$ 1 nM, $\leq$ 0.1 nM, $\leq$ 0.01 nM, or $\leq$ 0.001 nM (e.g., 10^{-8} M or less, e.g., from 10^{-8} M to 10^{-13} M, e.g., from 10^{-9} M to 10^{-13} M). The term "specific binding" as used in accordance with the present invention means that the molecules used in the invention do not or do not essentially cross-react with (poly-) peptides of similar structures, i.e., with a non-modified Fc domain. Accordingly, the antigen binding receptor (e.g., the CAR) used according to the invention specifically binds to/interacts with a recognition domain, e.g., an Fc domain, preferably a modified Fc domain. Cross-reactivity of a panel of constructs under investigation may be tested, for example, by assessing binding of a panel of antigen binding moieties under conventional conditions (see, e.g., Harlow and Lane, Antibodies: A Laboratory Manual, Cold

Spring Harbor Laboratory Press, (1988) and Using Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory Press, (1999)) to the recognition domain of interest, e.g., a modified Fc domain as well as to parent non-modified Fc domain. Only those constructs (i.e., Fab fragments, scFvs and the like) that bind to the domain of interest but do not or do not essentially bind to structurally closely related domain, e.g., a non-modified Fc domain, are considered specific for the recognition domain of interest and selected for further studies in accordance with the method provided herein. These methods may comprise, inter alia, binding studies, blocking and competition studies with structurally and/or functionally closely related domains. The binding studies also comprise FACS analysis, surface plasmon resonance (SPR, e.g., with BIAcore□), analytical ultracentrifugation, isothermal titration calorimetry, fluorescence anisotropy, fluorescence spectroscopy or by radiolabeled ligand binding assays.

The term "CDR" as employed herein relates to "complementary determining region", which is well known in the art. The CDRs are parts of immunoglobulins or antigen binding receptors that determine the specificity of said molecules and make contact with a specific ligand. The CDRs are the most variable part of the molecule and contribute to the antigen binding diversity of these molecules. There are three CDR regions CDR1, CDR2 and CDR3 in each V domain. CDR-H depicts a CDR region of a variable heavy chain and CDR-L relates to a CDR region of a variable light chain. VH means the variable heavy chain and VL means the variable light chain. The CDR regions of an Ig-derived region may be determined as described in "Kabat" (Sequences of Proteins of Immunological Interest", 5th edit. NIH Publication no. 91-3242 U.S. Department of Health and Human Services (1991); Chothia J. Mol. Biol. 196 (1987), 901-917) or "Chothia" (Nature 342 (1989), 877-883).

The term " CD3z" refers to T-cell surface glycoprotein CD3 zeta chain, also known as "T-cell receptor T3 zeta chain" and "CD247".

The term "chimeric antigen receptor" or "chimeric receptor" or "CAR" refers to an antigen binding receptor constituted of an extracellular portion of an antigen binding moiety (e.g., a scFv or a Fab) fused by a spacer sequence to intracellular signaling domains (e.g., of CD3z and CD28).

The "class" of an antibody or immunoglobulin refers to the type of constant domain or constant region possessed by its heavy chain. There are five major classes of antibodies: IgA, IgD, IgE, IgG, and IgM, and several of these may be further divided into subclasses (isotypes), e.g., IgG_1, IgG_2, IgG_3, IgG_4, IgA_1, and IgA_2. The heavy chain constant domains that correspond to the different classes of immunoglobulins are called α, δ, ε, γ, and μ, respectively.

By a "crossover Fab molecule" (also termed "crossFab" or "crossover Fab fragment") is meant a Fab molecule wherein either the variable regions or the constant regions of the Fab heavy and light chain are exchanged, i.e., the crossFab fragment comprises a peptide chain composed of the light chain variable region and the heavy chain constant region, and a peptide chain composed of the heavy chain variable region and the light chain constant region. For clarity, in a crossFab fragment wherein the variable regions of the Fab light chain and the Fab heavy chain are exchanged, the peptide chain comprising the heavy chain constant region is referred to herein as the heavy chain of the crossover Fab molecule. Conversely, in a crossFab fragment wherein the constant regions of the Fab light chain and the Fab heavy chain are exchanged, the peptide chain comprising the heavy chain variable region is referred to herein as the heavy chain of the crossFab fragment. Accordingly, a crossFab fragment comprises a heavy or light chain composed of the heavy chain variable and the light chain constant regions (VH-CL), and a heavy or light chain composed of the light chain variable and the heavy chain constant regions (VL-CH1). In contrast thereto, by a "Fab" or "conventional Fab molecule" is meant a Fab molecule in its natural format, i.e., comprising a heavy chain composed of the heavy chain variable and constant regions (VH-CH1), and a light chain composed of the light chain variable and constant regions (VL-CL).

The term "CSD" as used herein refers to co-stimulatory signaling domain.

The term "effector functions" refers to those biological activities attributable to the Fc region of an antibody, which vary with the antibody isotype. Examples of antibody effector functions include: C1q binding and complement dependent cytotoxicity (CDC), Fc receptor binding, antibody-dependent cell-mediated cytotoxicity (ADCC), antibody-dependent cellular phagocytosis (ADCP), cytokine secretion, immune complex-mediated antigen uptake by antigen presenting cells, down regulation of cell surface receptors (e.g., B cell receptor), and B cell activation.

As used herein, the terms "engineer", "engineered", "engineering", are considered to include any manipulation of the peptide backbone or the post-translational modifications of a naturally occurring or recombinant polypeptide or fragment thereof. Engineering includes modifications of the amino acid sequence, of the glycosylation pattern, or of the side chain group of individual amino acids, as well as combinations of these approaches.

The term "expression cassette" refers to a polynucleotide generated recombinantly or synthetically, with a series of specified nucleic acid elements that permit transcription of a particular nucleic acid in a target cell. The recombinant expression cassette can be incorporated into a plasmid, chromosome, mitochondrial DNA, plastid DNA, virus, or nucleic

acid fragment. Typically, the recombinant expression cassette portion of an expression vector includes, among other sequences, a nucleic acid sequence to be transcribed and a promoter.

A "Fab molecule" refers to a protein consisting of the VH and CH1 domain of the heavy chain (the "Fab heavy chain") and the VL and CL domain of the light chain (the "Fab light chain") of an antigen binding molecule.

The term "Fc domain" or "Fc region" herein is used to define a C-terminal region of an immunoglobulin heavy chain that contains at least a portion of the constant region. The term includes native sequence Fc regions and variant Fc regions. Although the boundaries of the Fc region of an IgG heavy chain might vary slightly, the human IgG heavy chain Fc region is usually defined to extend from Cys226, or from Pro230, to the carboxyl-terminus of the heavy chain. However, the C-terminal lysine (Lys447) of the Fc region may or may not be present. Unless otherwise specified herein, numbering of amino acid residues in the Fc region or constant region is according to the "EU numbering" system, also called the EU index, as described in Kabat et al., Sequences of Proteins of Immunological Interest, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, MD, 1991. A subunit of an Fc domain as used herein refers to one of the two polypeptides forming the dimeric Fc domain, i.e., a polypeptide comprising C-terminal constant regions of an immunoglobulin heavy chain, capable of stable self-association. For example, a subunit of an IgG Fc domain comprises an IgG CH2 and an IgG CH3 constant domain.

"Framework" or "FR" refers to variable domain residues other than hypervariable region (HVR) residues. The FR of a variable domain generally consists of four FR domains: FR1, FR2, FR3, and FR4. Accordingly, the HVR and FR sequences generally appear in the following sequence in VH (or VL): FR1-H1(L1)-FR2-H2(L2)-FR3-H3(L3)-FR4.

The term "full length antibody" denotes an antibody consisting of two "full length antibody heavy chains" and two "full length antibody light chains". A "full length antibody heavy chain" is a polypeptide consisting in N-terminal to C-terminal direction of an antibody heavy chain variable domain (VH), an antibody constant heavy chain domain 1 (CH1), an antibody hinge region (HR), an antibody heavy chain constant domain 2 (CH2), and an antibody heavy chain constant domain 3 (CH3), abbreviated as VH-CH1-HR-CH2-CH3; and optionally an antibody heavy chain constant domain 4 (CH4) in case of an antibody of the subclass IgE. Preferably the "full length antibody heavy chain" is a polypeptide consisting in N-terminal to C-terminal direction of VH, CH1, HR, CH2 and CH3. A "full length antibody light chain" is a polypeptide consisting in N-terminal to C-terminal direction of an antibody light chain variable domain (VL), and an antibody light chain constant domain (CL), abbreviated as VL-

CL. The antibody light chain constant domain (CL) can be κ (kappa) or λ (lambda). The two full length antibody chains are linked together via inter-polypeptide disulfide bonds between the CL domain and the CH1 domain and between the hinge regions of the full length antibody heavy chains. Examples of typical full length antibodies are natural antibodies like IgG (e.g., IgG 1 and IgG2), IgM, IgA, IgD, and IgE.) The full length antibodies used according to the invention can be from a single species e.g., human, or they can be chimerized or humanized antibodies. In some embodiments, the full length antibodies used according to the invention, comprise two antigen binding sites each formed by a pair of VH and VL, which both specifically bind to the same antigen. In further embodiments, the full length antibodies used according to the invention comprise two antigen binding sites each formed by a pair of VH and VL, wherein the two antigen binding sites bind to different antigens, e.g., wherein the antibodies are bispecific. The C-terminus of the heavy or light chain of said full length antibody denotes the last amino acid at the C-terminus of said heavy or light chain.

By "fused" is meant that the components (e.g., a Fab and a transmembrane domain) are linked by peptide bonds, either directly or via one or more peptide linkers.

The terms "host cell", "host cell line" and "host cell culture" are used interchangeably and refer to cells into which exogenous nucleic acid has been introduced, including the progeny of such cells. Host cells include "transformants" and "transformed cells" which include the primary transformed cell and progeny derived therefrom without regard to the number of passages. Progeny may not be completely identical in nucleic acid content to a parent cell, but may contain mutations. Mutant progeny that have the same function or biological activity as screened or selected for in the originally transformed cell are included herein. A host cell is any type of cellular system that can be used to generate an antibody used according to the present invention. Host cells include cultured cells, e.g., mammalian cultured cells, such as CHO cells, BHK cells, NS0 cells, SP2/0 cells, YO myeloma cells, P3X63 mouse myeloma cells, PER cells, PER.C6 cells or hybridoma cells, yeast cells, insect cells, and plant cells, to name only a few, but also cells comprised within a transgenic animal, transgenic plant or cultured plant or animal tissue.

The term "hypervariable region" or "HVR", as used herein, refers to each of the regions of an antibody variable domain which are hypervariable in sequence and/or form structurally defined loops ("hypervariable loops"). Generally, native four-chain antibodies comprise six HVRs; three in the VH (H1, H2, H3), and three in the VL (L1, L2, L3). HVRs generally comprise amino acid residues from the hypervariable loops and/or from the complementarity determining regions (CDRs), the latter being of highest sequence variability and/or involved

in antigen recognition. With the exception of CDR1 in VH, CDRs generally comprise the amino acid residues that form the hypervariable loops. Hypervariable regions (HVRs) are also referred to as complementarity determining regions (CDRs), and these terms are used herein interchangeably in reference to portions of the variable region that form the antigen binding regions. This particular region has been described by Kabat et al., U.S. Dept. of Health and Human Services, Sequences of Proteins of Immunological Interest (1983) and by Chothia et al., J Mol Biol 196:901-917 (1987), where the definitions include overlapping or subsets of amino acid residues when compared against each other. Nevertheless, application of either definition to refer to a CDR of an antibody and/or an antigen binding receptor or variants thereof is intended to be within the scope of the term as defined and used herein. The appropriate amino acid residues which encompass the CDRs as defined by each of the above cited references are set forth below in Table 1 as a comparison. The exact residue numbers which encompass a particular CDR will vary depending on the sequence and size of the CDR. Those skilled in the art can routinely determine which residues comprise a particular CDR given the variable region amino acid sequence of the antibody.

Table 1: CDR Definitions[1]

CDR	Kabat	Chothia	AbM[2]
V_H CDR1	31-35	26-32	26-35
V_H CDR2	50-65	52-58	50-58
V_H CDR3	95-102	95-102	95-102
V_L CDR1	24-34	26-32	24-34
V_L CDR2	50-56	50-52	50-56
V_L CDR3	89-97	91-96	89-97

[1] Numbering of all CDR definitions in Table 1 is according to the numbering conventions set forth by Kabat et al. (see below).
[2] "AbM" with a lowercase "b" as used in Table 1 refers to the CDRs as defined by Oxford Molecular's "AbM" antibody modeling software.

Kabat et al. also defined a numbering system for variable region sequences that is applicable to any antibody. One of ordinary skill in the art can unambiguously assign this system of Kabat numbering to any variable region sequence, without reliance on any experimental data beyond the sequence itself. As used herein, "Kabat numbering" refers to the numbering system set forth by Kabat et al., U.S. Dept. of Health and Human Services, "Sequence of Proteins of Immunological Interest" (1983). Unless otherwise specified, references to the numbering of specific amino acid residue positions in an antigen binding moiety variable region are according to the Kabat numbering system. The polypeptide sequences of the sequence listing are not numbered according to the Kabat numbering system. However, it is

well within the ordinary skill of one in the art to convert the numbering of the sequences of the Sequence Listing to Kabat numbering.

An "individual" or "subject" is a mammal. Mammals include, but are not limited to, domesticated animals (e.g., cows, sheep, cats, dogs, and horses), primates (e.g., humans and non-human primates such as monkeys), rabbits, and rodents (e.g., mice and rats). Particularly, the individual or subject is a human.

By "isolated nucleic acid" molecule or polynucleotide is intended a nucleic acid molecule, DNA or RNA, which has been removed from its native environment. For example, a recombinant polynucleotide encoding a polypeptide contained in a vector is considered isolated for the purposes of the present invention. Further examples of an isolated polynucleotide include recombinant polynucleotides maintained in heterologous host cells or purified (partially or substantially) polynucleotides in solution. An isolated polynucleotide includes a polynucleotide molecule contained in cells that ordinarily contain the polynucleotide molecule, but the polynucleotide molecule is present extrachromosomally or at a chromosomal location that is different from its natural chromosomal location. Isolated RNA molecules include *in vivo* or *in vitro* RNA transcripts of the present invention, as well as positive and negative strand forms, and double-stranded forms. Isolated polynucleotides or nucleic acids according to the present invention further include such molecules produced synthetically. In addition, a polynucleotide or a nucleic acid may be or may include a regulatory element such as a promoter, ribosome binding site, or a transcription terminator.

By a nucleic acid or polynucleotide having a nucleotide sequence at least, for example, 95% "identical" to a reference nucleotide sequence of the present invention, it is intended that the nucleotide sequence of the polynucleotide is identical to the reference sequence except that the polynucleotide sequence may include up to five point mutations per each 100 nucleotides of the reference nucleotide sequence. In other words, to obtain a polynucleotide having a nucleotide sequence at least 95% identical to a reference nucleotide sequence, up to 5% of the nucleotides in the reference sequence may be deleted or substituted with another nucleotide, or a number of nucleotides up to 5% of the total nucleotides in the reference sequence may be inserted into the reference sequence. These alterations of the reference sequence may occur at the 5' or 3' terminal positions of the reference nucleotide sequence or anywhere between those terminal positions, interspersed either individually among residues in the reference sequence or in one or more contiguous groups within the reference sequence. As a practical matter, whether any particular polynucleotide sequence is at least 80%, 85%, 90%, 95%, 96%, 97%, 98% or 99% identical to a nucleotide sequence of the present invention can be

determined conventionally using known computer programs, such as the ones discussed below for polypeptides (e.g., ALIGN-2).

By an "isolated polypeptide" or a variant, or derivative thereof is intended a polypeptide that is not in its natural milieu. No particular level of purification is required. For example, an isolated polypeptide can be removed from its native or natural environment. Recombinantly produced polypeptides and proteins expressed in host cells are considered isolated for the purpose of the invention, as are native or recombinant polypeptides which have been separated, fractionated, or partially or substantially purified by any suitable technique.

"Percent (%) amino acid sequence identity" with respect to a reference polypeptide sequence is defined as the percentage of amino acid residues in a candidate sequence that are identical with the amino acid residues in the reference polypeptide sequence, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity, and not considering any conservative substitutions as part of the sequence identity. Alignment for purposes of determining percent amino acid sequence identity can be achieved in various ways that are within the skill in the art, for instance, using publicly available computer software such as BLAST, BLAST-2, ALIGN or Megalign (DNASTAR) software. Those skilled in the art can determine appropriate parameters for aligning sequences, including any algorithms needed to achieve maximal alignment over the full length of the sequences being compared. For purposes herein, however, % amino acid sequence identity values are generated using the sequence comparison computer program ALIGN-2. The ALIGN-2 sequence comparison computer program was authored by Genentech, Inc., and the source code has been filed with user documentation in the U.S. Copyright Office, Washington D.C., 20559, where it is registered under U.S. Copyright Registration No. TXU510087. The ALIGN-2 program is publicly available from Genentech, Inc., South San Francisco, California, or may be compiled from the source code. The ALIGN-2 program should be compiled for use on a UNIX operating system, including digital UNIX V4.0D. All sequence comparison parameters are set by the ALIGN-2 program and do not vary. In situations where ALIGN-2 is employed for amino acid sequence comparisons, the % amino acid sequence identity of a given amino acid sequence A to, with, or against a given amino acid sequence B (which can alternatively be phrased as a given amino acid sequence A that has or comprises a certain % amino acid sequence identity to, with, or against a given amino acid sequence B) is calculated as follows: 100 times the fraction X/Y; where X is the number of amino acid residues scored as identical matches by the sequence alignment program ALIGN-2 in that program's alignment of A and B, and where Y is the total number of amino acid residues in

B. It will be appreciated that where the length of amino acid sequence A is not equal to the length of amino acid sequence B, the % amino acid sequence identity of A to B will not equal the % amino acid sequence identity of B to A. Unless specifically stated otherwise, all % amino acid sequence identity values used herein are obtained as described in the immediately preceding paragraph using the ALIGN-2 computer program.

The term "nucleic acid molecule" relates to the sequence of bases comprising purine- and pyrimidine bases which are comprised by polynucleotides, whereby said bases represent the primary structure of a nucleic acid molecule. Herein, the term nucleic acid molecule includes DNA, cDNA, genomic DNA, RNA, synthetic forms of DNA and mixed polymers comprising two or more of these molecules. In addition, the term nucleic acid molecule includes both, sense and antisense strands. Moreover, the herein described nucleic acid molecule may contain non-natural or derivatized nucleotide bases, as will be readily appreciated by those skilled in the art.

As used herein "NFAT" refers to the "nuclear factor of activated T-cells" and is a family of transcription factors which is expressed in most immune cells. Activation of transcription factors of the NFAT family is dependent on calcium signaling. As an example, T cell activation through the T cell synapse results in calcium influx. Increased intracellular calcium levels activate the calcium-sensitive phosphatase, calcineurin, which rapidly dephosphorylates the serine-rich region (SRR) and SP-repeats in the amino termini of NFAT proteins. This results in a conformational change that exposes a nuclear localization signal promoting NFAT nuclear import and activation of target genes.

As used herein "NFAT pathway" refers to the stimuli that lead to modulation of activity of member of the NFAT family of transcription factors. NFAT DNA elements are known to the art and are herein also referred to as "response element of the NFAT pathway". Hence, a "receptor of the NFAT pathway" refers to a receptor which can trigger the modulation of activity of NFAT. Examples of a "receptor of the NFAT pathway" are e.g., T cell receptor and B cell receptor.

As used herein "NF-κB" refers to the "nuclear factor kappa-light-chain-enhancer of activated B cells" and is a transcription factor which is implicated in the regulation of many genes that code for mediators of apoptosis, viral replication, tumorigenesis, various autoimmune diseases and inflammatory responses. NFκB is present in almost all eukaryotic cells. Generally, it is located in the cytosol in an inactive state, since it forms a complex with inhibitory kappa B (IκB) proteins. Through the binding of ligands to integral membrane receptors (also referred to as "receptors of the NF-κB pathway", the IκB kinase (IKK) is activated. IKK is an enzyme

complex which consists of two kinases and a regulatory subunit. This complex phosphorylates the IκB proteins, which leads to ubiquitination and therefore degradation of those proteins by the proteasome. Finally, the free NFκB is in an active state, translocates to the nucleus and binds to the κB DNA elements and induces transcription of target genes.

As used herein "NF-κB pathway" refers to the stimuli that lead to modulation of activity of NF-κB. For example activation of the Toll-like receptor signaling, TNF receptor signaling, T cell receptor and B cell receptor signaling through either binding of a ligand or an antibody result in activation of NF-κB. Subsequently, phosphorylated NF-κB dimers bind to κB DNA elements and induce transcription of target genes. κB DNA elements are known in the art and herein also referred to as "response element of the NF-κB pathway". Hence, a "receptor of the NF-κB pathway" refers to a receptor which can trigger the modulation of activity of NF-κB. Examples of a "receptor of the NF-κB pathway" are Toll-like receptors, TNF receptors, T cell receptor and B cell receptor.

As used herein "AP-1" refers to the "activator protein 1" and is a transcription factor which is involved a number of cellular processes including differentiation, proliferation, and apoptosis. AP-1 functions are dependent on the specific Fos and Jun subunits contributing to AP-1 dimers. AP-1 binds to a palindromic DNA motif (5'-TGA G/C TCA-3') to regulate gene expression.

The term "pharmaceutical composition" refers to a preparation which is in such form as to permit the biological activity of an active ingredient contained therein to be effective, and which contains no additional components which are unacceptably toxic to a subject to which the formulation would be administered. A pharmaceutical composition usually comprises one or more pharmaceutically acceptable carrier(s).

A "pharmaceutically acceptable carrier" refers to an ingredient in a pharmaceutical composition, other than an active ingredient, which is nontoxic to a subject. A pharmaceutically acceptable carrier includes, but is not limited to, a buffer, excipient, stabilizer, or preservative.

As used herein, the term "polypeptide" refers to a molecule composed of monomers (amino acids) linearly linked by amide bonds (also known as peptide bonds). The term polypeptide refers to any chain of two or more amino acids, and does not refer to a specific length of the product. Thus, peptides, dipeptides, tripeptides, oligopeptides, protein, amino acid chain, or any other term used to refer to a chain of two or more amino acids, are included within the definition of polypeptide, and the term polypeptide may be used instead of, or interchangeably with any of these terms. The term polypeptide is also intended to refer to the products of post-

expression modifications of the polypeptide, including without limitation glycosylation, acetylation, phosphorylation, amidation, derivatization by known protecting/blocking groups, proteolytic cleavage, or modification by non-naturally occurring amino acids. A polypeptide may be derived from a natural biological source or produced by recombinant technology, but is not necessarily translated from a designated nucleic acid sequence. It may be generated in any manner, including by chemical synthesis. A polypeptide of the invention may be of a size of about 3 or more, 5 or more, 10 or more, 20 or more, 25 or more, 50 or more, 75 or more, 100 or more, 200 or more, 500 or more, 1,000 or more, or 2,000 or more amino acids. Polypeptides may have a defined three-dimensional structure, although they do not necessarily have such structure. Polypeptides with a defined three-dimensional structure are referred to as folded, and polypeptides which do not possess a defined three-dimensional structure, but rather can adopt a large number of different conformations, and are referred to as unfolded.

The term "polynucleotide" refers to an isolated nucleic acid molecule or construct, e.g., messenger RNA (mRNA), virally-derived RNA, or plasmid DNA (pDNA). A polynucleotide may comprise a conventional phosphodiester bond or a non-conventional bond (e.g., an amide bond, such as found in peptide nucleic acids (PNA). The term nucleic acid molecule refers to any one or more nucleic acid segments, e.g., DNA or RNA fragments, present in a polynucleotide.

The term "protein with intrinsic fluorescence" refers to a protein capable of forming a highly fluorescent, intrinsic chromophore either through the cyclization and oxidation of internal amino acids within the protein or via the enzymatic addition of a fluorescent co-factor. The term "protein with intrinsic fluorescence" includes wild-type fluorescent proteins and mutants that exhibit altered spectral or physical properties. The term does not include proteins that exhibit weak fluorescence by virtue only of the fluorescence contribution of non-modified tyrosine, tryptophan, histidine and phenylalanine groups within the protein. Proteins with intrinsic fluorescence are known in the art, e.g., green fluorescent protein (GFP),), red fluorescent protein (RFP), Blue fluorescent protein (BFP, Heim et al. 1994, 1996), a cyan fluorescent variant known as CFP (Heim et al. 1996; Tsien 1998); a yellow fluorescent variant known as YFP (Ormo et al. 1996; Wachter et al. 1998); a violet-excitable green fluorescent variant known as Sapphire (Tsien 1998; Zapata-Hommer et al. 2003); and a cyan-excitable green fluorescing variant known as enhanced green fluorescent protein or EGFP (Yang et al. 1996) and can be measured e.g., by live cell imaging (e.g., Incucyte) or fluorescent spectrophotometry.

"Reduced binding" refers to a decrease in affinity for the respective interaction, as measured for example by SPR. For clarity the term includes also reduction of the affinity to zero (or below the detection limit of the analytic method), i.e., complete abolishment of the interaction. Conversely, "increased binding" refers to an increase in binding affinity for the respective interaction.

The term "regulatory sequence" refers to DNA sequences, which are necessary to effect the expression of coding sequences to which they are ligated. The nature of such control sequences differs depending upon the organism. In prokaryotes, control sequences generally include promoter, ribosomal binding site, and terminators. In eukaryotes generally control sequences include promoters, terminators and, in some instances, enhancers, transactivators or transcription factors. The term "control sequence" is intended to include, at a minimum, all components the presence of which are necessary for expression, and may also include additional advantageous components.

As used herein, a "reporter gene" means a gene whose expression can be assayed. In one preferred embodiment a "reporter gene" is a gene that encodes a protein the production and detection of which is used as a surrogate to detect indirectly the activity of the antibody or ligand to be tested. The reporter protein is the protein encoded by the reporter gene. Preferably, the reporter gene encodes an enzyme whose catalytic activity can be detected by a simple assay method or a protein with a property such as intrinsic fluorescence or luminescence so that expression of the reporter gene can be detected in a simple and rapid assay requiring minimal sample preparation. Non-limiting examples of enzymes whose catalytic activity can be detected are Luciferase, beta Galactosidase, Alkaline Phosphatase. Luciferase is a monomeric enzyme with a molecular weight (MW) of 61 kDa. It acts as a catalysator and is able to convert D-luciferin in the presence of Adenosine triphosphate (ATP) and Mg2+ to luciferyl adenylate. In addition, pyrophosphate (PPi) and adenosine monophosphate (AMP) are generated as byproducts. The intermediate luciferyl adenylate is then oxidized to oxyluciferin, carbon dioxide (CO_2) and light. Oxyluciferin is a bioluminescent product which can be quantitatively measured in a luminometer by the light released from the reaction. Luciferase reporter assays are commercially available and known in the art, e.g., Luciferase 1000 Assay System and ONE-Glo™ Luciferase Assay System.

A "response element" refers to a specific transcription factor binding element, or cis acting element which can be activated or silenced on binding of a certain transcription factor. In one embodiment the response element is a cis-acting enhancer element located upstream of a

minimal promotor (e.g., a TATA box promotor) which drives expression of the reporter gene upon transcription factor binding.

As used herein, the term "single-chain" refers to a molecule comprising amino acid monomers linearly linked by peptide bonds. In certain embodiments, one of the antigen binding moieties is a scFv fragment, i.e., a VH domain and a VL domain connected by a peptide linker. In certain embodiments, one of the antigen binding moieties is a single-chain Fab molecule, i.e., a Fab molecule wherein the Fab light chain and the Fab heavy chain are connected by a peptide linker to form a single peptide chain. In a particular such embodiment, the C-terminus of the Fab light chain is connected to the N-terminus of the Fab heavy chain in the single-chain Fab molecule.

The term "SSD" as used herein refers to stimulatory signaling domain.

As used herein, "treatment" (and grammatical variations thereof such as "treat" or "treating") refers to clinical intervention in an attempt to alter the natural course of a disease in the individual being treated, and can be performed either for prophylaxis or during the course of clinical pathology. Desirable effects of treatment include, but are not limited to, preventing occurrence or recurrence of disease, alleviation of symptoms, diminishment of any direct or indirect pathological consequences of the disease, preventing metastasis, decreasing the rate of disease progression, amelioration or palliation of the disease state, and remission or improved prognosis.

In the context of the present invention, the term „tag" refers to a molecule attached or engrafted to or onto a biomolecule such as a protein, particularly an antigen binding molecule. The function of a tag is to mark or label the "tagged" protein (e.g., an immunoglobulin or fragment thereof) such that it can be recognized by a specific antigen binding moiety capable of binding to the tag but not capable of binding to the untagged protein. The term is synonymous to "molecular tag" and comprises without being limited to fluorescent tags, protein tags, affinity tags, solubilization tags, chromatography tags, epitope tags and small molecule tags such as hapten tags. Small molecule tags, e.g., haptens, can be chemically coupled covalently or non-covalently to the biomolecule whereas "protein tags" or "polypeptide tags" are peptide sequences which can be genetically grafted onto a protein and subsequently be recognized by specific antigen binding moieties capable of binding to the tag but not capable of binding to the untagged protein. Hapten tags are able to elicit an immune response when attached to a carrier protein, and, therefore, are suitable to generate specific antigen binding moieties capable of recognizing the tag on a carrier such as a protein. In preferred embodiments of the present invention, the tag is a hapten tag or a polypeptide tag.

As used herein, the term "target antigenic determinant" is synonymous with "target antigen", "target epitope" and "target cell antigen" and refers to a site (e.g., a contiguous stretch of amino acids or a conformational configuration made up of different regions of non-contiguous amino acids) on a polypeptide macromolecule to which an antibody binds, forming an antigen binding moiety-antigen complex. Useful antigenic determinants can be found, for example, on the surfaces of tumor cells, on the surfaces of virus-infected cells, on the surfaces of other diseased cells, on the surface of immune cells, free in blood serum, and/or in the extracellular matrix (ECM). The proteins referred to as antigens herein (e.g., CD20, CD38, CD138, CEA, EGFR, FolR1, HER2, LeY, MCSP, STEAP1, TYRP, and WT1) can be any native form of the proteins from any vertebrate source, including mammals such as primates (e.g., humans) and rodents (e.g., mice and rats), unless otherwise indicated. In a particular embodiment the target antigen is a human protein. Where reference is made to a specific target protein herein, the term encompasses the "full-length", unprocessed target protein as well as any form of the target protein that results from processing in the target cell. The term also encompasses naturally occurring variants of the target protein, e.g., splice variants or allelic variants. Exemplary human target proteins useful as antigens include, but are not limited to: CD20, CD38, CD138, CEA, EGFR, FolR1, HER2, LeY, MCSP, STEAP1, TYRP, and WT1.

Antibodies may have one, two, three or more binding domains and may be monospecific, bispecific or multispecific. The antibodies can be full length from a single species, or be chimerized or humanized. For an antibody with more than two antigen binding domains, some binding domains may be identical and/or have the same specificity.

"T cell activation" as used herein refers to one or more cellular response of a T lymphocyte, particularly a cytotoxic T lymphocyte, selected from: proliferation, differentiation, cytokine secretion, cytotoxic effector molecule release, cytotoxic activity, and expression of activation markers. Suitable assays to measure T cell activation are known in the art and described herein.

In accordance with this invention, the term "T cell receptor" or "TCR" is commonly known in the art. In particular, herein the term "T cell receptor" refers to any T cell receptor, provided that the following three criteria are fulfilled: (i) tumor specificity, (ii) recognition of (most) tumor cells, which means that an antigen or target should be expressed in (most) tumor cells and (iii) that the TCR matches to the HLA-type of the subjected to be treated. In this context, suitable T cell receptors which fulfill the above mentioned three criteria are known in the art such as receptors recognizing NY-ESO-1 (for sequence information(s) see, e.g., PCT/GB2005/001924) and/or HER2neu (for sequence information(s) see WO-A1

2011/0280894). Major histocompatibility complex (MHC) class I molecules present peptides from endogenous antigens to CD8+ cytotoxic T cells, and therefore, MHC-peptide complexes are a suitable target for immunotherapeutic approaches. The MHC-peptide complexes can be targeted by recombinant T-cell receptors (TCRs). However, most TCRs may have affinities which are too low for immunotherapy whereas high affinity binding moieties with TCR specificity would be beneficial. Towards this end, high-affinity soluble antibody molecules with TCR-like specificity can be generated, e.g., by generating phage display libraries (e.g., combinatorial libraries) and screening such libraries as further described herein. These soluble antigen binding moieties e.g., scFv or Fab, with TCR-like specificity as described herein are referred to as "T cell receptor like antigen binding moieties" or "TCRL antigen binding moieties".

A "therapeutically effective amount" of an agent, e.g., a pharmaceutical composition, refers to an amount effective, at dosages and for periods of time necessary, to achieve the desired therapeutic or prophylactic result. A therapeutically effective amount of an agent for example eliminates, decreases, delays, minimizes or prevents adverse effects of a disease.

The term "vector" or "expression vector" is synonymous with "expression construct" and refers to a DNA molecule that is used to introduce and direct the expression of a specific gene to which it is operably associated in a target cell. The term includes the vector as a self-replicating nucleic acid structure as well as the vector incorporated into the genome of a host cell into which it has been introduced. The expression vector of the present invention comprises an expression cassette. Expression vectors allow transcription of large amounts of stable mRNA. Once the expression vector is inside the target cell, the ribonucleic acid molecule or protein that is encoded by the gene is produced by the cellular transcription and/or translation machinery. In one embodiment, the expression vector of the invention comprises an expression cassette that comprises polynucleotide sequences that encode antigen binding receptors of the invention or fragments thereof.

In this context, provided herein are methods, particularly *in vitro* methods, for selecting novel target antigen binding moieties for further development according to their specificity, in particular in relation to activation of reporter cells (e.g., T cells) upon contact to a target cell. In the herein described methods and assays, the target antigen binding moiety mediates the contact between a target cell, in particular a cancer cell, and a reporter cell, in particular a T cell. In this context, the methods as described herein are useful to select a candidate target

antigen binding moiety according to specificity of binding to the target, e.g., on cancer cells, and activation of effector cells, e.g., T cells.

Accordingly, in one embodiment, provided is a method for assessing the specificity of a target antigen binding moiety capable of specific binding to a target antigen, the method comprising the steps of:

a) providing an antigen binding molecule comprising an antigen binding domain and a recognition domain, wherein the antigen binding domain comprises the target antigen binding moiety, and wherein the recognition domain comprises a tag;
b) contacting the antigen binding molecule with a target cell comprising the target antigen on the surface, particularly wherein the target cell is a cancer cell;
c) contacting the antigen binding molecule with a chimeric antigen receptor (CAR) expressing reporter T (CAR-T) cell wherein the reporter CAR-T cell comprises:
    i. a CAR capable of specific binding to the recognition domain comprising the tag, wherein the CAR is operationally coupled to a response element;
    ii. a reporter gene under the control of the response element; and
d) determining T cell activation by measuring the expression of the reporter gene to establish the specificity of the target antigen binding moiety.

In this context further described and used for the methods of the present invention are antigen binding receptors (e.g., CARs) capable of specific binding to the recognition domain of the antigen binding molecule comprising the (candidate) target antigen binding moiety. The recognition domain can be any polypeptide domain capable of stable folding into a protein domain which can be tagged by a molecular tag, e.g., a hapten tag or a polypeptide tag. In certain embodiments, the recognition domain is an immunoglobulin domain. Immunoglobulins typically comprise variable and constant domain capable of stable folding wherein the variable domains confer the specificity of the immunoglobulin molecule towards a target antigen. Accordingly, the variable domains are the parts of an immunoglobulin with the highest degree of sequence variance. On the other hand, the constant domains are parts of minimal variance among immunoglobulins of the same class and, therefore, are particularly suited in the context of this invention as recognition domain for methods of the present invention. However, it may also be favorable to reduce the size of the antigen binding molecule as far as possible, in such embodiments, the variable domain of an immunoglobulin, which confer the specificity to a target antigen, can also exert the function of the recognition domain, i.e., the antigen binding domain and the recognition domain can be the same domain,

e.g., the variable domain can be coupled with, e.g., a hapten tag or a polypeptide tag, or alternatively, the hapten tag is coupled to the constant region of a Fab fragment.

The antigen binding molecule comprising the (novel) target antigen binding moiety preferably is an IgG class antibody, particularly an IgG1 or IgG4 isotype antibody, or a fragment thereof. However, the antigen binding molecule can be of any class of immunoglobulins or other antigen binding proteins as long as it is capable of providing a stable scaffold for the antigen binding domain comprising the target antigen binding moiety and the recognition domain. In one embodiment, the antigen binding molecule comprises an Fc domain, particularly an IgG Fc region, most particularly an IgG1 Fc region. In a preferred embodiment, the antigen binding molecule comprises a modified Fc region, particularly an Fc region comprising a tag (e.g., a hapten tag or a polypeptide tag) for specific recognition by the CAR. In such embodiments, the CAR used according to the present invention is capable of specific binding to the modified Fc region, i.e., the Fc region comprising the tag.

In another embodiment, the antigen binding molecule comprises a Fab domain, particularly an IgG Fab domain, most particularly an IgG1 Fab domain. In a preferred embodiment, the antigen binding molecule comprises a modified Fab domain, particularly a Fab domain comprising a tag (e.g., a hapten tag or a polypeptide tag) for specific recognition by the CAR. In exemplary embodiments, the antigen binding domain comprising the target antigen binding moiety and the recognition domain are the same domain and the CAR used according to the present invention is capable of specific binding to the modified Fab domain, i.e., the Fab domain comprising the tag.

The present invention further describes the transduction and use of T cells, such as CD8+ T cells, CD4+ T cells, CD3+ T cells, γδ T cells or natural killer (NK) T cells and immortalized cell lines, e.g., Jurkat cells, to introduce a reporter system as described herein and (a) CAR(s) as described herein and their targeted recruitment and activation by the antigen binding molecule, comprising the target antigen binding moiety and the recognition domain, preferably an Fc domain or a Fab domain, e.g., a modified (tagged) Fc domain or Fab fragment as herein described. In one embodiment, the antigen binding molecule, e.g., the tagged IgG1 antibody or tagged Fab fragment, is capable of specific binding to a tumor-specific antigen that is naturally occurring on the surface of a target cell, e.g., a cancer cell.

Upon binding of the antigen binding molecule comprising the target antigen binding moiety (e.g., deriving from a phage display screening) to the target cell and binding of the CAR to the recognition domain (e.g., the tagged IgG1 antibody or tagged Fab fragment), the reporter CAR-T cell becomes activated and the reporter gene is expressed. Expression of the reporter

gene is therefore indicative for (specific) binding of the target antigen binding moiety in the context of T cell activation induced by an antigen binding molecule directed against a target antigen, e.g., on a tumor cell.

The approach of the present invention bears significant advantages over conventional binding assays, as the T cell based *in vitro* method as described herein, without being bound by theory, more closely resembles the *in vivo* situation encountered for or with, e.g., therapeutic antibodies engaging T cells (e.g., T cell bispecific antibodies).

Accordingly, the invention provides a versatile screening platform wherein antibodies, in particular IgG type antibodies comprising a target antigen binding moiety, may be used to mark or label target cells (e.g., tumor cells) as a guidance for immune cells (e.g., T cells), in particular wherein T cells are specifically targeted toward the tumor cells by the antibody comprising the target antigen binding moiety. After binding of the CAR to the tag on the recognition domain and binding of the target antigen binding moiety to the target antigen on the surface of a tumor cell, the reporter T cell becomes activated wherein the activation can be measured, e.g., by read-out of a fluorescent or luminescent signal. The platform is flexible and specific by allowing the use of diverse newly developed target antigen binding moieties or co-application of multiple antibodies with different antigen specificity but comprising the same recognition domain.

In certain embodiment, the target antigen binding moiety is a conventional Fab fragment, i.e., a Fab molecule consisting of a Fab light chain and a Fab heavy chain. A particular advantage of this screening formats is the straight-forward integration of novel library derived target antigen binding moieties without changing the format, e.g., a Fab antigen binder deriving from screening a phage display library can be included in the Fab and/or crossFab antigen binding molecule immunoglobulin format as described herein. Accordingly, target antigen binding moieties deriving from Fab displaying phage libraries can be included in an antibody for screening without changing the format which might affect the binding properties of the library derived binder negatively. In a preferred embodiment, the target antigen binding moiety is a Fab fragment, in particular a Fab fragment deriving from a phage display library screening. In a preferred embodiment, the target antigen binding moiety is a Fab fragment, in particular a Fab fragment deriving from a phage display library screening. Such embodiment, as indicated above, has the advantage that the format of the binder (Fab) does not have to be changed from the Fab fragment (format) phage display library screening throughout the assessment for specificity, and ultimately to therapeutic antibodies engaging T cells (e.g., T cell bispecific antibodies).

In further embodiments, the target antigen binding moieties is a crossFab fragment, i.e., a Fab molecule consisting of a Fab light chain and a Fab heavy chain, wherein either the variable regions or the constant regions of the Fab heavy and light chain are exchanged.

In the context of the present invention, the CAR comprises an extracellular domain that does not naturally occur in or on T cells. Thus, the CAR is capable of providing tailored binding specificity to the recognition domain, e.g., a (modified) Fc domain of a therapeutic antibody format used for screening according to the invention. Cells, e.g., T cells, transduced with a CAR and used according to the invention become capable of specific binding to the recognition domain. Specificity for the recognition domain is provided by the extracellular domain of the CAR comprising an antigen binding moiety capable of specific binding to the recognition domain. In a preferred embodiment, the recognition domain is a fragment crystalizable (Fc) region. In specific embodiments, the recognition domain is an IgG1 or an IgG4 Fc domain. In one embodiment, the recognition domain is a human IgG1 Fc domain. In further embodiments, the recognition domain is a modified Fc domain, e.g., comprising a tag. In such embodiments, the CAR as described herein is capable of specific binding to the recognition domain comprising the tag but not capable of specific binding to the recognition domain not comprising the tag.

Accordingly, the present invention also relates to the use of CARs comprising an extracellular domain comprising at least one antigen binding moiety capable of specific binding to a modified Fc domain, wherein the at least one antigen binding moiety is not capable of specific binding to the non-modified Fc domain. In such embodiments, the CAR is capable of specific binding to the modified Fc domain of an antigen binding molecule, e.g., an antibody. In a preferred embodiment, the modified Fc domain comprises at tag, e.g., a hapten tag coupled to the Fc domain or a protein tag comprised in the Fc domain.

The CAR is capable of specific binding to a modified immunoglobulin domain comprising the tag but not capable of specific binding to the non-modified parent immunoglobulin domain not comprising the tag, wherein the modification is introduction of a hapten tag or a polypeptide tag.

In one aspect of the invention, provided herein is the use of CARs comprising at least one antigen binding moiety capable of specific binding to a modified immunoglobulin domain. Transduced cells, e.g., T cells, expressing such a CAR are capable of specific binding to the modified immunoglobulin domain of an antigen binding molecule, i.e., of a therapeutic antibody. The present invention *inter alia* provides a straight-forward screening platform to

assess specificity of novel target antigen binding moieties in a therapeutically meaningful antigen binding molecule format. The methods according to the invention integrate relevant cellular and molecular components of activation cascades of known or potential effector cells in a high-throughput assay format.

Antigen binding moieties capable of specific binding to a target antigen, e.g., a tumor antigen or a recognition domain, e.g., a modified Fc domain, may be generated by immunization of e.g., a mammalian immune system. Such methods are known in the art and e.g., are described in Burns in Methods in Molecular Biology 295:1-12 (2005). Alternatively, antigen binding moieties of desired activity may be isolated by screening combinatorial libraries for antibodies with the desired activity or activities. Methods for screening combinatorial libraries are reviewed, e.g., in Lerner et al. in Nature Reviews 16:498-508 (2016). For example, a variety of methods are known in the art for generating phage display libraries and screening such libraries for antigen binding moieties possessing the desired binding characteristics. Such methods are reviewed, e.g., in Frenzel et al. in mAbs 8:1177-1194 (2016); Bazan et al. in Human Vaccines and Immunotherapeutics 8:1817-1828 (2012) and Zhao et al. in Critical Reviews in Biotechnology 36:276-289 (2016) as well as in Hoogenboom et al. in Methods in Molecular Biology 178:1-37 (O'Brien et al., ed., Human Press, Totowa, NJ, 2001) and further described, e.g., in the McCafferty et al., Nature 348:552-554; Clackson et al., Nature 352: 624-628 (1991); Marks et al., J. Mol. Biol. 222: 581-597 (1992) and in Marks and Bradbury in Methods in Molecular Biology 248:161-175 (Lo, ed., Human Press, Totowa, NJ, 2003); Sidhu et al., J. Mol. Biol. 338(2): 299-310 (2004); Lee et al., J. Mol. Biol. 340(5): 1073-1093 (2004); Fellouse, Proc. Natl. Acad. Sci. USA 101(34): 12467-12472 (2004); and Lee et al., J. Immunol. Methods 284(1-2): 119-132(2004). In certain phage display methods, repertoires of VH and VL genes are separately cloned by polymerase chain reaction (PCR) and recombined randomly in phage libraries, which can then be screened for antigen-binding phage as described in Winter et al. in Annual Review of Immunology 12: 433-455 (1994). Phage typically display antibody fragments, either as single-chain Fv (scFv) fragments or as Fab fragments. Libraries from immunized sources provide high-affinity antigen binding moieties to the immunogen without the requirement of constructing hybridomas. Alternatively, the naive repertoire can be cloned (e.g., from human) to provide a single source of antigen binding moieties to a wide range of non-self and also self antigens without any immunization as described by Griffiths et al. in EMBO Journal 12: 725-734 (1993). Finally, naive libraries can also be made synthetically by cloning unrearranged V-gene segments from stem cells, and using PCR primers containing random sequence to encode the highly variable CDR3 regions

and to accomplish rearrangement *in vitro*, as described by Hoogenboom and Winter in Journal of Molecular Biology 227: 381-388 (1992). Patent publications describing human antibody phage libraries include, for example: US Patent Nos. 5,750,373; 7,985,840; 7,785,903 and 8,679,490 as well as US Patent Publication Nos. 2005/0079574, 2007/0117126, 2007/0237764, 2007/0292936 and 2009/0002360. Further examples of methods known in the art for screening combinatorial libraries for antigen binding moieties with a desired activity or activities include ribosome and mRNA display, as well as methods for antibody display and selection on bacteria, mammalian cells, insect cells or yeast cells. Methods for yeast surface display are reviewed, e.g., in Scholler et al. in Methods in Molecular Biology 503:135-56 (2012) and in Cherf et al. in Methods in Molecular biology 1319:155-175 (2015) as well as in the Zhao et al. in Methods in Molecular Biology 889:73-84 (2012). Methods for ribosome display are described, e.g., in He et al. in Nucleic Acids Research 25:5132-5134 (1997) and in Hanes et al. in PNAS 94:4937-4942 (1997).

In illustrative embodiments of the present invention, as a proof of concept, CARs are provided comprising an extracellular domain comprising at least one antigen binding moiety, wherein the at least one antigen binding moiety is capable of specific binding to a modified immunoglobulin domain (e.g., a Fc domain or a Fab domain) but not capable of specific binding to the non-modified immunoglobulin domain, wherein the modified immunoglobulin domain comprises a hapten tag or a polypeptide tag.

In an illustrative embodiment of the present invention, as a proof of concept, provided are CARs capable of specific binding to a modified immunoglobulin domain comprising the hapten tag Digoxigenin (DIG).

In one embodiment the CAR capable of specific binding to an immunoglobulin domain comprising the hapten tag DIG comprises the heavy chain complementarity determining regions (CDRs) of SEQ ID NO:1, SEQ ID NO:2 and SEQ ID NO:3 and the light chain CDRs of SEQ ID NO:4, SEQ ID NO:5 and SEQ ID NO:6.

In one preferred embodiment the CAR capable of specific binding to an immunoglobulin domain comprising the hapten tag DIG comprises a heavy chain variable region comprising:

(a) a heavy chain complementarity determining region (CDR H) 1 amino acid sequence of DYAMS (SEQ ID NO:1);

(b) a CDR H2 amino acid sequence of SINIGATYIYYADSVKG (SEQ ID NO:2);

(c) a CDR H3 amino acid sequence of PGSPYEYDKAYYSMAY (SEQ ID NO:3);

and a light chain variable region comprising:

(d) a light chain (CDR L)1 amino acid sequence of RASQDIKNYLN (SEQ ID NO:4);

(e) a CDR L2 amino acid sequence of YSSTLLS (SEQ ID NO:5); and

(f) a CDR L3 amino acid sequence of QQSITLPPT (SEQ ID NO:6).

In one embodiment the CAR capable of specific binding to an immunoglobulin domain comprising the hapten tag DIG comprises a heavy chain variable region (VH) comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to an amino acid sequence selected from SEQ ID NO:8 and SEQ ID NO:32 and a light chain variable region (VL) comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to an amino acid sequence selected from SEQ ID NO:9 and SEQ ID NO:33.

In one embodiment the CAR capable of specific binding to an immunoglobulin domain comprising the hapten tag DIG comprises a heavy chain variable region (VH) comprising an amino acid sequence selected from SEQ ID NO:8 and SEQ ID NO:32, and a light chain variable region (VL) comprising an amino acid sequence selected from SEQ ID NO:9 and SEQ ID NO:33.

In one embodiment the CAR capable of specific binding to an immunoglobulin domain comprising the hapten tag DIG comprises a heavy chain variable region (VH) comprising the amino acid sequence of SEQ ID NO:32 and a light chain variable region (VL) comprising the amino acid sequence of SEQ ID NO:33.

In one preferred embodiment the CAR capable of specific binding to an immunoglobulin domain comprising the hapten tag DIG comprises a heavy chain variable region (VH) comprising the amino acid sequence of SEQ ID NO:8 and a light chain variable region (VL) comprising the amino acid sequence of SEQ ID NO:9.

In one embodiment, the at least one antigen binding moiety is a scFv, a Fab, a crossFab or a scFab fragment. In one embodiment the CAR capable of specific binding to an immunoglobulin domain comprising the hapten tag DIG comprises a Fab fragment. In a preferred embodiment the CAR capable of specific binding to an immunoglobulin domain comprising the hapten tag DIG comprises a Fab fragment comprising a heavy chain of SEQ ID NO:30 and a light chain of SEQ ID NO:31.

In one embodiment the CAR capable of specific binding to an immunoglobulin domain comprising the hapten tag DIG comprises a scFv fragment which is a polypeptide consisting of an heavy chain variable domain (VH), an light chain variable domain (VL) and a linker,

wherein said variable domains and said linker have one of the following configurations in N-terminal to C-terminal direction: a) VH-linker-VL or b) VL-linker-VH. In a preferred embodiment, the scFv fragment has the configuration VH-linker-VL.

In a preferred embodiment the CAR capable of specific binding to an immunoglobulin domain comprising the hapten tag DIG comprises an scFv fragment comprising the amino acid sequence of SEQ ID NO:10.

In another illustrative embodiment of the present invention, as a proof of concept, provided are CARs capable of specific binding to a modified immunoglobulin domain comprising the hapten tag Fluorescein isothiocyanate (FITC).

In one embodiment the CAR capable of specific binding to an immunoglobulin domain comprising the hapten tag FITC comprises the heavy chain complementarity determining regions (CDRs) of SEQ ID NO:42, SEQ ID NO:43 and SEQ ID NO:44 and the light chain CDRs of SEQ ID NO:45, SEQ ID NO:46 and SEQ ID NO:47.

In one preferred embodiment the CAR capable of specific binding to an immunoglobulin domain comprising the hapten tag FITC comprises a heavy chain variable region comprising:

(a) a heavy chain complementarity determining region (CDR H) 1 amino acid sequence of HYWMN (SEQ ID NO:42);

(b) a CDR H2 amino acid sequence of QFRNKPYNYETYYSDSVKG (SEQ ID NO:43);

(c) a CDR H3 amino acid sequence of ASYGMEY (SEQ ID NO:44);

and a light chain variable region comprising:

(d) a light chain (CDR L)1 amino acid sequence of RSSQSLVHSNGNTYLR (SEQ ID NO:45);

(e) a CDR L2 amino acid sequence of KVSNRVS (SEQ ID NO:46); and

(f) a CDR L3 amino acid sequence of SQSTHVPWT (SEQ ID NO:47).

In one embodiment the CAR capable of specific binding to an immunoglobulin domain comprising the hapten tag FITC comprises a heavy chain variable region (VH) comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO:60 and a light chain variable region (VL) comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO:61.

In one embodiment the CAR capable of specific binding to an immunoglobulin domain comprising the hapten tag FITC comprises a heavy chain variable region (VH) comprising the

amino acid sequence of SEQ ID NO:60, and a light chain variable region (VL) comprising the amino acid sequence of SEQ ID NO:61.

In one embodiment, the at least one antigen binding moiety is a scFv, a Fab, a crossFab or a scFab fragment.

In one embodiment the CAR capable of specific binding to an immunoglobulin domain comprising the hapten tag FITC comprises a scFv fragment which is a polypeptide consisting of an heavy chain variable domain (VH), an light chain variable domain (VL) and a linker, wherein said variable domains and said linker have one of the following configurations in N-terminal to C-terminal direction: a) VH-linker-VL or b) VL-linker-VH. In a preferred embodiment, the scFv fragment has the configuration VH-linker-VL.

In a preferred embodiment the CAR capable of specific binding to an immunoglobulin domain comprising the hapten tag FITC comprises an scFv fragment comprising the amino acid sequence of SEQ ID NO:49.

In another illustrative embodiment of the present invention, as a proof of concept, provided are CARs capable of specific binding to a modified immunoglobulin domain comprising a polypeptide tag from the influenza hemagglutinin (HA) glycoprotein. In one embodiment, the polypeptide tag from the HA protein comprises the amino acid sequence of YPYDVPDYA (SEQ ID NO:100).

In one embodiment the CAR capable of specific binding to an immunoglobulin domain comprising the HA tag comprises the heavy chain complementarity determining regions (CDRs) of SEQ ID NO:52, SEQ ID NO:53 and SEQ ID NO:54 and the light chain CDRs of SEQ ID NO:55, SEQ ID NO:56 and SEQ ID NO:57.

In one preferred embodiment the CAR capable of specific binding to an immunoglobulin domain comprising the HA tag comprises a heavy chain variable region comprising:

(a) a heavy chain complementarity determining region (CDR H) 1 amino acid sequence of NYDMA (SEQ ID NO:52);

(b) a CDR H2 amino acid sequence of TISHDGRNTNYRDSVKG (SEQ ID NO:53);

(c) a CDR H3 amino acid sequence of PGFAH (SEQ ID NO:54);

and a light chain variable region comprising:

(d) a light chain (CDR L)1 amino acid sequence of RSSKTLLNTRGITSLY (SEQ ID NO:55);

(e) a CDR L2 amino acid sequence of RMSNLAS (SEQ ID NO:56); and

(f) a CDR L3 amino acid sequence of AQFLEFPLT (SEQ ID NO:57).

In one embodiment the CAR capable of specific binding to an immunoglobulin domain comprising the HA tag comprises a heavy chain variable region (VH) comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to an amino acid sequence selected from SEQ ID NO:60 and SEQ ID NO:65 and a light chain variable region (VL) comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to an amino acid sequence selected from SEQ ID NO:61 and SEQ ID NO:66.

In one embodiment the CAR capable of specific binding to an immunoglobulin domain comprising the HA tag comprises a heavy chain variable region (VH) comprising an amino acid sequence selected from SEQ ID NO:60 and SEQ ID NO:65, and a light chain variable region (VL) comprising an amino acid sequence selected from SEQ ID NO:61 and SEQ ID NO:66.

In one embodiment the CAR capable of specific binding to an immunoglobulin domain comprising the HA tag comprises a heavy chain variable region (VH) comprising the amino acid sequence of SEQ ID NO:60 and a light chain variable region (VL) comprising the amino acid sequence of SEQ ID NO:61.

In one preferred embodiment the CAR capable of specific binding to an immunoglobulin domain comprising the HA tag comprises a heavy chain variable region (VH) comprising the amino acid sequence of SEQ ID NO:65 and a light chain variable region (VL) comprising the amino acid sequence of SEQ ID NO:66.

In one embodiment, the at least one antigen binding moiety is a scFv, a Fab, a crossFab or a scFab fragment. In one embodiment the CAR capable of specific binding to an immunoglobulin domain comprising the HA tag comprises a Fab fragment. In a preferred embodiment the CAR capable of specific binding to an immunoglobulin domain comprising the HA tag comprises a Fab fragment comprising a heavy chain of SEQ ID NO:63 and a light chain of SEQ ID NO:64.

In one embodiment the CAR capable of specific binding to an immunoglobulin domain comprising the HA tag comprises a scFv fragment which is a polypeptide consisting of an heavy chain variable domain (VH), an light chain variable domain (VL) and a linker, wherein said variable domains and said linker have one of the following configurations in N-terminal to C-terminal direction: a) VH-linker-VL or b) VL-linker-VH. In a preferred embodiment, the scFv fragment has the configuration VH-linker-VL.

In a preferred embodiment the CAR capable of specific binding to an immunoglobulin domain comprising the HA tag comprises an scFv fragment comprising the amino acid sequence of SEQ ID NO:59.

In another illustrative embodiment of the present invention, as a proof of concept, provided are CARs capable of specific binding to a modified immunoglobulin domain comprising a polypeptide tag from the human c-myc protein. In one embodiment, the polypeptide tag from the human c-myc protein comprises the amino acid sequence of EQKLISEEDL (SEQ ID NO:101).

In one embodiment the CAR capable of specific binding to an immunoglobulin domain comprising the myc tag comprises the heavy chain complementarity determining regions (CDRs) of SEQ ID NO:77, SEQ ID NO:78 and SEQ ID NO:79 and the light chain CDRs of SEQ ID NO:80, SEQ ID NO:81 and SEQ ID NO:82.

In one preferred embodiment the CAR capable of specific binding to an immunoglobulin domain comprising the myc tag comprises a heavy chain variable region comprising:

(a) a heavy chain complementarity determining region (CDR H) 1 amino acid sequence of HYGMS (SEQ ID NO:77);

(b) a CDR H2 amino acid sequence of TIGSRGTYTHYPDSVKG (SEQ ID NO:78);

(c) a CDR H3 amino acid sequence of RSEFYYYGNTYYYSAMDY (SEQ ID NO:79);

and a light chain variable region comprising:

(d) a light chain (CDR L)1 amino acid sequence of RASESVDNYGFSFMN (SEQ ID NO:80);

(e) a CDR L2 amino acid sequence of AISNRGS (SEQ ID NO:81); and

(f) a CDR L3 amino acid sequence of QQTKEVPWT (SEQ ID NO:82).

In one embodiment the CAR capable of specific binding to an immunoglobulin domain comprising the myc tag comprises a heavy chain variable region (VH) comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence selected of SEQ ID NO:86 and a light chain variable region (VL) comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO:87.

In one embodiment the CAR capable of specific binding to an immunoglobulin domain comprising the myc tag comprises a heavy chain variable region (VH) comprising the amino

acid sequence of SEQ ID NO:86, and a light chain variable region (VL) comprising the amino acid sequence of SEQ ID NO:87.

In one embodiment, the at least one antigen binding moiety is a scFv, a Fab, a crossFab or a scFab fragment. In one embodiment the CAR capable of specific binding to an immunoglobulin domain comprising the myc tag comprises a Fab fragment. In a preferred embodiment the CAR capable of specific binding to an immunoglobulin domain comprising the myc tag comprises a Fab fragment comprising a heavy chain of SEQ ID NO:84 and a light chain of SEQ ID NO:85.

In one embodiment the CAR capable of specific binding to an immunoglobulin domain comprising the myc tag comprises a scFv fragment which is a polypeptide consisting of an heavy chain variable domain (VH), an light chain variable domain (VL) and a linker, wherein said variable domains and said linker have one of the following configurations in N-terminal to C-terminal direction: a) VH-linker-VL or b) VL-linker-VH. In a preferred embodiment, the scFv fragment has the configuration VH-linker-VL.

In another illustrative embodiment of the present invention, as a proof of concept, provided are CARs capable of specific binding to a modified immunoglobulin domain comprising the hapten tag Biotin.

In one embodiment the CAR capable of specific binding to an immunoglobulin domain comprising the hapten tag Biotin comprises the heavy chain complementarity determining regions (CDRs) of SEQ ID NO:67, SEQ ID NO:68 and SEQ ID NO:69 and the light chain CDRs of SEQ ID NO:70, SEQ ID NO:71 and SEQ ID NO:72.

In one preferred embodiment the CAR capable of specific binding to an immunoglobulin domain comprising the hapten tag Biotin comprises a heavy chain variable region comprising:

(a) a heavy chain complementarity determining region (CDR H) 1 amino acid sequence of GFNNKDTFFQ (SEQ ID NO:67);

(b) a CDR H2 amino acid sequence of RIDPANGFTKYAQKFQG (SEQ ID NO:68);

(c) a CDR H3 amino acid sequence of WDTYGAAWFAY (SEQ ID NO:69);

and a light chain variable region comprising:

(d) a light chain (CDR L)1 amino acid sequence of RASGNIHNYLS (SEQ ID NO:70);

(e) a CDR L2 amino acid sequence of SAKTLAD (SEQ ID NO:71); and

(f) a CDR L3 amino acid sequence of QHFWSSIYT (SEQ ID NO:72).

In one embodiment the CAR capable of specific binding to an immunoglobulin domain comprising the hapten tag Biotin comprises a heavy chain variable region (VH) comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO:75 and a light chain variable region (VL) comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO:76.

In one embodiment the CAR capable of specific binding to an immunoglobulin domain comprising the hapten tag Biotin comprises a heavy chain variable region (VH) comprising the amino acid sequence of SEQ ID NO:75, and a light chain variable region (VL) comprising the amino acid sequence of SEQ ID NO:76.

In one embodiment, the at least one antigen binding moiety is a scFv, a Fab, a crossFab or a scFab fragment.

In one embodiment the CAR capable of specific binding to an immunoglobulin domain comprising the hapten tag Biotin comprises a scFv fragment which is a polypeptide consisting of an heavy chain variable domain (VH), an light chain variable domain (VL) and a linker, wherein said variable domains and said linker have one of the following configurations in N-terminal to C-terminal direction: a) VH-linker-VL or b) VL-linker-VH. In a preferred embodiment, the scFv fragment has the configuration VH-linker-VL.

In a preferred embodiment the CAR capable of specific binding to an immunoglobulin domain comprising the hapten tag Biotin comprises an scFv fragment comprising the amino acid sequence of SEQ ID NO:74.

In another illustrative embodiment of the present invention, as a proof of concept, provided are CARs capable of specific binding to a modified immunoglobulin domain comprising a polypeptide tag from the human GCN4 protein. In one embodiment, the polypeptide tag from the human GCN4 protein comprises the amino acid sequence of YHLENEVARLKK (SEQ ID NO:102).

In one embodiment the CAR capable of specific binding to an immunoglobulin domain comprising the GCN4 tag comprises the heavy chain complementarity determining regions (CDRs) of SEQ ID NO:90, SEQ ID NO:91 and SEQ ID NO:92 and the light chain CDRs of SEQ ID NO:93, SEQ ID NO:94 and SEQ ID NO:95.

In one preferred embodiment the CAR capable of specific binding to an immunoglobulin domain comprising the GCN4 tag comprises a heavy chain variable region comprising:

(a) a heavy chain complementarity determining region (CDR H) 1 amino acid sequence of DYGVN (SEQ ID NO:90);

(b) a CDR H2 amino acid sequence of VIWGDGITDHNSALKS (SEQ ID NO:91);

(c) a CDR H3 amino acid sequence of GLFDY (SEQ ID NO:92);

and a light chain variable region comprising:

(d) a light chain (CDR L)1 amino acid sequence of RSSTGAVTTSNYAS (SEQ ID NO:93);

(e) a CDR L2 amino acid sequence of GTNNRAP (SEQ ID NO:94); and

(f) a CDR L3 amino acid sequence of VLWYSNHWV (SEQ ID NO:95).

In one embodiment the CAR capable of specific binding to an immunoglobulin domain comprising the GCN4 tag comprises a heavy chain variable region (VH) comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence selected of SEQ ID NO:98 and a light chain variable region (VL) comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO:99.

In one embodiment the CAR capable of specific binding to an immunoglobulin domain comprising the GCN4 tag comprises a heavy chain variable region (VH) comprising the amino acid sequence of SEQ ID NO:98, and a light chain variable region (VL) comprising the amino acid sequence of SEQ ID NO:99.

In one embodiment, the at least one antigen binding moiety is a scFv, a Fab, a crossFab or a scFab fragment. In one embodiment the CAR capable of specific binding to an immunoglobulin domain comprising the GCN4 tag comprises a Fab fragment

In one embodiment the CAR capable of specific binding to an immunoglobulin domain comprising the GCN4 tag comprises a scFv fragment which is a polypeptide consisting of an heavy chain variable domain (VH), an light chain variable domain (VL) and a linker, wherein said variable domains and said linker have one of the following configurations in N-terminal to C-terminal direction: a) VH-linker-VL or b) VL-linker-VH. In a preferred embodiment, the scFv fragment has the configuration VL-linker-VH.

Fab and scFab fragments are stabilized via the natural disulfide bond between the CL domain and the CH1 domain. Antigen binding moieties comprising a heavy chain variable domain (VH) and a light chain variable domain (VL), such as the Fab, crossFab, scFv and scFab fragments as described herein might be further stabilized by introducing interchain disulfide

bridges between the VH and the VL domain. Accordingly, in one embodiment, the Fab fragment(s), the crossFab fragment(s), the scFv fragment(s) and/or the scFab fragment(s) comprised in the antigen binding receptors according to the invention might be further stabilized by generation of interchain disulfide bonds via insertion of cysteine residues (e.g., position 44 in the variable heavy chain and position 100 in the variable light chain according to Kabat numbering). Such stabilized antigen binding moieties are herein referred to by the term "ds".

Haptens can be coupled covalently or non-covalently to the recognition domain according to methods known in the art. For example biotinylation is widely used in the art to couple the hapten Biotin to a polypeptide, e.g., an immunoglobulin. Biotin is typically conjugated to proteins via primary amines (e.g., lysine). For IgG antibodies, usually, between 3 and 6 biotin molecules are conjugated per antibody molecule. Alternatively, carbohydrates can be biotinylated according to methods known in the art.

In one embodiment, the hapten molecule is coupled to the recognition domain using site-directed coupling. In one embodiment, introduction of a polypeptide tag in the antigen binding molecule is combined with site-directed coupling of a hapten molecule to the polypeptide tag. In such embodiments of the present invention, the number of hapten molecules coupled to the antigen binding molecule can be controlled, e.g., by providing a defined number of coupling sites in the polypeptide tag. An example of a site-directed coupling technology is the AviTag system which is known in the art. The polypeptide tag AviTag of GLNDIFEAQKIEWH (SEQ ID NO:103) comprises a natural biotinylation site which can be selectively biotinylated using the BirA Biotin-protein ligase. In one embodiment, the recognition domain comprises a defined number of hapten molecules. In one embodiment, the recognition domain does not comprise more than 1, 2, 3 or 4 hapten molecules. In a preferred embodiment, the recognition domain comprises two hapten molecules, e.g., the recognition domain is a Fc domain composed of two polypeptide molecules each comprising one hapten molecule. In another preferred embodiment, the recognition domain comprises one hapten molecule, e.g., the recognition domain is a Fab fragment comprising a hapten molecule either coupled to the heavy chain or the light chain fragment.

In one embodiment, the recognition domain comprises more than one species of hapten molecules. In such embodiments, the more than one species of hapten molecules can be either

coupled separately to the recognition domain or as one unit, e.g., a bridging adapter, comprising more than one hapten molecule. A non-limiting example of such embodiments of the invention, as a proof of concept, is provided in Example 6 and Figure 13. In such embodiments, one of the hapten molecules can be recognized by the respective anti-hapten CAR provided and used according to the invention, whereas the other hapten molecule can be coupled, non-covalently or covalently to the recognition domain. In one embodiment, the hapten molecule is non-covalently coupled to the recognition domain via an antigen binding moiety capable of specific binding to the hapten molecule. In one embodiment, the bridging adapter comprises a first hapten molecule and a second hapten molecule, wherein the first hapten molecule is capable of interacting with a CAR capable of specific binding to the first hapten molecule, and wherein the second hapten molecule is capable of interacting with to the recognition domain, wherein the recognition domain comprises an antigen binding moiety capable of specific binding to the second hapten molecule. In one embodiment, upon or after binding of the target antigen binding moiety to the target antigen, the CAR binds to the first hapten molecule and the antigen binding moiety attached to the recognition domain binds to the second hapten molecule. Accordingly, upon binding of the antigen binding molecule comprising the target antigen binding moiety to the target cell and binding of the CAR to the recognition domain via the bridging domain (e.g., the Biotin-Digoxigenin bridge), the reporter CAR-T cell becomes activated and the reporter gene is expressed. Expression of the reporter gene is therefore indicative for (specific) binding of the target antigen binding moiety in the context of T cell activation induced by an antigen binding molecule directed against a target antigen, e.g., on a tumor cell. Accordingly, in one embodiment, provided is a bridging Biotin-Digoxigenin adapter for use according to the invention.

The CARs as provided and used herein comprise an extracellular domain comprising an antigen binding moiety capable of specific binding to the recognition domain, an anchoring transmembrane domain and at least one intracellular signaling and/or at least one co-stimulatory signaling domain. The anchoring transmembrane domain mediates confinement of the CAR to the cell membrane of the effector cell, e.g., the T cell. The intracellular signaling and/or at least one co-stimulatory signaling domain transfer the binding of the CAR to the recognition domain to an intracellular signal, e.g., T cell activation, which can be assessed by measuring reporter gene expression. In the context of the present invention, expression of the reporter gene as described herein is indicative for binding of the target

antigen binding moiety to the target antigen and resulting T cell activation as described herein.

The anchoring transmembrane domain of the CAR may be characterized by not having a cleavage site for mammalian proteases. Proteases refer to proteolytic enzymes that are able to hydrolyze the amino acid sequence of a transmembrane domain comprising a cleavage site for the protease. The term proteases include both endopeptidases and exopeptidases. In the context of the present invention any anchoring transmembrane domain of a transmembrane protein as laid down among others by the CD-nomenclature may be used to generate a CAR suitable according to the invention, which activates T cells, upon binding to a recognition domain, e.g., a modified immunoglobulin domain, as defined herein.

Accordingly, in the context of the present invention, the anchoring transmembrane domain may comprise part of a murine/mouse or preferably of a human transmembrane domain. An example for such an anchoring transmembrane domain is a transmembrane domain of CD28, for example, having the amino acid sequence as shown herein in SEQ ID NO:11 (as encoded by the DNA sequence shown in SEQ ID NO:24). In the context of the present invention, the transmembrane domain of the CAR may comprise/consist of an amino acid sequence as shown in SEQ ID NO:11 (as encoded by the DNA sequence shown in SEQ ID NO:24).

In an illustrative embodiment of the present invention, as a proof of concept, a CAR is used which comprises an antigen binding moiety comprising an amino acid sequence of SEQ ID NO:10 (as encoded by the DNA sequence shown in SEQ ID NO:22), and a fragment/polypeptide part of CD28 (the Uniprot Entry number of the human CD28 is P10747 (with the version number 173 and version 1 of the sequence)) as shown herein as SEQ ID NO:95 (as encoded by the DNA sequence shown in SEQ ID NO:108). Alternatively, any protein having a transmembrane domain, as provided among others by the CD nomenclature, may be used as an anchoring transmembrane domain of the CAR provided and used in the invention. As described above, the CAR may comprise the anchoring transmembrane domain of CD28 which is located at amino acids 153 to 179, 154 to 179, 155 to 179, 156 to 179, 157 to 179, 158 to 179, 159 to 179, 160 to 179, 161 to 179, 162 to 179, 163 to 179, 164 to 179, 165 to 179, 166 to 179, 167 to 179, 168 to 179, 169 to 179, 170 to 179, 171 to 179, 172 to 179, 173 to 179, 174 to 179, 175 to 179, 176 to 179, 177 to 179 or 178 to 179 of the human full length CD28 protein as shown in SEQ ID NO:109 (as encoded by the cDNA shown in SEQ ID NO:108). Accordingly, in context of the present invention the anchoring transmembrane domain may comprise or consist of an amino acid sequence as shown in SEQ ID NO:11 (as encoded by the DNA sequence shown in SEQ ID NO:24).

As described herein, the CAR used according to the invention comprises at least one stimulatory signaling and/or co-stimulatory signaling domain. The stimulatory signaling and/or co-stimulatory signaling domain transduce the binding of the antigen binding molecule comprising the target antigen binding moiety to an intracellular signal in the reporter CAR-T cell. Accordingly, the CAR preferably comprises a stimulatory signaling domain, which provides T cell activation. In a preferred embodiment, binding of the target antigen binding moiety to the target antigen and binding of the reporter CAR-T cell to the antigen binding molecule comprising the target antigen binding moiety leads to activation of the intracellular signaling and/or co-signaling domain. In certain embodiments, the herein provided CAR comprises a stimulatory signaling domain which is a fragment/polypeptide part of murine/mouse or human CD3z (the UniProt Entry of the human CD3z is P20963 (version number 177 with sequence number 2; the UniProt Entry of the murine/mouse CD3z is P24161 (primary citable accession number) or Q9D3G3 (secondary citable accession number) with the version number 143 and the sequence number 1)), FCGR3A (the UniProt Entry of the human FCGR3A is P08637 (version number 178 with sequence number 2)), or NKG2D (the UniProt Entry of the human NKG2D is P26718 (version number 151 with sequence number 1); the UniProt Entry of the murine/mouse NKG2D is O54709 (version number 132 with sequence number 2)). Thus, the stimulatory signaling domain which is comprised in the CAR may be a fragment/polypeptide part of the full length of CD3z, FCGR3A or NKG2D. The amino acid sequence of the murine/mouse full length of CD3z is shown herein as SEQ ID NO:106 (murine/mouse as encoded by the DNA sequence shown in SEQ ID NO:107). The amino acid sequence of the human full length CD3z is shown herein as SEQ ID NO:104 (human as encoded by the DNA sequence shown in SEQ ID NO:105). The CAR provided and used according to the present invention may comprise fragments of CD3z, FCGR3A or NKG2D as stimulatory domain, provided that at least one signaling domain is comprised. In particular, any part/fragment of CD3z, FCGR3A, or NKG2D is suitable as stimulatory domain as long as at least one signaling motive is comprised. However, more preferably, the CAR comprises polypeptides which are derived from human origin. Preferably, the CAR comprises the amino acid sequence as shown herein as SEQ ID NO:104 (CD3z) (human as encoded by the DNA sequences shown in SEQ ID NO:105 (CD3z)). For example, the fragment/polypeptide part of the human CD3z which may be comprised in the CAR may comprise or consist of the amino acid sequence shown in SEQ ID NO:13 (as encoded by the DNA sequence shown in SEQ ID NO:26). Accordingly, in one embodiment the CAR comprises the sequence as shown in SEQ ID NO:13 or a sequence which has up to 1, 2, 3, 4,

5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 23, 24, 25, 26, 27, 28, 29 or 30 substitutions, deletions or insertions in comparison to SEQ ID NO:13 and which is characterized by having a stimulatory signaling activity. Specific configurations of CARs comprising a stimulatory signaling domain are provided herein below and in the Examples and Figures. The stimulatory signaling activity can be determined; e.g., by enhanced cytokine release, as measured by ELISA (IL-2, IFNγ, TNFα), enhanced proliferative activity (as measured by enhanced cell numbers), or enhanced lytic activity as measured by LDH release assays.

The CAR preferably comprises at least one co-stimulatory signaling domain which provides additional activity to the reporter CAR-T cell. The CAR may comprise a co-stimulatory signaling domain which is a fragment/polypeptide part of murine/mouse or human CD28 (the UniProt Entry of the human CD28 is P10747 (version number 173 with sequence number 1); the UniProt Entry of the murine/mouse CD28 is P31041 (version number 134 with sequence number 2)), CD137 (the UniProt Entry of the human CD137 is Q07011 (version number 145 with sequence number 1); the UniProt Entry of murine/mouse CD137 is P20334 (version number 139 with sequence number 1)), OX40 (the UniProt Entry of the human OX40 is P23510 (version number 138 with sequence number 1); the UniProt Entry of murine/mouse OX40 is P43488 (version number 119 with sequence number 1)), ICOS (the UniProt Entry of the human ICOS is Q9Y6W8 (version number 126 with sequence number 1)); the UniProt Entry of the murine/mouse ICOS is Q9WV40 (primary citable accession number) or Q9JL17 (secondary citable accession number) with the version number 102 and sequence version 2)), CD27 (the UniProt Entry of the human CD27 is P26842 (version number 160 with sequence number 2); the Uniprot Entry of the murine/mouse CD27 is P41272 (version number 137 with sequence version 1)), 4-1-BB (the UniProt Entry of the murine/mouse 4-1-BB is P20334 (version number 140 with sequence version 1); the UniProt Entry of the human 4-1-BB is Q07011 (version number 146 with sequence version)), DAP10 (the UniProt Entry of the human DAP10 is Q9UBJ5 (version number 25 with sequence number 1); the UniProt entry of the murine/mouse DAP10 is Q9QUJ0 (primary citable accession number) or Q9R1E7 (secondary citable accession number) with the version number 101 and the sequence number 1)) or DAP12 (the UniProt Entry of the human DAP12 is O43914 (version number 146 and the sequence number 1); the UniProt entry of the murine/mouse DAP12 is O054885 (primary citable accession number) or Q9R1E7 (secondary citable accession number) with the version number 123 and the sequence number 1). In certain embodiments the CAR may comprise one or more, i.e., 1, 2, 3, 4, 5, 6 or 7 of the herein defined co-stimulatory signaling domains.

Accordingly, in the context of the present invention, the CAR may comprise a fragment/polypeptide part of a murine/mouse or preferably of a human CD28 as first co-stimulatory signaling domain and the second co-stimulatory signaling domain is selected from the group consisting of the murine/mouse or preferably of the human CD27, CD28, CD137, OX40, ICOS, DAP10 and DAP12, or fragments thereof. Preferably, the CAR comprises a co-stimulatory signaling domain which is derived from a human origin. Thus, more preferably, the co-stimulatory signaling domain(s) which is (are) comprised in the CAR may comprise or consist of the amino acid sequence as shown in SEQ ID NO:12 (as encoded by the DNA sequence shown in SEQ ID NO:25).

Thus, the co-stimulatory signaling domain which may be optionally comprised in the CAR is a fragment/polypeptide part of the full length CD27, CD28, CD137, OX40, ICOS, DAP10 and DAP12. The amino acid sequence of the murine/mouse full length CD28 is shown herein as SEQ ID NO:111 (murine/mouse as encoded by the DNA sequences shown in SEQ ID NO:110). However, because human sequences are most preferred in the context of the present invention, the co-stimulatory signaling domain which may be optionally comprised in the CAR protein is a fragment/polypeptide part of the human full length CD27, CD28, CD137, OX40, ICOS, DAP10 or DAP12. The amino acid sequence of the human full length CD28 is shown herein as SEQ ID NO:109 (human as encoded by the DNA sequence shown in SEQ ID NO:108).

In one preferred embodiment, the CAR comprises CD28 or a fragment thereof as co-stimulatory signaling domain. The CAR may comprise a fragment of CD28 as co-stimulatory signaling domain, provided that at least one signaling domain of CD28 is comprised. In particular, any part/fragment of CD28 is suitable for the CAR as long as at least one of the signaling motives of CD28 is comprised. For example, the CD28 polypeptide which is comprised in the CAR may comprise or consist of the amino acid sequence shown in SEQ ID NO:12 (as encoded by the DNA sequence shown in SEQ ID NO:25). In the present invention the intracellular domain of CD28, which functions as a co-stimulatory signaling domain, may comprise a sequence derived from the intracellular domain of the CD28 polypeptide having the sequence(s) YMNM (SEQ ID NO:112) and/or PYAP (SEQ ID NO:113). Preferably, the CAR comprises polypeptides which are derived from human origin. For example, the fragment/polypeptide part of the human CD28 which may be comprised in the CAR may comprise or consist of the amino acid sequence shown in SEQ ID NO:12 (as encoded by the DNA sequence shown in SEQ ID NO:25). Accordingly, in one embodiment, the CAR comprises the sequence as shown in SEQ ID NO:12 or a sequence which has up to 1, 2, 3, 4,

5, 6, 7, 8, 9 or 10 substitutions, deletions or insertions in comparison to SEQ ID NO:12 and which is characterized by having a co-stimulatory signaling activity. Specific configurations of CARs comprising a co-stimulatory signaling domain (CSD) are provided herein below and in the Examples and Figures. The co-stimulatory signaling activity can be determined; e.g., by enhanced cytokine release, as measured by ELISA (IL-2, IFNγ, TNFα), enhanced proliferative activity (as measured by enhanced cell numbers), or enhanced lytic activity as measured by LDH release assays.

As mentioned above, in an embodiment of the present invention, the co-stimulatory signaling domain of the CAR may be derived from the human CD28 gene (Uni Prot Entry No: P10747 (accession number with the entry version: 173 and version 1 of the sequence)) and provides CD28 activity, defined as cytokine production, proliferation and lytic activity of the transduced cell described herein, like a transduced T cell. CD28 activity can be measured by release of cytokines by ELISA or flow cytometry of cytokines such as interferon-gamma (IFN-γ) or interleukin 2 (IL-2), proliferation of T cells measured e.g., by ki67-measurement, cell quantification by flow cytometry, or lytic activity as assessed by real time impedence measurement of the target cell (by using e.g., an ICELLligence instrument as described e.g., in Thakur et al., Biosens Bioelectron. 35(1) (2012), 503-506; Krutzik et al., Methods Mol Biol. 699 (2011), 179-202; Ekkens et al., Infect Immun. 75(5) (2007), 2291-2296; Ge et al., Proc Natl Acad Sci U S A. 99(5) (2002), 2983-2988; Düwell et al., Cell Death Differ. 21(12) (2014), 1825-1837, Erratum in: Cell Death Differ. 21(12) (2014), 161). The co-stimulatory signaling domains PYAP and YMNM are beneficial for the function of the CD28 polypeptide and the functional effects enumerated above. The amino acid sequence of the YMNM domain is shown in SEQ ID NO:112; the amino acid sequence of the PYAP domain is shown in SEQ ID NO:113. Accordingly, in the CAR as provided and used herein, the CD28 polypeptide preferably comprises a sequence derived from intracellular domain of a CD28 polypeptide having the sequences YMNM (SEQ ID NO:112) and/or PYAP (SEQ ID NO:113). These signaling motives may, be present at any site within the intracellular domain of the CARs.

The extracellular domain comprising at least one antigen binding moiety capable of specific binding to the recognition domain, the anchoring transmembrane domain that does not have a cleavage site for mammalian proteases, the co-stimulatory signaling domain and the stimulatory signaling domain may be comprised in a single-chain multi-functional polypeptide. A single-chain fusion construct e.g., may consist of (a) polypeptide(s) comprising (an) extracellular domain(s) comprising at least one antigen binding moiety

capable of specific binding to a modified immunoglobulin domain, (an) anchoring transmembrane domain(s), (a) co-stimulatory signaling domain(s) and/or (a) stimulatory signaling domain(s). In alternative embodiments, the CAR comprises an antigen binding moiety which is not a single chain fusion construct, i.e., the antigen binding moiety capable of specific binding to a modified immunoglobulin is a Fab or a crossFab fragment. In such embodiments the CAR is not a single chain fusion construct comprising only one polypeptide chain. Preferably such constructs will comprise a single chain heavy chain fusion polypeptide combined with an immunoglobulin light chain, e.g., the heavy chain fusion polypeptide comprises (an) immunoglobulin heavy chain(s), (an) anchoring transmembrane domain(s), (a) co-stimulatory signaling domain(s) and/or (a) stimulatory signaling domain(s) and is combined with (an) immunoglobulin light chain(s). Accordingly, the extracellular domain, the anchoring transmembrane domain, the co-stimulatory signaling domain and the stimulatory signaling domain may be connected by one or more identical or different peptide linker. For example, the linker between the extracellular domain comprising at least one antigen binding moiety capable of specific binding to the recognition domain and the anchoring transmembrane domain may comprise or consist of the amino acid sequence as shown in SEQ ID NO:17. Accordingly, the anchoring transmembrane domain, the co-stimulatory signaling domain and/or the stimulatory domain may be connected to each other by peptide linkers or alternatively, by direct fusion of the domains.

In some embodiments, the antigen binding moiety comprised in the extracellular domain is a single-chain variable fragment (scFv) which is a fusion protein of the variable regions of the heavy (VH) and light chains (VL) of an antibody, connected with a short linker peptide of ten to about 25 amino acids. The linker is usually rich in glycine for flexibility, as well as serine or threonine for solubility, and can either connect the N-terminus of the VH with the C-terminus of the VL, or *vice versa*. For example, the linker may have the amino and amino acid sequence as shown in SEQ ID NO:16. A scFv antigen binding moiety retains the specificity of the original antibody, despite removal of the constant regions and the introduction of the linker. scFv antibodies are, e.g., described in Houston, J.S., Methods in Enzymol. 203 (1991) 46-96).

The CAR or parts thereof may comprise a signal peptide. Such a signal peptide will bring the protein to the surface of the T cell membrane. For example, the signal peptide may have the amino and amino acid sequence as shown in SEQ ID NO:114 (as encoded by the DNA sequence shown in SEQ ID NO:115).

The components of the CARs can be fused to each other in a variety of configurations to generate T cell activating CARs.

In some embodiments, the CAR comprises an extracellular domain composed of a heavy chain variable domain (VH) and a light chain variable domain (VL) connected to an anchoring transmembrane domain. In some embodiments, the VH domain is fused at the C-terminus to the N-terminus of the VL domain, optionally through a peptide linker. In other embodiments, the CAR further comprises a stimulatory signaling domain and/or a co-stimulatory signaling domain. In a specific such embodiment, the CAR essentially consists of a VH domain and a VL domain, an anchoring transmembrane domain, and optionally a stimulatory signaling domain connected by one or more peptide linkers, wherein the VH domain is fused at the C-terminus to the N-terminus of the VL domain, and the VL domain is fused at the C-terminus to the N-terminus of the anchoring transmembrane domain, wherein the anchoring transmembrane domain is fused at the C-terminus to the N-terminus of the stimulatory signaling domain. Optionally, the CAR further comprises a co-stimulatory signaling domain. In one such specific embodiment, the antigen binding receptor essentially consists of a VH domain and a VL domain, an anchoring transmembrane domain, a stimulatory signaling domain and a co-stimulatory signaling domain connected by one or more peptide linkers, wherein the VH domain is fused at the C-terminus to the N-terminus of the VL domain, and the VL domain is fused at the C-terminus to the N-terminus of the anchoring transmembrane domain, wherein the anchoring transmembrane domain is fused at the C-terminus to the N-terminus of the stimulatory signaling domain, wherein the stimulatory signaling domain is fused at the C-terminus to the N-terminus of the co-stimulatory signaling domain. In an alternative embodiment, the co-stimulatory signaling domain is connected to the anchoring transmembrane domain instead of the stimulatory signaling domain. In a preferred embodiment, the CAR essentially consists of a VH domain and a VL domain, an anchoring transmembrane domain, a co-stimulatory signaling domain and a stimulatory signaling domain connected by one or more peptide linkers, wherein the VH domain is fused at the C-terminus to the N-terminus of the VL domain, and the VL domain is fused at the C-terminus to the N-terminus of the anchoring transmembrane domain, wherein the anchoring transmembrane domain is fused at the C-terminus to the N-terminus of the co-stimulatory signaling domain, wherein the co-stimulatory signaling domain is fused at the C-terminus to the N-terminus of the stimulatory signaling domain.

In preferred embodiments, one of the binding moieties is a Fab fragment or a crossFab fragment. In one preferred embodiment, the antigen binding moiety is fused at the C-terminus

of the Fab or crossFab heavy chain to the N-terminus of the anchoring transmembrane domain, optionally through a peptide linker. In an alternative embodiment, the antigen binding moiety is fused at the C-terminus of the Fab or crossFab light chain to the N-terminus of the anchoring transmembrane domain, optionally through a peptide linker. In other embodiments, the CAR further comprises a stimulatory signaling domain and/or a co-stimulatory signaling domain. In a specific such embodiment, the CAR essentially consists of a Fab or crossFab fragment, an anchoring transmembrane domain, and optionally a stimulatory signaling domain connected by one or more peptide linkers, wherein the Fab or crossFab fragment is fused at the C-terminus of the heavy or light chain to the N-terminus of the anchoring transmembrane domain, wherein the anchoring transmembrane domain is fused at the C-terminus to the N-terminus of the stimulatory signaling domain. Preferably, the CAR further comprises a co-stimulatory signaling domain. In one such embodiment, the CAR essentially consists of a Fab or crossFab fragment, an anchoring transmembrane domain, a stimulatory signaling domain and a co-stimulatory signaling domain connected by one or more peptide linkers, wherein the Fab or crossFab fragment is fused at the C-terminus of the heavy or light chain to the N-terminus of the anchoring transmembrane domain, wherein the stimulatory signaling domain is fused at the C-terminus to the N-terminus of the co-stimulatory signaling domain. In a preferred embodiment, the co-stimulatory signaling domain is connected to the anchoring transmembrane domain instead of the stimulatory signaling domain. In a most preferred embodiment, the CAR essentially consists of a Fab or crossFab fragment, an anchoring transmembrane domain, a co-stimulatory signaling domain and a stimulatory signaling domain, wherein the Fab or crossFab fragment is fused at the C-terminus of the heavy chain to the N-terminus of the anchoring transmembrane domain through a peptide linker, wherein the anchoring transmembrane domain is fused at the C-terminus to the N-terminus of the co-stimulatory signaling domain, wherein the co-stimulatory signaling domain is fused at the C-terminus to N-terminus of the stimulatory signaling domain.

The antigen binding moiety, the anchoring transmembrane domain and the stimulatory signaling and/or co-stimulatory signaling domains may be fused to each other directly or through one or more peptide linker, comprising one or more amino acids, typically about 2-20 amino acids. Peptide linkers are known in the art and are described herein. Suitable, non-immunogenic peptide linkers include, for example, $(G_4S)_n$, $(SG_4)_n$, $(G_4S)_n$ or $G_4(SG_4)_n$ peptide linkers, wherein "n" is generally a number between 1 and 10, typically between 2 and 4. A preferred peptide linker for connecting the antigen binding moiety and the anchoring

transmembrane moiety is GGGGS (G_4S) according to SEQ ID NO 17. An exemplary peptide linker suitable for connecting variable heavy chain (VH) and the variable light chain (VL) is GGGSGGGSGGGSGGGS $(G_4S)_4$ according to SEQ ID NO 16.

Additionally, linkers may comprise (a portion of) an immunoglobulin hinge region. Particularly where an antigen binding moiety is fused to the N-terminus of an anchoring transmembrane domain, it may be fused via an immunoglobulin hinge region or a portion thereof, with or without an additional peptide linker.

As described herein, the CARs provided and used according to the present invention comprise an extracellular domain comprising at least one antigen binding moiety. A CAR with a single antigen binding moiety capable of specific binding to a recognition domain is useful and preferred, particularly in cases where high expression of the CAR is needed. In such cases, the presence of more than one antigen binding moiety specific for the target cell antigen may limit the expression efficiency of the CAR. In other cases, however, it will be advantageous to have a CAR comprising two or more antigen binding moieties specific for a target cell antigen, for example to optimize targeting to the target site or to allow crosslinking of target cell antigens.

In the context of the methods according to the invention, contacting the antigen binding molecule comprising the target antigen binding moiety with a target cell comprising the target antigen on the surface and contacting the antigen binding molecule with the CAR comprising an antigen binding moiety capable of specific binding to the recognition domain leads to expression of the reporter gene as described herein. Accordingly, in one embodiment, activation of the intracellular signaling and/or co-signaling domain as described herein leads to activation of a response element as herein described. In a preferred embodiment, the response element controls the expression of the reporter gene. In one embodiment, upon or after binding of the target antigen binding moiety to the target antigen, the CAR binds to the recognition domain, e.g., the modified immunoglobulin domain, wherein the response element activates the expression of a reporter gene as described herein. In a preferred embodiment, activation of the response element leads to expression of the reporter gene. Accordingly, the reporter gene in the reporter cells (e.g., the reporter CAR-T cell) is expressed upon binding of the target antigen binding moiety to the target antigen and binding of the CAR to the recognition domain of the molecule comprising the (candidate) target antigen binding moiety. In one embodiment, the expression of the reporter gene is indicative for binding of the target antigen binding moiety to the target antigen. In this context, the

binding of the antigen binding molecule to the CAR elicits a cellular response which results in a modulation of the activity of the response element, either directly or through a cascade of cell signaling. The response element is a DNA element which can be silenced or activated by transcription factors or the like. Response elements are known in the art and are commercially available, e.g., in reporter vectors. Usually the response element comprises DNA repeat elements and is a cis-acting enhancer element located upstream of a minimal promotor which drives expression of a reporter gene upon transcription factor binding.

Binding of the CAR to the recognition domain, e.g., the modified Fc domain or Fab fragment, activates the response element. In one embodiment the response element is a nuclear response element located in the nucleus of the cell. In another embodiment said response element is located on a plasmid in the reporter cell. In one embodiment the assay comprises the preliminary step of transfection of the reporter cells, e.g., a CAR-T cell, with an expression vector comprising the DNA sequence coding for the reporter gene under the control of the response element. Additionally, the reporter cells can be transfected with an expression vector comprising the DNA sequence coding for the CAR. The reporter cells can be transfected with an expression vector comprising all elements of the signaling cascade or with different vectors individually expressing the different components. In one embodiment, the reporter cells comprise the DNA sequence coding for the reporter gene under the control of the response element, and the DNA sequence coding for the CAR.

Accordingly, as described herein, the CAR is functionally linked to a response element. In one embodiment, the response element controls the expression of the reporter gene. In one embodiment the response element is part of the NFAT pathway, the NF-κB pathway or the AP-1 pathway, preferably, the NFAT pathway.

In one embodiment the reporter gene is selected from a gene coding for a fluorescent protein or a gene coding for an enzyme whose catalytic activity can be detected. In one embodiment, the reporter gene is coding for a luminescent protein. In further embodiments the fluorescent protein is selected from the group consisting of green fluorescent protein (GFP), yellow fluorescent protein (YFP), red fluorescent protein (RFP), Blue fluorescent protein (BFP, Heim et al. 1994, 1996), a cyan fluorescent variant known as CFP (Heim et al. 1996; Tsien 1998); a yellow fluorescent variant known as YFP (Ormo et al. 1996; Wachter et al. 1998); a violet-excitable green fluorescent variant known as Sapphire (Tsien 1998; Zapata-Hommer et al. 2003); and a cyan-excitable green fluorescing variant known as enhanced green fluorescent protein or EGFP (Yang et al. 1996) enhanced green fluorescent protein (EGFP) and can be measured e.g., by live cell imaging (e.g., Incucyte) or fluorescent spectrophotometry. In one

embodiment the enzyme whose catalytic activity can be detected is selected from the group consisting of luciferase, beta Galactosidase and Alkaline Phosphatase. In one embodiment the reporter gene is encoding for GFP. In a preferred embodiment the reporter gene is encoding for luciferase. The activity of luciferase can be detected by commercially available assays, e.g., by the Luciferase 1000 Assay System or the ONE-Glo™ Luciferase Assay System (both Promega). The Luciferase 1000 Assay System contains coenzyme A (CoA) besides luciferin as a substrate, resulting in a strong light intensity lasting for at least one minute. For assaying the intracellular luciferase, it is necessary to lyse the cells prior to detection. The light which is produced as a by-product of the reaction is collected by the luminometer from the entire visible spectrum. In the examples shown herein the signal was proportional to the amount of produced luciferase and therefore proportional to the strength of the activation of the NFAT promotor. In another embodiment a Luciferase assay is used wherein the luciferase is secreted from the cells. Hence the assay can be performed without lysis of the cells.

As described herein, the expression of the reporter gene can be directly correlated with the binding of the target antigen binding moiety to be tested and the resulting activation of the T cell, e.g., the reporter CAR-T cell. For example when using a gene encoding for luciferase as a reporter gene, the amount of light detected from the cells correlates directly with the target antigen binding and is indicative for the specificity of the target antigen binding moiety to be tested when compared to appropriate control situations. In one embodiment the antigen binding molecule comprising the target antigen binding moiety is applied in different concentrations and the half maximal effective concentration (EC50) of reporter gene activation is determined. EC50 refers to the concentration of the antigen binding molecule (e.g. the antibody) or ligand at which the antigen binding molecule activates or inhibits the reporter gene halfway between the baseline and maximum after a specified exposure time. The EC50 of the dose response curve therefore represents the concentration of the target antigen binding moiety where 50% of its maximal activating or inhibitory effect on the target antigen is observed.

In one embodiment, the target antigen is a cell surface antigen or receptor. In one embodiment, the target antigen is selected from the group consisting of CD20, CD38, CD138, CEA, EGFR, FolR1, HER2, LeY, MCSP, STEAP1, TYRP, and WT1, or a fragment thereof. However, the target antigen is not limited to proteins located on the cell surface but may also derive from polypeptides or proteins which are temporarily or permanently located intracellularly. In such cases, the target antigen deriving from an intracellular polypeptide or

protein can be presented on the cell surface by one or several molecules of the major histocompatibility complex (MHC). In one embodiment, the target antigen is a peptide bound to a molecule of the MHC. In one embodiment, the MHC is a human MHC. In one embodiment, the peptide bound to a molecule of the MHC has an overall length of between 8 and 100, preferably between 8 and 30, and more preferred between 8 and 16 amino acids. In one embodiment, the target antigen derives from a protein which is exclusively or mainly expressed in tumor tissue. In one embodiment, the protein is an intracellular protein and the peptide is generated by the MHC-I or MHC-II pathway and presented by a MHC class I or MHC class II complex. In one embodiment, the peptide is generated by the MHC-I pathway and presented by a MHC class I complex. In one embodiment, the target antigen binding moiety is a T cell receptor like (TCRL) antigen binding moiety. A TCRL antigen binding moiety is capable of specific binding to a peptide antigen which is exclusively or mainly expressed in tumor tissue, wherein the peptide antigen is bound to a molecule of the MHC located on the surface of a cell, particularly a cancer cell. In this context, the methods of the present invention are suitable to assess specificity of established or novel TCRL target antigen binding moieties in a high-throughput assay format.

The binding of the antigen binding molecule comprising the target antigen binding moiety to the target antigen can be determined qualitatively or qualitatively, i.e., by the presence or absence of the expression of the reporter gene; with the absence of any fluorescence or luminescense being indicative of no binding. For quantitative measurement of binding and activation the amount of reporter gene activation can be compared to a reference. Accordingly, the method as described herein may additionally comprise the step of comparing the level of expression of the reporter gene to a reference. A suitable reference usually comprises a negative control which is substantially identical to the referenced assay omitting one or several essential component(s) of the assay or method. For the methods of the invention the omitted component may be, e.g., omitting addition of the antigen binding molecule or omitting the target cell. Alternatively, a reporter CAR-T cell not capable of binding to the recognition domain of the antigen binding molecule can be used. In a preferred embodiment, the reference is expression of the reporter gene in absence of the antigen binding molecule. In specific embodiments, the expression of the reporter gene is at least 2x, 3x, 4x, 5x, 10x, 100x, 1000x, or 10000x, higher compared to the expression of the reporter gene in absence of the antigen binding molecule.

Alternatively, the absence of reporter gene expression can be defined by a certain threshold, i.e., after deduction of a background signal. The background signal is usually determined by

performing the assay with all reagents but the antigen binding molecule to be tested or in absence of the target cells. A novel target antigen binding moiety can, e.g., be selected according to the method of the invention by defining a threshold for baseline activation of the reporter gene expression and selecting the novel target antigen binding moiety if the level of expression of the reporter gene in the presence of the antigen binding molecule in relation to the expression of the reporter gene in absence of the target cell is higher than a predefined threshold value. Accordingly, the method as described herein may additionally comprise the step of selecting the novel target antigen binding moiety if the level of expression of the reporter gene in the presence of the antigen binding molecule in relation to the expression of the reporter gene in absence of the antigen binding molecule is higher than a predefined threshold value. In specific embodiments, the threshold value is 2, 3, 4, 5, 10, 100, 1000, or 10000.

The novel assay as described herein is robust, suitable for use in high-throughput format and efficient in terms of hands-on time needed to accomplish the assay. Furthermore, the assay of the present invention tolerates the presence of dead cells in the sample to be analyzed. This is in contrast to cell assays wherein the binding and functionality of an antigen binding molecule is determined by measuring cell viability or cell death, e.g., a killing assay.

One further advantage of the new assay described herein is that no washing steps are required. The antigen binding molecule to be tested and the reporter cells can be added to the target cells, e.g., tumor cells, in either order or at the same time. In one embodiment, the antigen binding molecule is diluted in cell culture medium and the tumor sample is added to the cell culture medium containing the diluted antigen binding molecule in a suitable cell culture format, e.g., in a well of a 24 well plate or in a well of a 96 well plate. Preferably the testing medium is a medium that provides conditions for cells to be viable for up to 48 hours. In one embodiment the assay is performed in a microtiter plate. In one embodiment the microtiter plate is suitable for high throughput screening. The assay of the present invention can be performed in any format that allows for rapid preparation, processing, and analysis of multiple reactions. This can be, for example, in multi-well assay plates (e.g., 24 wells, 96 wells or 384 wells). Stock solutions for various agents can be made manually or robotically, and all subsequent pipetting, diluting, mixing, distribution, washing, incubating, sample readout, data collection and analysis can be done robotically using commercially available analysis software, robotics, and detection instrumentation capable of detecting fluorescent and/or luminescent signals.

In one embodiment about 100000 to about 1000000 reporter CAR-T cells per well of a 24-well plate are provided in step c). In a preferred embodiment about 300000 to about 700000 cells or about 400000 to about 600000 reporter CAR-T cells per well of a 24-well plate are provided in step c). In one embodiment about 500000 reporter CAR-T cells per well of a 24-well plate are provided in step c). In one embodiment about 10000 to about 100000 reporter CAR-T per well of a 96-well plate are provided in step c). In a preferred embodiment about 30000 to about 70000 reporter CAR-T or about 40000 to about 60000 reporter CAR-T per well of a 96-well plate are provided in step c). In one embodiment about 50000 reporter CAR-T per well of a 96-well plate are provided in step c). In one embodiment about 3000 to about 30000 reporter CAR-T cells per well of a 384-well plate are provided in step c). In a preferred embodiment about 5000 to about 15000 cells or about 8000 to about 12000 reporter CAR-T cells per well of a 384-well plate are provided in step c). In one embodiment about 10000 reporter CAR-T cells per well of a 384-well plate are provided in step c). In one embodiment about 200000 to about 2000000 reporter CAR-T per ml of cell culture medium are provided in step c). In a preferred embodiment about 600000 to about 1400000 reporter CAR-T or about 800000 to about 1200000 reporter CAR-T per ml of cell culture medium are provided in step c). In one embodiment about 1000000 reporter CAR-T per ml of cell culture medium are provided in step c).

In one embodiment the antigen binding molecule is provided in step b) to achieve a final concentration of about 0.1 fg/ml to 10 µg/ml. In further embodiments the antigen binding molecule is provided in step b) to achieve a final concentration of about 1 fg/ml to about 1 µg/ml or about 1 pg/ml to about 1 µg/ml. In further embodiments the antigen binding molecule is provided in step b) to achieve a final concentration of about 0.1 ng/ml. In one embodiment the antigen binding molecule is provided in step b) to achieve a final concentration of about 1 nM to about 1000 nM. In further embodiments the antigen binding molecule is provided in step b) to achieve a final concentration of about 5 nM to about 200 nM or about 10 nM to about 100 nM. In further embodiments the antigen binding molecule is provided in step b) to achieve a final concentration of about 50 nM. The antigen binding molecule can be diluted in cell culture medium. The antigen binding molecule diluted to the final concentration as described herein is added to the target cells before or after adding the reporter cells. In one embodiment, the antigen binding molecule diluted to the final concentration as described herein is added to the target cells before adding the reporter cells. In one embodiment, the target cells are provided in cell culture inserts. In one embodiment, the target cells, e.g., tumor cells, are embedded in Matrigel.

In certain embodiments methods of the invention can be used to assess specificity of a novel target antigen binding moiety to be included in a T cell bispecific (TCB) format. The methods according to the present invention are particularly suitable to assess and select novel target antigen binding moieties for TCBs because the methods of the present invention measure T cell activation. It is a drawback of assays known to the art (e.g., binding assays) that the measured affinity does not always reflect the specificity in the TCB format. TCBs are highly potent molecules able to mediate T cell activation and killing already through binding affinities in the micromolar range. TCBs comprising a novel target antigen binding moiety therefore need to be highly selective to avoid unspecific reactivity, e.g., killing of target cells or alloreactivity. The methods as described in the present invention satisfy the high demands of such formats since the assay is based on T cell activation, i.e., a comparable mechanism of action. Accordingly, provided is a method as described herein, wherein high level of expression of the reporter gene in the presence of the antigen binding molecule and low level of expression of the reporter gene in the absence of the antigen binding molecule is indicative for high specificity of the target antigen binding moiety, in particular when the target antigen binding moiety is transferred into a T cell bispecific (TCB) antibody format. Furthermore, provided is a method for generating a TCB antibody, wherein the TCB antibody format comprises a first antigen binding moiety specific for a target antigen and a second antigen binding moiety capable of specific binding to a T cell activating receptor, wherein the first antigen binding moiety is selected according to the method as described herein, i.e., the first antigen binding moiety is assayed and selected as (candidate) target antigen binding moiety in the method of the present invention. In preferred embodiments, the T cell activating receptor is CD3.

In one such embodiment the TCB antibody comprises

(a) a first antigen binding moiety which is a Fab molecule capable of specific binding to a target cell antigen;

(b) a second antigen binding moiety which is a Fab molecule capable of specific binding to CD3.

In one exemplary embodiment, as a proof of concept, the TCB antibody comprises

(a) a first antigen binding moiety which is a Fab molecule capable of specific binding to a target cell antigen;

(b) a second antigen binding moiety which is a Fab molecule capable of specific binding to CD3, and which comprises the heavy chain complementarity determining

regions (CDRs) of SEQ ID NO:118, SEQ ID NO:119 and SEQ ID NO:120 and the light chain CDRs of SEQ ID NO:121, SEQ ID NO:122, SEQ ID NO:123.

A TCB antibody with a single antigen binding moiety capable of specific binding to a target cell antigen is useful, particularly in cases where internalization of the target cell antigen is to be expected following binding of a high affinity antigen binding moiety. In such cases, the presence of more than one antigen binding moiety specific for the target cell antigen may enhance internalization of the target cell antigen, thereby reducing its availability.

In many other cases, however, it will be advantageous to have a bispecific antibody comprising two or more antigen binding moieties specific for a target cell antigen, for example to optimize targeting to the target site.

Accordingly, in certain embodiments, the TCB antibody comprises a third antigen binding moiety capable of specific binding to a target cell antigen. In further embodiments, the third antigen binding moiety is a conventional Fab molecule, or a crossover Fab molecule wherein either the variable or the constant regions of the Fab light chain and the Fab heavy chain are exchanged. In one embodiment, the third antigen binding moiety is capable of specific binding to the same target cell antigen as the first antigen binding moiety. In a particular embodiment, the second antigen binding moiety is capable of specific binding to CD3, and the first and third antigen binding moieties are capable of specific binding to a target cell antigen. In a particular embodiment, the first and the third antigen binding moiety are identical (i.e., they comprise the same amino acid sequences) and are selected according to the method as described herein.

Furthermore provided are transduced T cells, i.e., reporter CAR-T cells, capable of expressing a CAR as described herein and their use in the methods according to the invention. The CAR relates to a molecule which is naturally not comprised in and/or on the surface of T cells and which is not (endogenously) expressed in or on normal (non-transduced) T cells. Thus, the CAR as used herein in and/or on T cells is artificially introduced into T cells. The CAR molecule, artificially introduced and subsequently presented in and/or on the surface of said T cells, e.g., reporter CAR-T cells, comprises domains comprising one or more antigen binding moiety accessible (*in vitro* or *in vivo*) to (Ig-derived) immunoglobulins, preferably antibodies, in particular to the Fc domain or Fab fragment of the antigen binding molecules used according to the invention. In this context, these artificially introduced molecules are presented in and/or on the surface of said T cells after transduction as described herein below. Accordingly, after transduction, T cells according to the disclosure can be activated by

immunoglobulins, preferably (therapeutic) antibodies comprising an antigen binding domain and a recognition domain.

Herein provided are also transduced T cells expressing a CAR encoded by (a) nucleic acid molecule(s) encoding the CAR as described herein. Accordingly, in the context of the present invention, the transduced cell may comprise a nucleic acid molecule encoding the CAR as provided and used herein.

In the context of the present invention, the term "transduced T cell" relates to a genetically modified T cell (i.e., a T cell wherein a nucleic acid molecule has been introduced deliberately). In particular, the nucleic acid molecule encoding the CAR as described herein can be stably integrated into the genome of the T cell by using a retroviral or lentiviral transduction. The extracellular domain of the CAR may comprise the complete extracellular domain of an antigen binding moiety as described herein but also parts thereof. The minimal size required being the antigen binding site of the antigen binding moiety in the CAR. The extracellular portion of the CAR (i.e., the extracellular domain comprising the antigen binding moiety) can be detected on the cell surface, while the intracellular portion (i.e., the co-stimulatory signaling domain(s) and the stimulatory signaling domain) are not detectable on the cell surface. The detection of the extracellular domain of the CAR can be carried out by using an antibody which specifically binds to this extracellular domain or by the recognition domain, e.g., the modified immunoglobulin domain, which the extracellular domain is capable to bind. The extracellular domain can be detected using these antibodies or recognition domains by flow cytometry or microscopy.

The transduced cells may be any immune cell. These include but are not limited to B-cells, T cells, Natural Killer (NK) cells, Natural Killer (NK) T cells, γδ T cells, innate lymphoid cells, macrophages, monocytes, dendritic cells, or neutrophils and immortalized cell lines thereof. Preferentially, said immune cell would be a lymphocyte, preferentially a NK or T cells. The said T cells include CD4 T cells and CD8 T cells. Triggering of the CAR on the surface of the leukocyte will render the cell responsive against a target cell in conjunction with an antigen binding molecule, e.g., a therapeutic antibody, comprising the recognition domain, e.g., a modified immunoglobulin domain, irrespective of the lineage the cell originated from. Activation will happen irrespective of the stimulatory signaling domain or co-stimulatory signaling domain chosen for the CAR and is not dependent on the exogenous supply of additional cytokines.

The transduced cell may be co-transduced with further nucleic acid molecules, e.g., with a nucleic acid molecule encoding a response element as described herein.

Specifically, the present disclosure relates to a method for the production of a reporter CAR-T cell expressing one or more CAR(s) and one or more response elements and reporter genes, comprising the steps of transducing a T cell with one or several vectors as described herein and culturing the transduced T cell under conditions allowing the expressing of the antigen binding receptor in or on said transduced cell.

Methods for transducing cells are known in the art and include, without being limited, in a case where nucleic acid or a recombinant nucleic acid is transduced, for example, an electroporation method, calcium phosphate method, cationic lipid method or liposome method. The nucleic acid to be transduced can, e.g., be transduced by using a commercially available transfection reagent, for example, Lipofectamine (manufactured by Invitrogen, catalogue no.: 11668027). In a case where a vector is used, the vector can be transduced in the same manner as the above-mentioned nucleic acid as long as the vector is a plasmid vector (i.e., a vector which is not a viral vector).

The transduced cell/cells is/are preferably grown under controlled conditions, outside of their natural environment. In particular, the term "culturing" means that cells (e.g., the transduced cell(s)) are *in vitro*. Culturing cells is a laboratory technique of keeping cells alive which are separated from their original tissue source. Herein, the transduced cell used according to the present invention is cultured under conditions allowing the expression of the introduce gene in or on said transduced cells. Conditions which allow the expression of a transgene are commonly known in the art.

A further aspect of the present disclosure is nucleic acids and vectors encoding one or several CARs used according to the present invention. The nucleic acid molecules may be under the control of regulatory sequences. For example, promoters, transcriptional enhancers and/or sequences which allow for induced expression of the CARs may be employed. In the context of the present invention, the nucleic acid molecules are expressed under the control of constitutive or inducible promoter. Suitable promoters are e.g., the CMV promoter (Qin et al., PLoS One 5(5) (2010), e10611), the UBC promoter (Qin et al., PLoS One 5(5) (2010), e10611), PGK (Qin et al., PLoS One 5(5) (2010), e10611), the EF1A promoter (Qin et al., PLoS One 5(5) (2010), e10611), the CAGG promoter (Qin et al., PLoS One 5(5) (2010), e10611), the SV40 promoter (Qin et al., PLoS One 5(5) (2010), e10611), the COPIA promoter (Qin et al., PLoS One 5(5) (2010), e10611), the ACT5C promoter (Qin et al., PLoS One 5(5) (2010), e10611), the TRE promoter (Qin et al., PLoS One. 5(5) (2010), e10611), the Oct3/4 promoter (Chang et al., Molecular Therapy 9 (2004), S367–S367 (doi: 10.1016/j.ymthe.2004.06.904)), or the Nanog promoter (Wu et al., Cell Res. 15(5) (2005),

317-24). Herein the term vector relates to a circular or linear nucleic acid molecule which can autonomously replicate in a cell (i.e., in a transduced cell) into which it has been introduced. Many suitable vectors are known to those skilled in molecular biology, the choice of which would depend on the function desired and include plasmids, cosmids, viruses, bacteriophages and other vectors used conventionally in genetic engineering. Methods which are well known to those skilled in the art can be used to construct various plasmids and vectors; see, for example, the techniques described in Sambrook et al. (loc cit.) and Ausubel, Current Protocols in Molecular Biology, Green Publishing Associates and Wiley Interscience, N.Y. (1989), (1994). Alternatively, the polynucleotides and vectors of the invention can be reconstituted into liposomes for delivery to target cells. As discussed in further details below, a cloning vector was used to isolate individual sequences of DNA. Relevant sequences can be transferred into expression vectors where expression of a particular polypeptide is required. Typical cloning vectors include pBluescript SK, pGEM, pUC9, pBR322, pGA18 and pGBT9. Typical expression vectors include pTRE, pCAL-n-EK, pESP-1, pOP13CAT.

In the context of the present invention the vector can be polycistronic. Such regulatory sequences (control elements) are known to the skilled person and may include a promoter, a splice cassette, translation initiation codon, translation and insertion site for introducing an insert into the vector(s). In the context of the present invention, said nucleic acid molecule(s) is (are) operatively linked to said expression control sequences allowing expression in eukaryotic or prokaryotic cells. It is envisaged that said vector(s) is (are) an expression vector(s) comprising the nucleic acid molecule(s) encoding the CAR as defined herein. Operably linked refers to a juxtaposition wherein the components so described are in a relationship permitting them to function in their intended manner. A control sequence operably linked to a coding sequence is ligated in such a way that expression of the coding sequence is achieved under conditions compatible with the control sequences. In case the control sequence is a promoter, it is obvious for a skilled person that double-stranded nucleic acid is preferably used.

In the context of the present invention the recited vector(s) is (are) an expression vector(s). An expression vector is a construct that can be used to transform a selected cell and provides for expression of a coding sequence in the selected cell. An expression vector(s) can for instance be cloning (a) vector(s), (a) binary vector(s) or (a) integrating vector(s). Expression comprises transcription of the nucleic acid molecule preferably into a translatable mRNA. Regulatory elements ensuring expression in prokaryotes and/or eukaryotic cells are well known to those skilled in the art. In the case of eukaryotic cells they comprise normally promoters ensuring

initiation of transcription and optionally poly-A signals ensuring termination of transcription and stabilization of the transcript. Possible regulatory elements permitting expression in prokaryotic host cells comprise, e.g., the PL, lac, trp or tac promoter in E. coli, and examples of regulatory elements permitting expression in eukaryotic host cells are the AOX1 or GAL1 promoter in yeast or the CMV-, SV40 , RSV-promoter (Rous sarcoma virus), CMV-enhancer, SV40-enhancer or a globin intron in mammalian and other animal cells.

Beside elements which are responsible for the initiation of transcription such regulatory elements may also comprise transcription termination signals, such as the SV40-poly-A site or the tk-poly-A site, downstream of the polynucleotide. Furthermore, depending on the expression system used leader sequences encoding signal peptides capable of directing the polypeptide to a cellular compartment or secreting it into the medium may be added to the coding sequence of the recited nucleic acid sequence and are well known in the art; see also, e.g., appended Examples.

The leader sequence(s) is (are) assembled in appropriate phase with translation, initiation and termination sequences, and preferably, a leader sequence capable of directing secretion of translated protein, or a portion thereof, into the periplasmic space or extracellular medium. Optionally, the heterologous sequence can encode a CAR including an N-terminal identification peptide imparting desired characteristics, e.g., stabilization or simplified purification of expressed recombinant product; see supra. In this context, suitable expression vectors are known in the art such as Okayama-Berg cDNA expression vector pcDV1 (Pharmacia), pCDM8, pRc/CMV, pcDNA1, pcDNA3 (Invitrogene), pEF-DHFR, pEF-ADA or pEF-neo (Raum et al. Cancer Immunol Immunother 50 (2001), 141-150) or pSPORT1 (GIBCO BRL).

The described nucleic acid molecule(s) or vector(s) which is (are) introduced in the T cell or its precursor cell may either integrate into the genome of the cell or it may be maintained extrachromosomally.

Exemplary embodiments

1. A method for assessing the specificity of a target antigen binding moiety capable of specific binding to a target antigen, the method comprising the steps of:
   a) providing an antigen binding molecule comprising an antigen binding domain and a recognition domain, wherein the antigen binding domain comprises the target antigen binding moiety, and wherein the recognition domain comprises a tag;

b) contacting the antigen binding molecule with a target cell comprising the target antigen on the surface, particularly wherein the target cell is a cancer cell;

c) contacting the antigen binding molecule with a chimeric antigen receptor (CAR) expressing reporter T (CAR-T) cell wherein the reporter CAR-T cell comprises:

i. a CAR capable of specific binding to the recognition domain comprising the tag, wherein the CAR is operationally coupled to a response element;

ii. a reporter gene under the control of the response element; and

d) determining T cell activation by measuring the expression of the reporter gene to establish the specificity of the target antigen binding moiety.

2. The method of embodiment 1, wherein the antigen binding domain and the recognition domain are immunoglobulin domains, or fragments thereof.
3. The method of any one of embodiments 1 or 2, wherein the antigen binding domain and the recognition domain are individually selected from the group consisting of an antibody, an Fc domain, a Fab fragment, a crossover Fab fragment, a single chain Fab fragment, a Fv fragment, a scFv fragment, a single-domain antibody, an aVH, or fragments thereof.
4. The method of any one of embodiments 1 to 3, wherein the antigen binding molecule is an IgG class antibody, particularly an IgG1 or IgG4 isotype antibody, or a fragment thereof.
5. The method of any one of embodiments 1 to 4, wherein the antigen binding domain is a Fab fragment and the recognition domain is an Fc domain.
6. The method of any one of embodiments 1 to 5, wherein the antigen binding domain and the recognition domain are the same domain, in particular a Fab fragment.
7. The method of any one of embodiments 1 to 6, wherein the CAR is capable of specific binding to the recognition domain comprising the tag but not capable of specific binding to the recognition domain not comprising the tag.
8. The method of any one of embodiments 1 to 7, wherein the tag is a hapten molecule.
9. The method of embodiment 8, wherein the hapten molecule is coupled to the recognition domain.
10. The method of any one of embodiments 1 to 9, wherein the hapten molecule is covalently coupled to the recognition domain.
11. The method of any one of embodiments 1 to 10, wherein the hapten molecule is non-covalently coupled to the recognition domain.

12. The method of any one of embodiments 1 to 11, wherein the recognition domain comprises a defined number of hapten molecules.
13. The method of any one of embodiments 1 to 12, wherein the recognition domain does not comprise more than 1, 2, 3 or 4 hapten molecules.
14. The method of any one of embodiments 1 to 13, wherein the recognition domain comprises more than one species of hapten molecules.
15. The method of any one of embodiments 1 to 14, wherein the hapten molecule is selected from the group consisting of Biotin, Digoxigenin (DIG) and Fluorescein (FITC).
16. The method of any one of embodiments 1 to 7, wherein the tag is a polypeptide tag.
17. The method of embodiment 16, wherein the polypeptide tag has a length of from 1 to 30 amino acids, from 1 to 25 amino acids, from 1 to 20 amino acids, from 1 to 15 amino acids or from 1 to 10 amino acids.
18. The method of any one of embodiments 16 or 17, wherein the polypeptide tag is connected at the C-terminus to the N-terminus of the recognition domain, optionally through a peptide linker.
19. The method of any one of embodiments 16 or 17, wherein the polypeptide tag is connected at the N-terminus to the C-terminus of the recognition domain, optionally through a peptide linker.
20. The method of any one of embodiments 16 to 19, wherein the polypeptide tag is selected from the group consisting of myc-tag, HA-tag, AviTag, FLAG-tag, His-tag, GCN4-tag, and NE-tag.
21. The method of any one of embodiments 1 to 20, wherein the target antigen binding moiety is a Fab fragment, in particular a Fab fragment deriving from a phage display library screening.
22. The method of any one of embodiments 1 to 21, wherein the CAR comprises at least one intracellular stimulatory signaling and/or co-stimulatory signaling domain.
23. The method of embodiment 22, wherein binding of the target antigen binding moiety to the target antigen and binding of the reporter CAR-T cell to the antigen binding molecule comprising the target antigen binding moiety leads to expression of the reporter gene.
24. The method of embodiment 22, wherein binding of the target antigen binding moiety to the target antigen and binding of the reporter CAR-T cell to the antigen binding

molecule comprising the target antigen binding moiety leads to activation of the intracellular signaling and/or co-signaling domain.

25. The method of any one of embodiments 22 or 24, wherein activation of the intracellular signaling and/or co-signaling domain leads to activation of the response element.
26. The method of any one of embodiments 1 to 25, wherein the response element controls the expression of the reporter gene.
27. The method of any one of embodiments 1 to 26, wherein activation of the response element leads to expression of the reporter gene.
28. The method of any one of embodiments 1 to 27, wherein the response element is part of the NFAT pathway, the NF-κB pathway or the AP-1 pathway.
29. The method of any one of embodiments 1 to 28, wherein the reporter gene is coding for a luminescent protein.
30. The method of any one of embodiments 1 to 29, wherein the reporter gene is coding for green fluorescent protein (GFP) or luciferase.
31. The method of any one of embodiments 1 to 30, wherein the target antigen is a cell surface antigen or receptor.
32. The method of any one of embodiments 1 to 31, wherein the target antigen is selected from the group consisting of CD20, CD38, CD138, CEA, EGFR, FolR1, HER2, LeY, MCSP, STEAP1, TYRP, and WT1, or a fragment thereof.
33. The method of any one of embodiments 1 to 32, wherein the target antigen is a peptide bound to a molecule of the human major histocompatibility complex (MHC).
34. The method of embodiment 33, wherein the target antigen binding moiety is a T cell receptor like (TCRL) antigen binding moiety.
35. The method of any one of embodiments 1 to 34, additionally comprising the step of:
    e) comparing the expression of the reporter gene to a reference.
36. The method of embodiment 35, wherein the reference is expression of the reporter gene in absence of the antigen binding molecule.
37. The method of embodiment 36, wherein the expression of the reporter gene in the presence of the antigen binding molecule is at least 2x, 3x, 4x, 5x, 10x, 100x, 1000x, or 10000x, higher compared to the expression of the reporter gene in absence of the antigen binding molecule.
38. The method of embodiment 35, additionally comprising the step of:

f) selecting the target antigen binding moiety if the expression of the reporter gene in the presence of the antigen binding molecule in relation to the expression of the reporter gene in absence of the antigen binding molecule is higher than a predefined threshold value.

39. The method of embodiment 38, wherein the threshold value is 2, 3, 4, 5, 10, 100, 1000, or 10000.
40. The method of any one of embodiments 1 to 39, wherein high level of expression of the reporter gene in the presence of the antigen binding molecule and low level of expression of the reporter gene in the absence of the antigen binding molecule is indicative for high specificity of the target antigen binding moiety.
41. The method of any one of embodiments 1 to 40, wherein high level of expression of the reporter gene in the presence of the antigen binding molecule and low level of expression of the reporter gene in the absence of the antigen binding molecule is indicative for high specificity of a T cell bispecific (TCB) antibody comprising the target antigen binding moiety.
42. A method for generating a TCB antibody, wherein the TCB antibody comprises a first antigen binding moiety specific for a target antigen and a second antigen binding moiety capable of specific binding to a T cell activating receptor, wherein the first antigen binding moiety is selected according to the method of any one of embodiments 1 to 41.
43. The method of embodiment 42, wherein the T cell activating receptor is CD3.
44. The method of any one of embodiment 1 to 43, wherein the method is an *in vitro* method.
45. A chimeric antigen receptor (CAR) comprising an anchoring transmembrane domain and an extracellular domain comprising an antigen binding moiety, wherein the antigen binding moiety is capable of specific binding to a recognition domain comprising a tag but not capable of specific binding to the recognition domain not comprising the tag.
46. The CAR of embodiment 45, wherein the tag is a hapten molecule.
47. The CAR of embodiment 46, wherein the hapten molecule is selected from the group consisting of Biotin, Digoxigenin (DIG) and Fluorescein (FITC).
48. The CAR of embodiment 46, wherein the hapten molecule is Biotin or DIG.
49. The CAR of embodiment 46, wherein the hapten molecule is DIG.
50. The CAR of embodiment 45, wherein the tag is a polypeptide tag.

51. The CAR of embodiment 50, wherein the polypeptide tag is selected from the group consisting of myc-tag, HA-tag, AviTag, FLAG-tag, his-tag, GCN4-tag, and NE-tag.
52. The CAR of embodiment 51, wherein the polypeptide tag is selected from the group consisting of myc-tag, HA-tag, GCN4-tag and his-tag.
53. The CAR of any one of embodiments 45 to 52, wherein the anchoring transmembrane domain is a transmembrane domain selected from the group consisting of the CD8, the CD3z, the FCGR3A, the NKG2D, the CD27, the CD28, the CD137, the OX40, the ICOS, the DAP10 or the DAP12 transmembrane domain or a fragment thereof, in particular wherein the anchoring transmembrane domain is the CD28 transmembrane domain or a fragment thereof.
54. The CAR of any one of embodiments 45 to 53 further comprising at least one stimulatory signaling domain and/or at least one co-stimulatory signaling domain.
55. The CAR of any one of embodiments 45 to 54, wherein the at least one stimulatory signaling domain is individually selected from the group consisting of the intracellular domain of CD3z, of FCGR3A and of NKG2D, or fragments thereof, in particular wherein the at least one stimulatory signaling domain is the CD3z intracellular domain or a fragment thereof.
56. The CAR of any one of embodiments 45 to 55, wherein the at least one co-stimulatory signaling domain is individually selected from the group consisting of the intracellular domain of CD27, of CD28, of CD137, of OX40, of ICOS, of DAP10 and of DAP12, or fragments thereof, in particular wherein the at least one co-stimulatory signaling domain is the CD28 intracellular domain or a fragment thereof.
57. The CAR of any one of embodiments 45 to 56, wherein the antigen binding receptor comprises one stimulatory signaling domain comprising the intracellular domain of CD28, or a fragment thereof, and wherein the antigen binding receptor comprises one co-stimulatory signaling domain comprising the intracellular domain of CD3z, or a fragment thereof.
58. The CAR of any one of embodiments 45 to 57, wherein the antigen binding moiety is a scFv fragment, wherein the scFv fragment is connected at the C-terminus to the N-terminus of the anchoring transmembrane domain, optionally through a peptide linker.
59. The CAR of any one of embodiments 45 to 57, wherein the antigen binding moiety is a Fab or a crossFab fragment, wherein the Fab or crossFab fragment is connected at the C-terminus of the heavy chain to the N-terminus of the anchoring transmembrane domain, optionally through a peptide linker.

60. The CAR of any one of embodiments 45 to 49 or 53 to 59, wherein the hapten molecule is DIG.
61. The CAR of embodiment 60, wherein the CAR capable of specific binding to the recognition domain comprising DIG but not capable of specific binding to the recognition domain not comprising DIG comprises:

(i) a heavy chain variable region (VH) comprising

(a) the heavy chain complementarity-determining region (CDR H) 1 amino acid sequence DYAMS (SEQ ID NO:1);

(b) the CDR H2 amino acid sequence SINIGATYIYYADSVKG (SEQ ID NO:2); and

(c) the CDR H3 amino acid sequence PGSPYEYDKAYYSMAY (SEQ ID NO:3); and

(ii) a light chain variable region (VL) comprising

(d) the light chain complementary-determining region (CDR L) 1 amino acid sequence RASQDIKNYLN (SEQ ID NO:4);

(e) the CDR L2 amino acid sequence YSSTLLS (SEQ ID NO:5); and

(f) the CDR L3 amino acid sequence QQSITLPPT (SEQ ID NO:6).

62. The CAR of any one of embodiments 45 to 47 or 53 to 59, wherein the hapten molecule is FITC.
63. The CAR of embodiment 62, wherein the CAR capable of specific binding to the recognition domain comprising FITC but not capable of specific binding to the recognition domain not comprising FITC comprises:

(i) a heavy chain variable region (VH) comprising

(a) the heavy chain complementarity-determining region (CDR H) 1 amino acid sequence HYWMN (SEQ ID NO:42);

(b) the CDR H2 amino acid sequence QFRNKPYNYETYYSDSVKG (SEQ ID NO:43); and

(c) the CDR H3 amino acid sequence ASYGMEY (SEQ ID NO:44); and

(ii) a light chain variable region (VL) comprising

(d) the light chain complementary-determining region (CDR L) 1 amino acid sequence RSSQSLVHSNGNTYLR (SEQ ID NO:45);

(e) the CDR L2 amino acid sequence KVSNRVS (SEQ ID NO:46); and

(f) the CDR L3 amino acid sequence SQSTHVPWT (SEQ ID NO:47).

64. The CAR of any one of embodiments 45 to 48 or 53 to 59, wherein the hapten molecule is Biotin.

65. The CAR of embodiment 64, wherein the CAR capable of specific binding to the recognition domain comprising Biotin but not capable of specific binding to the recognition domain not comprising Biotin comprises:

(i) a heavy chain variable region (VH) comprising

(a) the heavy chain complementarity-determining region (CDR H) 1 amino acid sequence GFNNKDTFFQ (SEQ ID NO:67);

(b) the CDR H2 amino acid sequence RIDPANGFTKYAQKFQG (SEQ ID NO:68); and

(c) the CDR H3 amino acid sequence WDTYGAAWFAY (SEQ ID NO:69); and

(ii) a light chain variable region (VL) comprising

(d) the light chain complementary-determining region (CDR L) 1 amino acid sequence RASGNIHNYLS (SEQ ID NO:70);

(e) the CDR L2 amino acid sequence SAKTLAD (SEQ ID NO:71); and

(f) the CDR L3 amino acid sequence QHFWSSIYT (SEQ ID NO:72).

66. The CAR of any one of embodiments 45 or 50 to 59, wherein the polypeptide tag is the HA tag.

67. The CAR of embodiment 66, wherein the CAR capable of specific binding to the recognition domain comprising the HA tag but not capable of specific binding to the recognition domain not comprising the HA tag comprises:

(i) a heavy chain variable region (VH) comprising

(a) the heavy chain complementarity-determining region (CDR H) 1 amino acid sequence NYDMA (SEQ ID NO:52);

(b) the CDR H2 amino acid sequence TISHDGRNTNYRDSVKG (SEQ ID NO:53); and

(c) the CDR H3 amino acid sequence PGFAH (SEQ ID NO:54); and

(ii) a light chain variable region (VL) comprising

(d) the light chain complementary-determining region (CDR L) 1 amino acid sequence RSSKTLLNTRGITSLY (SEQ ID NO:55);

(e) the CDR L2 amino acid sequence RMSNLAS (SEQ ID NO:56); and

(f) the CDR L3 amino acid sequence AQFLEFPLT (SEQ ID NO:57).

68. The CAR of any one of embodiments 45 or 50 to 59, wherein the polypeptide tag is the myc tag.

69. The CAR of embodiment 68, wherein the CAR capable of specific binding to the recognition domain comprising the myc tag but not capable of specific binding to the recognition domain not comprising the myc tag comprises:

(i) a heavy chain variable region (VH) comprising

(a) the heavy chain complementarity-determining region (CDR H) 1 amino acid sequence HYGMS (SEQ ID NO:77);

(b) the CDR H2 amino acid sequence TIGSRGTYTHYPDSVKG (SEQ ID NO:78); and

(c) the CDR H3 amino acid sequence RSEFYYYGNTYYYSAMDY (SEQ ID NO:79); and

(ii) a light chain variable region (VL) comprising

(d) the light chain complementary-determining region (CDR L) 1 amino acid sequence RASESVDNYGFSFMN (SEQ ID NO:80);

(e) the CDR L2 amino acid sequence AISNRGS (SEQ ID NO:81); and

(f) the CDR L3 amino acid sequence QQTKEVPWT (SEQ ID NO:82).

70. The CAR of any one of embodiments 45 or 50 to 59, wherein the polypeptide tag is the GCN4 tag (SEQ ID NO:102).

71. The CAR of embodiment 70, wherein the CAR capable of specific binding to the recognition domain comprising the GCN4 tag but not capable of specific binding to the recognition domain not comprising the GCN4 tag comprises:

(i) a heavy chain variable region (VH) comprising

(a) the heavy chain complementarity-determining region (CDR H) 1 amino acid sequence DYGVN (SEQ ID NO:90);

(b) the CDR H2 amino acid sequence VIWGDGITDHNSALKS (SEQ ID NO:91); and

(c) the CDR H3 amino acid sequence GLFDY (SEQ ID NO:92); and

(ii) a light chain variable region (VL) comprising

(d) the light chain complementary-determining region (CDR L) 1 amino acid sequence RSSTGAVTTSNYAS (SEQ ID NO:93);

(e) the CDR L2 amino acid sequence GTNNRAP (SEQ ID NO:94); and

(f) the CDR L3 amino acid sequence VLWYSNHWV (SEQ ID NO:95).

72. The methods as hereinbefore described.

Examples

The following are examples of methods and compositions of the invention. It is understood that various other embodiments may be practiced, given the general description provided above.

Recombinant DNA techniques

Standard methods were used to manipulate DNA as described in Sambrook et al., Molecular cloning: A laboratory manual; Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1989. The molecular biological reagents were used according to the manufacturer's instructions. General information regarding the nucleotide sequences of human immunoglobulin light and heavy chains is given in: Kabat, E.A. et al., (1991) Sequences of Proteins of Immunological Interest, Fifth Ed., NIH Publication No 91-3242.

DNA sequencing

DNA sequences were determined by double strand sequencing.

Gene synthesis

Desired gene segments were either generated by PCR using appropriate templates or were synthesized by Geneart AG (Regensburg, Germany) from synthetic oligonucleotides and PCR products by automated gene synthesis. The gene segments flanked by singular restriction endonuclease cleavage sites were cloned into standard cloning / sequencing vectors. The plasmid DNA was purified from transformed bacteria and concentration determined by UV spectroscopy. The DNA sequence of the subcloned gene fragments was confirmed by DNA sequencing. Gene segments were designed with suitable restriction sites to allow sub-cloning into the respective expression vectors. All constructs were designed with a 5'-end DNA sequence coding for a leader peptide which targets proteins for secretion in eukaryotic cells.

Protein purification

Proteins were purified from filtered cell culture supernatants referring to standard protocols. In brief, antibodies were applied to a Protein A Sepharose column (GE healthcare) and washed with PBS. Elution of antibodies was achieved at pH 2.8 followed by immediate neutralization of the sample. Aggregated protein was separated from monomeric antibodies by size exclusion chromatography (Superdex 200, GE Healthcare) in PBS or in 20 mM Histidine, 150 mM NaCl pH 6.0. Monomeric antibody fractions were pooled, concentrated (if required) using e.g., a MILLIPORE Amicon Ultra (30 MWCO) centrifugal concentrator, frozen and stored at -20°C or -80°C. Part of the samples were provided for subsequent protein

analytics and analytical characterization e.g., by SDS-PAGE and size exclusion chromatography (SEC).

SDS-PAGE

The NuPAGE® Pre-Cast gel system (Invitrogen) was used according to the manufacturer's instruction. In particular, 10% or 4-12% NuPAGE® Novex® Bis-TRIS Pre-Cast gels (pH 6.4) and a NuPAGE® MES (reduced gels, with NuPAGE® Antioxidant running buffer additive) or MOPS (non-reduced gels) running buffer was used.

Analytical size exclusion chromatography

Size exclusion chromatography (SEC) for the determination of the aggregation and oligomeric state of antibodies was performed by HPLC chromatography. Briefly, Protein A purified antibodies were applied to a Tosoh TSKgel G3000SW column in 300 mM NaCl, 50 mM KH_2PO_4/K_2HPO_4, pH 7.5 on an Agilent HPLC 1100 system or to a Superdex 200 column (GE Healthcare) in 2 x PBS on a Dionex HPLC-System. The eluted protein was quantified by UV absorbance and integration of peak areas. BioRad Gel Filtration Standard 151–1901 served as a standard.

Antibody production

The respective antibodies were produced by co-transfecting HEK293-EBNA cells with the mammalian expression vectors using polyethylenimine. The cells were transfected with the corresponding expression vectors for heavy and light chains in a 1:1 ratio.

Lentiviral transduction of Jurkat NFAT CAR-T cells

To produce lentiviral vectors, respective DNA sequences for the correct assembly of the CAR were cloned in frame in a lentiviral polynucleotide vector under a constitutively active human cytomegalovirus immediate early promoter (CMV). The retroviral vector contained a woodchuck hepatitis virus posttranscriptional regulatory element (WPRE), a central polypurine tract (cPPT) element, a pUC origin of replication and a gene encoding for antibiotic resistance facilitating the propagation and selection in bacteria.

To produce functional virus particles, Lipofectamine LTX™ based transfection was performed using 60-70% confluent Hek293T cells (ATCC CRL3216) and CAR containing vectors as well as pCMV-VSV-G:pRSV-REV:pCgpV transfer vectors at 3:1:1:1 ratio. After 48h supernatant was collected, centrifuged for 5 minutes at 250 g to remove cell debris and filtrated through 0.45 µm or 0.22 µm polyethersulfon filters. Concentrated virus particles (Lenti-x- Concentrator, Takara) were used to transduce Jurkat NFAT cells (Signosis). Positive

transduced cells were sorted as pool or single clones using a FACS-ARIA sorter (BD Bioscience). After cell expansion to appropriate density Jurkat NFAT reporter CAR-T cells were used for experiments.

Example 1

The anti-CD20 antibody GA101 was digoxigenylated and the incorporation of Digoxigenin (DIG) molecules verified by Western Blot analysis. For the coupling reaction of antibody and Digoxigenin, the antibody, which was dissolved in 20mM His 140 mM NaCl, pH6 was first de-salted and the buffer exchanged to 0.1M sodium bicarbonate (pH8) buffer using Zeba™ Spin Desalting Columns (ThermoFisher Cat.-No 89889). Equimolar or higher (1:3 ratio) amounts of antibody and Digoxigenin-3-O-methylcarbonyl-e-aminocaproic acid-N-hydroxysuccinimide ester (Sigma Aldrich Cat-No. 11333054001) were incubated for 1 hour at room temperature on a shaker at 300 rpm. Antibody-Digoxigenin conjugates were desalted again and the buffer was exchanged to 20 mM His 140mM NaCl pH6. Unconjugated Dig-NHS was removed in the same step (cut-off 7 kDa).

Digoxigeninylation was detected by anti-Digoxigenin-AP Fab fragments (Sigma Aldrich Cat.-No 11093274910) in a Western Blot. 1µg of the respective (un-)conjugated antibody was mixed with NuPAGE™ LDS Sample Buffer (4X (ThermoFisher Cat.-No. NP0007) in a total volume of 20 µl and boiled for 5 min at 95°C. 10 µl were loaded on a NuPAGE™ 4-12% Bis-Tris Protein Gel, 1.0 mm, 10-well (ThermoFisher Cat.-No. NP0321) and run for 1 hour at 170V in 1x NuPAGE™ MES SDS Running Buffer (Cat. No. NP0002). Subsequently, the gel was blotted onto a 0,2 µm PVDF membrane (Trans-Blot® Turbo™ Pack, Bio-Rad Cat.-No. 1704156) using the Trans-Blot® Turbo™ Transfer System (Bio-Rad, Cat.-No 1704150, mixed molecular weight standard protocol). The membrane was blocked with 5% milk in 1x TBS-T buffer for 1 hour at RT on an orbital shaker. Anti-Digoxigenin-AP Fab fragments were diluted 1:2000 in 5% milk/TBS-T and incubated for 1 hour on an orbital shaker at room temperature. The membrane was washed three times with 1x TBS-T for 10 minutes each. The membrane was then incubated for 1 min in 2 ml of BCIP®/NBT-Blue Liquid Substrate System for Membranes (Sigma Aldrich Cat.-No. B3804). After washing three times with bidistilled, the membrane was dried and documented (Figure 6).

Example 2

The expression of anti-Digoxigenin-ds-scFv-CD28ATD-CD28CSDCD3zSSD in Jurkat NFAT reporter CAR-T cells and the binding of Digoxigenin-Cy5 to the CAR was confirmed by FACS. Anti-Digoxigenin-ds-scFv-CD28ATD-CD28CSDCD3zSSD transduced Jurkat

NFAT reporter CAR-T cells were pelleted at 300 g for 3 min at room temperature and resuspended in fresh RPMI- 1640+10% FCS+1% Glutamax (growth medium) in an appropriate volume. $3x10^5$ cells were then added to each well in a 96-well plate, spun down once at 300 g for 5 min and resuspended in 100 µl in PBS with 2% FCS. Dig-Cy5 was added to a final concentration of 20 nM and incubated on ice for 45 minutes. Cells were then pelleted and resuspended in ice-cold PBS. The washing step was repeated two more times. Cells were then analyzed for Cy5 signal (APC channel) via flow cytometry (Figure 7). As a negative control, untransduced Jurkat NFAT cells were treated and analyzed equally.

Example 3

Described herein is a reporter CAR-T cell assay using CD20 expressing SUDHDL4 tumor cells as target cells and a sorted pool of anti-Digoxigenin-ds-scFv-CD28ATD-CD28CSDCD3zSSD expressing Jurkat NFAT reporter CAR-T cells as reporter cells (Figure 9). Digoxygeninylated GA101 IgG (antibody:Dig-NHS ratio 1:10) was used as IgG, which on one hand recognizes the tumor antigen and on the other hand is recognized by the transduced Jurkat NFAT reporter CAR-T cells. As positive control a 96 well plate (Cellstar Greiner-bio-one, CAT-No. 655185) was coated with 10 µg/ml CD3 antibody (from Biolegend®) in phosphate buffered saline (PBS) either for 4°C over night or for at least 1h at 37°C. The CD3 coated wells were washed twice with PBS, after the final washing step PBS was fully removed. Reporter cells or Jurkat NFAT wild type cells were counted and checked for their viability using Cedex HiRes. The cell number was adjusted to $1x10^6$ viable cells/ml. Therefore, an appropriate aliquot of the cell suspension was pelleted at 210 g for 5 min at room temperature (RT) and resuspended in fresh RPMI- 1640+10% FCS+1% Glutamax (growth medium). Target cells expressing the antigen of interest, were counted and checked for their viability as well. The cell number was adjusted to $1x10^6$ viable cells/ml in growth medium. Target cells and reporter (effector) cells were plated in either 5:1 E:T ratio (110.000 cells per well in total) in triplicates in a 96- well suspension culture plate (Greiner-bio one). As a next step a serial dilution of digoxigeninylated GA101 antibody, targeting the antigen of interest, was prepared in growth medium using a 2 ml deep well plate (Axygen®). To obtain final concentrations ranging from 1 µg/ml to 0.01 pg/ml in a final volume of 200 µl per well, a 50 µl aliquot of the different dilutions was pipetted to the respective wells. The 96 well plate was centrifuged for 2 min at 190 g and RT. Sealed with Parafilm®, the plate was incubated at 37°C and 5% CO_2 in a humidity atmosphere. After 20 hours incubation the content of each well was mixed by pipetting up and down 10 times using a multichannel pipette. 100 µl cell

suspension was transferred to a new white flat clear bottom 96 well plate (Greiner-bio-one) and 100 µl ONE-Glo™ Luciferase Assay (Promega) was added. After 15 min incubation in the dark on a rotary shaker at 300 rpm and room temperature, luminescence was measured using a Tecan® Spark10M plate reader, at 1 sec/well as detection time. Upon co-cultivation of target and reporter cells in a ratio 5:1 (grey dots) for 20 hours, the graphs show a dose-dependent activation of anti-Digoxigenin-ds-scFv-CD28ATD-CD28CSDCD3zSSD expressing Jurkat NFAT reporter CAR-T cells when digoxigenylated GA101 IgG was used as antibody (Figure 9). When the GA101 IgG without Digoxigeninylation (Figures 9, depicted in grey squares) was used, no activation of the transduced Jurkat NFAT reporter CAR-T cells was detectable. Further Jurkat NFAT wild type cells incubated with 1µg/ml digoxigeninylated GA101 but without target cells did not show any activation (Figure 9 black square). In constrast, anti-Digoxigenin-ds-scFv-CD28ATD-CD28CSDCD3zSSD expressing Jurkat NFAT reporter CAR-T cells incubated with 1µg/ml digoxigeninylated GA101 IgG but without target cells showed activation (Figure 9 black triangle).

Each point represents the mean value of technical triplicates. All values are depicted as baseline corrected. Standard deviation is indicated by error bars.

Example 4

Described herein is a reporter CAR-T cell assay using a sorted pool of anti-Digoxigenin-ds-scFv-CD28ATD-CD28CSDCD3zSSD expressing Jurkat NFAT reporter CAR-T cells (Figure 10) as reporter cells and anti-CD20 IgG antibody (GA101) coupled with different amounts of Digoxigenin (DIG) molecules. Effector cells were counted and checked for their viability using Cedex HiRes. The cell number was adjusted to $1x10^6$ viable cells/ml. Therefore, an appropriate aliquot of the cell suspension was pelleted at 210 g for 5 min at room temperature (RT) and resuspended in fresh RPMI- 1640+10% FCS+1% Glutamax (growth medium). $1x10^5$ reporter cells were plated in triplicates in a 96- well suspension culture plate (Greiner-bio one). A serial dilution of anti GA101 antibodies was prepared in growth medium using a 2 ml deep well plate (Axygen®). The anti GA101 antibodies used feature either one (1:1 GA101-Dig), three (1:3 GA101-Dig) or ten (1:10 GA101-Dig) Digoxigenin molecules on average. As a control, a non-digoxigeninylated antibody (GA101 wt) was used.

Final antibody concentrations were ranging from 1 µg/ml to 1 pg/ml in a final volume of 200 µl per well, a 100 µl aliquot of the different dilutions was pipetted to the respective wells containing the reporter cells. The 96 well plate was centrifuged for 2 min at 190g and room temperature. Sealed with Parafilm®, the plate was incubated at 37°C and 5% CO_2 in a

humidity atmosphere. After 20 hours incubation the content of each well was mixed by pipetting up and down 10 times using a multichannel pipette. 100 µl cell suspension was transferred to a new white flat clear bottom 96 well plate (Greiner-bio-one) and 100 µl ONE-Glo™ Luciferase Assay (Promega) was added. After 15 min incubation in the dark on a rotary shaker at 300 rpm and room temperature, luminescence was measured using a Tecan® Spark10M plate reader, at 1 sec/well as detection time.

The graphs show a dose-dependent activation of anti-Digoxigenin-ds-scFv-CD28ATD-CD28CSDCD3zSSD expressing Jurkat NFAT reporter CAR-T cells when digoxigeninylated GA101 IgG was used as antibody (Figure 10). The graph further shows, the more Digoxigenin molecules are coupled to the antibody, the stronger the activation of anti-Digoxigenin-ds-scFv-CD28ATD-CD28CSDCD3zSSD expressing Jurkat NFAT reporter CAR-T cells is. If the GA101 IgG without Digoxigeninylation (Figures 10, depicted in black triangle) was used, no activation of the transduced Jurkat NFAT reporter CAR-T cells was detectable.

Each point represents the mean value of technical triplicates. All values are depicted as baseline corrected.

Example 5

Described herein is a reporter CAR-T cell assay using CD20 expressing SUDHDL4 tumor cells as target cells and a sorted pool of anti-Digoxigenin-ds-scFv-CD28ATD-CD28CSDCD3zSSD expressing Jurkat NFAT reporter CAR-T cells (Figure 11) as target cells. 1:1 digoxigeninylated GA101 IgG was used as IgG, which on one hand recognizes the tumor antigen and on the other hand is recognized by the transduced Jurkat NFAT reporter CAR-T cells. Effector cells were counted and checked for their viability using Cedex HiRes. The cell number was adjusted to $1x10^6$ viable cells/ml. An appropriate aliquot of the cell suspension was pelleted at 210 g for 5 min at room temperature and resuspended in fresh RPMI- 1640+10% FCS+1% Glutamax (growth medium). Target cells expressing the antigen of interest, were counted and checked for their viability as well. The cell number was adjusted, analogous as described for the reporter cells, to $1x10^6$ viable cells/ml in growth medium. Target cells and reporter (effector) cells were plated in 5:1 E:T ratio (110.000 cells per well in total) in triplicates in a 96- well suspension culture plate (Greiner-bio one). As a next step a serial dilution of digoxigenylated GA101 antibody, targeting the antigen of interest, was prepared in growth medium using a 2 ml deep well plate (Axygen®). To obtain final concentrations ranging from 0.01 µg/ml to 0.1 fg/ml in a final volume of 200 µl per well,

a 50 µl aliquot of the different dilutions was pipetted to the respective wells. The 96 well plate was centrifuged for 2 min at 190g and room temperature. Sealed with Parafilm®, the plate was incubated at 37°C and 5% CO_2 in a humidity atmosphere. After 20 hours incubation the content of each well was mixed by pipetting up and down 10 times using a multichannel pipette. 100 µl cell suspension was transferred to a new white flat clear bottom 96 well plate (Greiner-bio-one) and 100 ul ONE-Glo™ Luciferase Assay (Promega) was added. After 15 min incubation in the dark on a rotary shaker at 300 rpm and room temperature, luminescence was measured using Tecan® Spark10M plate reader, at 1 sec/well as detection time. Upon co-cultivation of target and reporter cells in a ratio 5:1 (Figure 11, black triangle) for 20 hours the graphs show a dose-dependent activation of anti-digenylated-ds-scFv-CD28ATD-CD28CSDCD3zSSD expressing Jurkat NFAT reporter CAR-T cells when 1:1 digoxigeninylated GA101 IgG was used as antibody. If the GA101 IgG without Digoxigeninylation (Figures 11, depicted in black dots) was used, no activation of the transduced Jurkat NFAT reporter CAR-T cells was detectable.

Each point represents the mean value of technical triplicates. All values are depicted as baseline corrected. Standard deviation is indicated by error bars.

Example 6

Described herein is reporter CAR-T cell assay using LeY expressing MCF7 tumor cells as target cells and a sorted pool of anti-Digoxigenin-ds-scFv-CD28ATD-CD28CSDCD3zSSD expressing Jurkat NFAT reporter CAR-T cells. Two anti-LeY/Biotin antibodies (2+1, 2+2) and a bridging Biotin-Digoxigenin adapter were used. The antibody recognizes on one hand the tumor associated antigen and on the other hand the Biotinylated adapter molecule. The adapter-bound Digoxigenin is recognized by the Jurkat NFAT reporter CAR-T cells expressing CARs according to the invention (Figure 13).

On day 0 target cells were counted and checked for their viability using CASY® Model TTC. Cells were plated in 96 well plates (20.000 cells/well in 100µl). An appropriate aliquot of the cell suspension was pelleted at 300 g for 3 min at room temperature (RT) and resuspended in the appropriate amount of fresh RPMI- 1640+10% FCS+1% Glutamax (growth medium).

On day 1 a serial dilution of the LeY/Biotin antibody derivatives, targeting the antigen of interest, and equimolar amounts of Biotin-Digoxigenin adapter molecules were prepared in growth medium using a 2 ml deep well plate (Axygen®). To obtain final concentrations ranging from 0,01nM - 10 nM, the required volume was pipetted to the respective wells.

After 1 hour incubation at 37°C and 5% CO_2, media including unbound antibodies/adapters was removed. Reporter (effector) cells were counted and checked for their viability using CASY® Modell TTC and cell suspension adjusted to $1x10^6$ cells/ml . An appropriate aliquot of the cell suspension was pelleted at 300g for 3 min at room temperature (RT) and resuspended in the appropriate amount of fresh RPMI- 1640+10% FCS+1% Glutamax (growth medium). 100 µl of reporter cell suspension ($1x10^5$cells/well (5:1 E:T ratio)) were added to each well.

The plate was incubated at 37°C and 5% CO_2 in a humidity atmosphere. After 20 hours incubation 100 µl of ONE-Glo™ Luciferase Assay (Promega) was added. After 5 min incubation in the dark on a rotary shaker at 300 rpm and room temperature, luminescence was measured using a Tecan® Infinite F200 Pro, at 1 sec/well as detection time. Upon co-cultivation of target and reporter cells in a ratio 5:1 (Figure 13) for 20 hours the graphs show a dose-dependent activation of anti-Digoxigenin-ds-scFv-CD28ATD-CD28CSDCD3zSSD expressing Jurkat NFAT reporter CAR-T cells when the adapter molecule and the targeting LeY antibody derivative was used. If the non-targeting CD33/Biotin antibody + adapter, or the adapter alone was used, no activation of the transduced Jurkat NFAT reporter CAR-T cells was detectable.

Exemplary sequences

Table 2: Anti-DIG-ds-scFv amino acid sequences:

Construct	Amino acid sequence	SEQ ID NO
Anti-DIG CDR H1 Kabat	DYAMS	1
Anti-DIG CDR H2 Kabat	SINIGATYIYYADSVKG	2
Anti-DIG CDR H3 Kabat	PGSPYEYDKAYYSMAY	3
Anti-DIG CDR L1 Kabat	RASQDIKNYLN	4
Anti-DIG CDR L2 Kabat	YSSTLLS	5
Anti-DIG CDR L3 Kabat	QQSITLPPT	6
Anti-DIG-ds-scFv-CD28ATD-CD28CSD-CD3zSSD fusion	QVQLVESGGGLVKPGGSLRLSCAASGFTFSDYAMSWIR QAPGKCLEWVSSINIGATYIYYADSVKGRFTISRDNAK NSLYLQMNSLRAEDTAVYYCARPGSPYEYDKAYYSM AYWGQGTTVTVSSGGGGSGGGGSGGGGSGGGGSDIQ MTQSPSSLSASVGDRVTITCRASQDIKNYLNWYQQKPG KAPKLLIYYSSTLLSGVPSRFSGSGSGTDFTLTISSLQPE DFATYYCQQSITLPPTFGCGTKVEIKGGGGSFWVLVVV GGVLACYSLLVTVAFIIFWVRSKRSRLLHSDYMNMTPR RPGPTRKHYQPYAPPRDFAAYRSRVKFSRSADAPAYQ QGQNQLYNELNLGRREEYDVLDKRRGRDPEMGGKPR RKNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGH DGLYQGLSTATKDTYDALHMQALPPR	7
Anti-DIG-ds VH	QVQLVESGGGLVKPGGSLRLSCAASGFTFSDYAMSWIR QAPGKCLEWVSSINIGATYIYYADSVKGRFTISRDNAK NSLYLQMNSLRAEDTAVYYCARPGSPYEYDKAYYSM AYWGQGTTVTVSS	8
Anti-DIG-ds VL	DIQMTQSPSSLSASVGDRVTITCRASQDIKNYLNWYQQ KPGKAPKLLIYYSSTLLSGVPSRFSGSGSGTDFTLTISSL	9

	QPEDFATYYCQQSITLPPTFGCGTKVEIK	
Anti-DIG-ds-scFv	QVQLVESGGGLVKPGGSLRLSCAASGFTFSDYAMSWIR QAPGKCLEWVSSINIGATYIYYADSVKGRFTISRDNAK NSLYLQMNSLRAEDTAVYYCARPGSPYEYDKAYYSM AYWGQGTTVTVSSGGGGSGGGGSGGGGSGGGGSDIQ MTQSPSSLSASVGDRVTITCRASQDIKNYLNWYQQKPG KAPKLLIYYSSTLLSGVPSRFSGSGSGTDFTLTISSLQPE DFATYYCQQSITLPPTFGCGTKVEIK	10
CD28ATD	FWVLVVVGGVLACYSLLVTVAFIIFWV	11
CD28CSD	RSKRSRLLHSDYMNMTPRRPGPTRKHYQPYAPPRDFA AYRS	12
CD3zSSD	RVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDK RRGRDPEMGGKPRRKNPQEGLYNELQKDKMAEAYSEI GMKGERRRGKGHDGLYQGLSTATKDTYDALHMQALP PR	13
CD28ATD-CD28CSD-CD3zSSD	FWVLVVVGGVLACYSLLVTVAFIIFWVRSKRSRLLHSD YMNMTPRRPGPTRKHYQPYAPPRDFAAYRSRVKFSRS ADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGRDP EMGGKPRRKNPQEGLYNELQKDKMAEAYSEIGMKGE RRRGKGHDGLYQGLSTATKDTYDALHMQALPPR	14
eGFP	VSKGEELFTGVVPILVELDGDVNGHKFSVSGEGEGDAT YGKLTLKFICTTGKLPVPWPTLVTTLTYGVQCFSRYPD HMKQHDFFKSAMPEGYVQERTIFFKDDGNYKTRAEVK FEGDTLVNRIELKGIDFKEDGNILGHKLEYNYNSHNVYI MADKQKNGIKVNFKIRHNIEDGSVQLADHYQQNTPIG DGPVLLPDNHYLSTQSALSKDPNEKRDHMVLLEFVTA AGITLGMDELYK	15
(G4S)4 linker	GGGGSGGGGSGGGGSGGGGS	16
G4S linker	GGGGS	17
T2A linker	GEGRGSLLTCGDVEENPGP	18

Table 3: anti-DIG-ds- scFv DNA sequences:

Construct	DNA sequence	SEQ ID NO
Anti-DIG-ds-scFv-CD28ATD-CD28CSD-CD3zSSD fusion	ATGGGATGGAGCTGTATCATCCTCTTCTTGGTAGCAA CAGCTACCGGTGTGCATTCCCAGGTGCAGCTCGTGG AGTCAGGGGGAGGCCTGGTCAAGCCTGGCGGCTCCC TGAGACTGTCTTGCGCCGCCTCTGGCTTCACATTCTC CGACTACGCCATGAGCTGGATCAGACAGGCTCCCGG CAAATGCCTCGAGTGGGTGTCCAGCATCAACATCGG CGCCACCTACATCTACTATGCCGACTCCGTGAAGGG CCGGTTCACCATCTCCAGAGACAACGCCAAGAATAG CCTCTATCTCCAGATGAACTCCCTGCGGGCCGAAGAT ACCGCTGTGTATTACTGCGCCAGACCCGGCAGCCCCT ACGAGTACGACAAGGCCTACTACAGCATGGCCTACT GGGGCCAGGGCACCACCGTGACAGTGTCATCTGGAG GGGGCGGAAGTGGTGGCGGGGGAAGCGGCGGGGGT GGCAGCGGAGGGGGCGGATCTGACATCCAGATGACC CAGTCCCCAAGCAGCCTGAGCGCCAGCGTGGGCGAC AGAGTGACCATCACCTGTCGGGCCAGCCAGGACATC AAGAACTACCTGAATTGGTATCAGCAGAAACCTGGC AAAGCCCCTAAGCTGCTCATCTACTACAGCTCCACCC TGCTGAGCGGCGTGCCCAGCAGATTTTCCGGCAGCG GGAGCGGCACAGATTTCACACTGACAATCTCCAGCC TGCAGCCTGAGGACTTCGCCACCTACTATTGTCAGCA GAGCATCACCCTGCCCCCCACCTTTGGCTGTGGCACA AAAGTCGAGATCAAGGGAGGGGGCGGATCCTTCTGG GTGCTGGTGGTGGTGGGCGGCGTGCTGGCCTGCTAC AGCCTGCTGGTGACCGTGGCCTTCATCATCTTCTGGG TGAGGGTGAAGTTCAGCAGGAGCGCCGACGCCCCCG CCTACCAGCAGGGCCAGAACCAGCTGTATAACGAGC	19

	TGAACCTGGGCAGGAGGGAGGAGTACGACGTGCTGG ACAAGAGGAGGGGCAGGGACCCCGAGATGGGCGGC AAGCCCAGGAGGAAGAACCCCCAGGAGGGCCTGTAT AACGAGCTGCAGAAGGACAAGATGGCCGAGGCCTA CAGCGAGATCGGCATGAAGGGCGAGAGGAGGAGGG GCAAGGGCCACGACGGCCTGTACCAGGGCCTGAGCA CCGCCACCAAGGACACCTACGACGCCCTGCACATGC AGGCCCTGCCCCCCAGG	
Anti-DIG-ds VH	AGGTGCAGCTCGTGGAGTCAGGGGGAGGCCTGGTCA AGCCTGGCGGCTCCCTGAGACTGTCTTGCGCCGCCTC TGGCTTCACATTCTCCGACTACGCCATGAGCTGGATC AGACAGGCTCCCGGCAAATGCCTCGAGTGGGTGTCC AGCATCAACATCGGCGCCACCTACATCTACTATGCC GACTCCGTGAAGGGCCGGTTCACCATCTCCAGAGAC AACGCCAAGAATAGCCTCTATCTCCAGATGAACTCC CTGCGGGCCGAAGATACCGCTGTGTATTACTGCGCC AGACCCGGCAGCCCCTACGAGTACGACAAGGCCTAC TACAGCATGGCCTACTGGGGCCAGGGCACCACCGTG ACAGTGTCATCT	20
Anti-DIG-ds VL	GACATCCAGATGACCCAGTCCCCAAGCAGCCTGAGC GCCAGCGTGGGCGACAGAGTGACCATCACCTGTCGG GCCAGCCAGGACATCAAGAACTACCTGAATTGGTAT CAGCAGAAACCTGGCAAAGCCCCTAAGCTGCTCATC TACTACAGCTCCACCCTGCTGAGCGGCGTGCCCAGC AGATTTTCCGGCAGCGGGAGCGGCACAGATTTCACA CTGACAATCTCCAGCCTGCAGCCTGAGGACTTCGCC ACCTACTATTGTCAGCAGAGCATCACCCTGCCCCCCA CCTTTGGCTGTGGCACAAAAGTCGAGATCAAG	21
Anti-DIG-ds-scFv	ATGGGATGGAGCTGTATCATCCTCTTCTTGGTAGCAA CAGCTACCGGTGTGCATTCCCAGGTGCAGCTCGTGG AGTCAGGGGGAGGCCTGGTCAAGCCTGGCGGCTCCC TGAGACTGTCTTGCGCCGCCTCTGGCTTCACATTCTC CGACTACGCCATGAGCTGGATCAGACAGGCTCCCGG CAAATGCCTCGAGTGGGTGTCCAGCATCAACATCGG CGCCACCTACATCTACTATGCCGACTCCGTGAAGGG CCGGTTCACCATCTCCAGAGACAACGCCAAGAATAG CCTCTATCTCCAGATGAACTCCCTGCGGGCCGAAGAT ACCGCTGTGTATTACTGCGCCAGACCCGGCAGCCCCT ACGAGTACGACAAGGCCTACTACAGCATGGCCTACT GGGGCCAGGGCACCACCGTGACAGTGTCATCTGGAG GGGGCGGAAGTGGTGGCGGGGGAAGCGGCGGGGGT GGCAGCGGAGGGGGCGGATCTGACATCCAGATGACC CAGTCCCCAAGCAGCCTGAGCGCCAGCGTGGGCGAC AGAGTGACCATCACCTGTCGGGCCAGCCAGGACATC AAGAACTACCTGAATTGGTATCAGCAGAAACCTGGC AAAGCCCCTAAGCTGCTCATCTACTACAGCTCCACCC TGCTGAGCGGCGTGCCCAGCAGATTTTCCGGCAGCG GGAGCGGCACAGATTTCACACTGACAATCTCCAGCC TGCAGCCTGAGGACTTCGCCACCTACTATTGTCAGCA GAGCATCACCCTGCCCCCCACCTTTGGCTGTGGCACA AAAGTCGAGATCAAG	22
eGFP	GTGAGCAAGGGCGAGGAGCTGTTCACCGGGGTGGTG CCCATCCTGGTCGAGCTGGACGGCGACGTAAACGGC CACAAGTTCAGCGTGTCCGGCGAGGGCGAGGGCGAT GCCACCTACGGCAAGCTGACCCTGAAGTTCATCTGC ACCACCGGCAAGCTGCCCGTGCCCTGGCCCACCCTC GTGACCACCCTGACCTACGGCGTGCAGTGCTTCAGC CGCTACCCCGACCACATGAAGCAGCACGACTTCTTC AAGTCCGCCATGCCCGAAGGCTACGTCCAGGAGCGC ACCATCTTCTTCAAGGACGACGGCAACTACAAGACC CGCGCCGAGGTGAAGTTCGAGGGCGACACCCTGGTG	23

	AACCGCATCGAGCTGAAGGGCATCGACTTCAAGGAG GACGGCAACATCCTGGGGCACAAGCTGGAGTACAAC TACAACAGCCACAACGTCTATATCATGGCCGACAAG CAGAAGAACGGCATCAAGGTGAACTTCAAGATCCGC CACAACATCGAGGACGGCAGCGTGCAGCTCGCCGAC CACTACCAGCAGAACACCCCCATCGGCGACGGCCCC GTGCTGCTGCCCGACAACCACTACCTGAGCACCCAG TCCGCCCTGAGCAAAGACCCCAACGAGAAGCGCGAT CACATGGTCCTGCTGGAGTTCGTGACCGCCGCCGGG ATCACTCTCGGCATGGACGAGCTGTACAAGTGA	
CD28ATD	TTTTGGGTGCTGGTGGTGGTTGGTGGAGTCCTGGCTT GCTATAGCTTGCTAGTAACAGTGGCCTTTATTATTTT CTGGGTG	24
CD28CSD	AGGAGTAAGAGGAGCAGGCTCCTGCACAGTGACTAC ATGAACATGACTCCCCGCCGCCCCGGGCCCACCCGC AAGCATTACCAGCCCTATGCCCCACCACGCGACTTC GCAGCCTATCGCTCC	25
CD3zSSD	AGAGTGAAGTTCAGCAGGAGCGCAGACGCCCCCGCG TACCAGCAGGGCCAGAACCAGCTCTATAACGAGCTC AATCTAGGACGAAGAGAGGAGTACGATGTTTTGGAC AAGAGACGTGGCCGGGACCCTGAGATGGGGGGAAA GCCGAGAAGGAAGAACCCTCAGGAAGGCCTGTACA ATGAACTGCAGAAAGATAAGATGGCGGAGGCCTACA GTGAGATTGGGATGAAAGGCGAGCGCCGGAGGGGC AAGGGGCACGATGGCCTTTACCAGGGTCTCAGTACA GCCACCAAGGACACCTACGACGCCCTTCACATGCAG GCCCTGCCCCCTCGC	26
CD28ATD-CD28CSD-CD3zSSD	TTCTGGGTGCTGGTGGTGGTGGGCGGCGTGCTGGCCT GCTACAGCCTGCTGGTGACCGTGGCCTTCATCATCTT CTGGGTGAGGAGCAAGAGGAGCAGGCTGCTGCACA GCGACTACATGAACATGACCCCCAGGAGGCCCGGCC CCACCAGGAAGCACTACCAGCCCTACGCCCCCCCA GGGACTTCGCCGCCTACAGGAGCAGGGTGAAGTTCA GCAGGAGCGCCGACGCCCCCGCCTACCAGCAGGGCC AGAACCAGCTGTATAACGAGCTGAACCTGGGCAGGA GGGAGGAGTACGACGTGCTGGACAAGAGGAGGGGC AGGGACCCCGAGATGGGCGGCAAGCCCAGGAGGAA GAACCCCCAGGAGGGCCTGTATAACGAGCTGCAGAA GGACAAGATGGCCGAGGCCTACAGCGAGATCGGCAT GAAGGGCGAGAGGAGGAGGGGCAAGGGCCACGACG GCCTGTACCAGGGCCTGAGCACCGCCACCAAGGACA CCTACGACGCCCTGCACATGCAGGCCCTGCCCCCCA GG	27
T2A element	TCCGGAGAGGGCAGAGGAAGTCTTCTAACATGCGGT GACGTGGAGGAGAATCCCGGCCCTAGG	28

Table 4: Anti-DIG-Fab amino acid sequences:

Construct	Amino acid sequence	SEQ ID NO
Anti-DIG CDR H1 Kabat	see Table 2	1
Anti-DIG CDR H2 Kabat	see Table 2	2
Anti-DIG CDR H3 Kabat	see Table 2	3
Anti-DIG CDR L1 Kabat	see Table 2	4
Anti-DIG CDR L2 Kabat	see Table 2	5
Anti-DIG CDR L3 Kabat	see Table 2	6
Anti-DIG-Fab-heavy chain-CD28ATD-CD28CSD-CD3zSSD fusion	QVQLVESGGGLVKPGGSLRLSCAASGFTFSDYAMSWIR QAPGKGLEWVSSINIGATYIYYADSVKGRFTISRDNAK NSLYLQMNSLRAEDTAVYYCARPGSPYEYDKAYYSM AYWGQGTTVTVSSASTKGPSVFPLAPSSKSTSGGTAAL GCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLY SLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKS	29

	CGGGGSFWVLVVVGGVLACYSLLVTVAFIIFWVRSKR SRLLHSDYMNMTPRRPGPTRKHYQPYAPPRDFAAYRS RVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDK RRGRDPEMGGKPRRKNPQEGLYNELQKDKMAEAYSEI GMKGERRRGKGHDGLYQGLSTATKDTYDALHMQALP PR	
Anti-DIG-Fab heavy chain	QVQLVESGGGLVKPGGSLRLSCAASGFTFSDYAMSWIR QAPGKGLEWVSSINIGATYIYYADSVKGRFTISRDNAK NSLYLQMNSLRAEDTAVYYCARPGSPYEYDKAYYSM AYWGQGTTVTVSSASTKGPSVFPLAPSSKSTSGGTAAL GCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLY SLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKS C	30
Anti-DIG-Fab light chain	DIQMTQSPSSLSASVGDRVTITCRASQDIKNYLNWYQQ KPGKAPKLLIYYSSTLLSGVPSRFSGSGSGTDFTLTISSL QPEDFATYYCQQSITLPPTFGGGTKVEIKRTVAAPSVFIF PPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQ SGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYA CEVTHQGLSSPVTKSFNRGEC	31
Anti-DIG VH	QVQLVESGGGLVKPGGSLRLSCAASGFTFSDYAMSWIR QAPGKGLEWVSSINIGATYIYYADSVKGRFTISRDNAK NSLYLQMNSLRAEDTAVYYCARPGSPYEYDKAYYSM AYWGQGTTVTVSS	32
Anti-DIG VL	DIQMTQSPSSLSASVGDRVTITCRASQDIKNYLNWYQQ KPGKAPKLLIYYSSTLLSGVPSRFSGSGSGTDFTLTISSL QPEDFATYYCQQSITLPPTFGGGTKVEIK	33
CL	RTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKV QWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKA DYEKHKVYACEVTHQGLSSPVTKSFNRGEC	34
CH1 (human)	ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTV SWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGT QTYICNVNHKPSNTKVDKKVEPKSC	35

Table 5: Anti-DIG-Fab DNA sequences:

Construct	DNA Sequenz	SEQ ID NO
Anti-DIG-Fab-heavy chain-CD28ATD-CD28CSD-CD3zSSD fusion	ATGGGATGGAGCTGTATCATCCTCTTCTTGGTAGCAA CAGCTACCGGTGTGCACTCCGATATTCAGATGACCC AGAGCCCGAGCAGCCTGAGCGCGAGCGTGGGCGATC GCGTGACCATTACCTGCCGCGCGAGCCAGGATATTA AAAACTATCTGAACTGGTATCAGCAGAAACCGGGCA AAGCGCCGAAACTGCTGATTTATTATAGCAGCACCC TGCTGAGCGGCGTGCCGAGCCGCTTTAGCGGCAGCG GCAGCGGCACCGATTTTACCCTGACCATTAGCAGCCT GCAGCCGGAAGATTTTGCGACCTATTATTGCCAGCA GAGCATTACCCTGCCGCCGACCTTTGGCGGCGGCAC CAAAGTGGAAATTAAACGCACTGTCGCCGCTCCCTC TGTGTTCATTTTTCCTCCAAGTGATGAGCAGCTCAAA AGCGGTACCGCATCCGTTGTGTGCCTGCTTAACAACT TCTATCCCCGGGAAGCCAAGGTCCAATGGAAGGTGG ACAATGCTCTGCAGTCAGGAAACAGTCAGGAGAGCG TAACCGAGCAGGATTCCAAAGACTCTACTTACTCATT GAGCTCCACCCTGACACTCTCTAAGGCAGACTATGA AAAGCATAAAGTGTACGCCTGTGAGGTTACCCACCA GGGCCTGAGTAGCCCTGTGACAAAGTCCTTCAATAG GGGAGAGTGCTAGAATAGAATTCCCCGAAGTAACTT AGAAGCTGTAAATCAACGATCAATAGCAGGTGTGGC ACACCAGTCATACCTTGATCAAGCACTTCTGTTTCCC CGGACTGAGTATCAATAGGCTGCTCGCGCGGCTGAA GGAGAAAACGTTCGTTACCCGACCAACTACTTCGAG AAGCTTAGTACCACCATGAACGAGGCAGGGTGTTTC GCTCAGCACAACCCCAGTGTAGATCAGGCTGATGAG	36

	TCACTGCAACCCCCATGGGCGACCATGGCAGTGGCT GCGTTGGCGGCCTGCCCATGGAGAAATCCATGGGAC GCTCTAATTCTGACATGGTGTGAAGTGCCTATTGAGC TAACTGGTAGTCCTCCGGCCCCTGATTGCGGCTAATC CTAACTGCGGAGCACATGCTCACAAACCAGTGGGTG GTGTGTCGTAACGGGCAACTCTGCAGCGGAACCGAC TACTTTGGGTGTCCGTGTTTCCTTTTATTCCTATATTG GCTGCTTATGGTGACAATCAAAAAGTTGTTACCATAT AGCTATTGGATTGGCCATCCGGTGTGCAACAGGGCA ACTGTTTACCTATTTATTGGTTTTGTACCATTATCACT GAAGTCTGTGATCACTCTCAAATTCATTTTGACCCTC AACACAATCAAACGCCACCATGGGATGGAGCTGTAT CATCCTCTTCTTGGTAGCAACAGCTACTGGTGTGCAT TCCCAGGTGCAGCTGGTGGAAAGCGGCGGCGGCCTG GTGAAACCGGGCGGCAGCCTGCGCCTGAGCTGCGCG GCGAGCGGCTTTACCTTTAGCGATTATGCGATGAGCT GGATTCGCCAGGCGCCGGGCAAAGGCCTGGAATGGG TGAGCAGCATTAACATTGGCGCGACCTATATTTATTA TGCGGATAGCGTGAAAGGCCGCTTTACCATTAGCCG CGATAACGCGAAAAACAGCCTGTATCTGCAGATGAA CAGCCTGCGCGCGGAAGATACCGCGGTGTATTATTG CGCGCGCCCGGGCAGCCCGTATGAATATGATAAAGC GTATTATAGCATGGCGTATTGGGGCCAGGGCACCAC CGTGACCGTGAGCAGCGCGTCGACTAAGGGCCCTTC AGTTTTTCCACTCGCCCCCAGTAGCAAGTCCACATCT GGGGGTACCGCTGCCCTGGGCTGCCTTGTGAAAGAC TATTTCCCTGAACCAGTCACTGTGTCATGGAATAGCG GAGCCCTGACCTCCGGTGTACACACATTCCCCGCTGT GTTGCAGTCTAGTGGCCTGTACAGCCTCTCCTCTGTT GTGACCGTCCCTTCAAGCTCCCTGGGGACACAGACC TATATCTGTAACGTGAATCATAAGCCATCTAACACTA AAGTAGATAAAAAAGTGGAGCCCAAGAGTTGCGGA GGGGGCGGATCCTTCTGGGTGCTGGTGGTGGTGGGC GGCGTGCTGGCCTGCTACAGCCTGCTGGTGACCGTG GCCTTCATCATCTTCTGGGTGAGGGTGAAGTTCAGCA GGAGCGCCGACGCCCCCGCCTACCAGCAGGGCCAGA ACCAGCTGTATAACGAGCTGAACCTGGGCAGGAGGG AGGAGTACGACGTGCTGGACAAGAGGAGGGGCAGG GACCCCGAGATGGGCGGCAAGCCCAGGAGGAAGAA CCCCCAGGAGGGCCTGTATAACGAGCTGCAGAAGGA CAAGATGGCCGAGGCCTACAGCGAGATCGGCATGAA GGGCGAGAGGAGGAGGGGCAAGGGCCACGACGGCC TGTACCAGGGCCTGAGCACCGCCACCAAGGACACCT ACGACGCCCTGCACATGCAGGCCCTGCCCCCCAGGT CCGGAGAGGGCAGAGGAAGTCTTCTAACATGCGGTG ACGTGGAGGAGAATCCCGGCCCTAGGGTGAGCAAGG GCGAGGAGCTGTTCACCGGGGTGGTGCCCATCCTGG TCGAGCTGGACGGCGACGTAAACGGCCACAAGTTCA GCGTGTCCGGCGAGGGCGAGGGCGATGCCACCTACG GCAAGCTGACCCTGAAGTTCATCTGCACCACCGGCA AGCTGCCCGTGCCCTGGCCCACCCTCGTGACCACCCT GACCTACGGCGTGCAGTGCTTCAGCCGCTACCCCGA CCACATGAAGCAGCACGACTTCTTCAAGTCCGCCAT GCCCGAAGGCTACGTCCAGGAGCGCACCATCTTCTT CAAGGACGACGGCAACTACAAGACCCGCGCCGAGGT GAAGTTCGAGGGCGACACCCTGGTGAACCGCATCGA GCTGAAGGGCATCGACTTCAAGGAGGACGGCAACAT CCTGGGGCACAAGCTGGAGTACAACTACAACAGCCA CAACGTCTATATCATGGCCGACAAGCAGAAGAACGG CATCAAGGTGAACTTCAAGATCCGCCACAACATCGA GGACGGCAGCGTGCAGCTCGCCGACCACTACCAGCA	

	GAACACCCCCATCGGCGACGGCCCCGTGCTGCTGCC CGACAACCACTACCTGAGCACCCAGTCCGCCCTGAG CAAAGACCCCAACGAGAAGCGCGATCACATGGTCCT GCTGGAGTTCGTGACCGCCGCCGGGATCACTCTCGG CATGGACGAGCTGTACAAGTGAT	
Anti-DIG VL	GATATTCAGATGACCCAGAGCCCGAGCAGCCTGAGC GCGAGCGTGGGCGATCGCGTGACCATTACCTGCCGC GCGAGCCAGGATATTAAAAACTATCTGAACTGGTAT CAGCAGAAACCGGGCAAAGCGCCGAAACTGCTGATT TATTATAGCAGCACCCTGCTGAGCGGCGTGCCGAGC CGCTTTAGCGGCAGCGGCAGCGGCACCGATTTTACC CTGACCATTAGCAGCCTGCAGCCGGAAGATTTTGCG ACCTATTATTGCCAGCAGAGCATTACCCTGCCGCCGA CCTTTGGCGGCGGCACCAAAGTGGAAATTAAA	37
CL	CGCACTGTCGCCGCTCCCTCTGTGTTCATTTTTCCTCC AAGTGATGAGCAGCTCAAAAGCGGTACCGCATCCGT TGTGTGCCTGCTTAACAACTTCTATCCCCGGGAAGCC AAGGTCCAATGGAAGGTGGACAATGCTCTGCAGTCA GGAAACAGTCAGGAGAGCGTAACCGAGCAGGATTCC AAAGACTCTACTTACTCATTGAGCTCCACCCTGACAC TCTCTAAGGCAGACTATGAAAAGCATAAAGTGTACG CCTGTGAGGTTACCCACCAGGGCCTGAGTAGCCCTG TGACAAAGTCCTTCAATAGGGGAGAGTGC	38
Anti-DIG VH	CAGGTGCAGCTGGTGGAAAGCGGCGGCGGCCTGGTG AAACCGGGCGGCAGCCTGCGCCTGAGCTGCGCGGCG AGCGGCTTTACCTTTAGCGATTATGCGATGAGCTGGA TTCGCCAGGCGCCGGGCAAAGGCCTGGAATGGGTGA GCAGCATTAACATTGGCGCGACCTATATTTATTATGC GGATAGCGTGAAAGGCCGCTTTACCATTAGCCGCGA TAACGCGAAAAACAGCCTGTATCTGCAGATGAACAG CCTGCGCGCGGAAGATACCGCGGTGTATTATTGCGC GCGCCCGGGCAGCCCGTATGAATATGATAAAGCGTA TTATAGCATGGCGTATTGGGGCCAGGGCACCACCGT GACCGTGAGCAGC	39
CH1	GCGTCGACTAAGGGCCCTTCAGTTTTTCCACTCGCCC CCAGTAGCAAGTCCACATCTGGGGGTACCGCTGCCC TGGGCTGCCTTGTGAAAGACTATTTCCCTGAACCAGT CACTGTGTCATGGAATAGCGGAGCCCTGACCTCCGG TGTACACACATTCCCCGCTGTGTTGCAGTCTAGTGGC CTGTACAGCCTCTCCTCTGTTGTGACCGTCCCTTCAA GCTCCCTGGGGACACAGACCTATATCTGTAACGTGA ATCATAAGCCATCTAACACTAAAGTAGATAAAAAAG TGGAGCCCAAGAGTTGC	40
IRES EV71, internal ribosomal entry side	CCCGAAGTAACTTAGAAGCTGTAAATCAACGATCAA TAGCAGGTGTGGCACACCAGTCATACCTTGATCAAG CACTTCTGTTTCCCCGGACTGAGTATCAATAGGCTGC TCGCGCGGCTGAAGGAGAAAACGTTCGTTACCCGAC CAACTACTTCGAGAAGCTTAGTACCACCATGAACGA GGCAGGGTGTTTCGCTCAGCACAACCCCAGTGTAGA TCAGGCTGATGAGTCACTGCAACCCCCATGGGCGAC CATGGCAGTGGCTGCGTTGGCGGCCTGCCCATGGAG AAATCCATGGGACGCTCTAATTCTGACATGGTGTGA AGTGCCTATTGAGCTAACTGGTAGTCCTCCGGCCCCT GATTGCGGCTAATCCTAACTGCGGAGCACATGCTCA CAAACCAGTGGGTGGTGTGTCGTAACGGGCAACTCT GCAGCGGAACCGACTACTTTGGGTGTCCGTGTTTCCT TTTATTCCTATATTGGCTGCTTATGGTGACAATCAAA AAGTTGTTACCATATAGCTATTGGATTGGCCATCCGG TGTGCAACAGGGCAACTGTTTACCTATTTATTGGTTT TGTACCATTATCACTGAAGTCTGTGATCACTCTCAAA TTCATTTTGACCCTCAACACAATCAAAC	41

Table 6: Anti-FITC- scFv amino acid sequences

Construct	Amino acid sequence	SEQ ID NO
Anti-FITC CDR H1 Kabat	HYWMN	42
Anti-FITC CDR H2 Kabat	QFRNKPYNYETYYSDSVKG	43
Anti-FITC CDR H3 Kabat	ASYGMEY	44
Anti-FITC CDR L1 Kabat	RSSQSLVHSNGNTYLR	45
Anti-FITC CDR L2 Kabat	KVSNRVS	46
Anti-FITC CDR L3 Kabat	SQSTHVPWT	47
Anti-FITC-scFv-CD28ATD-CD28CSD-CD3zSSD fusion	GVKLDETGGGLVQPGGAMKLSCVTSGFTFGHYWMNW VRQSPEKGLEWVAQFRNKPYNYETYYSDSVKGRFTISR DDSKSSVYLQMNNLRVEDTGIYYCTGASYGMEYLGQG TSVTVSSGGGGSGGGGSGGGGSGGGGSDVVMTQTPLS LPVSLGDQASISCRSSQSLVHSNGNTYLRWYLQKPGQS PKVLIYKVSNRVSGVPDRFSGSGSGTDFTLKINRVEAED LGVYFCSQSTHVPWTFGGGTKLEIKRGGGGSFWVLVV VGGVLACYSLLVTVAFIIFWVRSKRSRLLHSDYMNMTP RRPGPTRKHYQPYAPPRDFAAYRSRVKFSRSADAPAYQ QGQNQLYNELNLGRREEYDVLDKRRGRDPEMGGKPR RKNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGH DGLYQGLSTATKDTYDALHMQALPPR	48
Anti-FITC-scFv	GVKLDETGGGLVQPGGAMKLSCVTSGFTFGHYWMNW VRQSPEKGLEWVAQFRNKPYNYETYYSDSVKGRFTISR DDSKSSVYLQMNNLRVEDTGIYYCTGASYGMEYLGQG TSVTVSSGGGGSGGGGSGGGGSGGGGSDVVMTQTPLS LPVSLGDQASISCRSSQSLVHSNGNTYLRWYLQKPGQS PKVLIYKVSNRVSGVPDRFSGSGSGTDFTLKINRVEAED LGVYFCSQSTHVPWTFGGGTKLEIK	49
Anti-FITC VH	GVKLDETGGGLVQPGGAMKLSCVTSGFTFGHYWMNW VRQSPEKGLEWVAQFRNKPYNYETYYSDSVKGRFTISR DDSKSSVYLQMNNLRVEDTGIYYCTGASYGMEYLGQG TSVTVSS	50
Anti-FITC VL	DVVMTQTPLSLPVSLGDQASISCRSSQSLVHSNGNTYL RWYLQKPGQSPKVLIYKVSNRVSGVPDRFSGSGSGTDF TLKINRVEAEDLGVYFCSQSTHVPWTFGGGTKLEIK	51

Table 7: Anti-HA- scFv amino acid sequences

Construct	Amino acid sequence	SEQ ID NO
Anti-HA CDR H1 Kabat	NYDMA	52
Anti-HA CDR H2 Kabat	TISHDGRNTNYRDSVKG	53
Anti-HA CDR H3 Kabat	PGFAH	54
Anti-HA CDR L1 Kabat	RSSKTLLNTRGITSLY	55
Anti-HA CDR L2 Kabat	RMSNLAS	56
Anti-HA CDR L3 Kabat	AQFLEFPLT	57
Anti-HA-scFv-CD28ATD-CD28CSD-CD3zSSD fusion	EVQLVESGGGLVQPGRSMKLSCAVSGFIFSNYDMAWV RQAPKKCLEWVATISHDGRNTNYRDSVKGRFTGSRDS AQSTLYLQMDSLRSEDTAIYFCAGPGFAHWGQGTLVT VSSGGGGSGGGGSGGGGSGGGGSDIVLTQAPLSVSVSP GESASISCRSSKTLLNTRGITSLYWYLQKPGKSPQLLIYR MSNLASGIPDRFSGSGSETHFTLQISKVETEDVGIYYCA QFLEFPLTFGCGTKLEIKGGGGSFWVLVVVGGVLACYS LLVTVAFIIFWVRSKRSRLLHSDYMNMTPRRPGPTRKH YQPYAPPRDFAAYRSRVKFSRSADAPAYQQGQNQLYN ELNLGRREEYDVLDKRRGRDPEMGGKPRRKNPQEGLY NELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLST ATKDTYDALHMQALPPR	58
Anti-HA-scFv	EVQLVESGGGLVQPGRSMKLSCAVSGFIFSNYDMAWV RQAPKKCLEWVATISHDGRNTNYRDSVKGRFTGSRDS AQSTLYLQMDSLRSEDTAIYFCAGPGFAHWGQGTLVT VSSGGGGSGGGGSGGGGSGGGGSDIVLTQAPLSVSVSP	59

	GESASISCRSSKTLLNTRGITSLYWYLQKPGKSPQLLIYR MSNLASGIPDRFSGSGSETHFTLQISKVETEDVGIYYCA QFLEFPLTFGCGTKLEIK	
Anti-HA VH	EVQLVESGGGLVQPGRSMKLSCAVSGFIFSNYDMAWV RQAPKKCLEWVATISHDGRNTNYRDSVKGRFTGSRDS AQSTLYLQMDSLRSEDTAIYFCAGPGFAHWGQGTLVT VSS	60
Anti-HA VL	DIVLTQAPLSVSVSPGESASISCRSSKTLLNTRGITSLYW YLQKPGKSPQLLIYRMSNLASGIPDRFSGSGSETHFTLQI SKVETEDVGIYYCAQFLEFPLTFGCGTKLEIK	61

Table 8: Anti-HA-Fab amino acid sequences

Construct	Protein Sequence	SEQ ID NO
Anti-HA CDR H1 Kabat	see table 7	52
Anti-HA CDR H2 Kabat	see table 7	53
Anti-HA CDR H3 Kabat	see table 7	54
Anti-HA CDR L1 Kabat	see table 7	55
Anti-HA CDR L2 Kabat	see table 7	56
Anti-HA CDR L3 Kabat	see table 7	57
Anti-HA-Fab-heavy chain-CD28ATD-CD28CSD-CD3zSSD fusion	EVQLVESGGGLVQPGRSMKLSCAVSGFIFSNYDMAWV RQAPKKGLEWVATISHDGRNTNYRDSVKGRFTGSRDS AQSTLYLQMDSLRSEDTAIYFCAGPGFAHWGQGTLVT VSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEP VTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSS SLGTQTYICNVNHKPSNTKVDKKVEPKSCGGGGSFWV LVVVGGVLACYSLLVTVAFIIFWVRSKRSRLLHSDYMN MTPRRPGPTRKHYQPYAPPRDFAAYRSRVKFSRSADAP AYQQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGG KPRRKNPQEGLYNELQKDKMAEAYSEIGMKGERRRGK GHDGLYQGLSTATKDTYDALHMQALPPR	62
Anti-HA-Fab heavy chain	EVQLVESGGGLVQPGRSMKLSCAVSGFIFSNYDMAWV RQAPKKGLEWVATISHDGRNTNYRDSVKGRFTGSRDS AQSTLYLQMDSLRSEDTAIYFCAGPGFAHWGQGTLVT VSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEP VTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSS SLGTQTYICNVNHKPSNTKVDKKVEPKSC	63
Anti-HA-Fab light chain	DIVLTQAPLSVSVSPGESASISCRSSKTLLNTRGITSLYW YLQKPGKSPQLLIYRMSNLASGIPDRFSGSGSETHFTLQI SKVETEDVGIYYCAQFLEFPLTFGSGTKLEIKRTVAAPS VFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDN ALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHK VYACEVTHQGLSSPVTKSFNRGEC	64
Anti-HA VH	EVQLVESGGGLVQPGRSMKLSCAVSGFIFSNYDMAWV RQAPKKGLEWVATISHDGRNTNYRDSVKGRFTGSRDS AQSTLYLQMDSLRSEDTAIYFCAGPGFAHWGQGTLVT VSS	65
Anti-HA VL	DIVLTQAPLSVSVSPGESASISCRSSKTLLNTRGITSLYW YLQKPGKSPQLLIYRMSNLASGIPDRFSGSGSETHFTLQI SKVETEDVGIYYCAQFLEFPLTFGSGTKLEIK	66
CL	see Table 4	33
CH1 (human)	see Table 4	35

Table 9: Anti-Biotin- scFv amino acid sequences

Construct	Amino acid sequence	SEQ ID NO
Anti-Biotin CDR H1 Kabat	GFNNKDTFFQ	67
Anti-Biotin CDR H2 Kabat	RIDPANGFTKYAQKFQG	68
Anti-Biotin CDR H3 Kabat	WDTYGAAWFAY	69
Anti-Biotin CDR L1 Kabat	RASGNIHNYLS	70
Anti-Biotin CDR L2 Kabat	SAKTLAD	71
Anti-Biotin CDR L3 Kabat	QHFWSSIYT	72

Anti-Biotin-scFv-CD28ATD-CD28CSD-CD3zSSD fusion	QVQLVQSGAEVKKPGSSVKVSCKSSGFNNKDTFFQWV RQAPGQCLEWMGRIDPANGFTKYAQKFQGRVTITADT STSTAYMELSSLRSEDTAVYYCARWDTYGAAWFAYW GQGTLVTVSSGGGGSGGGGSGGGGSGGGGSDIQMTQS PSSLSASVGDRVTITCRASGNIHNYLSWYQQKPGKVPK LLIYSAKTLADGVPSRFSGSGSGTDFTLTISSLQPEDVAT YYCQHFWSSIYTFGCGTKLEIKRGGGGSFWVLVVVGG VLACYSLLVTVAFIIFWVRSKRSRLLHSDYMNMTPRRP GPTRKHYQPYAPPRDFAAYRSRVKFSRSADAPAYQQG QNQLYNELNLGRREEYDVLDKRRGRDPEMGGKPRRK NPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDG LYQGLSTATKDTYDALHMQALPPR	73
Anti-Biotin-scFv	QVQLVQSGAEVKKPGSSVKVSCKSSGFNNKDTFFQWV RQAPGQCLEWMGRIDPANGFTKYAQKFQGRVTITADT STSTAYMELSSLRSEDTAVYYCARWDTYGAAWFAYW GQGTLVTVSSGGGGSGGGGSGGGGSGGGGSDIQMTQS PSSLSASVGDRVTITCRASGNIHNYLSWYQQKPGKVPK LLIYSAKTLADGVPSRFSGSGSGTDFTLTISSLQPEDVAT YYCQHFWSSIYTFGCGTKLEIK	74
Anti-Biotin VH	QVQLVQSGAEVKKPGSSVKVSCKSSGFNNKDTFFQWV RQAPGQCLEWMGRIDPANGFTKYAQKFQGRVTITADT STSTAYMELSSLRSEDTAVYYCARWDTYGAAWFAYW GQGTLVTVSS	75
Anti-Biotin VL	DIQMTQSPSSLSASVGDRVTITCRASGNIHNYLSWYQQ KPGKVPKLLIYSAKTLADGVPSRFSGSGSGTDFTLTISSL QPEDVATYYCQHFWSSIYTFGCGTKLEIK	76

Table 10: Anti-myc-Fab amino acid sequences

Construct	Protein Sequence	SEQ ID NO
Anti-myc CDR H1 Kabat	HYGMS	77
Anti-myc CDR H2 Kabat	TIGSRGTYTHYPDSVKG	78
Anti-myc CDR H3 Kabat	RSEFYYYGNTYYYSAMDY	79
Anti-myc CDR L1 Kabat	RASESVDNYGFSFMN	80
Anti-myc CDR L2 Kabat	AISNRGS	81
Anti-myc CDR L3 Kabat	QQTKEVPWT	82
Anti-myc-Fab-heavy chain-CD28ATD-CD28CSD-CD3zSSD fusion	EVHLVESGGDLVKPGGSLKLSCAASGFTFSHYGMSWV RQTPDKRLEWVATIGSRGTYTHYPDSVKGRFTISRDND KNALYLQMNSLKSEDTAMYYCARRSEFYYYGNTYYY SAMDYWGQGASVTVSSAKTTPPSVYPLAPGSAAQTNS MVTLGCLVKGYFPEPVTVTWNSGSLSSGVHTFPAVLQS DLYTLSSSVTVPSSTWPSETVTCNVAHPASSTKVDKKIV PRDCGGGGSFWVLVVVGGVLACYSLLVTVAFIIFWVR SKRSRLLHSDYMNMTPRRPGPTRKHYQPYAPPRDFAA YRSRVKFSRSADAPAYQQGQNQLYNELNLGRREEYDV LDKRRGRDPEMGGKPRRKNPQEGLYNELQKDKMAEA YSEIGMKGERRRGKGHDGLYQGLSTATKDTYDALHM QALPPR	83
Anti-myc-Fab heavy chain	EVHLVESGGDLVKPGGSLKLSCAASGFTFSHYGMSWV RQTPDKRLEWVATIGSRGTYTHYPDSVKGRFTISRDND KNALYLQMNSLKSEDTAMYYCARRSEFYYYGNTYYY SAMDYWGQGASVTVSSAKTTPPSVYPLAPGSAAQTNS MVTLGCLVKGYFPEPVTVTWNSGSLSSGVHTFPAVLQS DLYTLSSSVTVPSSTWPSETVTCNVAHPASSTKVDKKIV PRDC	84
Anti-myc-Fab light chain	DIVLTQSPASLAVSLGQRATISCRASESVDNYGFSFMN WFQQKPGQPPKLLIYAISNRGSGVPARFSGSGSGTDFSL NIHPVEEDDPAMYFCQQTKEVPWTFGGGTKLEIKRAD AAPTVSIFPPSSEQLTSGGASVVCFLNNFYPKDINVKWK IDGSERQNGVLNSWTDQDSKDSTYSMSSTLTLTKDEYE RHNSYTCEATHKTSTSPIVKSFNRNEC	85

Anti-myc VH	EVHLVESGGDLVKPGGSLKLSCAASGFTFSHYGMSWV RQTPDKRLEWVATIGSRGTYTHYPDSVKGRFTISRDND KNALYLQMNSLKSEDTAMYYCARRSEFYYYGNTYYY SAMDYWGQGASVTVSS	86
Anti-myc VL	DIVLTQSPASLAVSLGQRATISCRASESVDNYGFSFMN WFQQKPGQPPKLLIYAISNRGSGVPARFSGSGSGTDFSL NIHPVEEDDPAMYFCQQTKEVPWTFGGGTKLEIK	87
C kappa	RADAAPTVSIFPPSSEQLTSGGASVVCFLNNFYPKDINV KWKIDGSERQNGVLNSWTDQDSKDSTYSMSSTLTLTK DEYERHNSYTCEATHKTSTSPIVKSFNRNEC	88
CH1 (mouse)	AKTTPPSVYPLAPGSAAQTNSMVTLGCLVKGYFPEPVT VTWNSGSLSSGVHTFPAVLQSDLYTLSSSVTVPSSTWPS ETVTCNVAHPASSTKVDKKIVPRDC	89

Table 11: Anti-GCN4- scFv amino acid sequences

Construct	Amino acid sequence	SEQ ID NO
Anti-GCN4 CDR H1 Kabat	DYGVN	90
Anti-GCN4 CDR H2 Kabat	VIWGDGITDHNSALKS	91
Anti-GCN4 CDR H3 Kabat	GLFDY	92
Anti-GCN4 CDR L1 Kabat	RSSTGAVTTSNYAS	93
Anti-GCN4 CDR L2 Kabat	GTNNRAP	94
Anti-GCN4 CDR L3 Kabat	VLWYSNHWV	95
Anti-GCN4-scFv-CD28ATD-CD28CSD-CD3zSSD fusion	DAVVTQESALTSSPGETVTLTCRSSTGAVTTSNYASWV QEKPDHLFTGLIGGTNNRAPGVPARFSGSLIGDKAALTI TGAQTEDEAIYFCVLWYSNHWVLGGGTKLTVLGGGG GSGGGGSGGGGSGGGGSDVQLQQSGPGLVAPSQSLSIT CTVSGFSLTDYGVNWVRQSPGKGLEWLGVIWGDGITD HNSALKSRLSVTKDNSKSQVFLKMSSLQSGDSARYYC VTGLFDYWGQGTTLTVSSGGGGSFWVLVVVGGVLAC YSLLVTVAFIIFWVRSKRSRLLHSDYMNMTPRRPGPTR KHYQPYAPPRDFAAYRSRVKFSRSADAPAYQQGQNQL YNELNLGRREEYDVLDKRRGRDPEMGGKPRRKNPQEG LYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGL STATKDTYDALHMQALPPR	96
Anti-GCN4-scFv	DAVVTQESALTSSPGETVTLTCRSSTGAVTTSNYASWV QEKPDHLFTGLIGGTNNRAPGVPARFSGSLIGDKAALTI TGAQTEDEAIYFCVLWYSNHWVLGGGTKLTVLGGGG GSGGGGSGGGGSGGGGSDVQLQQSGPGLVAPSQSLSIT CTVSGFSLTDYGVNWVRQSPGKGLEWLGVIWGDGITD HNSALKSRLSVTKDNSKSQVFLKMSSLQSGDSARYYC VTGLFDYWGQGTTLTVSS	97
Anti-GCN4 VH	DVQLQQSGPGLVAPSQSLSITCTVSGFSLTDYGVNWVR QSPGKGLEWLGVIWGDGITDHNSALKSRLSVTKDNSKS QVFLKMSSLQSGDSARYYCVTGLFDYWGQGTTLTVSS	98
Anti-GCN4 VL	DAVVTQESALTSSPGETVTLTCRSSTGAVTTSNYASWV QEKPDHLFTGLIGGTNNRAPGVPARFSGSLIGDKAALTI TGAQTEDEAIYFCVLWYSNHWVLGGGTKLTVL	99

Table 12: Polypeptide tag sequencesAnti-GCN4- scFv amino acid sequences

Construct	Amino acid sequence	SEQ ID NO
HA tag	YPYDVPDYA	100
Myc tag	EQKLISEEDL	101
GCN4 tag	YHLENEVARLKK	102
AviTag	GLNDIFEAQKIEWH	103

Table 13

Construct	Amino acid sequence	SEQ ID NO
Human CD3z	MKWKALFTAAILQAQLPITEAQSFGLLDPKLCYLLDGI LFIYGVILTALFLRVKFSRSADAPAYQQGQNQLYNELN LGRREEYDVLDKRRGRDPEMGGKPQRRKNPQEGLYNE	104

	LQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTAT KDTYDALHMQALPPR	
Human CD3z	ATGAAGTGGAAGGCGCTTTTCACCGCGGCCATCCTG CAGGCACAGTTGCCGATTACAGAGGCACAGAGCTTT GGCCTGCTGGATCCCAAACTCTGCTACCTGCTGGATG GAATCCTCTTCATCTATGGTGTCATTCTCACTGCCTT GTTCCTGAGAGTGAAGTTCAGCAGGAGCGCAGAGCC CCCCGCGTACCAGCAGGGCCAGAACCAGCTCTATAA CGAGCTCAATCTAGGACGAAGAGAGGAGTACGATGT TTTGGACAAGAGACGTGGCCGGGACCCTGAGATGGG GGGAAAGCCGAGAAGGAAGAACCCTCAGGAAGGCC TGTACAATGAACTGCAGAAAGATAAGATGGCGGAGG CCTACAGTGAGATTGGGATGAAAGGCGAGCGCCGGA GGGGCAAGGGGCACGATGGCCTTTACCAGGGTCTCA GTACAGCCACCAAGGACACCTACGACGCCCTTCACA TGCAGGCCCTGCCCCCTCGCTAA	105
Murine CD3z	MKWKVSVLACILHVRFPGAEAQSFGLLDPKLCYLLDGI LFIYGVIITALYLRAKFSRSAETAANLQDPNQLYNELNL GRREEYDVLEKKRARDPEMGGKQQRRRNPQEGVYNA LQKDKMAEAYSEIGTKGERRRGKGHDGLYQGLSTATK DTYDALHMQTLAPR	106
Murine CD3z	ATGAAGTGGAAAGTGTCTGTTCTCGCCTGCATCCTCC ACGTGCGGTTCCCAGGAGCAGAGGCACAGAGCTTTG GTCTGCTGGATCCCAAACTCTGCTACTTGCTAGATGG AATCCTCTTCATCTACGGAGTCATCATCACAGCCCTG TACCTGAGAGCAAAATTCAGCAGGAGTGCAGAGACT GCTGCCAACCTGCAGGACCCCAACCAGCTCTACAAT GAGCTCAATCTAGGGCGAAGAGAGGAATATGACGTC TTGGAGAAGAAGCGGGCTCGGGATCCAGAGATGGG AGGCAAACAGCAGAGGAGGAGGAACCCCCAGGAAG GCGTATACAATGCACTGCAGAAAGACAAGATGGCAG AAGCCTACAGTGAGATCGGCACAAAAGGCGAGAGG CGGAGAGGCAAGGGGCACGATGGCCTTTACCAGGGT CTCAGCACTGCCACCAAGGACACCTATGATGCCCTG CATATGCAGACCCTGGCCCCTCGCTAA	107
Human CD28	ATGCTGCGCCTGCTGCTGGCGCTGAACCTGTTTCCGA GCATTCAGGTGACCGGCAACAAAATTCTGGTGAAAC AGAGCCCGATGCTGGTGGCGTATGATAACGCGGTGA ACCTGAGCTGCAAATATAGCTATAACCTGTTTAGCCG CGAATTTCGCGCGAGCCTGCATAAAGGCCTGGATAG CGCGGTGGAAGTGTGCGTGGTGTATGGCAACTATAG CCAGCAGCTGCAGGTGTATAGCAAAACCGGCTTTAA CTGCGATGGCAAACTGGGCAACGAAAGCGTGACCTT TTATCTGCAGAACCTGTATGTGAACCAGACCGATATT TATTTTTGCAAAATTGAAGTGATGTATCCGCCGCCGT ATCTGGATAACGAAAAAAGCAACGGCACCATTATTC ATGTGAAAGGCAAACATCTGTGCCCGAGCCCGCTGT TTCCGGGCCCGAGCAAACCGTTTTGGGTGCTGGTGGT GGTGGGCGGCGTGCTGGCGTGCTATAGCCTGCTGGT GACCGTGGCGTTTATTATTTTTTGGGTGCGCAGCAAA CGCAGCCGCCTGCTGCATAGCGATTATATGAACATG ACCCCGCGCCGCCCGGGCCCGACCCGCAAACATTAT CAGCCGTATGCGCCGCCGCGCGATTTTGCGGCGTATC GCAGC	108
Human CD28	MLRLLLALNLFPSIQVTGNKILVKQSPMLVAYDNAVNL SCKYSYNLFSREFRASLHKGLDSAVEVCVVYGNYSQQ LQVYSKTGFNCDGKLGNESVTFYLQNLYVNQTDIYFC KIEVMYPPPYLDNEKSNGTIIHVKGKHLCPSPLFPGPSK PFWVLVVVGGVLACYSLLVTVAFIIFWVRSKRSRLLHS DYMNMTPRRPGPTRKHYQPYAPPRDFAAYRS	109
Murine CD28	ATGACCCTGCGCCTGCTGTTTCTGGCGCTGAACTTTT	110

	TTAGCGTGCAGGTGACCGAAAACAAAATTCTGGTGA AACAGAGCCCGCTGCTGGTGGTGGATAGCAACGAAG TGAGCCTGAGCTGCCGCTATAGCTATAACCTGCTGGC GAAAGAATTTCGCGCGAGCCTGTATAAAGGCGTGAA CAGCGATGTGGAAGTGTGCGTGGGCAACGGCAACTT TACCTATCAGCCGCAGTTTCGCAGCAACGCGGAATTT AACTGCGATGGCGATTTTGATAACGAAACCGTGACC TTTCGCCTGTGGAACCTGCATGTGAACCATACCGATA TTTATTTTTGCAAAATTGAATTTATGTATCCGCCGCC GTATCTGGATAACGAACGCAGCAACGGCACCATTAT TCATATTAAAGAAAAACATCTGTGCCATACCCAGAG CAGCCCGAAACTGTTTTGGGCGCTGGTGGTGGTGGC GGGCGTGCTGTTTTGCTATGGCCTGCTGGTGACCGTG GCGCTGTGCGTGATTTGGACCAACAGCCGCCGCAAC CGCCTGCTGCAGAGCGATTATATGAACATGACCCCG CGCCGCCCGGGCCTGACCCGCAAACCGTATCAGCCG TATGCGCCGGCGCGCGATTTTGCGGCGTATCGCCCG	
Murine CD28	MTLRLLFLALNFFSVQVTENKILVKQSPLLVVDSNEVSL SCRYSYNLLAKEFRASLYKGVNSDVEVCVGNGNFTYQ PQFRSNAEFNCDGDFDNETVTFRLWNLHVNHTDIYFCK IEFMYPPPYLDNERSNGTIIHIKEKHLCHTQSSPKLFWAL VVVAGVLFCYGLLVTVALCVIWTNSRRNRLLQSDYMN MTPRRPGLTRKPYQPYAPARDFAAYRP	111
CD28 YMNM	YMNM	112
CD28 PYAP	PYAP	113
Signal peptide	ATMGWSCIILFLVATATGVHS	114
Signal peptide DNA sequence	ATGGGATGGAGCTGTATCATCCTCTTCTTGGTAGCAA CAGCTACCGGTGTGCACTCC	115
Anti-CD20 (GA101) heavy chain	QVQLVQSGAEVKKPGSSVKVSCKASGYAFSYSWINWV RQAPGQGLEWMGRIFPGDGDTDYNGKFKGRVTITADK STSTAYMELSSLRSEDTAVYYCARNVFDGYWLVYWG QGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVK DYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSV VTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKT HTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCV VVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNS TYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTI SKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYP SDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTV DKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK	116
Anti-CD20 (GA101) light chain	DIVMTQTPLSLPVTPGEPASISCRSSKSLLHSNGITYLYW YLQKPGQSPQLLIYQMSNLVSGVPDRFSGSGSGTDFTL KISRVEAEDVGVYYCAQNLELPYTFGGGTKVEIKRTVA APSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWK VDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYE KHKVYACEVTHQGLSSPVTKSFNRGEC	117
Anti-CD3 HCDR1 Kabat	TYAMN	118
Anti-CD3 HCDR2 Kabat	RIRSKYNNYATYYADSVKG	119
Anti-CD3 HCDR3 Kabat	HGNFGNSYVSWFAY	120
Anti-CD3 LCDR1 Kabat	GSSTGAVTTSNYAN	121
Anti-CD3 LCDR2 Kabat	GTNKRAP	122
Anti-CD3 LCDR3 Kabat	ALWYSNLWV	123

* * *

CLAIMS

1. A method for assessing the specificity of a target antigen binding moiety capable of specific binding to a target antigen, the method comprising the steps of:
   a) providing an antigen binding molecule comprising an antigen binding domain and a recognition domain, wherein the antigen binding domain comprises the target antigen binding moiety, and wherein the recognition domain comprises a tag;
   b) contacting the antigen binding molecule with a target cell comprising the target antigen on the surface, particularly wherein the target cell is a cancer cell;
   c) contacting the antigen binding molecule with a chimeric antigen receptor (CAR) expressing reporter T (CAR-T) cell wherein the reporter CAR-T cell comprises:
      i. a CAR capable of specific binding to the recognition domain comprising the tag, wherein the CAR is operationally coupled to a response element;
      ii. a reporter gene under the control of the response element; and
   d) determining T cell activation by measuring the expression of the reporter gene to establish the specificity of the target antigen binding moiety.

2. The method of claim 1, wherein the antigen binding molecule is an IgG class antibody, particularly an IgG1 or IgG4 isotype antibody, or a fragment thereof.

3. The method of any one of claims 1 or 2, wherein the antigen binding domain is a Fab fragment and the recognition domain is an Fc domain.

4. The method of any one of claims 1 or 2, wherein the antigen binding domain and the recognition domain are the same domain, in particular a Fab fragment.

5. The method of any one of claims 1 to 4, wherein the tag is a hapten molecule.

6. The method of any one of claims 1 to 5, wherein the hapten molecule is Digoxigenin (DIG).

7. The method of any one of claims 1 to 4, wherein the tag is a polypeptide tag.

8. The method of claim 7, wherein the polypeptide tag is selected from the group consisting of myc-tag, HA-tag, AviTag, FLAG-tag, His-tag, GCN4-tag, and NE-tag.

9. The method of any one of claims 1 to 8, wherein the target antigen is a cell surface antigen or receptor.

10. The method of any one of claims 1 to 9, wherein the target antigen is a peptide bound to a molecule of the human major histocompatibility complex (MHC), wherein the target antigen binding moiety is a T cell receptor like (TCRL) antigen binding moiety.

11. A method for generating a TCB antibody, wherein the TCB antibody comprises a first antigen binding moiety specific for a target antigen and a second antigen binding moiety capable of specific binding to a T cell activating receptor, wherein the first antigen binding moiety is selected according to the method of any one of claims 1 to 10.

12. The method of claim 11, wherein the T cell activating receptor is CD3.

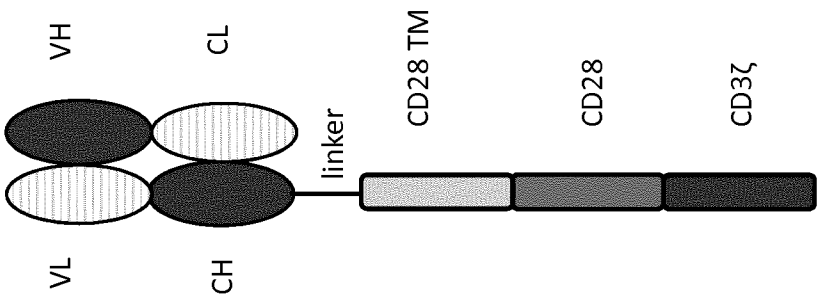
13. A chimeric antigen receptor (CAR) comprising an anchoring transmembrane domain and an extracellular domain comprising an antigen binding moiety, wherein the antigen binding moiety is capable of specific binding to a recognition domain comprising a tag but not capable of specific binding to the recognition domain not comprising the tag.

14. The CAR of claim 13, wherein the tag is a hapten molecule.

15. The CAR of claim 14, wherein the hapten molecule is Digoxigenin (DIG).

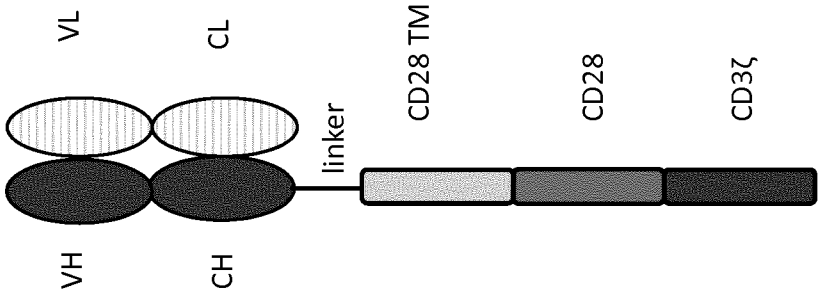
* * *

Figure 1C



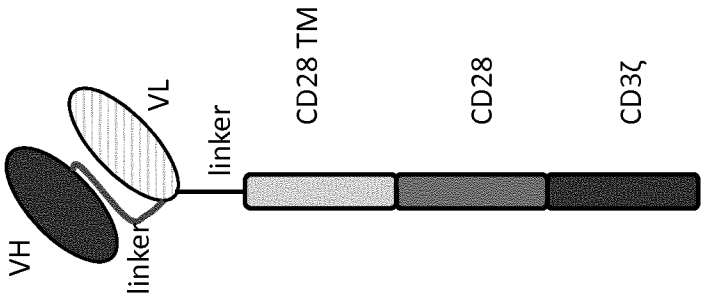
crossFab Format

Figure 1B



Fab Format

Figure 1A



scFv Format

Figure 2A

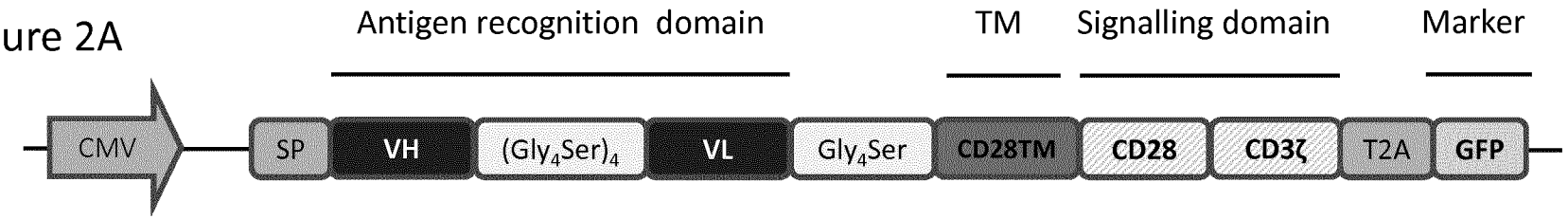


Figure 2B

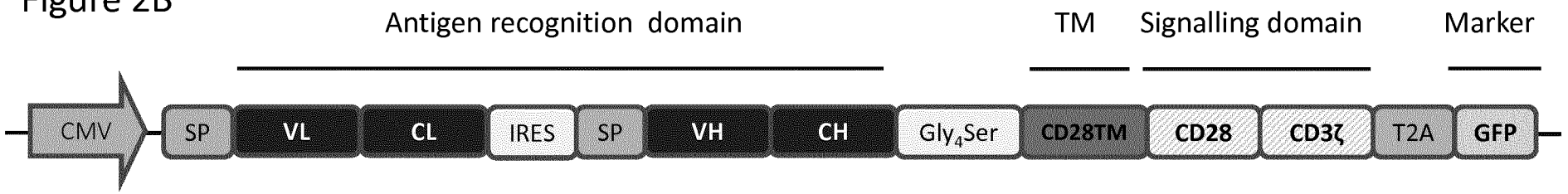
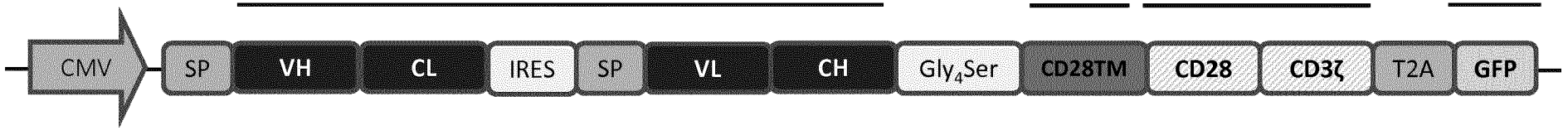
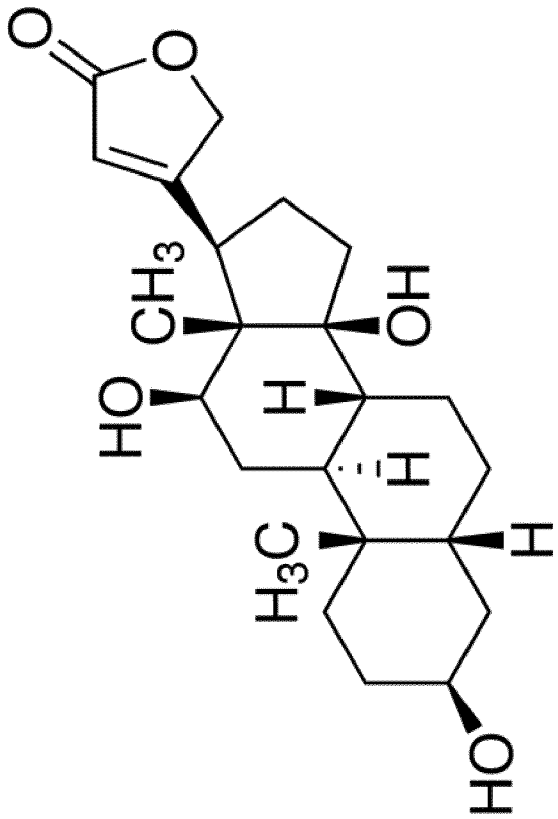


Figure 2C



CMV = Cytomegalovirus promotor
SP= Signal peptide
VH = variable heavy chain
VL = variable light chain
TM = transmembrane domain
IRES= internal ribosomal entry site



Digoxigenin

Synonym: *3β,12β,14β,21-Tetrahydroxy-20(22)-norcholenic acid lactone, 3β,12β,14-Trihydroxy-5β,20(22)-cardenolide, 5β,20(22)-Cardenolide-3β,12β,14-triol, Lanadigigenin*

Figure 3

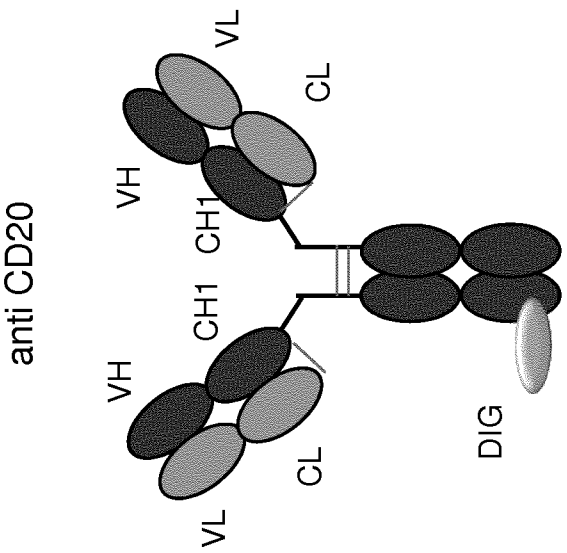


Figure 4

Figure 5B

Digoxigeninylated scFv fragment

Figure 5A

Digoxigeninylated Fab fragment

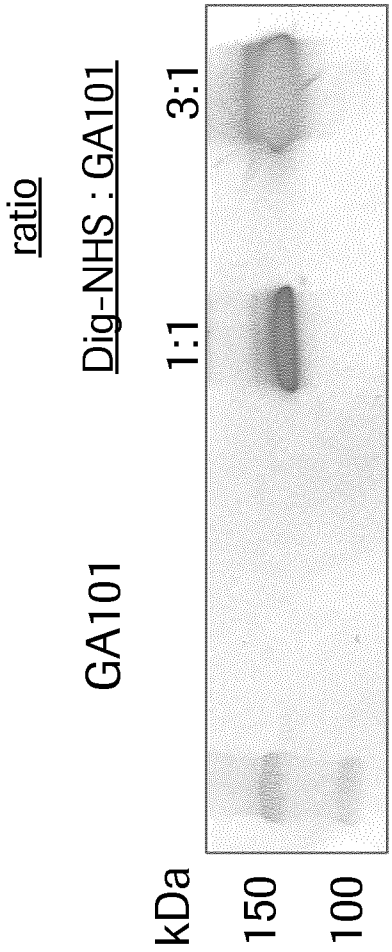


Figure 6

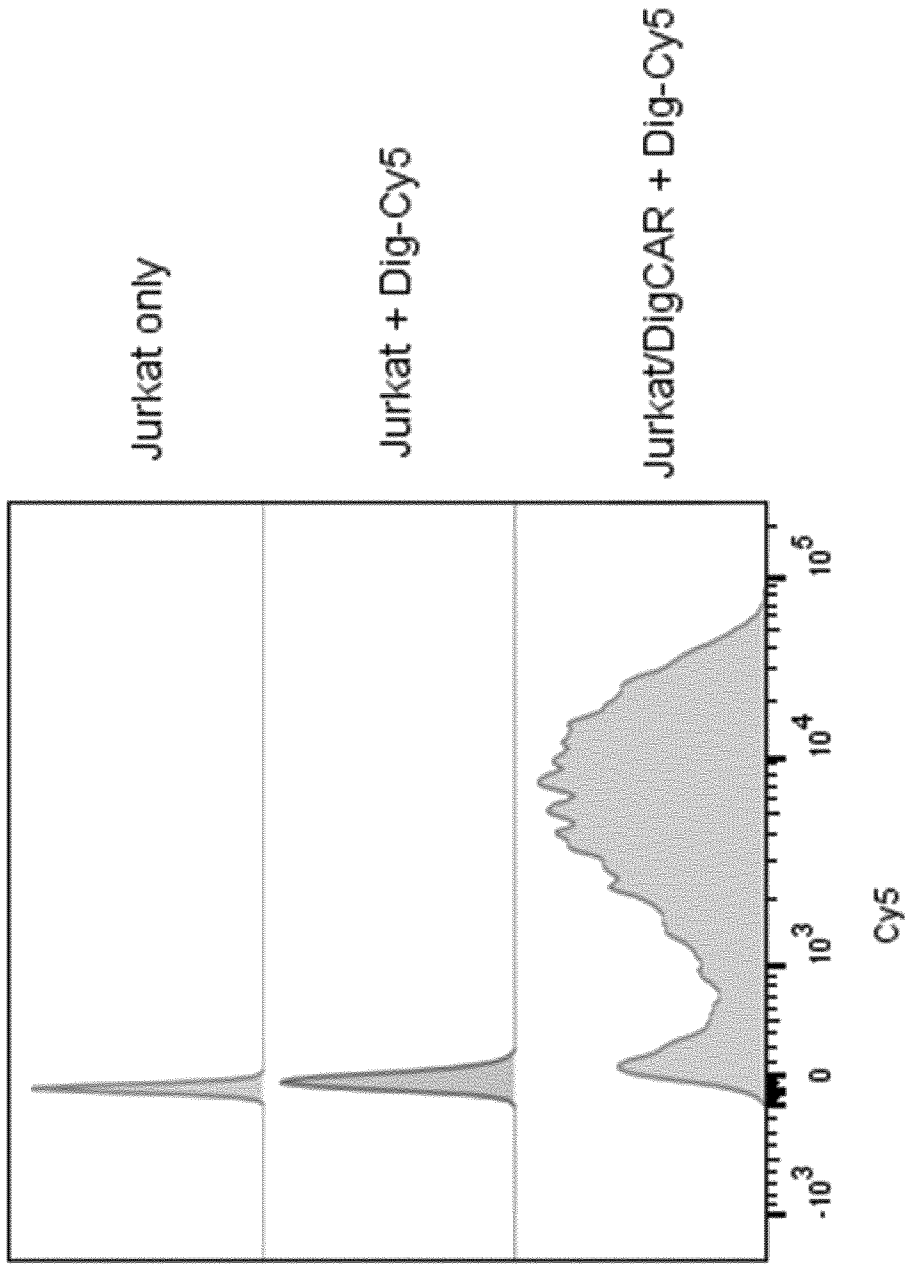

Jurkat only
Jurkat + Dig-Cy5
Jurkat/DigCAR + Dig-Cy5
-10^3
0
10^3
10^4
10^5
Cy5

Figure 7

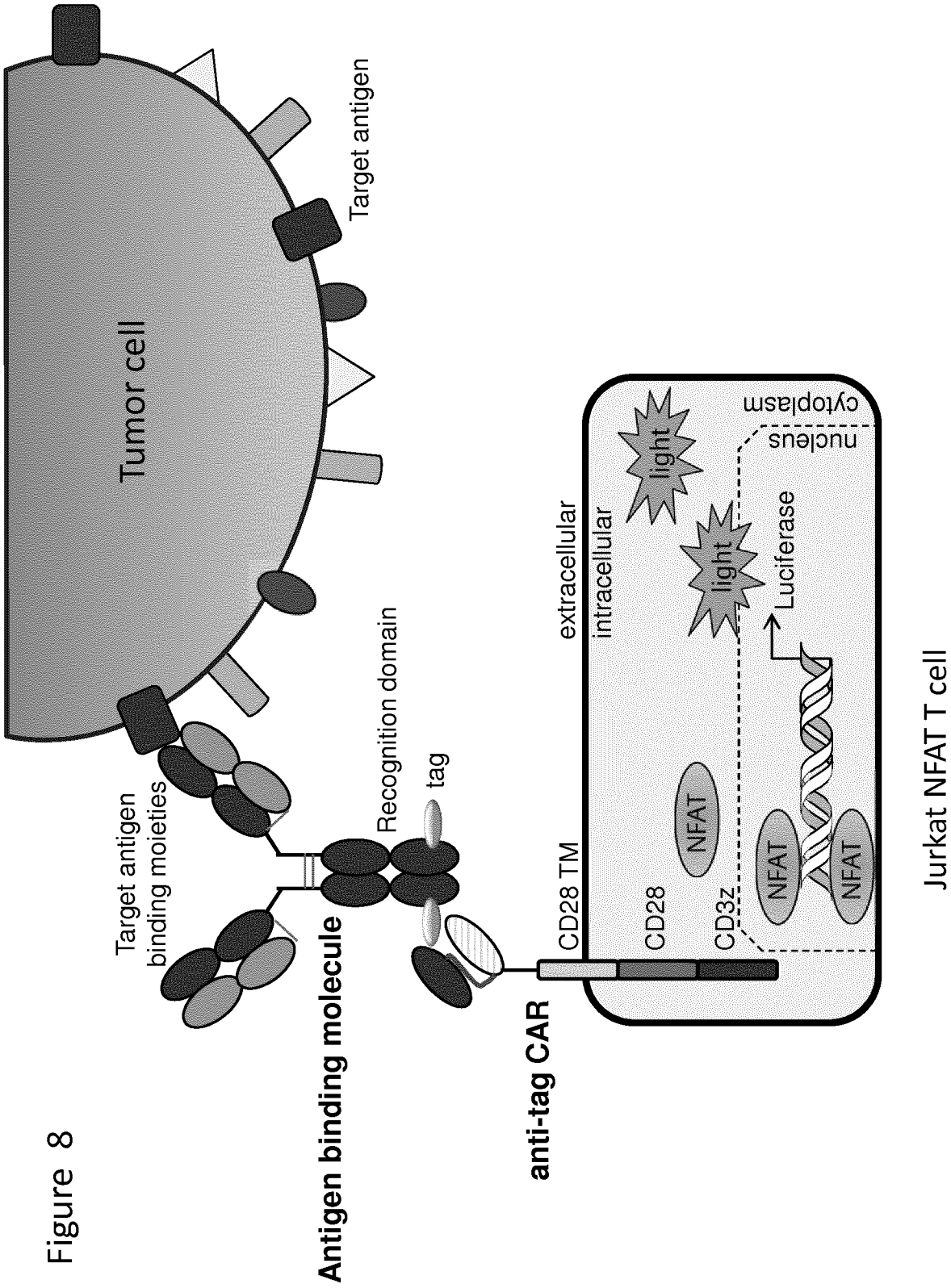
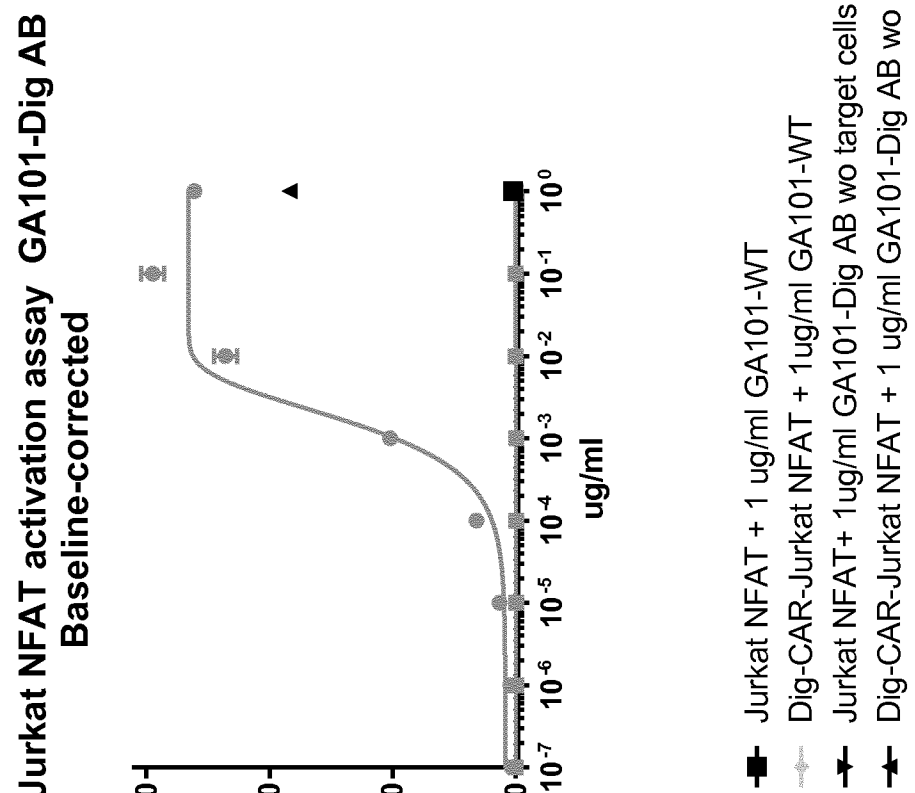

Tumor cell
Target antigen
Target antigen binding moieties
Antigen binding molecule
Recognition domain
tag
anti-tag CAR
CD28 TM
extracellular
intracellular
CD28
CD3z
NFAT
NFAT
NFAT
light
light
Luciferase
nucleus
cytoplasm
Jurkat NFAT T cell

Figure 8

Figure 9


anti-Dig Jurkat NFAT activation assay GA101-Dig AB
Baseline-corrected
CPS
15000
10000
5000
0
10-7 10-6 10-5 10-4 10-3 10-2 10-1 100
ug/ml
Jurkat NFAT + 1 ug/ml GA101-WT
Dig-CAR-Jurkat NFAT + 1ug/ml GA101-WT
Jurkat NFAT+ 1ug/ml GA101-Dig AB wo target cells
Dig-CAR-Jurkat NFAT + 1 ug/ml GA101-Dig AB wo target cells
Dig-CAR-Jurkat NFAT + GA101-Dig AB
Jurkat NFAT + GA101-Dig AB

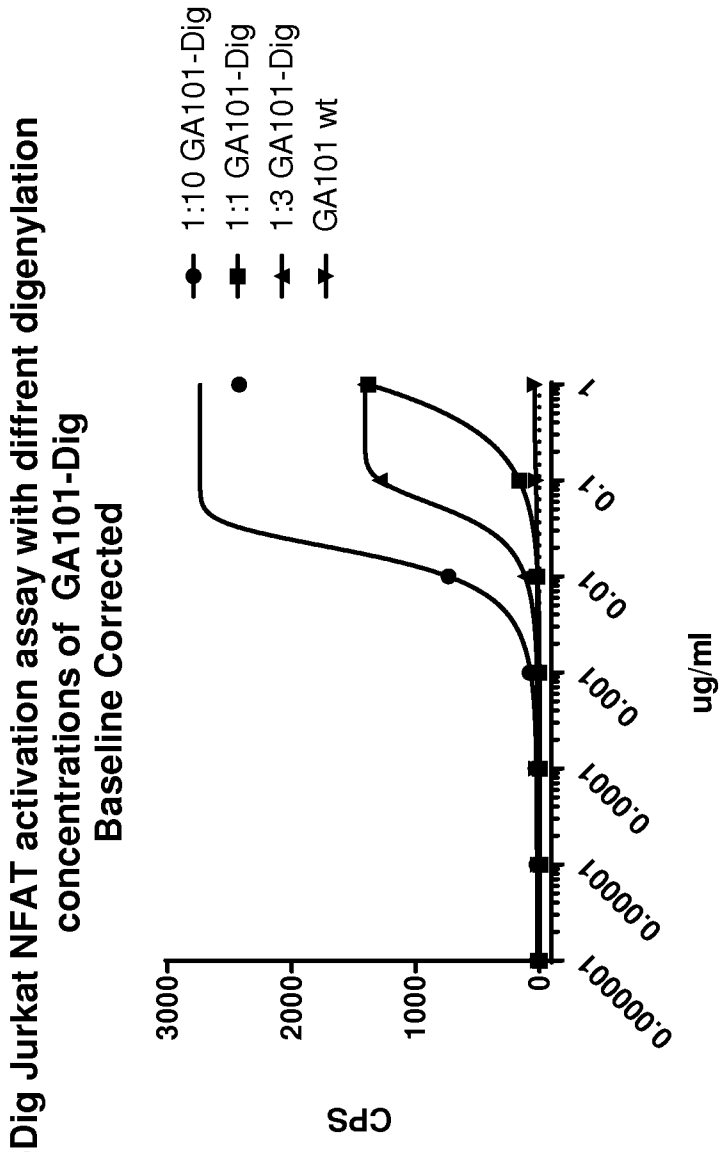
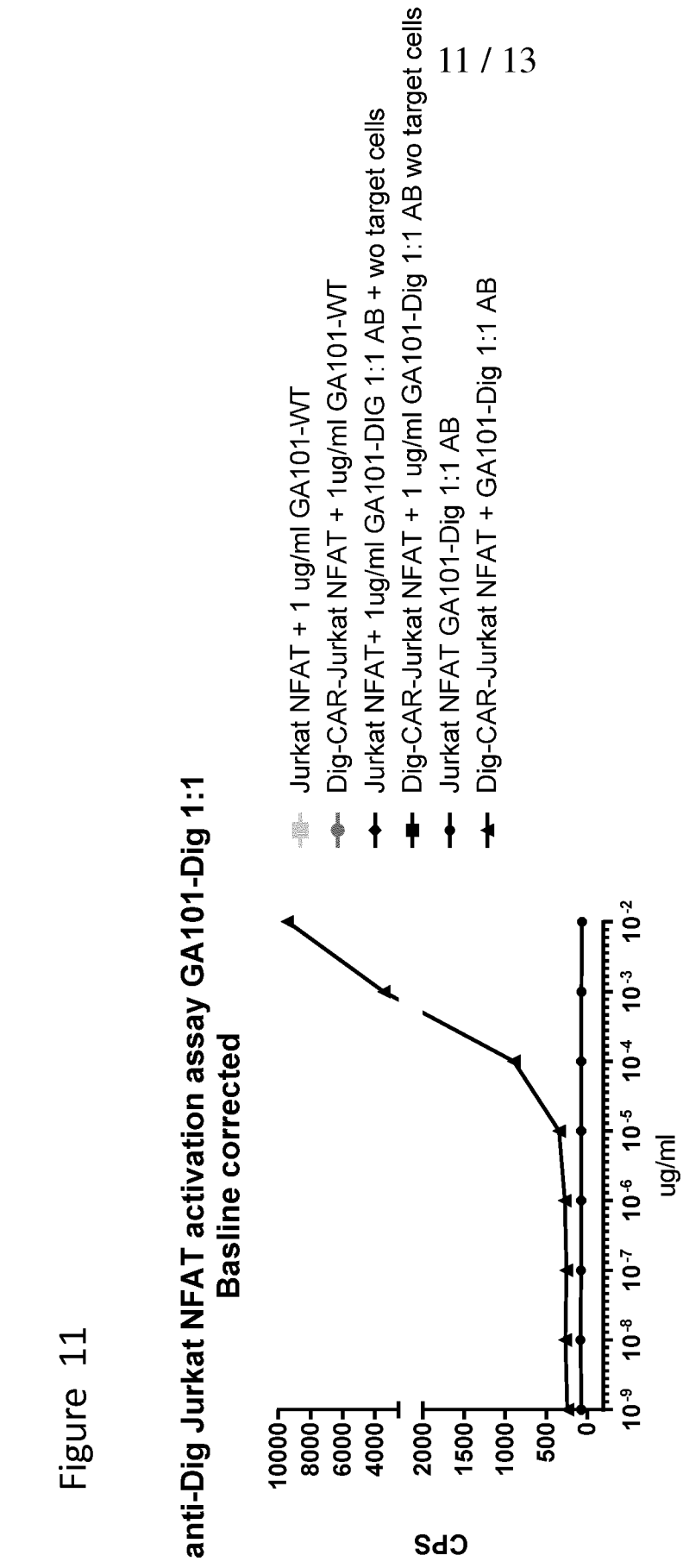

anti-Dig Jurkat NFAT activation assay with diffrent digenylation concentrations of GA101-Dig
Baseline Corrected
1:10 GA101-Dig
1:1 GA101-Dig
1:3 GA101-Dig
GA101 wt
CPS
3000
2000
1000
0
ug/ml
1
0,1
0,01
0,001
0,0001
0,00001
0,000001

Figure 10

Figure 11


anti-Dig Jurkat NFAT activation assay GA101-Dig 1:1
Basline corrected
Jurkat NFAT + 1 ug/ml GA101-WT
Dig-CAR-Jurkat NFAT + 1ug/ml GA101-WT
Jurkat NFAT+ 1ug/ml GA101-DIG 1:1 AB + wo target cells
Dig-CAR-Jurkat NFAT + 1 ug/ml GA101-Dig 1:1 AB wo target cells
Jurkat NFAT GA101-Dig 1:1 AB
Dig-CAR-Jurkat NFAT + GA101-Dig 1:1 AB
CPS
10000
8000
6000
4000
2000
1500
1000
500
0
10^-9 10^-8 10^-7 10^-6 10^-5 10^-4 10^-3 10^-2
ug/ml

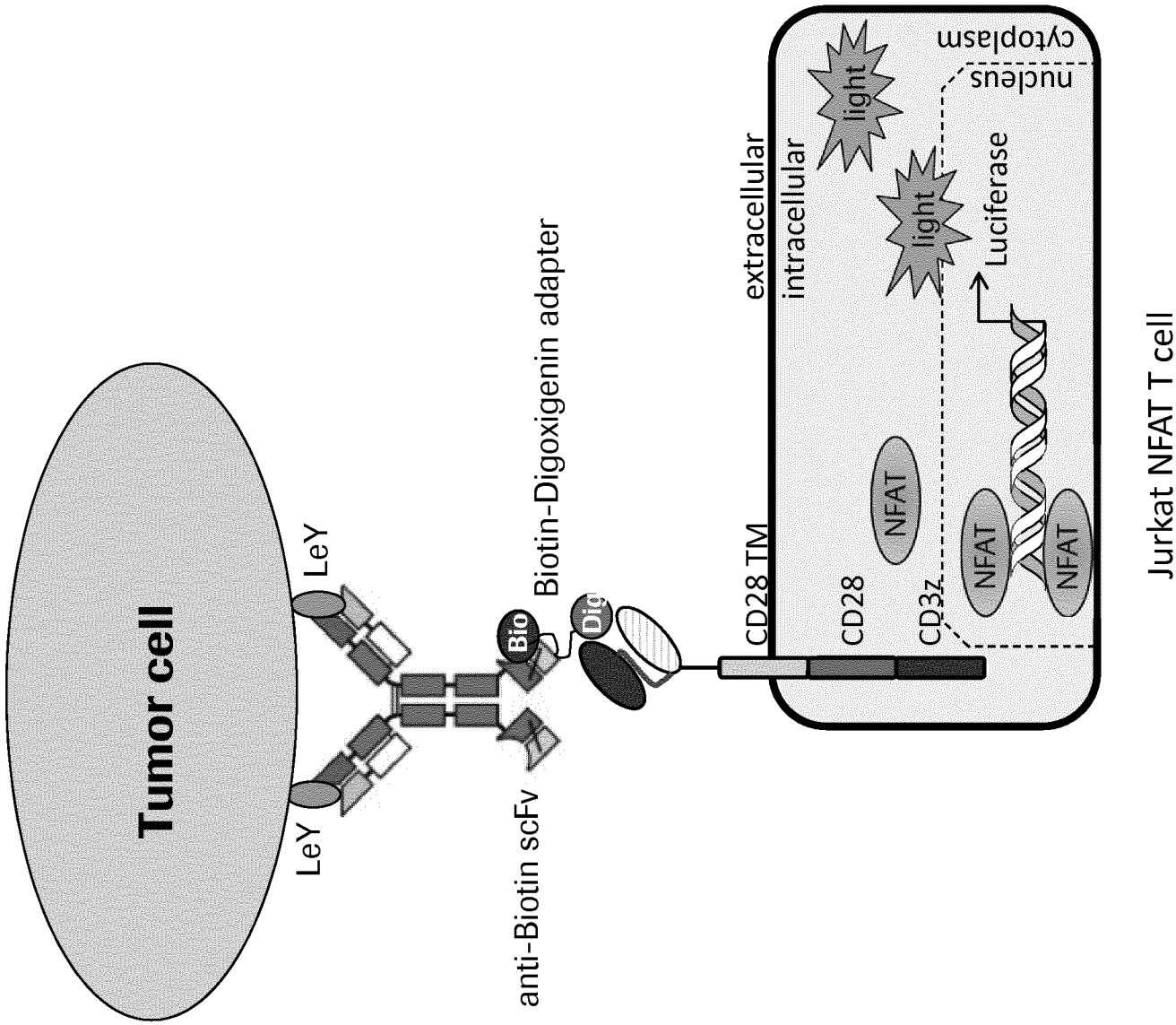

Tumor cell
LeY
LeY
anti-Biotin scFv
Bio
Biotin-Digoxigenin adapter
Dig
CD28 TM
extracellular
intracellular
CD28
CD3z
NFAT
NFAT
NFAT
light
light
Luciferase
nucleus
cytoplasm
Jurkat NFAT T cell

Figure 12

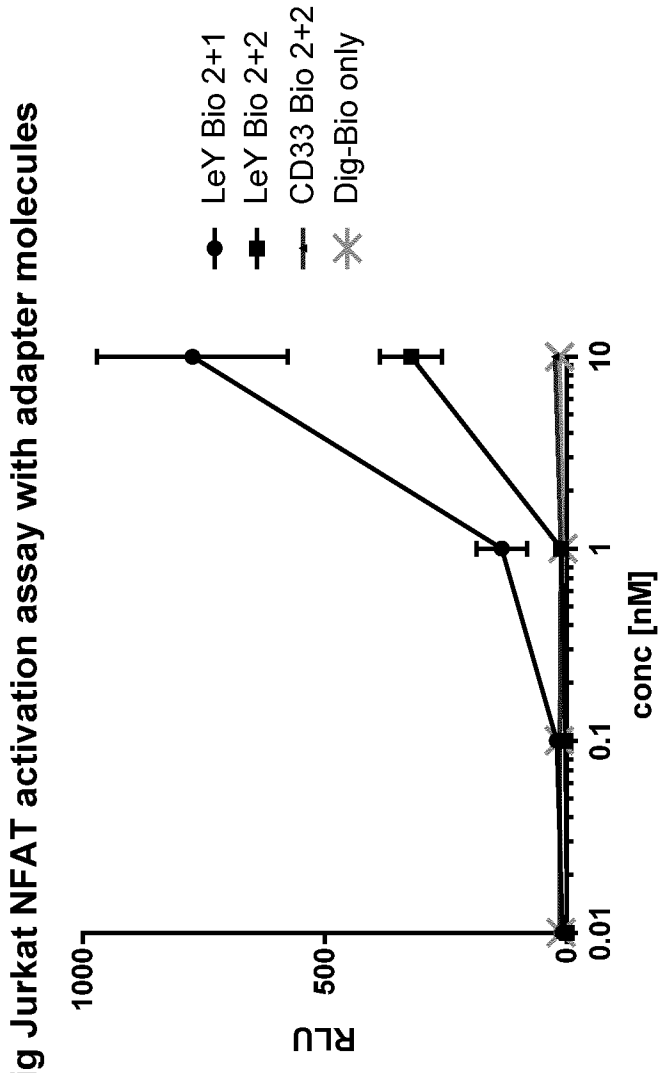

anti-Dig Jurkat NFAT activation assay with adapter molecules
LeY Bio 2+1
LeY Bio 2+2
CD33 Bio 2+2
Dig-Bio only
RLU
1000
500
0
conc [nM]
0.01
0.1
1
10

Figure 13

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2019/054786

A. CLASSIFICATION OF SUBJECT MATTER
INV. G01N33/566 C07K14/725 C07K16/46 G01N33/577
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
G01N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, BIOSIS, CHEM ABS Data, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2013/044225 A1 (UNIV PENNSYLVANIA [US]; SCHOLLER NATHALIE [US]; URBANSKA KATARZYNA [US]) 28 March 2013 (2013-03-28)	1-5, 7-10,13, 14
Y	figure 1 page 5, line 19 - line 21 page 24, paragraph 2 - paragraph 3 page 62, line 31 - page 63, line 3 page 74, line 15 - line 31 page 35, paragraph 3 - page 36, paragraph 2	6,15

Y	WO 2014/100615 A1 (PURDUE RESEARCH FOUNDATION [US]) 26 June 2014 (2014-06-26) claims 20,21,26	6,15

	-/--	

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
15 March 2019	05/04/2019

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Schwachtgen, J

1

Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	HANNE L. P. TYTGAT ET AL: "Endogenous biotin-binding proteins: an overlooked factor causing false positives in streptavidin-based protein detection : Endogenous biotin causes false positive signals", MICROBIAL BIOTECHNOLOGY, vol. 8, no. 1, 1 January 2015 (2015-01-01) , pages 164-168, XP055570056, GB ISSN: 1751-7915, DOI: 10.1111/1751-7915.12150 abstract -----	6,15
X	M. BACAC ET AL: "A Novel Carcinoembryonic Antigen T-Cell Bispecific Antibody (CEA TCB) for the Treatment of Solid Tumors", CLINICAL CANCER RESEARCH, vol. 22, no. 13, 9 February 2016 (2016-02-09), pages 3286-3297, XP055522007, US ISSN: 1078-0432, DOI: 10.1158/1078-0432.CCR-15-1696 figure 1 -----	11,12

1

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2019/054786

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2013044225 A1	28-03-2013	US 2014234348 A1	21-08-2014
		US 2017342124 A1	30-11-2017
		WO 2013044225 A1	28-03-2013
WO 2014100615 A1	26-06-2014	EP 2934532 A1	28-10-2015
		HK 1215791 A1	15-09-2016
		US 2015320799 A1	12-11-2015
		US 2017290900 A1	12-10-2017
		WO 2014100615 A1	26-06-2014