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(54) Title: TNF SUPERFAMILY MEMBER IMMUNOCYTOKINE AND USES THEREOF

(57) Abstract: Disclosure relates to antibody-cytokine fusion proteins, in particular, TNF superfamily member fusion proteins (immunocytokines) and uses thereof. The fusion proteins exhibit anti-tumor activity.

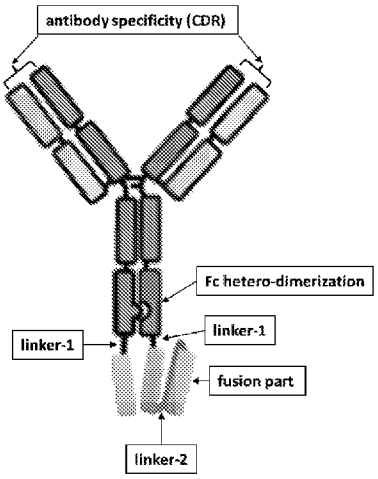

Figure 4A

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TNF SUPERFAMILY MEMBER IMMUNOCYTOKINE AND USES THEREOF

TECHNICAL FIELD

The present disclosure relates to the technical field of biomedicine or biopharmacy, and relates to antibody-cytokine fusion proteins, in particular, TNF superfamily member fusion proteins (immunocytokines) and uses thereof.

BACKGROUND

The statements in this section merely provide background information related to the present disclosure and do not necessarily constitute prior art.

Cytokines are a class of small proteins (5-20 kDa) that act as cell signaling molecules to regulate inflammation and modulate cellular activities. They belong to different families and can have different functions, such as attracting and stimulating immune cells, inducing antibody production, and promoting lymph node formation. Cytokines are a vast and varied group of pro-inflammatory or anti-inflammatory factors, classified into families based on structural similarities or those of their receptors. Cytokines encompass chemokines, interferons, interleukins, lymphokines, tumor necrosis factors, hormones, growth factors, and others.

TNFSF14 (TNF superfamily member 14), also known as LIGHT (homologous to Lymphotoxin, exhibits inducible expression and competes with Herpes Simplex Virus glycoprotein D for Herpes Virus Entry Mediator, a receptor expressed by T cells) is an inducible inflammatory cytokine that can bind to TNFRSF14 (TNF receptor superfamily member 14, also known as HVEM) and LTβR (lymphotoxin beta receptor), and the decoy receptor DcR3. HVEM is expressed on the surface of various immune cells, such as T cells, B cells, NK cells, and dendritic cells. LIGHT can bind to HVEM and subsequently stimulate T cells and promote inflammation. Another receptor, LTβR is expressed on the surface of epithelial, stromal, immature DC, and other myeloid cells but not on lymphocytes. Activation of LTβR signaling is critical for the recruitment and organization of immune cells, leading to the development of lymphoid organs and tertiary lymphoid structures (TLS). TLS consists of B and T cell zones, dendritic cells, and other immune cells, and their presence in tumor tissues has been associated with improved clinical outcomes in a variety of cancer indications, including breast, lung, and colon cancer, suggesting that TLS may play an important role in the immune response against cancer.

Although cytokine-based immunotherapy has shown promising efficacies in treating cancer, it suffers from a lack of specificity in targeting tumor tissues, which may result in systemic immune responses and severe side effects. A potential solution is to conjugate cytokines with antibodies that have tumor-targeting specificity. For example, three hmLIGHT copies were fused

to the N-terminal of Fc and then assembled with an anti-EGFR half-body (Tang et al., 2016). In a second study, LIGHT was conjugated to anti-fibronectin F8 antibodies in a variety of formats. For example, tandem-linked three murine LIGHT copies were fused to the C-terminal of the heavy chain or the light chain of the mAbs or scFv. Robust protein expression without significant aggregation was observed only when the tandem-linked murine LIGHTs were connected to a single-chain diabody, despite several antibody-LIGHT formats being evaluated (Stringhini et al., 2021). In addition, a murine LIGHT fused with a vascular targeting peptide (VTP) has been shown to induce lymphoid neogenesis in solid tumors (Johansson-Percival et al., 2017). However, many of these formats have limitations in clinical practice due to lackluster efficacies or challenging manufacturing processes. Therefore, there is an ongoing need to provide an antibody-cytokine fusion protein platform for robust manufacture and tumor-specific cytokine delivery to mitigate potential toxicity.

SUMMARY

The present disclosure provides an antibody-cytokine fusion protein platform for tumor-specific cytokine delivery. The platform achieves correct assembly and molecules generated from the platform show robust formation of T cell and B cell zones in the tumor microenvironment and lead to promising antitumor efficacies.

In the first aspect of the present disclosure, a fusion protein is provided, including:

a first cytokine fragment linked to the C-terminal of a first CH2-CH3 fragment, including a first cytokine molecule, or a first cytokine molecule and a third cytokine molecule that are tandem linked, and a second cytokine fragment linked to the C-terminal of a second CH2-CH3 fragment, including a second cytokine molecule, or a second cytokine molecule and a fourth cytokine molecule that are tandem linked, the first CH2-CH3 fragment and the second CH2-CH3 fragment form a dimer, such as a homodimer, or a heterodimer, totally three cytokine molecules are linked to the dimer.

In some embodiments, the first CH2-CH3 fragment and the second CH2-CH3 fragment are modified to improve the correct assembly of the fusion protein.

In some embodiments, the first cytokine fragment comprises the first cytokine molecule, and the second cytokine fragment comprises the second cytokine molecule and the fourth cytokine molecule that are tandem linked. In some embodiments, the first cytokine fragment comprises the first cytokine molecule and a third cytokine molecule that are tandem linked, and the second cytokine fragment comprises the second cytokine molecule.

In some embodiments, the first antigen-binding fragment is an scFv that specifically recognizes a first antigen, and/or the second antigen-binding fragment is an scFv or a Fab that specifically recognizes a second antigen. In some embodiments, the first antigen-binding

fragment is a Fab that specifically recognizes a first antigen; and/or the second antigen-binding fragment is a Fab or an scFv that specifically recognizes a second antigen.

The fusion protein provided herein includes a first heavy chain, from the N-terminus to the C-terminus, of which the first antigen-binding fragment, the first CH2-CH3 fragment, and the first cytokine fragment are operably linked; and a second heavy chain, from the N terminus to the C terminus, of which the second antigen-binding fragment, the second CH2-CH3 fragment, and the second cytokine fragment are operably linked.

The fusion protein provided herein includes a first light chain, which pairs with the first antigen-binding fragment to form a heterodimer; and/ or a second light chain, which pairs with the second antigen-binding fragment to form a heterodimer.

In the second aspect of the present disclosure, there is provided an isolated polynucleotide encoding the first heavy chain, the first light chain, the second heavy chain or the second light chain of the fusion protein according to the present disclosure. The present disclosure further provides a set of isolated polynucleotides, including the polynucleotide encoding the first heavy chain, the polynucleotide encoding the first light chain, the polynucleotide encoding the second heavy chain and the polynucleotide encoding the second light chain of the fusion protein according to the present disclosure.

In the third aspect of the present disclosure, there is provided an isolated vector including the isolated polynucleotide according to the present disclosure.

In the fourth aspect of the present disclosure, there is provided a host cell line harboring the isolated polynucleotide, the set of isolated polynucleotides or the isolated vector according to the present disclosure.

In the fifth aspect of the present disclosure, there is provided a pharmaceutical composition including the fusion protein, the isolated polynucleotide, the set of isolated polynucleotides, the isolated vector or the host cell line according to the present disclosure, and a pharmaceutically acceptable carrier.

In the sixth aspect of the present disclosure, there is provided a kit including the fusion protein, the isolated polynucleotide, the set of isolated polynucleotides, the isolated vector or the host cell line or the pharmaceutical composition according to the present disclosure.

In the seventh aspect of the present disclosure, there is provided use of the fusion protein according to the present disclosure or the isolated polynucleotide according to the present disclosure or the set of isolated polynucleotides according to the present disclosure or the isolated vector according to the present disclosure or the host cell line according to the present disclosure or the pharmaceutical composition according to the present disclosure in the manufacture of a drug for preventing or treating a disease, or in the manufacture of a kit for diagnosing a disease.

In the eighth aspect of the present disclosure, there is provided a method of preventing or treating a disease in a subject in need thereof, including administrating to the subject a therapeutically effective amount of the fusion protein according to the present disclosure or the isolated polynucleotide according to the present disclosure or the set of isolated polynucleotides according to the present disclosure or the isolated vector according to the present disclosure or the host cell according to the present disclosure or the pharmaceutical composition according to the present disclosure.

In the ninth aspect of the present disclosure, there is provided a method for the production of the fusion protein according to the present disclosure, including introducing a first expression vector encoding the first heavy chain, a second expression vector encoding the first light chain, a third expression vector encoding the second heavy chain, and a fourth expression vector encoding the second light chain together into one host cell line, or into separate host cell lines, and expressing the first, second, third and fourth expression vectors under proper conditions.

BRIEF DESCRIPTION OF THE DRAWINGS

The following is a brief description of the drawings, which are presented for the purposes of illustrating the exemplary embodiments disclosed herein and not for the purposes of limiting the same.

Figure 1 illustrates the different formats of LIGHT-based immunocytokines that have been evaluated.

Figure 2 shows the SDS-PAGE analysis of LIGHT-based immunocytokines in Format D. The analysis was performed under reducing (R) and non-reducing (NR) conditions and a protein marker (M) was included for comparison.

Figure 3A and 3B show the HPLC analysis of LIGHT-based immunocytokines ABC233 and ABC234 after one-step protein A purification, respectively.

Figure 4A and 4B present the schematic format of LIGHT-based immunocytokines and the extended designs of LIGHT-based immunocytokines, respectively.

Figure 5 shows the SDS-PAGE analysis of LIGHT-based immunocytokines (A-D) under reducing (R) and non-reducing (NR) conditions, along with the protein marker (M).

Figure 6 shows the SEC-HPLC analysis of LIGHT-based immunocytokines ABC538 and ABC539 after purification.

Figure 7 shows the SEC-HPLC analysis of LIGHT-based immunocytokines ABC770-ABC773 after purification.

Figure 8 demonstrates ELISA analysis of LIGHT-based immunocytokines (A-E) binding to the human LTβR extracellular domain, n=2.

Figure 9 demonstrates ELISA analysis of LIGHT-based immunocytokines (A-E) binding to the mouse LTβR extracellular domain, n=2.

Figures 10A-D show the ELISA analysis of LIGHT-based immunocytokines binding to the human HVEM extracellular domain; Figures 10E-H show the ELISA analysis of LIGHT-based immunocytokines binding to the mouse HVEM extracellular domain.

Figure 11 shows the ELISA analysis of LIGHT-based immunocytokines (A-F) binding to the human DcR3.

Figure 12 illustrates the binding of LIGHT-based immunocytokines (A-G) to 293T cells overexpressing human LTβR. The mean fluorescence intensity (MFI) and concentration (Conc.) are indicated.

Figure 13 shows the binding of LIGHT-based immunocytokines (A-G) to 293T cells overexpressing mouse LTβR. MFI: mean fluorescence intensity.

Figure 14 shows the binding of LIGHT-based immunocytokines (A-D) to 293T cells overexpressing human HVEM. MFI: mean fluorescence intensity.

Figure 15 shows the binding of LIGHT-based immunocytokines (A-D) to 293T cells overexpressing mouse HVEM. MFI: mean fluorescence intensity.

Figure 16 illustrates the NF-κB activities induced by LIGHT-based immunocytokines on HeLa-NF-κB cells in the absence (Figures 16A-D) and presence (Figures 16E-H) of 293T cells overexpressing FAP (293T-FAP). Relative luciferase activity (RLU) is reported.

Figure 17 shows the NF-κB activities induced by LIGHT-based immunocytokines (A-C) on 293T-NF-κB cells overexpressing human HVEM. Relative luciferase activity (RLU) is reported.

Figure 18 demonstrates the NF-κB activities induced by LIGHT-based immunocytokines (A-C) on 293T-NF-κB cells overexpressing mouse HVEM. Relative luciferase activity (RLU) is reported.

Figure 19 shows the NF-κB activities induced by LIGHT-based immunocytokines (A-D) on HeLa-NF-κB cells in the absence and presence of 293T-FAP cells.

Figure 20 demonstrates the impact of DcR3 on the activities of LIGHT-based immunocytokines (A-D).

Figure 21 shows the binding of LIGHT-based immunocytokines to BALB/c-3T3 cells (Figure 21A) and BALB/c-3T3 cells overexpressing FAP (Figure 21B).

Figure 22 shows the binding of LIGHT-based immunocytokines to primary human $CD4^+$ T cells (Figure 22A), primary human $CD8^+$ T cells (Figure 22B), primary mouse $CD4^+$ T cells (Figure 22C), and primary mouse $CD8^+$ T cells (Figure 22D).

Figure 23 shows the induction of CCL2 by LIGHT-based immunocytokines in BALB/c-3T3-WT (wild type) (Figure 23A), and BALB/c-3T3-FAP cells (Figure 23B). Figure 23C presents the same results that are grouped by different molecules.

Figure 24 shows the transactivation of LTβR on 3T3 cells in the presence of CT26-FAP or CT26-WT upon addition of ABC233 (Figure 24A), ABC538 (Figure 24B), or ABC539 (Figure 24C).
Figure 25 demonstrates the in vivo anti-tumor activity of anti-FAP×LIGHT immunocytokines in the CT26-FAP model, presenting the treatment timeline (Figure 25A), tumor volume (Figure 25B), survival curve (Figure 25C), and the body weight (Figure 25D).
Figure 26 shows the detected ratio of LIGHT to hIgG1 upon immunocytokine administration, which indicates the cleavage that releases LIGHT moieties from the immunocytokines in vivo.
Figure 27 demonstrates the in vivo anti-tumor activity of anti-FAP×LIGHT immunocytokines in the KPC model. Figure 27A outlines the treatment timeline for Example 13; Figure 27B shows individual tumor volumes of each group; Figure 27C displays the average tumor volumes of each group; Figure 27D indicates the tumor growth inhibition (TGI) of each group.
Figures 28A-28B show the NF-κB activities induced by ABC890 and ABC892, respectively, on HeLa-NF-κB cells in the absence or presence of 293T-FAP cells. Figures 28C-28E show the NF-κB activities induced by ABC890, ABC892, and ABC538, respectively, on HEK293T-NF-κB cells overexpressing human HVEM in the absence or presence of 293T-FAP cells.
Figure 29 demonstrates HeLa-NF-κB activities induced by ABC653.
Figure 30 shows in vivo anti-tumor activity of ABC653 in the KPC0826 model.

DETAILED DESCRIPTION

The present disclosure is explained in greater detail below. This description is not intended to be a detailed catalog of all the different ways in which the invention may be implemented, or all the features that may be added to the instant invention. For example, features illustrated with respect to one embodiment may be incorporated into other embodiments, and features illustrated with respect to a particular embodiment may be deleted from that embodiment. In addition, numerous variations and additions to the embodiments suggested herein will be apparent to those skilled in the art in light of the instant disclosure which does not depart from the instant invention. Hence, the following description is intended to illustrate some particular embodiments of the present disclosure, and not to exhaustively specify all permutations, combinations and variations thereof.

As used herein, the singular forms "a," "an," and "the" include plural references unless the context clearly dictates otherwise. Thus, for example, references to "the method" includes one or more methods, and/or steps of the type described herein which will become apparent to those persons skilled in the art upon reading this disclosure and so forth.

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which the disclosure pertains. Although any methods and materials similar or equivalent to those described herein may be used in the practice for testing of the present disclosure, the preferred materials and

methods are described herein. In describing and claiming the present disclosure, the following terminology will be used.

[term]

As used herein, the term "fusion" refers to connecting a protein with another biological material, such as a protein, a peptide, a nucleic acid molecule, or any other biological molecule or part thereof.

The term "protein", "peptide" and "polypeptide" are used interchangeably herein to refer to a biological polymer including units derived from amino acids linked by peptide bonds. A protein can be composed of one or more chains.

As used herein, the term "domain" refers to a part of a molecule or structure that shares common physical, chemical or structural characteristics, such as similar hydrophobic or polar properties. The exemplary domain may include a protein binding domain, a DNA binding domain, an ATP binding domain, or a similar folding structure such as globular or helical features. The domains can be identified according to their homology with conserved structural or functional motifs.

As used herein, the term "antibody" is used in its broadest sense and encompasses various antibody structures, including but not limited to monoclonal antibodies, polyclonal antibodies, multi-specific antibodies (e.g., bispecific antibodies), full-length antibodies and antigen-binding fragments thereof, so long as they exhibit the desired antigen-binding activity. The term "antibody" may refer to a full-length antibody or an antigen-binding fragment thereof. A full-length antibody includes two heavy chains and two light chains. The variable regions/ domains of the light and heavy chains are responsible for antigen binding. The "variable region" or "variable domain" of an antibody refers to the amino-terminal domains of the heavy or light chain of the antibody. The variable domains of the heavy chain and light chain may be referred to as "VH" and "VL", respectively. These domains are generally the most variable parts of the antibody (relative to other antibodies of the same class) and contain the antigen-binding sites. In some embodiments, from the N- to the C-terminus, the variable domain of heavy chain/domain/region, was followed by three constant heavy domains (CH1, CH2, and CH3). Similarly, from the N- to the C-terminus, the variable domain of light chain/domain/region, was followed by a constant light (CL) domain.

As used herein, the term "antigen-binding fragment" refers to an antibody fragment including a diabody, a Fab, a Fab', a F(ab')2, an Fv fragment, a disulfide stabilized Fv fragment (dsFv), a (dsFv)2, a bispecific dsFv (dsFv-dsFv'), a disulfide stabilized diabody (ds diabody), a single-chain Fv (scFv), an scFv dimer (bivalent diabody), a multi-specific antibody formed from a portion of an antibody including one or more CDRs, a camelized single domain antibody, a nanobody, a domain antibody, a bivalent domain antibody, or any other antibody fragment that

binds to an antigen but does not include a complete antibody structure. An antigen-binding fragment is capable of binding to the same antigen to which the parent antibody or a parent antibody fragment (e.g., a parent scFv) binds.

A Fab fragment corresponds to one of the two identical arms of an antibody molecule and contains the complete light chain paired with the VH and CH1 domains of a heavy chain.

"Fv" is the minimum antibody fragment that contains a complete antigen-recognition and antigen-binding site. This fragment consists of a dimer of one heavy-chain variable region domain and one light-chain variable region domain in a tight, non-covalent association. The folding of these two domains emanates six hypervariable loops (3 loops each from the heavy and light chain) that provide a conformation for antigen binding and confer antigen binding specificity to the antibody.

As used herein, the term "single-chain variable fragment" or "scFv" is a fusion protein of the variable regions of the heavy (VH) and light chains (VL) of an immunoglobulin (e.g., mouse or human) covalently linked to form a VH::VL heterodimer. The heavy (VH) and light chains (VL) are either joined directly or by a peptide-encoding linker or spacer, which connects the N-terminal of the VH with the C-terminal of the VL, or the C-terminal of the VH with the N-terminal of the VL.

As used herein, the term "CH2-CH3 fragment" refers to a portion of the constant region and at least encompasses CH2 and CH3 domains. In some embodiments, two CH2-CH3 fragments can form an antibody Fc domain. "Fc domain" refers to a pair of associated Fc regions. The two Fc regions dimerize to create the Fc domain. The two Fc regions within the Fc domain can be the same or different from one another.

The term "Fc region" or "Fc chain" as used herein is meant the polypeptide comprising the CH2-CH3 domains of an IgG molecule, and in some cases, inclusive of the hinge. In EU numbering for human IgG1, the CH2-CH3 domain comprises amino acids 231 to 447, and the hinge is 216 to 230. Human IgG Fc regions are of particular use in the present disclosure, and can be the Fc region from human IgG1, IgG2, IgG3 or IgG4.

As used herein, the term "specifically binds" refers to measurable and reproducible interactions, such as binding between a target and an antibody, which is determinative of the presence of the target in the presence of a heterogeneous population of molecules, including biological molecules. For example, an antibody that specifically recognizes a target (which can be an epitope) is an antibody that binds this target with greater affinity, avidity, more readily interaction, and/or with greater duration than its bindings to other targets. Binding specificity of the antibody or antigen-binding domain can be determined experimentally by methods known in the art. Such methods include, but are not limited to Octet, Western blots, ELISA-, RIA-, ECL-, IRMA-, EIA-, BIACORE™ -tests and peptide scans.

As used herein, the term "linker" means a polypeptide used to couple two molecules. The linker typically is a stretch of amino acids, e.g., predominantly glycine and/or serine. The linker may have a length of up to 100 amino acids, such as from about 1, 2, 5, 7, 10, 15 amino acid(s) up to about 15, 20, 25, 30, 35, 50, 75, or 100 amino acids.

As used herein, the term "VH-CH1 linker" refers to a polypeptide connecting VH and CH1 in a naturally occurring antibody, and derivatives thereof.

As used herein, the term "VL-CL linker" refers to a polypeptide connecting VL and CL in a naturally occurring antibody, and derivatives thereof.

As used herein, the term "CH2-CH3 linker" refers to a polypeptide connecting CH2 and CH3 in a naturally occurring antibody, and derivatives thereof.

As used herein, the term "IgM tail linker" refers to a polypeptide in a naturally occurring IgM antibody, and derivatives thereof.

When describing a linker, the term "derivative" refers to a linker with an amino acid sequence that differs from a naturally occurring sequence by one or more amino acids, for example, containing one or more amino acid insertions, deletions, or substitutions relative to a naturally occurring sequence.

As used herein, the term "cytokine" is understood to mean any protein, analog or functional fragment thereof, which is capable of stimulating or inducing a biological response against a preselected cell type. Accordingly, it is contemplated that a variety of cytokines can be incorporated into the present application. Useful cytokines include, for example, tumor necrosis factors (TNFs), interleukins (ILs), lymphokines (Ls), colony stimulating factors (CSFs), interferons (IFNs), chemokine, growth factors including species variants, truncated analogs thereof which are capable of stimulating or inducing such biological responses, TNF family members such as LIGHT, lymphotoxin α, lymphotoxin β, 4-1BBL and the like. The term "cytokine" is also understood to encompass any derivative of a wild-type cytokine that includes modification and maintains at least a significant portion (such as at least about 50%) of any of its desired functions.

When describing a cytokine, the term "wild-type (WT)" refers to a cytokine with an amino acid sequence that is naturally occurring and encoded by a germline genome of a given species. A species can have one or more wild-type sequences (for example, with one canonical wild-type sequence and one or more non-canonical wild-type sequences). A wild-type cytokine sequence can include a sequence that is truncated at the N and/or the C terminus relative to the sequence encoded by an open reading frame. A wild-type cytokine sequence can be a mature form of a cytokine that has been processed to remove the N-terminal and/or the C-terminal residues. A wild-type cytokine can lack a signal peptide or can include a signal peptide (e.g., a signal peptide can be added to the N-terminus of the wild-type cytokine).

When describing a cytokine, the term "derivative" "variant" "mutein" and "mutant" are used interchangeably herein to refer to a cytokine with an amino acid sequence that differs from a wild-type sequence by one or more amino acids, for example, containing one or more amino acid insertions, deletions, or substitutions relative to a wild-type sequence.

As used herein, the term "naturally occurring" refers to a sequence of natural origin which means that the whole or parts thereof are not synthetic and exist or are produced in nature. More preferably, the term "naturally occurring" as used herein refers to a sequence of natural origin which means that the whole sequence is not synthetic and exists or is produced in nature.

As used herein, the term "LIGHT" has its general meaning in the art and refers to a protein transiently expressed on activated T cells, dendritic cells (DCs), monocytes, natural killer cells (NK), and others. LIGHT can stimulate HVEM-expressing cells, such as T cells, B cells, NK cells, and dendritic cells, leading to cell activation and inflammation. LIGHT binding to another receptor LTβR, which is expressed on stromal cells, myeloid cells and some epithelial cells, can lead to the development of lymphoid organs and tertiary lymphoid structures (TLS). The term "LIGHT" is also understood to encompass WT-LIGHT and any derivative of a WT-LIGHT.

As used herein, the term "knobs-into-holes" is used in its broadest sense and encompasses various situations, such as the CH1 domain of one heavy chain with the knob mutations and the CH1 domain of the other heavy chain with the hole mutations, the CH2 domain of one heavy chain with the knob mutations and the CH2 domain of the other heavy chain with the hole mutations, and/or the CH3 domain of one heavy chain with the knob mutations and the CH3 domain of the other heavy chain with the hole mutations. For example, and generally, "knobs-into-holes" may refer to an intra-interface modification between two antibody heavy chains in the CH3 domains: i) in the CH3 domain of one heavy chain (first CH3 domain), an amino acid residue is substituted with another amino acid residue bearing a large side chain, thereby creating a protrusion ("knob") in the interface in the first CH3 domain; ii) in the CH3 domain of the other heavy chain (second CH3 domain), an amino acid residue is substituted with another amino acid residue bearing a smaller side chain, thereby creating a cavity ("hole") within the interface in the second CH3 domain, in which a protrusion ("knob") in the first CH3 domain can be placed.

As used herein, the term "DDKK" refers to a modification mediating electrostatic steering effect that is used to enhance antibody Fc heterodimer formation as inter alia described by Gunasekaran et al., (J. Biol. Chem. 2010,19637-19646). The Fc part of one heavy chain includes the mutations K392D and K409D (termed DD mutations) and the Fc part of the other heavy chain includes the mutations E356K and D399K (termed KK mutations). The numbering is according to the EU index.

An "isolated" polynucleotide encoding a protein, or portion or fragment thereof described herein is a nucleic acid molecule that is identified and separated from at least one contaminant

nucleic acid molecule with which it is ordinarily associated in the environment in which it was produced. Preferably, the isolated polynucleotide is free of association with all components associated with the production environment. The isolated polynucleotide encoding the protein, or portion or fragment thereof described herein is in a form other than in the form or setting in which it is found in nature. Isolated polynucleotides, therefore, are distinguished from polynucleotides encoding the protein, or portion or fragment thereof s described herein existing naturally in cells. An isolated polynucleotide includes a nucleic acid molecule contained in cells that ordinarily contain the nucleic acid molecule, but the nucleic acid molecule is present extra chromosomally or at a chromosomal location that is different from its natural chromosomal location.

As used herein, the term "vector" refers to a nucleic acid molecule capable of propagating another nucleic acid to which it is linked. 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. Certain vectors are capable of directing the expression of nucleic acids to which they are operatively linked. Such vectors are referred to herein as "expression vectors."

As used herein, the term "host cells" or "host cell line" refers to cells into which exogenous nucleic acids have been introduced, including the progeny of such cells. Host cells include "transfected cells", which include the primary transfected cells 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, and (or) may contain mutations. Mutant progeny having the same function or biological activity as screened or selected for in the originally transfected cell are included herein. The host cell may be a mammalian cell, plant cell, yeast cell, and (or) a bacterial cell. The host cell may contain the nucleic acid molecule or vector of the present disclosure as an extra-chromosomally (episomal) replicating molecule, or more preferably, includes the nucleic acid molecule or vector of the present disclosure integrated into the genome of the host cell.

As used herein, the term "transfected" or "transfection" refers to a process by which exogenous nucleic acid is transferred or introduced into the host cell. A "transfected" cell is one which has been transfected with exogenous nucleic acids. The cell includes the primary subject cell and its progeny.

As used herein, the terms "pharmaceutical composition" refers to a preparation which is in such form as to permit the biological activity of the active ingredient(s) to be effective, and which contains no additional components which are unacceptably toxic to an individual to which the formulation would be administered. Such formulations may be sterile.

A "pharmaceutically acceptable carrier" refers to a non-toxic solid, semisolid, or liquid filler, diluent, encapsulating material, formulation auxiliary, or carrier conventional in the art for

use with a therapeutic agent that together includes a "pharmaceutical composition" for administration to an individual. A pharmaceutically acceptable carrier is non-toxic to recipients at the dosages and concentrations employed and is compatible with other ingredients of the formulation. The pharmaceutically acceptable carrier is appropriate for the formulation employed.

"Cancer", and "tumor" are used interchangeably herein to refer to any of several diseases that are characterized by an uncontrolled and abnormal proliferation of cells, the ability of the affected cells to spread locally or through the bloodstream and the lymphatic system to other parts of the body (metastasize), as well as any of several characteristic structural and/or molecular features. Examples of cancers that can be treated using the fusion protein of the present disclosure include solid tumors and hematologic cancers. Additional examples of cancers that can be treated using the fusion protein of the present disclosure include breast, lung, brain, bone, liver, kidney, colon, head and neck, ovarian, hematopoietic (e.g., leukemia) and prostate cancers. Additional examples of cancers that can be treated using multivalent and multi-specific antibodies include, but are not limited to, carcinoma, lymphoma, blastoma, sarcoma, and leukemia. Among the most particular examples of such cancers include squamous cell cancer, small cell lung cancer, non-small cell lung cancer, lung adenocarcinoma, squamous cell carcinoma of the lung, peritoneal cancer, hepatocellular cancer, gastrointestinal cancer, pancreatic cancer, glioblastoma, cancer cervical cancer, ovarian cancer, liver cancer, bladder cancer, hepatoma, breast cancer, colon cancer, colorectal cancer, endometrial or uterine carcinoma, carcinoma of the salivary glands, kidney cancer, liver cancer, prostate cancer, vulvar cancer, thyroid cancer, hepatic carcinoma and various types of cancers of the head and neck. Other cancers and tumors that can be treated using the fusion protein of the present disclosure are described herein or are otherwise known in the art.

As used herein, the term "agent" encompasses the fusion protein according to the present disclosure, the isolated polynucleotide according to the present disclosure, the set of isolated polynucleotides according to the present disclosure, the isolated vector according to the present disclosure, the host cell according to the present disclosure, and the pharmaceutical composition according to the present disclosure.

An "effective amount" of an agent of the present disclosure is an amount sufficient to achieve a specifically indicated purpose in order to cause an observable change in the level of one or more biological activities related to the target cell to which the fusion protein binds. The change can increase the activity level of the target. The change can reduce the activity level of the target. An "effective amount" can be determined empirically and in a routine manner, in relation to the stated purpose.

The term "therapeutically effective amount" refers to an amount of an agent of the present disclosure or another drug effective to "treat" a disease or disorder in a patient or mammal. In the case of cancer, the therapeutically effective amount of the agent of the present disclosure can reduce angiogenesis and neovascularization; reduce the number of cancer cells; reduce the size of the tumor; inhibit (i.e., slow down to some extent or stop) the infiltration of cancer cells into peripheral organs; inhibit (i.e., slow down to a certain point or stop) tumor metastasis; inhibit, to some extent, tumor growth or the incidence of tumors; stimulate immune responses against cancer cells and/or alleviate to some extent one or more of the symptoms associated with cancer. See the definition in this document of "treat." A "therapeutically effective amount" may also refer to an effective amount, at the dosages and for the periods of time necessary, to achieve a desired therapeutic result. A therapeutically effective amount of the agent of the present disclosure may vary depending on factors such as the pathology, age, sex and weight of the individual and the ability of the composition to elicit a desired response in the individual. A therapeutically effective amount is also one in which the toxic or detrimental effects of the therapeutic composition are overcome by the therapeutically beneficial effects.

As used herein, "treatment" or "treating" is an approach for obtaining beneficial or desired results, including clinical results. For purposes of this application, beneficial or desired clinical results include, but are not limited to, one or more of the following: alleviating one or more symptoms resulting from the disease, diminishing the extent of the disease, stabilizing the disease (e.g., preventing or delaying the worsening of the disease), preventing or delaying the spread (e.g., metastasis) of the disease, preventing or delaying the recurrence of the disease, delay or slowing the progression of the disease, ameliorating the disease state, providing a remission (partial or total) of the disease, decreasing the dose of one or more other medications required to treat the disease, delaying the progression of the disease, increasing or improving the quality of life, increasing weight gain, and/or prolonging survival. Also encompassed by "treatment" is a reduction of pathological consequences of cancer (such as, for example, tumor volume). The methods of the application contemplate any one or more of these aspects of treatment.

In the context of cancer, the term "treating" includes any or all of the inhibiting growth of cancer cells, inhibiting replication of cancer cells, lessening of overall tumor burden and ameliorating one or more symptoms associated with the disease.

The terms "inhibition" or "inhibit" refer to a decrease or cessation of any phenotypic characteristic or to the decrease or cessation in the incidence, degree, or likelihood of that characteristic. To "reduce" or "inhibit" is to decrease, reduce or arrest an activity, function, and/or amount as compared to that of a reference. In certain embodiments, by "reduce" or "inhibit" is meant the ability to cause an overall decrease of 20% or greater. In another embodiment, by "reduce" or "inhibit" is meant the ability to cause an overall decrease of 50%

or greater. In yet another embodiment, by "reduce" or "inhibit" is meant the ability to cause an overall decrease of 75%, 85%, 90%, 95%, or greater.

As used herein, the term "preventing" includes providing prophylaxis with respect to the occurrence or recurrence of a disease in an individual that may be predisposed to the disease but has not yet been diagnosed with the disease.

[Antibody-cytokine Fusion Protein]

The present disclosure provides the immunocytokines, which are antibody-cytokine fusion proteins. The fusion protein includes a first cytokine fragment and a second cytokine fragment, wherein the first cytokine fragment and the second cytokine fragment are linked to the C-terminal of a first CH2-CH3 fragment and a second CH2-CH3 fragment, respectively.

In some embodiments, the first CH2-CH3 fragment and the second CH2-CH3 fragment form a dimer, such as a homodimer or a heterodimer.

In some embodiments, the first CH2-CH3 fragment and the second CH2-CH3 fragment form an Fc domain. Optionally, the Fc domain is derived from IgG, such as human IgG molecules, which encompass IgG1, IgG2, IgG3, and IgG4 subclass. In some embodiments, the first CH2-CH3 fragment and the second CH2-CH3 fragment form a human IgG1 Fc domain or IgG4 Fc domain.

In some embodiments, the first CH2-CH3 fragment and the second CH2-CH3 fragment form a human IgG1 Fc with mutation N297A, which refers to the amino acid N (Asparagine) at position 297 numbering by EU numbering is mutated to amino acid (Alanine). Other mutations like this have a similar instruction.

The amino acid in the present disclosure refers to an organic compound containing amine (-NH2) and carboxyl (-COOH) functional groups, along with a side chain specific to each amino acid. Preferably, the amino acid in the present disclosure is natural amino acids, such as Alanine (A), Cysteine (C), Aspartic Acid (D), Glutamic Acid (E), Phenylalanine(F), Glycine(G), Histidine (H), Isoleucine (I), Lysine (K), Leucine (L), Methionine (M), Asparagine (N), Proline (P), Glutamine (Q), Arginine (R), Serine (S), Threonine (T), Valine (V), Tryptophan (W), Tyrosine (Y).

Optionally, the Fc domain is modified to improve the correct assembly of the fusion protein when needed. In some embodiments, the first CH2-CH3 fragment and the second CH2-CH3 fragment include one or more modifications selected from the group consisting of knobs-into-holes, electrostatic steering of CH3 (e.g., DDKK), DuoBody, SEEDbodies, cFAE, XmAb, Azymetric, and BEAT®. In some embodiments, the first CH2-CH3 fragment and the second CH2-CH3 fragment include one or more modifications selected from the group consisting of knobs-into-holes, and DDKK.

In some embodiments, the first CH2-CH3 fragment includes knob mutations, and the second CH2-CH3 fragment includes hole mutations. In some embodiments, the first CH2-CH3 fragment includes hole mutations, and the second CH2-CH3 fragment includes knob mutations.

In some embodiments, the first CH2-CH3 fragment includes DD mutations, and the second CH2-CH3 fragment includes the KK mutations. In some embodiments, the first CH2-CH3 fragment includes the KK mutations, and the second CH2-CH3 fragment includes the mutations DD mutations.

In some embodiments, one of the first CH2-CH3 fragment and the second CH2-CH3 fragment includes one or more mutations selected from the group consisting of Y349C, T366S, L368A, and Y407V; and the other one includes mutations S354C and/or T366W, defined by EU numbering.

In some embodiments, the first CH2-CH3 fragment includes mutations Y349C, T366S, L368A, and Y407V to form a "hole", and the second CH2-CH3 fragment includes mutations S354C and T366W to form a "knob". In some embodiments, the first CH2-CH3 fragment includes mutations S354C and T366W to form a "knob", and the second CH2-CH3 fragment includes mutations Y349C, T366S, L368A, and Y407V to form a "hole".

The C-terminal of the dimer is totally linked to three cytokine molecules. In some embodiments, the first cytokine fragment includes at least one wild type, truncated or mutated cytokine molecule. In some embodiments, the first cytokine fragment includes one or two wild type, truncated or mutated cytokine molecules. In some embodiments, the second cytokine fragment includes at least one wild type, truncated or mutated cytokine molecule. In some embodiments, the second cytokine fragment includes one or two wild type, truncated or mutated cytokine molecules.

In some embodiments, the first cytokine fragment includes a first cytokine molecule. In some embodiments, the second cytokine fragment includes a second cytokine molecule. In some embodiments, the first cytokine fragment includes a tandem linked first cytokine molecule and a third cytokine molecule. In some embodiments, the second cytokine fragment includes a tandem linked second cytokine molecule and a fourth cytokine molecule.

In some embodiments, the first cytokine fragment includes the first cytokine molecule, and the second cytokine fragment includes the second cytokine molecule and the fourth cytokine molecule that are tandem linked. In some embodiments, the first cytokine fragment includes the first cytokine molecule and a third cytokine molecule that are tandem linked, and the second cytokine fragment includes the second cytokine molecule.

In some embodiments, the cytokine molecule(s) in the first cytokine fragment and the cytokine molecule(s) in the second cytokine fragment are the same. In some embodiments, the first cytokine molecule, the second cytokine molecule, and the third cytokine molecule are the

same. In some embodiments, the first cytokine molecule, the second cytokine molecule, and the fourth cytokine molecule are the same.

In some embodiments, the cytokine molecule(s) in the first cytokine fragment and the cytokine molecule(s) in the second cytokine fragment are different from each other. In some embodiments, the first cytokine molecule, the second cytokine molecule, and the third cytokine molecule are different from each other. In some embodiments, the first cytokine molecule, the second cytokine molecule, and the fourth cytokine molecule are different from each other.

The first cytokine molecule, second cytokine molecule, third cytokine molecule and fourth cytokine molecule independently of each other are selected from the group consisting of tumor necrosis factor, interleukin, lymphokine, interferon, colony stimulating factor, chemokine and growth factor. In some embodiments, the first cytokine molecule, second cytokine molecule, third cytokine molecule and fourth cytokine molecule independently of each other are selected from the group consisting of LIGHT, lymphotoxin α, lymphotoxin β, and 4-1BBL, for example, wild type LIGHT (preferably human LIGHT), truncated LIGHT or mutein thereof.

In some embodiments, the first cytokine molecule, second cytokine molecule, third cytokine molecule and fourth cytokine molecule independently of each other are truncated LIGHT (i.e., the amino acid sequence is short than wild type). Exemplary truncated LIGHT is WT-LIGHT (74-240) (SEQ ID NO: 68), WT-LIGHT (91-240) (SEQ ID NO: 78).

In some embodiments, the first cytokine molecule, second cytokine molecule, third cytokine molecule and fourth cytokine molecule independently of each other include a truncated LIGHT amino acid sequence set forth in SEQ ID NO: 68 or SEQ ID NO: 78.

In some embodiments, the first cytokine molecule, second cytokine molecule and the third cytokine include or have amino acid sequence set forth in SEQ ID NO: 68 or SEQ ID NO: 78. In some embodiments, the first cytokine molecule, second cytokine molecule and the fourth cytokine include or have amino acid sequence set forth in SEQ ID NO: 68 or SEQ ID NO: 78.

In some embodiments, the first cytokine molecule, second cytokine molecule, third cytokine molecule and fourth cytokine molecule independently of each other are LIGHT mutein. Exemplary LIGHT mutein are LIGHT MUTEIN-1 (74-240), LIGHT MUTEIN-2 (74-240), LIGHT MUTEIN-9 (74-240), LIGHT MUTEIN-11 (74-240), LIGHT MUTEIN-18 (74-240), LIGHT MUTEIN-22 (74-240), LIGHT MUTEIN-29 (74-240), LIGHT MUTEIN-37 (74-240), LIGHT MUTEIN-52 (74-240), (SEQ ID NOs: 69-77); LIGHT MUTEIN-1 (59-240), LIGHT MUTEIN-1 (91-240), LIGHT MUTEIN-1 (87-240), LIGHT MUTEIN-90 (74-240), LIGHT MUTEIN-63 (74-240), LIGHT MUTEIN-86 (74-240), LIGHT MUTEIN-88 (74-240), LIGHT MUTEIN-60 (74-240), LIGHT MUTEIN-92 (74-240), (SEQ ID NOs: 126-134); LIGHT MUTEIN-93 (74-240), LIGHT MUTEIN-41 (74-240), (SEQ ID NOs: 25-26); LIGHT MUTEIN-42 (74-240), LIGHT MUTEIN-2 (91-240), (SEQ ID NOs: 58-59); LIGHT MUTEIN-

95 (91-240), LIGHT MUTEIN-96 (91-240), LIGHT MUTEIN-97 (91-240), LIGHT MUTEIN-98 (91-240), LIGHT MUTEIN-99 (91-240), LIGHT MUTEIN-100 (91-240), LIGHT1(91-240)(M150V), (SEQ ID NOs: 135-141); LIGHT MUTEIN-101 (91-240) (SEQ ID NO: 158).

In some embodiments, the first cytokine molecule, second cytokine molecule, third cytokine molecule and fourth cytokine molecule independently of each other comprise a mutated LIGHT amino acid sequence set forth in SEQ ID NO: 25, 26, 58, 59, 69-77, 126-141, 158, 177, 178 and 180.

In some embodiments, the first cytokine molecule, the second cytokine molecule and the third cytokine include or have amino acid sequence set forth in SEQ ID NO: 25, 26, 58, 59, 69-77, 126-141, 158, 177, 178 and 180. In some embodiments, the first cytokine molecule, second cytokine molecule and the fourth cytokine include or have amino acid sequence set forth in SEQ ID NO: 25, 26, 58, 59, 69-77, 126-141, 158, 177, 178 and 180.

In some embodiments, the first cytokine molecule, the second cytokine molecule, the third cytokine molecule and the fourth cytokine molecule independently are wild type 4-1BBL (preferably human 4-1BBL), truncated 4-1BBL or mutein thereof. In some embodiments, the wild type 4-1BBL, truncated 4-1BBL or mutein thereof includes amino acid sequence of SEQ ID NO:179.

In some embodiments, the first cytokine molecule, the second cytokine molecule, the third cytokine molecule and the fourth cytokine molecule independently are wild, truncated lymphotoxin α or lymphotoxin β, or mutein thereof. In some embodiments, the wild, truncated lymphotoxin α or lymphotoxin β, or mutein thereof includes amino acid sequence of SEQ ID NO: 181, 182.

In some the first cytokine molecule, the second cytokine molecule, and the third cytokine molecule have the same amino acid sequence. In some embodiments, the first cytokine molecule, the second cytokine molecule, and the fourth cytokine molecule have the same amino acid sequence.

In some embodiments, the first cytokine fragment links to the C-terminal of the first CH2-CH3 fragment directly or via a linker. In some embodiments, the second cytokine fragment link to the C-terminal of the second CH2-CH3 fragment directly or via a linker.

In some embodiments, the C terminal of the CH3 domain in the first CH2-CH3 fragment is fused to the N terminal of a first cytokine fragment directly or via a first linker A, and the C terminal of the CH3 domain in the second CH2-CH3 fragment is fused to N terminal of a second cytokine fragment directly or via a first linker B.

In some embodiments, the first cytokine molecule is fused to a third cytokine molecule directly or via a second linker A. In some embodiments, the second cytokine molecule is fused to a fourth cytokine molecule directly or via a second linker B.

In some embodiments, the C terminal of the first cytokine molecule is fused to the N terminal of a third cytokine molecule directly or via a second linker A. In some embodiments, the C terminal of the second cytokine molecule is fused to the N terminal of a fourth cytokine molecule directly or via a second linker B.

In some embodiments, the first linker A, the first linker B, the second linker A, and the second linker B independently of each other are absent or are selected from the group consisting of VH-CH1 linker ASTKGPSVFPLAPS (SEQ ID NO: 82); VL-CL linker RTVAAPSVFIFPPS (SEQ ID NO: 83); CH2-CH3 linker ISKAKGQPREPQ (SEQ ID NO: 81); IgM tail linker KSTGKPTLYNVSLVMSDTAGTCY (SEQ ID NO: 84); GGGGSGGGGSGGGGSGGGGT (SEQ ID NO: 89), G; and (GGGGS)n, n=1, 2, 3, 4, 5, 6, 7, 8, 9, or 10. In some embodiments, the first linker A, the first linker B, the second linker A, and the second linker B independently of each other are absent or are selected from the group consisting of VH-CH1 linker; VL-CL linker; CH2-CH3 linker; IgM tail linker; G; and (GGGGS)n, wherein n=1, 2, 3, 4, 5, or 6. In some embodiments, the first linker A, the first linker B, the second linker A, and the second linker B independently of each other are absent or are selected from the group consisting of VH-CH1 linker; VL-CL linker; CH2-CH3 linker; IgM tail linker; GGGGSGGGGSGGGGSGGGGT (SEQ ID NO: 89), G; and (GGGGS)n, n=1, 2, 3, or 4.

In some embodiments, the first linker A and the first linker B independently of each other are absent or are selected from the group consisting of VH-CH1 linker; VL-CL linker; CH2-CH3 linker; IgM tail linker; and (GGGGS)n, n=1, 2, 3, 4, 5, 6, 7, 8, 9, or 10. In some embodiments, the first linker A and the first linker B independently of each other are absent or are selected from the group consisting of VH-CH1 linker; VL-CL linker; CH2-CH3 linker; IgM tail linker; and (GGGGS)n, n=1, 2, 3, 4, 5, or 6. In some embodiments, the first linker A and the first linker B independently of each other are absent or are selected from the group consisting of VH-CH1 linker; VL-CL linker; CH2-CH3 linker; IgM tail linker; GGGGSGGGGSGGGGSGGGGT (SEQ ID NO: 89), G, and (GGGGS)n, n=1, 2, 3, or 4.

In some embodiments, the first linker A and the first linker B independently of each other are absent or include the amino acid sequence as set forth in any one of SEQ ID NOs: 81-90. In some embodiments, the first linker A and the first linker B independently of each other are absent or have the amino acid sequence as set forth in any one of SEQ ID NOs: 81-90.

In some embodiments, the first linker A and the first linker B are the same. In some embodiments, the first linker A and the first linker B are different.

In some embodiments, the second linker A and the second linker B independently of each other are absent or are selected from the group consisting of G; and (GGGGS)n, wherein n=1, 2, 3, 4, 5, 6, 7, 8, 9, or 10. In some embodiments, the second linker A and the second linker B independently of each other are absent or are selected from the group consisting of G; and

(GGGGS)n, wherein n=1, 2, 3, 4, 5, or 6. In some embodiments, the second linker A and the second linker B independently of each other are absent or are selected from the group consisting of G; and (GGGGS)n, wherein n=1, 2, 3, or 4.

In some embodiments, the second linker A and the second linker B independently of each other are absent or include the amino acid sequence of G or the amino acid sequence as set forth in any one of SEQ ID NOs: 85-88. In some embodiments, the second linker A and the second linker B independently of each other are absent or have the amino acid sequence of G or the amino acid sequence as set forth in any one of SEQ ID NOs: 85-89.

In some embodiments, the fusion protein provided herein includes a first antigen-binding fragment and a second antigen-binding fragment, which are connected to N terminal of the first CH2-CH3 fragment and the second CH2-CH3 fragment, respectively.

In some embodiments, the first antigen-binding fragment and the second antigen-binding fragment independently of each other are selected from the group consisting of a diabody, a Fab, a Fab', a F(ab')2, an Fv fragment, a disulfide stabilized Fv fragment (dsFv), a (dsFv)2, a bispecific dsFv (dsFv-dsFv'), a disulfide stabilized diabody (ds diabody), a single-chain Fv (scFv), an scFv dimer (bivalent diabody), a multispecific antibody formed from a portion of an antibody including one or more CDRs, a camelized single domain antibody, a nanobody, a domain antibody, a bivalent domain antibody, or any other antibody fragment.

In some embodiments, the first antigen-binding fragment is an scFv that specifically binds to a first antigen. In some embodiments, the second antigen-binding fragment is an scFv that specifically binds to a second antigen.

In some embodiments, the scFv includes a heavy chain variable region (VH) and a light chain variable region (VL) which can pair with each other to form an Fv fragment that specifically binds to an antigen. In the scFv, the N-terminal of the VH is linked to the C-terminal of the VL directly or via a linker, or the C-terminal of the VH is linked to the N-terminal of the VL directly or via a linker. Those skilled in the art would be able to select the appropriate linker for use in the present disclosure, for example, the linker between the VH and the VL is (GGGGS)n, wherein n=1, 2, 3, 4, 5, 6, 7, 8, 9, or 10. In some embodiments, the linker between the VH and the VL is (GGGGS)4 (SEQ ID NO: 88).

In some embodiments, the first antigen-binding fragment is a Fab that specifically binds to a first antigen. In some embodiments, the second antigen-binding fragment is a Fab that specifically binds to a second antigen.

In some embodiments, the first antigen-binding fragment is an scFv that specifically binds to a first antigen; and the second antigen-binding fragment is an scFv or a Fab that specifically binds to a second antigen. In some embodiments, the first antigen-binding fragment is a Fab that specifically binds to a first antigen; and the second antigen-binding fragment is a Fab or an scFv

that specifically binds to a second antigen. In some embodiments, the first antigen-binding fragment is an scFv or a Fab that specifically binds to a first antigen; and the second antigen-binding fragment is a Fab that specifically binds to a second antigen. In some embodiments, the first antigen-binding fragment and the second antigen-binding fragment are Fab. In some embodiments, the first antigen-binding fragment is an scFv and the second antigen-binding fragment is Fab. In some embodiments, the first antigen-binding fragment is a Fab and the second antigen-binding fragment is an scFv.

In some embodiments, the first antigen and the second antigen independently of each other are selected from the group consisting of FAP, HER2, PDL-1, PD-1, EGFR, VEGFR, VEGF, CCR8, OX-40, 41BB, Angiopoietin-2, IL-4Rα, BCMA, Blys, BTNO2, C5, CD122, CD13, CD133, CD137, CD138, CD16a, CD19, CD20, CD22, CD27, CD28, CD3, CD30, CD33, CD38, CD40, CD47, CD-8, CEA, CGPR/CGRPR, CSPGs, CTLA4, CTLA-4, DLL-4, EpCAM, factor IXa, factor X, GITR, GP130, Her3, HSG, ICOS, IGF1, IGF1/2, IGF-1R, IGF2, IGFR, IL-1, IL-12, IL-12p40, IL-13, IL-17A, IL-1β, IL-23, IL-5, IL-6, IL-6R, Lag-3, LAG3, MAG, Met, NgR, NogoA, OMGp, OX40, PDGFR, PSMA, RGMA, RGMB, SARS-CoV-2, Te38, TIM-3, TNF, TNFα, TROP-2, and TWEAK. In some embodiments, the first antigen and the second antigen independently of each other are selected from the group consisting of FAP, HER2, PDL-1, and EGFR.

In some embodiments, the first antigen and the second antigen are different antigens or different epitopes on the same antigen. In some embodiments, the first antigen and the second antigen are the same or the same epitope on the same antigen.

The fusion protein herein is monovalent or bivalent, monospecific or bispecific.

In some embodiments, both of the first antigen and the second antigen are FAP, or both of the first antigen and the second antigen are HER2, or both of the first antigen and the second antigen are EGFR, or both of the first antigen and the second antigen are PDL-1, or the first antigen is HER2 and the second antigen are FAP.

In some embodiments, the fusion protein provided herein includes: a first heavy chain, from the N terminal to the C terminal, including the operably linked first antigen-binding fragment, the first CH2-CH3 fragment, and the first cytokine fragment; and a second heavy chain, from the N terminal to the C terminal, including the operably linked second antigen-binding fragment, the second CH2-CH3 fragment, and the second cytokine fragment.

As Figure 4A shows, the fusion protein is with the "Y" shape. The first heavy chain used herein is referred to the heavy chain of the left arm of "Y" shape, and the second heavy chain used herein is referred to the heavy chain of the right arm of "Y" shape.

In some embodiments, the first heavy chain and the second heavy chain are the same. In some embodiments, the first heavy chain and the second heavy chain are different.

In some embodiments, the fusion protein provided herein includes a first light chain, which pairs with the first antigen-binding fragment to form a heterodimer. In some embodiments, the fusion protein provided herein includes a second light chain, which pairs with the second antigen-binding fragment to form a heterodimer.

In some embodiments, the first light chain and the second light chain are the same, under which circumstance they are collectively referred to as "light chain". In some embodiments, the first light chain and the second light chain are different.

In some embodiments, the first antigen-binding fragment is an scFv, in this case, the first light chain is absent. In some embodiments, the second antigen-binding fragment is an scFv, in this case, the second light chain is absent.

In some embodiments, the first light chain and the second light chain independently of each other include the amino acid sequence as set forth in any one of SEQ ID NOs: 1, 29, 91 and 94. In some embodiments, the first light chain and the second light chain independently of each other have the amino acid sequence as set forth in any one of SEQ ID NOs: 1, 29, 91 and 94. In some embodiments, both the first light chain and the second light chain have the amino acid sequence as set forth in SEQ ID NO: 1. In some embodiments, both the first light chain and the second light chain have the amino acid sequence as set forth in SEQ ID NO: 29. In some embodiments, both the first light chain and the second light chain have the amino acid sequence as set forth in SEQ ID NO: 91. In some embodiments, both the first light chain and the second light chain have the amino acid sequence as set forth in SEQ ID NO: 94. In some embodiments, the first light chain has the amino acid sequence as set forth in SEQ ID NO: 1, and the second light chain has the amino acid sequence as set forth in SEQ ID NO: 29. In some embodiments, the second light chain has the amino acid sequence as set forth in SEQ ID NO: 1, and the first light chain has the amino acid sequence as set forth in SEQ ID NO: 29. In some embodiments, the first light chain has the amino acid sequence as set forth in SEQ ID NO: 1, and the second light chain has the amino acid sequence as set forth in SEQ ID NO: 91. In some embodiments, the second light chain has the amino acid sequence as set forth in SEQ ID NO: 1, and the first light chain has the amino acid sequence as set forth in SEQ ID NO: 91. In some embodiments, the first light chain has the amino acid sequence as set forth in SEQ ID NO: 1, and the second light chain has the amino acid sequence as set forth in SEQ ID NO: 94. In some embodiments, the second light chain has the amino acid sequence as set forth in SEQ ID NO: 1, and the first light chain has the amino acid sequence as set forth in SEQ ID NO: 94.

In some embodiments, the second heavy chain includes or has the amino acid sequence as set forth in SEQ ID NO: 2, 5, 7, 9, 11, 13, 15, 17, 19, 21, 24, 27, 30, 33, 34, 36, 38, 40, 42, 44, 46, 48, 50, 54, 56, 60, 62, 64, 66, 92, 95, 97, 101, 103, 105, 106, 108, 110, 112, 115, 116, 118,

120, 122, 124, 142, 144, 146, 148, 150, 152, 154, 156, 159, 161, 163, 165, 167, 169, 171, 173 or 175.

In some embodiments, the first heavy chain includes or has the amino acid sequence as set forth in SEQ ID NO: 3, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 23, 28, 31, 32, 35, 37, 39, 41, 43, 45, 47, 49, 51, 52, 53, 55, 57, 61, 63, 65, 67, 93, 96, 98, 99, 100, 102, 104, 107, 109, 111, 113, 114, 117, 119, 121, 123, 125, 143, 145, 147, 149, 151, 153, 157, 155, 160, 162, 164, 166, 168, 170, 172, 174 or 176.

In some embodiments, the fusion protein is select from the following:

(1) both of the first light chain and the second light chain include or have the amino acid sequence as set forth in SEQ ID NO: 1, the first heavy chain includes or has the amino acid sequence as set forth in SEQ ID NO: 3, and the second heavy chain includes or has the amino acid sequence as set forth in SEQ ID NO: 3; (2) both of the first light chain and the second light chain include or have the amino acid sequence as set forth in SEQ ID NO: 1, the first heavy chain includes or has the amino acid sequence as set forth in SEQ ID NO: 4, and the second heavy chain includes or has the amino acid sequence as set forth in SEQ ID NO: 2; (3) both of the first light chain and the second light chain include or have the amino acid sequence as set forth in SEQ ID NO: 1, the first heavy chain includes or has the amino acid sequence as set forth in SEQ ID NO: 6, and the second heavy chain includes or has the amino acid sequence as set forth in SEQ ID NO: 5; (4) both of the first light chain and the second light chain include or have the amino acid sequence as set forth in SEQ ID NO: 1, the first heavy chain includes or has the amino acid sequence as set forth in SEQ ID NO: 8, and the second heavy chain includes or has the amino acid sequence as set forth in SEQ ID NO: 7; (5) both of the first light chain and the second light chain include or have the amino acid sequence as set forth in SEQ ID NO: 1, the first heavy chain includes or has the amino acid sequence as set forth in SEQ ID NO: 10, and the second heavy chain includes or has the amino acid sequence as set forth in SEQ ID NO: 9; (6) both of the first light chain and the second light chain include or have the amino acid sequence as set forth in SEQ ID NO: 1, the first heavy chain includes or has the amino acid sequence as set forth in SEQ ID NO: 12, and the second heavy chain includes or has the amino acid sequence as set forth in SEQ ID NO: 11; (7) both of the first light chain and the second light chain include or have the amino acid sequence as set forth in SEQ ID NO: 1, the first heavy chain includes or has the amino acid sequence as set forth in SEQ ID NO: 14, and the second heavy chain includes or has the amino acid sequence as set forth in SEQ ID NO: 13; (8) both of the first light chain and the second light chain include or have the amino acid sequence as set forth in SEQ ID NO: 1, the first heavy chain includes or has the amino acid sequence as set forth in SEQ ID NO: 16, and the second heavy chain includes or has the amino acid sequence as set forth in SEQ ID NO: 15; (9) both of the first light chain and the second light chain include or have the amino acid

sequence as set forth in SEQ ID NO: 1, the first heavy chain includes or has the amino acid sequence as set forth in SEQ ID NO: 18, and the second heavy chain includes or has the amino acid sequence as set forth in SEQ ID NO: 17; (10) both of the first light chain and the second light chain include or have the amino acid sequence as set forth in SEQ ID NO: 1, the first heavy chain includes or has the amino acid sequence as set forth in SEQ ID NO: 20, and the second heavy chain includes or has the amino acid sequence as set forth in SEQ ID NO: 19; (11) both of the first light chain and the second light chain include or have the amino acid sequence as set forth in SEQ ID NO: 1, the first heavy chain includes or has the amino acid sequence as set forth in SEQ ID NO: 22, and the second heavy chain includes or has the amino acid sequence as set forth in SEQ ID NO: 21; (12) both of the first light chain and the second light chain include or have the amino acid sequence as set forth in SEQ ID NO: 1, the first heavy chain includes or has the amino acid sequence as set forth in SEQ ID NO: 23, and the second heavy chain includes or has the amino acid sequence as set forth in SEQ ID NO: 24; (13) both of the first light chain and the second light chain include or have the amino acid sequence as set forth in SEQ ID NO: 1, the first heavy chain includes or has the amino acid sequence as set forth in SEQ ID NO: 27, and the second heavy chain includes or has the amino acid sequence as set forth in SEQ ID NO: 28; (14) both of the first light chain and the second light chain include or have the amino acid sequence as set forth in SEQ ID NO: 29, the first heavy chain includes or has the amino acid sequence as set forth in SEQ ID NO: 30, and the second heavy chain includes or has the amino acid sequence as set forth in SEQ ID NO: 31; (15) both of the first light chain and the second light chain include or have the amino acid sequence as set forth in SEQ ID NO: 1, the first heavy chain includes or has the amino acid sequence as set forth in SEQ ID NO: 32, and the second heavy chain includes or has the amino acid sequence as set forth in SEQ ID NO: 33; (16) both of the first light chain and the second light chain include or have the amino acid sequence as set forth in SEQ ID NO: 1, the first heavy chain includes or has the amino acid sequence as set forth in SEQ ID NO: 34, and the second heavy chain includes or has the amino acid sequence as set forth in SEQ ID NO: 35; (17) both of the first light chain and the second light chain include or have the amino acid sequence as set forth in SEQ ID NO: 1, the first heavy chain includes or has the amino acid sequence as set forth in SEQ ID NO: 37, and the second heavy chain includes or has the amino acid sequence as set forth in SEQ ID NO: 36; (18) both of the first light chain and the second light chain include or have the amino acid sequence as set forth in SEQ ID NO: 1, the first heavy chain includes or has the amino acid sequence as set forth in SEQ ID NO: 39, and the second heavy chain includes or has the amino acid sequence as set forth in SEQ ID NO: 38; (19) both of the first light chain and the second light chain include or have the amino acid sequence as set forth in SEQ ID NO: 1, the first heavy chain includes or has the amino acid sequence as set forth in SEQ ID NO: 41, and the second heavy chain includes or has the amino acid sequence as set

forth in SEQ ID NO: 40; (20) both of the first light chain and the second light chain include or have the amino acid sequence as set forth in SEQ ID NO: 29, the first heavy chain includes or has the amino acid sequence as set forth in SEQ ID NO: 42, and the second heavy chain includes or has the amino acid sequence as set forth in SEQ ID NO: 43; (21) both of the first light chain and the second light chain include or have the amino acid sequence as set forth in SEQ ID NO: 1, the first heavy chain includes or has the amino acid sequence as set forth in SEQ ID NO: 45, and the second heavy chain includes or has the amino acid sequence as set forth in SEQ ID NO: 44; (22) both of the first light chain and the second light chain include or have the amino acid sequence as set forth in SEQ ID NO: 1, the first heavy chain includes or has the amino acid sequence as set forth in SEQ ID NO: 47, and the second heavy chain includes or has the amino acid sequence as set forth in SEQ ID NO: 46; (23) both of the first light chain and the second light chain include or have the amino acid sequence as set forth in SEQ ID NO: 1, the first heavy chain includes or has the amino acid sequence as set forth in SEQ ID NO: 49, and the second heavy chain includes or has the amino acid sequence as set forth in SEQ ID NO: 48; (24) both of the first light chain and the second light chain include or have the amino acid sequence as set forth in SEQ ID NO: 1, the first heavy chain includes or has the amino acid sequence as set forth in SEQ ID NO: 51, and the second heavy chain includes or has the amino acid sequence as set forth in SEQ ID NO: 50; (25) both of the first light chain and the second light chain include or have the amino acid sequence as set forth in SEQ ID NO: 1, the first heavy chain includes or has the amino acid sequence as set forth in SEQ ID NO: 52, and the second heavy chain includes or has the amino acid sequence as set forth in SEQ ID NO: 2; (26) both of the first light chain and the second light chain include or have the amino acid sequence as set forth in SEQ ID NO: 1, the first heavy chain includes or has the amino acid sequence as set forth in SEQ ID NO:53, and the second heavy chain includes or has the amino acid sequence as set forth in SEQ ID NO: 2; (27) both of the first light chain and the second light chain include or have the amino acid sequence as set forth in SEQ ID NO: 1, the first heavy chain includes or has the amino acid sequence as set forth in SEQ ID NO: 55, and the second heavy chain includes or has the amino acid sequence as set forth in SEQ ID NO: 54; (28) both of the first light chain and the second light chain include or have the amino acid sequence as set forth in SEQ ID NO: 1, the first heavy chain includes or has the amino acid sequence as set forth in SEQ ID NO: 57, and the second heavy chain includes or has the amino acid sequence as set forth in SEQ ID NO: 56; (29) both of the first light chain and the second light chain include or have the amino acid sequence as set forth in SEQ ID NO: 1, the first heavy chain includes or has the amino acid sequence as set forth in SEQ ID NO: 61, and the second heavy chain includes or has the amino acid sequence as set forth in SEQ ID NO: 60; (30) both of the first light chain and the second light chain include or have the amino acid sequence as set forth in SEQ ID NO: 1, the first heavy chain includes or has the amino acid

sequence as set forth in SEQ ID NO: 63, and the second heavy chain includes or has the amino acid sequence as set forth in SEQ ID NO: 62; (31) both of the first light chain and the second light chain include or have the amino acid sequence as set forth in SEQ ID NO: 1, the first heavy chain includes or has the amino acid sequence as set forth in SEQ ID NO: 65, and the second heavy chain includes or has the amino acid sequence as set forth in SEQ ID NO: 64; (32) both of the first light chain and the second light chain include or have the amino acid sequence as set forth in SEQ ID NO: 1, the first heavy chain includes or has the amino acid sequence as set forth in SEQ ID NO: 67, and the second heavy chain includes or has the amino acid sequence as set forth in SEQ ID NO: 66;(33) both of the first light chain and the second light chain include or have the amino acid sequence as set forth in SEQ ID NO: 91, the first heavy chain includes or has the amino acid sequence as set forth in SEQ ID NO: 92, and the second heavy chain includes or has the amino acid sequence as set forth in SEQ ID NO: 93; (34) both of the first light chain and the second light chain include or have the amino acid sequence as set forth in SEQ ID NO: 94, the first heavy chain includes or has the amino acid sequence as set forth in SEQ ID NO: 95, and the second heavy chain includes or has the amino acid sequence as set forth in SEQ ID NO: 96; (35) both of the first light chain and the second light chain include or have the amino acid sequence as set forth in SEQ ID NO: 1, the first heavy chain includes or has the amino acid sequence as set forth in SEQ ID NO: 98, and the second heavy chain includes or has the amino acid sequence as set forth in SEQ ID NO: 97; (36) both of the first light chain and the second light chain include or have the amino acid sequence as set forth in SEQ ID NO: 1, the first heavy chain includes or has the amino acid sequence as set forth in SEQ ID NO: 99, and the second heavy chain includes or has the amino acid sequence as set forth in SEQ ID NO: 97; (37) both of the first light chain and the second light chain include or have the amino acid sequence as set forth in SEQ ID NO: 1, the first heavy chain includes or has the amino acid sequence as set forth in SEQ ID NO: 100, and the second heavy chain includes or has the amino acid sequence as set forth in SEQ ID NO: 10; (38) both of the first light chain and the second light chain include or have the amino acid sequence as set forth in SEQ ID NO: 1, the first heavy chain includes or has the amino acid sequence as set forth in SEQ ID NO: 102, and the second heavy chain includes or has the amino acid sequence as set forth in SEQ ID NO: 103; (39) both of the first light chain and the second light chain include or have the amino acid sequence as set forth in SEQ ID NO: 1, the first heavy chain includes or has the amino acid sequence as set forth in SEQ ID NO: 104, and the second heavy chain includes or has the amino acid sequence as set forth in SEQ ID NO: 105; (40) both of the first light chain and the second light chain include or have the amino acid sequence as set forth in SEQ ID NO: 1, the first heavy chain includes or has the amino acid sequence as set forth in SEQ ID NO: 107, and the second heavy chain includes or has the amino acid sequence as set forth in SEQ ID NO: 106; (41) both of the first light chain and the second

light chain include or have the amino acid sequence as set forth in SEQ ID NO: 1, the first heavy chain includes or has the amino acid sequence as set forth in SEQ ID NO: 109, and the second heavy chain includes or has the amino acid sequence as set forth in SEQ ID NO: 108; (42) both of the first light chain and the second light chain include or have the amino acid sequence as set forth in SEQ ID NO: 1, the first heavy chain includes or has the amino acid sequence as set forth in SEQ ID NO: 111, and the second heavy chain includes or has the amino acid sequence as set forth in SEQ ID NO: 110; (43) both of the first light chain and the second light chain include or have the amino acid sequence as set forth in SEQ ID NO: 1, the first heavy chain includes or has the amino acid sequence as set forth in SEQ ID NO: 113, and the second heavy chain includes or has the amino acid sequence as set forth in SEQ ID NO: 112; (44) both of the first light chain and the second light chain include or have the amino acid sequence as set forth in SEQ ID NO: 1, the first heavy chain includes or has the amino acid sequence as set forth in SEQ ID NO: 114, and the second heavy chain includes or has the amino acid sequence as set forth in SEQ ID NO: 115; (45) both of the first light chain and the second light chain include or have the amino acid sequence as set forth in SEQ ID NO: 1, the first heavy chain includes or has the amino acid sequence as set forth in SEQ ID NO: 117, and the second heavy chain includes or has the amino acid sequence as set forth in SEQ ID NO: 116; (46) both of the first light chain and the second light chain include or have the amino acid sequence as set forth in SEQ ID NO: 1, the first heavy chain includes the amino acid sequence as set forth in SEQ ID NO: 119, and the second heavy chain includes the amino acid sequence as set forth in SEQ ID NO: 118; (47) both of the first light chain and the second light chain include or have the amino acid sequence as set forth in SEQ ID NO: 1, the first heavy chain includes or has the amino acid sequence as set forth in SEQ ID NO: 121, and the second heavy chain includes or has the amino acid sequence as set forth in SEQ ID NO: 120; (48) both of the first light chain and the second light chain include or have the amino acid sequence as set forth in SEQ ID NO: 1, the first heavy chain includes or has the amino acid sequence as set forth in SEQ ID NO: 123, and the second heavy chain includes or has the amino acid sequence as set forth in SEQ ID NO: 122; (49) the second light chain includes or has the amino acid sequence as set forth in SEQ ID NO: 1, the first heavy chain includes or has the amino acid sequence as set forth in SEQ ID NO: 124, and the second heavy chain includes or has the amino acid sequence as set forth in SEQ ID NO: 4; (50) the second light chain includes or has the amino acid sequence as set forth in SEQ ID NO: 1, the first heavy chain includes or has the amino acid sequence as set forth in SEQ ID NO: 2, and the second heavy chain includes or has the amino acid sequence as set forth in SEQ ID NO: 125; (51) both of the first light chain and the second light chain include or have the amino acid sequence as set forth in SEQ ID NO: 1, the first heavy chain includes or has the amino acid sequence as set forth in SEQ ID NO: 143, and the second heavy chain includes or has the amino acid sequence as set forth in SEQ ID NO:

142; (52) both of the first light chain and the second light chain include or have the amino acid sequence as set forth in SEQ ID NO: 1, the first heavy chain includes or has the amino acid sequence as set forth in SEQ ID NO: 145, and the second heavy chain includes or has the amino acid sequence as set forth in SEQ ID NO: 144; (53) both of the first light chain and the second light chain include or have the amino acid sequence as set forth in SEQ ID NO: 1, the first heavy chain includes or has the amino acid sequence as set forth in SEQ ID NO: 147, and the second heavy chain includes or has the amino acid sequence as set forth in SEQ ID NO: 146; (54) both of the first light chain and the second light chain include or have the amino acid sequence as set forth in SEQ ID NO: 1, the first heavy chain includes or has the amino acid sequence as set forth in SEQ ID NO: 149, and the second heavy chain includes or has the amino acid sequence as set forth in SEQ ID NO: 148; (55) both of the first light chain and the second light chain include or have the amino acid sequence as set forth in SEQ ID NO: 1, the first heavy chain includes or has the amino acid sequence as set forth in SEQ ID NO: 151, and the second heavy chain includes or has the amino acid sequence as set forth in SEQ ID NO: 152; (56) both of the first light chain and the second light chain include or have the amino acid sequence as set forth in SEQ ID NO: 1, the first heavy chain includes or has the amino acid sequence as set forth in SEQ ID NO: 153, and the second heavy chain includes or has the amino acid sequence as set forth in SEQ ID NO: 154; (57) both of the first light chain and the second light chain include or have the amino acid sequence as set forth in SEQ ID NO: 1, the first heavy chain includes or has the amino acid sequence as set forth in SEQ ID NO: 155, and the second heavy chain includes or has the amino acid sequence as set forth in SEQ ID NO: 154; (58) both of the first light chain and the second light chain include or have the amino acid sequence as set forth in SEQ ID NO: 1, the first heavy chain includes or has the amino acid sequence as set forth in SEQ ID NO: 157, and the second heavy chain includes or has the amino acid sequence as set forth in SEQ ID NO: 156; (59) both of the first light chain and the second light chain include or have the amino acid sequence as set forth in SEQ ID NO: 1, the first heavy chain includes or has the amino acid sequence as set forth in SEQ ID NO: 160, and the second heavy chain includes or has the amino acid sequence as set forth in SEQ ID NO: 159; (60) both of the first light chain and the second light chain include or have the amino acid sequence as set forth in SEQ ID NO: 1, the first heavy chain includes or has the amino acid sequence as set forth in SEQ ID NO: 162, and the second heavy chain includes or has the amino acid sequence as set forth in SEQ ID NO: 161; (61) both of the first light chain and the second light chain include or have the amino acid sequence as set forth in SEQ ID NO: 1, the first heavy chain includes or has the amino acid sequence as set forth in SEQ ID NO: 164, and the second heavy chain includes t or has he amino acid sequence as set forth in SEQ ID NO: 163; (62) both of the first light chain and the second light chain include or have the amino acid sequence as set forth in SEQ ID NO: 1, the first heavy chain includes or has the amino acid

sequence as set forth in SEQ ID NO: 166, and the second heavy chain includes or has the amino acid sequence as set forth in SEQ ID NO: 165; (63) both of the first light chain and the second light chain include the amino acid sequence as set forth in SEQ ID NO: 1, the first heavy chain includes the amino acid sequence as set forth in SEQ ID NO: 168, and the second heavy chain includes the amino acid sequence as set forth in SEQ ID NO: 167; (64) both of the first light chain and the second light chain include the amino acid sequence as set forth in SEQ ID NO: 1, the first heavy chain includes the amino acid sequence as set forth in SEQ ID NO: 170, and the second heavy chain includes the amino acid sequence as set forth in SEQ ID NO: 169; (65) both of the first light chain and the second light chain include the amino acid sequence as set forth in SEQ ID NO: 1, the first heavy chain includes the amino acid sequence as set forth in SEQ ID NO: 172, and the second heavy chain includes the amino acid sequence as set forth in SEQ ID NO: 171; (66) both of the first light chain and the second light chain include the amino acid sequence as set forth in SEQ ID NO: 1, the first heavy chain includes the amino acid sequence as set forth in SEQ ID NO: 174, and the second heavy chain includes the amino acid sequence as set forth in SEQ ID NO: 173; (67) both of the first light chain and the second light chain include the amino acid sequence as set forth in SEQ ID NO: 1, the first heavy chain includes the amino acid sequence as set forth in SEQ ID NO: 176, and the second heavy chain includes the amino acid sequence as set forth in SEQ ID NO: 175.

[Polynucleotide]

The present disclosure provides an isolated polynucleotide encoding the first heavy chain, the first light chain, the second heavy chain or the second light chain of the fusion protein according to the present disclosure. In some embodiments, the isolated polynucleotide encodes the first heavy chain of the fusion protein according to the present disclosure. In some embodiments, the isolated polynucleotide encodes the second heavy chain of the fusion protein according to the present disclosure.

In another aspect, the present disclosure provides a set of isolated polynucleotides, including the polynucleotide encoding the first heavy chain, the polynucleotide encoding the first light chain, the polynucleotide encoding the second heavy chain and the polynucleotide encoding the second light chain of the fusion protein according to the present disclosure.

The polynucleotide is polymers of DNA, RNA, DNA/RNA hybrids, or modifications thereof. In some embodiments, the polynucleotide is polymers of DNA. The polynucleotide is polymers of RNA. DNA or RNA encoding an anti-SIRPα antibody or antigen binding fragment thereof or a bi-specific molecule described above is readily isolated and sequenced using conventional procedures (e.g., by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of the antibody). The encoding DNA or RNA may also be obtained by synthetic methods.

The present disclosure provides an isolated vector including the isolated polynucleotide.

The present disclosure further provides a host cell including the isolated polynucleotide, the set of insolated polynucleotides or the isolated vector according to the present disclosure.

The present disclosure further provides a method for the production of the fusion protein according to the present disclosure, including introducing a first expression vector encoding the first heavy chain, a second expression vector encoding the first light chain, a third expression vector encoding the second heavy chain and a fourth expression vector encoding the second light chain together into one host cell, or into separate host cells, and expressing the first, second, third and fourth expression vectors under a proper condition. The host cell includes prokaryotic cell and eukaryotic cell.

In some embodiments, the host cells are eukaryotic cells. In some embodiments, the host cells are mammalian cells, such as CHO, COS, HEK293 cells, or Bowes melanoma cells, or a combination thereof.

[Pharmaceutical Composition]

The present disclosure provides a pharmaceutical composition including the fusion protein, the isolated polynucleotide, the set of isolated polynucleotides, the isolated vector or the host cell according to the present disclosure, and a pharmaceutically acceptable carrier.

The pharmaceutical composition can be administered in a suitable manner according to the specific applicable form, physicochemical characteristics, etc. of the pharmaceutically acceptable excipient. In some embodiments, the pharmaceutical composition may be formulated in a form of a freeze-dried formulation or a liquid formulation, which may contain appropriate formulation additives in the art. For example, the above pharmaceutical composition typically contains more than one pharmaceutical carrier, for example, sterile liquid such as water and oil (including petroleum, oils of animal, vegetable, or synthetic origin (e.g., peanut oil, soybean oil, mineral oil, and sesame oil, etc.)). In the case of intravenous administration of the above pharmaceutical composition, water is a more representative carrier. In addition, saline solution, aqueous glucose, and glycerol solution may also be used as a liquid carrier, especially for an injectable solution. Suitable pharmaceutical excipients are known in the art. The above pharmaceutical composition may also contain a trace amount of wetting agent, emulsifier, or pH buffering agent as required. The administration mode of the pharmaceutical composition is usually parenteral administration, which can be intradermal, intramuscular, intraperitoneal, intravenous or subcutaneous injection, but is not limited thereto, for example, the pharmaceutical composition may be administered by infusion or bolus injection. See, e.g., the Handbook of Pharmaceutical Excipients, Third Edition, A. H. Kibbe (Pharmaceutical Press, London, UK, 2000), which is incorporated by reference in its entirety. Remington's Pharmaceutical Sciences,

Sixteenth Edition, E. W. Martin (Mack Publishing Co., Easton, Pa., 1980), which is incorporated by reference in its entirety.

With regard to the present disclosure, the active agent, or pharmaceutical composition comprising the same, can be administered to the subject via any suitable route of administration. For example, the active agent can be administered to a subject via parenteral, nasal, oral, pulmonary, topical, vaginal, or rectal administration. The following discussion on routes of administration is merely provided to illustrate various embodiments and should not be construed as limiting the scope in any way.

[Kit]

The present disclosure provides kits including the fusion protein, the isolated polynucleotide, the set of isolated polynucleotides, the isolated vector, the host cell or the pharmaceutical composition described above. Such kits can further include, if desired, one or more of various conventional pharmaceutical kit components, such as, for example, containers with one or more pharmaceutically acceptable carriers, additional containers etc., as will be readily apparent to a person skilled in the art. Instructions, either as inserts or a labels, indicating quantities of the components to be administered, guidelines for administration, and/or guidelines for mixing the components, can also be included in the kit.

[Method Of Uses]

The present disclosure provides use of the fusion protein, the isolated polynucleotide, the set of isolated polynucleotides, the isolated vector, the host cell or the pharmaceutical composition according to the present disclosure in the manufacture of a drug for preventing or treating a disease, or in the manufacture of a kit for diagnosing a disease.

The present disclosure provides a method of preventing or treating a disease in a subject in need thereof, including administrating to the subject a therapeutically effective amount of the fusion protein, the isolated polynucleotide, the set of isolated polynucleotides, the isolated vector, the host cell or the pharmaceutical composition according to the present disclosure.

The terms "subject", "individual," and "patient" are used interchangeably herein to refer to a mammal, including, but not limited to, human, bovine, horse, feline, canine, rodent, or primate. In some embodiments, the individual is a human.

In some embodiments, the disease includes tumor or cancer. In some embodiments, the tumor is a solid tumor.

Examples

Example 1: Design and verification of antibody-LIGHT immunocytokine formats

A variety of LIGHT-based immunocytokine (antibody-LIGHT fusion proteins) formats (Figure 1) were developed for tumor-specific delivery. The designs incorporated the extracellular

domain (residues 59-240, residues 74-240, residues 87-240 and residue 91-240) of wild-type LIGHT (Uniprot, #O43557) and an anti-FAP antibody clone 3F2 (patent No. WO 2012020006A). Some formats linked three LIGHT subunits with polypeptide linkers and connected to the N-terminal or the C-terminal of the heavy chain or light chain of the antibody (Format A, E). Some formats fused two subunits of LIGHT to the N-terminal or the C-terminal of the heavy chain or the light chain connected with "G" or "GGGGS" linker(s), with the remaining LIGHT subunit connected to the corresponding site on the opposite chain (Format B, C, D). In Format C, the heavy chain CH1 and light chain CL were switched.

The codon-optimized genes for LIGHT-based immunocytokines were synthesized by Genewiz and subcloned into the pCI vector (Promega #E1731) and co-transfected into Expi293F cells (ThermoFisher A14527) for protein production. The cells were grown in suspension culture for about six days after transfection and then harvested by centrifugation (7,000 RPM) at 4°C for 20 minutes. The supernatant was filtered through a 0.22 μm filter, and affinity-purified using protein A resins. The protein was then eluted by elution buffer (1 M Glycine pH 3.8, 10% glycerol) and neutralized to pH 6.0 with 1M Tris (pH 7.5). After size exclusion chromatography (SEC) was performed on AKTA equipment, concentrations of the refined products were measured using NanoDrop at 280 nm. The antibody-LIGHT fusion proteins were subjected to electrophoresis on 4-20% Tris-glycine gels (Bio-Rad) under reducing (R) and non-reducing (NR) conditions, by mixing with sample dye with or without DTT (Dithiothreitol) respectively and heated at 95 ℃ for 5 min. The purity of the antibody-LIGHT fusion proteins was further characterized by High Performance Liquid Chromatography (HPLC).

Among all the tested formats, only Format D (ABC232, ABC233) successfully generated high-quality wild-type LIGHT (residues 74-240) fusion proteins using either the "GGGGS-GGGGSGGGGSGGGGSGGGGT" or "G" linker (Figure 2), as seen in the HPLC-SEC analysis (Figure 3A). In contrast, ABC243, the same molecular format expressing a shorter version of LIGHT (residues 87-240), exhibited poorer expression and higher aggregation than ABC233 on HPLC-SEC (Figure 3B).

Example 2: Extended design of LIGHT-based immunocytokines

Once the molecular format of the antibody-LIGHT fusion proteins (ABC232 and ABC233) identified, more constructs were generated to test different parameters (Figures 4A-4B): (1) the linker-1 between CH3 and cytokine was substituted with ISKAKGQPREPQ, ASTKGPSVFPLAPS, RTVAAPSVFIFPPS, KSTGKPTLYNVSLVMSDTAGTCY, G4S, (G4S)2 (SEQ ID NO: 86), (G4S)3, GGGGSGGGGSGGGGSGGGGT or without linker; (2) the linker-2 between the two cytokine units conjugated on the same heavy chain, was substituted with GGGGSGGGGSGGGGSGGGGSGGGGT, G, (G4S)2; (3) different LIGHT variants, including human WT-LIGHT, human LIGHT mutants, were tested; (4) different IgG Fc hetero-

dimerization technologies, such as knobs-into-holes (hereinafter referred to as KIH) and electrostatic steering of CH3 were evaluated; (5) different antibodies, such as anti-FAP, anti-HER2, anti-EGFR, and anti-PDL1 were assessed; (6) bispecific antibodies such as anti-FAP×anti-HER2 were examined; (7) wild-type and a glycosylated IgG1 with N297A mutation were investigated; (8) various IgG subclasses, such as IgG1 and IgG4, were tested. As a result, LIGHT-based immunocytokines ABC475, ABC476, ABC477, ABC538, ABC539, ABC540, ABC541, ABC542, ABC543, ABC580, ABC584, ABC581, ABC639, ABC582, ABC640, ABC641, ABC677, ABC678, ABC679, ABC680, ABC232, ABC233, ABC649, ABC651, ABC650, ABC652, ABC566, ABC578, ABC567, ABC653, ABC568, ABC579, ABC569, ABC583, ABC690, ABC691, ABC770, ABC771, ABC772, ABC773, ABC890, ABC892, ABC687, ABC688, ABC689, ABC985, ABC986, ABC987, ABC988, ABC1054, ABC1056, ABC234, ABC548, ABC549, ABC676, ABC831, ABC893, ABC894, ABC1209, ABC1210, ABC1030, ABC1031, ABC1032, ABC1033, ABC1034, ABC1035, ABC1047, ABC1048, and ABC1056 were expressed and purified, and the amino acid sequences of these LIGHT-based immunocytokines are listed in the table below.

The formats of the immunocytokines produced above were listed as D1, D2, D3, D4, D5 and D6. The schematic diagram of these constructs was shown in Figure 4B.

Fusion proteins	cytokine (SEQ ID NO:)	linker-1 (SEQ ID NO:)	linker-2 (SEQ ID NO:)	Antigen	IgG Fc	Format	light chain (SEQ ID NO:)	first heavy chain (SEQ ID NO:)	second heavy chain (SEQ ID NO:)
ABC232	68	89	90	FAP	IgG1 KIH	D2	1	3	2
ABC233	68	89	Linker G	FAP	IgG1 KIH	D2	1	4	2
ABC475	71	89	Linker G	FAP	IgG1 KIH	D2	1	6	5
ABC476	72	89	Linker G	FAP	IgG1 KIH	D2	1	8	7
ABC477	75	89	Linker G	FAP	IgG1KIH	D2	1	10	9
ABC538	69	89	Linker G	FAP	IgG1KIH	D2	1	12	11
ABC539	70	89	Linker G	FAP	IgG1KIH	D2	1	14	13
ABC540	73	89	Linker G	FAP	IgG1KIH	D2	1	16	15
ABC541	74	89	Linker G	FAP	IgG1KIH	D2	1	18	17
ABC542	76	89	Linker G	FAP	IgG1KIH	D2	1	20	19
ABC543	77	89	Linker G	FAP	IgG1KIH	D2	1	22	21
ABC566	68	89	Linker G	FAP	IgG1KIH	D1	1	23	24
ABC568	68	89	Linker G	FAP	IgG1 DDKK	D3	1	27	28
ABC569	68	89	Linker G	HER2	IgG1 DDKK	D4	29	30	31
ABC578	69	89	Linker G	FAP	IgG1 KIH	D1	1	32	33
ABC579	69	89	Linker G	FAP	IgG1 DDKK	D3	1	34	35
ABC580	126	No linker	Linker G	FAP	IgG1 KIH	D2	1	35	36
ABC581	68	85	Linker G	FAP	IgG1 KIH	D2	1	39	38
ABC582	68	86	Linker G	FAP	IgG1 KIH	D2	1	41	40
ABC583	69	89	Linker G	HER2	IgG1DDKK	D4	29	42	43
ABC584	69	No linker	Linker G	FAP	IgG1KIH	D2	1	45	44
ABC639	69	85	Linker G	FAP	IgG1KIH	D2	1	47	46
ABC640	69	86	Linker G	FAP	IgG1 KIH	D2	1	49	48
ABC641	69	87	Linker G	FAP	IgG1 KIH	D2	1	51	50

Fusion proteins	cytokine (SEQ ID NO:)	linker-1 (SEQ ID NO:)	linker-2 (SEQ ID NO:)	Antigen	IgG Fc	Format	light chain (SEQ ID NO:)	first heavy chain (SEQ ID NO:)	second heavy chain (SEQ ID NO:)
ABC649	68	89	No linker	FAP	IgG1 KIH	D2	1	52	2
ABC650	68	89	86	FAP	IgG1 KIH	D2	1	53	2
ABC651	69	89	No linker	FAP	IgG1 KIH	D2	1	55	54
ABC652	69	89	86	FAP	IgG1 KIH	D2	1	57	56
ABC677	68	81	90	FAP	IgG1 KIH	D2	1	61	60
ABC678	68	H1: 82 H2: 83	90	FAP	IgG1 KIH	D2	1	63	62
ABC679	68	H1: 83 H2: 82	90	FAP	IgG1 KIH	D2	1	65	64
ABC680	68	84	Linker G	FAP	IgG1 KIH	D2	1	67	66
ABC770	68	89	Linker G	EGFR	IgG1 DDKK	D4	91	92	93
ABC771	68	89	Linker G	PDL1	IgG1 DDKK	D4	94	95	96
ABC890	127	86	No linker	FAP	IgG1.N297A KIH	D2	1	98	97
ABC892	127	86	86	FAP	IgG1.N297A KIH	D2	1	99	97
ABC893	68	89	Linker G	FAP	IgG1 KIH	D2	1	160	159
ABC894	69	89	Linker G	FAP	IgG1 KIH	D2	1	162	161
ABC234	128	89	Linker G	FAP	IgG1.N297A KIH	D2	1	100	101
ABC690	129	89	Linker G	FAP	IgG1.N297A KIH	D2	1	102	103
ABC691	130	89	Linker G	FAP	IgG1.N297A KIH	D2	1	104	105
ABC687	131	89	Linker G	FAP	IgG1.N297A KIH	D2	1	107	106

Fusion proteins	cytokine (SEQ ID NO:)	linker-1 (SEQ ID NO:)	linker-2 (SEQ ID NO:)	Antigen	IgG Fc	Format	light chain (SEQ ID NO:)	first heavy chain (SEQ ID NO:)	second heavy chain (SEQ ID NO:)
ABC688	132	89	Linker G	FAP	IgG1.N297A KIH	D2	1	109	108
ABC689	133	89	Linker G	FAP	IgG1.N297A KIH	D2	1	111	110
ABC985	134	89	Linker G	FAP	IgG1.N297A KIH	D2	1	113	112
ABC986	25	89	Linker G	FAP	IgG1.N297A KIH	D2	1	114	115
ABC987	26	89	Linker G	FAP	IgG1.N297A KIH	D2	1	117	116
ABC988	58	89	Linker G	FAP	IgG1.N297A KIH	D2	1	119	118
ABC1054	59	86	86	FAP	IgG1.N297A KIH	D2	1	121	120
ABC1056	78	86	86	FAP	IgG1.N297A KIH	D2	1	123	122
ABC772	68	89	Linker G	FAPxHER2	IgG1.N297A KIH	D5	1	124	4
ABC773	68	89	Linker G	FAPxHER2	IgG1.N297A KIH	D6	1	125	2
ABC1030	135	86	No linker	FAP	IgG1.N297A KIH	D2	1	143	142
ABC1031	136	86	No linker	FAP	IgG1.N297A KIH	D2	1	145	144
ABC1032	137	86	No linker	FAP	IgG1.N297A KIH	D2	1	147	146

Fusion proteins	cytokine (SEQ ID NO:)	linker-1 (SEQ ID NO:)	linker-2 (SEQ ID NO:)	Antigen	IgG Fc	Format	light chain (SEQ ID NO:)	first heavy chain (SEQ ID NO:)	second heavy chain (SEQ ID NO:)
ABC1033	138	86	No linker	FAP	IgG1.N297A KIH	D2	1	149	148
ABC1034	139	86	No linker	FAP	IgG1.N297A KIH	D2	1	151	150
ABC1035	140	86	No linker	FAP	IgG1.N297A KIH	D2	1	153	152
ABC1047	158	86	No linker	FAP	IgG1.N297A KIH	D2	1	157	156
ABC1048	141	86	No linker	FAP	IgG1.N297A KIH	D2	1	155	154
ABC1209	127	86	86	FAP	IgG4 KIH	D2	1	164	163
ABC1210	78	86	86	FAP	IgG4 KIH	D2	1	166	165
ABC548	177	G	G	FAP	IgG4 KIH	D2	1	168	167
ABC549	178	G	No linker	FAP	IgG4 KIH	D2	1	170	169
ABC676	179	89	G	FAP	IgG4 KIH	D	1	172	171
ABC831	180	89	G	FAP	IgG4 KIH	D2	1	174	173
ABC653	181,182	89	No linker	FAP	IgG4 KIH	D	1	176	175

First heavy chain is abbreviated to H1, and second heavy chain is abbreviated to H2.

The heavy chain of the left arm of antibody with Y shape is defined as the first heavy chain, and the heavy chain of the right arm of antibody with Y shape is defined as the second heavy chain.

Anti-FAP monoclonal antibody ABC002 had the light chain as shown in SEQ ID NO: 79 and the heavy chain as shown in SEQ ID NO: 80.

The above LIGHT-based immunocytokines were expressed and purified using similar protocols described in Example 1.

Successful expression and correct assembly of all the LIGHT-based immunocytokines were demonstrated by the presence of a dominant band in the non-reducing gel that corresponds to the molecular weight of correctly assembled molecules. This was further supported by SDS-PAGE analysis of reduced proteins, which showed two heavy chains and one light chain, providing additional evidence of correct assembly (Figures 5A-5D). Additionally, after one-step protein A affinity purification and size-exclusion chromatography (SEC) polishing, each immunocytokine displayed as a single peak upon SEC-HPLC analysis, such as ABC538, ABC539, ABC770, ABC771, ABC772, and ABC773 (Figure 6 and Figure 7). Thus, this platform presents a promising approach for generating a variety of LIGHT-based immunocytokines.

Example 3: Binding affinities of LIGHT-based immunocytokines to LTβR, HVEM and DcR3 proteins

Binding affinity to LTβR protein measured by ELISA

LIGHT binding to its cell surface receptor LTβR leads to downstream NF-kB signaling. To evaluate the binding of LIGHT-based immunocytokines to the human-LTβR-Fc (Novoprotein #CX78) and mouse-LTβR-Fc proteins (Acrobiosystems #LTR-H5251), 96-well ELISA plates (Thermo Fisher) were coated with the LTβR proteins at a concentration of 0.5 μg/mL, and then blocked with 2% BSA-PBS buffer for one hour. Serial dilutions of the LIGHT-based immunocytokines were added with a maximum concentration of 10 nM. The plates were incubated for one hour, washed for four times with PBST, and then incubated with a biotinylated mouse anti-human Ig light chain κ antibody (Biolegend, #316504). HRP-conjugated streptavidin (Sino Biological) was added, and the plates were further incubated before being washed for three times with PBST and treated with TMB substrate (#34029, Thermo Fisher). The plates were read at 450 nM on a SpectraMax M5 microplate reader (Molecule Devices). The ELISA results were analyzed using GraphPad Prism 9.0 software and the EC50s (half maximal effective concentration) were summarized in Table 1 and Table 2.

Table 1: EC50s of LIGHT-based immunocytokines binding to the human LTβR.

Immunocytokine	EC50(nM)	Immunocytokine	EC50(nM)
ABC233	0.1193	ABC581	0.106
ABC538	0.08855	ABC582	0.08262
ABC539	0.1006	ABC639	0.1094
ABC543	0.1668	ABC640	0.08604
ABC690	0.07742	ABC641	0.0796
ABC691	0.1584	ABC580	0.1798
ABC566	0.4989	ABC584	0.09628

ABC578	0.1244	ABC650	0.09535
ABC568	0.1022	ABC651	0.1016
ABC579	0.1058	ABC652	0.1038
ABC569	0.1451	ABC583	0.2127
ABC890	0.1428	ABC892	0.1712
ABC1048	0.2713	ABC1209	0.1936
ABC1210	0.2475	ABC1056	0.2656

Table 2. EC50s of LIGHT-based immunocytokines binding to the mouse LTβR.

Immunocytokine	EC50(nM)	Immunocytokine	EC50(nM)
ABC233	0.1484	ABC581	0.1513
ABC538	0.1407	ABC582	0.1367
ABC539	1.181	ABC639	0.2334
ABC543	0.2468	ABC640	0.2013
ABC690	0.1192	ABC641	0.1572
ABC691	0.5964	ABC580	0.3643
ABC566	0.6374	ABC584	0.209
ABC578	0.2206	ABC650	0.1624
ABC568	0.1192	ABC651	0.2395
ABC579	0.1597	ABC652	0.2768
ABC569	0.1579	ABC583	0.3424
ABC890	0.1582	ABC892	0.1989
ABC1048	0.7586	ABC1209	0.9820
ABC1210	0.6566	ABC1056	0.5689

The LIGHT-based immunocytokines show specific dose-dependent binding to both human LTβR and mouse LTβR proteins, as shown in Figures 8A-8E and Figures 9A-9E. Fc heterodimerization technologies, such as knobs-into-holes and electronic steering, can facilitate the production and boost activities of LIGHT-based immunocytokine. The holes-LIGHT/knobs-LIGHT-LIGHT and knobs-LIGHT/holes-LIGHT-LIGHT (knobs and holes exchange) designs show similar affinities to LTβR.

The results also suggest that the binding affinities to LTβR were comparable across different linker-1 and linker-2 options, including no linker, G4S, (G4S)2, (G4S)3, (G4S)4, GGGGSGGGGSGGGGSGGGGT, ISKAKGQPREPQ, ASTKGPSVFPLAPS, RTVAAPSVFIFPPS, and KSTGKPTLYNVSLVMSDTAGTCY. Additionally, the binding can be fine-tuned by using different linker-1 and linker-2 options.

Binding affinities to HVEM and DcR3 protein measured by ELISA

Recombinant HVEM proteins were produced by fusing the C-terminal of human HVEM (referred to as hHVEM) residues 39-202 or mouse HVEM (referred to as mHVEM) residues 38-207 with human Fc, while the recombinant DcR3 protein was generated by linking human DcR3 residues 33-300 to the N-terminal of rabbit Fc. The recombinant proteins were expressed in expi293F and affinity-purified using protein A resins and polished using size exclusion column.

Human HVEM, mouse HVEM or human DcR3 proteins were immobilized onto maxiSorp 96-well ELISA plates (Thermo Fisher) at a concentration of 0.5 μg/mL and then blocked with 2% BSA-PBS buffer for one hour. The LIGHT-based immunocytokines were added to the plates at various dilutions, with a maximum concentration of 10 nM. The plates were incubated for an hour, washed four times with PBST, and further incubated with a biotinylated mouse anti-human Ig light chain κ antibody (Biolegend, #316504). After three washes with PBST, the plates were further incubated with HRP-conjugated streptavidin (Sino Biological) and washed again with PBST. The TMB substrate (#34029, Thermo Fisher) was added to the plates, which were read at 450 nM on a SpectraMax M5 microplate reader (Molecule Devices). The ELISA results were analyzed using GraphPad Prism 9.0 software and the EC50s (half maximal effective concentration) were summarized in Table 3.

Table 3. EC50s for LIGHT-based immunocytokines binding to human and mouse HVEM and human DcR3.

Immunocytokine	Human HVEM	Mouse HVEM	Human DcR3
ABC233	1.553	1.567	0.3168
ABC538	N.D	27.14	25.72
ABC539	N.D	N.D	N.D
ABC543	-	-	0.5685
ABC687	2.355	0.1857	2.070
ABC688	9.758	16.10	8.053
ABC689	0.3128	0.1165	0.2256
ABC690	0.5185	0.1235	0.2129
ABC985	-	-	29.04
ABC986	-	-	19.71
ABC987	-	-	149.6
ABC988	-	-	91.70
ABC890	N.D	N.D	N.D
ABC892	N.D	N.D	N.D
ABC1030	-	-	0.7123
ABC1031	-	-	1.018
ABC1032	-	-	N.D
ABC1033	-	-	N.D
ABC1034	-	-	0.7673
ABC1035	-	-	N.D
ABC1048	-	-	N.D
ABC1054	-	-	N.D
ABC1056	-	-	0.359
ABC549	1.527	0.9288	-
ABC831	N.D	0.2411	-
ABC893	0.2158	1.008	-
ABC894	N.D	N.D	-
ABC1047	N.D	N.D	-
ABC1048	N.D	N.D	N.D
ABC1209	N.D	N.D	N.D

ABC1210	0.4798	0.4853	/
ABC1056	0.5156	0.4274	/
N.D: not determined due to low binding affinity. "-": not performed/not available.			

The binding of LIGHT-based immunocytokines to the other two LIGHT receptors, HVEM and DcR3, was also evaluated. As described in patent application PCT/CN2022/097735, compared to wild-type LIGHT, the LIGHT-1 (SEQ ID NO: 69) homotrimer, and the LIGHT-2 (SEQ ID NO: 70) homotrimer have lower affinities with HVEM. Consistently, the anti-FAP×LIGHT-1 (ABC538) and the anti-FAP×LIGHT-2 (ABC539) immunocytokines also displayed reduced binding capabilities to HVEM (Figure 10B and 10F).

DcR3 is a soluble protein expressed in humans and cynomolgus macaques but is absent in mice. DcR3 negatively regulates the abilities of LIGHT to activate LTβR and HVEM (Liu et al., 2021) and is upregulated in certain autoinflammatory diseases and cancer patients (Fuchsberger et al., 2021). Consistent with the lower binding affinities of DcR3 to LIGHT-1 or LIGHT-2 than wild-type LIGHT, DcR3 had significantly weaker binding to ABC538 and ABC539 than to ABC233 (Figures 11A-11F).

Example 4: Cell binding affinities of LIGHT-based immunocytokines

Cell line generation:

Full-length human or mouse HVEM or LTβR (listed in Table 4) was transfected into HEK-293T cells (ATCC) using lipofectamine 3000 (Thermo Fisher, L3000001). Three days after transfection, the cells were treated with hygromycin B (Millipore Sigma) for fourteen days to obtain stable cell lines. The stable cells were used to evaluate the binding of LIGHT-based immunocytokines to HVEM or LTβR.

Table 4. Protein sequence accession/source.

Protein name	Sequence source
Human HVEM	Uniport Q92956
Mouse HVEM	Uniport Q80WM9
Human LTβR	Uniport P36941
Mouse LTβR	Uniport P50284
Human FAP	Uniport Q12884

Cell binding analysis by FACS:

The stably transfected HEK-293T cells expressing LTβR or HVEM were used to evaluate the binding activities of LIGHT-based immunocytokines. The cells were incubated with serially diluted fusion proteins for 30 mins on ice, with a maximum concentration of 50 nM in flow cytometry buffer (PBS, 0.5% BSA, 1 mM EDTA). The cells were then washed three times with PBS, and the bound immunocytokines were stained with FITC-conjugated mouse anti-human IgG antibody for 15 minutes on ice. The cells were further washed with PBS three times, and the

fluorescence was measured using a Cytek Aurora cytometer (Cytek) and analyzed using FlowJo and GraphPad Prism 9.0 software to determine the EC50. Results were summarized in Tables 5, 6, and 7.

Table 5: EC50s of LIGHT-based immunocytokines binding to 293T cells expressing human LTβR

Immunocytokine	EC50 (nM)	Immunocytokine	EC50 (nM)
ABC566	1.163	ABC651	0.2842
ABC578	0.1476	ABC652	0.3149
ABC568	0.09827	ABC1054	0.570
ABC579	0.09392	ABC1056	0.4737
ABC569	0.1354	ABC475	0.5698
ABC583	0.2639	ABC476	0.7160
ABC581	0.07292	ABC477	0.3295
ABC582	0.103	ABC538	2.291
ABC580	0.1168	ABC539	0.7604
ABC584	0.09526	ABC540	0.3953
ABC639	0.09368	ABC541	1.087
ABC640	0.1153	ABC542	0.6354
ABC641	0.3363	ABC543	0.5824
ABC233	0.4178	ABC677	0.212
ABC649	0.2013	ABC678	0.1867
ABC650	0.5593	ABC679	0.3431
ABC770	1.780	ABC680	0.3612
ABC771	0.407	ABC985	5.562
ABC772	2.018	ABC986	4.621
ABC773	1.934	ABC987	0.334
ABC890	0.353	ABC988	0.309
ABC892	0.372	ABC1034	0.4401
ABC1030	0.6026	ABC1035	N.D.
ABC1031	0.4417	ABC1048	0.451
ABC1032	0.7415	ABC1047	N.D.
ABC1033	N.D.	ABC690	0.2516
ABC689	0.2769	/	/

Table 6: EC50s of LIGHT-based immunocytokines binding to 293T cells expressing mouse LTβR

Immunocytokine	EC50 (nM)	Immunocytokine	EC50 (nM)
ABC566	9.281	ABC650	0.9535
ABC578	1.152	ABC651	0.8582
ABC568	0.8239	ABC652	0.9841

ABC579	0.6791	ABC1054	5.612
ABC569	0.7478	ABC890	0.4560
ABC583	1.033	ABC475	0.5267
ABC581	0.6781	ABC476	1.671
ABC582	0.7283	ABC477	0.9506
ABC580	1.435	ABC538	5.717
ABC584	0.7066	ABC539	7.452
ABC639	0.7577	ABC540	1.2
ABC640	0.7916	ABC541	1.258
ABC641	1.07	ABC542	2.795
ABC233	0.9440	ABC543	1.354
ABC649	0.9948	ABC985	9.140
ABC770	0.651	ABC986	0.209
ABC771	0.669	ABC987	0.086
ABC772	0.959	ABC988	0.249
ABC773	1.090	ABC892	0.274
ABC1030	1.338	ABC1034	0.6433
ABC1031	0.9857	ABC1035	N.D.
ABC1032	3.377	ABC1047	N.D.
ABC1033	N.D.	ABC1048	1.022
ABC689	0.2610	ABC690	0.2288
N.D.: not determined due to low binding affinity.			

Table 7: EC50s of LIGHT-based immunocytokines binding to 293T cells expressing human or mouse HVEM

Immunocytokine	EC50 (nM)	
	human HVEM	mouse HVEM
ABC233	2.487	0.4848
ABC475	N.D.	0.7456
ABC476	N.D.	0.7933
ABC477	4.38	11
ABC538	N.D.	17
ABC539	N.D.	N.D.
ABC543	6.092	16.07
ABC580	N.D.	1.435
ABC689	0.7388	0.8154
ABC690	1.341	1.414
ABC770	1.640	0.151
ABC771	1.009	0.384
ABC772	0.992	0.705
ABC773	0.761	1.265

ABC890	N.D	N.D
ABC892	N.D	N.D
ABC985	0.627	1.379
ABC986	1.526	1.276
ABC987	6.80	2.892
ABC988	14.62	3.969
ABC1030	N.D	1.951
ABC1031	N.D	0.9959
ABC1032	N.D	N.D
ABC1033	N.D	N.D
ABC1034	N.D	2.176
ABC1035	N.D	N.D
ABC1048	N.D.	N.D
N.D.: not determined due to low binding affinity.		

FACS analysis suggested that the LIGHT-based immunocytokines exhibited dose-dependent binding to cells overexpressing human or mouse LTβR (Figures 12A-12G and Figures 13A-13G). Additionally, the binding affinities of these immunocytokines to cells expressing HVEM were dominated by the LIGHT mutein conjugated to the antibodies (Figures 14A-14D and Figures 15A-15D). Some LIGHT-based immunocytokines, such as ABC477 and ABC543, exhibited similar binding to human or mouse HVEM-expressing cells as that of the wild-type immunocytokine (ABC233), while others, such as ABC539, had minimal binding to both types of cells.

Example 5: Pathway activation of anti-FAP×LIGHT immunocytokines in the absence or presence of FAP-expressing cells in trans

The 293T-FAP cell line was generated by transfecting full-length human FAP (Table 4) into HEK-293T cells using lipofectamine 3000 following standard protocols. After three days, the cells were treated with hygromycin (Millipore Sigma) for 14 days to generate stable cell lines.

HeLa-NF-kB and HEK-293T-NF-kB cells were generated by transfecting the cells with the pNL3.2.NF-κB-RE[NlucP/NF-κB-RE/Hygro] vector (Promega # N1111) using lipofectamine 3000. After three days, the cells were treated with hygromycin B (Sigma) and cultured for 14 days in 37°C, 5% CO_2. The resulting HeLa-NF-κB reporter cells were used to evaluate the downstream signaling of LTβR activation by the treatment of LIGHT-based immunocytokines.

To evaluate the ability of LIGHT-based immunocytokines to activate HVEM, the HEK293T-NF-κB cells were further transfected with full-length human or mouse HVEM (listed in Table 4) using lipofectamine 3000. After fourteen-day incubation in the presence of hygromycin B and puromycin (Millipore Sigma), the resulting HEK293T-HVEM-NF-κB stable cells were collected for further analysis.

To evaluate the effect of FAP on LIGHT-induced pathway activation, the reporter cells were either cultured alone or co-cultured with 293T-FAP cells and treated with indicated molecules. Twenty-four hours later, cells were lysed with lysis buffer (Promega #E397A), and luciferase activity was measured (Promega #E4500) using SpectraMax M5 microplate reader (Molecule Devices).

LIGHT-based immunocytokines were able to activate NF-κB signaling in HeLa cells harboring endogenous LTβR (Figures 16A-16H and Table 8). Immunocytokines equipped with the same LIGHT mutein also showed similar capabilities to activate LTβR. Coculturing with 293T-FAP cells enhanced LTβR activation for most immunocytokines (Table 8), except for ABC543 (Figures 19A-19D). For instance, when cultured alone, ABC538 and ABC539 had weaker induction capabilities for NF-κB signaling than ABC233 with the wild-type LIGHT (Figures 16A-16D, 19 and 28A-28B, Table 8). However, they were able to activate NF-κB signaling similarly to the wild-type molecule when co-cultured with HeLa-NF-κB reporter cells and 293T-FAP cells. These results suggest that immunocytokines such as ABC538 and ABC539 can be selectively activated in the tumor microenvironment when FAP-expressing fibroblasts are present.

Table 8: EC50s of LIGHT-based immunocytokines to activate HeLa-NF-κB cells

Immunocytokines	without 293T-FAP EC50	with 293T-FAP EC50	Immunocytokines	without 293T-FAP EC50	with 293T-FAP EC50
ABC566	0.08414	0.04397	ABC651	0.132	0.0293
ABC578	0.2498	0.03267	ABC652	0.04424	0.03279
ABC568	0.03133	0.01519	ABC475	0.04547	0.01037
ABC579	0.06908	0.02685	ABC476	0.07302	0.02645
ABC569	0.01096	0.01024	ABC477	0.03407	0.02337
ABC583	0.02508	0.01103	ABC538	0.08118	0.02498
ABC581	0.03948	0.01327	ABC539	0.3421	0.02352
ABC582	0.04317	0.01361	ABC540	0.0349	0.00521
ABC580	0.1088	0.03591	ABC541	0.09108	0.02452
ABC584	0.09802	0.03306	ABC542	0.06545	0.01781
ABC639	0.1298	0.02855	ABC543	0.2594	0.01177
ABC640	0.1506	0.02998	ABC677	0.05062	0.01279
ABC641	0.1404	0.02914	ABC678	0.07749	0.01112
ABC233	0.04741	0.01911	ABC679	0.06419	0.007281
ABC649	0.04199	0.02915	ABC680	0.0582	0.01793
ABC650	0.2191	0.04408	ABC890	0.03796	0.01339
ABC892	0.03271	0.01946	/	/	/

The abilities of LIGHT-based immunocytokines to activate the HVEM downstream signaling were evaluated with 293T-NF-κB cells overexpressing human or mouse HVEM. The results showed that while the wild-type LIGHT (ABC233) effectively activated downstream NF-

κB signaling (Figures 17A-17C, 18A-18C and 28C-28E, Table 9), ABC538 ABC539, ABC890, and ABC892 exhibited reduced activities to activate the pathway, consistent with their reduced binding to HVEM.

Table 9: EC50s of the LIGHT-based immunocytokines to activate HEK293T-HVEM-NF-κB cells.

LIGHT-based immunocytokine	EC50 (nM)	
	human HVEM	mouse HVEM
ABC233	0.3551	0.0765
ABC538	N.D	N.D
ABC539	13.72	4.87
ABC475	14.45	0.0686
ABC476	35.86	0.0789
ABC477	0.410	0.0404
ABC540	0.666	0.0735
ABC541	0.505	0.0422
ABC542	0.516	0.0562
ABC543	0.681	0.0832
ABC566	1.434	0.105
ABC578	N.D	1.837
ABC771	0.757	0.497
ABC890	N.D	N.D
ABC892	N.D	N.D
ABC985	0.268	0.108
ABC986	0.649	0.072
ABC987	17.60	0.128
ABC988	130.0	0.639
N.D: not determined due to low activity		

Example 6: Characterization of DcR3's impact on the activity of LIGHT-based immunocytokines

In addition to HVEM and LTβR, LIGHT also interacts with a decoy receptor, DcR3, which lacks the transmembrane and cytoplasmic segments. This interaction has the potential to disrupt the signaling pathways by sequestering LIGHT away from HVEM and LTbR. While DcR3 expression is typically low in healthy human tissues, it is often significantly upregulated in cancer patients (Wu et al., 2003; Yoo et al., 2022).

To minimize the binding of LIGHT-based immunocytokines to DcR3, ABC538 and ABC539 were developed from LIGHT-1 and LIGHT-2, two muteins with considerably weaker affinities to DcR3. To investigate the impact of DcR3 on LIGHT-induced LTβR activation, HeLa-NF-κB reporter cells were exposed to 200 μg/ml soluble DcR3-Fc protein, when treated

with LIGHT-based immunocytokines. After 24 hours, the cells were lysed (Promega #E397A) and the luciferase activity was measured with a SpectraMax M5 microplate reader (Molecule Devices) using Promega #E4500.

The addition of DcR3-Fc protein reduced the ABC233-induced LTβR activation by 12 to 26 folds, regardless of the presence or absence of FAP-expressing cells, whereas DcR3 had a minimum effect on ABC538-induced LTβR activation (Figures 20A-20D). These results suggest that ABC538 is less likely to be affected by soluble DcR3, leading to potentially improved pharmacokinetics (PK) properties and potentially higher levels of active molecules in the tumor microenvironment.

Example 7: Binding of anti-FAP×LIGHT immunocytokines to endogenous mouse LTβR in the presence or absence of coexpressed FAP

Two murine cell lines, BALB/c-3T3-FAP ($LT\beta R^+$ $HVEM^-$ FAP^+) and BALB/c-3T3 ($LT\beta R^+$ $HVEM^-$ FAP^-) were used to investigate the effect of LIGHT muteins to activate endogenous mouse LTβR. BALB/c-3T3-FAP cell lines were generated by transfecting BALB/c-3T3 cells (ATCC) with mouse Fap (referred to as mFAP) and screened for mFAP expression. The immunocytokines were incubated with 0.1 million purified cells for 30 mins on ice in FACS buffer (PBS, 0.5%BSA, and 1 mM EDTA). Then the cells were washed with FACS buffer for 3 times, and live/dead cell discrimination was performed using Live/Dead Fixable Near-IR Dead Cell Stain Kit (Life Technologies). Cells then were stained with 1 μg/mL goat anti-human IgG Fc (FITC) (ab97224, Abcam) on ice for 30 mins in FACS buffer. Cells were then washed with FACS buffer for 3 times before the final flow cytometric analysis. All flow cytometry data were acquired on a Cytek Aurora cytometer (Cytek) and analyzed by FlowJo software (v.10.5.3, Treestar, Inc.). GraphPad Prism 9.0 software was used to determine the EC50.

In the absence of FAP, the binding affinities of all three LIGHT-based immunocytokines to endogenous mLTβR were relatively weak, with EC50 values exceeding 10 nM, despite ABC538 and ABC233 showing better binding than ABC539. However, co-expression of FAP and LTβR on the same cells resulted in a significantly decreased in EC50 values of all three molecules to the nanomolar range (Figures 21A-21B). Therefore, anti-FAP×LIGHT immunocytokines allowed for the preferential targeting of FAP-expressing cells over those not expressing FAP.

Example 8: Binding of LIGHT-based immunocytokines to endogenous mouse HVEM

Primary human and mouse T cells were utilized to evaluate the interaction between anti-FAP×LIGHT immunocytokines and h/mHVEM. The T cells were negative for LTβR and FAP but positive for HVEM. Human $CD3^+$ T cells were purified from human PBMC with a purity above 97% through negative selection following the manufacturer's protocol (480134, Biolegend). Mouse $CD3^+$ T cells were purified from pooled splenocytes of BALB/c mice with a purity above 97% by negative selection following the manufacturer's protocol (480031,

Biolegend). The cells were incubated with twelve 3-fold serial dilutions of LIGHT-based immunocytokines on ice for 30mins in FACS buffer (PBS, 0.5%BSA, and 1 mM EDTA). The cells were washed with FACS buffer for 3 times, and live/dead cell discrimination was performed using the Live/Dead Fixable Near-IR Dead Cell Stain Kit (Life Technologies). Afterward, human T cells were stained with either anti-hCD4 (RPA-T4, Biolegend) or anti-hCD8a (RPA-T8, Biolegend) along with 1 μg/mL goat anti-human IgG Fc (FITC) (ab97224, Abcam) on ice for 30 mins in FACS buffer. Mouse T cells were stained with either anti-mCD4 (RM4-5, Biolegend) or anti-mCD8a (53-6.7, Biolegend) along with 1 μg/mL goat anti-human IgG Fc (FITC) (ab97224, Abcam) on ice for 30 mins in FACS buffer. Cells were then washed for three times with FACS buffer, and the cytometry data was collected using a Cytek Aurora cytometer (Cytek) and analyzed using FlowJo. The GraphPad Prism 9.0 software was utilized to determine the EC50, which refers to the concentration of protein required to achieve half of the maximum binding.

The results in Figure 22 showed that ABC538 displayed weaker binding to endogenous HVEM on primary CD4 and CD8 T cells than ABC233, whereas ABC539 exhibited minimal binding to HVEM. These results suggest that ABC538, with its reduced binding to HVEM, may predominantly activate the LTBR rather than the HVEM pathway within the tumor microenvironment.

Example 9: Activation of the LTβR pathway by anti-FAP×LIGHT immunocytokines in the absence or presence of co-expressed FAP

BALB/c-3T3-FAP ($LT\beta R^+$ $HVEM^-$ FAP^+) and BALB/c-3T3 ($LT\beta R^+$ $HVEM^-$ FAP^-) were used to investigate the effect of LIGHT muteins to activate endogenous LTβR. The cells were plated at 3,000 cells per well in a 96-well tissue culture plate and incubated with different concentrations of anti-FAP×LIGHT immunocytokines for 24 hours at 37℃. After centrifugation, supernatants were collected to quantify the level of CCL2 protein by ELISA (Cat#432704, Biolegend) with a Tecan Spark reader.

Figure 23A showed that in the absence of FAP expression in 3T3 cells, the activation of the LTβR pathway and the induction of CCL2 protein were primarily determined by the binding affinity of the immunocytokines, with ABC233 being the most effective followed by ABC538 and ABC539. However, the co-expressed FAP significantly increased the capabilities of all three molecules to induce CCL2 expression, particularly at lower concentrations (Figure 23B and 23C). Since cancer-associate fibroblasts (CAFs) in the tumor stroma play an important role in tumor biology and exhibit abundant expression of both FAP and LTβR, the anti-FAP×LIGHT immunocytokines are designed to preferentially deliver FAP-mediated avidity-driven LTβR stimulation to CAFs in the tumor microenvironment.

Example 10: Activation of the LTβR pathway by anti-FAP×LIGHT immunocytokines in the absence or presence of trans-expressed FAP

To study the effect of trans-expressed FAP on the activation of endogenous LTβR pathway in BALB/c-3T3-WT ($LT\beta R^+ HVEM^- FAP^-$) cells, a CT26-FAP cell line was generated by stably transfecting CT26 cells (ATCC) with a plasmid containing mouse FAP gene using lipofectamine 3000 and antibiotic selection. We confirmed that only 3T3 cells, but not CT26-WT or CT26-FAP, could induce CCL2 after stimulation by LIGHT-based immunocytokines.

CT26-WT or CT26-FAP cells were plated at 2.5K/well in a 96-well tissue culture plate and incubated with serially diluted LIGHT-based immunocytokines for one hour at 37℃, 5% CO_2. Then 2.5K BALB/c-3T3 cells were added to the culture as responder cells. ELISA assay (Cat#432704, Biolegend) was performed to measure the CCL2 protein level in the media, as a surrogate of the NF-κB pathway activation.

In the presence of CT26-FAP cells, ABC233, ABC538 and ABC539 enhanced the activation of LTβR signaling and the production of CCL2 from 3T3 cells at low concentrations, suggesting that the immunocytokine's anti-FAP arm binding to the CT26-FAP cells could assist LIGHT-LTβR signaling in trans (Figure 24).

Example 11: Assessment of in vivo anti-tumor activity of anti-FAP×LIGHT immunocytokines in the CT26-FAP tumor model

To assess the anti-tumor activity of the immunocytokines, 0.4 million CT26-FAP cells in 50 μl of HBSS were mixed with 50 μl of matrigel and implanted subcutaneously into the flank of BALB/c mice. When tumor volumes reached 60-93 mm^3 on day 8, mice were grouped based on body weight and tumor volume to ensure similar distribution. Mice were divided into three groups: G1_buffer (negative control), G2_ABC002 (anti-FAP, negative control) and G3_ABC538, n=9/group. Mice were treated twice a week at 1 nanomole/mouse for the first 3 doses, and 1.5 nanomole/mouse for the next 2 doses. One nanomole of ABC538 is equivalent to 200 μg of protein, while 1.5 nanomoles are equal to 300 μg of protein. ABC002's equivalent protein for 1 nanomole and 1.5 nanomoles is 144 μg and 200 μg, respectively. Tumor growth was monitored by measuring the tumor volume using calipers at specific time points until day 22. The tumor volume was calculated using the formula: length × $width^2/2$. The mouse's body weight was also recorded at the indicated time points.

The survival of the mice was calculated using the Kaplan-Meier method. The mice were considered expired if one of the following criteria was met: (1) the tumor volume reached ~2000 mm^3, (2) it experienced a body weight loss of 20%, or (3) it died. The results showed that ABC538 had single-agent anti-tumor activity, as indicated by both tumor growth inhibition and mouse survival, as demonstrated in Figure 25.

Example 12: Assessment of in vivo cleavage of LIGHT-based immunocytokines

It has been shown that the metalloprotease family members (MMPs) can cleave the surface LIGHT expressed on activated T cells. Indeed, inhibition of MMPs leads to an increased LIGHT expression on activated T cells (Morel et al., 2000). For enhanced the efficacy and half-life of the LIGHT-based immunocytokines, the LIGHT moiety in the immunocytokines was further truncated to residues 91-240 to generate cleavage-resistant immunocytokines, such as ABC890, ABC892, ABC1054, ABC1056, ABC1032 and ABC1048.

These cleavage-resistant immunocytokines maintain their binding specificity and functionality. For instance, ABC890 and ABC892, which share the same mutations as ABC538 (LIGHT1, residues 74-240), exhibit similar binding affinities to human and mouse LTβR, and reduced binding to human and mouse HVEM and human DcR3 (Figure 8D, 9D, 10B, 10F).

These cleavage-resistant immunocytokines can activate LTβR with comparable EC50s as shown in Table 8, and their activities of activating LTβR were augmented by clustering with cells expressing the antigen. However, since all three molecules have reduced binding for HVEM, their activities of stimulating HVEM were only observed in the presence of cells expressing the antigen, such as 293T-hFAP cells (Figures 28A-28E). These results suggest that molecules such as ABC538 and ABC539 can be selectively activated in the tumor microenvironment when FAP-expressing fibroblasts are present.

To evaluate the in vivo cleavage of the anti-FAP×LIGHT immunocytokines, 6-week-old female C57BL/6J mice were given a single dose (i.p.) of 1.5 nanomoles of ABC538, ABC890, or ABC892. Plasma samples were collected 24h and 96h after the injection and then analyzed for cleavage. The plasma cells were diluted 100-fold and incubated with 0.2 million 293T-mFAP cells for 30 mins on ice, followed by washing three times with FACS buffer. The Live/Dead Fixable Near-IR Dead Cell Stain Kit (Life Technologies) was used to determine the live and dead cells, which were then stained with a mixture of 1 μg/mL goat anti-human IgG Fc (FITC) (ab97224, Abcam) and 1 μg/mL anti-hLIGHT (PE) (T5-39, Biolegend) for 30 mins on ice. After washing three times with FACS buffer, the cells were analyzed using flow cytometry on a Cytek Aurora cytometer (Cytek) and the results were analyzed by FlowJo software. Twelve 3-fold serial dilutions of the indicated antibodies were used with the 4-parameter logistics curve-fitting algorithm to generate standard curves. The ratio of LIGHT concentration to hIgG1 concentration was used to evaluate cleavage, with a ratio of 1 indicating no cleavage.

The results showed that ABC538 underwent partial cleavage in vivo, resulting in a 50% ratio of detected LIGHT to detected hIgG1 in mouse plasma at both 24 and 96 hours. In contrast, ABC890 and ABC892, with shorter LIGHT subunit, exhibited higher ratios, suggesting less cleavage, improved stability, and increased bioavailability in vivo (Figure 26).

Example 13: In vivo anti-tumor activity of anti-FAP×LIGHT immunocytokines in the KPC0826 tumor model

The KPC0826 cell line was derived from a genetically modified KPC mouse model using a previously published protocol (Beatty et al., 2011). To establish a subcutaneous KPC0826 tumor model, 2.5 million KPC0826 cells in 50 μl HBSS were mixed with 50 μl of matrigel and implanted into the flank of BALB/c mice. The established subcutaneous KPC0826 tumor model showed a relatively high presence of FAP^+ CAF cells, around 1-5% of total dissociated tumor cells (data not shown). On day 0, when tumor volumes reached 70-102 mm^3 with an average ~87 mm^3, the mice were divided into 6 groups based on body weight and tumor volume: G1_ABC002 (anti-FAP, negative control), G2_ABC233 (anti-FAP×LIGHT-wt), G3_ABC538 (anti-FAP×LIGHT-1), G4_ABC539 (anti-FAP×LIGHT-2), G5_ABC890 and G6_ABC892, with eight mice per group. Mice were treated twice a week for 6 doses with 1.5 nanomoles of the respective molecules, corresponding to a dose of 200 μg for ABC002 and 300 μg for the other molecules. Tumor growth was monitored by measuring tumor volumes using calipers at indicated time points until day 21. The tumor volume was calculated using the formula: length x $width^2/2$. Tumor growth inhibition (TGI) was compared to the control group (G1_ABC002) and calculated using the DRAP R package (J Transl Med 17, 39, 2019).

The results demonstrated that all tested anti-FAP×LIGHT immunocytokines exhibited single-agent anti-tumor activity in the KPC0826 model. Among them, ABC890 and ABC892 showed the highest tumor growth inhibition (TGI), followed by ABC538, ABC233, and ABC539. The cleavage-resistant derivatives, ABC890 and ABC892, demonstrated superior anti-tumor activity compared to ABC538 (shown in Figures 27A-27D).

Example 14: other TNFSF14 superfamily member immunocytokine

The platform can also be applied to other TNFSF14 superfamily member. 4-1BBL is another TNF superfamily member and its interaction with CD137/TNFRSF9/4-1BB can promote T lymphocyte activation and proliferation. Antibody-4-1BBL fusion protein (ABC676) was successfully expressed using the format D. Lymphotoxin-αββ, similar to engineered LIGHT1, binds to LTβR without engaging HVEM and DcR3, potentially offering enhanced safety and efficacy in providing an anti-tumor effect. With Format D, an immunocytokine equipped with Lymphotoxin-αββ can also be generated (ABC653).

HeLa-NF-κB reporter cells were utilized to assess the downstream signaling upon LTβR activation following treatment with Lymphotoxin-based immunocytokines. The Lymphotoxin-based immunocytokine (ABC653) demonstrated the ability to activate NF-κB signaling in HeLa cells containing endogenous LTβR, with an EC50 of 0.135 nM (Figure 29).

ABC653 was evaluated for anti-tumor efficacy in genetically modified KPC mice. When tumor volumes reached 70-102 mm^3, mice were treated twice a week for 8 doses with 1.5 nanomoles of ABC653 and the mAb control (ABC002). Tumor growth was monitored by measuring tumor volumes using calipers, and the tumor volume was calculated using the formula: length x

width^2/2. The results demonstrated that anti-FAP×Lymphotoxin (ABC653) immunocytokines exhibited single-agent anti-tumor activity in the KPC0826 model (Figure 30).

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Claims

1. A fusion protein, comprising:

a first cytokine fragment linked to the C-terminal of a first CH2-CH3 fragment, comprising a first cytokine molecule, or a first cytokine molecule and a third cytokine molecule that are tandem linked, and

a second cytokine fragment linked to the C-terminal of a second CH2-CH3 fragment, comprising a second cytokine molecule, or a second cytokine molecule and a fourth cytokine molecule that are tandem linked,

the first CH2-CH3 fragment and the second CH2-CH3 fragment form a dimer, totally three cytokine molecules are linked to the dimer.

2. The fusion protein according to claim 1, wherein the first cytokine fragment comprises the first cytokine molecule, and the second cytokine fragment comprises the second cytokine molecule and the fourth cytokine molecule that are tandem linked, or

the first cytokine fragment comprises the first cytokine molecule and a third cytokine molecule that are tandem linked, and the second cytokine fragment comprises the second cytokine molecule.

3. The fusion protein according to claim 1 or 2, wherein the first cytokine molecule, the second cytokine molecule, the third cytokine molecule and the fourth cytokine molecule independently are selected from the group consisting of tumor necrosis factor, interleukin, lymphokine, interferon, colony stimulating factor, chemokine and growth factor.

4. The fusion protein according to any one of claims 1-3, wherein the first cytokine molecule, the second cytokine molecule, the third cytokine molecule and the fourth cytokine molecule independently are LIGHT, lymphotoxin α, lymphotoxin β, or 4-1BBL.

5. The fusion protein according to any one of claims 1-4, wherein the first cytokine molecule, the second cytokine molecule, the third cytokine molecule and the fourth cytokine molecule independently are wild type LIGHT (preferably human LIGHT), truncated LIGHT or mutein thereof; optionally, wild type LIGHT, truncated LIGHT or mutein thereof comprises amino acid sequence of any one of SEQ ID NOs: 25, 26, 58, 59, 68-78, 126-141, 158, 177, 178 and 180;

the first cytokine molecule, the second cytokine molecule, the third cytokine molecule and the fourth cytokine molecule independently are wild type 4-1BBL (preferably human 4-1BBL), truncated 4-1BBL or mutein thereof; optionally, wild type 4-1BBL, truncated 4-1BBL or mutein thereof comprises amino acid sequence of SEQ ID NO:179;

the first cytokine molecule, the second cytokine molecule, the third cytokine molecule and the fourth cytokine molecule independently are wild, truncated lymphotoxin α or lymphotoxin β, or mutein thereof; optionally, the wild, truncated lymphotoxin α or lymphotoxin β, or mutein thereof comprises amino acid sequence of SEQ ID NO: 181, 182.

6. The fusion protein according to any one of claims 1-5, wherein the first cytokine molecule, the second cytokine molecule, the third cytokine molecule and the fourth cytokine molecule independently are truncated LIGHT, optionally, the truncated LIGHT includes an amino acid sequence set forth in SEQ ID NO: 68 or SEQ ID NO: 78.

7. The fusion protein according to any one of claims 1-6, wherein the first cytokine molecule, the second cytokine molecule, the third cytokine molecule and the fourth cytokine molecule independently are LIGHT mutein; optionally, the LIGHT mutein includes an amino acid sequence set forth in one of SEQ ID NOs: 25-26, 58-59, 69-77, 126-141, 158, 177, 178 and 180.

8. The fusion protein according to any one of claims 1-7, wherein the dimer is an Fc domain, which is modified to improve the correct assembly of the fusion protein.

9. The fusion protein according to claim 8, wherein the Fc domain is derived from IgG, which includes IgG1, IgG2, IgG3, and IgG4, optionally the Fc domain is derived from human IgG1 or IgG4 Fc domain.

10. The fusion protein according to any one of claims 1-9, wherein the first CH2-CH3 fragment and the second CH2-CH3 fragment comprise one or more modifications selected from the group consisting of knobs-into-holes, electrostatic steering of CH3 (such as DDKK), DuoBody, SEEDbodies, cFAE, XmAb, Azymetric, and BEAT®,

optionally, the modifications comprise knobs-into-holes, and/or DDKK.

11. The fusion protein according to any one of claims 1-10, wherein the first CH2-CH3 fragment and the second CH2-CH3 fragment form a human IgG1 Fc domain with mutation N297A.

12. The fusion protein according to any one of claims 1-11, wherein the first CH2-CH3 fragment or the second CH2-CH3 fragment further comprises one or more mutations selected from the group consisting of Y349C, T366S, L368A, and Y407V; and the other one comprises mutations S354C and/or T366W.

13. The fusion protein according to any one of claims 1-12, wherein the first cytokine fragment links to the C-terminal of the first CH2-CH3 fragment directly or via a linker; and/or

the second cytokine fragment link to the C-terminal of the second CH2-CH3 fragment directly or via a linker.

14. The fusion protein according to any one of claims 1-13, wherein the C terminal of the CH3 domain in the first CH2-CH3 fragment is fused to the N terminal of the first cytokine fragment directly or via a first linker A; and/or the C terminal of the CH3 domain in the second CH2-CH3 fragment is fused to the N- terminal of the second cytokine fragment directly or via a first linker B.

15. The fusion protein according to any one of claims 1-14, wherein the first cytokine molecule is fused to the third cytokine molecule directly or via a second linker A; and/or

the second cytokine molecule is fused to the fourth cytokine molecule directly or via a second linker B.

16. The fusion protein according to any one of claims 1-15, wherein the C terminal of the first cytokine molecule is fused to the N terminal of the third cytokine molecule directly or via a second linker A; and/or the C terminal of the second cytokine is fused to the N terminal of the fourth cytokine directly or via a second linker B.

17. The fusion protein according to any one of claims 1-16, wherein the first linker A, the first linker B, the second linker A, and the second linker B independently are absent or are selected from the group consisting of VH-CH1 linker (SEQ ID NO: 82), VL-CL linker (SEQ ID NO: 83), CH2-CH3 linker (SEQ ID NO: 81), IgM tail linker (SEQ ID NO: 84), GGGGSGGGGSGGGGSGGGGT (SEQ ID NO: 89), G, and (GGGGS)n, wherein n=1, 2, 3, 4, 5, 6, 7, 8, 9, or 10.

optionally, the first linker A and the first linker B independently are absent or are selected from the group consisting of SEQ ID NOs: 81, 82, 83, 84 and 89, G, and (GGGGS)n, wherein n=1, 2, 3, 4, 5, 6, 7, 8, 9, or 10.

18. The fusion protein according to any one of claims 1-17, wherein the first linker A and the first linker B independently are absent or comprise the amino acid sequence as set forth in any one of SEQ ID NOs: 81-90.

19. The fusion protein according to any one of claims 1-18, wherein the first linker A and the first linker B are the same or different.

20. The fusion protein according to any one of claims 1-19, wherein the second linker A and the second linker B independently are absent or are G, GGGGSGGGGSGGGGSGGGGT, or (GGGGS)n, wherein n=1, 2, 3, 4, 5, 6, 7, 8, 9, or 10.

21. The fusion protein according to any one of claims 1-20, wherein the second linker A and the second linker B independently of each other are absent or comprise the amino acid G or the amino acid sequence as set forth in any one of SEQ ID NOs: 85-89.

22. The fusion protein according to any one of claims 1-21, comprising a first antigen-binding fragment and a second antigen-binding fragment, which are connected to the N-terminal of the first CH2-CH3 fragment and the second CH2-CH3 fragment, respectively.

23. The fusion protein according to claim 22, wherein the first antigen-binding fragment and the second antigen-binding fragment independently are selected from the group consisting of a diabody, a Fab, a Fab', a F(ab')2, an Fv fragment, a disulfide stabilized Fv fragment (dsFv), a (dsFv)2, a bispecific dsFv (dsFv-dsFv'), a disulfide stabilized diabody (ds diabody), a single-chain Fv (scFv), an scFv dimer (bivalent diabody), a multispecific antibody formed from a portion of an antibody including one or more CDRs, a camelized single domain antibody, a nanobody, a domain antibody, a bivalent domain antibody, or any other antibody fragment.

24. The fusion protein according to claim 22 or 23, wherein the first antigen-binding fragment is an scFv or a Fab that specifically binds to a first antigen; and the second antigen-binding fragment is a Fab that specifically binds to a second antigen.

25. The fusion protein according to any one of claims 22-24, comprising

a first heavy chain, from N terminal to C terminal, including the operably linked first antigen-binding fragment, the first CH2-CH3 fragment, and the first cytokine fragment;

a second heavy chain, from the N terminal to the C terminal, including the operably linked second antigen-binding fragment, the second CH2-CH3 fragment, and the second cytokine fragment.

26. The fusion protein according to any one of claims 22-25, further comprising

a first light chain, which pairs with the first antigen-binding fragment to form a heterodimer; and/or

a second light, which pairs with the second antigen-binding fragment to form a heterodimer.

27. The fusion protein according to any one of claims 22-26, wherein the first antigen and the second antigen independently of each other are selected from the group consisting of FAP, HER2, PDL-1, PD-1, EGFR, VEGFR, VEGF, CCR8, OX-40, 41BB, Angiopoietin-2, IL-4Rα, BCMA, Blys, BTNO2, C5, CD122, CD13, CD133, CD137, CD138, CD16a, CD19, CD20, CD22, CD27, CD28, CD3, CD30, CD33, CD38, CD40, CD47, CD-8, CEA, CGPR/CGRPR, CSPGs, CTLA4, CTLA-4, DLL-4, EpCAM, factor IXa, factor X, GITR, GP130, Her3, HSG, ICOS, IGF1, IGF1/2, IGF-1R, IGF2, IGFR, IL-1, IL-12, IL-12p40, IL-13, IL-17A, IL-1β, IL-23, IL-5, IL-6, IL-6R, Lag-3, LAG3 , MAG, Met, NgR, NogoA, OMGp, OX40, PDGFR, PSMA, RGMA, RGMB, SARS-CoV-2, Te38, TIM-3, TNF, TNFα, TROP-2, and TWEAK.

28. The fusion protein according to any one of claims 22-27, wherein the first antigen and the second antigen independently of each other are selected from the group consisting of FAP, HER2, PDL-1, and EGFR.

29. The fusion protein according to any one of claims 22-28, wherein both the first antigen and the second antigen are FAP, HER2, EGFR, or PDL-1, or the first antigen is HER2 and the second antigen is FAP.

30. The fusion protein according to any one of claims 1-29, wherein the fusion protein is monovalent or bivalent, monospecific or bispecific.

31. The fusion protein according to any one of claims 1-30, wherein the first cytokine molecule, the second cytokine molecule, and the third cytokine molecule have the same amino acid sequence; or

the first cytokine molecule, the second cytokine molecule, and the fourth cytokine molecule have the same amino acid sequence.

32. The fusion protein according to any one of claims 26-31, wherein the first light chain and the second light chain independently comprise an amino acid sequence as set forth in SEQ ID NO: 1, 29, 91 or 94.

33. The fusion protein according to any one of claims 22-32, wherein the first heavy chain comprises the amino acid sequence as set forth in SEQ ID NO: 3, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 23, 28, 31, 32, 35, 37, 39, 41, 43, 45, 47, 49, 51, 52, 53, 55, 57, 61, 63, 65, 67, 93, 96, 98, 99, 100, 102, 104, 107, 109, 111, 113, 114, 117, 119, 121, 123, 125, 143, 145, 147, 149, 151, 153, 157, 155, 160, 162, 164, 166, 167, 169, 171, 173 or 175.

34. The fusion protein according to any one of claims 22-33, wherein the second heavy chain comprises the amino acid sequence as set forth in SEQ ID NO: 2, 5, 7, 9, 11, 13, 15, 17, 19, 21, 24, 27, 30, 33, 34, 36, 38, 40, 42, 44, 46, 48, 50, 54, 56, 60, 62, 64, 66, 92, 95, 97, 101, 103, 105, 106, 108, 110, 112, 115, 116, 118, 120, 122, 124, 142, 144, 146, 148, 150, 152, 154, 156, 159, 161, 163, 165, 168, 170, 172, 174 or 176.

35. The fusion protein according to any one of claims 22-34, wherein the fusion protein is select from the following:

(1) both of the first light chain and the second light chain comprise the amino acid sequence as set forth in SEQ ID NO: 1, the first heavy chain comprises the amino acid sequence as set forth in SEQ ID NO: 3, and the second heavy chain comprises the amino acid sequence as set forth in SEQ ID NO: 2;

(2) both of the first light chain and the second light chain comprise the amino acid sequence as set forth in SEQ ID NO: 1, the first heavy chain comprises the amino acid sequence as set forth in SEQ ID NO: 4, and the second heavy chain comprises the amino acid sequence as set forth in SEQ ID NO: 2;

(3) both of the first light chain and the second light chain comprise the amino acid sequence as set forth in SEQ ID NO: 1, the first heavy chain comprises the amino acid sequence as set forth in SEQ ID NO: 6, and the second heavy chain comprises the amino acid sequence as set forth in SEQ ID NO: 5;

(4) both of the first light chain and the second light chain comprise the amino acid sequence as set forth in SEQ ID NO: 1, the first heavy chain comprises the amino acid sequence as set forth in SEQ ID NO: 8, and the second heavy chain comprises the amino acid sequence as set forth in SEQ ID NO: 7;

(5) both of the first light chain and the second light chain comprise the amino acid sequence as set forth in SEQ ID NO: 1, the first heavy chain comprises the amino acid sequence as set forth in SEQ ID NO: 10, and the second heavy chain comprises the amino acid sequence as set forth in SEQ ID NO: 9;

(6) both of the first light chain and the second light chain comprise the amino acid sequence as set forth in SEQ ID NO: 1, the first heavy chain comprises the amino acid sequence as set forth in SEQ ID NO: 11, and the second heavy chain comprises the amino acid sequence as set forth in SEQ ID NO: 11;

(7) both of the first light chain and the second light chain comprise the amino acid sequence as set forth in SEQ ID NO: 1, the first heavy chain comprises the amino acid sequence as set forth in SEQ ID NO: 14, and the second heavy chain comprises the amino acid sequence as set forth in SEQ ID NO: 13;

(8) both of the first light chain and the second light chain comprise the amino acid sequence as set forth in SEQ ID NO: 1, the first heavy chain comprises the amino acid sequence as set forth in SEQ ID NO: 16, and the second heavy chain comprises the amino acid sequence as set forth in SEQ ID NO: 15;

(9) both of the first light chain and the second light chain comprise the amino acid sequence as set forth in SEQ ID NO: 1, the first heavy chain comprises the amino acid sequence as set forth in SEQ ID NO: 18, and the second heavy chain comprises the amino acid sequence as set forth in SEQ ID NO: 17;

(10) both of the first light chain and the second light chain comprise the amino acid sequence as set forth in SEQ ID NO: 1, the first heavy chain comprises the amino acid sequence as set forth in SEQ ID NO: 20, and the second heavy chain comprises the amino acid sequence as set forth in SEQ ID NO: 19;

(11) both of the first light chain and the second light chain comprise the amino acid sequence as set forth in SEQ ID NO: 1, the first heavy chain comprises the amino acid sequence as set forth in SEQ ID NO: 22, and the second heavy chain comprises the amino acid sequence as set forth in SEQ ID NO: 21;

(12) both of the first light chain and the second light chain comprise the amino acid sequence as set forth in SEQ ID NO: 1, the first heavy chain comprises the amino acid sequence as set forth in SEQ ID NO: 23, and the second heavy chain comprises the amino acid sequence as set forth in SEQ ID NO: 24;

(13) both of the first light chain and the second light chain comprise the amino acid sequence as set forth in SEQ ID NO: 1, the first heavy chain comprises the amino acid sequence as set forth in SEQ ID NO: 27, and the second heavy chain comprises the amino acid sequence as set forth in SEQ ID NO: 28;

(14) both of the first light chain and the second light chain comprise the amino acid sequence as set forth in SEQ ID NO: 29, the first heavy chain comprises the amino acid sequence as set forth in SEQ ID NO: 30, and the second heavy chain comprises the amino acid sequence as set forth in SEQ ID NO: 31;

(15) both of the first light chain and the second light chain comprise the amino acid sequence as set forth in SEQ ID NO: 1, the first heavy chain comprises the amino acid sequence as set forth in SEQ ID NO: 32, and the second heavy chain comprises the amino acid sequence as set forth in SEQ ID NO: 33;

(16) both of the first light chain and the second light chain comprise the amino acid sequence as set forth in SEQ ID NO: 1, the first heavy chain comprises the amino acid sequence as set forth in SEQ ID NO: 34, and the second heavy chain comprises the amino acid sequence as set forth in SEQ ID NO: 35;

(17) both of the first light chain and the second light chain comprise the amino acid sequence as set forth in SEQ ID NO: 1, the first heavy chain comprises the amino acid sequence as set forth in SEQ ID NO: 37, and the second heavy chain comprises the amino acid sequence as set forth in SEQ ID NO: 36;

(18) both of the first light chain and the second light chain comprise the amino acid sequence as set forth in SEQ ID NO: 1, the first heavy chain comprises the amino acid sequence as set forth in SEQ ID NO: 39, and the second heavy chain comprises the amino acid sequence as set forth in SEQ ID NO: 38;

(19) both of the first light chain and the second light chain comprise the amino acid sequence as set forth in SEQ ID NO: 1, the first heavy chain comprises the amino acid sequence as set forth in SEQ ID NO: 41, and the second heavy chain comprises the amino acid sequence as set forth in SEQ ID NO: 40;

(20) both of the first light chain and the second light chain comprise the amino acid sequence as set forth in SEQ ID NO: 29, the first heavy chain comprises the amino acid sequence as set forth in SEQ ID NO: 42, and the second heavy chain comprises the amino acid sequence as set forth in SEQ ID NO: 43; or

(21) both of the first light chain and the second light chain comprise the amino acid sequence as set forth in SEQ ID NO: 1, the first heavy chain comprises the amino acid sequence as set forth in SEQ ID NO: 45, and the second heavy chain comprises the amino acid sequence as set forth in SEQ ID NO: 44;

(22) both of the first light chain and the second light chain comprise the amino acid sequence as set forth in SEQ ID NO: 1, the first heavy chain comprises the amino acid sequence as set forth in SEQ ID NO: 47, and the second heavy chain comprises the amino acid sequence as set forth in SEQ ID NO: 46;

(23) both of the first light chain and the second light chain comprise the amino acid sequence as set forth in SEQ ID NO: 1, the first heavy chain comprises the amino acid sequence as set forth in SEQ ID NO: 49, and the second heavy chain comprises the amino acid sequence as set forth in SEQ ID NO: 48;

(24) both of the first light chain and the second light chain comprise the amino acid sequence as set forth in SEQ ID NO: 1, the first heavy chain comprises the amino acid sequence as set forth in SEQ ID NO: 51, and the second heavy chain comprises the amino acid sequence as set forth in SEQ ID NO: 50;

(25) both of the first light chain and the second light chain comprise the amino acid sequence as set forth in SEQ ID NO: 1, the first heavy chain comprises the amino acid sequence as set forth in SEQ ID NO: 52, and the second heavy chain comprises the amino acid sequence as set forth in SEQ ID NO: 2;

(26) both of the first light chain and the second light chain comprise the amino acid sequence as set forth in SEQ ID NO: 1, the first heavy chain comprises the amino acid sequence as set forth in SEQ ID NO: 53, and the second heavy chain comprises the amino acid sequence as set forth in SEQ ID NO: 2;

(27) both of the first light chain and the second light chain comprise the amino acid sequence as set forth in SEQ ID NO: 1, the first heavy chain comprises the amino acid sequence as set forth in SEQ ID NO: 55, and the second heavy chain comprises the amino acid sequence as set forth in SEQ ID NO: 54;

(28) both of the first light chain and the second light chain comprise the amino acid sequence as set forth in SEQ ID NO: 1, the first heavy chain comprises the amino acid sequence as set forth in SEQ ID NO: 57, and the second heavy chain comprises the amino acid sequence as set forth in SEQ ID NO: 56;

(29) both of the first light chain and the second light chain comprise the amino acid sequence as set forth in SEQ ID NO: 1, the first heavy chain comprises the amino acid sequence as set forth in SEQ ID NO: 61, and the second heavy chain comprises the amino acid sequence as set forth in SEQ ID NO: 60;

(30) both of the first light chain and the second light chain comprise the amino acid sequence as set forth in SEQ ID NO: 1, the first heavy chain comprises the amino acid sequence as set forth in SEQ ID NO: 63, and the second heavy chain comprises the amino acid sequence as set forth in SEQ ID NO: 62;

(31) both of the first light chain and the second light chain comprise the amino acid sequence as set forth in SEQ ID NO: 1, the first heavy chain comprises the amino acid sequence as set forth in SEQ ID NO: 65, and the second heavy chain comprises the amino acid sequence as set forth in SEQ ID NO: 64;

(32) both of the first light chain and the second light chain comprise the amino acid sequence as set forth in SEQ ID NO: 1, the first heavy chain comprises the amino acid sequence as set forth in SEQ ID NO: 67, and the second heavy chain comprises the amino acid sequence as set forth in SEQ ID NO: 66;

(33) both of the first light chain and the second light chain comprise the amino acid sequence as set forth in SEQ ID NO: 91, the first heavy chain comprises the amino acid sequence as set forth in SEQ ID NO: 92, and the second heavy chain comprises the amino acid sequence as set forth in SEQ ID NO: 93;

(34) both of the first light chain and the second light chain comprise the amino acid sequence as set forth in SEQ ID NO: 94, the first heavy chain comprises the amino acid sequence as set forth in SEQ ID NO: 95, and the second heavy chain comprises the amino acid sequence as set forth in SEQ ID NO: 96;

(35) both of the first light chain and the second light chain comprise the amino acid sequence as set forth in SEQ ID NO: 1, the first heavy chain comprises the amino acid sequence as set forth in SEQ ID NO: 98, and the second heavy chain comprises the amino acid sequence as set forth in SEQ ID NO: 97;

(36) both of the first light chain and the second light chain comprise the amino acid sequence as set forth in SEQ ID NO: 1, the first heavy chain comprises the amino acid sequence as set forth in SEQ ID NO: 99, and the second heavy chain comprises the amino acid sequence as set forth in SEQ ID NO: 97;

(37) both of the first light chain and the second light chain comprise the amino acid sequence as set forth in SEQ ID NO: 1, the first heavy chain comprises the amino acid sequence as set forth in SEQ ID NO: 100, and the second heavy chain comprises the amino acid sequence as set forth in SEQ ID NO: 101;

(38) both of the first light chain and the second light chain comprise the amino acid sequence as set forth in SEQ ID NO: 1, the first heavy chain comprises the amino acid sequence as set forth in SEQ ID NO: 102, and the second heavy chain comprises the amino acid sequence as set forth in SEQ ID NO: 103;

(39) both of the first light chain and the second light chain comprise the amino acid sequence as set forth in SEQ ID NO: 1, the first heavy chain comprises the amino acid sequence as set forth in SEQ ID NO: 104, and the second heavy chain comprises the amino acid sequence as set forth in SEQ ID NO: 105;

(40) both of the first light chain and the second light chain comprise the amino acid sequence as set forth in SEQ ID NO: 1, the first heavy chain comprises the amino acid sequence as set forth in SEQ ID NO: 107, and the second heavy chain comprises the amino acid sequence as set forth in SEQ ID NO: 106;

(41) both of the first light chain and the second light chain comprise the amino acid sequence as set forth in SEQ ID NO: 1, the first heavy chain comprises the amino acid sequence as set forth in SEQ ID NO: 109, and the second heavy chain comprises the amino acid sequence as set forth in SEQ ID NO: 108;

(42) both of the first light chain and the second light chain comprise the amino acid sequence as set forth in SEQ ID NO: 1, the first heavy chain comprises the amino acid sequence as set forth in SEQ ID NO: 111, and the second heavy chain comprises the amino acid sequence as set forth in SEQ ID NO: 110;

(43) both of the first light chain and the second light chain comprise the amino acid sequence as set forth in SEQ ID NO: 1, the first heavy chain comprises the amino acid sequence as set forth in SEQ ID NO: 113, and the second heavy chain comprises the amino acid sequence as set forth in SEQ ID NO: 112;

(44) both of the first light chain and the second light chain comprise the amino acid sequence as set forth in SEQ ID NO: 1, the first heavy chain comprises the amino acid sequence as set forth in SEQ ID NO: 114, and the second heavy chain comprises the amino acid sequence as set forth in SEQ ID NO: 115;

(45) both of the first light chain and the second light chain comprise the amino acid sequence as set forth in SEQ ID NO: 1, the first heavy chain comprises the amino acid sequence as set forth in SEQ ID NO: 117, and the second heavy chain comprises the amino acid sequence as set forth in SEQ ID NO: 116;

(46) both of the first light chain and the second light chain comprise the amino acid sequence as set forth in SEQ ID NO: 1, the first heavy chain comprises the amino acid sequence as set forth in SEQ ID NO: 119, and the second heavy chain comprises the amino acid sequence as set forth in SEQ ID NO: 118;

(47) both of the first light chain and the second light chain comprise the amino acid sequence as set forth in SEQ ID NO: 1, the first heavy chain comprises the amino acid sequence as set forth in SEQ ID NO: 121, and the second heavy chain comprises the amino acid sequence as set forth in SEQ ID NO: 120;

(48) both of the first light chain and the second light chain comprise the amino acid sequence as set forth in SEQ ID NO: 1, the first heavy chain comprises the amino acid sequence as set forth in SEQ ID NO: 123, and the second heavy chain comprises the amino acid sequence as set forth in SEQ ID NO: 122;

(49) the second light chain comprises the amino acid sequence as set forth in SEQ ID NO: 1, the first heavy chain comprises the amino acid sequence as set forth in SEQ ID NO: 124, and the second heavy chain comprises the amino acid sequence as set forth in SEQ ID NO: 4;

(50) the second light chain comprises the amino acid sequence as set forth in SEQ ID NO: 1, the first heavy chain comprises the amino acid sequence as set forth in SEQ ID NO: 2, and the second heavy chain comprises the amino acid sequence as set forth in SEQ ID NO: 125;

(51) both of the first light chain and the second light chain comprise the amino acid sequence as set forth in SEQ ID NO: 1, the first heavy chain comprises the amino acid sequence as set

forth in SEQ ID NO: 143, and the second heavy chain comprises the amino acid sequence as set forth in SEQ ID NO: 142;

(52) both of the first light chain and the second light chain comprise the amino acid sequence as set forth in SEQ ID NO: 1, the first heavy chain comprises the amino acid sequence as set forth in SEQ ID NO: 145, and the second heavy chain comprises the amino acid sequence as set forth in SEQ ID NO: 144;

(53) both of the first light chain and the second light chain comprise the amino acid sequence as set forth in SEQ ID NO: 1, the first heavy chain comprises the amino acid sequence as set forth in SEQ ID NO: 147, and the second heavy chain comprises the amino acid sequence as set forth in SEQ ID NO: 146;

(54) both of the first light chain and the second light chain comprise the amino acid sequence as set forth in SEQ ID NO: 1, the first heavy chain comprises the amino acid sequence as set forth in SEQ ID NO: 149, and the second heavy chain comprises the amino acid sequence as set forth in SEQ ID NO: 148;

(55) both of the first light chain and the second light chain comprise the amino acid sequence as set forth in SEQ ID NO: 1, the first heavy chain comprises the amino acid sequence as set forth in SEQ ID NO: 151, and the second heavy chain comprises the amino acid sequence as set forth in SEQ ID NO: 150;

(56) both of the first light chain and the second light chain comprise the amino acid sequence as set forth in SEQ ID NO: 1, the first heavy chain comprises the amino acid sequence as set forth in SEQ ID NO: 153, and the second heavy chain comprises the amino acid sequence as set forth in SEQ ID NO: 152;

(57) both of the first light chain and the second light chain comprise the amino acid sequence as set forth in SEQ ID NO: 1, the first heavy chain comprises the amino acid sequence as set forth in SEQ ID NO: 155, and the second heavy chain comprises the amino acid sequence as set forth in SEQ ID NO: 154;

(58) both of the first light chain and the second light chain comprise the amino acid sequence as set forth in SEQ ID NO: 1, the first heavy chain comprises the amino acid sequence as set forth in SEQ ID NO: 157, and the second heavy chain comprises the amino acid sequence as set forth in SEQ ID NO: 156;

(59) both of the first light chain and the second light chain comprise the amino acid sequence as set forth in SEQ ID NO: 1, the first heavy chain comprises the amino acid sequence as set forth in SEQ ID NO: 160, and the second heavy chain comprises the amino acid sequence as set forth in SEQ ID NO: 159;

(60) both of the first light chain and the second light chain comprise the amino acid sequence as set forth in SEQ ID NO: 1, the first heavy chain comprises the amino acid sequence as set

forth in SEQ ID NO: 162, and the second heavy chain comprises the amino acid sequence as set forth in SEQ ID NO: 161;

(61) both of the first light chain and the second light chain comprise the amino acid sequence as set forth in SEQ ID NO: 1, the first heavy chain comprises the amino acid sequence as set forth in SEQ ID NO: 164, and the second heavy chain comprises the amino acid sequence as set forth in SEQ ID NO: 163;

(62) both of the first light chain and the second light chain comprise the amino acid sequence as set forth in SEQ ID NO: 1, the first heavy chain comprises the amino acid sequence as set forth in SEQ ID NO: 166, and the second heavy chain comprises the amino acid sequence as set forth in SEQ ID NO: 165;

(63) both of the first light chain and the second light chain comprise the amino acid sequence as set forth in SEQ ID NO: 1, the first heavy chain comprises the amino acid sequence as set forth in SEQ ID NO: 168, and the second heavy chain comprises the amino acid sequence as set forth in SEQ ID NO: 167;

(64) both of the first light chain and the second light chain comprise the amino acid sequence as set forth in SEQ ID NO: 1, the first heavy chain comprises the amino acid sequence as set forth in SEQ ID NO: 170, and the second heavy chain comprises the amino acid sequence as set forth in SEQ ID NO: 169;

(65) both of the first light chain and the second light chain comprise the amino acid sequence as set forth in SEQ ID NO: 1, the first heavy chain comprises the amino acid sequence as set forth in SEQ ID NO: 172, and the second heavy chain comprises the amino acid sequence as set forth in SEQ ID NO: 171;

(66) both of the first light chain and the second light chain comprise the amino acid sequence as set forth in SEQ ID NO: 1, the first heavy chain comprises the amino acid sequence as set forth in SEQ ID NO: 174, and the second heavy chain comprises the amino acid sequence as set forth in SEQ ID NO: 173;

(67) both of the first light chain and the second light chain comprise the amino acid sequence as set forth in SEQ ID NO: 1, the first heavy chain comprises the amino acid sequence as set forth in SEQ ID NO: 176, and the second heavy chain comprises the amino acid sequence as set forth in SEQ ID NO: 175.

36. An isolated polynucleotide encoding the first heavy chain, the first light chain, the second heavy chain or the second light chain of the fusion protein according to any one of claims 1-35.

37. A set of isolated polynucleotides, comprising the polynucleotide encoding the first heavy chain, the polynucleotide encoding first light chain, the polynucleotide encoding second

heavy chain and the polynucleotide encoding second light chain of the fusion protein according to any one of claims 1-35.

38. An isolated vector comprising the isolated polynucleotide according to claim 36 or 37.

39. A host cell comprising the isolated polynucleotide according to claim 36, the set of isolated polynucleotides according to claim 37 or the isolated vector according to claim 38.

40. A pharmaceutical composition comprising the fusion protein according to any one of claims 1-35, the isolated polynucleotide according to claim 36, the set of isolated polynucleotides according to claim 37, the isolated vector according to claim 38 or the host cell according to claim 39, and a pharmaceutically acceptable carrier.

41. A kit, comprising the fusion protein according to any one of claims 1-35, the isolated polynucleotide according to claim 36, the set of isolated polynucleotides according to claim 37, the isolated vector according to claim 38, the host cell according to claim 39 or the pharmaceutical composition according to claim 40.

42. Use of the fusion protein according to any one of claims 1-35, the isolated polynucleotide according to claim 36, the set of isolated polynucleotides according to claim 37, the isolated vector according to claim 38, the host cell according to claim 39, the pharmaceutical composition according to claim 40 or the kit according to claim 41 in the manufacture of a drug for preventing or treating a disease, or in the manufacture of a kit for diagnosing a disease.

43. The use according to claim 42, wherein the disease comprises tumor, optionally solid tumor.

44. A method of diagnosing, preventing or treating a disease in a subject in need thereof, comprising administrating to the subject a therapeutically effective amount of the fusion protein according to any one of claims 1-36, the isolated polynucleotide according to claim 36, the set of isolated polynucleotides according to claim 37, the isolated vector according to claim 38, the host cell according to claim 39, the pharmaceutical composition according to claim 40 or the kit of claim 41.

45. The method according to claim 44, wherein the disease comprises tumor, optionally solid tumor.

46. A method for production of the fusion protein according to any one of claims 1-35, comprising introducing a first expression vector encoding the first heavy chain, a second expression vector encoding the first light chain, a third expression vector encoding the second heavy chain and/or a fourth expression vector encoding the second light chain together into one host cell, or into separate host cells, and expressing the first, second, third and fourth expression vectors under a proper condition.

47. The method according to claim 46, wherein the host cells are eukaryotic cells.

48. The method according to claim 46 or 47, wherein the host cells are mammalian cells.

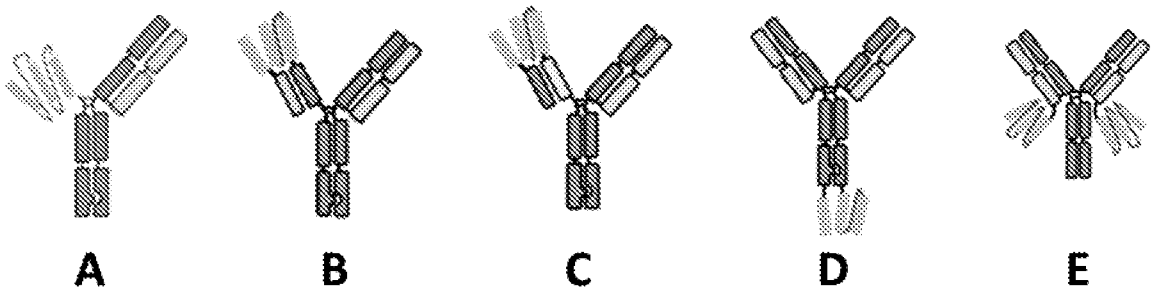


Figure 1

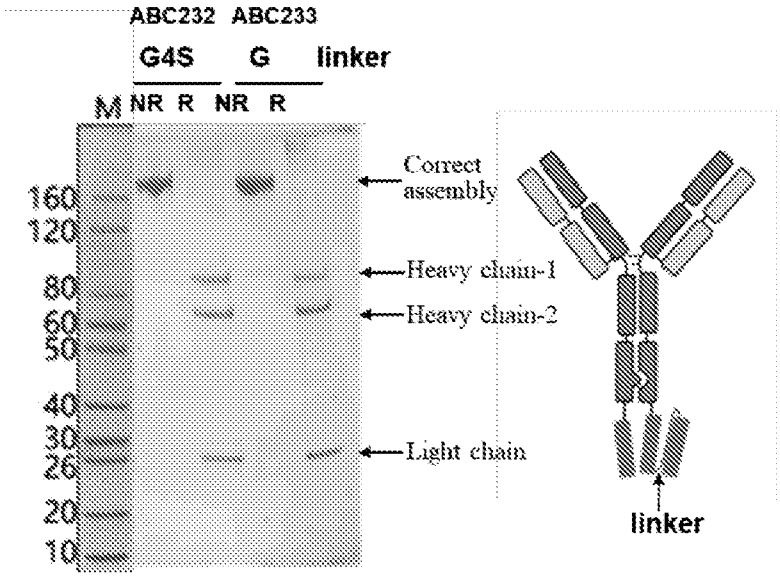


Figure 2

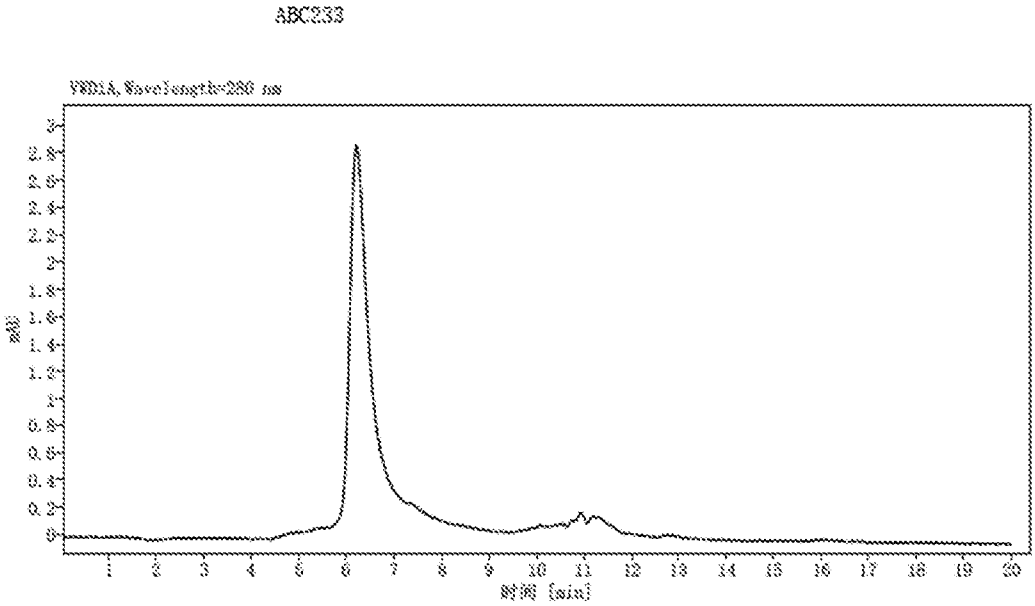


Figure 3A

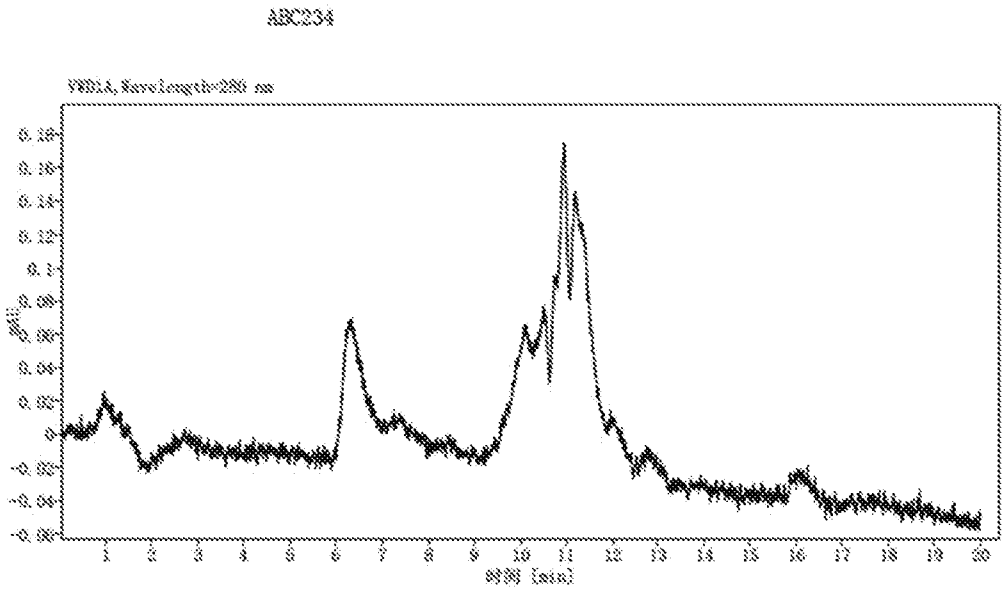


Figure 3B

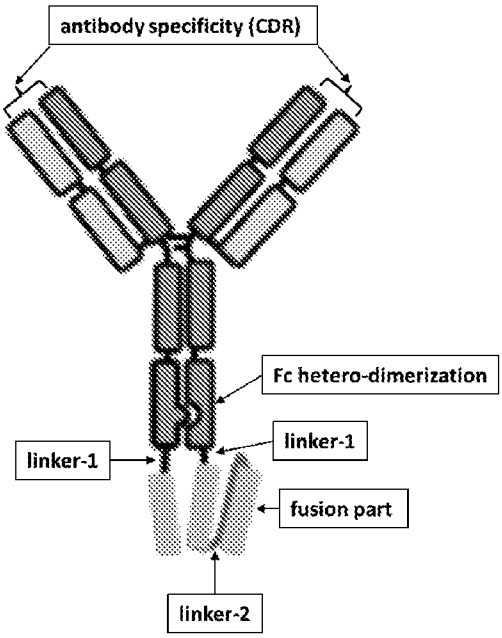


Figure 4A

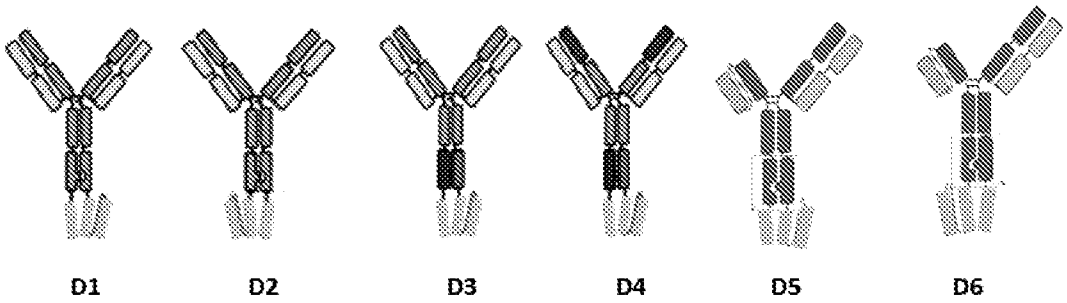


Figure 4B

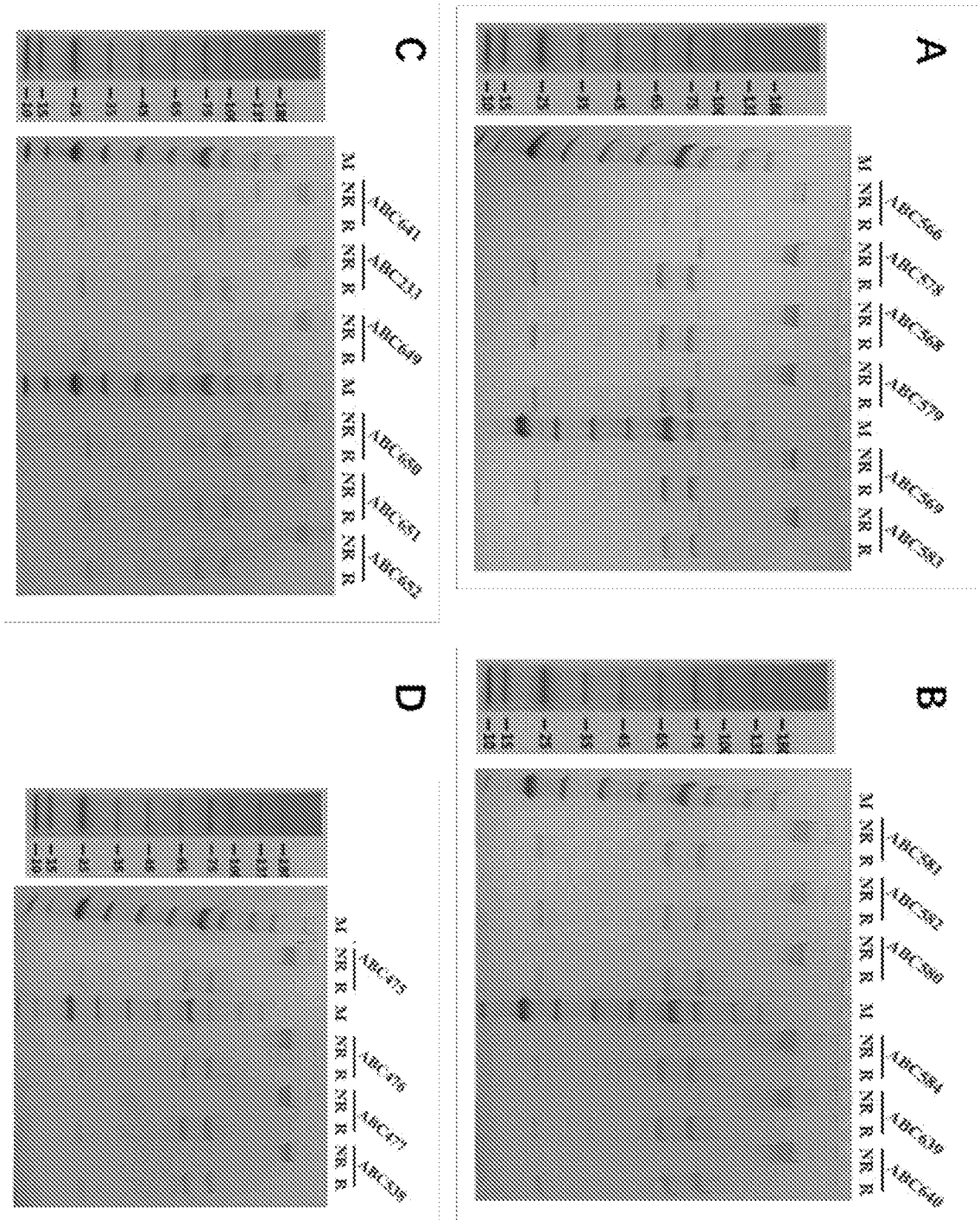


Figure 5

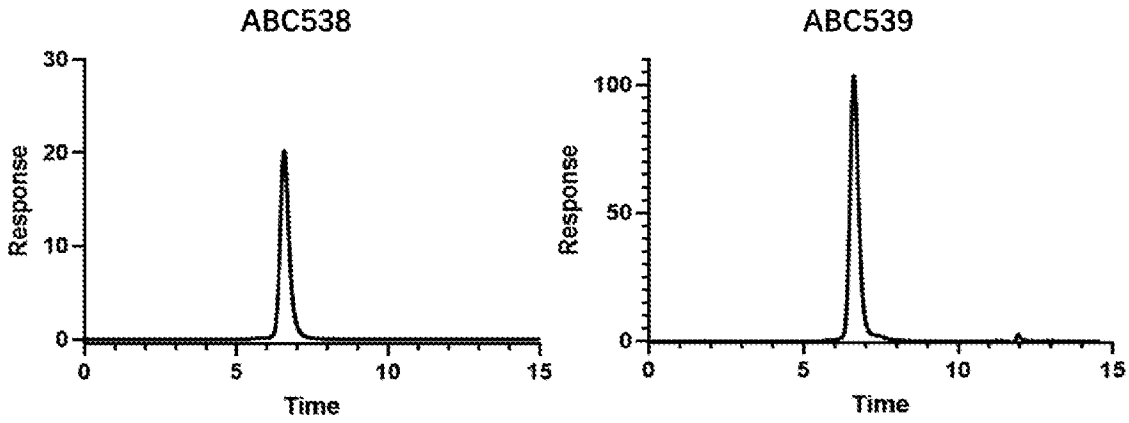


Figure 6

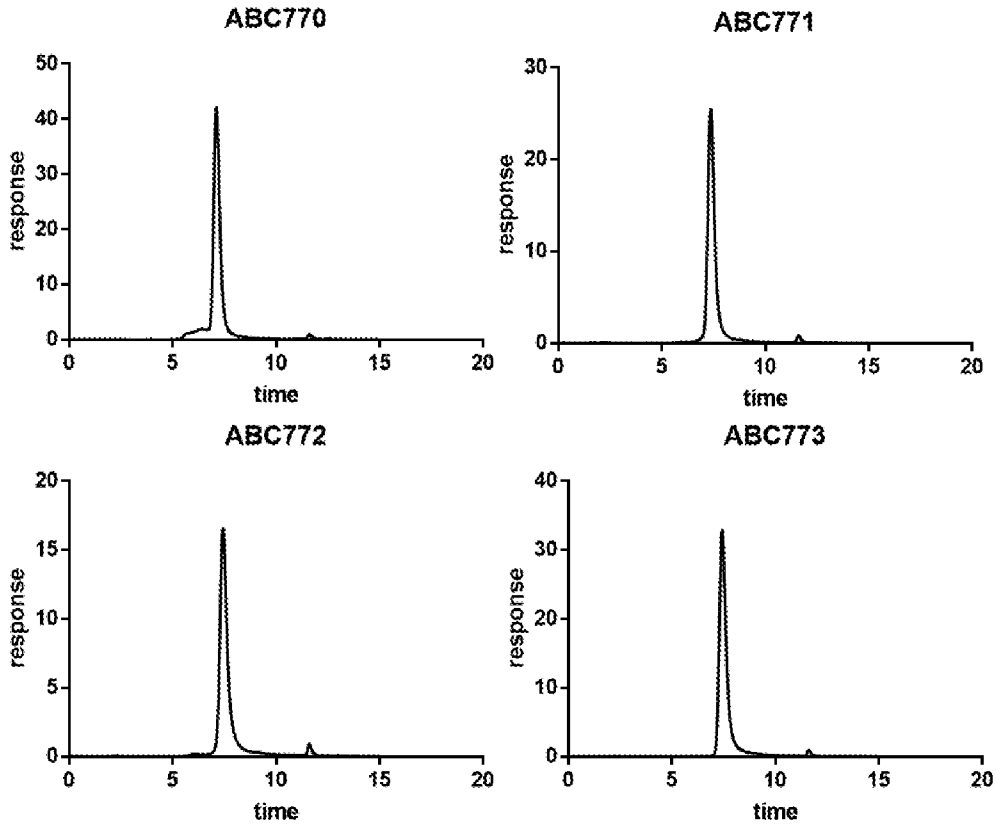
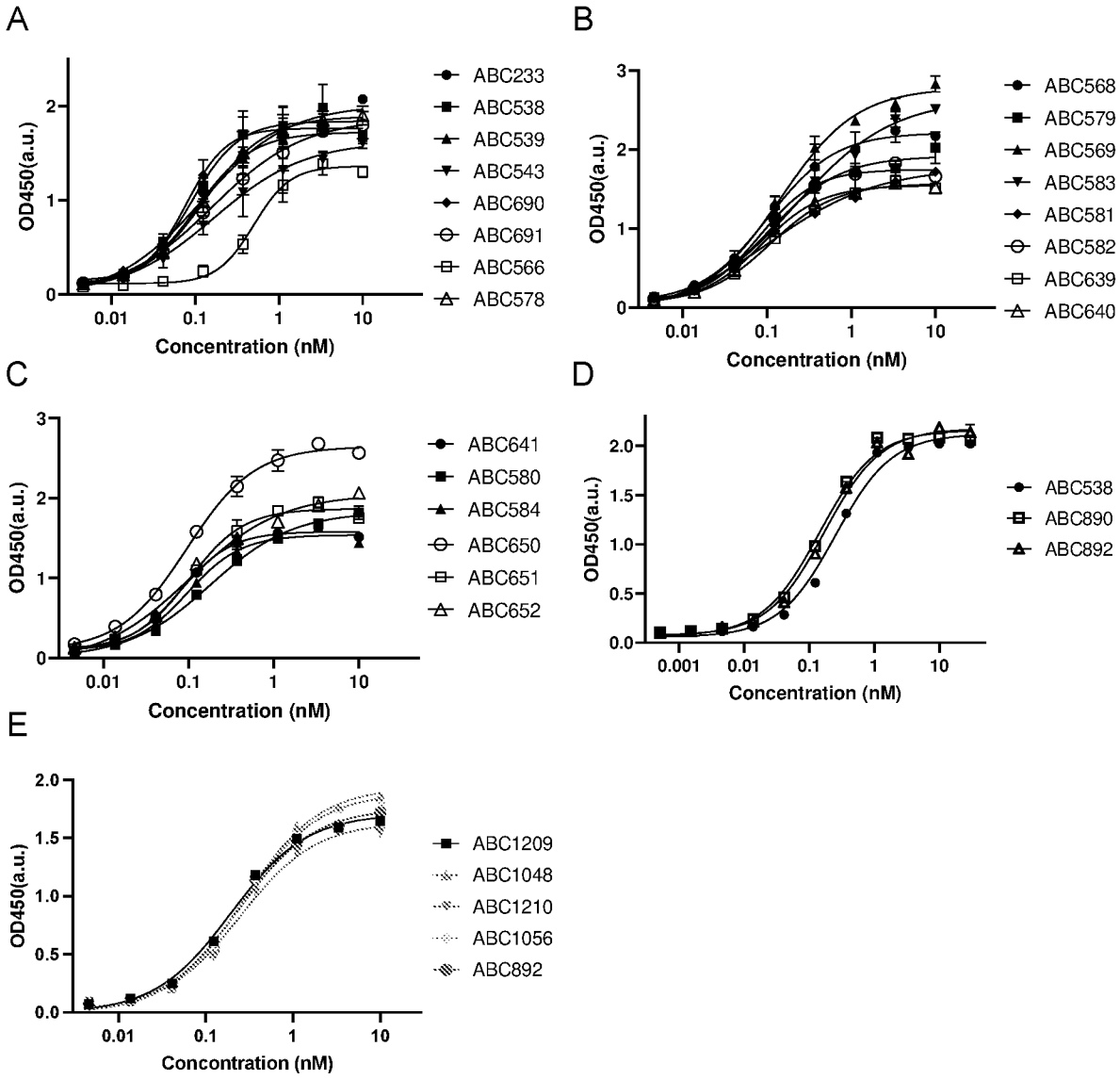


Figure 7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OD450(a.u.)
Concentration (nM)
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ABC539
ABC543
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ABC691
ABC566
ABC578
B
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Concentration (nM)
ABC568
ABC579
ABC569
ABC583
ABC581
ABC582
ABC639
ABC640
C
OD450(a.u.)
Concentration (nM)
ABC641
ABC580
ABC584
ABC650
ABC651
ABC652
D
OD450(a.u.)
Concentration (nM)
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OD450(a.u.)
Concontration (nM)
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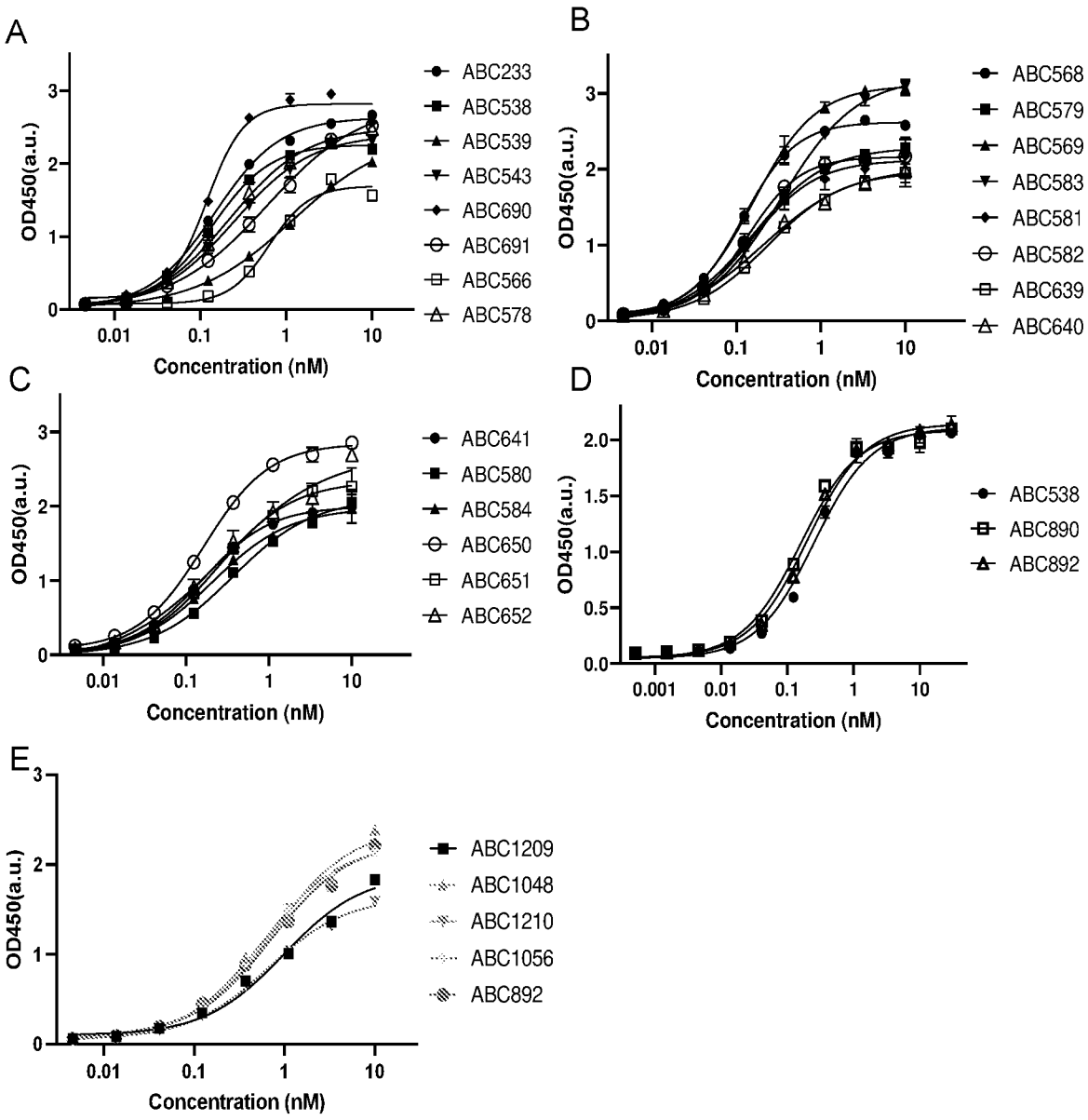
ABC1210
ABC1056
ABC892

Figure 8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Concentration (nM)
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ABC640
C
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Concentration (nM)
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Concentration (nM)
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E
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Concentration (nM)
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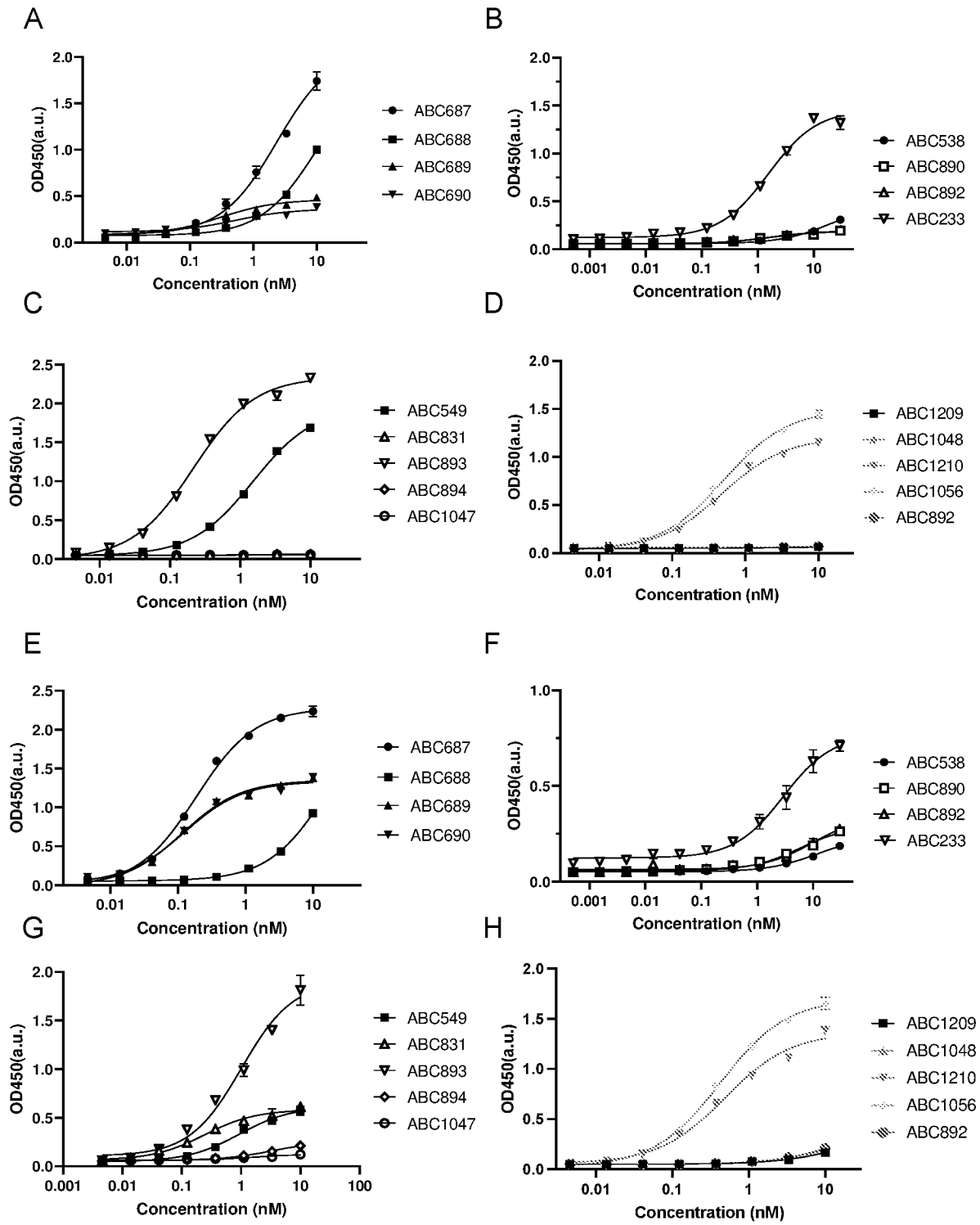
ABC1048
ABC1210
ABC1056
ABC892

Figure 9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Concentration (nM)
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OD450(a.u.)
Concentration (nM)
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Concentration (nM)
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Concentration (nM)
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Concentration (nM)
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ABC892
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Concentration (nM)
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Concentration (nM)
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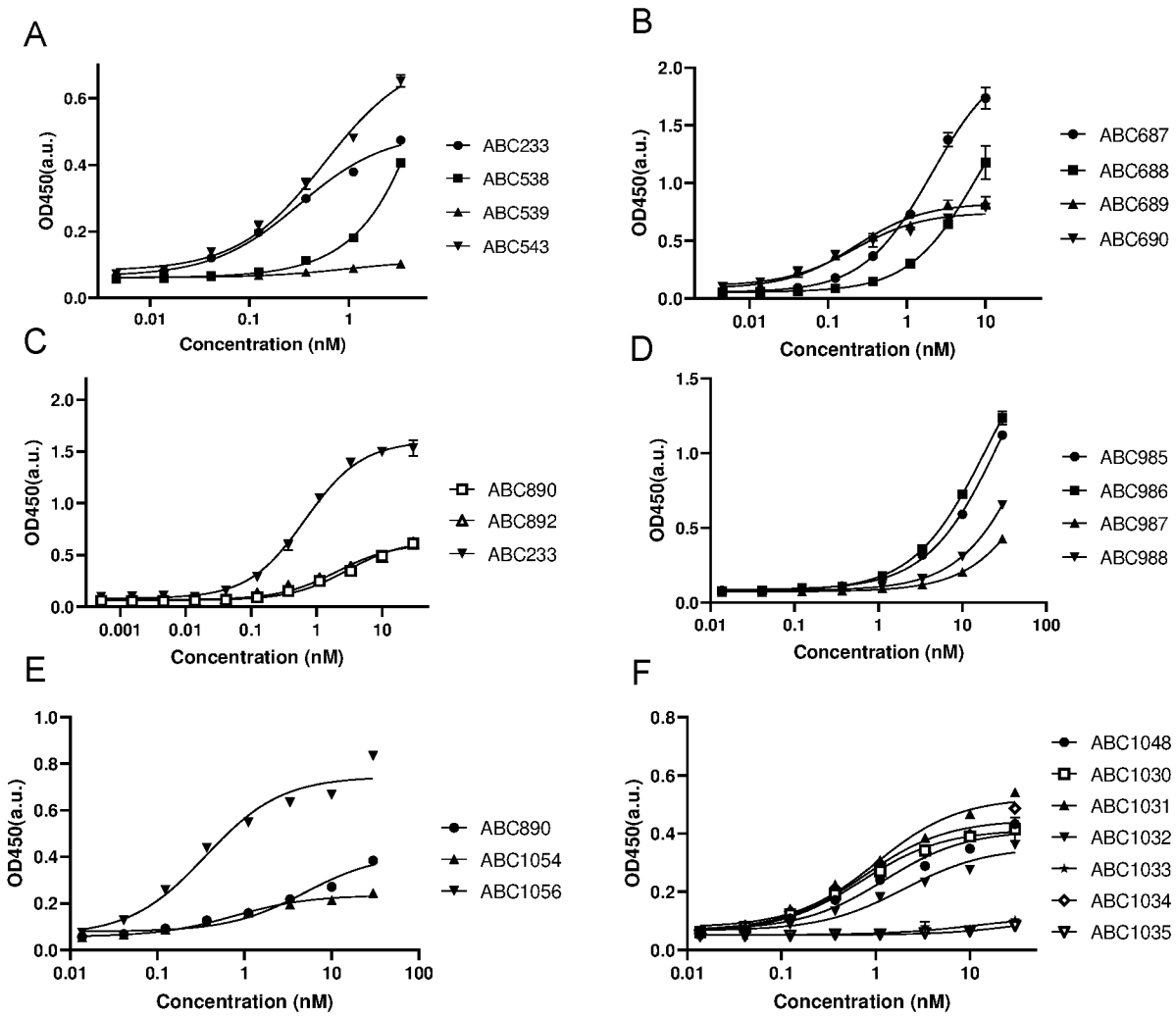
ABC1048
ABC1210
ABC1056
ABC892

Figure 10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ABC1056
F
OD450(a.u.)
Concentration (nM)
ABC1048
ABC1030
ABC1031
ABC1032
ABC1033
ABC1034
ABC1035

Figure 11

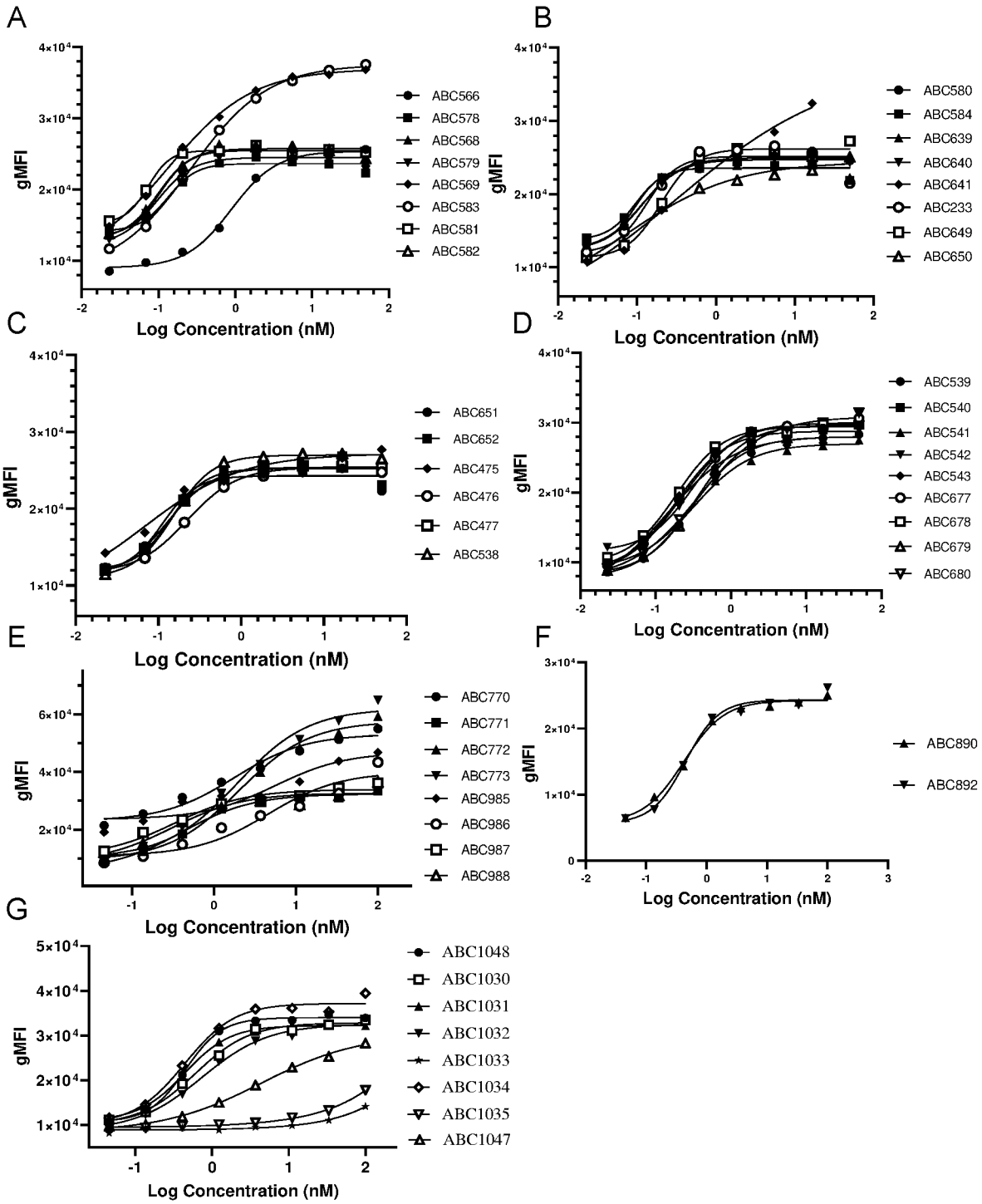

A
gMFI
Log Concentration (nM)
ABC566
ABC578
ABC568
ABC579
ABC569
ABC583
ABC581
ABC582
B
gMFI
Log Concentration (nM)
ABC580
ABC584
ABC639
ABC640
ABC641
ABC233
ABC649
ABC650
C
gMFI
Log Concentration (nM)
ABC651
ABC652
ABC475
ABC476
ABC477
ABC538
D
gMFI
Log Concentration (nM)
ABC539
ABC540
ABC541
ABC542
ABC543
ABC677
ABC678
ABC679
ABC680
E
gMFI
Log Concentration (nM)
ABC770
ABC771
ABC772
ABC773
ABC985
ABC986
ABC987
ABC988
F
gMFI
Log Concentration (nM)
ABC890
ABC892
G
gMFI
Log Concentration (nM)
ABC1048
ABC1030
ABC1031
ABC1032
ABC1033
ABC1034
ABC1035
ABC1047

Figure 12

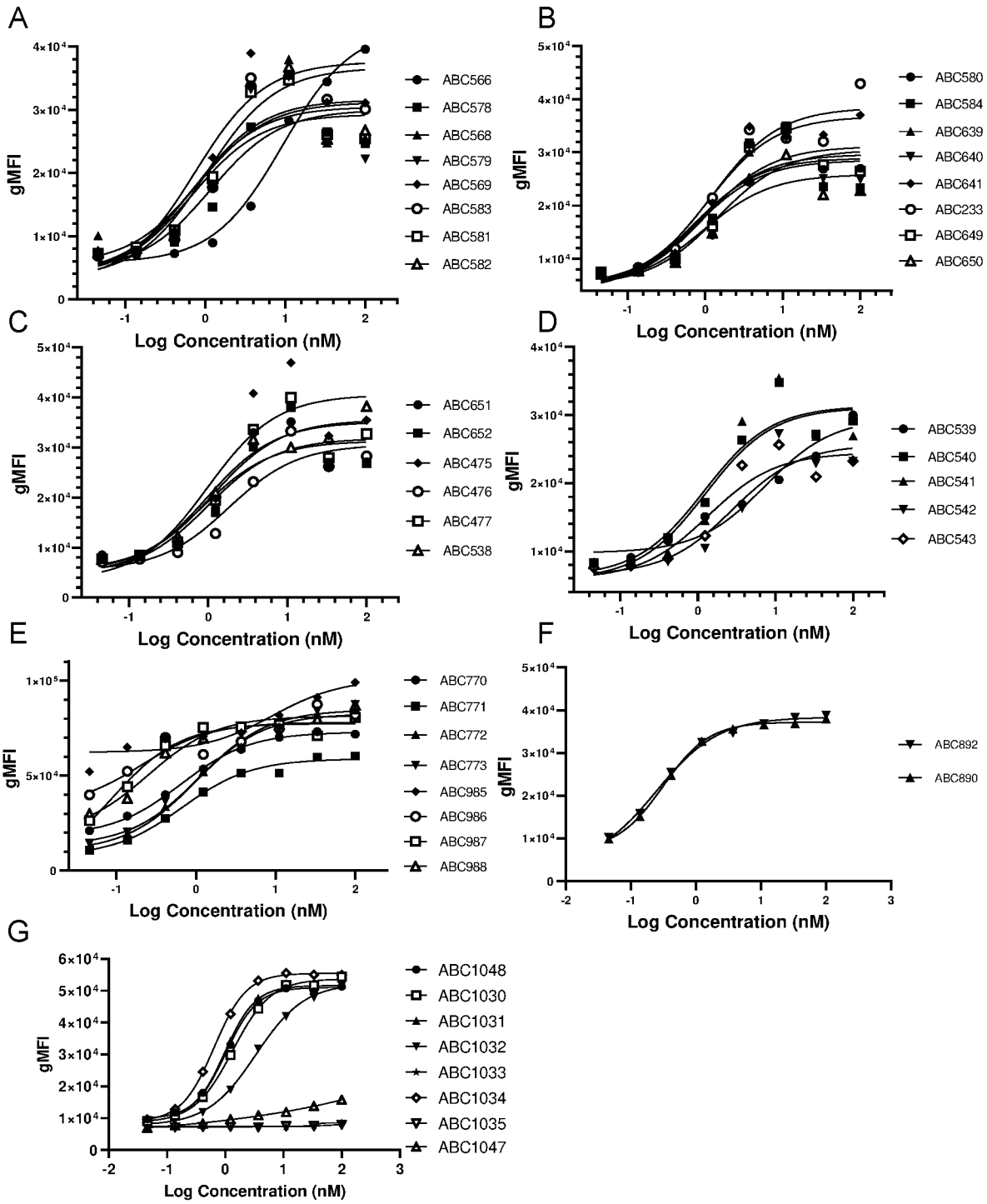

A
gMFI
Log Concentration (nM)
ABC566
ABC578
ABC568
ABC579
ABC569
ABC583
ABC581
ABC582
B
gMFI
Log Concentration (nM)
ABC580
ABC584
ABC639
ABC640
ABC641
ABC233
ABC649
ABC650
C
gMFI
Log Concentration (nM)
ABC651
ABC652
ABC475
ABC476
ABC477
ABC538
D
gMFI
Log Concentration (nM)
ABC539
ABC540
ABC541
ABC542
ABC543
E
gMFI
Log Concentration (nM)
ABC770
ABC771
ABC772
ABC773
ABC985
ABC986
ABC987
ABC988
F
gMFI
Log Concentration (nM)
ABC892
ABC890
G
gMFI
Log Concentration (nM)
ABC1048
ABC1030
ABC1031
ABC1032
ABC1033
ABC1034
ABC1035
ABC1047

Figure 13

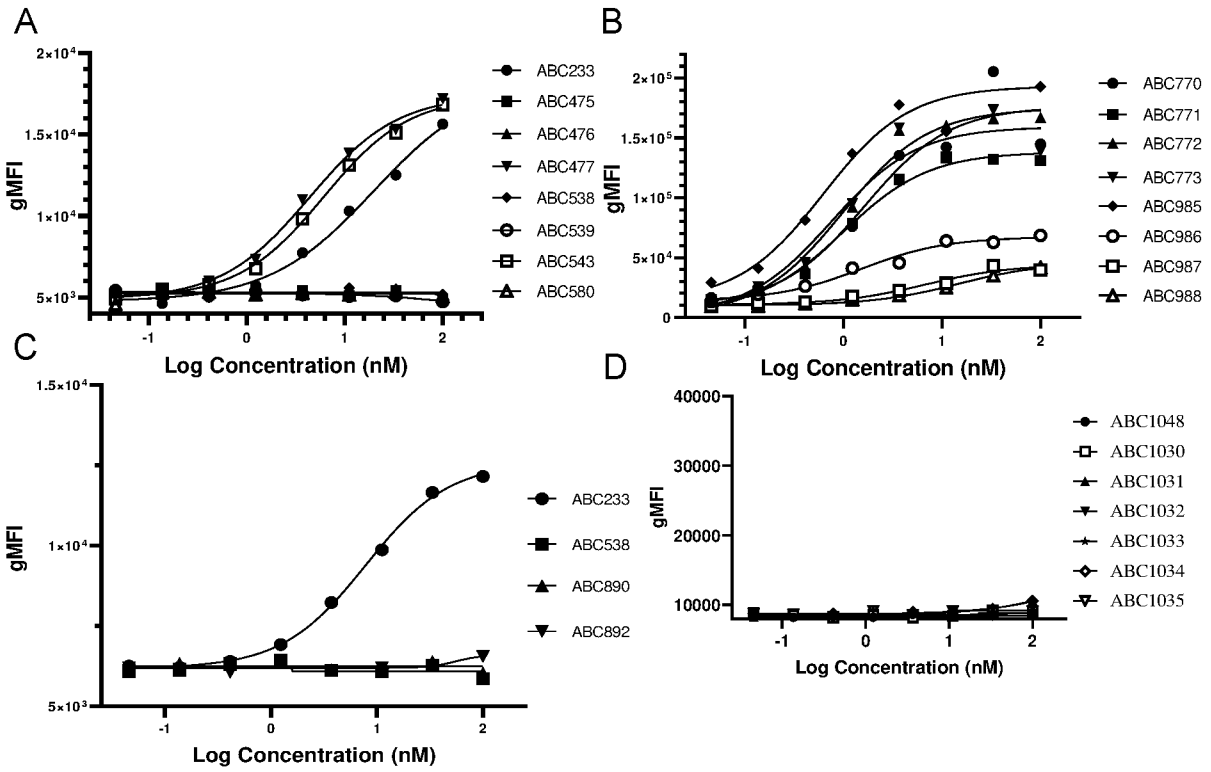


Figure 14

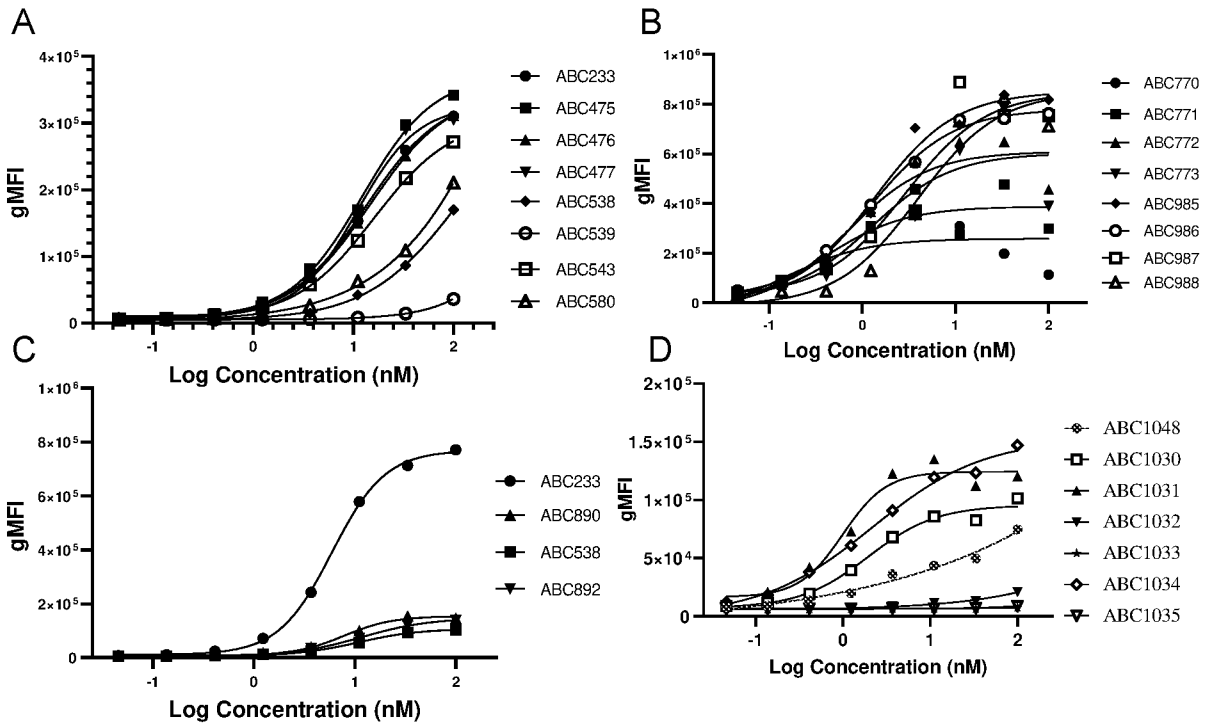


Figure 15

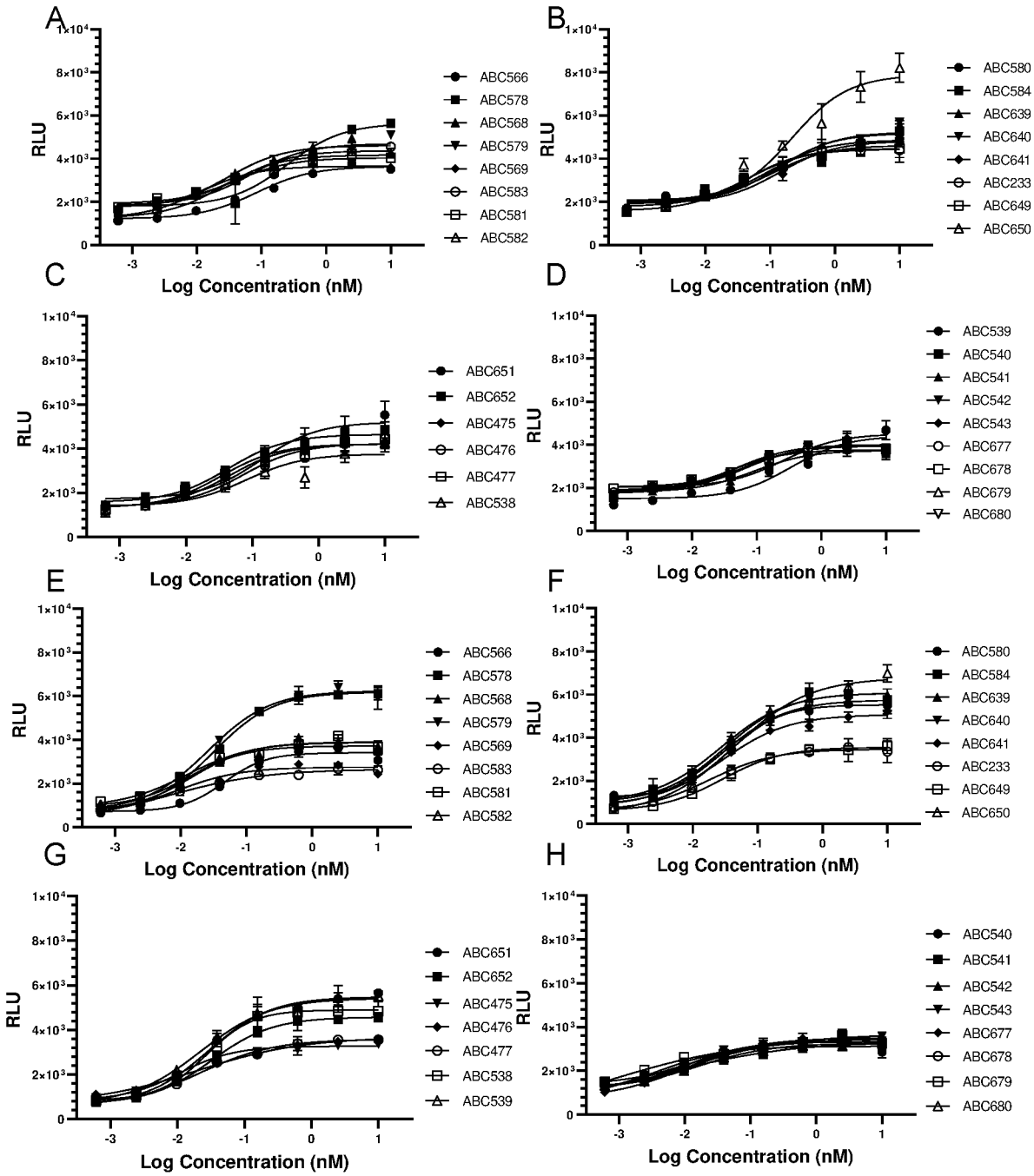

A
B
C
D
E
F
G
H
RLU
Log Concentration (nM)
ABC566
ABC578
ABC568
ABC579
ABC569
ABC583
ABC581
ABC582
ABC580
ABC584
ABC639
ABC640
ABC641
ABC233
ABC649
ABC650
ABC651
ABC652
ABC475
ABC476
ABC477
ABC538
ABC539
ABC540
ABC541
ABC542
ABC543
ABC677
ABC678
ABC679
ABC680

Figure 16

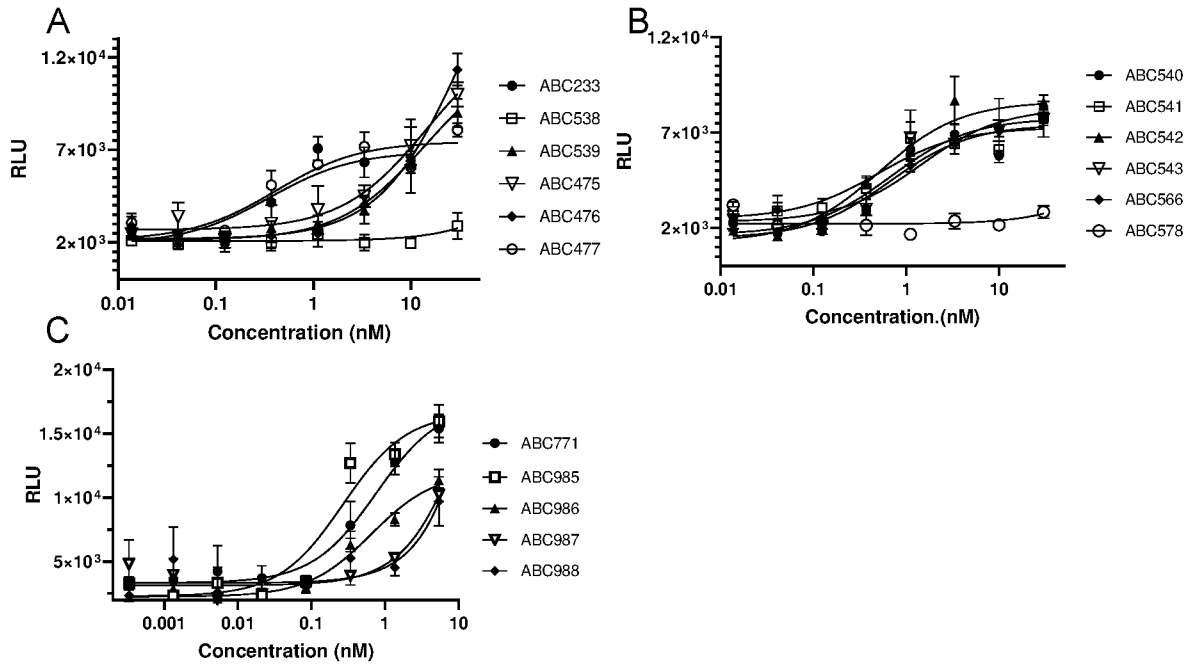


Figure 17

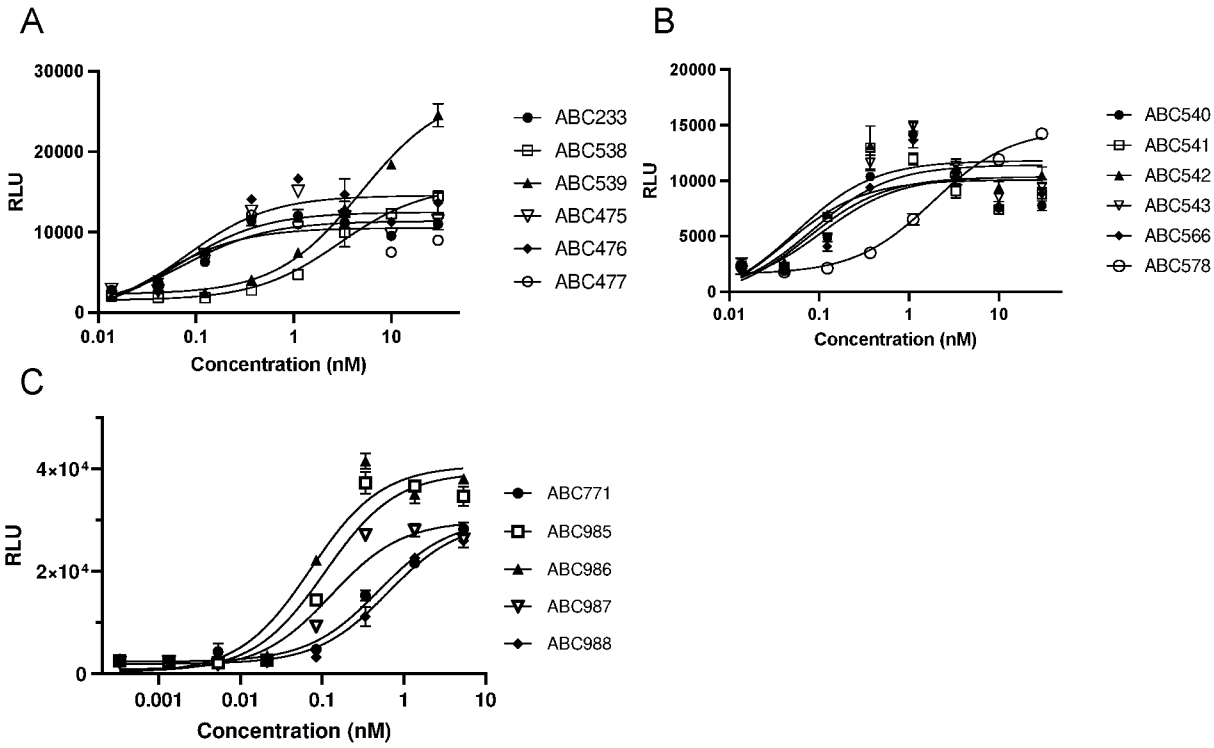


Figure 18

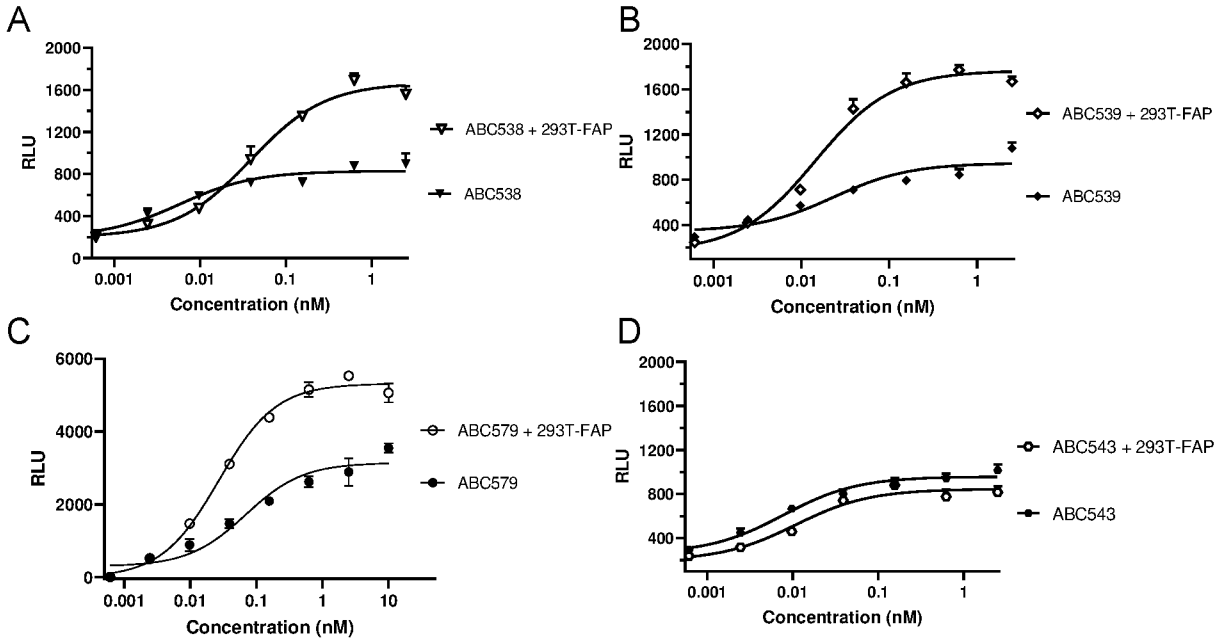


Figure 19

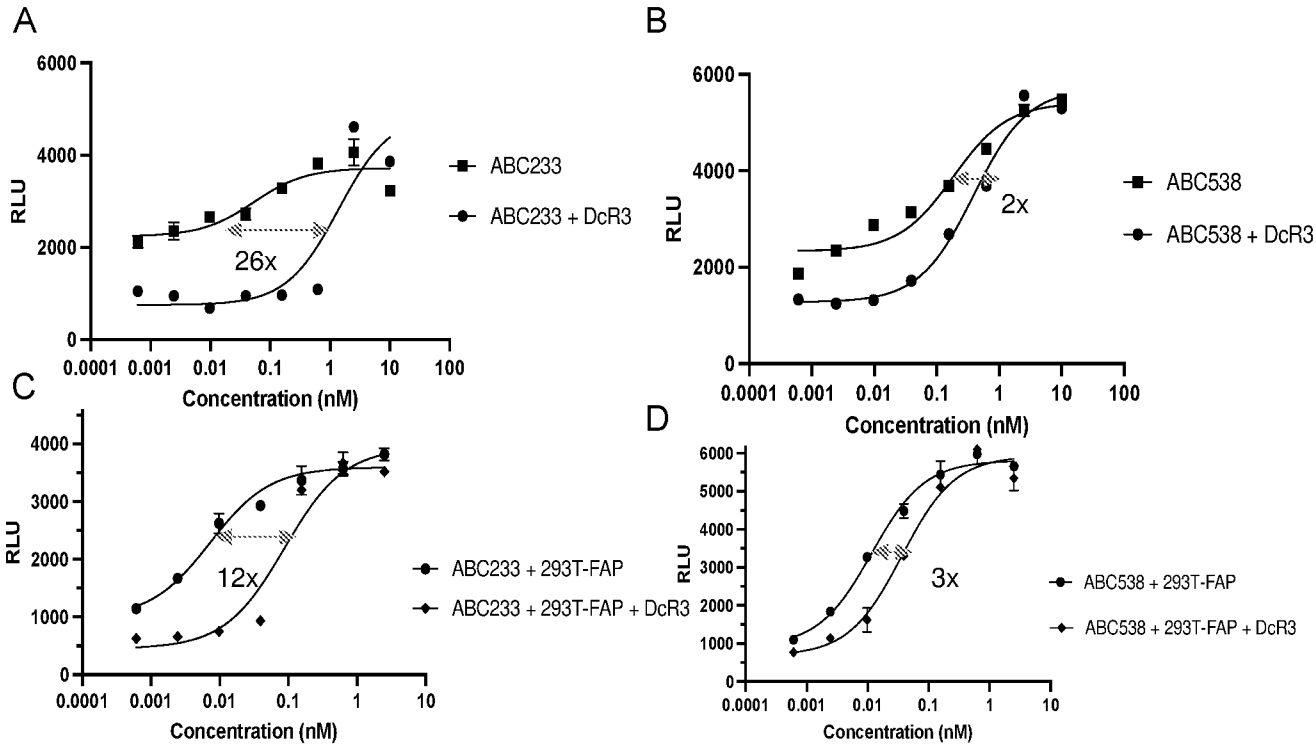
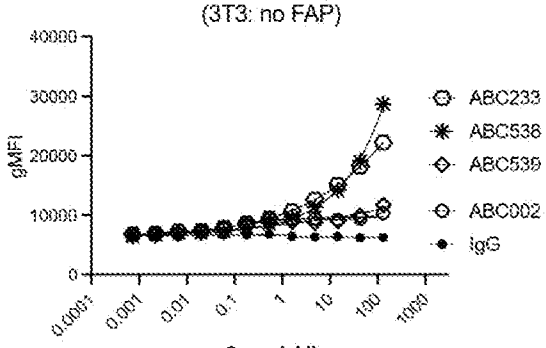


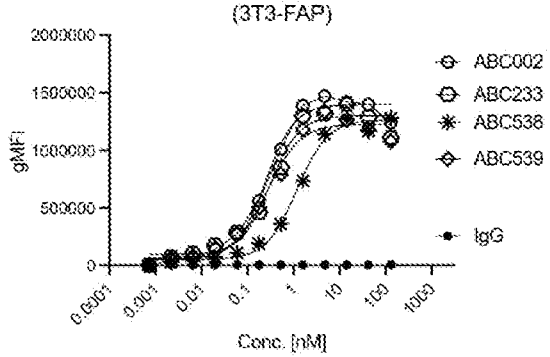
Figure 20

21A



	ABC233	ABC538	ABC539
EC50	1319	~ 9966737150	~ 6.566e+023

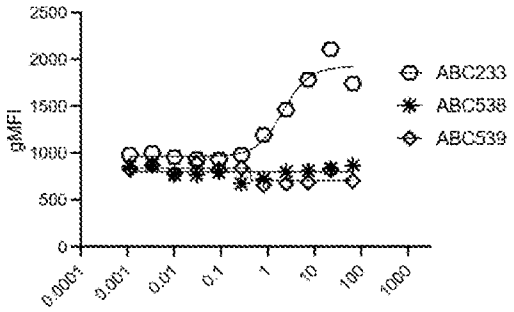
21B



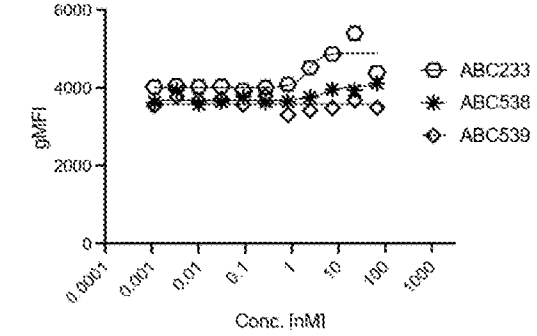
	ABC233	ABC538	ABC539
EC50	0.3081	1.195	0.2699

Figure 21

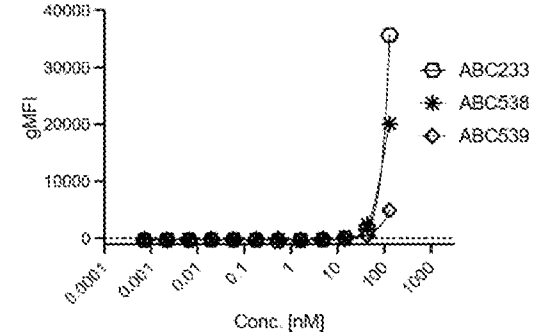
22A



22B



22C



22D

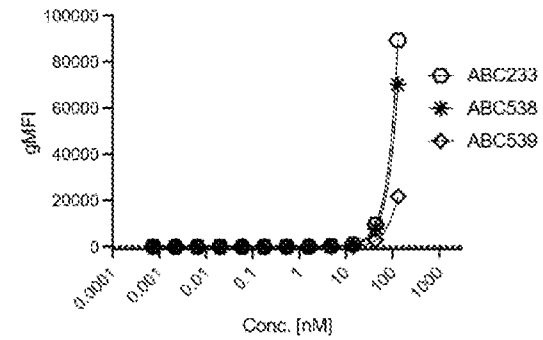


Figure 22

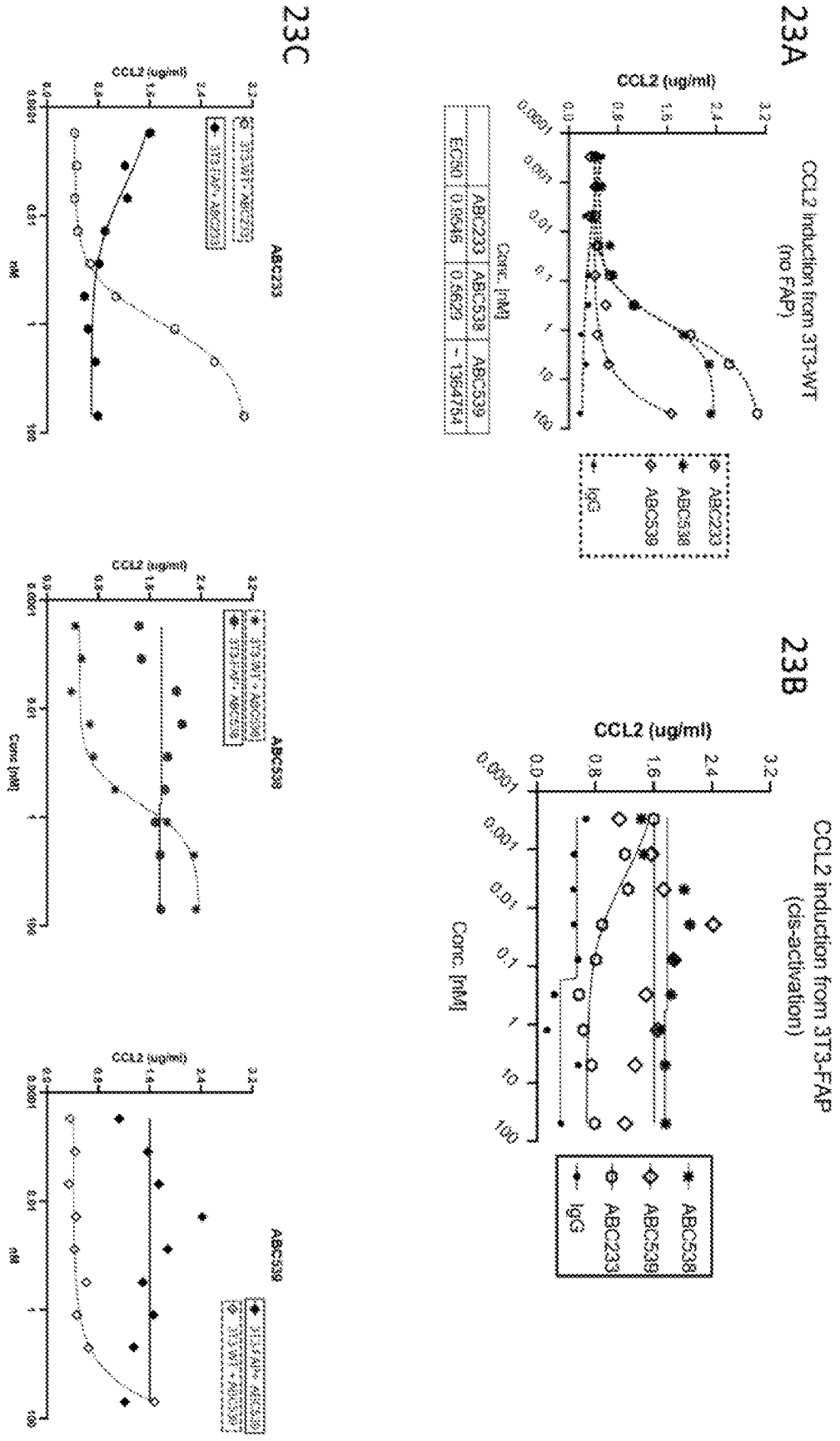

23A
CCL2 induction from 3T3-WT (no FAP)
CCL2 (ug/ml)
Conc. [nM]
ABC233
ABC538
ABC539
IgG
ABC233 ABC538 ABC539
EC50 0.8646 0.5623 -1364754
23B
CCL2 induction from 3T3-FAP (cis-activation)
CCL2 (ug/ml)
Conc. [nM]
ABC538
ABC539
ABC233
IgG
23C
ABC233
ABC538
ABC539
CCL2 (ug/ml)
Conc. [nM]

Figure 23

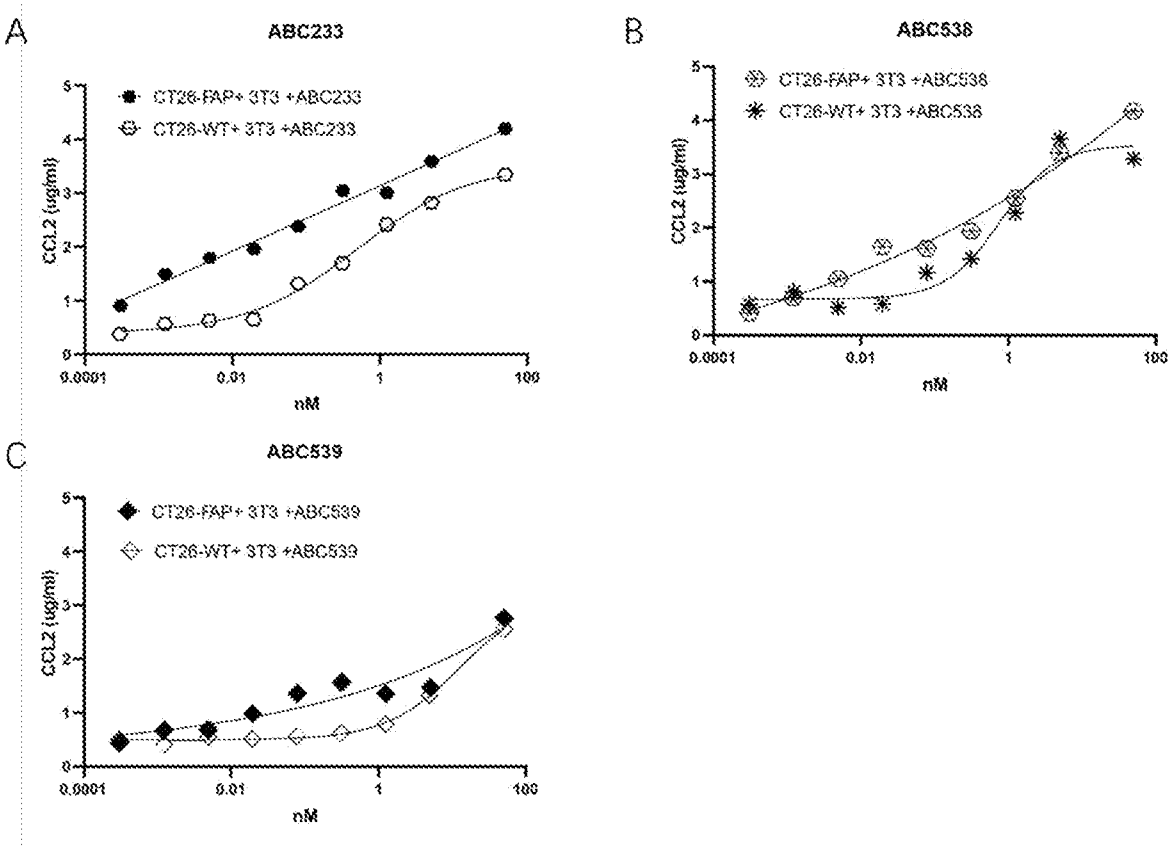

A
ABC233
CT26-FAP+ 3T3 +ABC233
CT26-WT+ 3T3 +ABC233
CCL2 (ug/ml)
nM
B
ABC538
CT26-FAP+ 3T3 +ABC538
CT26-WT+ 3T3 +ABC538
CCL2 (ug/ml)
nM
C
ABC539
CT26-FAP+ 3T3 +ABC539
CT26-WT+ 3T3 +ABC539
CCL2 (ug/ml)
nM

Figure 24

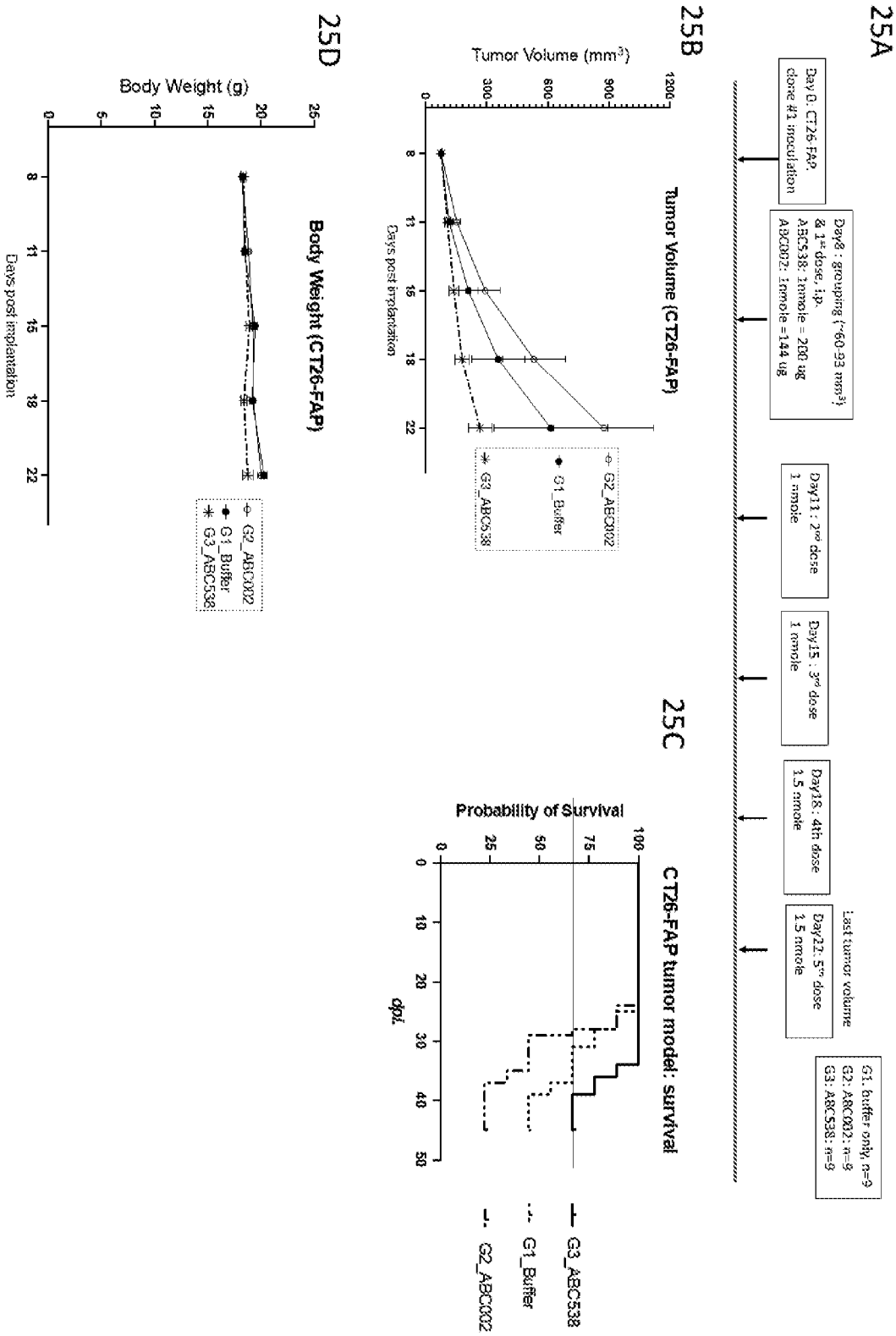

25A
Day 0: CT26-FAP clone #1 inoculation
Day8 : grouping (~60-93 mm³) & 1st dose, i.p.
A5C538: 1mmole = 200 ug
ABC002: 1mmole =144 ug
Day11 : 2nd dose 1 mmole
Day15 : 3rd dose 1 mmole
Day18 : 4th dose 1.5 mmole
Day22: 5th dose 1.5 mmole
Last tumor volume
G1: buffer only, n=9
G2: ABC002: n=9
G3: ABC538: n=9
25B
Tumor Volume (CT26-FAP)
Tumor Volume (mm³)
Days post implantation
G2_ABC002
G1_Buffer
G3_ABC538
25C
CT26-FAP tumor model: survival
Probability of Survival
dpi
G3_ABC538
G1_Buffer
G2_ABC002
25D
Body Weight (CT26-FAP)
Body Weight (g)
Days post implantation
G2_ABC002
G1_Buffer
G3_ABC538

Figure 25

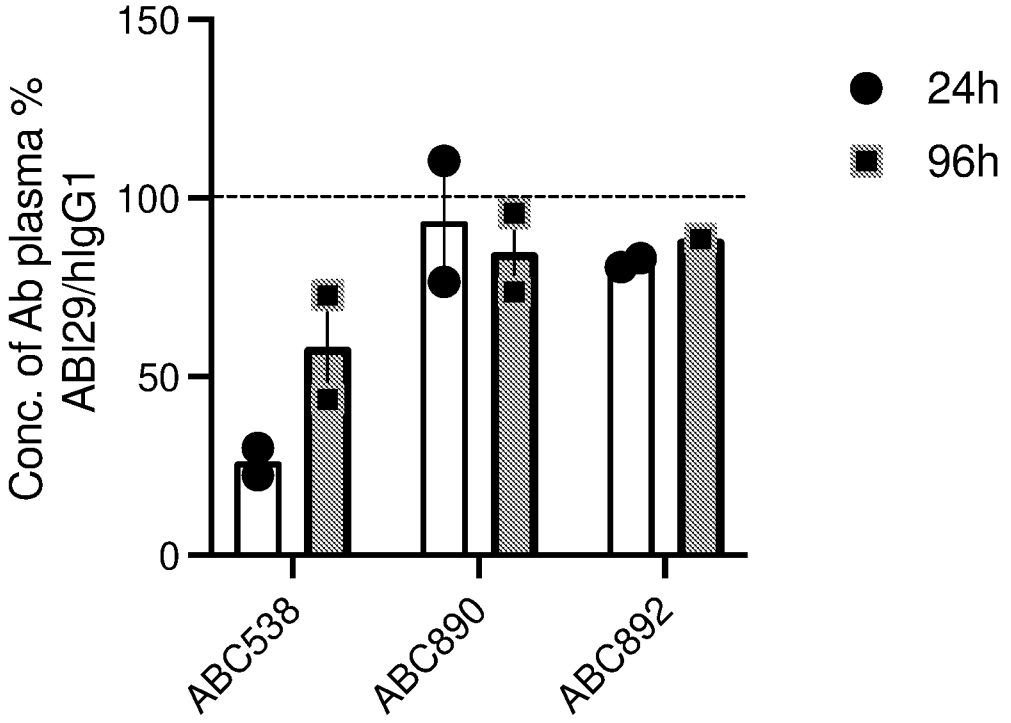

150
100
50
0
Conc. of Ab plasma %
ABI29/hIgG1
24h
96h
ABC538
ABC890
ABC892

Figure 26

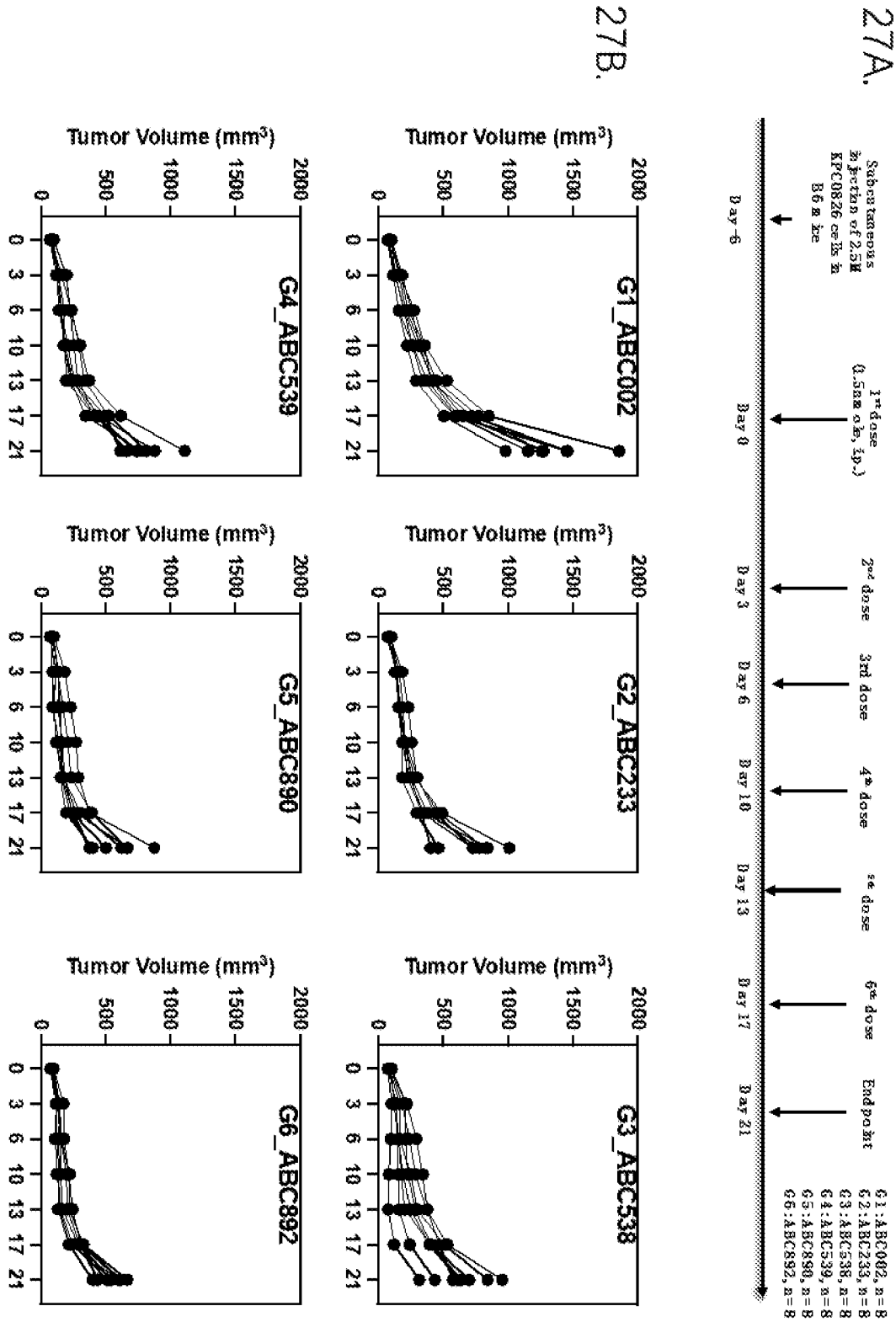


Figure 27A-27B

27C.

27D.

Arms	Times (Days)	TGI
G2_ABC233	21	45.1%
G3_ABC538	21	46.8%
G4_ABC539	21	36.6%
G5_ABC890	21	62.7%
G6_ABC892	21	62.4%

Figure 27C-27D

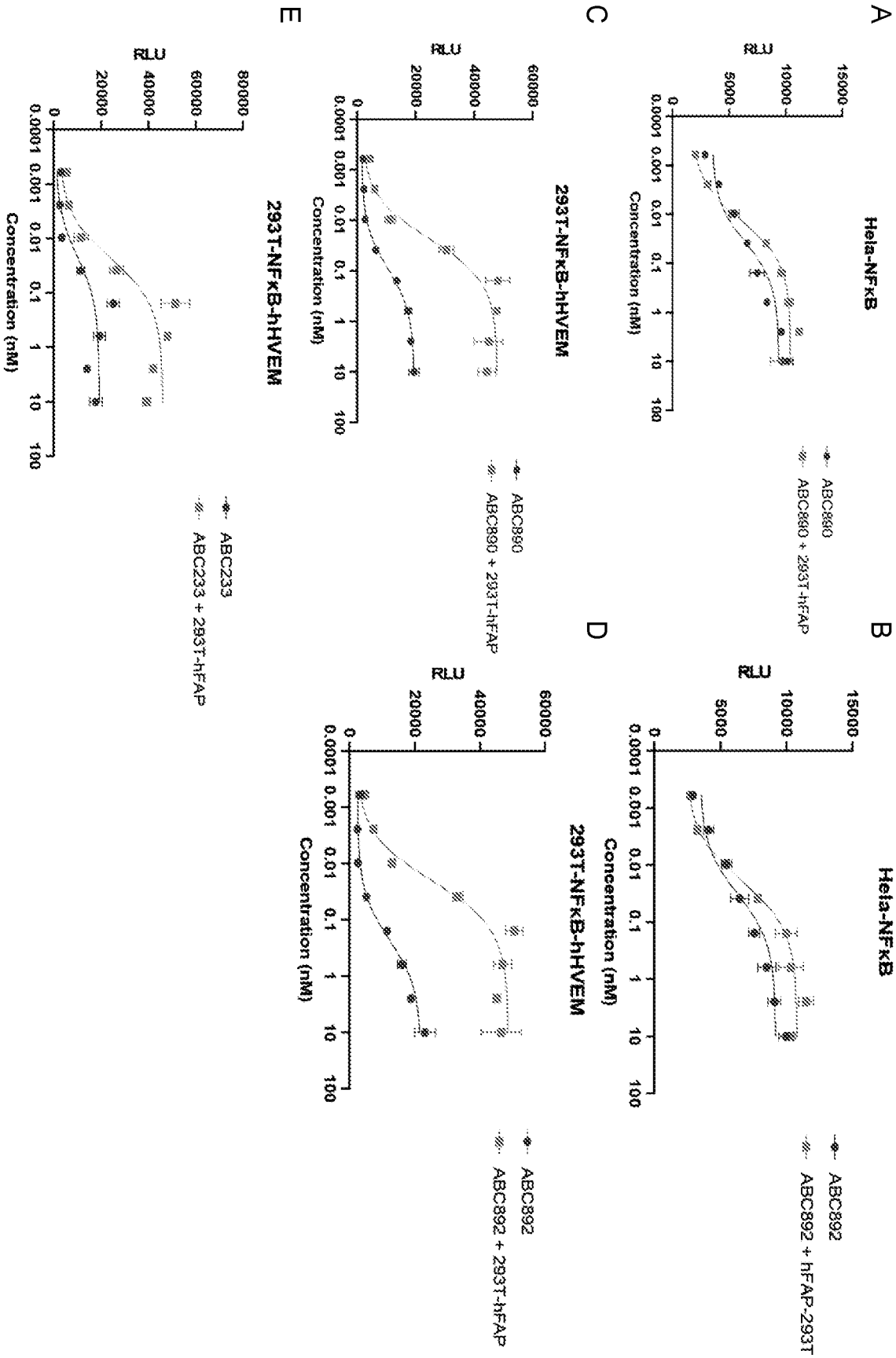

A
Hela-NFκB
RLU
Concentration (nM)
ABC890
ABC890 + 293T-hFAP
B
Hela-NFκB
RLU
Concentration (nM)
ABC892
ABC892 + hFAP-293T
C
293T-NFκB-hHVEM
RLU
Concentration (nM)
ABC890
ABC890 + 293T-hFAP
D
293T-NFκB-hHVEM
RLU
Concentration (nM)
ABC892
ABC892 + 293T-hFAP
E
293T-NFκB-hHVEM
RLU
Concentration (nM)
ABC233
ABC233 + 293T-hFAP

Figure 28

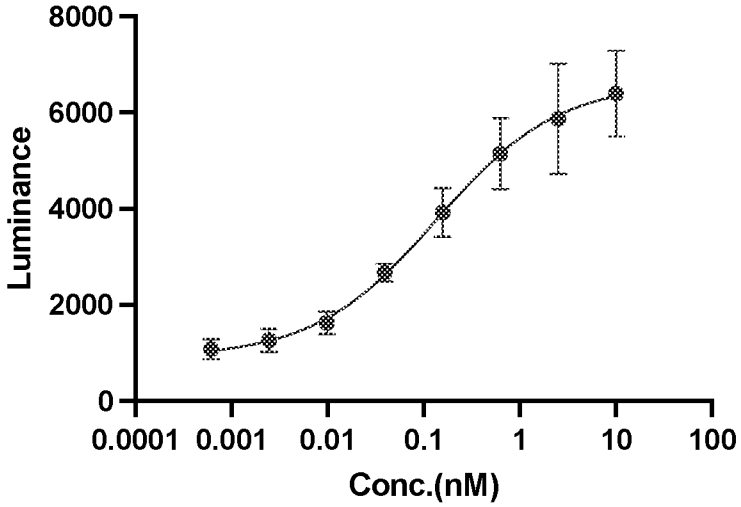


Figure 29

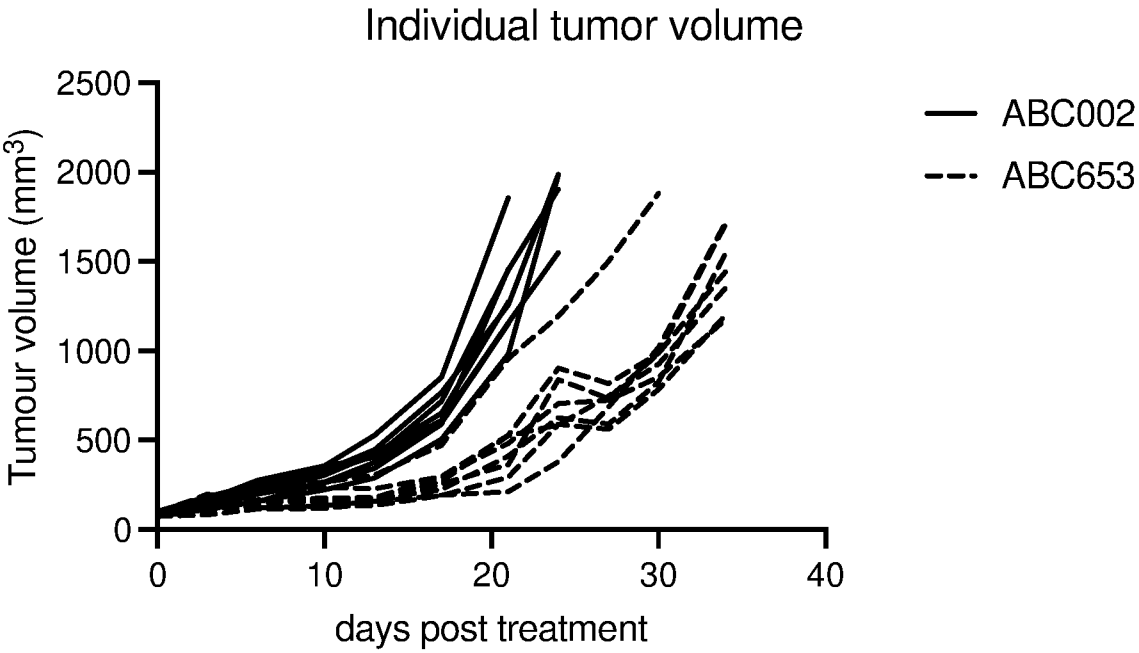


Figure 30

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CN2024/083357

A. CLASSIFICATION OF SUBJECT MATTER

C07K 19/00(2006.01)i; C12N15/62(2006.01)i; A61K47/68(2017.01)i; A61P35/00(2006.01)i

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)

IPC: C07K,C12N, A61K, A61P

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)

CNTXT, WPABSC, WPABS, ENTXTC, ENTXT, DPWI, VEN, CNKI, WANFANG DATA, Web of Science, Baidu Xueshu, HimmPat, incoPat, Bio-Sequence Database of Chinese Patent, NCBI, EBI, STNext: Applicants, Inventors, antibody, Fc, CH2, CH3, Fab, scFv, fusion protein, cytokine, 4-1BB, CD137, LIGHT, CD258, lymphotoxin, SEQ 1-182

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CN 111655716 A (ALTOR BIOSCIENCE LLC) 11 September 2020 (2020-09-11) description, paragraphs 7-9, 25-28,126-128,207-208, figure 1	1,2,36-48
Y	CN 111655716 A (ALTOR BIOSCIENCE LLC) 11 September 2020 (2020-09-11) description, paragraphs 7-9, 25-28,126-128,207-208, figure 1	3-48
Y	CN 101822840 A (FU, Y.X.) 08 September 2010 (2010-09-08) claims 1-5, 7-14 and 16, description, paragraphs 9-25 and 28-4	3-48
Y	WO 2022189377 A1 (F.HOFF-MANN-LA ROCHE AG) 15 September 2022 (2022-09-15) description, paragraphs 8-10, 14-16	3-48
Y	WO 2020135804 A1 (SHANGHAI YICHEN BIOMED CO. LTD) 02 July 2020 (2020-07-02) description, page 3 paragraph 1 to page 4 paragraph 1	8-48
Y	CN 103748114 A (ROCHE GLYCART AG) 23 April 2014 (2014-04-23) description, paragraphs 11-15, sequence 55 and sequence 59	22-48

☑ Further documents are listed in the continuation of Box C. ☑ 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
"D" document cited by the applicant in the international application
"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
18 June 2024	**25 June 2024**
Name and mailing address of the ISA/CN **CHINA NATIONAL INTELLECTUAL PROPERTY ADMINISTRATION** **6, Xitucheng Rd., Jimen Bridge, Haidian District, Beijing 100088, China**	Authorized officer **LIU,ChunJie** Telephone No. **(+86) 010-53961934**

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CN2024/083357

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	CN 107406878 A (ONCOMED PHARMACEUTICALS, INC.) 28 November 2017 (2017-11-28) description, paragraphs 12-13,115-120	1-48
A	ORTIZ-SÁNCHEZ, E. et al. "Antibody-cytokine fusion proteins: applications in cancer therapy" *EXPERT OPIN. BIOL. THER.*, Vol. 8, No. 5, 31 May 2008 (2008-05-31), 609-632 abstract,figure 2	1-48
A	HORNIG, N. et al. "Evaluating combinations of costimulatory antibody–ligand fusion proteins for targeted cancer immunotherapy" *CANCER IMMUNOL IMMUNOTHER.*, Vol. 62, No. 8, 31 August 2013 (2013-08-31), 1369-1380 abstract,figure 1	1-48

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CN2024/083357

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:
   a. ☑ forming part of the international application as filed.
   b. ☐ furnished subsequent to the international filing date for the purposes of international search (Rule 13*ter*.1(a)),
      ☐ accompanied by a statement to the effect that the sequence listing does not go beyond the disclosure in the international application as filed.

2. ☐ With regard to any nucleotide and/or amino acid sequence disclosed in the international application, this report has been established to the extent that a meaningful search could be carried out without a WIPO Standard ST.26 compliant sequence listing.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CN2024/083357

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☑ Claims Nos.: **44-45**
because they relate to subject matter not required to be searched by this Authority, namely:

The subject-matter of claims 44-45 relates to a method of diagnosing, preventing or treating a disease, which belong to the technical solution defined in PCT Rule 39. 1 (iv) that does not require international search units to search. This search assumes that it is amended into a claim for pharmaceutical use, and it is still searched.

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/CN2024/083357

Patent document cited in search report			Publication date (day/month/year)	Patent family member(s)			Publication date (day/month/year)
CN	111655716	A	11 September 2020	US	2022033455	A1	03 February 2022
				US	2024076340	A1	07 March 2024
				AU	2018323455	A1	19 March 2020
				AU	2018323455	B2	03 February 2022
				US	2019300591	A1	03 October 2019
				US	11161890	B2	02 November 2021
				EP	3676289	A1	08 July 2020
				EP	3676289	A4	05 May 2021
				CA	3074635	A1	07 March 2019
				WO	2019046313	A1	07 March 2019
				KR	20200090742	A	29 July 2020
				JP	2020533971	A	26 November 2020
CN	101822840	A	08 September 2010	None			
WO	2022189377	A1	15 September 2022	AU	2022231874	A1	27 July 2023
				BR	112023018117	A2	31 October 2023
				KR	20230156051	A	13 November 2023
				US	2024092856	A1	21 March 2024
				CA	3209640	A1	15 September 2022
				JP	2024508949	A	28 February 2024
				TW	202246358	A	01 December 2022
				IL	304376	A	01 September 2023
				EP	4304723	A1	17 January 2024
WO	2020135804	A1	02 July 2020	None			
CN	103748114	A	23 April 2014	CA	2837975	A1	28 February 2013
				CA	2837975	C	05 April 2022
				US	2018273643	A1	27 September 2018
				PL	3321286	T3	31 May 2021
				TW	201321413	A	01 June 2013
				TWI	633121	B	21 August 2018
				HUE	038225	T2	29 October 2018
				KR	20140034310	A	19 March 2014
				KR	101963923	B1	29 March 2019
				WO	2013026833	A1	28 February 2013
				JP	2018153188	A	04 October 2018
				UA	116192	C2	26 February 2018
				PT	2748201	T	08 February 2018
				ES	2659764	T3	19 March 2018
				RS	56879	B1	30 April 2018
				CR	20130669	A	12 February 2014
				EP	2748201	A1	02 July 2014
				EP	2748201	B1	13 December 2017
				BR	112014003769	A2	26 December 2017
				BR	112014003769	A8	17 April 2018
				BR	112014003769	B1	10 May 2022
				PE	20141521	A1	25 October 2014
				JP	2014529402	A	13 November 2014
				JP	6339015	B2	06 June 2018
				AU	2012298537	A1	12 December 2013
				AU	2012298537	A8	09 January 2014

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/CN2024/083357

Patent document cited in search report	Publication date (day/month/year)	Patent family member(s)			Publication date (day/month/year)
		AU	2012298537	B2	10 August 2017
		CO	6811817	A2	16 December 2013
		DK	2748201	T3	12 February 2018
		NO	2748201	T3	12 May 2018
		AR	087608	A1	03 April 2014
		IL	229765	A0	30 January 2014
		IL	229765	B	26 September 2019
		ECSP	14013220	A	31 March 2015
		EA	201400254	A1	29 August 2014
		EA	030147	B1	29 June 2018
		US	2013078249	A1	28 March 2013
		US	2023340160	A1	26 October 2023
		SI	2748201	T1	30 March 2018
		ZA	201309742	B	28 August 2019
		MX	2014002099	A	28 May 2014
		MX	352101	B	08 November 2017
		CL	2014000126	A1	01 August 2014
		EP	3892640	A1	13 October 2021
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