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(71) Applicants: **PROMAB BIOTECHNOLOGIES, INC.** [US/US]; 2600 Hilltop Drive, Suite 320, Richmond, California 94806 (US). **FOREVERTEK BIOTECHNOLOGY CO., LTD** [CN/CN]; Room A7245, 7 Floor, No.9 Building, CEC Software Park Phase I, No.39 Jianshan Road, Changsha Hi-tech Industrial Development Zone, Changsha, Hunan 410205 (CN).

(72) Inventors: **WU, Lijun**; c/o Promab Biotechnologies, Inc., 2600 Hilltop Drive, Suite 320, Richmond, California 94806 (US). **GOLUBOVSKAYA, Vita**; c/o Promab Biotechnologies, Inc., 2600 Hilltop Drive, Suite 320, Richmond, California 94806 (US).

(74) Agent: **KUNG, Viola T.** et al.; P.O. Box 1247, Seattle, Washington 98111-1247 (US).

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(54) Title: PLAP-CD3 EPSILON BISPECIFIC ANTIBODIES

(57) Abstract: The present invention is directed to bispecific humanized PLAP (placental alkaline phosphatase)-CD3 epsilon chain (CD3e) antibodies. The present invention is further directed to a method for treating PLAP-positive cancer cells by administering the bispecific PLAP-CD3e antibody to the patients.

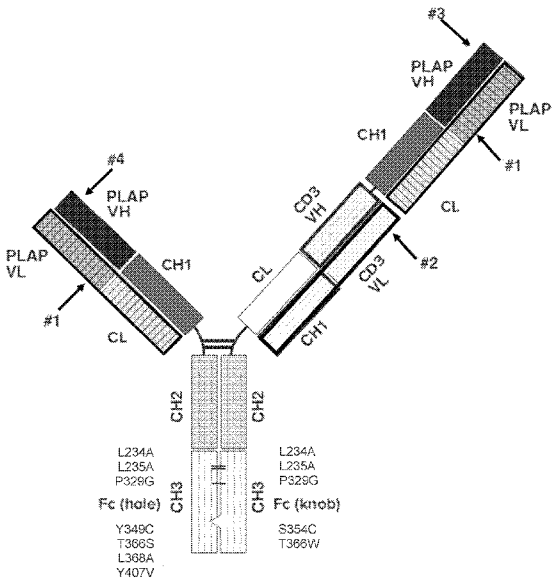

FIG. 1A

[Continued on next page]

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PLAP-CD3 EPSILON BISPECIFIC ANTIBODIES

REFERENCE TO SEQUENCE LISTING, TABLE OR COMPUTER PROGRAM

The Sequence Listing is concurrently submitted herewith with the specification as an ASCII formatted text file via EFS-Web with a file name of Sequence Listing.txt with a creation date of January 14, 2021, and a size of 72.1 kilobytes. The Sequence Listing filed via EFS-Web is part of the specification and is hereby incorporated in its entirety by reference herein.

FIELD OF THE INVENTION

The present invention relates to PLAP (placental alkaline phosphatase)-CD3 epsilon chain (CD3e) bispecific antibodies. The present invention is also directed to a method for killing PLAP-positive cancer cells by administering PLAP-CD3e bispecific antibody with T cells to the patients.

BACKGROUND OF THE INVENTION

Immunotherapy is emerging as a highly promising approach for the treatment of cancer. T cells or T lymphocytes, the armed forces of our immune system, constantly look for foreign antigens and discriminate abnormal (cancer or infected cells) from normal cells. Using bispecific antibodies binding T cells and tumor associated antigen is the most common approach to design bispecific antibody by bringing cytotoxic T cells to kill cancer cells. Bispecific antibodies can be infused into patients by different routes. The advantage of bispecific antibodies compared with chemotherapy or antibody is that it specifically targets antigen-positive cancer cells and simultaneously activates T cells.

Redirecting the activity of T cells by bispecific antibodies against tumor cells, independently of their TCR specificity, is a potent approach to treat cancer. The concept is based on recognition of a cell surface tumor antigen and simultaneous binding to the CD3 epsilon chain (CD3e) within the T-cell receptor (TCR) complex on T cells. This triggers T-cell activation, including release of cytotoxic molecules, cytokines and chemokines, and induction of T-cell proliferation.

PLAP

PLAP is a placental alkaline phosphatase that is encoded by *ALPP* gene. PLAP is a metalloenzyme enzyme that catalyzes the hydrolysis of phosphoric acid monoesters. PLAP is

expressed mainly in placental and endometrial tissues, it is not expressed in normal tissues.

PLAP has high expression in placenta (1), and it is not expressed in most normal tissues except of testis (2). It was found to be overexpressed in malignant seminoma, teratoma (2), (3), ovarian and cervical carcinoma (3), (4),(5), and colon adenocarcinoma (6). PLAP was detected in lung, pancreas, stomach tumors (7). PLAP was also detected among several other membrane-bound proteins in exosomes of non-small cell lung cancer patients with a potential to be prognostic marker (8).

Human PLAP is a 535 amino-acid glycosylated protein encoded by *ALPP* gene with 1-22 signaling peptide, then extracellular domain (23-506), 513-529 transmembrane domain (sequence is shown below, transmembrane domain is underlined) Uniprot database (www.uniprot.org /uniprot/P05187; NM_001632). Its sequence is shown below (SEQ ID NO: 1).

```
          10         20         30         40         50
MLGPCMLLLL LLLGLRLQLS LGIIPVEEEN PDFWNREAAE ALGAAKKLQP
          60         70         80         90        100
AQTAAKNLII FLGDGMGVST VTAARILKGQ KKDKLGPEIP LAMDRFPYVA
         110        120        130        140        150
LSKTYNVDKH VPDSGATATA YLCGVKGNFQ TIGLSAAARF NQCNTTRGNE
         160        170        180        190        200
VISVMNRAKK AGKSVGVVTT TRVQHASPAG TYAHTVNRNW YSDADVPASA
         210        220        230        240        250
RQEGCQDIAT QLISNMDIDV ILGGGRKYMF PMGTPDPEYP DDYSQGGTRL
         260        270        280        290        300
DGKNLVQEWL AKRQGARYVW NRTELMQASL DPSVTHLMGL FEPGDMKYEI
         310        320        330        340        350
HRDSTLDPSL MEMTEAALRL LSRNPRGFFL FVEGGRIDHG HHESRAYRAL
         360        370        380        390        400
TETIMFDDAI ERAGQLTSEE DTLSLVTADH SHVFSFGGYP LRGSSIFGLA
         410        420        430        440        450
PGKARDRKAY TVLLYGNGPG YVLKDGARPD VTESESGSPE YRQQSAVPLD
         460        470        480        490        500
EETHAGEDVA VFARGPQAHL VHGVQEQTFI AHVMAFAACL EPYTACDLAP
         510        520        530
PAGTTDAAHP GRSVVPALLP LLAGTLLLLE TATAP
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There are four distinct but related alkaline phosphatases: intestinal (encoded by ALPI) (NM_001631); placental (ALPP); placental-like (ALPPL2) (NM_031313) which are all encoded by gene on at chromosome 2 and liver/bone/kidney (ALPL) (tissue-nonspecific) (NM_000478) encoded by gene on chromosome 1.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGs. 1A-1C show the structures of bi-specific humanized PLAP and CD3 antibodies. FIG. 1A shows # 1-4 DNA constructs encoding four polypeptides. FIG. 1B shows # 1-3 DNA constructs encoding 3 polypeptides of bivalent PLAP-CD3 antibody. FIG. 1C shows # 1-3 DNA constructs encoding 3 polypeptides of humanized univalent PLAP-CD3 antibody. The antibody of FIGs. 1A and 1B have two PLAP binding moieties and one CD3 binding moiety. The antibody of FIG. 1C has one PLAP binding moiety and one CD3 binding moiety. The knobs-in-hole structure and silent Fc mutations P329G and leucine to alanine (L234A, L235A or LA-LA) mutations are shown in structures FIGs. 1A and 1B; and LA-LA only for FIG. 1C. The amino acid numbers in CH3 are counted from human IgG1 according to [10].

FIG. 2 shows expression of PLAP-h2-CD3 and PLAP-h4-CD3 antibodies on SDS gel. The supernatant shows higher 206 kDa band at non-reducing conditions (B) and lower molecular bands at reducing conditions (C). A shows molecular weight marker (KDa) with proteins marked in kDa.

FIG. 3 shows purification of PLAP-h2-CD3 antibody. PLAP h2 (chimeric form was used with Fc nucleotide sequence with different codon optimization)-CD3 antibody. A-non-reduced; B-reduced conditions; C-molecular marker, molecular weight is shown in kDa.

FIG. 4 shows binding of PLAP-CD3 antibody with CD3 and PLAP antigens by FACS. Bispecific antibodies used with PLAP-positive and PLAP-negative cell lines. CD3-positive T cells were used for testing binding. Bispecific antibodies had positive binding with both PLAP and Cd3 antigens. PLAP h2 -CD3 antibody is shown, the same was observed for PLAP h4-CD3 antibody (not shown).

FIGs. 5A-5B show real-time cytotoxicity assay. PLAP h2-CD3 bispecific antibody with T cells killed Lovo (PLAP-positive) cells and did not kill HT29 (PLAP-negative) cells. T cells ratio to target cells was 5:1 (E:T).

FIGs. 6A-6B show real-time cytotoxicity assay. PLAP h4-CD3 antibody with T cells killed Lovo (PLAP-positive) cells and did not kill PLAP-negative cells. T cells were used at E:T ratio 5:1 (T to target cells)

FIG. 7 shows that PLAP h2-CD3 antibody plus T cells significantly decreased Lovo xenograft tumor growth. P=0.007 at day 18 versus Mock T cells, Student's t-test.

FIG. 8 shows that bivalent PLAP h4-CD3 Ab PBM0015 (Fig. 1B structure) runs as a single band on SDS gel with Molecular Weight 130 kDa.

FIG. 9 shows that bivalent PLAP h4-CD3 (PBM0015) antibody with T cells caused dose-dependent killing of PLAP-positive cells,

FIG. 10 shows that bivalent humanized PLAPh4-CD3 antibody (PBM0015) with T cells secreted significant level of IFN-gamma with Lovo cells but not with HCT116 cells. Concentration of Ab is expressed in ng/ml.

FIGs. 11A-11D show that univalent PLAP h2-3 (PBM008, FIG. 1C structure) with T cells specifically killed PLAP-positive Lovo cells and secrete IFN-gamma. FIGs. 11A-11B: RTCA was performed with PLAP h2-3 and compared with PLAP h2 and PLAP h4 (FIG. 1A structure). PLAPh2-3 had similar e high activity in Lovo cells and low activity in PLAP-negative cells. FIGs. 11C-11D: PLAP h2-3 had high secretion of IFN-gamma with PLAP-positive Lovo target cells, but not with PLAP-negative HCT116 cells.

DETAILED DESCRIPTION OF THE INVENTION

Definitions

As used herein, “affinity” is the strength of binding of a single molecule to its ligand. Affinity is typically measured and reported by the equilibrium dissociation constant (K_D or Kd), which is used to evaluate and rank order strengths of bimolecular interactions.

As used herein, “bispecific antibody” is an artificial protein that can simultaneously bind to two different types of antigen or different epitopes of the same antigen.

As used herein, “CD3 epsilon (CD3e)” is a polypeptide encoded by the CD3E gene which resides on chromosome 11 in human. CD3-epsilon polypeptide, which together with CD3-gamma, -delta and -zeta, and the T-cell receptor alpha/beta and gamma/delta heterodimers, forms the T cell receptor-CD3 complex. This complex plays an important role in coupling antigen recognition to several intracellular signal-transduction pathways. The CD3 epsilon polypeptide plays an essential role in T-cell development. CD3 epsilon, CD3e, and CD3 are used interchangeably in this application.

As used herein, a "domain" means one region in a polypeptide which is folded into a particular structure independently of other regions.

As used herein, a "single chain variable fragment (scFv)" means a single chain polypeptide derived from an antibody which retains the ability to bind to an antigen. An example of the scFv includes an antibody polypeptide which is formed by a recombinant DNA technique and in which Fv regions of immunoglobulin heavy chain (H chain) and light chain (L chain) fragments are linked via a spacer sequence. Various methods for preparing an scFv are known to a person skilled in the art.

As used herein, a "tumor antigen" means a biological molecule having antigenicity, expression of which causes cancer.

The inventors have discovered that human PLAP is a unique tumor marker. Unlike other tumor markers that are expressed in low levels in normal tissues, human PLAP is not expressed in most normal tissues but only in placenta and testis. Therefore, PLAP-CD3e bispecific antibodies do not react against normal tissues and they are safe and have low toxicity.

The present invention is directed to bispecific antibodies that specifically binds to both human PLAP and human CD3e. The PLAP-CD3e bispecific antibody targets PLAP tumor antigen which is highly overexpressed in many types of cancer such as ovarian, seminoma, and colon cancer. The PLAP-CD3 bispecific antibodies of the present invention have high cytotoxic activity against several colon cancer cell lines. The bispecific antibody activates T cells and re-directs T cells to PLAP-positive cancer cells.

Three bispecific antibody structures of the present invention are shown in FIGs. 1A-1C. FIGs. 1A and 1B shows a heterodimeric antibody that binds with one arm to human CD3e chain expressed on T cells and with two arms to human PLAP expressed on PLAP-positive cancer cells. FIG. 1C shows a heterodimeric antibody that binds with one arm to human CD3e chain and one arm to human PLAP.

Bispecific Antibody Structure of FIG. 1A

The present invention is directed to a bispecific antigen-binding molecule having structure of FIG. 1A. In one aspect, the PLAP antibody is humanized h2, and the bispecific antibody comprises: (a) a first and a second antigen-binding moiety each of which is a humanized Fab molecule capable of specific binding to human PLAP, and each comprises a heavy chain variable region (PALP VH) having the amino acid sequence of SEQ ID NO: 10 and a light chain variable region (PLAP VL) having the amino acid sequence of SEQ ID NO: 5; (b) a third antigen-binding moiety which is a Fab molecule capable of specific binding to human CD3 epsilon, the third antigen-binding moiety comprises a heavy chain variable region (CD3 VH) having the amino acid sequence of SEQ ID NO: 11 and a light chain variable region (CD3 VL) having the amino acid sequence of SEQ ID NO: 7, wherein the third antigen-binding moiety is a crossover Fab molecule, in which the constant regions of the Fab light chain and the Fab heavy chain are exchanged; and (c) an human IgG Fc domain comprising a first subunit and a second subunit capable of stable association; wherein the Fab heavy chain of the third antigen-binding moiety is (i) fused at the N-terminus to the C-terminus of the Fab heavy chain of the first antigen-binding moiety (CH1), and (ii) fused at the C-terminus to the N-terminus of the first subunit of the Fc knob domain, and wherein the second antigen-binding moiety is fused at the C-terminus of the Fab heavy chain (CH1) to the

N-terminus of the second subunit of the Fc hole domain.

In another aspect, the PLAP antibody is humanized h4, and the bispecific antibody comprises: (a) a first and a second antigen-binding moiety each of which is a humanized Fab molecule capable of specific binding to human PLAP, and each comprises a heavy chain variable region (PALP VH) having the amino acid sequence of SEQ ID NO: 19 and a light chain variable region (PLAP VL) having the amino acid sequence of SEQ ID NO: 16; (b) a third antigen-binding moiety which is a Fab molecule capable of specific binding to human CD3 epsilon, the third antigen-binding moiety comprises a heavy chain variable region (CD3 VH) having the amino acid sequence of SEQ ID NO: 11 and a light chain variable region (CD3 VL) having the amino acid sequence of SEQ ID NO: 7, wherein the third antigen-binding moiety is a crossover Fab molecule, in which the constant regions of the Fab light chain and the Fab heavy chain are exchanged; and (c) an human IgG Fc domain comprising a first subunit and a second subunit capable of stable association; wherein the Fab heavy chain of the third antigen-binding moiety is (i) fused at the N-terminus to the C-terminus of the Fab heavy chain of the first antigen-binding moiety (CH1), and (ii) fused at the C-terminus to the N-terminus of the first subunit of the Fc knob domain, and wherein the second antigen-binding moiety is fused at the C-terminus of the Fab heavy chain (CH1) to the N-terminus of the second subunit of the Fc hole domain.

The bispecific antibody of the present invention uses CROSSFAB approach, which crossovers the constant domain and variable domain and switches the CH1 domain and CL domain in the CD3e Fab molecule, which reduces undesired mis-paring.

In one embodiment, the bispecific antibody of the present invention comprises: (1) humanized PLAP light chain, (2) CD3e cross FAB, CD3VL-CH1; (3) humanized PLAP VH-CH1-CD3e CROSSFAB (VH-CL) –Fc (knob), and (4) humanized PLAP VH-CH1- Fc (hole). (FIG. 1A)

In one embodiment, the VH of the humanized PLAP antibody has the amino acid sequence of SEQ ID NO: 10 and the VL has the amino acid sequence of SEQ ID NO: 4.

In another embodiment, the VH of the humanized PLAP antibody has the amino acid sequence of SEQ ID NO: 19 and the VL has the amino acid sequence of SEQ ID NO: 16.

In one embodiment, the Fc domain comprises a modification promoting the association of the first and the second subunit of the Fc domain.

In one embodiment, in the CH3 domain of the first subunit of the Fc domain, an amino acid residue is replaced with an amino acid residue having a larger side chain volume, thereby generating a protuberance within the CH3 domain of the first subunit which fits in a

cavity within the CH3 domain of the second subunit, and in the CH3 domain of the second subunit of the Fc domain an amino acid residue is replaced with an amino acid residue having a smaller side chain volume, thereby generating a cavity within the CH3 domain of the second subunit within which the protuberance within the CH3 domain of the first subunit fits.

In one embodiment, the Fc domain exhibits reduced binding affinity to an Fc receptor and/or reduced effector function, as compared to a native IgG Fc domain.

In one embodiment, the Fc domain comprises one or more amino acid substitution that reduces binding to an Fc receptor and/or effector function. In one embodiment, the one or more amino acid substitution in the Fc domain are selected from the group of L234, L235, and P329 (Kabat numbering). In one embodiment, said amino acid substitutions are L234A, L235A and P329G.

In one embodiment, silent Fc mutations P329G, and L234A and L235A mutations are used to prevent Fc-dependent immune reactions.

In one embodiment, only silent mutations L234A and L235A mutations are used to prevent Fc-dependent immune reactions.

In a specific embodiment, the Fc domain is modified with a so-called "knob-into-hole" modification, comprising a "knob" modification in one of the two subunits of the Fc domain and a "hole" modification in the other one of the two subunits of the Fc domain. The knob-into-hole technology is described e.g. in U.S. Pat. No. 5,731,168. Generally, the method involves introducing a protuberance ("knob") at the interface of a first polypeptide and a corresponding cavity ("hole") in the interface of a second polypeptide, such that the protuberance can be positioned in the cavity to promote heterodimer formation and hinder homodimer formation. Protuberances are constructed by replacing small amino acid side chains from the interface of the first polypeptide with larger side chains (e.g. tyrosine or tryptophan). Compensatory cavities of identical or similar size to the protuberances are created in the interface of the second polypeptide by replacing large amino acid side chains with smaller ones (e.g. alanine or threonine).

In one embodiment, a “knob” is made by mutations of S354C and T366W on one Fc, and the corresponding “hole” is made by mutations of Y349C, T366S, L368A and Y407V on the partner Fc.

In one embodiment, the bispecific antigen-binding molecule comprising two binding moieties to PLAP, and one binding moiety to CD3 epsilon, the molecules comprises the amino acid sequences of SEQ ID NO: 5, 8, 12, and 14, in a molar ratio of 2:1:1:1; optionally each amino acid sequence has at least 95%, 96%, 97%, 98%, or 99% sequence identity

thereof, provided that the sequence variation is in the non-CDR framework regions.

In one embodiment, the bispecific antigen-binding molecule comprising two binding moieties to PLAP, and one binding moiety to CD3 epsilon, the molecules comprises the amino acid sequences of SEQ ID NO: 17, 8, 20, and 22, in a molar ratio of 2:1:1:1; optionally each amino acid sequence has at least 95%, 96%, 97%, 98%, or 99% sequence identity thereof, provided that the sequence variation is in the non-CDR framework regions.

Bispecific Antibody Structure of FIG. 1B

FIG. 1B shows the structure of humanized bivalent bispecific PLAP-CD3e antibody consisting of 3 DNA constructs. This structure comprises two binding moieties to PLAP and one binding moiety to CD3 epsilon.

In one embodiment, the antibody comprises the amino acid sequences of SEQ ID NO: 17, 24, and 22, in a molar ratio of 2:1:1; optionally each amino acid sequence has at least 95%, 96%, 97%, 98%, or 99% sequence identity thereof, provided that the sequence variation is in the non-CDR framework regions.

Bispecific Antibody Structure of FIG. 1C

FIG. 1C shows a bispecific antibody structure of monovalent humanized PLAP and monovalent CD3e; the structure consists of 3 DNA constructs. The structure does not have CD3 CROSS FAB, but is has a CD3e scFv. The bispecific antibody comprises one binding moiety to PLAP, and one binding moiety to CD3 epsilon.

In one embodiment, the bispecific antibody comprises the amino acid sequences of SEQ ID NO: 5, 28, and 30, in a molar ratio of 2:1:1; optionally each amino acid sequence has at least 95%, 96%, 97%, 98%, or 99% sequence identity thereof, provided that the sequence variation is in the non-CDR framework regions.

In another embodiment, the bispecific antibody comprises the amino acid sequences of SEQ ID NO: 17, 28, and 30, in a molar ratio of 2:1:1; optionally each amino acid sequence has at least 95%, 96%, 97%, 98%, or 99% sequence identity thereof, provided that the sequence variation is in the non-CDR framework regions.

The above sequence variations of structures of FIG. 1A-1C, i.e., the amino acid changes are preferably of a minor amino acid change such as a conservative amino acid substitution. A conservative amino acid substitution is well-known to a person skilled in the art.

The present invention is directed to a bispecific antibody method for treating cancer,

comprising the step of administering PLAP-CD3e antibody to a subject suffering from cancer, wherein the cancer is selected from the group consisting of colon cancer, lung cancer, pancreatic cancer, stomach cancer, testicular cancer, teratoma, seminoma, ovarian cancer, and cervical cancer, and the cancer is PLAP-positive.

The present invention is also directed to a pharmaceutical composition comprising the bispecific antigen-binding molecule and a pharmaceutically acceptable carrier.

The nucleic acid encoding the bispecific antibody of the present invention can be inserted into a vector and expressed in mammalian 293S or CHO cells using serum-free medium. The antibody can be purified with protein A or protein G column and used for the study.

This application demonstrates the efficacy of bispecific antibody targeting PLAP antigen that is overexpressed in colon cancer tumors. This application demonstrates that PLAP-CD3e antibody binds CD3e antigen and PLAP antigen. This antibody delivered with T cells specifically decreases viability of PLAP-positive colon cancer cells but not PLAP-negative cancer cells. PLAP-CD3e antibody delivered with T cells caused secretion of significant level of IFN-gamma after co-incubation with PLAP-positive colon cancer cells but not after co-incubation with PLAP-negative cancer cells. This application demonstrates that PLAP-CD3e antibody administered with T cells significantly decreased Lovo (positive PLAP-colon cancer cells) xenograft tumor growth *in vivo*.

The inventors demonstrate that PLAP-CD3 antibody with T cells significantly killed all PLAP-positive cancer cells, but not kill PLAP-negative colon cancers. This implies high specificity of PLAP-CD3 antibody.

The inventors demonstrated high efficacy of three different designs of bispecific antibodies of FIGs. 1A-1C.

The following examples further illustrate the present invention. These examples are intended merely to be illustrative of the present invention and are not to be construed as being limiting.

EXAMPLES

EXAMPLE 1. MATERIALS AND METHODS

Cells and culture medium

HEK293FT cells from *AlStem* (Richmond, CA) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) plus 10% FBS and 1% penicillin/streptomycin. Human peripheral

blood mononuclear cells (PBMC) were isolated from whole blood obtained from the Stanford Hospital Blood Center, Stanford, CA according to IRB-approved protocol using Ficoll-Paque solution (*GE Healthcare*). Colon cancer cell lines: PLAP-negative: HT29, and PLAP-positive: Lovo cells were used for the study. The cells were cultured in a humidified 5% CO_2 (9).

Antibodies

The (APC)-labeled anti-CD3 and secondary antibodies were described in (9).

PLAP-CD3 antibody constructs

The four constructs of Example 2A were designed according to Cross-Fab designed described in (10). The constructs had P329G mutation and Leucine 324,235 changed to alanine, called LA-LA to decrease Fc immune activity. In addition, Fc silent and knobs-in-hole mutations were used for engineering, as described (10). We also expressed three constructs of FIG. 1B and three constructs of FIG. 1C. All constructs for FIG. 1A and FIG. 1B were cloned into Nhe I and Nsi I sites of pYD11 vector.

Expression of PLAP-CD3 antibodies

For structure FIG. 1A, the four antibody constructs were mixed at weight ratio 2 (PLAP VL-CL):1:1:1 (μg/mL) with NanoFect transfection agent and used for 293S cell transformation. For structures 1B and 1C, the three antibody constructs were mixed at weight ratio 1:1:1 (μg/mL) with NanoFect transfection agent and used for 293S cell transformation. The cells were rotated in bottles on shaker in Freestyle F17 medium, containing 8mM L-Glutamine (or GlutaMAX), and 0.1% Pluoronic F-68 for one week at 37°C incubator. The supernatant or purified antibody on protein A column was analyzed on SDS gel, by FACS and functional assays.

PBMC

PBMC were resuspended at 1 x 10^6 cells/ml in AIM V-AlbuMAX medium (*Thermo Fisher*) containing 10% FBS with 300 U/ml IL-2 (*Thermo Fisher*). PBMC cells were activated with CD3/CD28 Dynabeads (*Invitrogen*), and used for cytotoxicity analysis with bi-specific antibodies.

Fluorescence-activated cell sorting (FACS) analysis

The allophycocyanin (APC)-labeled anti-CD3 (*eBioscience*, San Diego, CA) antibody was used for FACS analysis using FACSCalibur *(BD Biosciences*). For FACS with colon cancer cell lines to detect PLAP levels, either bi-specific PLAP-CD3 or mouse monoclonal PLAP antibody (H17E2) from *Ximbio* (London, UK) were used for FACS analysis which was performed on FACSCalibur, as described (9).

Real-time cytotoxicity assay (RTCA)

Adherent colon cancer target cells (10,000 cells per well) were seeded into 96-well E-plates (*Acea Biosciences,* San Diego, CA) and cultured overnight using the impedance-based real-time cell analysis (RTCA) iCELLigence system (*Acea Biosciences*). After 20-24 hours, the medium was replaced with 1 x 10^5 effector cells T cells, T cells with bispecific antibody or antibody alone in AIM V-AlbuMAX medium containing 10% FBS, in triplicate. The cells were monitored for >40 hours with the RTCA system, and impedance (proportional to cell index) was plotted over time. Cytotoxicity was calculated as (impedance of target cells without effector cells – impedance of target cells with effector cells) x100 /impedance of target cells without effector cells.

ELISA assay for cytokine secretion

The target cells were cultured with the effector cells or agents at in U-bottom 96-well plates with AIM V-AlbuMAX medium plus 10% FBS, in triplicate. After 16 h the supernatant was removed and centrifuged to remove residual cells. In some experiments, supernatant after RTCA assay was used for ELISA cytokine assays. The supernatant was transferred to a new 96-well plate and analyzed by ELISA for human cytokines using kits from *Thermo Fisher* according to the manufacturer's protocol.

Mouse *in vivo* xenograft study

Six-week old male NSG mice (*Jackson Laboratories*, Bar Harbor, ME) were housed in accordance with the Institutional Animal Care and Use Committee (IACUC) protocol. Each mouse was injected subcutaneously with 2 x 10^6 Lovo colon cancer cells in sterile 1x PBS. The bi-specific antibody 10 μg/mice with 1x10^7 T cells were injected intravenously into mice at different time points. Tumor sizes were measured with calipers twice weekly and tumor volume (in mm^3) was determined using the formula $W^2L/2$, where W is tumor width

and L is tumor length. At the end 0.1 ml of blood was collected and used for analysis of toxicology markers.

EXAMPLE 2. THE SEQUENCE OF PLAP H2-CD3E BISPECIFIC ANTIBODY (FIG. 1A)

FIG. 1A shows the structure of humanized PLAP-CD3 bivalent antibody consisting of 4 DNA constructs. The structure has CD3 CROSS-Fab.

PLAP h2-CD3e bispecific antibody of FIG. 1A comprises 4 constructs:

1. PLAP h2 light chain (VL-CL): PLAP VL (humanized h2 PLAP, WO2019/240934, which was codon optimized as below)
2. CD3 CROSSFAB, (VL-CH1)
3. PLAP h2 VH-CH1- CD3 CROSSFAB (VH-CL) – Fc (knob) P329GLA-LA
4. PLAP h2 VH- CH1 - Fc(hole) P329GLA-LA

P329G mutation abolishes interaction of FcγR and C1q interactions and thus eliminates elimination of targeted cells via antibody-dependent cellular-cytotoxicity (ADCC), antibody-dependent phagocytosis (ADCP) or complement-dependent cytotoxicity (CDC). P329G mutation removes FcγR-mediated immune effector functions when delivered to cells providing silent Fc region (11). Addition of two other mutations LA-LA mutation changes Leucine Leu 234 and Leu 235 to alanine (A) completely blocked binding of FcγR and C1q interactions and thus abolished Fc-mediated ADC, ADCC and other immunogenicity (10).

All sequences were codon optimized and synthesized as GBlocks and inserted into Nhe I and Nsi I site of pYD11 vector. In order not to have mispairing of light chain domains, CrossFAB technology was used whereCD3 VH is connected to CL, and CD3 VL is connected to CH1. We also used knobs-in-hole mutations proposed by Crick in 1952 in order to create the knob (T366W), and S354C mutations were used; or the hole (Y349C, T366S, L368A and Y407V) mutations were used to hold both Fc chains together. All sequences start with the signaling peptide (underlined): METDTLLLWVLLLWVPGSTGAAS (SEQ ID NO: 2).

Construct #1. PLAP h2 light chain: LC-PLAP

DNA artificial sequence LC (light chain) of humanized PLAP (PLAP h2 VL (bold)-CL (italics) is shown below. The nucleotide sequence of PLAP h2 VL is shown in WO2019/240934 which was codon-optimized and inserted with constant CL region into Nhe

I (*GCTAGC* site shown in italics, underlined) and Nsi I sites (*atgcat* shown in italics, underlined of pYD11 vector). The sequences started with signaling peptide (Signaling peptide is underlined+(AAS amino-acids after due to cloning site): METDTLLLWVLLLWVPGSTGAAS (SEQ ID NO: 2).

The two stop codons were added to the sequence before start of human Fc to express light chain with no Fc present in the vector. Signaling peptide in bold italics, underlined,; VL bold; CL italics.

ATGGAGACAGACACACTCCTGCTATGGGTACTGCTGCTCTGGGTTCCAGGTTC CACTGGCGCC*GCTAGC***GAT ATT CAA ATG ACC CAA TCC CCG AGC A GC CTG AGT GCC AGC GTA GGA GAT CGG GTT ACG ATA ACG TG TCGA GCA TCC GAA AAT ATC TAT AGC TAT GTA GCA TGG TAC CAA CAA AAA CCT GGC AAG GCA CCG AAA CTGTTG ATA TAC AA C GCC AAG AGT CTG GCA AGT GGG GTG CCT TCA CGC TTC AGT GGA AGC GGA AGT GGG ACAGAT TTT ACA CTT ACC ATT TCC T CC CTT CAG CCT GAA GAC TTT GCA ACA TAT TAT TGT CAA CA T CAC TATGTG AGT CCG TGG ACT TTC GGG GGA GGA ACC AAA TTG GAA ATA AAG CGC** *ACA GTC GCA GCG CCC AGT GTGTTT AT A TTC CCC CCT TCA GAC GAA CAG CTC AAA TCC GGC ACA GCA TCA GTG GTG TGT CTG TTG AAC AATTTC TAT CCT AGA GAG G CA AAG GTT CAA TGG AAA GTG GAC AAC GCG CTC CAA AGC GG G AAC TCC CAA GAGAGC GTC ACT GAA CAA GAT TCC AAA GAT AGC ACG TAC TCT CTT TCT TCA ACG CTC ACG CTC AGT AAG G CCGAT TAC GAG AAA CAT AAG GTA TAC GCT TGC GAG GTC ACG CAT CAA GGG CTG TCC TCA CCC GTG ACC AAGTCT TTC AAC C GG GGG GAA TGT TAA TAA (atgcat)*

(SEQ ID NO: 3)

Amino-acid sequence:

Signaling peptide (underlined)+AAS: METDTLLLWVLLLWVPGSTGAAS (SEQ ID NO: 2).

PALP h2 VL (SEQ ID NO: 4)
DIQMTQSPSSLSASVGDRVTITCRASENIYSYVAWYQQKPGKAPKLLIYNAKSLA SGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQHHYVSPWTFGGGTKLEIKR

PALP h2 VL-CL (SEQ ID NO: 5)
DIQMTQSPSSLSASVGDRVTITCRASENIYSYVAWYQQKPGKAPKLLIYNAKSLA SGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQHHYVSPWTFGGGTKLEIKR*TV AAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDS TYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC*

Construct #2. CD3 CROSSFAB (VL-CH1)

CD3 VL is shown in bold, CH1 is in italics font, the nucleotide sequence was codon optimized. The Nhe I and Nsi I sites are shown in italics. The stop codon TAA was added to terminate the sequence before Fc.

Nucleotide sequence: Signaling peptide underlined in italics in bold, then AAS in italics regular font; VL in bold, CH1, regular font italics.

ATGGAGACAGACACACTCCTGCTATGGGTACTGCTGCTCTGGGTTCCAGGTTC CACTGGCGCC*GCTAGC*CAG GCC GTA GTG ACA CAG GAA CCG TCT T TG ACG GTG TCT CCG GGA GGT ACC GTC ACC TTG ACG TGT GG GTCC AGC ACT GGA GCT GTA ACA ACG AGC AAT TAC GCG AAT TGG GTG CAG GAG AAG CCA GGT CAG GCT TTTAGG GGT CTT AT C GGA GGG ACT AAT AAA AGG GCT CCA GGC ACG CCG GCA AGA TTC TCA GGG TCC CTG CTGGGG GGG AAA GCG GCA CTC ACC C TT TCT GGT GCT CAG CCA GAG GAT GAG GCC GAA TAT TAT TG T GCC TTGTGG TAT TCT AAT TTG TGG GTC TTT GGA GGC GGG ACA AAA CTC ACT GTA TTG *TCA TCT GCG TCA ACG AAGGGA CC T TCT GTA TTC CCC TTG GCA CCA TCC AGT AAA TCT ACC AGT GGG GGT ACC GCT GCC CTC GGT TGCCTT GTA AAA GAT TAC T TT CCG GAG CCC GTC ACC GTG TCC TGG AAC AGC GGG GCA TT G ACC AGT GGT GTCCAC ACT TTT CCC GCA GTA CTC CAA AGC TCC GGC CTC TAC AGT CTC TCT TCA GTT GTG ACG GTT CCT A GCTCT TCC CTT GGT ACG CAG ACT TAT ATC TGC AAC GTC AAC CAC AAA CCT TCC AAT ACT AAG GTA GAC AAAAAG GTG GAG C CC AAA TCT TGT TAA*ATGCAT

(SEQ ID NO: 6)

Amino-acid sequence (not including signaling peptide)

CD3 VL (SEQ ID NO: 7)
Q A V V T Q E P S L T V S P G G T V T L T C G S S T G A V T T S N Y A N W V Q E K P G Q A F R G L I G G T N K R A P G T P A R F S G S L L G G K A A L T L S G A Q P E D E A E Y Y C A L W Y S N L W V F G G G T K L T V L

CD3 VL-CH1 (SEQ ID NO: 8)
Q A V V T Q E P S L T V S P G G T V T L T C G S S T G A V T T S N Y A N W V Q E K P G Q A F R G L I G G T N K R A P G T P A R F S G S L L G G K A A L T L S G A Q P E D E A E Y Y C A L W Y S N L W V F G G G T K L T V L S S *A S T K G P S V F P L A P S S K S T S G G T A A L G C L V K D Y F P E P V T V S W N S G A L T S G V H T F P A V L Q S S G L Y S L S S V V T V P S S S L G T Q T Y I C N V N H K P S N T K V D K K V E P K S C*

Construct #3.

PLAP h2 VH CH1- CD3 CROSSFAB VH-CL – Fc (knob) P329GLA-LA

Signaling peptide in bold, italics underlined, then 3 amino acids-AAS due to cloning sites; Cloning sites Nhe I *GCTAGC* and Nsi I *ATGCAT* are underlined, larger font

PLAP h2-VH-in bold; CH1-underlined; 2xG4S linker; ***CD3 VH bold italics***; CL in italics underlined; IgG Fc chain with LA-LA, (L234 and L235 changed to A) mutations shown in bold, underlined, and P329G mutation, P changed to G, bold underlined.

The knob mutations in Fc domain were S354C and T366W shown in bold larger font, italics.

Nucleotide sequence:

ATGGAGACAGACACACTCCTGCTATGGGTACTGCTGCTCTGGGTTCCAGGTTCCACTGGCGCC*GCTAGC*CAG
GTA CAA TTG CAG GAA TCA GGA CCC GGA TTG GTG AAG CCA AGT GAA AC
T CTG AGT TTG ACT TGC ACAGTC AGC GGC TTC TCA CTG ACA TCC TAT G
GG GTA AGT TGG ATT CGG CAA CCG GCA GGT AAG GGC CTT GAATGG ATC
GGG GTC ATC TGG GAA GAT GGG TCA ACT AAC TAC CAT TCT GCG CTG AT
A AGT CGC GTT ACC ATGTCA GTC GAT ACA AGC AAG AAT CAG TTC TCT C
TG AAA CTG AGC AGT GTG ACA GCC GCA GAC ACC GCA GTGTAC TAT TGC
GCA CGC CCT CAC TAC GGA TCC TCT TAT GTG GGA GCA ATG GAA TAT TG
G GGA GCG GGA ACAACA GTA ACA GTT TCT TCA GCG AGC ACT AAG GGC C
CG TCT GTA TTT CCC CTT GCC CCT TCA TCC AAG AGCACG AGT GGT GGA
ACG GCC GCA CTC GGA TGT TTG GTA AAA GAC TAT TTC CCA GAG CCC GT
G ACT GTG TCTTGG AAT TCC GGT GCA CTG ACT TCT GGT GTG CAT ACT T
TT CCG GCC GTG CTT CAA AGT TCA GGC CTT TATAGC TTG AGC TCA GTA
GTC ACC GTC CCT TCA TCT TCA TTG GGG ACA CAA ACC TAT ATC TGT AA
T GTT AATCAT AAA CCT TCC AAC ACC AAA GTC GAC AAG AAA GTG GAG C
CA AAG AGT TGC GAT GGG GGA GGG GGA AGCGGG GGA GGG GGT TCA GAG
GTA CAG CTG CTC GAA AGC GGC GGA GGT CTT GTG CAA CCA GGC GGG ***TC***
A CTCAGA CTC AGT TGT GCG GCG TCC GGC TTC ACT TTT AGT ACG TAT G
CC ATG AAC TGG GTG CGA CAA GCT CCTGGC AAG GGA CTG GAG TGG GTG
TCC CGG ATA AGA TCA AAA TAT AAC AAT TAC GCG ACC TAT TAT GCA GA
TAGT GTC AAA GGA AGG TTT ACA ATA TCT CGC GAT GAT TCT AAA AAT A
CG CTC TAT CTG CAG ATG AAT AGTTTG CGG GCC GAA GAT ACC GCG GTA
TAT TAT TGT GTC CGC CAC GGG AAT TTT GGC AAC TCT TAC GTT AGTTGG
TTC GCT TAT TGG GGG CAG GGA ACT CTG GTC ACA GTA TCC AGT GCA T
CA *GTT GCG GCC CCC TCA GTCTTC ATT TTT CCC CCT AGT GAT GAG CAA*
CTT AAG TCC GGA ACA GCC AGC GTG GTC TGC CTG CTG AAC AATTTT TAT
CCG AGG GAG GCG AAG GTT CAA TGG AAA GTC GAT AAC GCT CTG CAA T
CA GGT AAT TCT CAG GAATCT GTC ACT GAA CAA GAT AGT AAA GAC AGC
ACA TAC TCT TTG TCT TCT ACA TTG ACC TTG TCT AAG GCGGAT TAC GAA
AAG CAT AAG GTC TAT GCT TGC GAA GTG ACG CAT CAG GGG CTT AGT T
CC CCG GTC ACC AAGAGT TTC AAT AGG GGG GAG TGC GAT AAG ACC CAC
ACC TGT CCG CCA TGC CCT GCA CCT GAG **GCA GCG** GGAGGG CCG AGT GTA
TTC TTG TTC CCT CCA AAA CCG AAA GAT ACT CTG ATG ATT AGC CGG A
CC CCC GAA GTTACG TGT GTG GTT GTA GAC GTA AGT CAC GAA GAT CCT

GAA GTT AAG TTT AAC TGG TAT GTT GAT GGG GTGGAA GTT CAC AAT GCC AAA ACC AAA CCT AGA GAG GAG CAA TAC AAC TCC ACC TAT CGG GTT G TA AGC GTCTTG ACC GTG CTC CAC CAA GAC TGG CTG AAC GGT AAG GAG TAT AAG TGT AAG GTG AGC AAC AAG GCT TTGGGA GCA CCC ATC GAA AAA ACG ATC AGC AAA GCC AAA GGT CAG CCA CGC GAA CCC CAG GTG TAT A CC CTTCCG CCT *TGT* AGG GAT GAG CTT ACT AAG AAC CAA GTT TCA CTC *TGG* TGT CTG GTG AAG GGT TTT TAC CCCTCC GAT ATT GCT GTG GAG TGG GAG TCA AAC GGG CAG CCA GAA AAT AAC TAT AAG ACC ACG CCA CCT G TCCTT GAC AGT GAC GGA AGT TTT TTC CTG TAT TCT AAA TTG ACC GTA GAT AAG TCT CGA TGG CAG CAA GGAAAC GTG TTT TCA TGC TCT GTT ATG CAC GAA GCT CTC CAC AAC CAT TAT ACA CAA AAG TCA CTG AGC CTTAG T CCT GGT AAA*ATGCAT*GAGGCTCTGCACAACCACTACACGCAGAAGAGCCTCTCCCTG TCTCCCGGGAAA**TGA**

(SEQ ID NO: 9).

Amino-acid sequence PLAP h2 VH, SEQ ID NO: 10

QVQLQESGPGLVKPSETLSLTCTVSGFSLTSYGVSWIRQPAGKGLEWIGVIWED GSTNYHSALISRVTMSVDTSKNQFSLKLSSVTAADTAVYYCARPHYGSSYVGAM EYWGAGTTVTVSS

CD3 VH, SEQ ID NO: 11

EVQLLESGGGLVQPGGSLRLSCAASGFTFSTYAMNWVRQAPGKGLEWVSRIRSKYN NYATYYADSVKGRFTISRDDSKNTLYLQMNSLRAEDTAVYYCVRHGNFGNSYVSWFA YWGQGTLVTVSS

Amino-acid sequence of Construct #3 (not including signaling peptide):

QVQLQESGPGLVKPSETLSLTCTVSGFSLTSYGVSWIRQPAGKGLEWI GVIWEDGSTNYHSALISRVTMSVDTSKNQFSLKLSSVTAADTAVYYCA RPHYGSSYVGAMEYWGAGTTVTVSSAS<u>STKGPSVFPLAPSSKSTSGGTAA LGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSS LGTQTYICNVNHKPSNTKVDKKVEPKSCD</u>GGGGSGGGGS***EVQLLESGGGL VQPGGSLRLSCAASGFTFSTYAMNWVRQAPGKGLEWVSRIRSKYNNYATY YADSVKGRFTISRDDSKNTLYLQMNSLRAEDTAVYYCVRHGNFGNSYVSW FAYWGQGTLVTVSS***AS<u>*VAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKV QWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQ GLSSPVTKSFNRGEC*</u>DKTHTCPPCPAPE**AA**GGPSVFLFPPKPKDTLMISRTPE VTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLT VLHQDWLNGKEYKCKVSNKAL**G**APIEKTISKAKGQPREPQVYTLPP***C***RDE LTKNQVSL***W***CLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLY SKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGKMHEALHNHY TQKSLSLSPGK (SEQ ID NO: 12)

Construct #4. PLAP h2 VH- CH1 - Fc(hole) P329GLA-LA

Construct #4 used the same P329G and LA-LA mutations as in Construct #3, shown in bold. The hole mutations were Y349C, T366S, L368A and Y407V shown in bold, larger fond, italics. Cloning sites Nhe I *GCTAGC* and Nsi I *ATGCAT* are underlined

Signaling peptide underlined, bold, italics, then 9 nucleotides encoding 3 amino-acids AAS (cloning sites), regular font, italics; PLAP-VH-bold, CH1 underlined, then Fc with P329GLA-LA and hole mutations

Nucleotide:

ATGGAGACAGACACACTCCTGCTATGGGTACTGCTGCTCTGGGTTCCAGGTTCCACTGGCGCC
*GCTAGC*CAG GTC CAG CTG CAA GAG TCC GGA CCT GGG CTG GTC AAA CCC
AGT GAG ACG TTG TCC CTT ACG TGT ACTGTC TCC GGC TTC AGT TTG ACG
TCT TAT GGA GTT TCT TGG ATA CGG CAG CCC GCC GGT AAG GGC CTC G
AGTGG ATT GGA GTT ATA TGG GAA GAT GGG TCC ACT AAT TAT CAT AGC
GCC CTT ATT AGC AGG GTA ACC ATGTCT GTC GAT ACT AGC AAA AAT CAG
TTC AGC CTT AAA TTG TCA AGT GTG ACC GCT GCA GAT ACA GCA GTATA
T TAC TGT GCG AGA CCA CAT TAT GGA TCC AGT TAT GTC GGA GCG ATG
GAG TAT TGG GGC GCG GGC ACCACT GTT ACG GTT AGC AGC ACA AAG GGA
CCG TCT GTG TTC CCC CTT GCG CCG TCT TCC AAG TCC ACT AGTGGG GG
G ACC GCG GCA CTG GGA TGC CTG GTT AAG GAC TAC TTC CCG GAA CCG
GTG ACC GTT AGT TGG AACAGT GGC GCC CTT ACG TCA GGG GTT CAT ACG
TTT CCC GCG GTG CTG CAA AGC AGT GGA CTC TAT AGT CTTTCT TCA GT
A GTT ACA GTG CCT AGC AGC AGT CTC GGC ACG CAG ACA TAT ATA TGT
AAT GTT AAT CAT AAACCC TCA AAT ACA AAG GTA GAC AAG AAA GTA GAG
CCA AAG AGT TGC GAC AAG ACA CAT ACT TGC CCT CCGTGC CCT GCA CC
C GAA **GCG GCA** GGC GGC CCA TCA GTA TTT TTG TTT CCT CCT AAA CCT
AAA GAC ACT CTTATG ATA TCA CGG ACA CCT GAA GTC ACT TGT GTA GTT
GTG GAC GTT TCA CAT GAG GAT CCC GAA GTC AAGTTC AAC TGG TAC GT
C GAT GGC GTA GAA GTT CAT AAC GCA AAA ACA AAG CCG CGG GAG GAG
CAG TAT AACTCA ACC TAT CGA GTA GTC TCT GTT CTT ACG GTT TTG CAT
CAA GAC TGG CTC AAT GGT AAG GAG TAT AAATGT AAA GTG AGC AAT AA
A GCT CTG **GGT** GCT CCT ATA GAG AAA ACG ATC TCT AAG GCG AAA GGG
CAA CCTCGG GAG CCG CAG GTG ***TGT*** ACA TTG CCC CCC AGC CGA GAT GAA
CTC ACT AAA AAC CAA GTT TCC CTC ***AGC***TGC ***GCA*** GTC AAG GGA TTC T
AC CCG AGC GAC ATT GCA GTA GAG TGG GAG TCA AAT GGT CAG CCA GAA
AATAAT TAT AAA ACA ACC CCT CCC GTC CTG GAC AGC GAT GGA TCA TT
C TTT CTG ***GTG*** TCT AAA CTT ACA GTGGAT AAA TCC CGA TGG CAA CAA G
GC AAT GTG TTT TCT TGT TCT GTA ATG CAC GAG GCA TTG CAT AAT CAC
TAC ACT CAG AAA TCT CTC AGC CTT AGT CCA GGG AAA *ATGCAT*
GAGGCTCTGCACAACCACTACACGCAGAAGAGCCTCTCCCTGTCTCCCGGGAAA**TGA**
(SEQ ID NO: 13)

Amino-acid of Construct #4 (not including signaling peptide), SEQ ID NO: 14

QVQLQESGPGLVKPSETLSLTCTVSGFSLTSYGVSWIRQPAGKGLEWIGVIWED GSTNYHSALISRVTMSVDTSKNQFSLKLSSVTAADTAVYYCARPHYGSSYVGAM EYWGAGTTVTVSSTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGA LTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSC DKTHTCPPCPAPE**AA**GGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNW YVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKAL**G**API EKTISKAKGQPREPQV***C***TLPPSRDELTKNQVSL***S***C***A***VKGFYPSDIAVEWESNGQPEN NYKTTPPVLDSDGSFFL***V***SKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSP GKMHEALHNHYTQKSLSLSPGK

EXAMPLE 3. THE SEQUENCE OF PLAP H4-CD3 ANTIBODY (FIG. 1A)

PLAP h4-CD3e bispecific comprises 4 constructs:

1. PLAP h4 light chain (VL-CL): LC PLAP
2. CD3 CROSSFAB, (CD3e VL-CH1), same as Example 2.
3. PLAP h4 VH-CH1-CD3 CROSSFAB (CD3e VH-CL) – Fc (knob) P329GLA-LA
4. PLAP h4 VH-CH1-Fc(hole) P329G, LA-LA

Construct #1

PLAP h4 light chain: LC PLAP (humanized h4 PLAP, WO2019/240934, which was codon optimized as below), signaling peptide in bold, italics, underlined; followed by 9 nucleotides, cloning sites in italics regular font; Nhe I and Nsi I sites underlined. PLAP h4 VL is shown in bold, then CL in regular font

Nucleotide sequence:

ATGGAGACAGACACACTCCTGCTATGGGTACTGCTGCTCTGGGTTCCAGGTTCCACTGGCGCC*GCTAGC***GA**
C ATACAG ATG ACT CAA AGC CCC TCT TCA CTG TCT GCA TCA GTC GGG GAC AGA GTC ACA ATA ACC TGC AGA GCGAGC GAG AAT ATC TAC TCT TAT GTA GCC TGG TAT CAG CAA AAA CCC GGC AAG GCG CCG AAA TTG CTC ATCTAT AAT GCG AAA TCC TTG GCC AGT GGG GTC CCA TCA CGG TTC AGT GGC TCC GGC TCT GGA ACC GAT TTCACA CTC ACA ATC TCT AGC CTC CAG CCC GAA GAC TTC GCC ACA TAC TAT TGC CAA CAT CAC TAT GTC AGCCCA TGG ACA TTT GGG GGA GGT ACG AAA CTT GAA ATT AAACGT ACA GTA GCT GCT CCG TCC GTC TTT ATT TTC CCG CCG TCT GAC GAA CAG CTC AAA AGC GGG ACT GCATCA GTT GTC TGT CTC CTC AAC AAT TTT TAC CCG CGA GAG 18A ACTT GTT CAA TGG AAA GTT GAT AAC GCCCTC CAG AGT GGA AAC TCT CAG GAG AGT GTA ACT GAG CAA GAT TCC AAA GAT TCA ACC TAT AGT CTT TCAAGT ACC TTG ACT CTT TCT AAA GCG GAT TAT GAG AAA CAT AAA GTG TAT GCC TGC GAA GTG ACC CAT CAGGGG CTT TCA TCA CCC GTG ACG AAG TCC 18A ACTT CGA GGC GAA TGC**TAA***ATGCAT*
(SEQ ID NO: 15)

Amino-acid sequence of PLAP h4 VL, SEQ ID NO: 16

DIQMTQSPSSLSASVGDRVTITCRASENIYSYVAWYQQKPGKAPKLLIYNAKSLA
SGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQHHYVSPWTFGGGTKLEIKR

Amino-acid sequence of Construct #1, PLAP h4 VL-CL (without signaling peptide), SEQ ID NO: 17

DIQMTQSPSSLSASVGDRVTITCRASENIYSYVAWYQQKPGKAPKLLIYNAKSLA
SGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQHHYVSPWTFGGGTKLEIKRTV
AAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQD
SKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC

Construct # 2 (light chain of CD3 CrossFAB) was same as Example 2.

Construct #3 PLAP h4 VH-CH1- CD3 CROSSFAB (VH-CL) – Fc (knob).

Signaling peptide in italics, underlined, in bold, with following 9 nucleotides (cloning sites); PLAPh4 VH in bold; CH1 underlined; CD3 VH bold in italics; CL in italics, underlined; Fc with P329GLA-LA mutations in bold underlined; knob mutations in bold italics, larger font.

ATGGAGACAGACACACTCCTGCTATGGGTACTGCTGCTCTGGGTTCCAGGTTCCACTGGCGCCGCTAGC**CAG**
GTT CAA CTT CAA GAA TCA GGA CCG GGC TTG GTT AAA CCT TCC GAA AC
T CTG AGC CTT ACT TGT ACAGTG TCT GGT GGA TCT ATT ACG AGC TAC G
GA GTA AGT TGG ATC CGG CAA CCA CCC GGG AAA GGG CTC GAATGG ATA
GGG GTG ATA TGG GAG GAT GGT TCA ACC AAC TAC CAT AGC GCT CTG AT
C AGC CGG GTG ACC ATTAGT GTC GAC ACT TCC AAA AAC CAG TTT TCA T
TG AAG CTC TCA AGC GTA ACT GCG GCG GAT ACC GCC GTATAC TAT TGT
GCG CGG CCA CAT TAC GGG TCC TCT TAT GTT GGG GCG ATG GAA TAT TG
G GGG GCA GGT ACAACG GTC ACG GTG TCT TCA ACA AAA GGT CCT TCC
GTA TTT CCG CTC GCA CCC AGC TCT AAG TCA ACC TCT GGC GGT ACT
GCA GCC CTG GGT TGC CTC GTA AAG GAC TAT TTT CCT GAG CCA GTA
ACA GTT TCT TGG AAC AGC GGG GCA CTT ACG AGC GGT GTT CAT ACG
TTC CCT GCA GTG TTG CAA TCC AGC GGC CTT TAT TCA TTG TCT TCA
GTT GTA ACG GTT CCT TCT AGT AGT TTG GGG ACC CAG ACA TAT ATC
TGC AAC GTG AAC CAT AAG CCA AGC AAT ACC AAA GTT GAT AAG AAG
GTC GAA CCT AAG TCC TGC GAC *GGC GGG GGA GGA TCT GGC GGG GGA*
GGC AGT ***GAG GTA CAA TTG TTG GAG TCA GGC GGC GGT CTT GTC CAA***
CCG GGA GGG TCC CTG AGA CTG TCC TGT GCG GCC AGC GGG TTT ACT
TTT TCA ACA TAT GCC ATG AAC TGG GTT CGG CAA GCA CCA GGT AAG
GGA CTG GAA TGG GTT AGT CGA ATT AGG TCC AAG TAT AAT AAT TAC
GCA ACC TAC TAC GCT GAC TCT GTC AAG GGG CGG TTT ACC ATA TCT
AGG GAT GAC TCC AAA AAC ACA TTG TAC TTG CAA ATG AAC AGC CTG
AGG GCA GAA GAC ACC GCA GTC TAC TAT TGT GTA CGC CAC GGA AAC
TTC GGG AAT AGT TAT GTC TCC TGG TTC GCT TAC TGG GGT CAG GGA
ACA CTG GTC ACA GTC TCA TCA GCC AGT GTA GCG GCC CCG TCC GTT
TTC ATA TTC CCT CCT TCC GAC GAG CAG TTG AAA AGC GGT ACG GCG

AGC GTT GTG TGC TTG TTG AAC AAC TTC TAC CCA CGC GAA GCC AAG
GTC CAA TGG AAG GTA GAC AAC GCA CTG CAG AGT GGT AAC TCA CAG
GAA TCA GTG ACG GAA CAG GAC TCA AAA GAT AGT ACT TAC AGT CTT
TCT TCC ACA CTG ACA CTC AGT AAG GCC GAT TAT GAG AAA CAT AAA
GTA TAC GCA TGT GAA GTA ACT CAC CAG GGT CTC AGT TCA CCA GTA
ACT AAG TCT TTC AAT CGC GGG GAA TGC GAC AAA ACA CAC ACC TGT
CCC CCC TGT CCA GCC CCA GAG **GCA GCT** GGC GGC CCT AGT GTG TTC
TTG TTC CCG CCC AAG CCA AAA GAT ACA CTG ATG ATT AGC CGG ACC
CCT GAG GTA ACT TGT GTG GTG GTG GAC GTG TCT CAT GAG GAC CCA
GAG GTA AAA TTC AAC TGG TAC GTA GAC GGC GTC GAG GTC CAT AAT
GCC AAA ACC AAG CCA CGG GAG GAG CAG TAT AAT TCC ACT TAT CGC
GTA GTC TCT GTA CTT ACA GTT CTT CAC CAA GAT TGG TTG AAC GGA
AAA GAA TAC AAG TGT AAA GTT AGC AAT AAG GCG CTC **GGA** GCT CCG
ATC GAA AAA ACA ATC TCC AAA GCA AAA GGG CAA CCC CGA GAA CCA
CAG GTA TAC ACC CTG CCG CCG ***TGC*** CGA GAC GAG CTG ACG AAA AAC
CAA GTG TCC CTG ***TGG*** TGC TTG GTG AAG GGC TTT TAT CCA AGT GAC
ATT GCA GTT GAA TGG GAG TCT AAC GGA CAG CCT GAA AAT AAC TAT
AAG ACC ACG CCA CCA GTC CTT GAT AGC GAT GGA TCT TTT TTT CTC
TAT AGC AAG TTG ACT GTA GAT AAA TCA CGA TGG CAA CAA GGC AAT
GTC TTT TCA TGC AGC GTT *ATG CAT*
GAGGCTCTGCACAACCACTACACGCAGAAGAGCCTCTCCCTGTCTCCCGGGAAA**TGA**

(SEQ ID NO: 18)

Amino Acid Sequence of PLAP h4 VH, SEQ ID NO: 19

QVQLQESGPGLVKPSETLSLTCTVSGGSITSYGVSWIRQPPGKGLEWIGVIWEDGSTN
YHSALISRVTISVDTSKNQFSLKLSSVTAADTAVYYCARPHYGSSYVGAMEYWGAG
TTVTVSS

Amino-acid Sequence of signal peptide underlined + AAS
METDTLLLWVLLLWVPGSTGAAS (SEQ ID NO: 2)

Amino Acid Sequence of Construct #3 (without signaling peptide)

**QVQLQESGPGLVKPSETLSLTCTVSGGSITSYGVSWIRQPPGKGLEWIGVIWED
GSTNYHSALISRVTISVDTSKNQFSLKLSSVTAADTAVYYCARPHYGSSYVGAME
YWGAGTTVTVSS**TKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGAL
TSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCD
*GGGGSGGGGS**EVQLLESGGGLVQPGGSLRLSCAASGFTFSTYAMNWVRQAPGKGLE
WVSRIRSKYNNYATYYADSVKGRFTISRDDSKNTLYLQMNSLRAEDTAVYYCVRHGN
FGNSYVSWFAYWGQGTLVTVSS***AS*VAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAK
VQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVT
KSFNRGEC*DKTHTCPPCPAPE**AA**GGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHED
PEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVS
NKAL**G**APIEKTISKAKGQPREPQVYTLPP***C***RDELTKNQVSL***W***CLVKGFYPSDIAVE
WESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNH
YTQKSLSLSPGK (SEQ ID NO: 20)

Construct #4, PLAP h4 VH- CH1 - Fc(hole) P329GLA-LA

Signaling peptide in italics, bold underlined+9 nucleotides cloning sites encoding AAS in italics regular font; PLAP h4 bold, CH1-underlined, Hole mutations shown in bold italics, larger font; 329GLA-LA bold underlined

Nucleotide sequence:

ATGGAGACAGACACACTCCTGCTATGGGTACTGCTGCTCTGGGTTCCAGGTTCCACTGGCGC
C*GCTAGC*CAG GTT CAA CTT CAA GAA TCA GGA CCG GGC TTG GTT AAA CCT
TCC GAA ACT CTG AGC CTT ACT TGT ACAGTG TCT GGT GGA TCT ATT AC
G AGC TAC GGA GTA AGT TGG ATC CGG CAA CCA CCC GGG AAA GGG CTC
GAATGG ATA GGG GTG ATA TGG GAG GAT GGT TCA ACC AAC TAC CAT AGC
GCT CTG ATC AGC CGG GTG ACC ATTAGT GTC GAC ACT TCC AAA AAC CA
G TTT TCA TTG AAG CTC TCA AGC GTA ACT GCG GCG GAT ACC GCC GTAT
AC TAT TGT GCG CGG CCA CAT TAC GGG TCC TCT TAT GTT GGG GCG ATG
GAA TAT TGG GGG GCA GGT ACAACG GTC ACG GTG TCT TCA
ACC AAA GGT CCA AGC GTA TTT CCA CTC GCA CCG TCC TCC AAA TCA AC
G AGT GGA GGT ACT GCG GCG TTG GGA TGC TTG GTG AAAGAC TAT TTC C
CA GAG CCG GTG ACA GTT AGT TGG AAC TCA GGC GCG CTG ACT AGT GGT
GTT CAC ACA TTT CCT GCA GTT TTG CAATCC TCA GGC CTC TAT AGC CT
G TCA AGC GTC GTG ACA GTA CCT AGC AGC TCA CTT GGC ACG CAG ACA
TAT ATT TGT AAT GTC AACCAT AAG CCC TCT AAC ACA AAG GTA GAT AAG
AAG GTT GAA CCC AAG TCC TGT GAC AAA ACG CAC ACT TGT CCA CCA T
GT CCA GCGCCC GAA **GCA GCC** GGG GGC CCA AGC GTG TTC CTC TTC CCT
CCC AAG CCA AAA GAC ACC CTT ATG ATC TCA AGG ACT CCA GAA GTGACA
TGC GTA GTC GTT GAC GTA AGT CAC GAG GAT CCG GAA GTG AAG TTC A
AC TGG TAC GTG GAC GGT GTG GAG GTA CAT AAC GCGAAG ACT AAG CCC
AGA GAA GAA CAA TAT AAC TCA ACC TAC CGG GTC GTT TCT GTG CTC AC
A GTG CTC CAC CAG GAC TGG CTT AACGGA AAA GAG TAT AAA TGC AAA G
TA TCT AAT AAA GCG CTC **GGA** GCG CCC ATA GAG AAA ACT ATT TCT AAA
GCA AAA GGT CAA CCACGG GAG CCG CAG GTT ***TGC*** ACA CTT CCA CCG TC
C AGG GAT GAA CTT ACT AAG AAC CAG GTA TCT CTT ***TCT*** TGT ***GCC*** GTG
AAA GGTTTT TAT CCT AGT GAC ATC GCT GTC GAG TGG GAG AGC AAC GG
T CAG CCG GAG AAT AAC TAT AAG ACC ACA CCT CCG GTT CTG GATTCT G
AC GGC TCT TTC TTC CTG ***GTA*** TCT AAG CTT ACA GTC GAT AAA AGT CGA
TGG CAA CAA GGG AAT GTT TTT AGC TGC TCT GTG atgcat
GAGGCTCTGCACAACCACTACACGCAGAAGAGCCTCTCCCTGTCTCCCGGGAAA**TGA**

(SEQ ID NO: 21)

Amino-acid of signaling peptide underlined + AAS

METDTLLLWVLLLWVPGSTGAAS (SEQ ID NO: 2)

Amino-acid of Construct #4 (without signaling peptide)

QVQLQESGPGLVKPSETLSLTCTVSGGSITSYGVSWIRQPPGKGLEWIGVIWED GSTNYHSALISRVTISVDTSKNQFSLKLSSVTAADTAVYYCARPHYGSSYVGAME YWGAGTTVTVSSTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGAL TSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCD

KTHTCPPCPAPE**AA**GGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWY
VDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKAL**G**APIE
KTISKAKGQPREPQV***C***TLPPSRDELTKNQVSL***S***C***A***VKGFYPSDIAVEWESNGQPENN
YKTTPPVLDSDGSFFL***V***SKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPG
K (SEQ ID NO: 22)

EXAMPLE 4. THE SEQUENCE OF PLAP H4-CD3 ANTIBODY (FIG. 1B)

FIG. 1B shows the structure of humanized bivalent PLAP consisting of 3 DNA constructs. The structure has CD3 scFv (VH-linker-VL) attached to the C-terminal end of CH3. There is with no CROSS-Fab CD3.

PLAP h4-CD3e bivalent antibody (PBM0015) comprises 3 constructs:

1. PLAP h4 light chain, VL-CL: same as in example 3, construct #1.
2. PLAP h4 VH-CH1– Fc (knob) P329GLA-LA-CD3VH-linker-VL

   Amino acids of PLAP h4 VH-CH1, see Example 3, part of Construct 3.
3. PLAP h4 VH- CH1 - Fc (hole) same as construct #4 in example 3.

Construct DNA#2

Construct #2: PLAP h4 VH-CH1– Fc (knob) P329GLA-LA-G4Sx3 linker-CD3VH-linker-VL

DNA was cloned to the same sites as in Example 3 to pYD11 vector.

Nucleotide sequence

Signaling peptide in italics bold, underlined+9 nucleotides cloning sites encoding AAS (italics, regular font); PLAPh4 VH (bold underlined); CH1 regular font, FC with (knob); P329GLA-LA mutations, regular font underlined; G4Sx2 linker bold, italics; CD3scFV (VH-G4Sx3-VL) is shown in bold italics, underlined

ATGGAGACAGACACACTCCTGCTATGGGTACTGCTGCTCTGGGTTCCAGGTTCCACTGGC*GCCGCTAGC*
CAG GTT CAA CTT CAA GAA TCA GGA CCG GGC TTG GTT AAA CCT TCC GA
A ACT CTG AGC CTT ACT TGT ACAGTG TCT GGT GGA TCT ATT ACG AGC T
AC GGA GTA AGT TGG ATC CGG CAA CCA CCC GGG AAA GGG CTC GAATGG
ATA GGG GTG ATA TGG GAG GAT GGT TCA ACC AAC TAC CAT AGC GCT CT
G ATC AGC CGG GTG ACC ATTAGT GTC GAC ACT TCC AAA AAC CAG TTT T
CA TTG AAG CTC TCA AGC GTA ACT GCG GCG GAT ACC GCC GTATAC TAT
TGT GCG CGG CCA CAT TAC GGG TCC TCT TAT GTT GGG GCG ATG GAA TA
T TGG GGG GCA GGT ACAACG GTC ACG GTG TCT TCAACA AAA GGT CCT
TCC GTA TTT CCG CTC GCA CCC AGC TCT AAG TCA ACC TCT GGC GGT
ACT GCA GCC CTG GGT TGC CTC GTA AAG GAC TAT TTT CCT GAG CCA
GTA ACA GTT TCT TGG AAC AGC GGG GCA CTT ACG AGC GGT GTT CAT
ACG TTC CCT GCA GTG TTG CAA TCC AGC GGC CTT TAT TCA TTG TCT

TCA GTT GTA ACG GTT CCT TCT AGT AGT TTG GGG ACC CAG ACA TAT
ATC TGC AAC GTG AAC CAT AAG CCA AGC AAT ACC AAA GTT GAT AAG
AAG GTC GAA CCT AAG TCC TGC GAC AAA ACA CAC ACC TGT CCC CCC
TGT CCA GCC CCA GAG GCA GCT GGC GGC CCT AGT GTG TTC TTG TTC
CCG CCC AAG CCA AAA GAT ACA CTG ATG ATT AGC CGG ACC CCT GAG
GTA ACT TGT GTG GTG GTG GAC GTG TCT CAT GAG GAC CCA GAG GTA
AAA TTC AAC TGG TAC GTA GAC GGC GTC GAG GTC CAT AAT GCC AAA
ACC AAG CCA CGG GAG GAG CAG TAT AAT TCC ACT TAT CGC GTA GTC
TCT GTA CTT ACA GTT CTT CAC CAA GAT TGG TTG AAC GGA AAA GAA
TAC AAG TGT AAA GTT AGC AAT AAG GCG CTC GGA GCT CCG ATC GAA
AAA ACA ATC TCC AAA GCA AAA GGG CAA CCC CGA GAA CCA CAG GTA
TAC ACC CTG CCG CCG TGC CGA GAC GAG CTG ACG AAA AAC CAA GTG
TCC CTG TGG TGC TTG GTG AAG GGC TTT TAT CCA AGT GAC ATT GCA
GTT GAA TGG GAG TCT AAC GGA CAG CCT GAA AAT AAC TAT AAG ACC
ACG CCA CCA GTC CTT GAT AGC GAT GGA TCT TTT TTT CTC TAT AGC
AAG TTG ACT GTA GAT AAA TCA CGA TGG CAA CAA GGC AAT GTC TTT
TCA TGC AGC GTT ATG CAT
gaggctctgcacaaccactacacgcagaagagcctctccctgtctcccgggaaa
GGC GGG GGA GGA TCT GGC GGG GGA GGC AGT GGC GGG GGA GGA TCT
GAG GTA CAA TTG TTG GAG TCA GGC GGC GGT CTT GTC CAA CCG GGA
GGG TCC CTG AGA CTG TCC TGT GCG GCC AGC GGG TTT ACT TTT TCA
ACA TAT GCC ATG AAC TGG GTT CGG CAA GCA CCA GGT AAG GGA CTG
GAA TGG GTT AGT CGA ATT AGG TCC AAG TAT AAT AAT TAC GCA ACC
TAC TAC GCT GAC TCT GTC AAG GGG CGG TTT ACC ATA TCT AGG GAT
GAC TCC AAA AAC ACA TTG TAC TTG CAA ATG AAC AGC CTG AGG GCA
GAA GAC ACC GCA GTC TAC TAT TGT GTA CGC CAC GGA AAC TTC GGG
AAT AGT TAT GTC TCC TGG TTC GCT TAC TGG GGT CAG GGA ACA CTG
GTC ACA GTC TCA TCA GGC GGG GGA GGA TCT GGC GGG GGA GGC AGT
GGC GGG GGA GGA TCT
CAG GCC GTA GTG ACA CAG GAA CCG TCT TTG ACG GTG TCT CCG GGA GG
T ACC GTC ACC TTG ACG TGT GGGTCC AGC ACT GGA GCT GTA ACA ACG A
GC AAT TAC GCG AAT TGG GTG CAG GAG AAG CCA GGT CAG GCT TTTAGG
GGT CTT ATC GGA GGG ACT AAT AAA AGG GCT CCA GGC ACG CCG GCA AG
A TTC TCA GGG TCC CTG CTGGGG GGG AAA GCG GCA CTC ACC CTT TCT G
GT GCT CAG CCA GAG GAT GAG GCC GAA TAT TAT TGT GCC TTGTGG TAT
TCT AAT TTG TGG GTC TTT GGA GGC GGG ACA AAA CTC ACT GTA TTG TA
A (SEQ ID NO: 23)

Construct 2, amino-acid without signaling peptide in front

QVQLQESGPGLVKPSETLSLTCTVSGGSITSYGVSWIRQPPGKGLEWIGVIWEDGSTN YHSALISRVTISVDTSKNQFSLKLSSVTAADTAVYYCARPHYGSSYVGAMEYWGAGTT VTVSSTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPA VLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCD*KTHTCPPCP APEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTK PREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALGAPIEKTISKAKGQPREPQVYTL PPCRDELTKNQVSLWCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTV DKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK**GGGGSGGGGSGGGGS*EVQLLES GGGLVQPGGSLRLSCAASGFTFSTYAMNWVRQAPGKGLEWVSRIRSKYNNYATYYA***

DSVKGRFTISRDDSKNTLYLQMNSLRAEDTAVYYCVRHGNFGNSYVSWFAYWGQGT LVTVSSGGGGSGGGGSGGGGSQAVVTQEPSLTVSPGGTVTLTCGSSTGAVTTSNYAN WVQEKPGQAFRGLIGGTNKRAPGTPARFSGSLLGGKAALTLSGAQPEDEAEYYCAL WYSNLWVFGGGTKLTVL

(SEQ ID NO: 24)

CD3 ScFV (VH underlined, linker italicized, VL, bold)

EVQLLESGGGLVQPGGSLRLSCAASGFTFSTYAMNWVRQAPGKGLEWVSRIRS KYNNYATYYADSVKGRFTISRDDSKNTLYLQMNSLRAEDTAVYYCVRHGNFGN SYVSWFAYWGQGTLVTVSS*GGGGSGGGGSGGGGS***QAVVTQEPSLTVSPGGTVTL TCGSSTGAVTTSNYANWVQEKPGQAFRGLIGGTNKRAPGTPARFSGSLLGGKA ALTLSGAQPEDEAEYYCALWYSNLWVFGGGTKLTVL**

(SEQ ID NO: 25)

EXAMPLE 5. THE SEQUENCE OF PLAP H2-CD3 ANTIBODY (FIG. 1C)

FIG. 1C shows the structure of monovalent humanized PLAP and monovalent CD3, which consists 3 DNA constructs. The structure does not have CD3 CROSSFAB, but is has CD3 scFv to bind CD3.

PLAP h2-CD3e monovalent antibody comprises 3 constructs:

Signaling peptide same as SEQ ID NO 2 except no AAS amino-acids at the end; METDTLLLWVLLLWVPGSTG (SEQ ID NO: 26).

1. PLAP h2 VL-CL, the amino-acid sequence is the same as that in EXAMPLE 2, Construct #1. The nucleotide sequence is different due to codon optimized.
2. PLAP h2 VH-CH1– Fc (knob)
3. CD3scFv-Fc (hole)

Nucleotide Sequence of Construct 2

Signaling peptide underlined bold italics; PLAP h2 VH (bold)-CH1-Fc(knob): L234A; L235A mutations are shown in larger font underlined, bold, two knob mutations are in italics, larger font, bold shown on FIG. 1C.

ATGGAGACAGACACACTCCTGCTATGGGTACTGCTGCTCTGGGTTCCAGGGTCGAC TGGC**GAAGTGCAGCAGGTGCAGCTTCAGGAAAGTGGACCGGGCCTTGTCAA ACCGTCAGAGACCCTTTCACTGACTTGCACTGTAAGTGGTTTCTCCCTGACA AGCTACGGAGTCTCCTGGATACGCCAGCCAGCGGGGAAAGGGCTTGAGTGG ATCGGTGTGATCTGGGAAGACGGGAGTACAAACTATCACTCAGCACTCATTA GTCGAGTAACAATGTCCGTTGACACTTCCAAGAATCAATTCAGTTTGAAACT GTCTAGTGTGACGGCTGCGGATACAGCGGTTTATTACTGTGCCAGGCCTCAT TACGGAAGTTCTTATGTTGGTGCAATGGAGTATTGGGGAGCCGGCACAACT**

GTCACTGTGAGCTCCGTCACCGTCTCAAGCGCCTCCACCAAGGGCCCATCGGT
CTTCCCGCTAGCACCCTCCTCCAAGAGCACCTCTGGGGGCACAGCGGCCCTGGGC
TGCCTGGTCAAGGACTACTTCCCCGAACCGGTGACGGTGTCGTGGAACTCAGGCG
CCCTGACCAGCGGCGTCCACACCTTCCCGGCTGTCCTACAGTCCTCCGGACTCTA
CTCCCTCAGCAGCGTAGTGACCGTGCCCTCCAGCAGCTTGGGCACCCAGACCTAC
ATCTGCAACGTGAATCACAAGCCCAGCAACACCAAGGTGGACAAGAAAGTTGAG
CCCAAATCTTGTGACAAAACTCACACATGCCCACCGTGCCCAGCACCTGAA**gcc**
gcgGGGGGACCGTCAGTCTTCCTCTTCCCCCCAAAACCCAAGGACACCCTCATG
ATCTCCCGGACCCCTGAGGTCACATGCGTGGTGGTGGACGTGAGCCACGAAGAC
CCTGAGGTCAAGTTCAACTGGTACGTGGACGGCGTGGAGGTGCATAATGCCAAG
ACAAAGCCGCGGGAGGAGCAGTACAACAGCACGTACCGTGTGGTCAGCGTCCTC
ACCGTCCTGCACCAGGACTGGCTGAATGGCAAGGAGTACAAGTGCAAGGTCTCC
AACAAAGCCCTCCCAGCCCCCATCGAGAAAACCATCTCCAAAGCCAAAGGGCAG
CCCCGAGAACCACAGGTGTACACCCTGCCCCCA***tgc***CGGGATGAGCTGACCAAG
AACCAGGTCAGCCTG***tgg***TGCCTGGTCAAAGGCTTCTATCCCAGCGACATCGCC
GTGGAGTGGGAGAGCAATGGGCAGCCGGAGAACAACTACAAGACCACGCCTCCC
GTGCTGGACTCCGACGGCTCCTTCTTCCTCTACAGCAAGCTCACCGTGGACAAGA
GCAGGTGGCAGCAGGGGAACGTCTTCTCATGCTCCGTGATGCATGAGGCTCTGCA
CAACCACTACACGCAGAAGAGCCTCTCCCTGTCTCCGGGTAAA**TGA**
(SEQ ID NO: 27).

Amino-acid of Construct 2, PLAP h2 VH-CH1-Fc (knob) (no signaling peptide):

PLAP h2 VH, underlined CH1; Fc in italics with mutations LA-LA in larger font and knock mutations underlined.

QVQLQESGPGLVKPSETLSLTCTVSGFSLTSYGVSWIRQPAGKGLEWIGVIWEDGSTN
YHSALISRVTMSVDTSKNQFSLKLSSVTAADTAVYYCARPHYGSSYVGAMEYWGAGT
TVTVSS*VTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVH*
TFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCP
APEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKT
KPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYT
LPPCRDELTKNQVSLWCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKL
TVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK

(SEQ ID NO: 28)

Nucleotide sequence of Construct 3, CD3scFv-Fc (hole)

CD3 scFv in italics bold, then Fc (hole) with LA-LA mutations in bold underlined; hole mutations in italics larger font, bold

ATGGAGACAGACACACTCCTGCTATGGGTACTGCTGCTCTGGGTTCCAGGGTCGA
CTGGC***GAGGTGCAGCTGGTCGAGTCTGGAGGAGGATTGGTGCAGCCTGGAGGGTC***
ATTGAAACTCTCATGTGCAGCCTCTGGATTCACCTTCAATAAGTACGCCATGAACT
GGGTCCGCCAGGCTCCAGGAAAGGGTTTGGAATGGGTTGCTCGCATAAGAAGTAA

ATATAATAATTATGCAACATATTATGCCGATTCAGTGAAAGACAGGTTCACCATCTC
CAGAGATGATTCAAAAAACACTGCCTATCTACAAATGAACAACTTGAAAACTGAGG
ACACTGCCGTGTACTACTGTGTGAGACATGGGAACTTCGGTAATAGCTACATATCC
TACTGGGCTTACTGGGGCCAAGGGACTCTGGTCACCGTCTCCTCAGGTGGTGGTG
GTTCTGGCGGCGGCGGCTCCGGTGGTGGTGGTTCTCAGACTGTTGTGACTCAGGA
ACCTTCACTCACCGTATCACCTGGTGGAACAGTCACACTCACTTGTGGCTCCTCGA
CTGGGGCTGTTACATCTGGCAACTATCCAAACTGGGTCCAACAAAAACCAGGTCAG
GCACCCCGTGGTCTAATAGGTGGGACTAAGTTCCTCGCCCCCGGTACTCCTGCCAG
ATTCTCAGGCTCCCTGCTTGGAGGCAAGGCTGCCCTCACCCTCTCAGGGGTACAGC
CAGAGGATGAGGCAGAATATTACTGTGTTCTATGGTACAGCAACCGCTGGGTGTTC
*GGTGGAGGAACCAAACTGACTGTCCTA*GGCGGCGGAGGAAGTGCGGCCGCG
ACTCACACATGCCCACCGTGCCCAGCACCTGAA**GCCGCG**GGGGGACCGTCAGTCTTCCTCTTCC
CCCCAAAACCCAAGGACACCCTCATGATCTCCCGGACCCCTGAGGTCACATGCGTGGTGGTGGAC
GTGAGCCACGAAGACCCTGAGGTCAAGTTCAACTGGTACGTGGACGGCGTGGAGGTGCATAATGC
CAAGACAAAGCCGCGGGAGGAGCAGTACAACAGCACGTACCGTGTGGTCAGCGTCCTCACCGTCC
TGCACCAGGACTGGCTGAATGGCAAGGAGTACAAGTGCAAGGTCTCCAACAAAGCCCTCCCAGCC
CCCATCGAGAAAACCATCTCCAAAGCCAAAGGGCAGCCCCGAGAACCACAGGTG***TGC***ACCCTGC
CCCCATCCCGGGATGAGCTGACCAAGAACCAGGTCAGCCTG***TCC***TGC***GCC***GTCAAAGGCTTCTAT
CCCAGCGACATCGCCGTGGAGTGGGAGAGCAATGGGCAGCCGGAGAACAACTACAAGACCACGC
CTCCCGTGCTGGACTCCGACGGCTCCTTCTTCCTC***GTG***AGCAAGCTCACCGTGGACAAGAGCAGG
TGGCAGCAGGGGAACGTCTTCTCATGCTCCGTGATGCATGAGGCTCTGCACAACCACTACACGCAG
AAGAGCCTCTCCCTGTCTCCCGGGAAATGA
(SEQ ID NO: 29)

Amino-acid of Construct 3, CD3scFv-Fc (hole), without signaling peptide:

CD3 scFv in bold (linker underlined between CD3 VH and VL), in italics, FC in italics, L234A; L235A mutations in larger font; hole mutations (Y349C; T366S; L368A; Y407V underlined in bold, larger font as shown on FIG. 1C.

EVQLVESGGGLVQPGGSLKLSCAASGFTFNKYAMNWVRQAPGKGLEWVARIRSKYN
NYATYYADSVKDRFTISRDDSKNTAYLQMNNLKTEDTAVYYCVRHGNFGNSYISYWA
***YWGQGTLVTVSS*GGGGSGGGGSGGGGSQTVVTQEPSLTVSPGGTVTLTCGSSTGAVT**
SGNYPNWVQQKPGQAPRGLIGGTKFLAPGTPARFSGSLLGGKAALTLSGVQPEDEAE
YYCVLWYSNRWVFGGGTKLTVL*GGGGSAAATHTCPPCPAPE**AA**GGPSVFLFPPKPKD
TLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLH
*QDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQV**C**TLPPSRDELTKNQVSL**S**C**A**V*
*KGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFL**V**SKLTVDKSRWQQGNVFSCSVM*
HEALHNHYTQKSLSLSPGK (SEQ ID NO: 30)

EXAMPLE 6. EXPRESSION OF PLAP H2 AND PLAP H4-CD3 ANTIBODIES (FIG. 1A)

293S cells were used that were grown in Freestyle F17 Expression serum free medium with 8mM L-Glutamine (or GlutaMAX); 0.1% Pluoronic F-68. For transfection

NanoFect Transfection Reagent was used at ratio 3:1 (3 microliters for 1 microg DNA). Harvest the supernatant after 3-7 days of transfection.

The antibody protein supernatants were expressed and run on the SDS gel at reduced and non-reduced condition (adding beta-mercaptoethanol to lysis buffer) (FIG. 2). The gel showed 4 bands.

The protein was also purified using protein A or G columns. The purification was done with Millipore Sigma Protein A beads and Thermo IgG Elution buffer (Catalog number: 21004). After collection the samples were dialyzed using the Thermo Fisher Slide-A-Lyzer MINI Dialysis Devices. FIG. 3 shows purified PLAP h2-CD3 antibodies on SDS gel. The purified PLAP h2 antibody shows upper 206 kDA band at non-reducing conditions (A), this band disappears at reducing conditions (B).

EXAMPLE 7. BINDING OF CD3 AND PLAP ANTIGENS BY FACS

The FACS using bispecific PLAh2 and PLAP h4 antibodies (FIG. 1A) demonstrates that both antibodies bind to PLAP in PLAP-positive cells, and CD3 using T cells (FIG. 4).

The bispecific antibodies were tested with PLAP-positive and PLAP-negative cell lines. CD3-positive T cells were used for testing binding to CD3. Bispecific antibodies had positive binding with both PLAP and CD3 antigens. FIG. 4 shows the results of PLAP h2 - CD3 antibody. Similar result was observed for PLAP h4-Cd3 antibody (data not shown).

EXAMPLE 8. CYTOTOXIC ACTIVITY OF PLAP-CD3 ANTIBODY WITH T CELLS ON PLAP-POSITIVE CELL TARGET LINE

The antibody supernatants together with T cells were used for RTCA assay. Both bispecific antibodies added with activated T cells killed PLAP-positive cells and did not kill without T cells. PLAP-h2-CD3 plus T cells killed PLAP-positive cells and did not kill PLAP-negative HT29 cells (FIGs. 5A-5B). Antibody alone did not kill colon cancer cell line. T cells alone also did not kill target cells. This demonstrates high specificity of bispecific antibody when used together with T cells confirming mechanism of bringing T cells to cancer cells through bispecific antibody binding to CD3 antigen in T cells and to PLAP antigen.

PLAP h4-CD3 antibody when used with activated T cells killed PLAP-positive cells and did not kill PLAP-negative cells (FIGs. 6A=6B). PLAP h4-CD3 antibody alone did not kill PLAP-positive target cells. In addition, bispecific antibodies demonstrated dose-dependent activity (not shown).

EXAMPLE 9. *IN VIVO* ACTIVITY IN MICE

We administered bispecific antibody PLAP h2-CD3 (FIG. 1A structure) with T cells in Lovo xenograft mouse model (FIG. 7). The first injection of $1x10^{\wedge 7}$ T cells was done at day 4, and bispecific antibody (10 micrograms to each mice or 0.5 mg/kg) was injected intravenously (iv) at day 7; then T cells with antibody were injected together by iv on days 7, 10, 14 and 17. Bispecific PLAP h2-CD3 antibody with T cells significantly decreased xenograft tumor growth (FIG. 7).

EXAMPLE 10. BIVALENT HUMANIZED PLAP H4-CD3 SCFV PLUS T CELLS SPECIFICALLY KILLED PLAP-POSITIVE CELLS AND SECRETE IFN-GAMMA

The bivalent bispecific humanized PLAPh4 with CD3 ScFv antibody (see FIG. 1B, PBM0015) showed as a single band on SDS gel (FIG 8) with molecular weight around 130 kDa. PBM0015 antibody specifically bound to PLAP in Lovo cells and not to HCT116 (PLAP-negative cells); it also bound to CD3 as detected by FACS (not shown). PBM0015 antibody and T cells specifically killed PLAP-positive Lovo target cells in a dose-dependent manner (FIG 9) and had minimal killing of PLAP-negative HCT116 cells (not shown). PBM0015 antibody with T cells secreted high level of IFN-gamma with Lovo cells but not with PLAP-negative HCT116 (FIG 10). The results demonstrate high and specific activity of this antibody.

EXAMPLE 11. UNIVALENT PLAP H2-CD3 SCFV ANTIBODY WITH T CELLS SPECIFICALLY KILLED PLAP-POSITIVE CELLS AND SECRETED IFN-GAMMA

The bispecific univalent humanized PLAP h2 with CD3 Scfv antibody with structure as shown in FIG 1 C (PLAPh2-3) was run as one band on SDS gel (MW>100 kDa) (not shown). Humanized PLAP h2-CD3 antibody bound to PLAP in PLAP-positive Lovo, LS123 cells and not in HCT116 cells, it also bound to CD3 by FACS analysis (not shown). PLAPh2-3 antibody and T cells specifically killed PLAP-positive Lovo target cells and did not kill PLAP-negative cells (FIGs. 11A-B). The cytotoxic activity was similar or higher than PLAPh2 and PLAPh4 having the structure of FIG. 1A. The PLAPh2-3 Ab with T cells also secreted significant level of IFN-gamma with PLAP-positive cells but not with PLAP-negative cells (FIGs. 11C-D).

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WHAT IS CLAIMED IS:

1. A bispecific antigen-binding molecule comprising: (a) a first and a second antigen-binding moiety each of which is a humanized Fab molecule capable of specific binding to human PLAP, and each comprises a heavy chain variable region (PALP VH) having the amino acid sequence of SEQ ID NO: 10 and a light chain variable region (PLAP VL) having the amino acid sequence of SEQ ID NO: 5; (b) a third antigen-binding moiety which is a Fab molecule capable of specific binding to human CD3 epsilon, the third antigen-binding moiety comprises a heavy chain variable region (CD3 VH) having the amino acid sequence of SEQ ID NO: 11 and a light chain variable region (CD3 VL) having the amino acid sequence of SEQ ID NO: 7, wherein the third antigen-binding moiety is a crossover Fab molecule, in which the constant regions of the Fab light chain and the Fab heavy chain are exchanged; and (c) an human IgG Fc domain comprising a first subunit and a second subunit capable of stable association;

wherein the Fab heavy chain of the third antigen-binding moiety is (i) fused at the N-terminus to the C-terminus of the Fab heavy chain of the first antigen-binding moiety (CH1), and (ii) fused at the C-terminus to the N-terminus of the first subunit of the Fc knob domain, and wherein the second antigen-binding moiety is fused at the C-terminus of the Fab heavy chain (CH1) to the N-terminus of the second subunit of the Fc hole domain.

2. A bispecific antigen-binding molecule comprising: (a) a first and a second antigen-binding moiety each of which is a humanized Fab molecule capable of specific binding to human PLAP, and each comprises a heavy chain variable region (PLAP VH) having the amino acid sequence of SEQ ID NO: 19 and a light chain variable region (PLAP VL) having the amino acid sequence of SEQ ID NO: 16; (b) a third antigen-binding moiety which is a Fab molecule capable of specific binding to human CD3 epsilon, the third antigen-binding moiety comprises a heavy chain variable region (CD3 VH) having the amino acid sequence of SEQ ID NO: 11 and a light chain variable region (CD3 VL) having the amino acid sequence of SEQ ID NO: 7, wherein the third antigen-binding moiety is a crossover Fab molecule, in which the constant regions of the Fab light chain and the Fab heavy chain are exchanged; and (c) an human IgG Fc domain comprising a first subunit and a second subunit capable of stable association;

wherein the Fab heavy chain of the third antigen-binding moiety is (i) fused at the N-terminus to the C-terminus of the Fab heavy chain of the first antigen-binding moiety (CH1), and (ii) fused at the C-terminus to the N-terminus of the first subunit of the Fc knob domain,

and wherein the second antigen-binding moiety is fused at the C-terminus of the Fab heavy chain (CH1) to the N-terminus of the second subunit of the Fc hole domain.

3. The bispecific antigen-binding molecule of claim 1 or 2, wherein the human Fc domain comprises one or more amino acid substitutions promoting the association of the first and the second subunit of the Fc domain.

4. The bispecific antigen-binding molecule of claim 1 or 2, wherein said one or more amino acid substitutions are at one or more positions selected from the group of L234, L235, and P329 (EU numbering).

5. A bispecific antigen-binding molecule comprising two binding moieties to PLAP, and one binding moiety to CD3 epsilon, the molecules comprises the amino acid sequences of SEQ ID NO: 5, 8, 12, and 14, in a molar ratio of 2:1:1:1.

6. A bispecific antigen-binding molecule comprising two binding moieties to PLAP, and one binding moiety to CD3 epsilon, the molecules comprises the amino acid sequences of SEQ ID NO: 17, 8, 20, and 22, or at least 95% sequence identity thereof, in a molar ratio of 2:1:1:1.

7. A bispecific antigen-binding molecule comprising two binding moieties to PLAP, and one binding moiety to CD3 epsilon, the molecules comprises the amino acid sequences of SEQ ID NO: 17, 24, and 22, or at least 95% sequence identity thereof, in a molar ratio of 2:1:1.

8. A bispecific antigen-binding molecule comprising one binding moiety to PLAP, and one binding moiety to CD3 epsilon, the molecules comprises the amino acid sequences of SEQ ID NO: 5, 28, and 30, or at least 95% sequence identity thereof, in a molar ratio of 2:1:1.

9. A bispecific antigen-binding molecule comprising one binding moiety to PLAP, and one binding moiety to CD3 epsilon, the molecules comprises the amino acid sequences of SEQ ID NO: 17, 28, and 30, or at least 95% sequence identity thereof, in a molar ratio of 2:1:1.

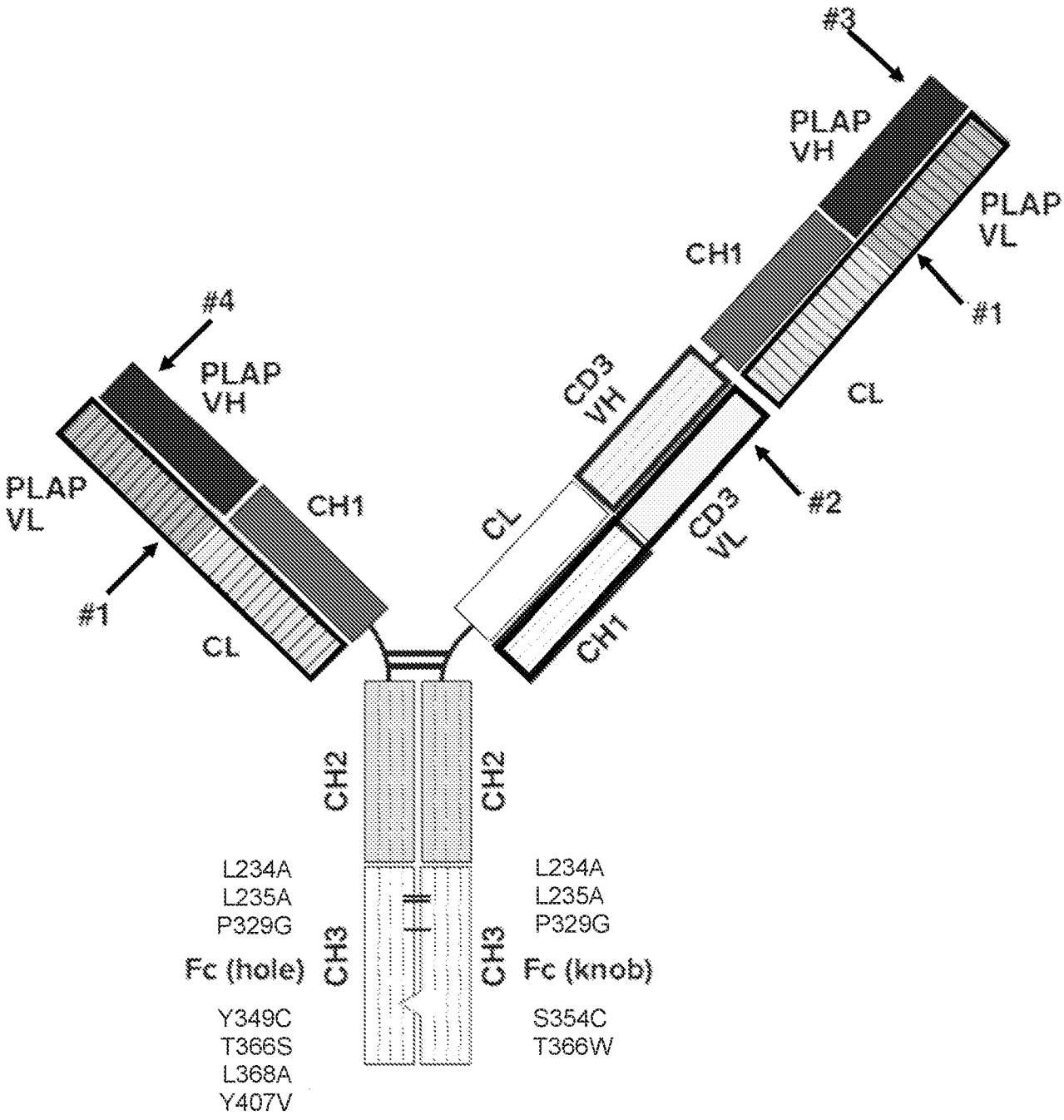
#3
PLAP
VH
PLAP
VL
CH1
#1
#4
PLAP
VH
CD3
VH
CL
PLAP
VL
CH1
#2
CL
CD3
VL
#1
CH1
CL
CH2
CH2
L234A
L235A
P329G
Fc (hole)
CH3
CH3
Fc (knob)
L234A
L235A
P329G
Y349C
T366S
L368A
Y407V
S354C
T366W

FIG. 1A

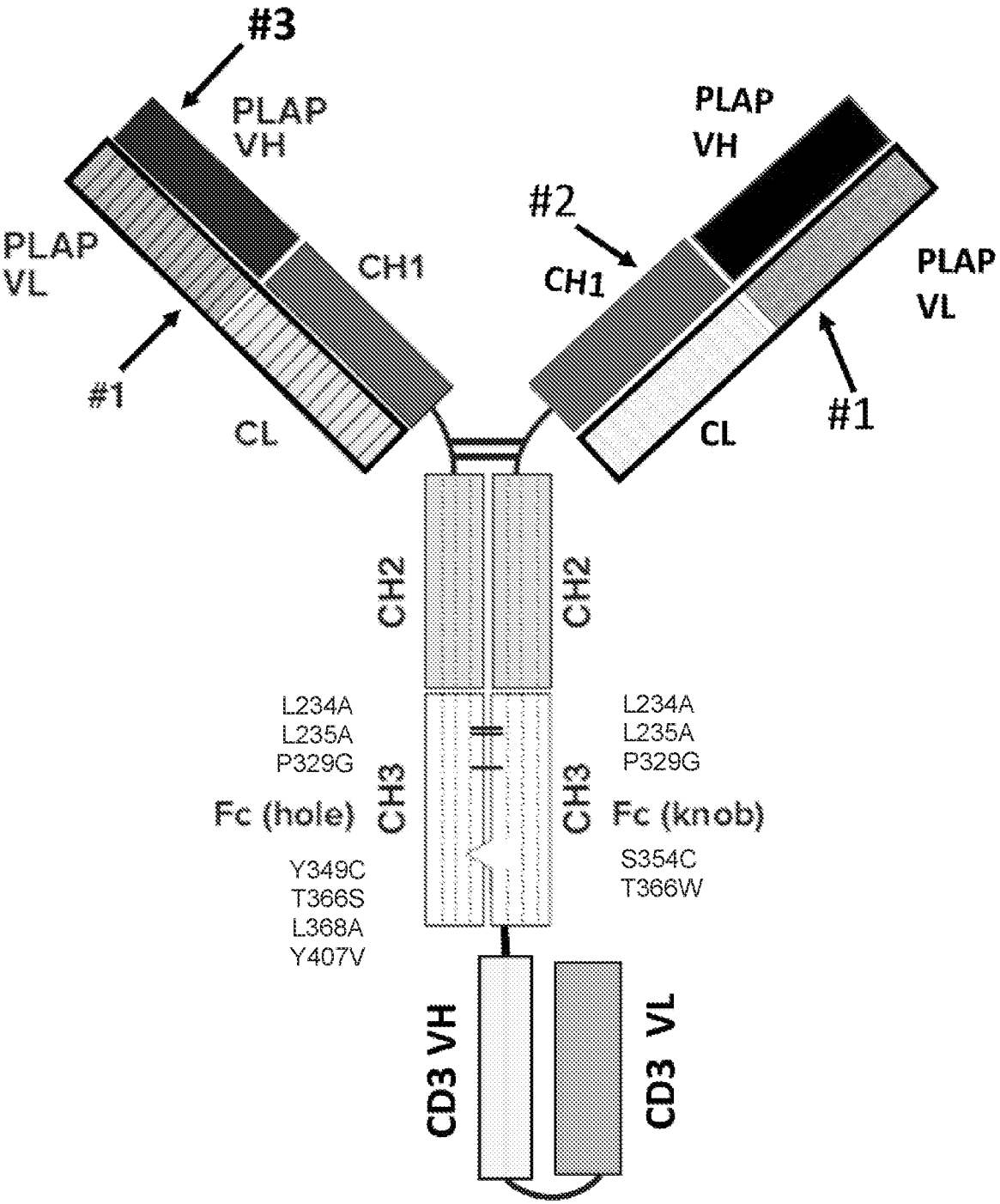
#3
PLAP
VH
PLAP
VL
CH1
#1
CL
PLAP
VH
#2
CH1
PLAP
VL
#1
CL
CH2
CH2
L234A
L235A
P329G
Fc (hole)
CH3
Y349C
T366S
L368A
Y407V
CH3
L234A
L235A
P329G
Fc (knob)
S354C
T366W
CD3 VH
CD3 VL

FIG. 1B

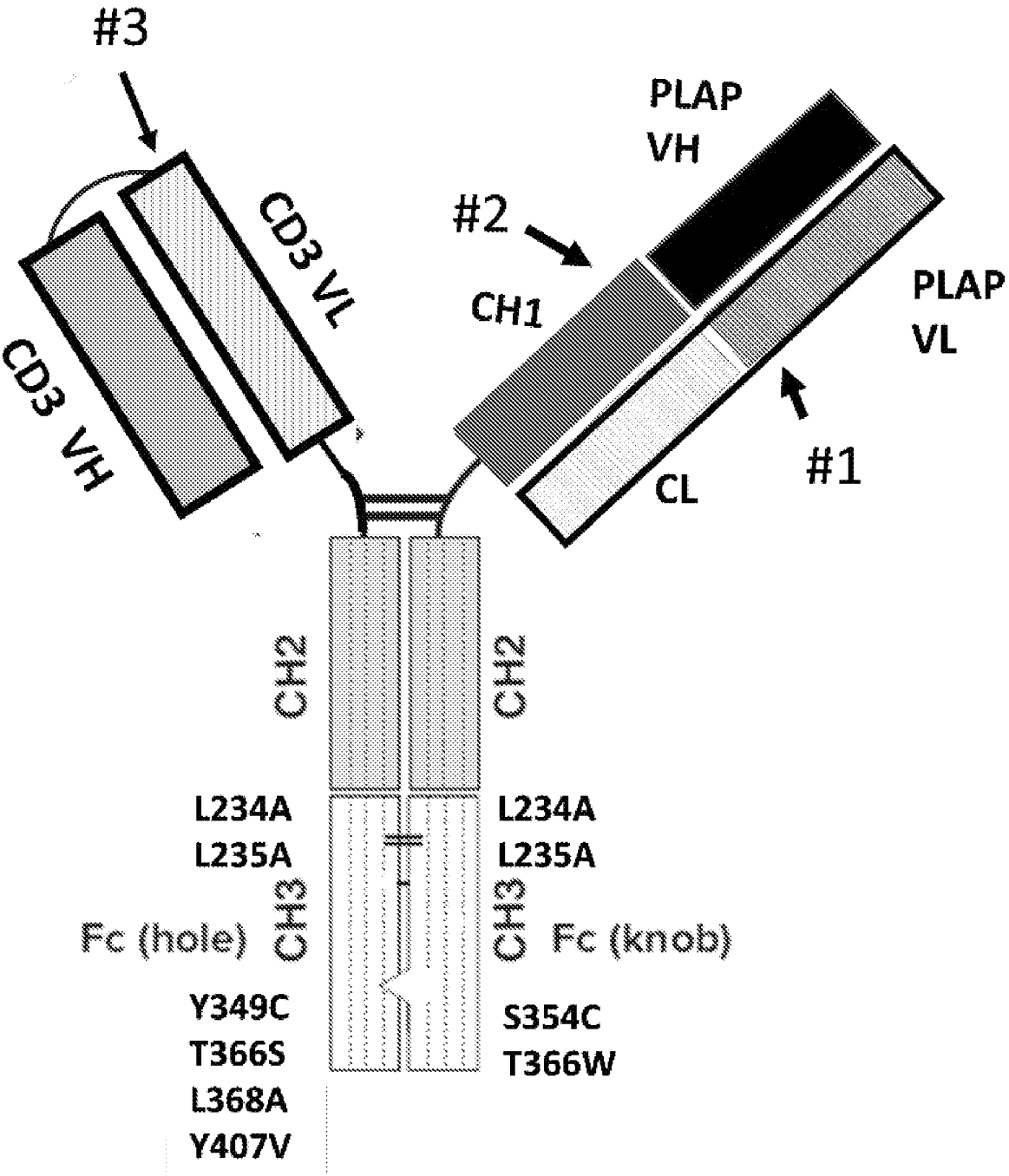
#3
CD3 VL
CD3 VH
PLAP
VH
#2
CH1
PLAP
VL
#1
CL
CH2
CH2
L234A
L235A
Fc (hole)
CH3
CH3
Fc (knob)
L234A
L235A
Y349C
T366S
L368A
Y407V
S354C
T366W

FIG. 1C

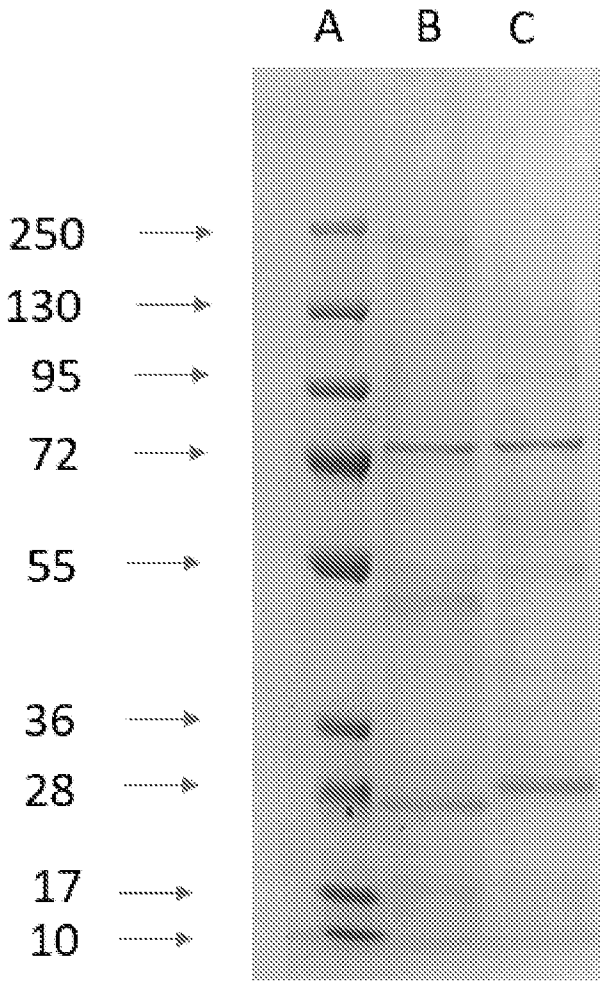

FIG. 2

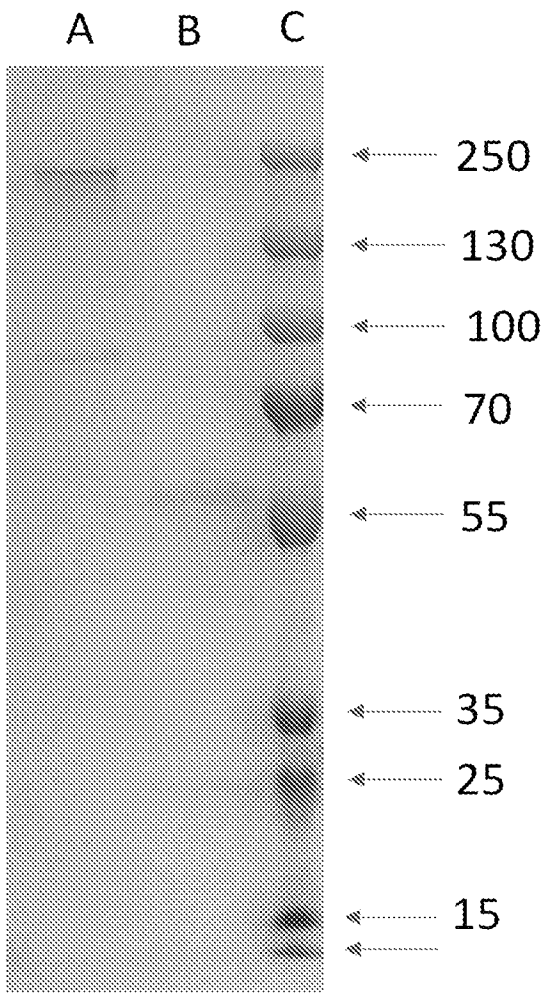

FIG. 3

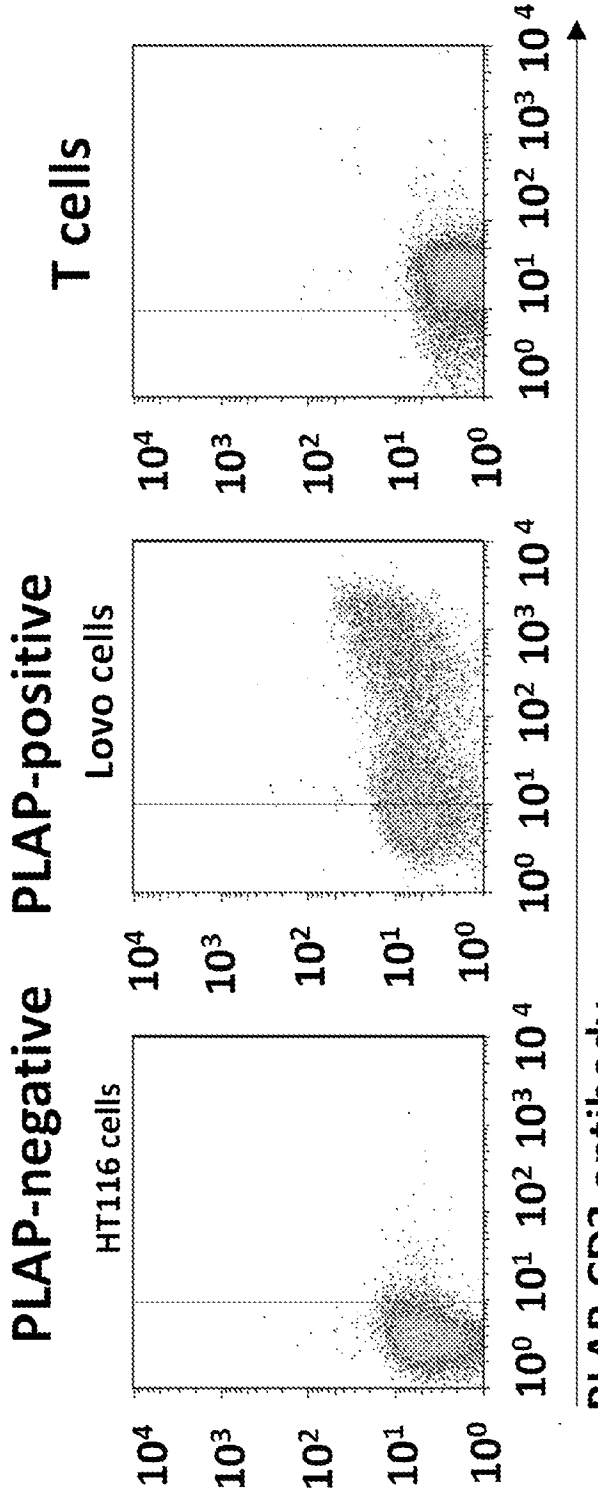
PLAP-negative
PLAP-positive
HT116 cells
Lovo cells
T cells
PLAP-CD3 antibody
10^0 10^1 10^2 10^3 10^4

FIG. 4

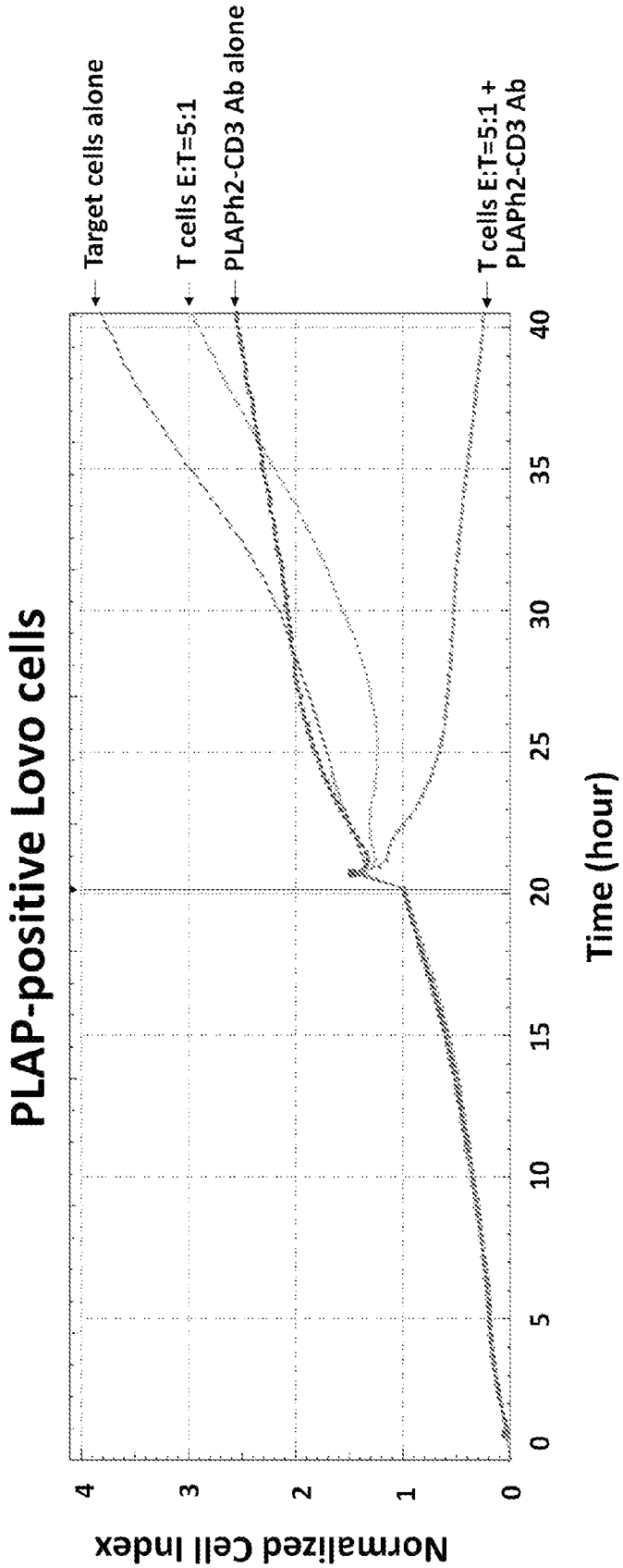
PLAP-positive Lovo cells
Target cells alone
T cells E:T=5:1
PLAPh2-CD3 Ab alone
T cells E:T=5:1 + PLAPh2-CD3 Ab
Normalized Cell Index
Time (hour)
0
1
2
3
4
0
5
10
15
20
25
30
35
40

FIG. 5A

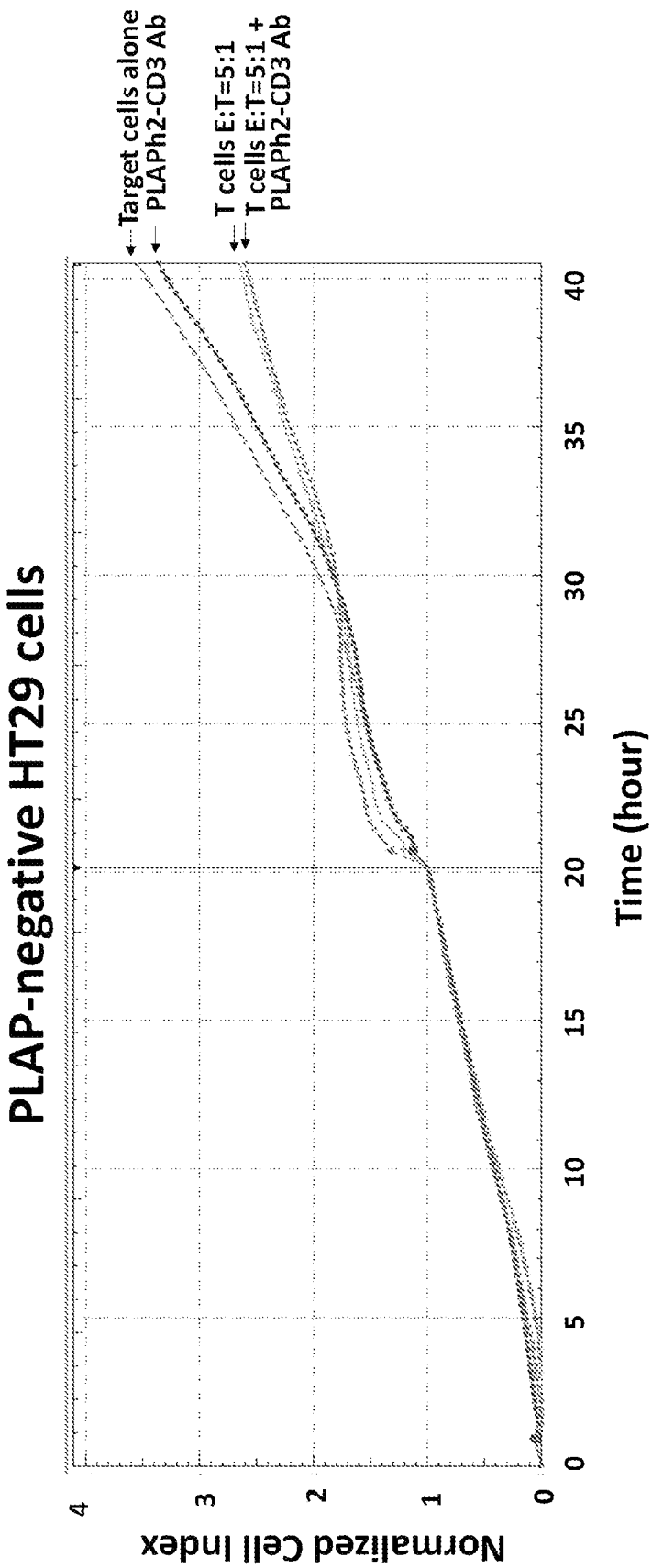
PLAP-negative HT29 cells
Target cells alone
PLAPh2-CD3 Ab
T cells E:T=5:1
T cells E:T=5:1 +
PLAPh2-CD3 Ab
Normalized Cell Index
Time (hour)
0
1
2
3
4
0
5
10
15
20
25
30
35
40

FIG. 5B

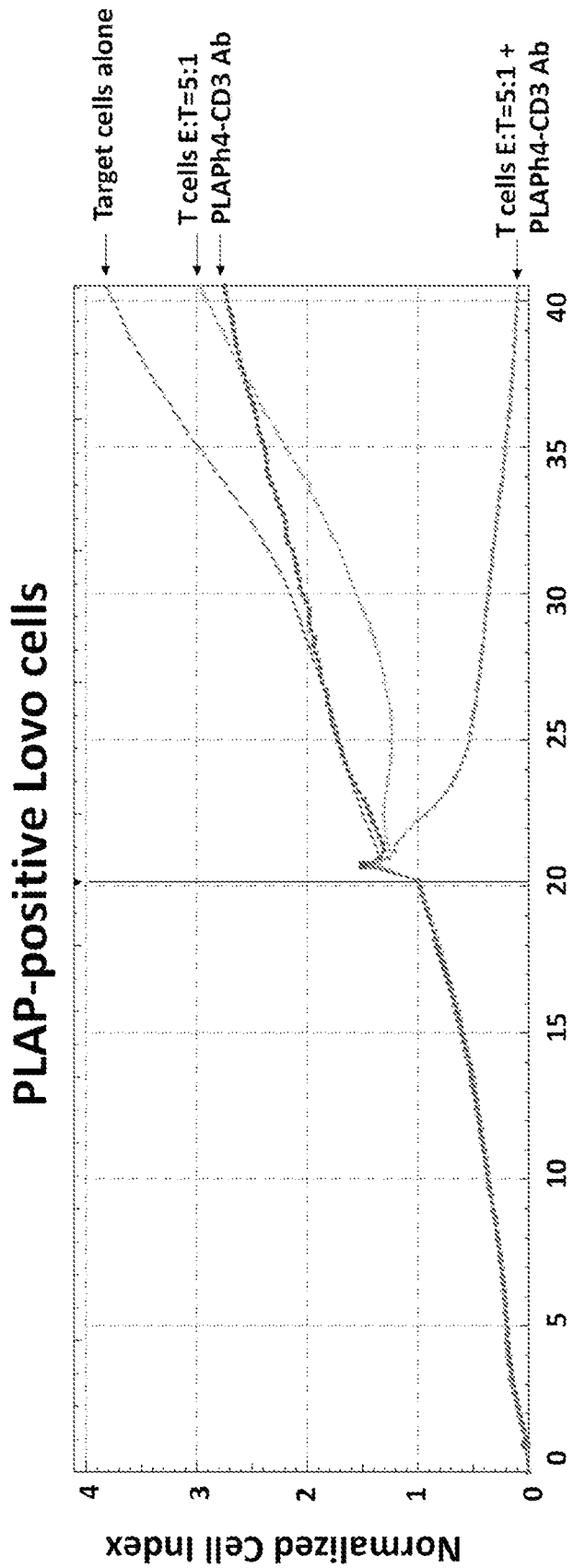
PLAP-positive Lovo cells
Target cells alone
T cells E:T=5:1
PLAPh4-CD3 Ab
T cells E:T=5:1 +
PLAPh4-CD3 Ab
Normalized Cell Index
Time (hour)
0
1
2
3
4
0
5
10
15
20
25
30
35
40

FIG. 6A

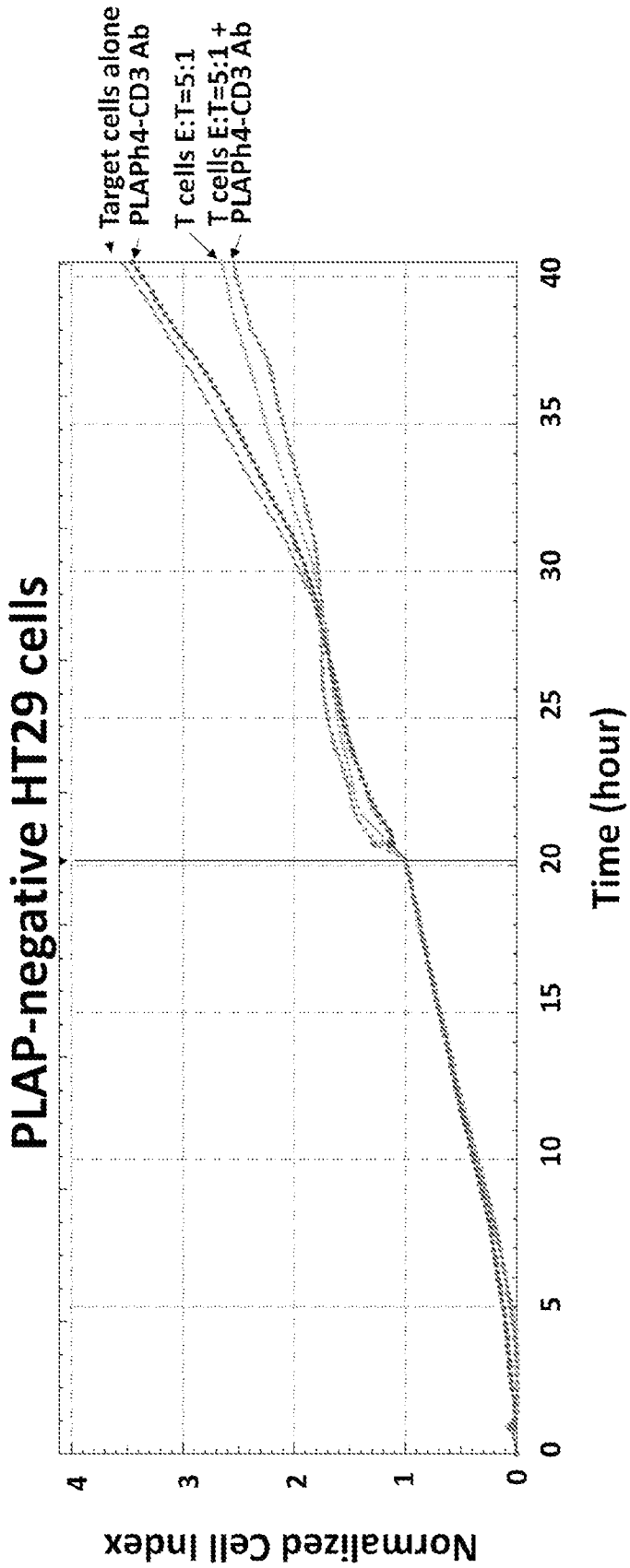
PLAP-negative HT29 cells
Target cells alone
PLAPh4-CD3 Ab
T cells E:T=5:1
T cells E:T=5:1 +
PLAPh4-CD3 Ab
Normalized Cell Index
Time (hour)
0
1
2
3
4
0
5
10
15
20
25
30
35
40

FIG. 6B

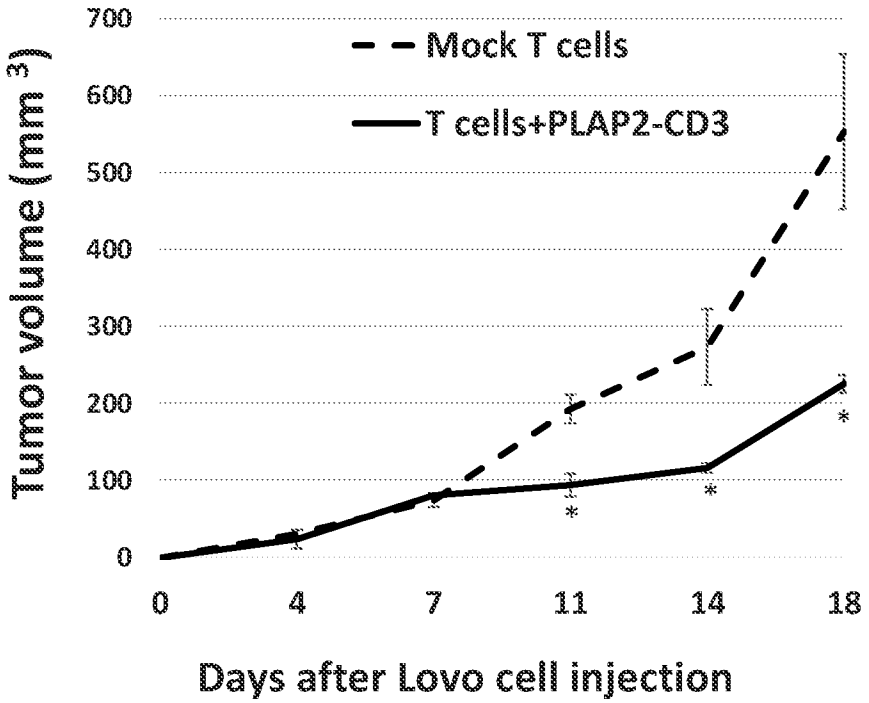
Tumor volume (mm 3)
700
600
500
400
300
200
100
0
Mock T cells
T cells+PLAP2-CD3
0
4
7
11
14
18
Days after Lovo cell injection

FIG. 7

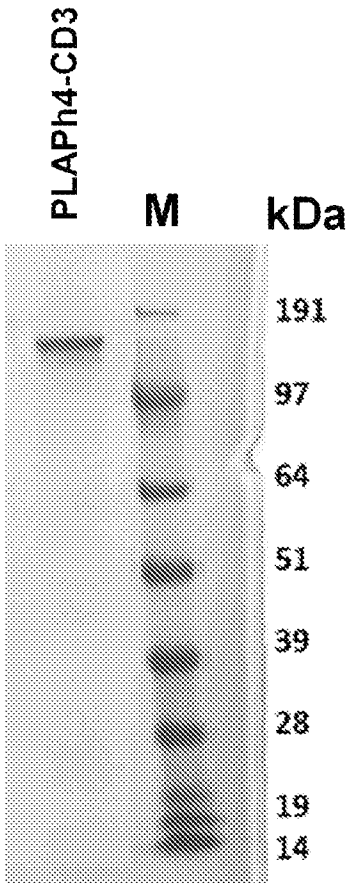

FIG. 8

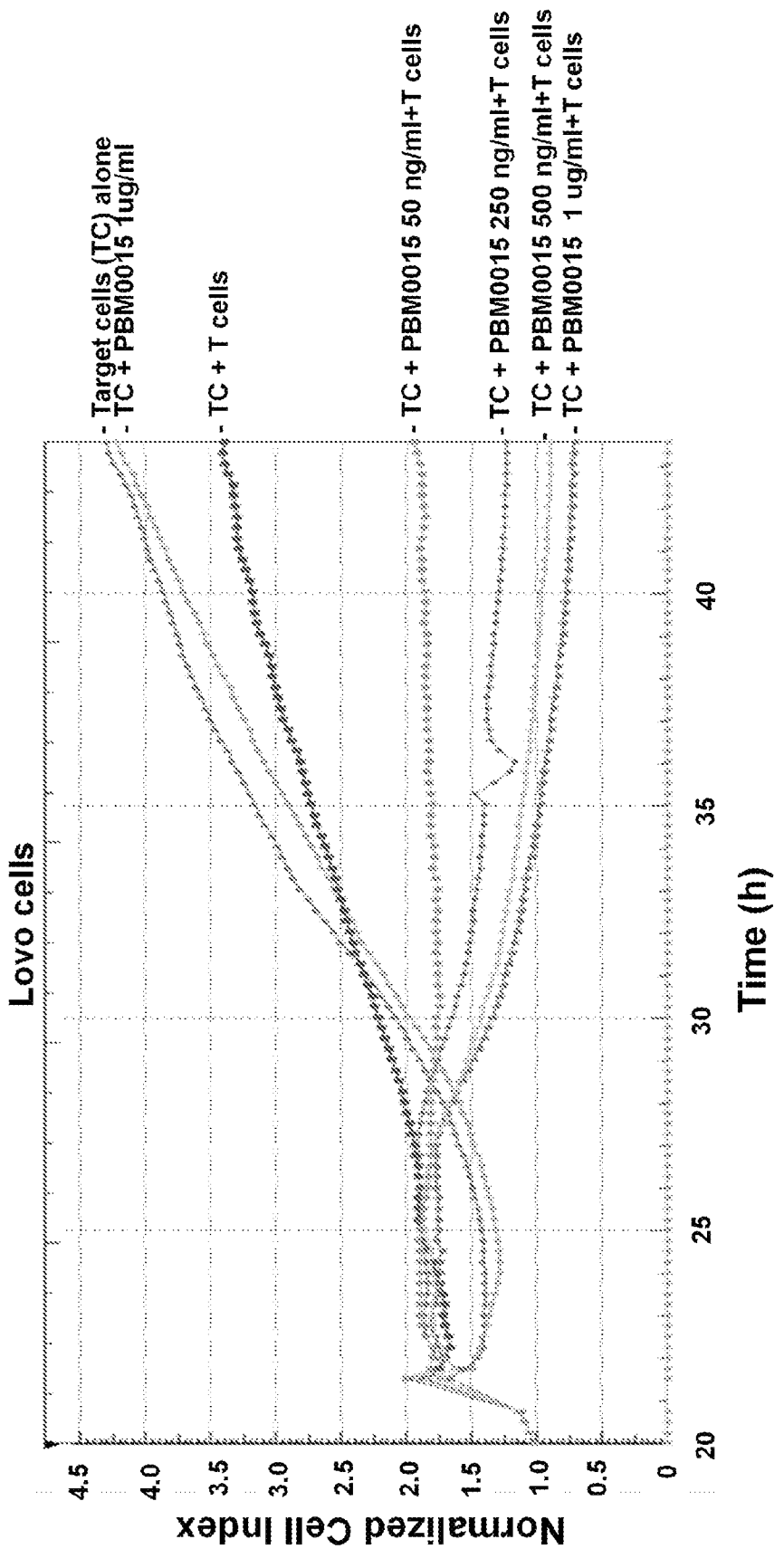
Lovo cells
Normalized Cell Index
Time (h)
- Target cells (TC) alone
- TC + PBM0015 1ug/ml
- TC + T cells
- TC + PBM0015 50 ng/ml+T cells
- TC + PBM0015 250 ng/ml+T cells
- TC + PBM0015 500 ng/ml+T cells
- TC + PBM0015 1 ug/ml+T cells
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
20
25
30
35
40

FIG. 9

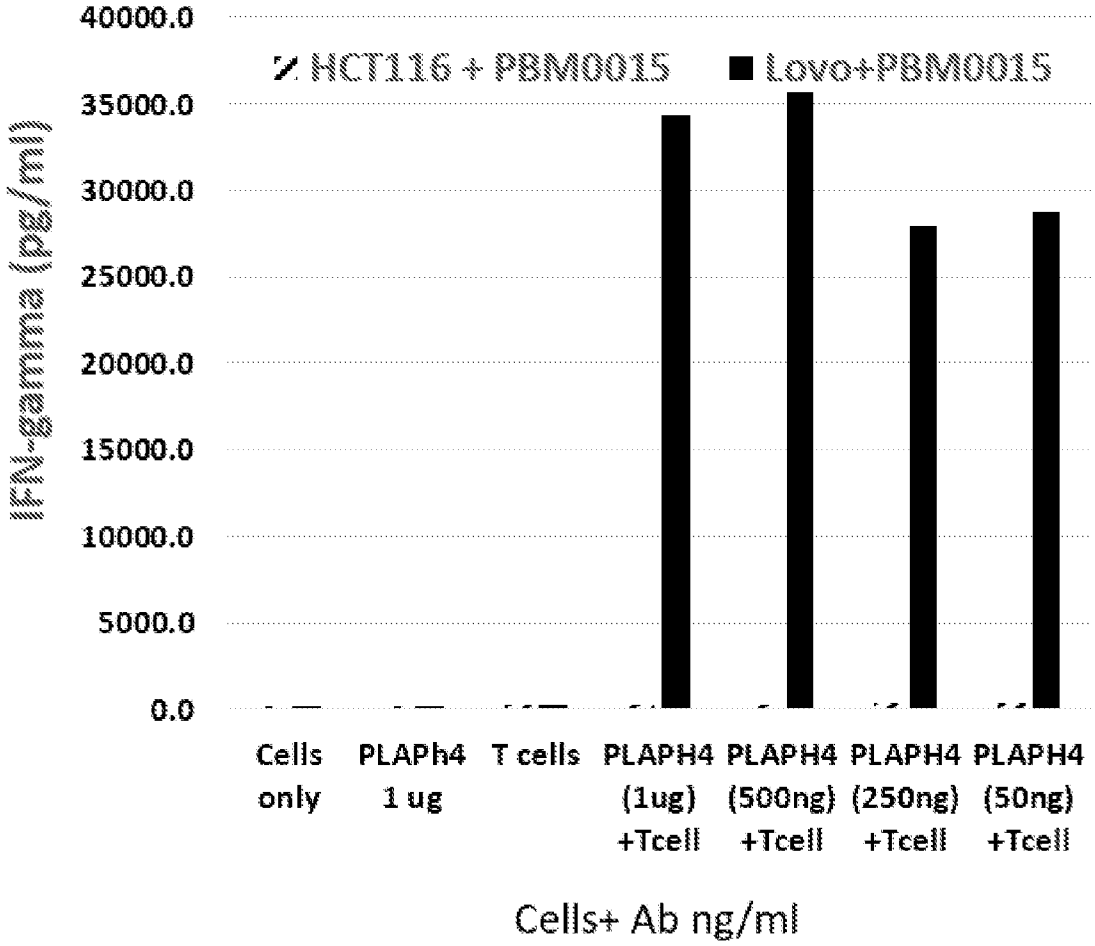
40000.0
35000.0
30000.0
25000.0
20000.0
15000.0
10000.0
5000.0
0.0
IFN-gamma (pg/ml)
HCT116 + PBM0015
Lovo+PBM0015
Cells only
PLAPh4 1 ug
T cells
PLAPH4 (1ug) +Tcell
PLAPH4 (500ng) +Tcell
PLAPH4 (250ng) +Tcell
PLAPH4 (50ng) +Tcell
Cells+ Ab ng/ml

FIG. 10

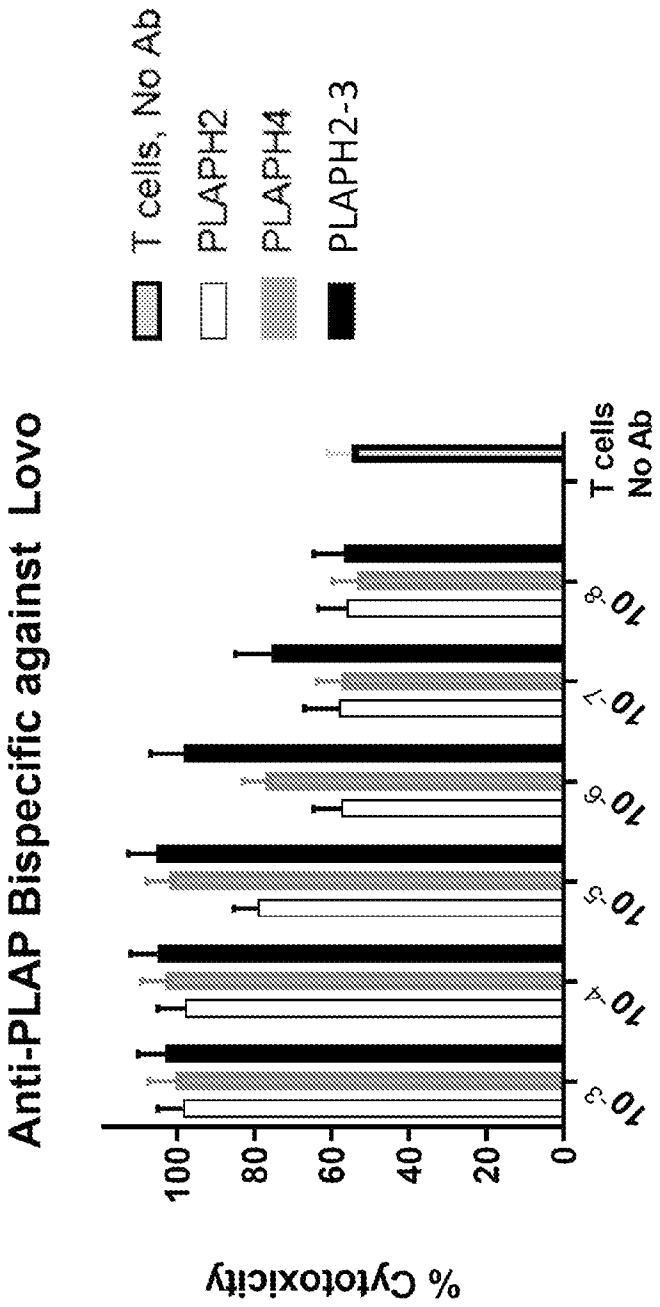
Anti-PLAP Bispecific against Lovo
T cells, No Ab
PLAPH2
PLAPH4
PLAPH2-3
% Cytotoxicity
100
80
60
40
20
0
10-3
10-4
10-5
10-6
10-7
10-8
T cells No Ab
Bispecific Ab mg/ml
T cells

FIG. 11A

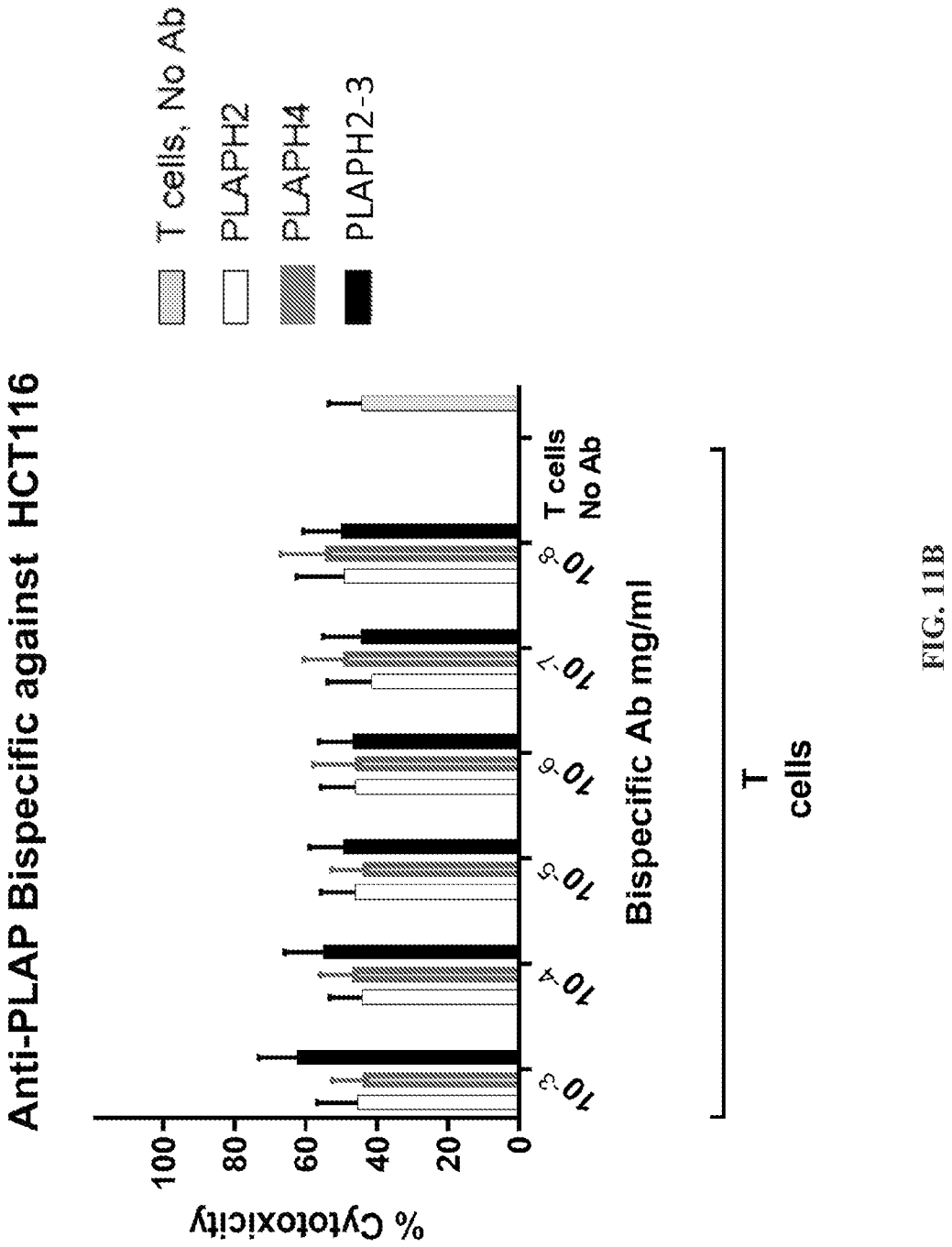
Anti-PLAP Bispecific against HCT116
T cells, No Ab
PLAPH2
PLAPH4
PLAPH2-3
% Cytotoxicity
0
20
40
60
80
100
T cells No Ab
10-3
10-4
10-5
10-6
10-7
10-8
Bispecific Ab mg/ml
T cells

FIG. 11B

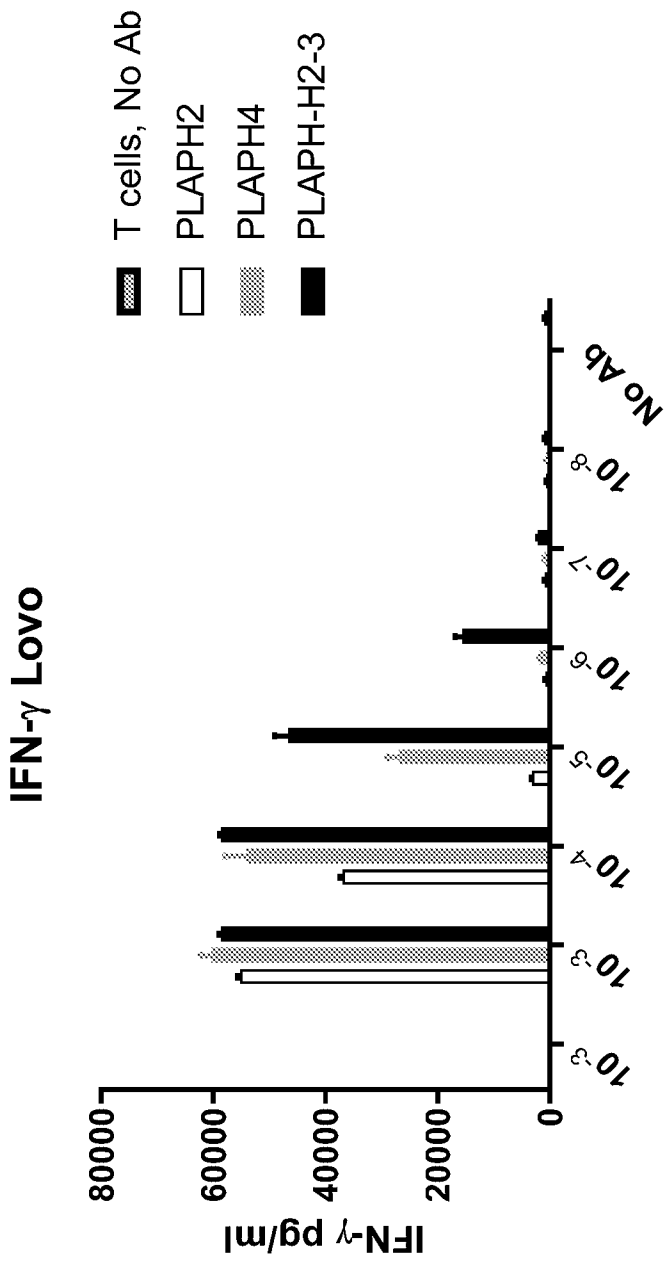
IFN-γ Lovo
T cells, No Ab
PLAPH2
PLAPH4
PLAPH-H2-3
IFN-γ pg/ml
80000
60000
40000
20000
0
10-3
10-2
10-4
10-5
10-6
10-7
10-8
No Ab
Bispecific Ab mg/ml
No T cells
T cells

FIG. 11C

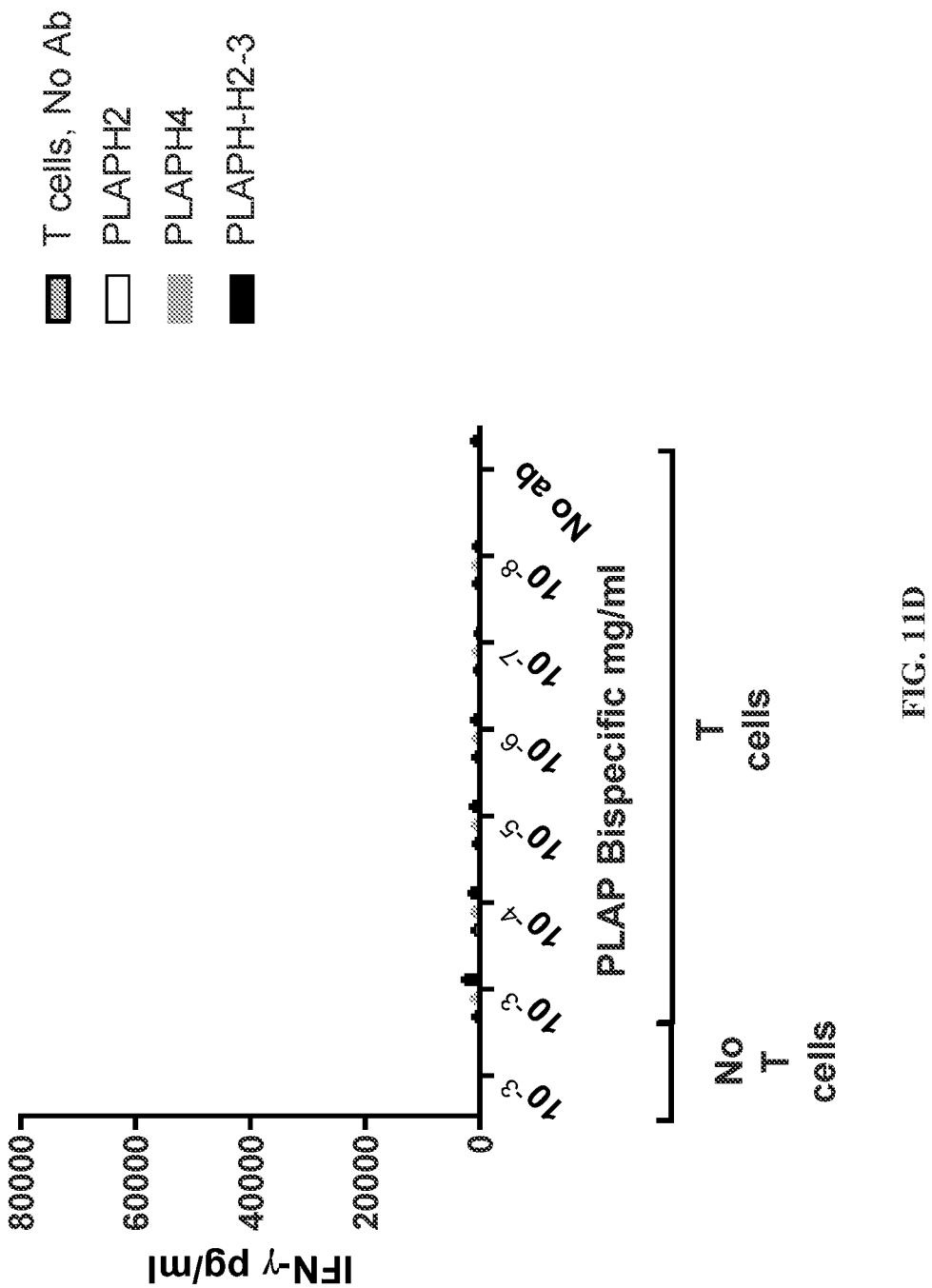
IFN-γ HCT116
T cells, No Ab
PLAPH2
PLAPH4
PLAPH-H2-3
IFN-γ pg/ml
80000
60000
40000
20000
0
No T cells
T cells
PLAP Bispecific mg/ml
10-3
10-4
10-5
10-6
10-7
10-8
No ab

FIG. 11D

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 21/13916

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:

      ☒ in the form of an Annex C/ST.25 text file.

      ☐ on paper or in the form of an image file.

   b. ☐ furnished together with the international application under PCT Rule 13*ter*.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.

   c. ☐ furnished subsequent to the international filing date for the purposes of international search only:

      ☐ in the form of an Annex C/ST.25 text file (Rule 13*ter*.1(a)).

      ☐ on paper or in the form of an image file (Rule 13*ter*.1(b) and Administrative Instructions, Section 713).

2. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 21/13916

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.:
because they relate to subject matter not required to be searched by this Authority, namely:

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).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:
This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

----------Please see continuation in first extra sheet----------

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1 and 3-4, limited to SEQ ID NOs: 5, 7, 10-11

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 21/13916

A. CLASSIFICATION OF SUBJECT MATTER

IPC - C07K 16/30, C07K 16/28, C07K 14/705 (2021.01)

CPC - C07K 14/7051, C07K 16/2809, C07K 16/2875, C07K 2317/31, C07K 2317/522

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)
See Search History document

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
See Search History document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 2018/208864 A1 (ADIMAB, LLC) 15 November 2018 (15.11.2018) para [0003], [0007], [0010], [0026], [0069], [0096], [0217], [0220], [0235], [0343], Clause 55, Clause 68	1, 3-4
A	WO 2019/240934 A1 (PROMAB BIOTECHNOLOGIES, INC) 19 December 2019 (19.12.2019) Claim 3, Seq ID No: 21, Abstract, claim 3, SEQ ID NO: 21	1, 3-4
A	WO 2011/002968 A2 (GLAXO GROUP LIMITED) 06 January 2011 (06.01.2011) Claim 38, Claim 44, SEQ ID NO: 98.	1, 3-4
A	WO 2014/056783 A1 (ROCHE GLYCART AG) 17 April 2014 (17.04.2014) pg 5 ln 25-30, pg 20 ln 20-25, Seq ID No: 169, Seq ID No: 173, Abstract	1, 3-4

☐ 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
31 March 2021 (31.03.2021)	JUN 03 2021
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300	Authorized officer Lee Young Telephone No. PCT Helpdesk: 571-272-4300

Form PCT/ISA/210 (second sheet) (July 2019)

Continuation of Box No. III. Observations where unity of invention is lacking

Group I+: Claims 1-9 directed to a bispecific antigen-binding molecule composition comprising: (a) a first and a second antigen binding moiety each of which is a humanized Fab molecule capable of specific binding to human PLAP, and each comprises heavy and light chain variable regions (PALP VH, VL); (b) a third antigen-binding moiety which is optionally a crossover Fab molecule (constant regions of light and heavy chains are exchanged) capable of specific binding to human CD3 epsilon, comprising anti-CD3 VH and VL and (c) an human IgG Fc domain comprising first and second subunits capable of stable association; wherein the Fab heavy chain of the third antigen-binding moiety is (i) fused at the N-terminus to the C-terminus of the Fab heavy chain of the first antigen-binding moiety (CH1), and (ii) fused at the C-terminus to the N-terminus of the first subunit of the Fc knob domain, and the second antigen-binding moiety is fused at the C-terminus of the Fab heavy chain (CH1) to the N-terminus of the second subunit of the Fc hole domain. The bispecific antigen-binding molecule (humanized h2) composition will be searched to the extent that the PALP VH and VL comprise SEQ ID NOs: 10 and 5, the (crossover Fab) CD3 VH and VH comprise SEQ ID NOs: 11 and 7, respectively, and wherein the human Fc domain comprises an L234 substitution. It is believed that claims 1 and 3-4, limited to the antigen-binding molecule having said sequence features encompass this first named invention, and thus these claims will be searched without fee to the extent that the bispecific molecule comprises said sequences. Additional PALP-CD3 bispecific antigen-binding molecules will be searched upon the payment of additional fees. Applicants must specify the claims that encompass any additionally elected PALP-CD3 bispecific antigen-binding molecules. Applicants must further indicate, if applicable, the claims which encompass the first named invention, if different than what was indicated above for this group. Failure to clearly identify how any paid additional invention fees are to be applied to the "+" group(s) will result in only the first claimed invention to be searched. An exemplary election would be a bispecific antigen-binding molecule (humanized h4) composition will be searched to the extent that the PALP VH and VL comprise SEQ ID NOs: 19 and 16, the (crossover Fab) CD3 VH and VL comprise SEQ ID NOs: 11 and 7, respectively, and wherein the human Fc domain comprises an L235 substitution (claims 2-4). Another exemplary election would be a bispecific antigen-binding molecule comprising two binding moieties to PLAP, and one binding moiety to CD3 epsilon, the molecules comprises the amino acid sequences of SEQ ID NOs: 17, 8, 20, and 22, or at least 95% sequence identity thereof, in a molar ratio of 2:1:1:1, provided that the sequence variation is in the non-CDR framework regions (see instant specification, pg 8 ln 6) (claim 6).

The inventions listed as Groups I+ do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

Special Technical Features

No technical features are shared between the bispecific binding molecule amino acid sequences of Group I+, accordingly, these groups lack unity a priori.

Additionally, even if the inventions listed as Group I+ were considered to share technical features, these shared technical features are previously disclosed by the prior art, as further discussed below.

Common Technical Features

The inventions of Group I+ share the technical feature of a bispecific antigen-binding molecule composition comprising: (a) a first and a second antigen binding moiety each of which is a humanized Fab molecule capable of specific binding to human PLAP, and each Fab comprises a VH and a VL, (b) a third antigen-binding moiety having a VH and VL capable of specific binding to human CD3 epsilon, optionally having a crossover Fab, and (c) a human IgG Fc domain comprising a first second subunits capable of stable association; wherein the Fab heavy chain of the third antigen-binding moiety is (i) fused at the N-terminus to the C-terminus of the Fab heavy chain of the first antigen-binding moiety (CH1), and (ii) fused at the C-terminus to the N-terminus of the first subunit of the Fc knob domain, and the second antigen-binding moiety is fused at the C-terminus of the Fab heavy chain (CH1) to the N-terminus of the second subunit of the Fc hole domain.

********** See Next Extra Sheet to continue **********

Continuation of Box No. III. Observations where unity of invention is lacking

continuation of previous extra sheet:

However, this shared technical feature does not represent a contribution over prior art, because the shared technical feature is anticipated by WO 2018/208864 A1 to Adimab, LLC (hereinafter 'Adimab'). Adimab discloses a bispecific antigen-binding molecule comprising: (a) a first and a second antigen binding moiety each of which is a humanized Fab molecule capable of specific binding to human PLAP (para [0069] - "the inventive CD3 binding domains and antibodies comprising them comprise a multispecific antibody, for example, a bispecific antibody wherein the multispecific antibody comprises a second binding domain having specificity for a second antigen selected from...placental alkaline phosphatase (PLAP)"; para [0217]; Clause 55), and each comprises a heavy chain variable region (PALP VH) having the amino acid sequence and a light chain variable region (PLAP VL), (b) a third antigen-binding moiety which is a Fab molecule capable of specific binding to human CD3 epsilon (para [0003] - "The invention relates...to anti-Cluster of Differentiation 3 (CD3)- binding domains and antibodies comprising them, including multispecific and bispecific antibodies") optionally having a crossover Fab, (para [0220] - "Multispecific antibodies may also be engineered using immunoglobulin crossover (also known as Fab domain exchange or CrossMab format) technology"), in which the constant regions of the Fab light chain and the Fab heavy chain are exchanged (para [0220] - "Multispecific antibodies...(also known as Fab domain exchange or CrossMab format) technology"); and (c) an human IgG Fc domain comprising a first subunit and a second subunit capable of stable association (para [0235] - "The Fc region variant may comprise a human Fc region sequence"); wherein the Fab heavy chain of the third antigen-binding moiety is (i) fused at the N-terminus to the C-terminus of the Fab heavy chain of the first antigen-binding moiety (CH1), and (ii) fused at the C-terminus to the N-terminus of the first subunit of the Fc knob domain, and wherein the second antigen-binding moiety is fused at the C-terminus of the Fab heavy chain (CH1) to the N-terminus of the second subunit of the Fc hole domain (para [0220] - ""Knob-in-hole" engineering of multispecific antibodies may be utilized to generate a first arm containing a knob and a second arm containing the hole into which the knob of the first arm may bind. The knob of the multispecific antibodies of the invention may be an anti-CD3 arm in one embodiment. Alternatively, the knob of the multispecific antibodies of the invention may be an anti-target/antigen arm in one embodiment. The hole of the multispecific antibodies of the invention may be an anti-CD3 arm in one embodiment. Alternatively, the hole of the multispecific antibodies of the invention may be an anti-target/antigen arm in one embodiment.").

As the technical feature was known in the art at the time of the invention, this cannot be considered a special technical feature that would otherwise unify the inventions.

Group I+ therefore lacks unity under PCT Rule 13 because they do not share the same or corresponding special technical feature.