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(54) **Title:** PLAP-CAR-EFFECTOR CELLS

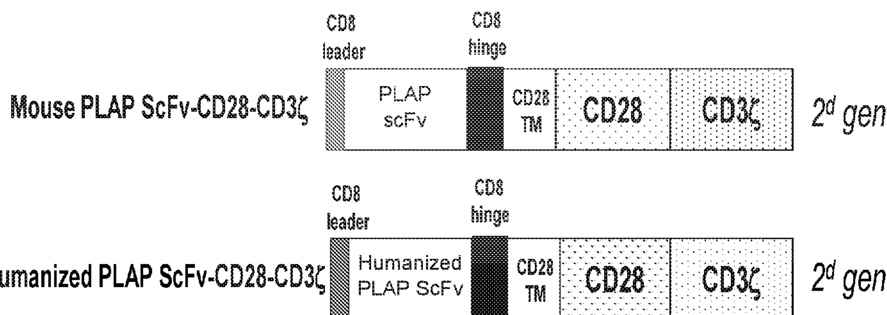

FIG. 2

(57) **Abstract:** The present invention is directed to a chimeric antigen receptor (CAR) fusion protein comprising from N-terminus to C-terminus: (i) a single-chain variable fragment (scFv) comprising VH and VL, wherein scFv binds to human PLAP (placental alkaline phosphatase), (ii) a transmembrane domain, (iii) a co-stimulatory domain of CD28, OX-40, GITR, or 4-1BB, and (iv) CD3 an activating domain. The present invention is also directed to T cells, natural killer (NK) cells, or macrophages, modified to express the CAR of the present invention. The present invention is further directed to a method for treating PLAP-positive cancer cells by administering PLAP-CAR-T cells, PLAP-CAR-NK cells, or PLAP-CAR-macrophages to the patients.

[Continued on next page]

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PLAP-CAR-EFFECTOR CELLS

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 May 23, 2019, and a size of 66.1 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)-CAR. The present invention is also directed to a method for treating PLAP-positive cancer cells by administering PLAP-CAR-T cells, PLAP-CAR-natural killer cells, or PLAP-CAR-macrophages 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. Genetically modifying T cells with CARs is the most common approach to design tumor-specific T cells. CAR-T cells targeting tumor-associated antigens (TAA) can be infused into patients (called adoptive cell transfer or ACT) representing an efficient immunotherapy approach. The advantage of CAR-T technology compared with chemotherapy or antibody is that reprogrammed engineered T cells can proliferate and persist in the patient ("a living drug").

CARs (Chimeric antigen receptors) usually consist of a monoclonal antibody-derived single-chain variable fragment (scFv) linked by a hinge and transmembrane domain to a variable number of intracellular signaling co-stimulatory domains: (i) CD28, Ox-40, CD137 (4-1BB), GITR or other co-stimulatory domains; and (ii) a single, cellular activating, CD3-zeta domain after co-stimulatory domains (FIG. 1). The evolution of CARs went from first generation (with no co-stimulatory domains) to second generation (with one co-stimulatory domain) to third generation CAR (with several co-stimulatory domains). Generating CARs with multiple costimulatory domains (the so-called 3rd generation CAR) have led to increased

cytolytic activity, and significantly improved persistence of CAR-T cells that demonstrate augmented antitumor activity.

Natural-killer (NK) cells are CD56+CD3− large granular lymphocytes that can kill virally infected and transformed cells, and constitute a critical cellular subset of the innate immune system. Unlike cytotoxic CD8+ T lymphocytes, NK cells launch cytotoxicity against tumor cells without the requirement for prior sensitization, and can also eradicate MHC-I negative cells.

CAR-T cell therapy had successful clinical results in the treatment of hematological cancer patients [1-5]. Chimeric antigen receptor contains single chain fragment variant (ScFv) of antibody targeting cancer cell surface antigen fused to a hinge, transmembrane domain, co-stimulatory (CD28, 41-BB or other domains) and CD3 activation domain [1,6],[7,8]. Recently two CD19-CAR-T cell therapies (Kymriah and Yescarta) were approved by FDA for the treatment of hematological cancers based on their high response rate in acute lymphoblastic leukemia and other hematological cancers in clinical trials [3], [9-11]. There are also several other CAR-T cells that are tested in clinical trials such as CD22-CAR-T cells [12] for B-cell lymphoma, BCMA-CAR-T cells for multiple myeloma [13-14].

In terms of solid tumors, CAR-T cell therapy still has many challenges for targeting solid cancers due to on-target off-tumor effects, suppressive tumor microenvironment, decreased CAR-T cell access to the tumor, T cell exhaustion and low persistency [15], [16-18]. The main challenge with CAR-T cells targeting solid tumors is that most tumor solid tumor antigens are expressed in normal tissues.

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 [19], and it is not expressed in most normal tissues except of testis [20]. It was found to be overexpressed in malignant seminoma, teratoma [20], [21], ovarian and cervical carcinoma [22], [23], [24], and colon adenocarcinoma [25]. PLAP was detected in lung, pancreas, stomach tumors [39]. 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 [26].

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 RMGTPDPEYP 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
```

There are four distinct but related alkaline phosphatases: intestinal (ALPI) (NM_001631); placental; 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

FIG. 1 shows the structure of CAR. On the left panel: the structure of first generation (no co-stimulation domains), on the middle panel: second generation (one co-stimulation domain CD28 or 4-BB) and on the right panel: third generation of CAR (two or several co-stimulation domains) are shown [7].

FIG. 2 shows the structure of mouse and humanized PLAP-CAR constructs.

FIG. 3 shows relapse free survival probability vs. months in follow up in patients with high and low PLAP expression.

FIG. 4A shows expression of PLAP in several colon cancer cell lines by FACS analysis. PLAP-negative and PLAP-positive cell lines are shown. MFI (Mean fluorescent intensity)/isotype ratio is shown for each cell line. FIG. 4B shows three positive and three

negative cell lines by mRNA and protein. PLAP-negative: HCT116, SW620 and HT-29 cell lines; and PLAP-positive: Lovo, Caco-2, LS123 cell lines.

FIG. 5A shows that PLAP-CAR-T cells specifically killed PLAP-positive colon cancer cells more significantly compared to T and Mock-CAR-T cells. Real-time cytotoxicity assay (RTCA) was used as described in Materials and Methods. CAR-T cells to target cells ratio (E:T) is10:1. FIG. 5B shows that PLAP-CAR-T cells had significant killing activity compared with normal T cells against Lovo and LS-123 colon cancer target cells but did not have significant killing activity with PLAP-negative HCT116 and HT29 colon cancer cell lines. FIG. 5C shows that CAR-T secreted significant level of IFN-gamma against PLAP-positive cells. Bars show average level of IFN-gamma from three independent experiments. $p<0.05$, Student's t-test.

FIG. 6A shows that PLAP h2- and PLAPh4-CAR-T positive cells were detected with FAB antibody by FACS. FIG. 6B shows that PLAP CAR-T positive cells were detected by FACS with biotinylated recombinant PLAP protein. FIGs. 6C(1) and 6C(2) show quantification of real-time cytotoxicity as described in Materials and Methods. Humanized PLAP-CAR-T specifically killed PALP-positive colon cancer cells, but not PALP-negative colon cancer cells. $P<0.06$, Student's t-test, increased cytotoxicity of PLAP-CAR-T cells versus Mock-CAR-T cells. FIGs. 6D(1) and 6D(2) show that PLAP-CAR-T cells secreted significant level of IFN-gamma, IL-2 and IL-6 versus Mock-CAR-T cells against PLAP-positive colon cancer cell lines and not against PLAP-negative colon cancer cell lines. $p<0.05$, Student's t –test.

FIG. 7A shows that humanized PLAP-CAR-T cells significantly decreased Lovo xenograft tumor growth. The volume of CAR-T cell-treated tumors was significantly less than with Mock control treated cells. $p<0.05$, Student's t-test. FIG. 7B shows that the size of humanized PLAP-CAR-T cell treated tumors was significantly less than in control mice. $p<0.05$, Student's t-test. FIG. 7C shows that tumor weight was significantly less in hPLAP-CAR-T treated mice than in control mice. $P<0.05$, Student's t-test. FIG. 7D shows that AST, ALT, and amylase levels were not significantly affected in blood serum of humanized PLAP-CAR-T cell treated mice. The samples were analyzed as described in Materials and Methods.

FIGs. 8A(1)-8A(3) show that PLAP h5-CAR-T cells significantly killed PLAP-positive colon cancer cells (Caco-2 cells and Lovo cells), but not PLAP-negative colon cancer cells (HCT116). FIG. 8B shows that PLAP h5-CAR-T cells secreted significant higher level of IFN- γ, against PLAP-positive colon cancer cells (Caco-2 cells and Lovo cells), but not PLAP-negative colon cancer cells (HCT116).

FIG. 9A is the quantification of FACS data, which shows the PDL-1 expression in colon cancer cell lines before and after PLAP-CAR-T-treatment by FACS analysis. Addition of IFN-gamma (20/ng/ml) was used as a positive control for PDL-1 induction. PLAP-positive Lovo cells significantly induced PDL-1 expression compared with T and Mock-CAR-T cells in response to hPLAP-CAR-T cells while Caco-2, HCT116, HT29 cells did not. FIG. 9B shows the response of PDL-1 up-regulation to different doses of CAR-T cells. FIG. 9C shows the time and dose-dependent induction of PDL-1 in Lovo cancer cells induced by hPLAP-CAR-T cells. FIG. 9D shows that PD-1 expression was induced in CAR-T cells after co-incubation with PLAP-positive target cells. FACS analysis is shown with PD-1 antibody before and after co-incubation with target cells. PD-1 level is significantly increased. $p<0.05$, Student's t-test. FIG. 9E shows that LAG-3 expression was upregulated after co-incubation with PLAP-positive cells. LAG-3 level is significantly up-regulated versus Mock or CAR-T cells without target cells. $p<0.05$, Student's t-test. FIG. 9F shows that the combination of PLAP-CAR-T cells with PD-1 or LAG-3 antibody increases cytotoxicity of CAR-T cells against target cells. RTCA assay was performed with PLAPh2-CAR-T cells at 3:1 either alone or in combination with PD-1 or PDL-1 antibody. Quantification of RTCA is shown after overnight co-incubation with Lovo target cells. FIG. 9G shows that the secretion of IFN-gamma by PLAP-CAR-T cells in combination with PD-1 or LAG-3 antibody significantly increased versus PLAP-CAR-T cells alone or antibodies alone. $^*p<0.05$, Student's one-tailed t-test vs PLAP-CAR-T cells plus isotype antibody.

DETAILED DESCRIPTION OF THE INVENTION

Definitions

As used herein, "adoptive cell therapy" (ACT) is a treatment that uses a cancer patient's own T lymphocytes, or NK cells, or other hematopoietic cells such as macrophages, induced pluripotent cells, with anti-tumor activity, expanded in vitro and reinfused into the patient with cancer.

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, a "chimeric antigen receptor (CAR)" means a fused protein comprising an extracellular domain capable of binding to an antigen, a transmembrane domain derived from a polypeptide different from a polypeptide from which the extracellular domain is derived, and at least one intracellular domain. The "chimeric antigen receptor

(CAR)" is sometimes called a "chimeric receptor", a "T-body", or a "chimeric immune receptor (CIR)." The "extracellular domain capable of binding to an antigen" means any oligopeptide or polypeptide that can bind to a certain antigen. The "intracellular domain" means any oligopeptide or polypeptide known to function as a domain that transmits a signal
5 to cause activation or inhibition of a biological process in a cell.

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
10 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,
15 expression of which causes cancer.

The inventors have discovered that PLAP is a unique tumor marker and that PLAP can be advantageously used to prepare PLAP-CAR T cells or PLAP-NK cells, which can be used for CAR-T cell therapy or CAR-NK cell therapy, because PLAP is not expressed in normal tissues. Unlike
20 other tumor markers that are expressed in low levels in normal tissues, the advantage of PLAP target not expressed in most normal tissues but only in placenta and testis is that PLAP-CAR-T cells/PLAP-NK cells do not react against normal tissues and thus they are safe and have low toxicity.

The present invention provides CAR-T cells and NK cells that target PLAP tumor antigen which is highly overexpressed in many types of cancer such as ovarian, seminoma, and colon cancer.
25 The PLAP-CAR-T cells and PLAP-NK cells of the present invention have high cytotoxic activity against several cancer cells: colon and ovarian cancer cell lines.

The present invention is directed to a chimeric antigen receptor fusion protein comprising from N-terminus to C-terminus: (i) a single-chain variable fragment (scFv) comprising V_H and V_L, wherein scFv binds to human PLAP, (ii) a transmembrane domain,
30 (iii) a co-stimulatory domain of CD28, and (iv) an activating domain.

In one embodiment, the PLAP antibody is a mouse antibody, and V_H has the amino acid sequence of SEQ ID NO: 5 and V_L has the amino acid sequence of SEQ ID NO: 6.

In one embodiment, the PLAP antibody is a humanized antibody, and V_H has the amino acid sequence of SEQ ID NO: 16, 21, 26, 30, or 34, and V_L has the amino acid

sequence of SEQ ID NO: 22.

In one embodiment, the scFv comprises the amino acid sequence of SEQ ID NO: 8, 18, 23, 27, 31, or 35; or an amino acid sequence having 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 CAR comprises the amino acid sequence of SEQ ID NO: 5, 15, 20, 25, 29, or 33; or an amino acid sequence having at least 95%, 96%, 97%, 98%, or 99% sequence identity thereof, provided that the sequence variation is not in the CDR regions.

The sequence variation, 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 an adoptive cell therapy method for treating cancer, comprising the step of administering PLAP CAR-T cells, PLAP CAR-NK cells, or PLAP CAR-macrophages 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.

Suitable antibody useful for PLAP CAR includes mouse PLAP antibody against PLAP and humanized PLAP antibody against PLAP. In one embodiment, the antibody has a high affinity against PLAP.

The CAR of the present invention comprises a single chain variable fragment (scFv) that binds specifically to PLAP. The heavy chain (H chain) and light chain (L chain) fragments of an anti-PLAP antibody are linked via a linker sequence. For example, a linker can be 5-20 amino acids. The scFv structure can be VL-linker-VH, or VH-linker-VL, from N-terminus to C-terminus.

The CAR of the present invention comprises a transmembrane domain which spans the membrane. The transmembrane domain may be derived from a natural polypeptide, or may be artificially designed. The transmembrane domain derived from a natural polypeptide can be obtained from any membrane-binding or transmembrane protein. For example, a transmembrane domain of a T cell receptor α or β chain, a CD3 zeta chain, CD28, CD3-epsilon, CD45, CD4, CD5, CD8, CD9, CD16, CD22, CD33, CD37, CD64, CD80, CD86, CD134, CD137, ICOS, CD154, or a GITR can be used. The artificially designed transmembrane domain is a polypeptide mainly comprising hydrophobic residues such as

leucine and valine. It is preferable that a triplet of phenylalanine, tryptophan and valine is found at each end of the synthetic transmembrane domain. In preferred embodiments, the transmembrane domain is derived from CD28 or CD8, which give good receptor stability.

In the present invention, the co-stimulatory domain is selected from the group consisting of human CD28, 4-1BB (CD137), ICOS-1, CD27, OX 40 (CD137), DAP10, and GITR (AITR).

The endodomain (the activating domain) is the signal-transmission portion of the CAR. After antigen recognition, receptors cluster and a signal is transmitted to the cell. The most commonly used endodomain component is that of CD3-zeta (CD3 Z or CD3ζ), which contains 3 ITAMs. This transmits an activation signal to the T cell after antigen is bound. CD3-zeta may not provide a fully competent activation signal and additional co-stimulatory signaling may be needed. For example, one or more co-stimulating domains can be used with CD3-Zeta to transmit a proliferative/survival signal.

The CAR of the present invention may comprise a signal peptide N-terminal to the ScFv so that when the CAR is expressed inside a cell, such as a T-cell, NK cells, or macrophages, the nascent protein is directed to the endoplasmic reticulum and subsequently to the cell surface, where it is expressed. The core of the signal peptide may contain a long stretch of hydrophobic amino acids that has a tendency to form a single alpha-helix. The signal peptide may begin with a short positively charged stretch of amino acids, which helps to enforce proper topology of the polypeptide during translocation. At the end of the signal peptide there is typically a stretch of amino acids that is recognized and cleaved by signal peptidase. Signal peptidase may cleave either during or after completion of translocation to generate a free signal peptide and a mature protein. The free signal peptides are then digested by specific proteases. As an example, the signal peptide may derive from human CD8 or GM-CSF, or a variant thereof having 1 or 2 amino acid mutations provided that the signal peptide still functions to cause cell surface expression of the CAR.

The CAR of the present invention may comprise a spacer sequence as a hinge to connect scFv with the transmembrane domain and spatially separate antigen binding domain from the endodomain. A flexible spacer allows to the binding domain to orient in different directions to enable its binding to a tumor antigen. The spacer sequence may, for example, comprise an IgG1 Fc region, an IgG1 hinge or a CD8 stalk, or a combination thereof. A human CD28 or CD8 stalk is preferred.

The present invention provides a nucleic acid encoding the CAR described above. The nucleic acid encoding the CAR can be prepared from an amino acid sequence of the

specified CAR by a conventional method. A base sequence encoding an amino acid sequence can be obtained from the aforementioned NCBI RefSeq IDs or accession numbers of GenBenk for an amino acid sequence of each domain, and the nucleic acid of the present invention can be prepared using a standard molecular biological and/or chemical procedure. For example, based on the base sequence, a nucleic acid can be synthesized, and the nucleic acid of the present invention can be prepared by combining DNA fragments which are obtained from a cDNA library using a polymerase chain reaction (PCR).

The nucleic acid encoding the CAR of the present invention can be inserted into a vector, and the vector can be introduced into a cell. For example, a virus vector such as a retrovirus vector (including an oncoretrovirus vector, a lentivirus vector, and a pseudo type vector), an adenovirus vector, an adeno-associated virus (AAV) vector, a simian virus vector, a vaccinia virus vector or a Sendai virus vector, an Epstein-Barr virus (EBV) vector, and a HSV vector can be used. As the virus vector, a virus vector lacking the replicating ability so as not to self-replicate in an infected cell is preferably used.

For example, when a retrovirus vector is used, the process of the present invention can be carried out by selecting a suitable packaging cell based on a LTR sequence and a packaging signal sequence possessed by the vector and preparing a retrovirus particle using the packaging cell. Examples of the packaging cell include PG13 (ATCC CRL-10686), PA317 (ATCC CRL-9078), GP+E-86 and GP+envAm-12, and Psi-Crip. A retrovirus particle can also be prepared using a 293 cell or a 293T cell having high transfection efficiency. Many kinds of retrovirus vectors produced based on retroviruses and packaging cells that can be used for packaging of the retrovirus vectors are widely commercially available from many companies.

The present invention provides T cells, or NK cells, or macrophages, modified to express the chimeric antigen receptor fusion protein as described above. CAR-T cells, CAR-NK cells, or CAR-macrophages of the present invention bind to a specific antigen via the CAR, thereby a signal is transmitted into the cell, and as a result, the cell is activated. The activation of the cell expressing the CAR is varied depending on the kind of a host cell and an intracellular domain of the CAR, and can be confirmed based on, for example, release of a cytokine, improvement of a cell proliferation rate, change in a cell surface molecule, or the like as an index.

T cells, or NK cells, or macrophages, modified to express the CAR can be used as a therapeutic agent for a disease. The therapeutic agent comprises the T cells expressing the CAR as an active ingredient, and may further comprise a suitable excipient. Examples of the

excipient include pharmaceutically acceptable excipients known to a person skilled in the art.

This application demonstrates the efficacy of CAR-T cells targeting PLAP antigen that is overexpressed in colon cancer tumors. This application demonstrates that PLAP-CAR-T cells specifically decreases viability of PLAP-positive colon cancer cells but not PLAP-negative cancer cells. PLAP-CAR-T cells secrets 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-CAR-T cells significantly decreases Lovo (positive PLAP-colon cancer cells) xenograft tumor growth *in vivo*. There are no increase of AST, ALT or amylase enzyme levels in mouse blood and no decrease of mouse body weight after treating mice with hPLAP-CAR-T cells demonstrating no toxic effect of hPLAP-CAR-T cells *in vivo*. In addition, combination of hPLAP-CAR-T cells with PD-1 or LAG-3 antibodies increased efficacy of CAR-T cells against colon cancer cells.

The inventors found that PLAP-CAR-T cells significantly killed all PLAP-positive cancer cells, and did not kill PLAP-negative colon cancers. This implies high specificity of PLAP-CAR-T cells. In addition, Lovo and Caco-2 colon cancer cells differed in up-regulation of PDL-1 by CAR-T cells. Lovo colon cancer cell induced PDL-1 in response to PLAP-CAR-T cells, while Caco-2 cells did not. Both of cell lines were effectively killed by hPLAP-CAR-T cells independently of induction of PDL-1 expression. The humanized PLAP-CAR-T cells killed faster Lovo cells than Caco-2 cells and secreted more IFN-gamma against Lovo colon cancer cells than against Caco-2 cells. In addition, T cells and Mock CAR-T cells had more activity in Lovo cells that in Caco-2 cells. This show that hPLAP-CAR-T cells can overcome PDL-1 up-regulation in Lovo cells. This was shown when Lovo cells were pretreated with IFN-gamma to up-regulate PDL-1, PLAP-CAR-T cells effectively killed Lovo cells. Colon cancer with Kras mutations were shown to be resistant to therapies such as Cetuximab (Erbitux) [40], while hPLAP-CAR-T cells effectively killed two different colon cancer cell lines: Lovo (codon 13 mutation: G13D) and LS123 (codon 12 mutation: G12D). This is another advantage of hPLAP-CAR-T cells against solid tumors with Kras mutations responsible for resistance to other therapies.

PLAP-CAR-T cells up-regulated PD-1 and LAG-3 after co-culturing with PLAP-positive colon cancer cell lines but did not increase with PLAP-negative colon cancer cell lines. The inventors have found dose-dependent up-regulation of PDL-1 in response to PLAP-CAR-T cells in Lovo colon cancer cell lines. PD-1, PDL-1 or LAG-3 antibody in combination with PLAP-CAR-T cells significantly increased CAR-T induced cytotoxicity

and IFN-gamma secretion against Lovo cancer cells. Thus, checkpoint inhibitors can decrease exhaustion of CAR-T cells and provide basis for combination therapy.

PLAP scFv-(CD28, OX-40, 4-1BB, or GITR)-CD3 zeta CAR-T cells, CAR-NK cells, or CAR-macrophages can be used in combination with different chemotherapy: checkpoint inhibitors; targeted therapies, small molecule inhibitors, and antibodies.

Tags (Flag tag or other tags) conjugated PLAP scFv can be used for CAR generation.

Third generation CAR-T or other co-activation signaling domains can be used for the PLAP-scFv inside CAR.

Bispecific PLAP- and other antigens (EGFR, HER-2, VEGFR, NGFR) CAR-T cells, CAR-NK cells, or CAR-macrophages can be used for immunotherapy. The construct of the bispecific CAR-T cells contain a first scFv against PLAP, and a second scFv against a second tumor antigen. CAR-T cells with bispecific antibody can target cancer cells that overexpress two tumor antigens more effectively and specifically.

Combination of PLAP-CAR-T cells, CAR-NK cells, or CAR-macrophages with CAR-T cells, CAR-NK cells, or CAR-macrophages targeting other tumor antigens or tumor microenvironment (e.g. VEGFR-1-3), i.e., dual CAR-T cells, CAR-NK cells, or CAR-macrophages, can be used to enhance activity of monotherapy PLAP-CAR.

PLAP-CAR-T cells, CAR-NK cells, or CAR-macrophages can be used to activate phagocytosis and block “don’t eat” signaling.

PLAP-CAR-NK cells are safe effector cells, as they may avoid the potentially lethal complications of cytokine storms, tumor lysis syndrome, and on-target, off-tumor effects.

Anti-PLAP antibody h2, h4 and h5 VH and VL sequences can be used as one arm of a bi-specific antibody.

Both PLAP-CAR-T cells and bi-specific antibodies containing anti-PLAP VH and VL can be used in combination with checkpoint inhibitors (PDL-1 antibody, PD-1 antibody, LAG-3 antibody, TIM-3 antibody, TIGIT antibody, and other antibodies), and with chemotherapies to improve efficacy against cancer cells.

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
5 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: SW620, HT29, HCT116 and PLAP-positive: Lovo, Caco-2, LS123 were obtained from Dr. Walter Bodmer (Oxford,
10 UK), whose laboratory authenticated cell lines using SNPs, Sequenom MassARRAY iPLEX and HumanOmniExpress-24 BeadChip arrays, and tested for the absence of Mycoplasma as described [28-29]. The cell lines were cultured in DMEM plus 10% FBS and penicillin/streptomycin. The list of 117 colon cancer cell lines from W. Bodmer laboratory which were used for PLAP mRNA level detection is shown in supplementary

15 The cell lines were additionally authenticated by FACS using cell-specific surface markers and cultured in a humidified 5% CO_2 incubator.

Antibodies

Monoclonal PD-1 (EH122H7), PDL-1 (clone 29E2A3), TIGIT (clone A15152G),
20 LAG3 (clone 7H2C65), CD62L (clone DREG-56), CD45RO (clone UCHL1), CD4 (clone RPA-T4) and CD8 (clone RPA-T8) antibodies antibodies were from *Biolegend*. PLAP antibody (clone H17E2) was obtained from *Thermo Fisher*. Other antibodies were described in [30].

25 **CAR constructs**

The second generation CAR with CD8 alpha signaling peptide, PLAP Ab ScFv [21], CD8 hinge, CD28 co-stimulatory domain and CD3 activation domain was cloned down-stream of EF1 promoter into modified lentiviral vector pCD510 (*Systems Bioscience*). The same construct was generated with humanized PLAP ScFv (called humanized PLAP or
30 PLAPh2, h4 (clone 2 or 4), and Mock control with either ScFv of intracellular protein or Mock control with 45 amino-acid sequence containing three epitopes of transferrin antibody, called (Mock-CAR). The mouse PLAP-CAR was generated by *Synbio*. The humanized PLAP ScFv sequences was synthesized by *IDT* as gBlock sequence with Nhe I and Xho I restriction

sites flanking ScFv, and sub-cloned into these sites in lentiviral vector between CD8 alpha signaling peptide and CD8 hinge sequences.

Humanization of PLAP

5 Humanization of PLAP was performed as described in [31]. The human frames from human antibody clones with highest homology were used for humanized pairs using bioinformatics *in silico* methods as described [32,33]. Mouse CDR were inserted into these clones and different humanized ScFv variants were used for generating CAR constructs and performing CAR-T cell functional tests.

10

Lentivirus preparation in 293FT cells

The lentiviral CAR constructs were used for generation of lentivirus by transfecting 293 FT cells using transfection agent (*Alstem*) and Lentivirus Packaging Mix as described

15 [34]. The lentiviral titers in pfu/ml were detected by RT-PCR using the Lenti-X qRT-PCR kit (*Takara*) according to the manufacturer's protocol and the 7900HT thermal cycler (*Thermo Fisher*).

Transduction with CAR lentivirus and CAR-T cell expansion

20 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 were activated with CD3/CD28 Dynabeads (*Invitrogen*), and cultured in 24-well plates. CAR lentivirus was added to the PBMC cultures at 24 and 48 hours using TransPlus transduction enhancer (*AlStem*), as described [30,31,34]. The CAR-T cells were cultured and expanded for 14 days

25 by adding fresh medium to maintain the cell density at 1 x 10^6 cells/ml.

Fluorescence-activated cell sorting (FACS) analysis

To detect CAR expression, 5x10^5 cells were suspended in 1xPBS plus 0.5% BSA buffer and incubated on ice with human serum (*Jackson Immunoresearch*, West Grove, PA)

30 for 10 min. Then allophycocyanin (APC)-labeled anti-CD3 (*eBioscience*, San Diego, CA), 7-aminoactinomycin D (7-AAD, *BioLegend*, San Diego, CA), anti-F(ab)2 or its isotype control were added, and the cells were incubated on ice for 30 min. Then cells were rinsed with buffer, and analyzed on a FACSCalibur (*BD Biosciences*) first for light scatter versus 7-AAD staining, then the 7-AAD-negative live gated cells were plotted for CD3 staining versus

F(ab)2 staining or isotype control staining. For FACS with colon cancer cell lines to detect PLAP levels mouse monoclonal PLAP antibody (H17E2) from *Ximbio* (London, UK) was used, and analysis was performed on FACSCalibur.

5 **Blitz ForteBio binding assay**

The binding of PLAP antibody with recombinant PLAP extracellular domain protein from Sino Biological was performed using Blitz ForteBio system as described [30]. In brief, anti-mouse-capture (AMC) biosensors were soaked in kinetics buffer (PBS, 0.1% Tween, 0.05%BSA) for 10 min and then with mouse anti-PLAP antibody at 0.1mg/mL in same buffer 10 for 30 min. After washing, biosensors were used to bind the PLAP antigen at different concentrations. The Kd was detected with Blitz system software.

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 15 real-time cell analysis (RTCA) iCELLigence system (*Acea Biosciences*). After 20-24 hours, the medium was replaced with 1×10^5 effector cells (CAR-T cells, Mock CAR-T cells or non-transduced T cells) in AIM V-AlbuMAX medium containing 10% FBS, in triplicate. In some experiments checkpoint protein antibodies PD-1, LAG-3 or isotype at 10 μg/ml were added to the effector cells either alone or in combination with CAR-T cells. In some series of 20 experiments target cells were pre-treated with 20 ng/ml of IFN-γ for 24 h. The cells were monitored for 1-2 days 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.

25

ELISA assay for cytokine secretion

The target cells were cultured with the effector cells (CAR-T cells or non-transduced T cells) 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. 30 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 colon cancer cells in sterile 1x PBS.
5 The CAR-T cells ($1x10^7$ CAR-T cells/mice) were injected intravenously into mice at days 1, 7 and 13. 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.

10 **Toxicology markers.**

Mouse serum samples were processed with clinical chemistry analyzer (Beckman-Coulter AU680) by IDEX Bioanalytics (West Sacramento, CA) for detection levels of toxicology markers: ALT (alanine aminotransferase), AST (aspartate aminotransferase), amylase in U/ml.

15

Primary tumor samples

Samples with different types of normal tissues or tumor tissues were obtained from archived slides of Promab (Richmond, CA). The TMA slide with 106 primary colon cancer adenocarcinoma was obtained from *Biomax* (Rockville, MD) and used for IHC with PLAP
20 antibody.

Immunohistochemistry (IHC) staining

The primary tumor tissue or normal tissue section slides or primary TMA slides were incubated in xylene twice for 10 min, then hydrated in alcohol and rinsed in 1xPBS. Heat-
25 induced antigen retrieval was performed using a pressure cooker for 20 min in 10 mM citrate buffer, pH 6.0. The slides were rinsed with PBS, incubated in a 3% H_2O_2 solution for 10 min, then rinsed again with 1xPBS, and incubated in goat serum for 20 min. The tissue section slides were incubated with mouse monoclonal PLAP (H17E2) primary antibody overnight at 4 °C or 1.5 hours at 37°C. The slides were rinsed 3 times with PBS, incubated with biotin-
30 conjugated secondary antibody for 10 min, rinsed with PBS, incubated with streptavidin-conjugated peroxidase for 10 min, and rinsed 3 times with 1xPBS buffer. The slides were incubated in DAB substrate solution for 2-5 min under the microscope. The reaction was stopped by washing in water, counterstained with hematoxylin, rinsed with water, and

dehydrated in 75%, 80%, 95% and 100% ethanol and xylene. For negative control isotype antibody was used, and for positive control placenta samples were used. Images were acquired on the Motic DMB5-2231PL microscope using Images Plus 2.0. software (*Motic*, Xiamen, China). PLAP expression correlation with survival free prognosis was performed using R2 Genomics Analysis and Visualization platform (http://r2platform.com / http://r2.amc.nl).

Example 2. The sequence of mouse PLAP-CD28-CD3zeta CAR

The CAR structures were: Human CD8 signaling peptide, mouse scFv or humanized derived from antibody H17E2 (V_H-Linker- 3x(GGGGS) -V_L), CD8 hinge, CD28 transmembrane, co-activation domain, CD3 zeta activation domain (FIG. 2). The sequence of lentiviral vector with CAR construct inside Eco R1 and Xho I site is shown below. The scFv is flanked with Nhe I and Xho I sites for potential re-cloning to other constructs. The nucleotide sequence of PLAP-CD28-CD3 is shown below,

SEQ ID NO: 2, *tctaga*gccgccacc-flanking vector sequence starting with Xba I site (italics):

SEQ ID NO: 3 (Mouse PLAP CAR, called PMC262), starting with ATG and ending with a stop codon TAA (underlined), signaling peptide is in bold, VH with CDRs 1, 2, 3, bold underlined; linker in italics, VL with CDR 1,2,3 in bold, underlined); ScFV is flanked by 5' Nhe and 3' Xho sites, small font

ATGGCCTTACCAGTGACCGCCTTGCTCCTGCCGCTGGCCTTGCTGCTCCACGCCGCCAGGCCGgctagc
CAGGTGCAGCTGAAGGAGTCAGGACCTGGCCTGGTGGCGCCCTCACAGAGCCTG
TCCATCACATGCACTGTCTCAGGG**TTCTCATTAACCAGTTATGGTGTAAGC**TGG
GTTCGCCAGCCTCCAAGAAAGGGTCTGGAGTGGCTGGGA**GTAATATGGGAAGA**
CGGGAGCACAAATTATCATTCAGCTCTCATATCCAGACTGAGCATCAACAAGG
ATAACTCCAAGAGCCAAGTTTTCTTAAAACTGAACAGTCTGCAAACTGATGACAC
AGCCACGTACTACTGTGCCAAA**CCCCACTACGGTAGCAGCTACGTGGGGGCT**
ATGGAATACTGGGGTCAAGGAACCTCAGTCACCGTCTCCTCA
GGTGGCGGTGGTTCT GGTGGCGGTGGTTCT GGTGGCGGTGGTTCT
GAC ATC CAG ATG ACT CAG TCT CCA GCC TCC CTA ACT GCA TCT GTG
GGA GAA ACT GTC ACCATC ACC TGT CGA GCA AGT GAA AAT ATT TAC
AGT TAT GTA GCA TGG TAT CAG CAG AAA CAGGGA AAA TCT CCT CAG
TTC CTG GTC TAT AAT GCA AAA TCC TTA GCA GAG GGT GTG CCA
TCAAGG TTC AGT GGC AGY GGA TCA GGC ACA CAG TTT TCT CTG AAG
ATC AAC AGC CTG CAG CCTGAA GAT TTT GGG AAT TAT TAC TGT CAA
CAT CAT TAT GTT AGT CCG TGG ACG TTC GGT GGAGGC ACC AAG CTG
GAA ATC AGA CGG

ctcgagAAGCCCACCACGACGCCAGCGCCGCGACCACCAACACCGGCGCCCACCAT
CGCGTCGCAGCCCCTGTCCCTGCGCCCAGAGGCGAGCCGGCCAGCGGCGGGGGG
CGCAGTGCACACGAGGGGGCTGGACTTCGCCAGTGATaagcccttttgggtgctggtggtggttgg
tggagtcctggcttgctatagcttgctagtaacagtggcctttattattttctgggtgaggagtaagaggagcaggctcctgcacagtgac
5 tacatgaacatgactccccgccgccccgggcccacccgcaagcattaccagccctatgccccaccacgcgacttcgcagcctatcgc
tccAGAGTGAAGTTCAGCAGGAGCGCAGACGCCCCCGCGTACCAGCAGGGCC
AGAACCAGCTCTATAACGAGCTCAATCTAGGACGAAGAGAGGAGTACGATG
TTTTGGACAAGAGACGTGGCCGGGACCCTGAGATGGGGGGAAAGCCGCAGA
GAAGGAAGAACCCTCAGGAAGGCCTGTACAATGAACTGCAGAAAGATAAGA
10 TGGCGGAGGCCTACAGTGAGATTGGGATGAAAGGCGAGCGCCGGAGGGGC
AAGGGGCACGATGGCCTTTACCAGGGTCTCAGTACAGCCACCAAGGACACC
TACGACGCCCTTCACATGCAGGCCCTGCCCCCTCGCTAA

SEQ ID NO: 4: tag*gaattc* flanking vector with EcoR I site *(italics)*

15

SEQ ID NO: 5 is the amino acid sequence of SEQ ID NO: 3 (mouse PLAP-CD28-CD3zeta CAR): signaling peptide-VH-linker (in italics smaller font GSSSSx3)-VL-h-CD28 – CD3. Sequence in bold is mouse PLAP scFv; CDR 1,2,3 underlined; VH-linker in italics-VL.

20 M A L P V T A L L L P L A L L L H A A R P A S **Q V Q L K E S G P G L V A P S Q S L S I T C T V S G F S L T S Y G V S W V R Q P P R K G L E W L G V I W E D G S T N Y H S A L I S R L S I N K D N S K S Q V F L K L N S L Q T D D T A T Y Y C A K P H Y G S S Y V G A M E Y W G Q G T S V T V S S** *G G G G S G G G G S G G G G S* **D I Q M T Q S P A S L T A S V G E T V T I T C R A S E N I Y S Y V A W Y Q Q K Q G K S P**
25 **Q F L V Y N A K S L A E G V P S R F S G X G S G T Q F S L K I N S L Q P E D F G N Y Y C Q H H Y V S P W T F G G G T K L E I R R** L E K P T T T P A P R P P T P A P T I A S Q P L S L R P E A S R P A A G G A V H T R G L D F A S D K P F W V L V V V G G V L A C Y S L L V T V A F I I F W V R S K R S R L L H S D Y M N M T P R R P G P T R K H Y Q P Y A P P R D F A A Y R S R V K F S R S A D A P A Y Q Q G Q N Q L Y
30 N E L N L G R R E E Y D V L D K R R G R D P E Met G G K P Q R R K N P Q E G L Y N E L Q K D K M A E A Y S E I G M K G E R R R G K G H D G L Y Q G L S T A T K D T Y D A L H M Q A L P P R (SEQ ID NO: 5)

35 Mouse VH (with underlined CDR 1, 2, 3), SEQ ID NO: 6
Q V Q L K E S G P G L V A P S Q S L S I T C T V S G F S L T S Y G V S W V R Q P P R K G L E W L G V I W E D G S T N Y H S A L I S R L S I N K D N S K S Q V F L K L N S L Q T D D T A T Y Y C A K P H Y G S S Y V G A M E Y W G Q G T S V T V S S

40

Mouse VL (with underlined CDR 1, 2, 3), SEQ ID NO: 7

D I Q M T Q S P A S L T A S V G E T V T I T C R A S E N I Y S Y V A W Y Q Q K Q G K S P Q F L V Y N A K S L A E G V P S R F S G X G S G T Q F S L K I N S L Q P E D F G N Y Y C Q H H Y V S P W T F G G G T K L E I R R

Mouse PLAP scFv, SEQ ID NO: 8
Q V Q L K E S G P G L V A P S Q S L S I T C T V S G F S L T S Y G V S W V R Q P P R K G L E W L G V I W E D G S T N Y H S A L I S R L S I N K D N S K S Q V F L K L N S L Q T D D T A T Y Y C A K P H Y G S S Y V G A M E Y W G Q G T S V T V S S *G G G G S G G G G S G G G G S* **D I Q M T Q S P A S L T A S V G E T V T I T C R A S E N I Y S Y V A W Y Q Q K Q G K S P Q F L V Y N A K S L A E G V P S R F S G X G S G T Q F S L K I N S L Q P E D F G N Y Y C Q H H Y V S P W T F G G G T K L E I R R**

The scheme of CAR construct is shown below, which shows the sub-domain sequences of SEQ ID NO: 3.

<huCD8 signal peptide> SEQ ID NO: 9
ATGGCCTTACCAGTGACCGCCTTGCTCCTGCCGCTGGCCTTGCTGCTCCACGCCG
CCAGGCCG

<NheI restriction site>
GCTAGC

< Mouse PLAP scFv (VH-linker-VL)> SEQ ID NO: 10

ATGGCCTTACCAGTGACCGCCTTGCTCCTGCCGCTGGCCTTGCTGCTCCACGCCGCCAGGCCGgctagc
CAGGTGCAGCTGAAGGAGTCAGGACCTGGCCTGGTGGCGCCCTCACAGAGCCTG
TCCATCACATGCACTGTCTCAGGG**TTCTCATTAACCAGTTATGGTGTAAGC**TGG
GTTCGCCAGCCTCCAAGAAAGGGTCTGGAGTGGCTGGGA**GTAATATGGGAAGA**
CGGGAGCACAAATTATCATTCAGCTCTCATATCCAGACTGAGCATCAACAAGG
ATAACTCCAAGAGCCAAGTTTTCTTAAAACTGAACAGTCTGCAAACTGATGACAC
AGCCACGTACTACTGTGCCAAA**CCCCACTACGGTAGCAGCTACGTGGGGGCT**
ATGGAATACTGGGGTCAAGGAACCTCAGTCACCGTCTCCTCA
GGTGGCGGTGGTTCT GGTGGCGGTGGTTCT GGTGGCGGTGGTTCT
GAC ATC CAG ATG ACT CAG TCT CCA GCC TCC CTA ACT GCA TCT GTG GGA GAA ACT GTC ACCATC ACC TGT CGA GCA AGT GAA AAT ATT TAC AGT TAT GTA GCA TGG TAT CAG CAG AAA CAGGGA AAA TCT CCT CAG TTC CTG GTC TAT AAT GCA AAA TCC TTA GCA GAG GGT GTG CCA TCAAGG TTC AGT GGC AGY GGA TCA GGC ACA CAG TTT TCT CTG AAG ATC AAC AGC CTG CAG CCTGAA GAT TTT GGG AAT TAT TAC TGT CAA CAT CAT TAT GTT AGT CCG TGG ACG TTC GGT GGAGGC ACC AAG CTG GAA ATC AGA CGG

<XhoI restriction site>
CTCGAG

<CD8> SEQ ID NO: 11

AAGCCCACCACGACGCCAGCGCCGCGACCACCAACACCGGCGCCCACCATCGCG
TCGCAGCCCCTGTCCCTGCGCCCAGAGGCGAGCCGGCCAGCGGCGGGGGGCGCA
GTGCACACGAGGGGGCTGGACTTCGCCAGTGATaagccc

<CD28 TM/activation> SEQ ID NO: 12
TTTTGGGTGCTGGTGGTGGTTGGTGGAGTCCTGGCTTGCTATAGCTTGCTAGTAA
CAGTGGCCTTTATTATTTTCTGGGTGAGGAGTAAGAGGAGCAGGCTCCTGCACAG
TGACTACATGAACATGACTCCCCGCCGCCCCGGGCCCACCCGCAAGCATTACCAG
CCCTATGCCCCACCACGCGACTTCGCAGCCTATCGCTCC

<CD3zeta> SEQ ID NO: 13
AGAGTGAAGTTCAGCAGGAGCGCAGACGCCCCCGCGTACCAGCAGGGCCAGAAC
CAGCTCTATAACGAGCTCAATCTAGGACGAAGAGAGGAGTACGATGTTTTGGAC
AAGAGACGTGGCCGGGACCCTGAGATGGGGGGAAAGCCGAGAAGGAAGAACCC
TCAGGAAGGCCTGTACAATGAACTGCAGAAAGATAAGATGGCGGAGGCCTACAG
TGAGATTGGGATGAAAGGCGAGCGCCGGAGGGGCAAGGGGCACGATGGCCTTTA
CCAGGGTCTCAGTACAGCCACCAAGGACACCTACGACGCCCTTCACATGCAGGC
CCTGCCCCCTCGCTAATAG

<EcoRI restriction site>
Gaattc

Example 3. PLAP CAR with humanized antibody h1.

SEQ ID NO: 14 (human h1 PLAP CAR), starting with ATG and ending with a stop codon TAA (underlined). The sequence starts with a signaling peptide, then the humanized PLAP scFv h1. The nucleotide sequence has the same structure as SEQ ID NO: 2 except the scFv portion. The bold sequence is humanized h1 PLAP-1 scFv (CDRs 1, 2, 3 are underlined). Different nucleotides in humanized frame regions compared with mouse are underlined but not bolded.

ATGGCCTTACCAGTGACCGCCTTGCTCCTGCCGCTGGCCTTGCTGCTCCACGCCGCCAGGCCGgctagc
CAGGTCCAACTGCAGGAGAGCGGTCCAGGTCTTGTGAGACCTAGCCAGACC
CTGAGCCTGACCTGCACCGTGTCTGGCTTCACCTTCACCAGTTATGGTGTAA
GCTGGGTGAGACAGCCACCTGGACGAGGTCTTGAGTGGATTGGAGTAATAT
GGGAAGACGGGAGCACAAATTATCATTCAGCTCTCATATCCAGAGTGACAAT
GCTG GTAGACACCAGCAAGAACCAGTTCAGCCTGAGACTC
AGCAGCGTGACAGCCGCCGACACCGCGGTCTATTATTGTGCAAGACCCCACT
ACGGTAGCAGCTACGTGGGGGCTATGGAATACTGGGGTCAAGGCAGCCTCG
TCACAGTCTCCTCA
GGTGGCGGTGGTTCT GGTGGCGGTGGTTCT GGTGGCGGTGGTTCT
GAC ATC CAG ATG ACC CAG AGC CCA AGC AGC CTG AGC GCC AGC GTG GGT
GAC AGA GTG ACC
ATC ACC TGT **CGA GCA AGT GAA AAT ATT TAC AGT TAT GTA GCA** TGG
TAC CAG CAG AAG
CCA GGT AAG GCT CCA AAG CTG CTG ATC TAC **AAT GCA AAA TCC TTA GCA**
GAG GGT GTG CCA AGC
AGA TTC AGC GGT AGC GGT AGC GGT ACC GAC TTC ACC TTC ACC ATC AGC
AGC CTC CAG

CCA GAG GAC ATC GCC ACC TAC TAC TGC **CAA CAT CAT TAT GTT AGT CCG TGG ACG** TTC GGC CAA
GGG ACC AAG GTG GAA ATC AAA CGT

ctcgagAAGCCCACCACGACGCCAGCGCCGCGACCACCAACACCGGCGCCCACCATCGCGT
CGCAGCCCCTGTCCCTGCGCCCAGAGGCGAGCCGGCCAGCGGCGGGGGGCGCAGTGCAC
ACGAGGGGGCTGGACTTCGCCAGTGATaagcccttttgggtgctggtggtggttggtggagtcctggcttgctatagcttg
ctagtaacagtggcctttattattttctgggtgaggagtaagaggagcaggctcctgcacagtgactacatgaacatgactccccgccgccccgggc
ccacccgcaagcattaccagccctatgccccaccacgcgacttcgcagcctatcgctccAGAGTGAAGTTCAGCAGGAGCG
CAGACGCCCCCGCGTACCAGCAGGGCCAGAACCAGCTCTATAACGAGCTCAATCTAGGA
CGAAGAGAGGAGTACGATGTTTTGGACAAGAGACGTGGCCGGGACCCTGAGATGGGGG
GAAAGCCGCAGAGAAGGAAGAACCCTCAGGAAGGCCTGTACAATGAACTGCAGAAAGA
TAAGATGGCGGAGGCCTACAGTGAGATTGGGATGAAAGGCGAGCGCCGGAGGGGCAAG
GGGCACGATGGCCTTTACCAGGGTCTCAGTACAGCCACCAAGGACACCTACGACGCCCT
TCACATGCAGGCCCTGCCCCCTCGCTAA (SEQ ID NO: 14)

SEQ ID NO: 15 is humanized h1 PLAP-1 CAR amino-acid sequence; it has the same structure as mouse PLAP-CAR except the scFv portion; sequence in bold is humanized h1 PLAP-1 ScFv, CDR 1, 2, 3 are in italics and underlined; linker are in a smaller font; different amino-acids in CDR regions in regular font; different amino-acids from mouse sequence in frame region are underlined.

M A L P V T A L L L P L A L L L H A A R P A S **Q V Q L Q E S G P G L V R P S Q T L**
S L T C T V S ***G F T F T S Y G V S*** **W V R Q P P G R G L E W I G** ***V I W E D G S T N***
Y H S A L I S **R V T M L V D T S K N Q F S L R L S S V T A A D T A V Y Y C A** ***R P***
H Y G S S Y V G A M E Y **W G Q G S L V T V S S** *G G G G S G G G G S G G G G S* **D I Q M**
T Q S P S S L S A S V G D R V T I T C ***R A S E N I Y S Y V A*** **W Y Q Q K P G K A P K**
L L I Y ***N A K S L A*** **E G V P S R F S G S G S G T D F T F T I S S L Q P E D I A T Y Y**
C ***Q H H Y V S P W*** **T F G Q G T K V E I K R** L E K P T T T P A P R P P T P A P T I A S
Q P L S L R P E A S R P A A G G A V H T R G L D F A S D K P F W V L V V V G G V L
A C Y S L L V T V A F I I F W V R S K R S R L L H S D Y M N M T P R R P G P T R K
H Y Q P Y A P P R D F A A Y R S R V K F S R S A D A P A Y Q Q G Q N Q L Y N E L N
L G R R E E Y D V L D K R R G R D P E M G G K P Q R R K N P Q E G L Y N E L Q K
D K M A E A Y S E I G M K G E R R R G K G H D G L Y Q G L S T A T K D T Y D A L
H M Q A L P P R (SEQ ID NO: 15)

Humanized h1 PLAP-1 VH (SEQ ID NO: 16)

Q V Q L Q E S G P G L V R P S Q T L S L T C T V S *G F T F T S Y G* V S W V R Q P P G R G L E W I G V *I W E D G S T* N Y H S A L I S R V T M L V D T S K N Q F S L R L S S V T A A D T A V Y Y C *A R P H Y G S S Y V G A M E Y* W G Q G S L V T V S S

Humanized h1 PLAP-1 VL (SEQ ID NO: 17)

D I Q M T Q S P S S L S A S V G D R V T I T C R A S *E N I Y S Y* V A W Y Q Q K P G K A P K L L I Y N A K S L A E G V P S R F S G S G S G T D F T F T I S S L Q P E D I A T Y Y C *Q H H Y V S P W T F* G Q G T K V E I K R

Humanized h1 PLAP-1 scFv (SEQ ID NO: 18)

Q V Q L Q E S G P G L V R P S Q T L S L T C T V S *G F T F T S Y G* V S W V R Q P P G R G L E W I G V *I W E D G S T* N Y H S A L I S R V T M L V D T S K N Q F S L R L S S V T A A D T A V Y Y C *A R P H Y G S S Y V G A M E Y* W G Q G S L V T V S S *G G G G S G G G G S G G G G S* D I Q M T Q S P S S L S A S V G D R V T I T C R A S *E N I Y S Y* V A W Y Q Q K P G K A P K L L I Y N A K S L A E G V P S R F S G S G S G T D F T F T I S S L Q P E D I A T Y Y C *Q H H Y V S P W T F* G Q G T K V E I K R

Example 4. PLAP CAR with humanized antibody h2.

The bioinformatics approach was performed to generate additional humanized versions of PLAP CAR. The sequences were codon-optimized for higher expression of CAR.

The sequence starts with a signaling peptide (underlined, codon optimized), then the humanized PLAP scFv (bold). The nucleotide sequence has the same structure as SEQ ID NO: 3, except the scFv portion. The bold sequence is humanized PLAP-h2 (PMC409) scFv, the rest is same structure as mouse PLAP-CAR (SEQ ID NO: 5).

Humanized PLAP h2- CAR. Nucleotide sequence (codon optimized), SEQ ID NO: 19

<u>ATGGCCTTACCAGTGACCGCCTTGCTCCTGCCGCTGGCCTTGCTGCTCCACGCCGCCAGGCCG</u>gctagc

CAG GTG CAG CTT CAG GAA AGT GGA CCG GGC CTT GTC AAA CCG TCA GAG ACC CTT TCA CTG ACT TGC ACTGTA AGT GGT TTC TCC CTG ACA AGC TAC GGA GTC TCC TGG ATA CGC CAG CCA GCG GGG AAA GGG CTT GAGTGG ATC GGT GTG ATC TGG GAA GAC GGG AGT ACA AAC TAT CAC

TCA GCA CTC ATT AGT CGA GTA ACA ATGTCC GTT GAC ACT TCC AAG AAT CAA TTC AGT TTG AAA CTG TCT AGT GTG ACG GCT GCG GAT ACA GCG GTTTAT TAC TGT GCC AGG CCT CAT TAC GGA AGT TCT TAT GTT GGT GCA ATG GAG TAT TGG GGA GCC GGC ACAACT GTC ACT GTG AGC TCC GGC GGG GGC GGA AGT GGG GGA GGA GGC TCA GGC GGA GGT GGA AGT GAT ATACAG ATG ACC CAG AGT CCT AGC TCA CTC TCT GCG TCC GTA GGG GAC CGG GTA ACC ATC ACA TGC CGC GCCAGC GAG AAT ATA TAC AGT TAC GTT GCA TGG TAC CAG CAA AAA CCT GGC AAG GCG CCG AAG CTG TTG ATCTAC AAC GCC AAA AGT CTC GCT TCC GGG GTC CCC AGC CGA TTT TCT GGC TCA GGT AGT GGC ACA GAT TTCACA CTC ACA ATA AGC TCT CTC CAG CCC GAA GAC TTT GCG ACG TAC TAC TGC CAG CAT CAT TAT GTT AGTCCT TGG ACG TTT GGC GGA GGC ACA AAA TTG GAA ATA AAA

ctcgagAAGCCCACCACGACGCCAGCGCCGCGACCACCAACACCGGCGCCCACCAT CGCGTCGCAGCCCCTGTCCCTGCGCCCAGAGGCGAGCCGGCCAGCGGCGGGGG CGCAGTGCACACGAGGGGGCTGGACTTCGCCAGTGATaagcccttttgggtgctggtggtggttgg tggagtcctggcttgctatagcttgctagtaacagtggcctttattattttctgggtgaggagtaagaggagcaggctcctgcacagtgac tacatgaacatgactccccgccgccccgggcccacccgcaagcattaccagccctatgccccaccacgcgacttcgcagcctatcgc tccAGAGTGAAGTTCAGCAGGAGCGCAGACGCCCCCGCGTACCAGCAGGGCCAGA ACCAGCTCTATAACGAGCTCAATCTAGGACGAAGAGAGGAGTACGATGTTTTGG ACAAGAGACGTGGCCGGGACCCTGAGATGGGGGGAAAGCCGCAGAGAAGGAAG AACCCTCAGGAAGGCCTGTACAATGAACTGCAGAAAGATAAGATGGCGGAGGCC TACAGTGAGATTGGGATGAAAGGCGAGCGCCGGAGGGGCAAGGGGCACGATGG CCTTTACCAGGGTCTCAGTACAGCCACCAAGGACACCTACGACGCCCTTCACATG CAGGCCCTGCCCCCTCGCTAA

The humanized PLAP h2 CAR amino-acid sequence is shown in SEQ ID NO: 20. It has the same structure as Mouse PLAP-CAR except the scFv portion; sequence in bold is humanized PLAP ScFV consisting from VL-linker-VL.

M A L P V T A L L L P L A L L L H A A R P A S **Q V Q L Q E S G P G L V K P S E T L S L T C T V S G F S L T S Y G V S W I R Q P A G K G L E W I G V I W E D G S T N Y H S A L I S R V T M S V D T S K N Q F S L K L S S V T A A D T A V Y Y C A R P H Y G S S Y V G A M E Y W G A G T T V T V S S G G G G S G G G G S G G G G S**

D I Q M T Q S P S S L S A S V G D R V T I T C R A S E N I Y S Y V A W Y Q Q K P G
K A P K L L I Y N A K S L A S G V P S R F S G S G S G T D F T L T I S S L Q P E D
F A T Y Y C Q H H Y V S P W T F G G G T K L E I K L E K P T T T P A P R P P T P A
P T I A S Q P L S L R P E A S R P A A G G A V H T R G L D F A S D K P F W V L V V
V G G V L A C Y S L L V T V A F I I F W V R S K R S R L L H S D Y M N M T P R R P
G P T R K H Y Q P Y A P P R D F A A Y R S R V K F S R S A D A P A Y Q Q G Q N Q L
Y N E L N L G R R E E Y D V L D K R R G R D P E M G G K P Q R R K N P Q E G L Y
N E L Q K D K M A E A Y S E I G M K G E R R R G K G H D G L Y Q G L S T A T K D
T Y D A L H M Q A L P P R (SEQ ID NO: 20)

Humanized PLAP h2 VH (SEQ ID NO: 21)

Q V Q L Q E S G P G L V K P S E T L S L T C T V S G F S L T S Y G V S W I R Q P
A G K G L E W I G V I W E D G S T N Y H S A L I S R V T M S V D T S K N Q F S L
K L S S V T A A D T A V Y Y C A R P H Y G S S Y V G A M E Y W G A G T T V T V
S S

Humanized PLAP h2 VL (SEQ ID NO: 22), CDR 1, 2, 3, underlined

DIQMTQSPSSLSASVGDRVTITCRASENIYSYVAWYQQKPGKAPKLLIYNAKSLA
SGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQHHYVSPWTFGGGTKLEIK

Humanized PLAP h2 scFv (SEQ ID NO: 23)

Q V Q L Q E S G P G L V K P S E T L S L T C T V S G F S L T S Y G V S W I R Q P
A G K G L E W I G V I W E D G S T N Y H S A L I S R V T M S V D T S K N Q F S L
K L S S V T A A D T A V Y Y C A R P H Y G S S Y V G A M E Y W G A G T T V T V
S S G G G G S G G G G S G G G G S D I Q M T Q S P S S L S A S V G D R V T I T C
R A S E N I Y S Y V A W Y Q Q K P G K A P K L L I Y N A K S L A S G V P S R F S
G S G S G T D F T L T I S S L Q P E D F A T Y Y C Q H H Y V S P W T F G G G T K
L E I K

Example 5. PLAP CAR with humanized antibody h4.

The humanized PLAP h4 CAR (PMC410) codon optimized nucleotide sequence starts with a signaling peptide (underlined, SEQ ID NO: 9, codon optimized), then the humanized PLAP scFv (bold). The bold sequence is humanized PLAP-h4 (PMC410) scFv,

SEQ ID NO: 24 is the humanized PLAP h4-CAR nucleotide sequence (codon optimized).

ATGGCCTTACCAGTGACCGCCTTGCTCCTGCCGCTGGCCTTGCTGCTCCACGCCG
CCAGGCCGgctagc

5 **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 ACG 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**

10 **AAC CAG TTT TCA 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 TAT TGG GGG GCA GGT ACAACG GTC ACG GTG TCT TCA GGA GGA GGA GGG TCA GGT GGT GGT GGT TCA GGA GGC GGG GGT AGC GAC ATACAG ATG ACT CAA AGC CCC TCT TCA CTG TCT GCA TCA**

15 **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**

20 **CAT CAC TAT GTC AGCCCA TGG ACA TTT GGG GGA GGT ACG AAA CTT GAA ATT AAA**

ctcgagAAGCCCACCACGACGCCAGCGCCGCGACCACCAACACCGGCGCCCACCAT
CGCGTCGCAGCCCCTGTCCCTGCGCCCAGAGGCGAGCCGGCCAGCGGCGGGGGG
CGCAGTGCACACGAGGGGGCTGGACTTCGCCAGTGATaagcccttttgggtgctggtggtggttgg

25 tggagtcctggcttgctatagcttgctagtaacagtggcctttattattttctgggtgaggagtaagaggagcaggctcctgcacagtgac
tacatgaacatgactccccgccgccccgggcccacccgcaagcattaccagccctatgccccaccacgcgacttcgcagcctatcgc
tccAGAGTGAAGTTCAGCAGGAGCGCAGACGCCCCCGCGTACCAGCAGGGCCAGA
ACCAGCTCTATAACGAGCTCAATCTAGGACGAAGAGAGGAGTACGATGTTTTGG
ACAAGAGACGTGGCCGGGACCCTGAGATGGGGGGAAAGCCGCAGAGAAGGAAG

30 AACCCTCAGGAAGGCCTGTACAATGAACTGCAGAAAGATAAGATGGCGGAGGCC
TACAGTGAGATTGGGATGAAAGGCGAGCGCCGGAGGGGCAAGGGGCACGATGG
CCTTTACCAGGGTCTCAGTACAGCCACCAAGGACACCTACGACGCCCTTCACATG
CAGGCCCTGCCCCCTCGCTAAtag

SEQ ID NO: 25 is the humanized PLAP h4 CAR amino-acid sequence: ScFv sequence is in bold.

M A L P V T A L L L P L A L L L H A A R P A S **Q V Q L Q E S G P G L V K P S E T L**
S L T C T V S G G S I T S Y G V S W I R Q P P G K G L E W I G V I W E D G S T N
Y H S A L I S R V T I S V D T S K N Q F S L K L S S V T A A D T A V Y Y C A R P H
Y G S S Y V G A M E Y W G A G T T V T V S S G G G G S G G G G S G G G G S D I
Q M T Q S P S S L S A S V G D R V T I T C R A S E N I Y S Y V A W Y Q Q K P G K
A P K L L I Y N A K S L A S G V P S R F S G S G S G T D F T L T I S S L Q P E D F
A T Y Y C Q H H Y V S P W T F G G G T K L E I K L E K P T T T P A P R P P T P A P
T I A S Q P L S L R P E A S R P A A G G A V H T R G L D F A S D K P F W V L V V V
G G V L A C Y S L L V T V A F I I F W V R S K R S R L L H S D Y M N M T P R R P G
P T R K H Y Q P Y A P P R D F A A Y R S R V K F S R S A D A P A Y Q Q G Q N Q L Y
N E L N L G R R E E Y D V L D K R R G R D P E M G G K P Q R R K N P Q E G L Y N
E L Q K D K M A E A Y S E I G M K G E R R R G K G H D G L Y Q G L S T A T K D T
Y D A L H M Q A L P P R

Humanized PLAP-h4 VH (SEQ ID NO: 26). CDR 1, 2, 3 underlined

Q V Q L Q E S G P G L V K P S E T L S L T C T V S G *G S I T S Y G* V S W I R Q P P
G K G L E W I G V I W E D G S T N Y H S A L I S R V T I S V D T S K N Q F S L K
L S S V T A A D T A V Y Y C A R P H Y G S S Y V G A M E Y W G A G T T V T V S
S

Humanized PLAP-h4 VL (SEQ ID NO: 22)

Humanized PLAP-h4 scFv (SEQ ID NO: 27)

Q V Q L Q E S G P G L V K P S E T L S L T C T V S G G S I T S Y G V S W I R Q P P
G K G L E W I G V I W E D G S T N Y H S A L I S R V T I S V D T S K N Q F S L K
L S S V T A A D T A V Y Y C A R P H Y G S S Y V G A M E Y W G A G T T V T V S
S G G G G S G G G G S G G G G S D I Q M T Q S P S S L S A S V G D R V T I T C R
A S E N I Y S Y V A W Y Q Q K P G K A P K L L I Y N A K S L A S G V P S R F S G
S G S G T D F T L T I S S L Q P E D F A T Y Y C Q H H Y V S P W T F G G G T K L
E I K

Example 6. PLAP CAR with humanized antibody h3.

SEQ ID NO: 28 is the humanized PLAP-h3 (PMC407) nucleotide sequence:

ATGGCCTTACCAGTGACCGCCTTGCTCCTGCCGCTGGCCTTGCTGCTCCACGCCG
CCAGGCCG**gctagc**

**CAG GTT CAA TTG CAA GAA TCA GGC CCT GGG CTT GTT AAG CCG TCA
GAG ACG CTT TCA CTG ACC TGT ACCGTG AGC GGG TTC AGC CTC ACT
TCC TAT GGT GTT TCT TGG ATA CGA CAA CCA CCC GGA AAG GGC CTG
GAATGG ATC GGG GTC ATT TGG GAA GAT GGA TCC ACA AAC TAC AAT
CCT TCA CTT AAA TCC CGA GTT ACT ATCTCT GTT GAC ACC AGT AAA AAT
CAA TTC AGT CTC AAA CTG TCC AGT GTG ACA GCC GCC GAC ACA GCA
GTCTAC TAT TGC GCT CGC CCA CAT TAC GGC TCC AGC TAC GTT GGG
GCG ATG GAA TAT TGG GGA GCT GGT ACCACA GTC ACG GTT AGT AGT
GGA GGA GGT GGT TCC GGG GGA GGG GGG AGC GGC GGA GGT GGA TCT
GAT ATCCAG ATG ACT CAG TCT CCA AGT TCC CTT TCT GCA AGC GTA
GGT GAT CGA GTC ACT ATC ACA TGC AGG GCGTCC GAG AAC ATA TAC
AGT TAT GTT GCA TGG TAC CAA CAG AAG CCA GGT AAA GCG CCT AAG
CTG CTT ATTTAT AAC GCT AAA TCT CTT GCT TCT GGG GTA CCA TCC
CGA TTC TCA GGG TCT GGA AGT GGC ACT GAT TTCACG TTG ACT ATT
TCC TCC CTT CAA CCG GAG GAT TTT GCA ACG TAC TAC TGT CAG CAT
CAT TAT GTC AGCCCG TGG ACG TTC GGT GGC GGC ACG AAA CTT GAG
ATT AAA**

ctcgagAAGCCCACCACGACGCCAGCGCCGCGACCACCAACACCGGCGCCCACCAT
CGCGTCGCAGCCCCTGTCCCTGCGCCCAGAGGCGAGCCGGCCAGCGGCGGGGG
CGCAGTGCACACGAGGGGGCTGGACTTCGCCAGTGATaagcccttttgggtgctggtggtggttgg
tggagtcctggcttgctatagcttgctagtaacagtggcctttattattttctgggtgaggagtaagaggagcaggctcctgcacagtgac
tacatgaacatgactccccgccgccccgggcccacccgcaagcattaccagccctatgccccaccacgcgacttcgcagcctatcgc
tccAGAGTGAAGTTCAGCAGGAGCGCAGACGCCCCCGCGTACCAGCAGGGCCAGA
ACCAGCTCTATAACGAGCTCAATCTAGGACGAAGAGAGGAGTACGATGTTTTGG
ACAAGAGACGTGGCCGGGACCCTGAGATGGGGGGAAAGCCGCAGAGAAGGAAG
AACCCTCAGGAAGGCCTGTACAATGAACTGCAGAAAGATAAGATGGCGGAGGCC
TACAGTGAGATTGGGATGAAAGGCGAGCGCCGGAGGGGCAAGGGGCACGATGG
CCTTTACCAGGGTCTCAGTACAGCCACCAAGGACACCTACGACGCCCTTCACATG
CAGGCCCTGCCCCCTCGCTAAtag

SEQ ID NO: 29 is the PLAP h3 CAR amino-acid sequence (ScFv sequence bold).

M A L P V T A L L L P L A L L L H A A R P A S **Q V Q L Q E S G P G L V K P S E T L**
S L T C T V S G F S L T S Y G V S W I R Q P P G K G L E W I G V I W E D G S T N
Y N P S L K S R V T I S V D T S K N Q F S L K L S S V T A A D T A V Y Y C A R P
H Y G S S Y V G A M E Y W G A G T T V T V S S G G G G S G G G G S G G G G S D
I Q M T Q S P S S L S A S V G D R V T I T C R A S E N I Y S Y V A W Y Q Q K P G
K A P K L L I Y N A K S L A S G V P S R F S G S G S G T D F T L T I S S L Q P E D
F A T Y Y C Q H H Y V S P W T F G G G T K L E I K L E K P T T T P A P R P P T P A
P T I A S Q P L S L R P E A S R P A A G G A V H T R G L D F A S D K P F W V L V V
V G G V L A C Y S L L V T V A F I I F W V R S K R S R L L H S D Y M N M T P R R P
G P T R K H Y Q P Y A P P R D F A A Y R S R V K F S R S A D A P A Y Q Q G Q N Q L
Y N E L N L G R R E E Y D V L D K R R G R D P E M G G K P Q R R K N P Q E G L Y
N E L Q K D K M A E A Y S E I G M K G E R R R G K G H D G L Y Q G L S T A T K D
T Y D A L H M Q A L P P R

Humanized PLAP-h3 VH, SEQ ID NO: 30.

Q V Q L Q E S G P G L V K P S E T L S L T C T V S G F S L T S Y G V S W I R Q P
P G K G L E W I G V I W E D G S T N Y *N P S* L *K* S R V T I S V D T S K N Q F S L
K L S S V T A A D T A V Y Y C A R P H Y G S S Y V G A M E Y W G A G T T V T V
S S

Humanized PLAP h3 VL, SEQ ID NO: 22

Humanized PLAP h3 scFv, SEQ ID NO: 31

Q V Q L Q E S G P G L V K P S E T L S L T C T V S G F S L T S Y G V S W I R Q P
P G K G L E W I G V I W E D G S T N Y N P S L K S R V T I S V D T S K N Q F S L
K L S S V T A A D T A V Y Y C A R P H Y G S S Y V G A M E Y W G A G T T V T V
S S G G G G S G G G G S G G G G S D I Q M T Q S P S S L S A S V G D R V T I T C
R A S E N I Y S Y V A W Y Q Q K P G K A P K L L I Y N A K S L A S G V P S R F S
G S G S G T D F T L T I S S L Q P E D F A T Y Y C Q H H Y V S P W T F G G G T K
L E I K

Example 7. PLAP CAR with humanized antibody h5.

SEQ ID NO: 32 is the humanized PLAP-h5 scFv nucleotide sequence, which is inserted between Xho and NheI sites:

CAG GTC CAG CTG CAA GAA TCA GGA CCA GGA CTG GTA AAG CCG TCC GAA
5 ACG CTC AGT TTG ACG TGC ACCGTG TCA GGC GGC AGT ATA ACA TCC TAC G
GG GTC AGC TGG ATC CGC CAA CCG CCT GGG AAA GGC CTC GAATGG ATA G
GG GTG ATT TGG GAA GAC GGG AGT ACA AAC TAC AAT CCG AGT TTG AAG A
GC CGC GTG ACG ATAAGC GTT GAC ACA AGT AAG AAC CAG TTT AGT CTC AA
A CTC TCC AGT GTA ACA GCT GCT GAT ACA GCA GTGTAC TAC TGC GCT CGA
10 CCT CAC TAT GGC TCT AGT TAC GTC GGA GCT ATG GAA TAC TGG GGG GCT G
GC ACTACA GTT ACT GTG AGT TCC GGT GGC GGA GGA TCT GGT GGC GGT GG
T TCC GGT GGG GGA GGA TCC GAC ATACAG ATG ACG CAG TCC CCA AGT AGC
TTG AGC GCA TCA GTA GGA GAC AGA GTC ACC ATT ACA TGC CGA GCTTCC G
AG AAC ATC TAC AGT TAC GTA GCT TGG TAT CAG CAA AAA CCG GGG AAA G
15 CA CCT AAA CTT CTC ATCTAC AAC GCA AAA AGT CTG GCG AGT GGG GTT CC
C TCA AGG TTC TCT GGA AGC GGG AGC GGA ACG GAT TTTACT CTG ACT ATT
AGT AGT TTG CAA CCA GAA GAC TTT GCC ACG TAC TAC TGT CAG CAT CAC T
AT GTC TCCCCT TGG ACG TTC GGA GGA GGG ACC AAG CTC GAA ATC AAA
(SEQ ID NO: 31)
20

Humanized PLAP-h5 CAR amino-acid sequence (SEQ ID NO: 33)

M A L P V T A L L L P L A L L L H A A R P A S Q V Q L Q E S G P G L V K P S E T L S
L T C T V S G G S I T S Y G V S W I R Q P P G K G L E W I G V I W E D G S T N Y N P
25 S L K S R V T I S V D T S K N Q F S L K L S S V T A A D T A V Y Y C A R P H Y G S S
Y V G A M E Y W G A G T T V T V S S G G G G S G G G G S G G G G S D I Q M T Q S
P S S L S A S V G D R V T I T C R A S E N I Y S Y V A W Y Q Q K P G K A P K L L I Y
N A K S L A S G V P S R F S G S G S G T D F T L T I S S L Q P E D F A T Y Y C Q H H
Y V S P W T F G G G T K L E I K L E K P T T T P A P R P P T P A P T I A S Q P L S L R
30 P E A S R P A A G G A V H T R G L D F A S D K P F W V L V V V G G V L A C Y S L L
V T V A F I I F W V R S K R S R L L H S D Y M N M T P R R P G P T R K H Y Q P Y A
P P R D F A A Y R S R V K F S R S A D A P A Y Q Q G Q N Q L Y N E L N L G R R E E
Y D V L D K R R G R D P E M G G K P Q R R K N P Q E G L Y N E L Q K D K M A E A
Y S E I G M K G E R R R G K G H D G L Y Q G L S T A T K D T Y D A L H M Q A L P
35 P R

Humanized PLAP-h5 VH, SEQ ID NO: 34. CDR 1, 2, 3, underlined.

QVQLQESGPGLVKPSETLSLTCTVSG*GSITSYG*VSWIRQPPGKGLE*WIGVIWEDGS TNYNPSLKS*RVTISVDTSKNQFSLKLSSVTAADTAVYYCARPHYGSSYVGAMEYW
40 **GAGTTVTVSS**

Humanized PLAP h5 VL, SEQ ID NO: 22

Humanized PLAP h5 scFv, SEQ ID NO: 35

Q V Q L Q E S G P G L V K P S E T L S L T C T V S G G S I T S Y G V S W I R Q P P G K G L E W I G V I W E D G S T N Y N P S L K S R V T I S V D T S K N Q F S L K L S S V T A A D T A V Y Y C A R P H Y G S S Y V G A M E Y W G A G T T V T V S S G G G G S G G G G S G G G G S D I Q M T Q S P S S L S A S V G D R V T I T C R A S E N I Y S Y V A W Y Q Q K P G K A P K L L I Y N A K S L A S G V P S R F S G S G S G T D F T L T I S S L Q P E D F A T Y Y C Q H H Y V S P W T F G G G T K L E I K

Example 8. PLAP has negative expression in most normal tissues and expressed in gastro-intestinal cancers

We performed IHC staining with PLAP antibody on placenta, testis, colon cancer, ovarian cancer and other normal or malignant tissues from different types of cancer. Placenta had highest staining, testis, colon and ovarian cancer were positive, while other type of cancer (breast, lung, prostate cancer) were negative as well as normal tissues: pancreas, tonsil, rectum, muscle, esophagus, brain and other tissues. In addition, we evaluated mRNA expression of PLAP expression *in silico* across 1457 different malignant cell lines, including 63 colon cancer cell lines using the Cancer Cell Line Encyclopedia (CCLE). Expression of PLAP was high in gastro-intestinal (GI) cancers: cancers of esophagus, upper aerodigestive organs, stomach, pancreatic and colon cancers. We also performed analysis using Genotype-Tissue Expression (GTEx) database of PLAP expression in nonmalignant normal tissues. PLAP mRNA had minimal expression in many normal tissues (many had 0 TMP (transcript per million kb) mRNA level. In contrast when we analyzed EpCAM as a positive control, its expression was medium-high in many normal tissues with medium expression in colon 445 TMP (transcript per million kb), small intestine 391 and in thyroid 259. Thus, PLAP has negative expression in most normal tissues in contrast to other tumor-associated markers.

Example 9. PLAP is expressed in primary colon tumors and colon cancer cell lines

We performed IHC staining with mouse PLAP antibody using 106 primary colon cancer tumors, and found PLAP expression in 25 of 106 samples that is 23.8% of all colon cancer tumors. We also tested PLAP expression by R2 genomics analysis and visualization platform in 557 primary colon cancer tumors and performed correlation with patient outcome (FIG. 3). Patients with high PLAP expression had shorter survival than patients with low PLAP expression demonstrating that PLAP expression can correlate with poor prognosis in colon cancer. These data show that PLAP is overexpressed in primary colon cancer tumors.

In addition, we tested PLAP mRNA level in 117 colon cancer cell lines using microarray assay, and detected that 21.3% of colon cancer cell lines expressed PLAP mRNA. We performed FACS assay and detected PLAP in colon cancer cell lines with high PLAP mRNA expression: Lovo, Caco-2 and LS123 cell lines (FIG. 4A). We detected minimal
5 PLAP expression in PLAP-negative colon cancer cell lines such as HCT116, HT-29 and SW620 cell lines (FIG. 4B). Thus, PLAP mRNA and PLAP protein levels corresponded to each other (FIG. 4B). To confirm specificity of PLAP antibody H17E2 we detected that it recognized purified recombinant PLAP protein with Kd=3.2 nM by BLI BLITZ analysis. PLAP antibody H17E2 also recognized PLAP protein expressed in 293 cells. Thus, PLAP is
10 expressed in colon cancers and PLAP antibody detects PLAP antigen suggesting that it can be used for CAR-T therapy.

Example 10. PLAP-CAR-T cells specifically kill PLAP-positive cells but not PLAP-negative cells.

15 We designed second generation CAR construct using mouse monoclonal PLAP antibody ScFv, CD8 alpha hinge, CD28 transmembrane and co-stimulatory domain and CD3 activation domain (FIG. 2). We prepared lentiviral PLAP-CAR and Mock CAR with intracellular protein ScFv, and transduced T cells to generate CAR-T cells. The PLAP-CAR-T cells had >200-fold expansion that was similar as Mock-CAR-T cells or T cells. CAR-T
20 positive cells were detected by FACS with mouse FAB antibody (FIG. 5A).

PLAP-CAR-T cells were used in a Real-time cytotoxicity assay (RTCA) with PLAP-positive target colon cancer cell lines: Lovo, and LS-123; and with PLAP-negative colon cancer cell lines: HT29, and HCT116. PLAP-CAR-T cells had significant killing activity compared with normal T cells against Lovo and LS-123 colon cancer target cells but did not have
25 significant killing activity with PLAP-negative HCT116 and HT29 colon cancer cell lines (FIG. 5B). In addition, all CAR-T cells cell lines secreted significant level of IFN-gamma against PLAP-positive target colon cancer cells but not against PLAP-negative colon cancer cells (FIG. 5C). There were also no significant secretion of IFN-gamma against normal 293 and CHO cell lines (FIG. 5C). These data show specific functional activity of PLAP-CAR-T
30 cells against PLAP-positive colon cancer cell lines.

Example 11. Humanized PLAP-CAR-T cells (h2 and h4) specifically kill PLAP-positive cells

To improve mPLAP-CAR-T cells, we humanized mouse PLAP ScFv, and generated humanized PLAP-CAR cells (FIG. 2). The humanized PLAP h2 had 44.1% and PLAP h4 CAR-T cells had 50.6 % of CAR-positive cells which were detected by FACS with FAB antibody (FIG. 6A). To confirm specificity of PLAP-CAR-T cells to PLAP antigen, we performed FACS using biotinylated PLAP recombinant protein (FIG. 6B). Biotinylated PLAP protein recognized PLAP-CAR as well as FAB antibody demonstrating specific binding of humanized PLAP-ScFv to PLAP antigen (FIG. 6B).

PLAP-CAR-T cells (h2 and h4) significantly killed PLAP-positive cells compared to Mock control CAR-T cells and did not kill significantly PLAP-negative cells in RTCA assay (FIG. 6C). In addition, PLAP-CAR-T cells secreted significant level of IFN-gamma, IL-2 and IL-6 against PLAP-positive colon cancer cells but not against PLAP-negative colon cancer cells (FIG. 6D). These data show that humanized PLAP-CAR-T cells specifically and effectively killed PLAP-positive colon cancer cells.

Example 12. Humanized PLAP-CAR-T cells (h2 and h4) significantly decrease colon cancer xenograft tumor growth.

We analyzed PLAP-CAR-T cell efficacy in Lovo xenograft mouse model *in vivo* (FIG. 7). Lovo cancer cells were injected subcutaneously into NSG mice, and then CAR-T cells were injected at days 1, 7 and 13. Humanized PLAP h2 and PLAPh4-CAR-T cells significantly decreased Lovo xenograft tumor growth (FIG. 7A). The tumor size (FIG. 7B) and tumor weight (FIG. 7C) were significantly reduced by humanized PLAP-CAR-T cells. The mice body weight did not decrease by PLAP-CAR-T cells suggesting negative toxicity of CAR-T cells. Human T cells and CAR-T cells were detected in mouse blood with anti-human CD3 antibody at day 16 demonstrating persistence of humanized PLAP-CAR-T cells *in vivo*.

To test toxicity of CAR-T cells, we performed analysis of several enzymes from mouse blood serum: AST, ALT and amylase (FIG. 7D). There were no toxic effects of PLAP-CAR-T cells on these enzymes (FIG. 7D) suggesting no toxicity of PLAP-CAR-T cells *in vivo*. Thus, PLAP-CAR-T cells have high efficacy with no toxicity *in vivo*.

Example 13. Humanized PLAP-CAR-T cells (h5) specifically kill PLAP-positive cells

Real-time cytotoxicity assay (RCTA) and IFN-γ assay were performed according to Example 1.

FIG. 8A shows that PLAP h5-CAR-T cells significantly killed PLAP-positive colon cancer cells (Caco-2 cells and Lovo cells) compared to T cells and target cells alone. PLAP h5-CAR-T cells (h5) did not kill PLAP-negative colon cancer cells (HCT116) by RTCA. FIG. 8B shows that PLAP h5-CAR-T cells secreted significant higher level of IFN- γ, against PLAP-positive colon cancer cells (Caco-2 cells and Lovo cells), but not against PLAP-negative colon cancer cells (HCT116).

These data show that humanized PLAP h5-CAR-T cells specifically and effectively killed PLAP-positive colon cancer cells and specifically secreted IFN-gamma against PLAP-positive colon cancer cell line.

Example 14. Combination of PLAP-CAR-T cells with checkpoint inhibitors increased activity of CAR-T cells.

We tested expression of PDL-1 on colon cancer target cells in response to hPLAP-CAR-T cells when we co-cultured them for 24 hours (FIG. 9A). We also used IFN-γ, a known agent to induce PDL-1 in cancer cells [35] as a positive control for PDL-1 induction. The PLAP-negative cells HT29 and HCT116 cells activated PDL-1 in response to hPLAP-CAR-T cells similarly in response to T cells, Mock-CAR-T cells and IFN-γ (FIG. 9A). In contrast, PLAP-positive Lovo cells significantly up-regulated PDL-1 in response to CAR-T cells versus T and Mock CAR-T cells and more than in response to IFN-gamma (FIG. 9A). Caco-2 cells did not activate PDL-1 in response to IFN-gamma and also to PLAP-CAR-T cells (FIG. 9A). These data show that CAR-T cells caused significant up-regulation of PDL-1 in PLAP-positive cancer cells and that PLAP-positive cancer cells differ in their up-regulated PDL-1 levels, and that PLAP-CAR-T cells did not cause significant up-regulation of PDL-1 in PLAP-negative target colon cancer cells compared with Mock-Car-T cells and non-transduced T cells.

Since Lovo cells activated PDL-1 significantly more in response to PLAP-CAR-T cells than in response to IFN-gamma (FIG. 9A), we focused on PDL-1 up-regulation in this cell line in more detail. The expression of PDL-1 was low at one and 4 hours after addition of CAR-T cells and resulted in significant up-regulation of PDL-1 at 24 hours (FIG. 9B), and its level did not increase more at 49 hours (not shown). We added different doses of PLAP-CAR-T cells to Lovo cells, co-cultured for 24 hours, and detected dose-dependent response in terms of PDL-1 up-regulation in Lovo colon cancer target cells in response to hPLAP CAR-T cells (FIG. 9C). PDL-1 was significantly up-regulated even at small dose of PLAP-CAR-T cells added to target cancer cells (Effector to target cell ratio, E:T=0.3:1) (FIG. 9C).

To evaluate up-regulation of checkpoint proteins in CAR-T cells after co-incubation with colon cancer cells, we tested several checkpoint proteins: PD-1, TIM-3, TIGIT and LAG-3. Only PD-1 was significantly up-regulated in CAR-T cells after co-culture with PALP-positive colon cancer target cells than before co-culture (FIG. 9D). PD-1 protein level was up-regulated in co-culture with PLAP-positive cells (Caco-2 and Lovo cells) but not with PLAP-negative HCT116 and HT29 cells (FIG. 9D). LAG-3 was also significantly upregulated after co-culture with Lovo cancer cell line (FIG. 9E). Thus, PLAP-positive target cells up-regulated PDL-1, and PLAP-CAR-T cells up-regulated PD-1 or LAG-3 expression.

To test checkpoint inhibitors in combination with PLAP-CAR-T cells, we used PLAP-h2-CAR-T cells in combination with either PD-1 antibody or LAG-3 antibody and performed RTCA assay with Lovo target cells (FIG. 9F). There was significant up-regulation of cytotoxicity PLAP-CAR-T cells in combination with PD1 or LAG3 antibody versus PLAP-CAR-T cells alone with isotype antibody or versus PD-1 or LAG3 antibody alone (FIG. 9F). The combination of PLAP-CAR-T cells with either PD-1 antibody, or LAG-3 antibody significantly increased secretion of IFN-gamma versus each treatment alone in Lovo cells (FIG. 9G). The increased secretion of IFN-gamma was also observed when PLAP-CAR-T cells with PD-1 antibody were co-cultured with pre-treated IFN-g to up-regulate PDL-1 before treatment confirming data above (not shown). Thus, combination of hPLAP-CAR-T cells with checkpoint inhibitors (PD1 or LAG3 antibodies) can be an effective approach to induce efficacy of PLAP-CAR-T cells with increased IFN-gamma secretion against colon cancers.

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WHAT IS CLAIMED IS:

1. A chimeric antigen receptor (CAR) comprising from N-terminus to C-terminus:

(i) a single-chain variable fragment (scFv) comprising V_H and V_L, wherein scFv binds
5 to PLAP (placental alkaline phosphase),

(ii) a transmembrane domain,

(iii) a co-stimulatory domain of CD28, OX-40, GITR, or 4-1BB, and

(iv) an activating domain.

10 2. The CAR of Claim 1, wherein the V_H has the amino acid sequence of SEQ ID NO: 5 and the V_L has the amino acid sequence of SEQ ID NO: 6.

3. The CAR of Claim 1, wherein the V_H has the amino acid sequence of SEQ ID NO: 21, 26, 30, or 34, and the V_L has the amino acid sequence of SEQ ID NO: 22.

15

4. The CAR of Claim 3, wherein the V_H has the amino acid sequence of SEQ ID NO: 21, 26, or 34.

5. The CAR of Claim 1, wherein the scFv comprises SEQ ID NO: 8, 18, 23, 27, 31, or
20 35, or an amino acid sequence having at least 95% sequence identity thereof, provided that the sequence variation is in the non-CDR framework regions.

6. The CAR of Claim 1, wherein the scFv comprises SEQ ID NO: 8, 23, 27, or 35 or an amino acid sequence having at least 95% sequence identity thereof, provided that the
25 sequence variation is in the non-CDR framework regions.

7. The CAR of Claim 1, wherein the activating domain is CD3 zeta.

8. The CAR of Claim 1, wherein the co-stimulatory domain is CD28.

30

9. The CAR of Claim 1, having the amino acid sequence of SEQ ID NO: 5, 15, 20, 25, 29, or 33, or an amino acid sequence having at least 95% identity thereof, provided that the sequence variation is not in the CDR regions.

10. A nucleic acid sequence encoding the CAR of Claim 1.

11. T cells, nature-killer cells, or macrophages modified to express the CAR of Claim 1.

5 12. A method for treating cancer, comprising the step of administering the T cells, nature-killer cells, or macrophages of Claim 11 to a patient suffering from cancer selected from the group consisting of colon cancer, lung cancer, pancreatic cancer, stomach cancer, testicular cancer, teratoma, seminoma, ovarian cancer, and cervical cancer, wherein the cancer is PLAP-positive.

10

13. The method of Claim 12, further comprising administering to the patient a checkpoint inhibitor selected from the group consisting of: PD-1 antibody, PDL-1 antibody, and LAG-3 antibody.

15 14. An antibody or antigen-binding fragment thereof comprising VL having the amino acid sequence of SEQ ID NO: 22, and VH having the amino acid sequence of SEQ ID NO: 21, 26, or 34.

20

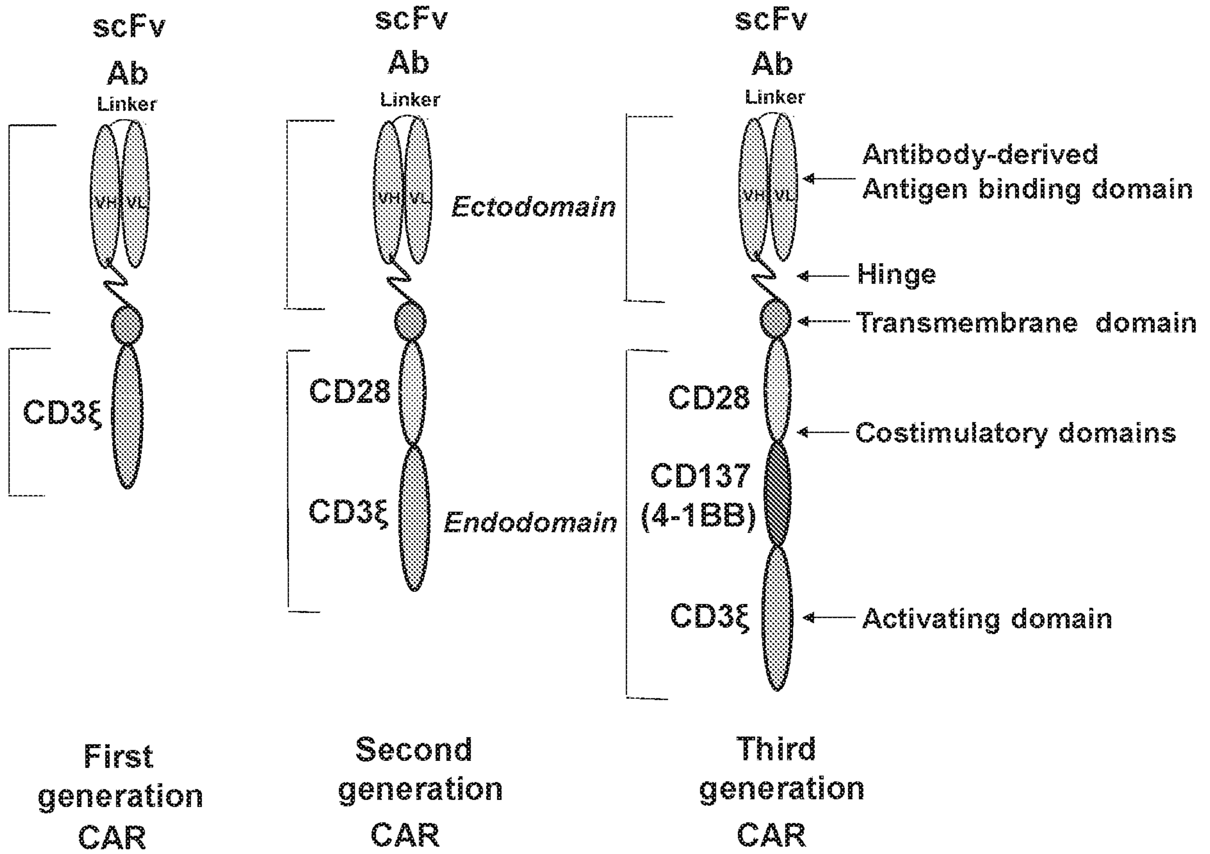


FIG. 1

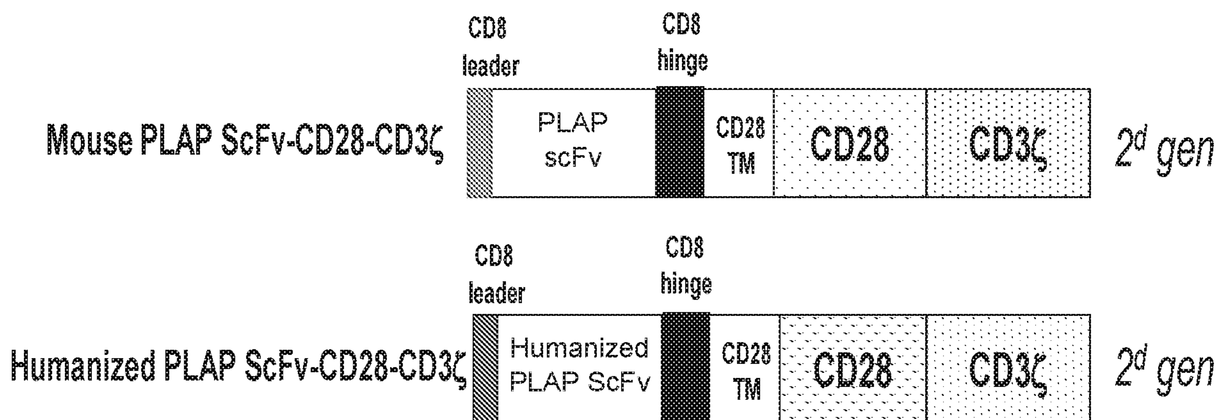


FIG. 2

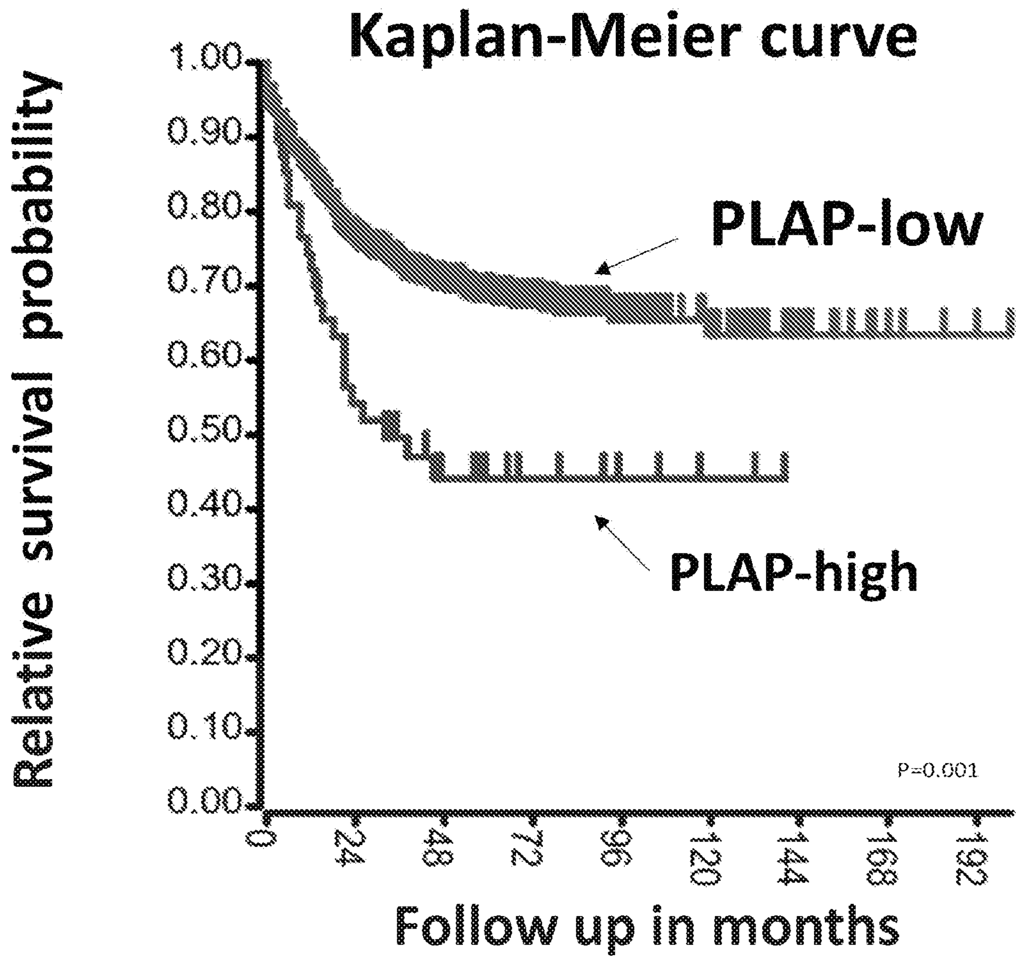


FIG. 3

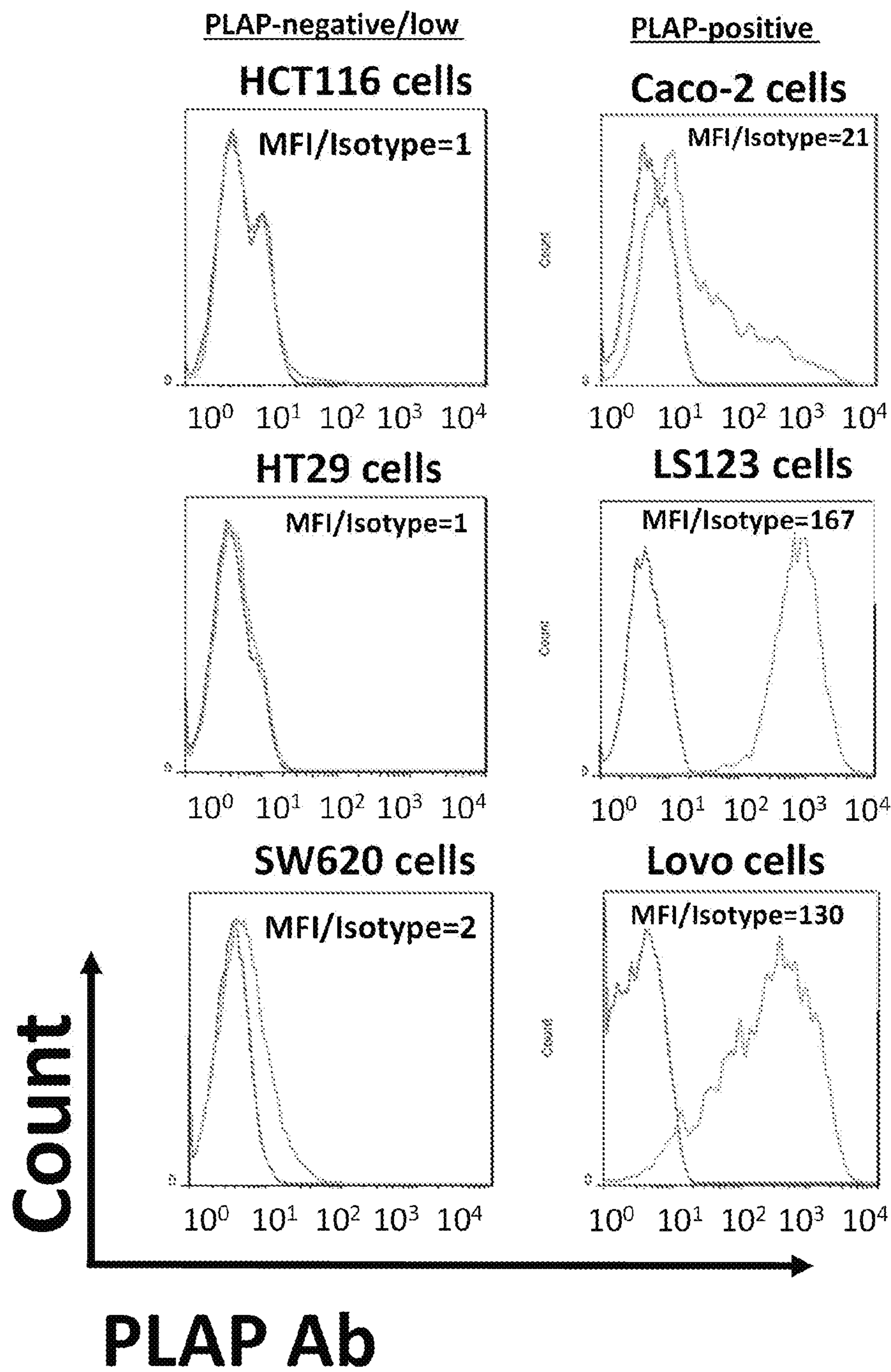


FIG. 4A

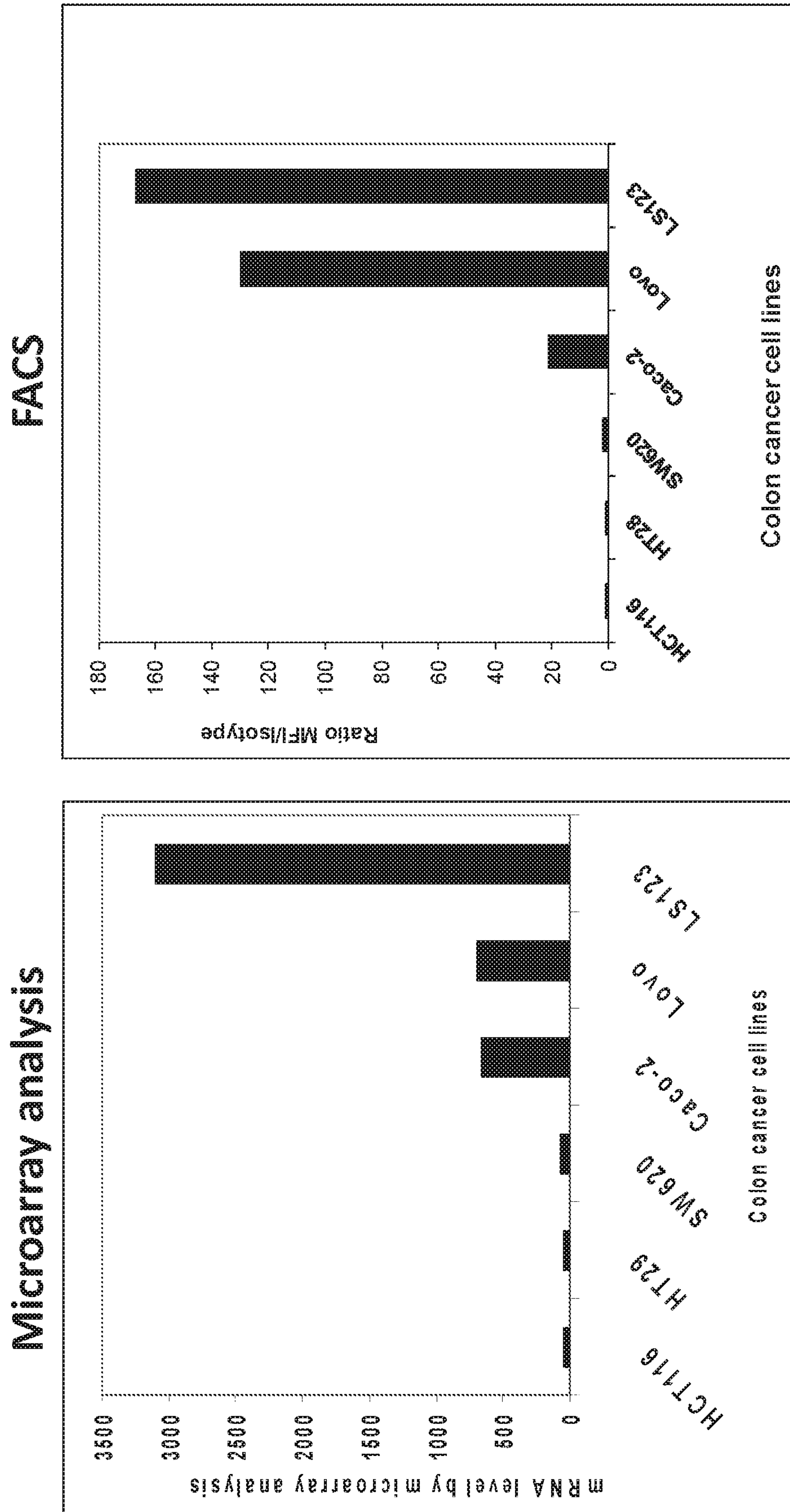


FIG. 4B

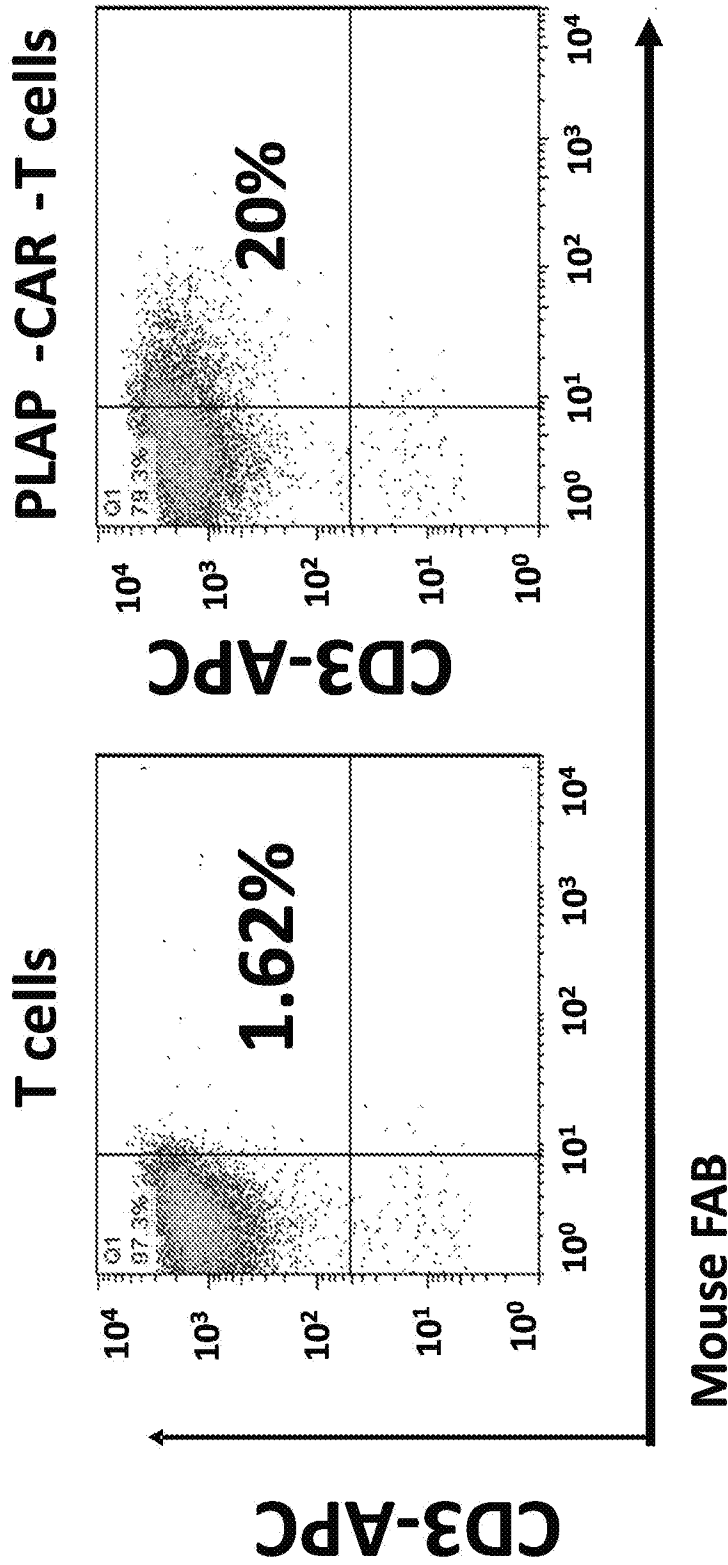


FIG. 5A

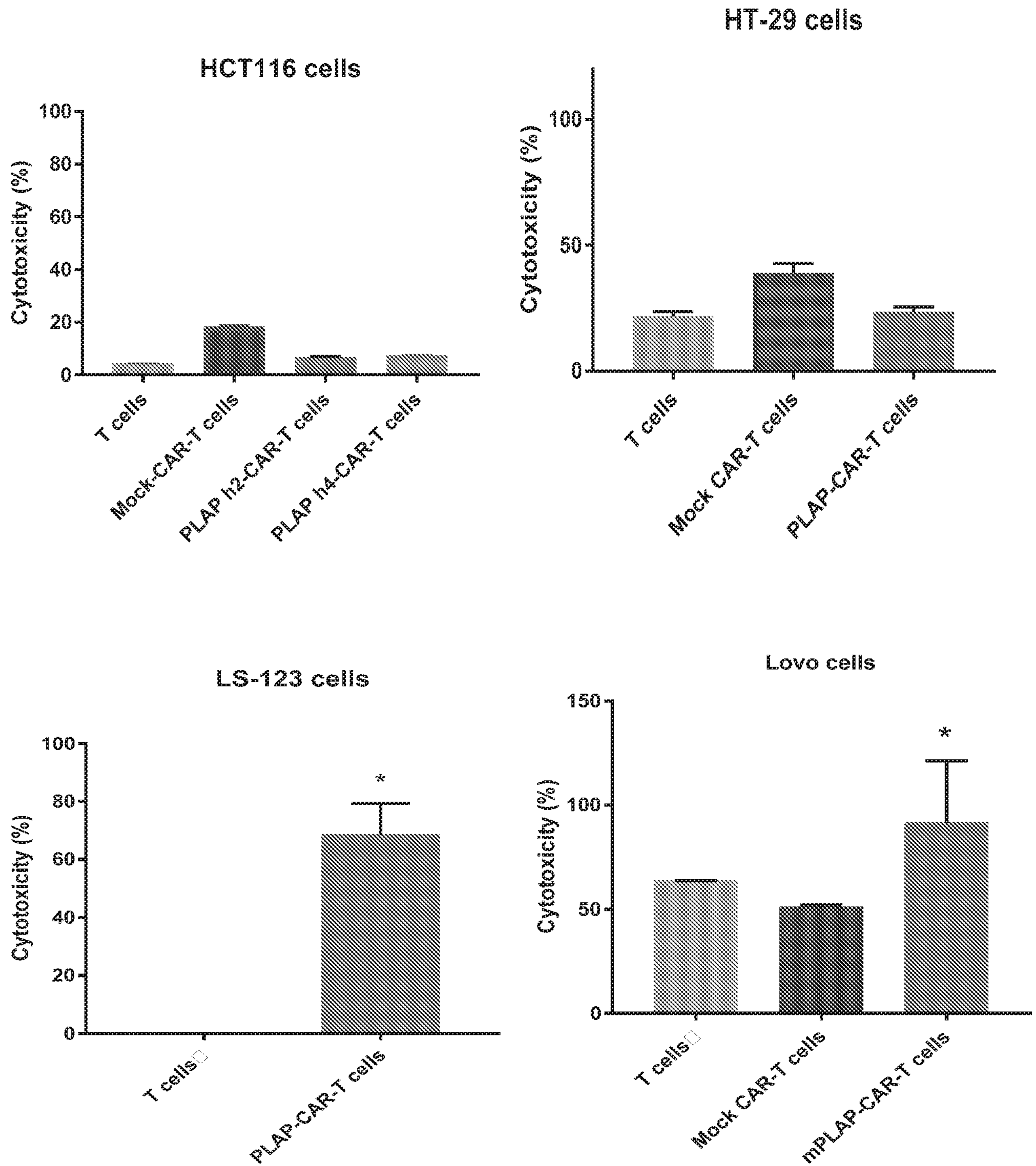


FIG. 5B

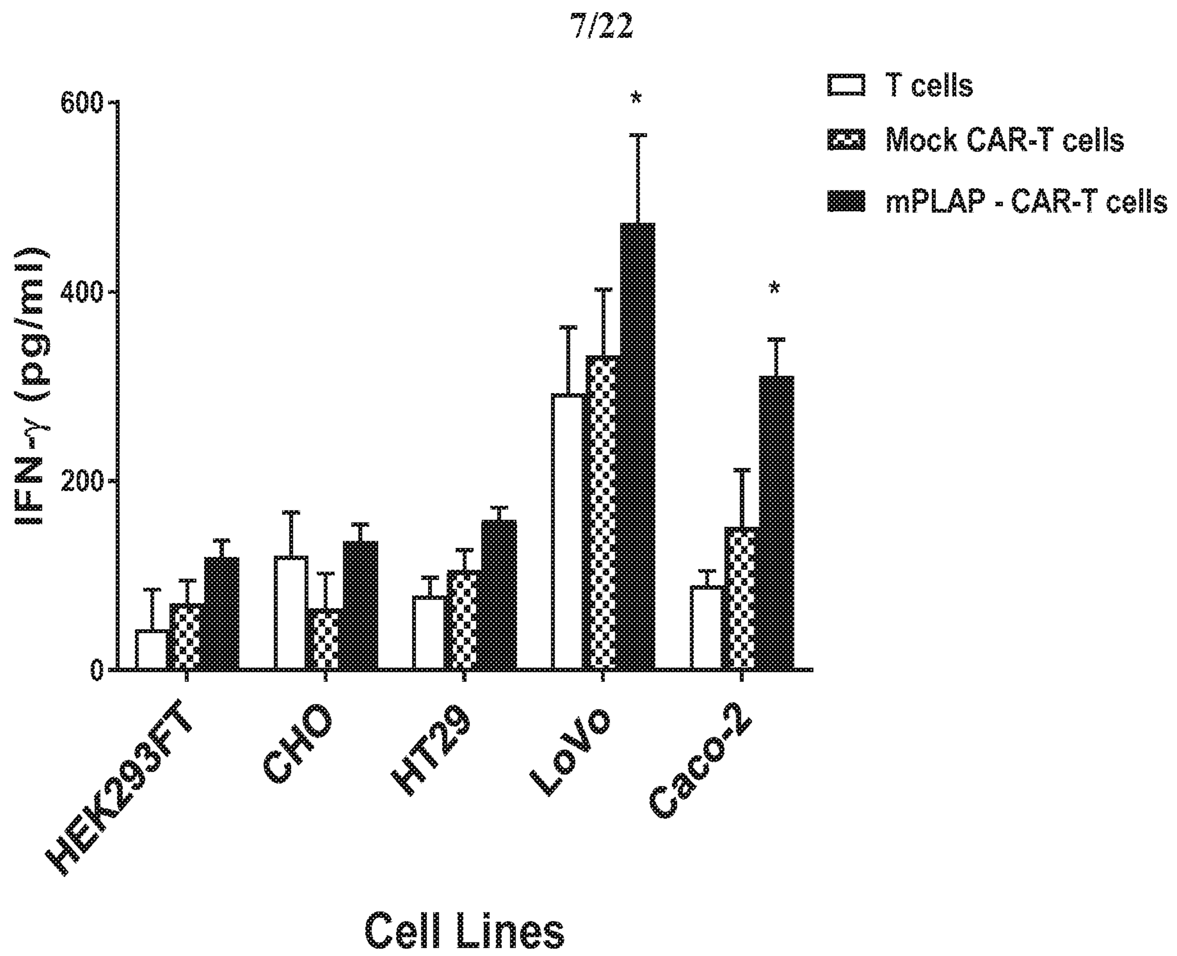


FIG. 5C

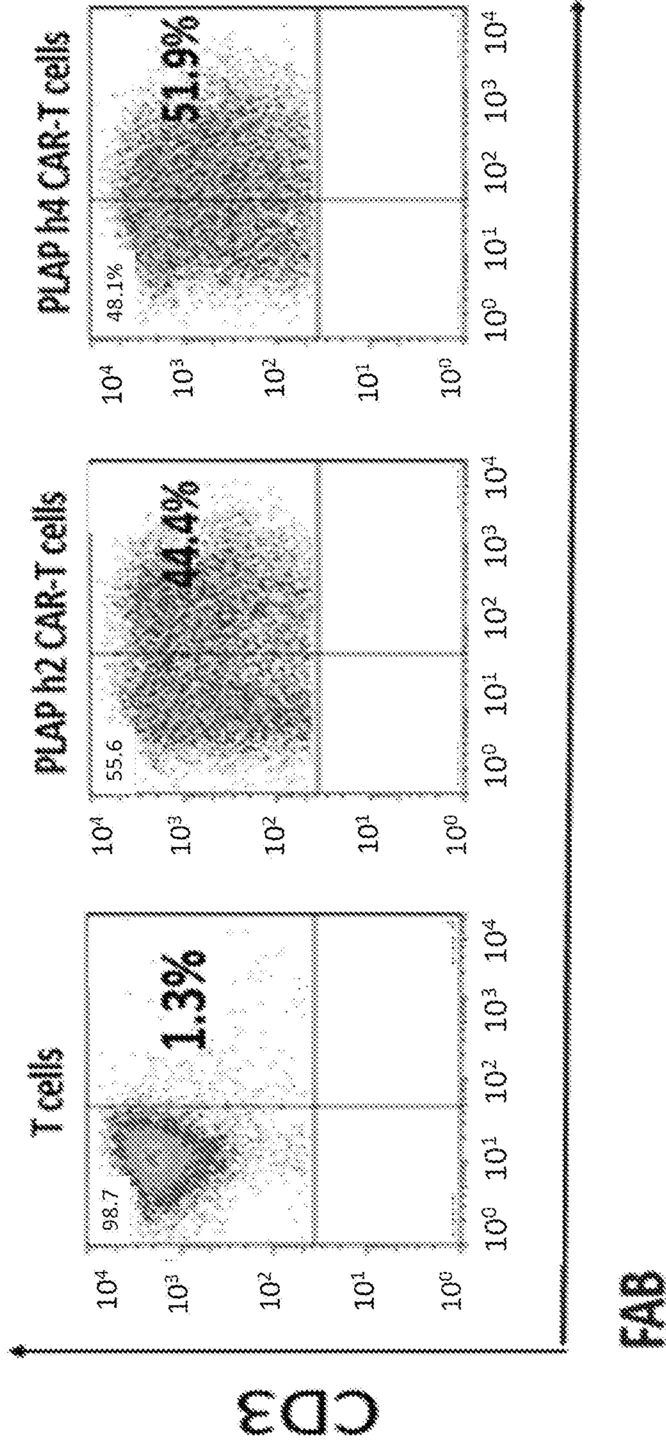


FIG. 6A

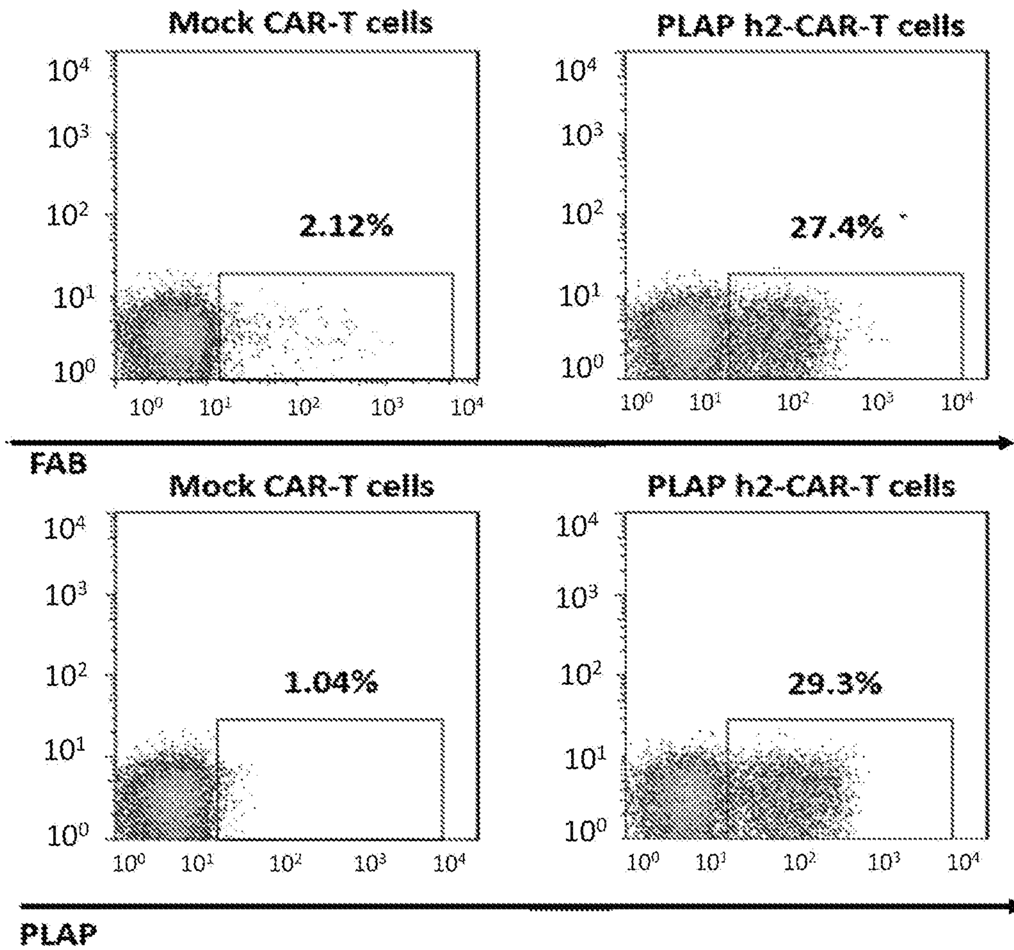


FIG. 6B

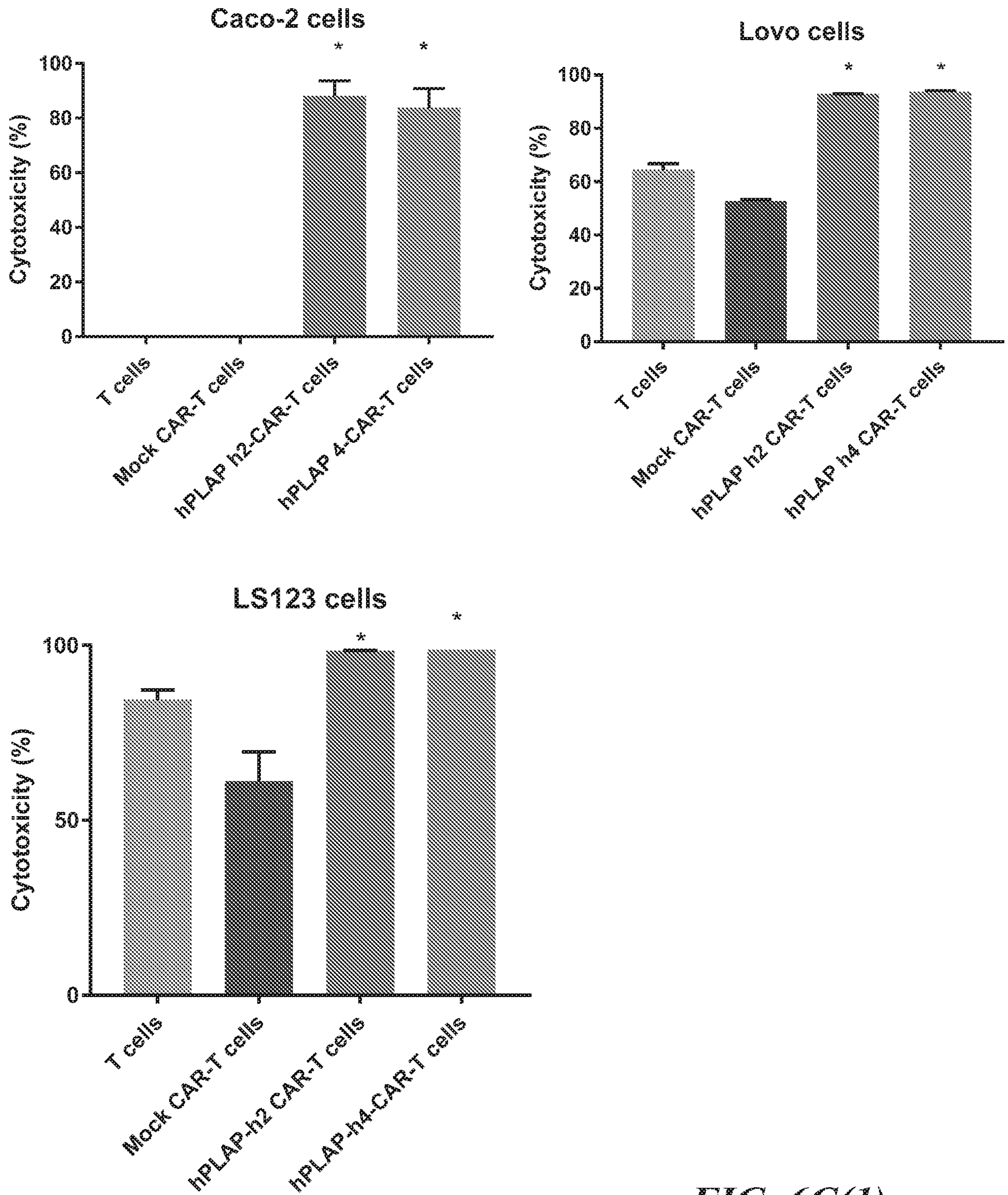


FIG. 6C(1)

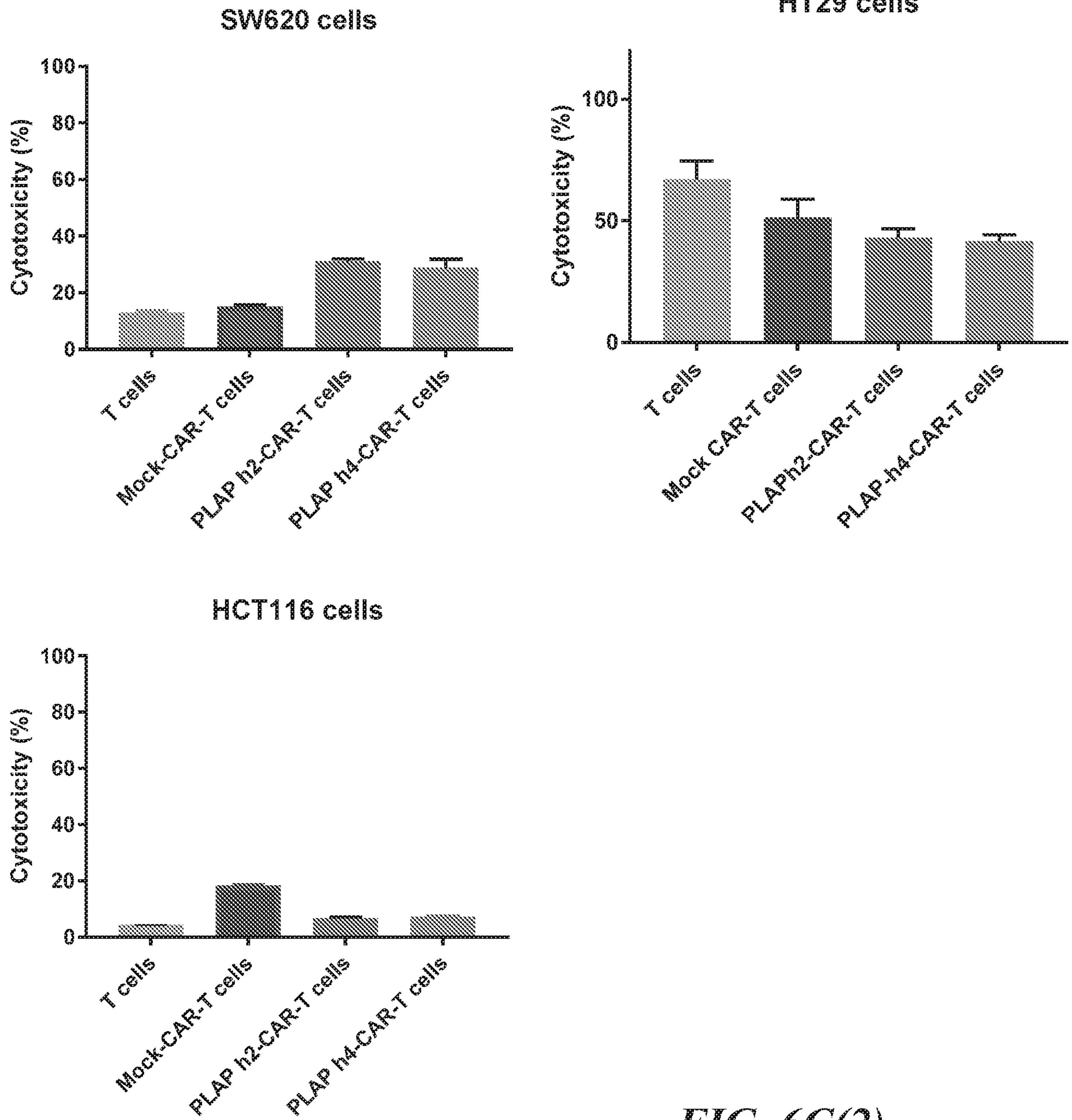
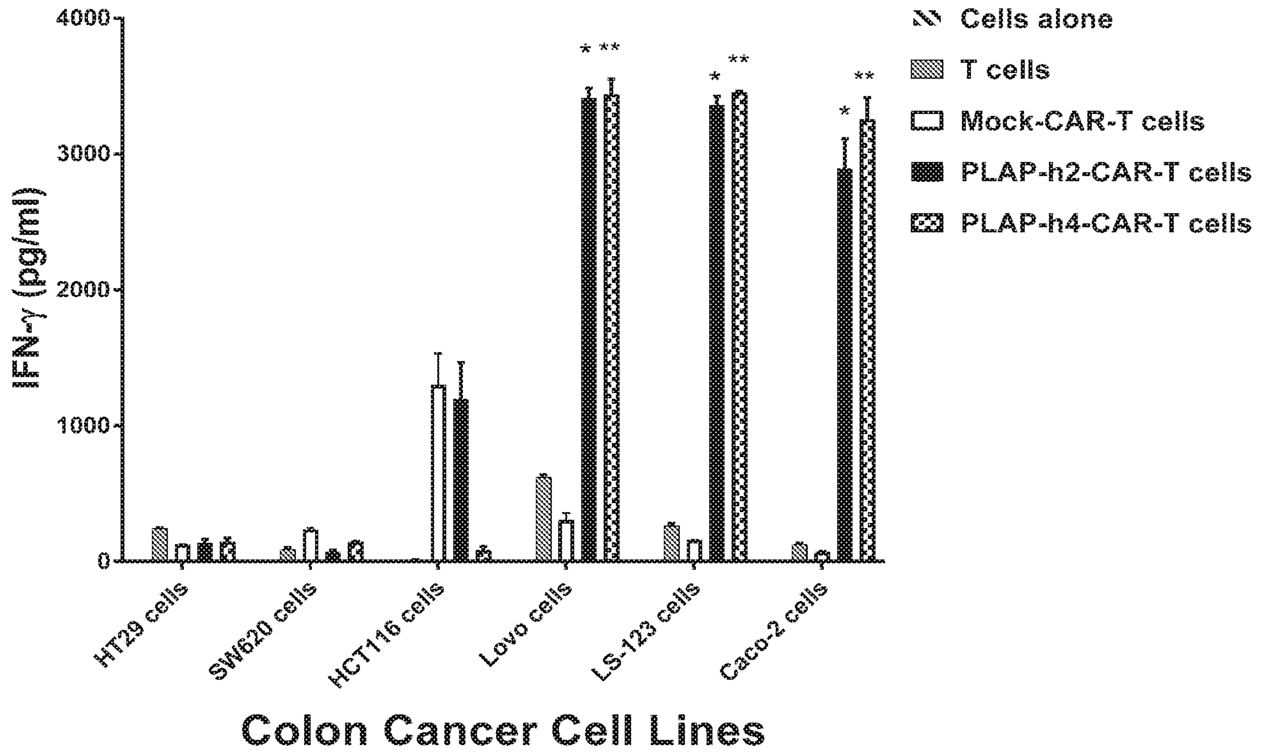


FIG. 6C(2)



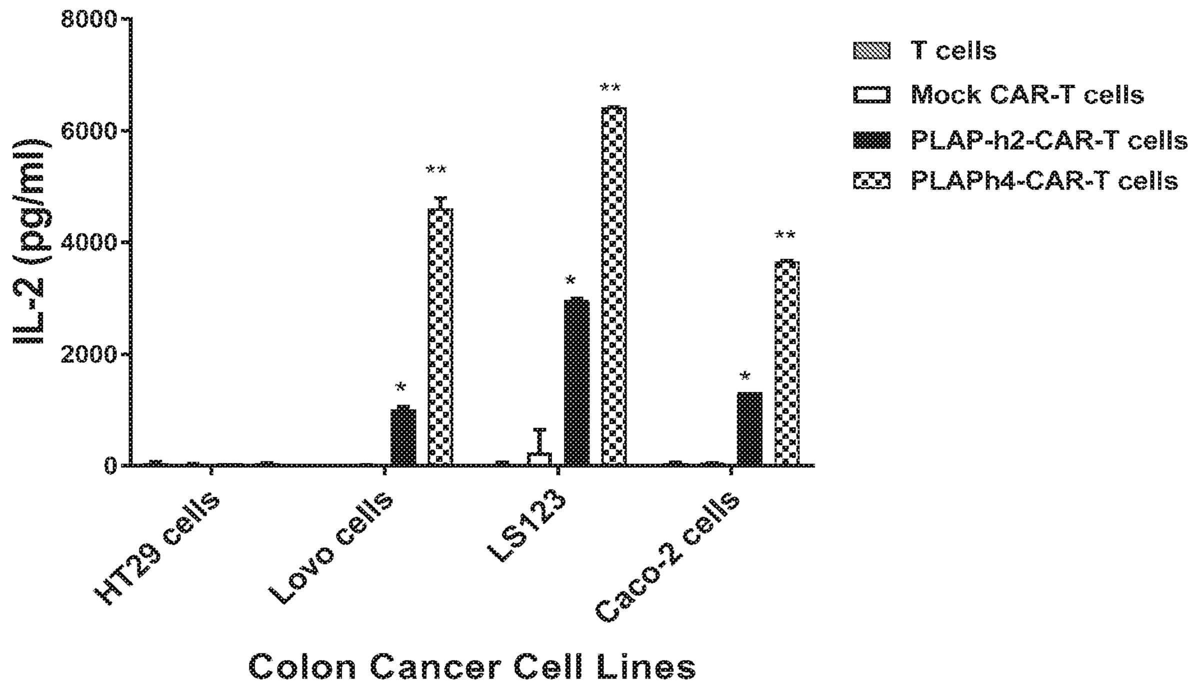


FIG. 6D(1)

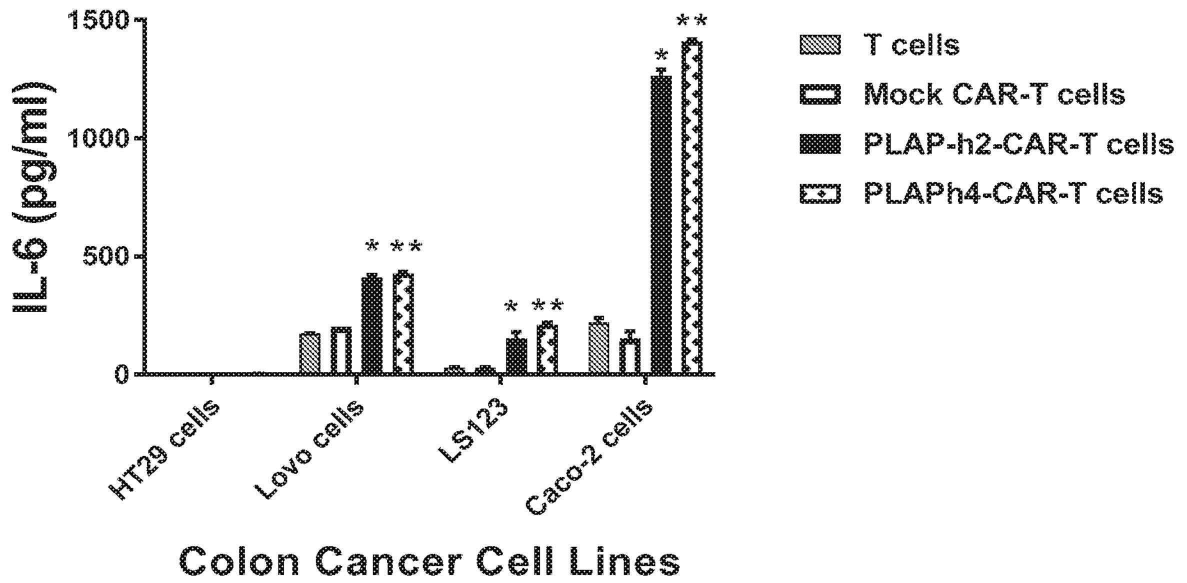


FIG. 6D(2)

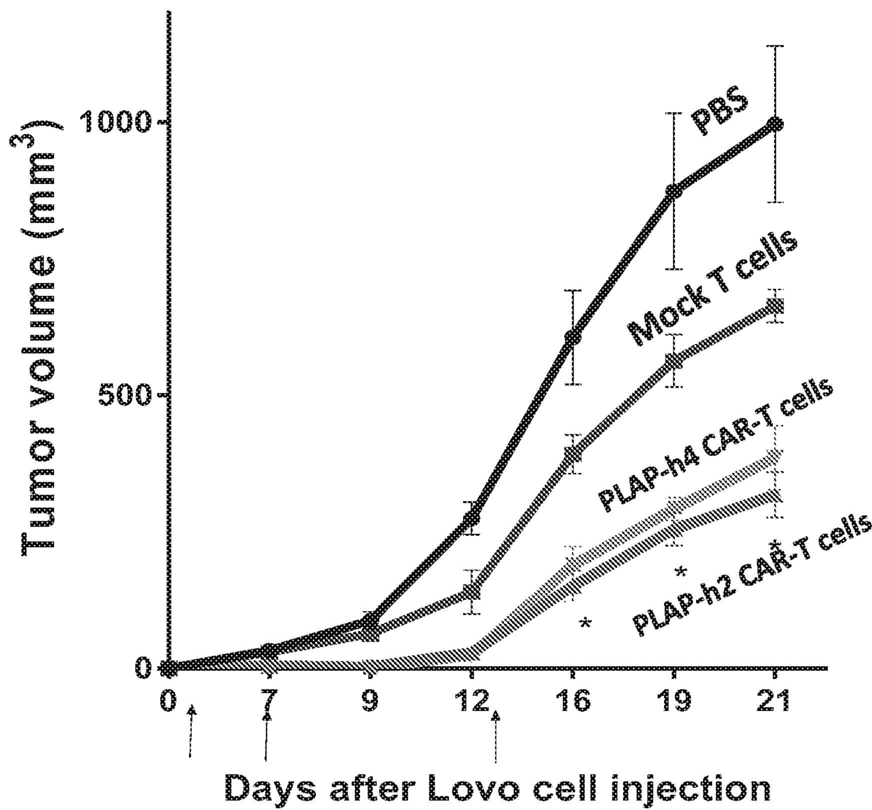


FIG 7A

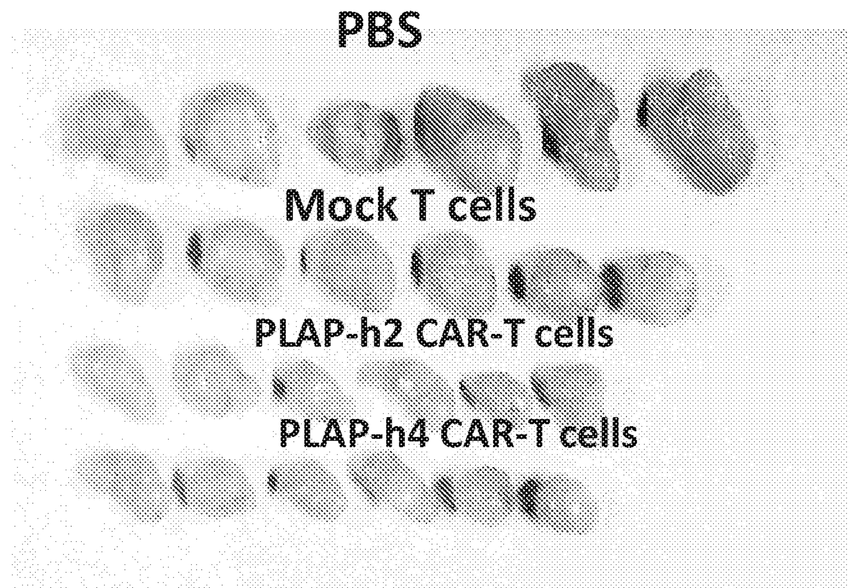


FIG. 7B

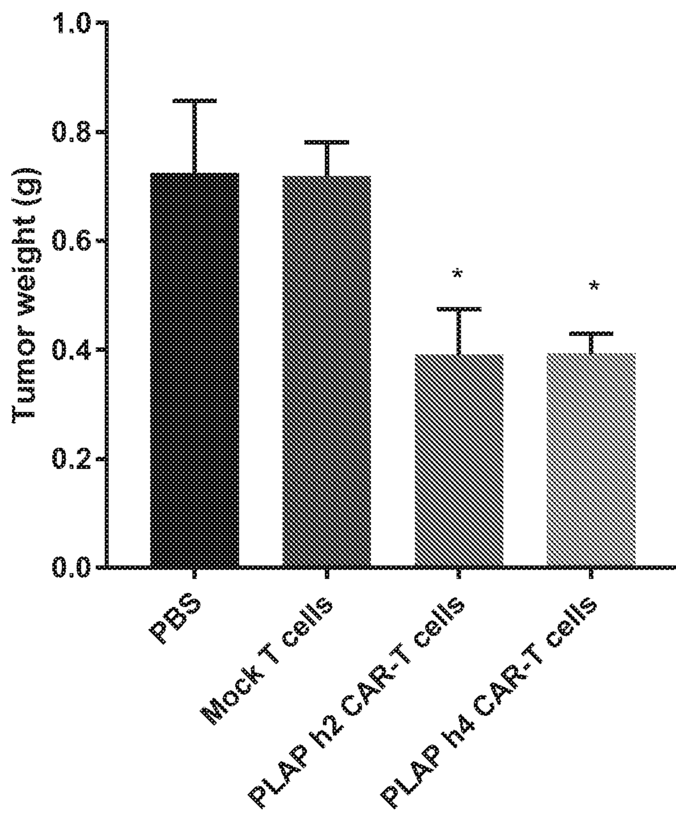


FIG. 7C

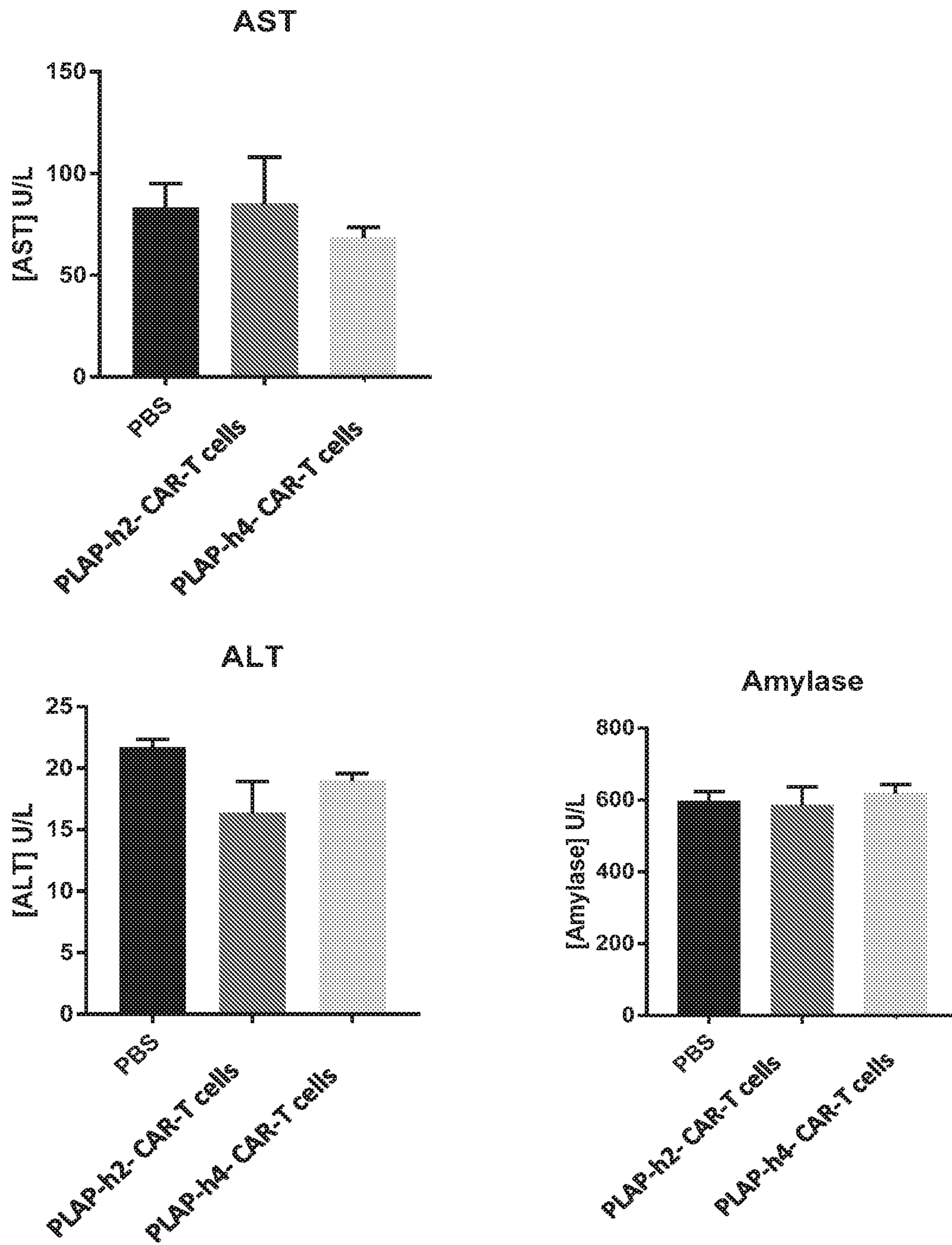


FIG. 7D

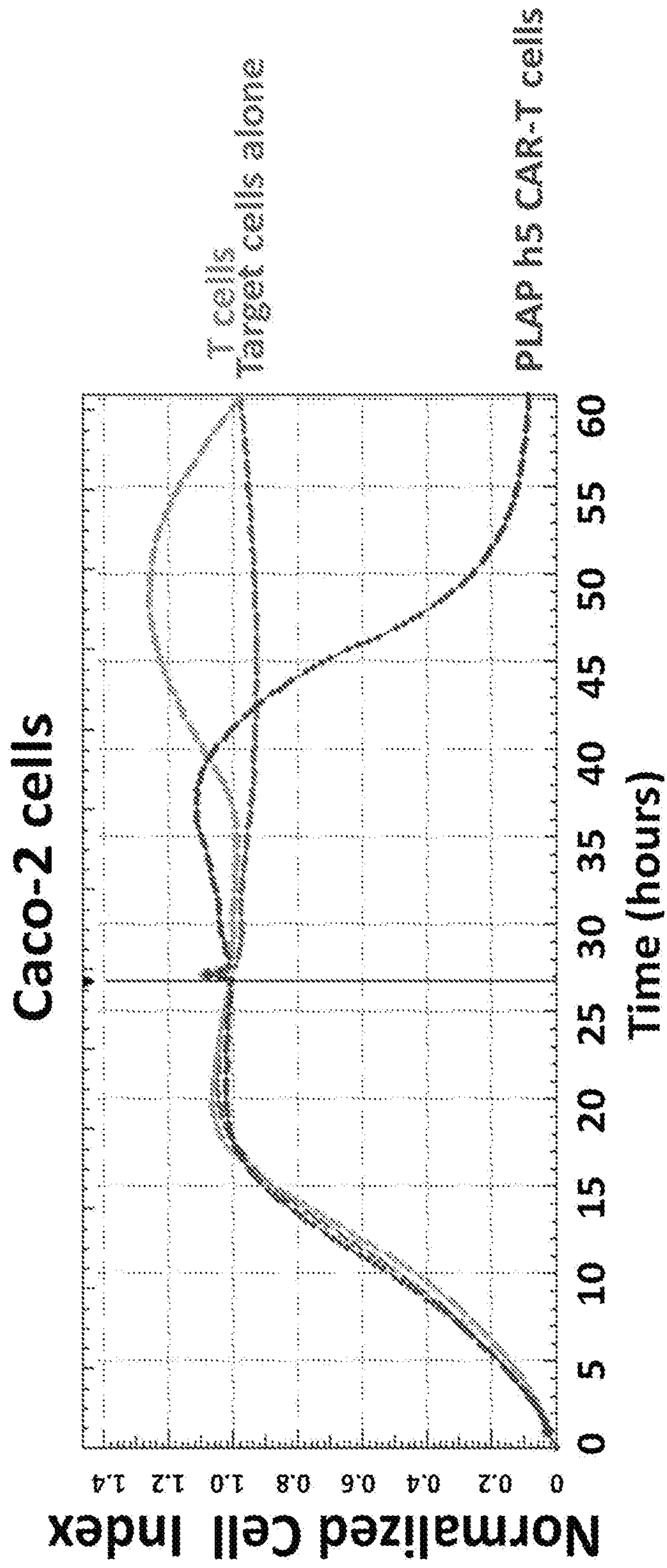


FIG. 8A(1)

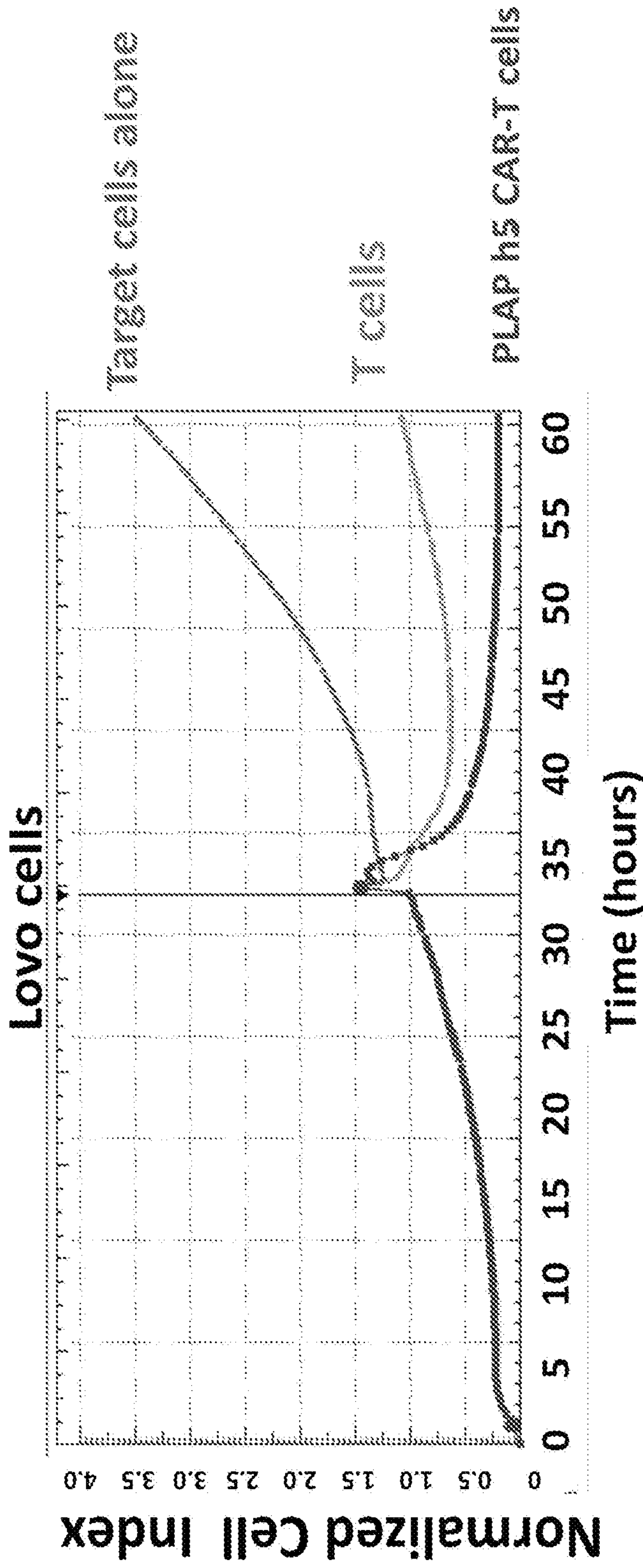


FIG. 8A(2)

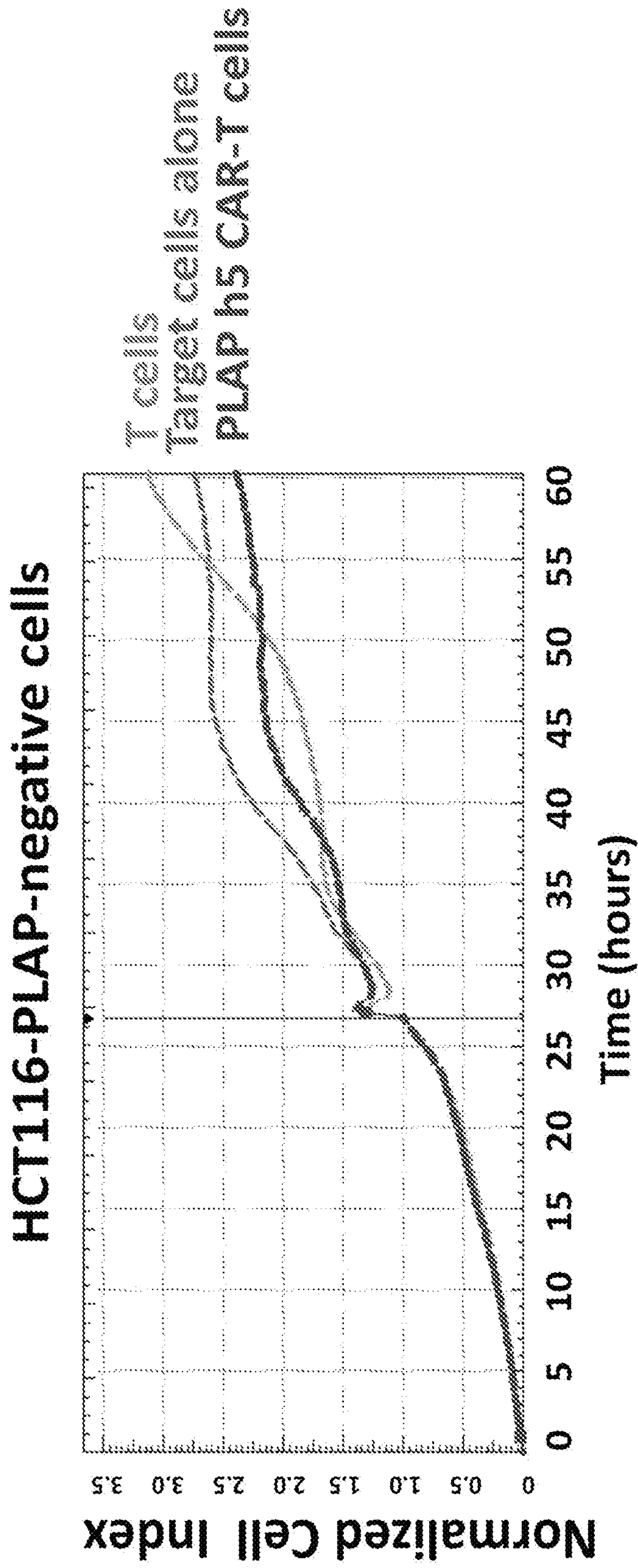


FIG. 8A(3)

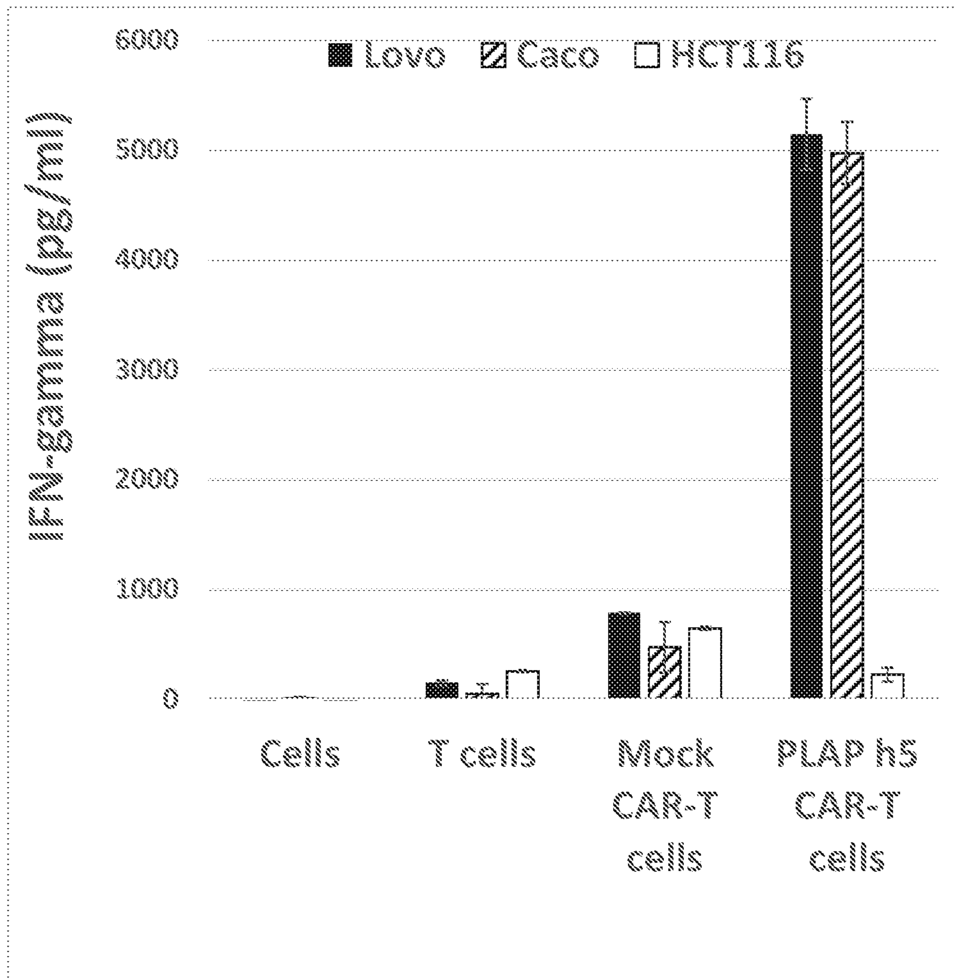


FIG. 8B

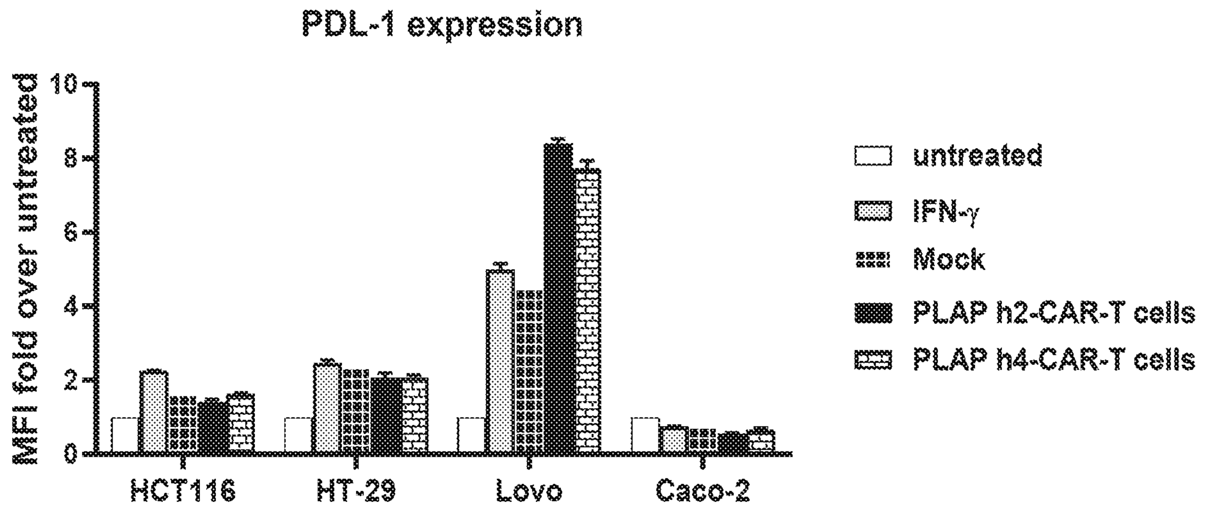


FIG. 9A

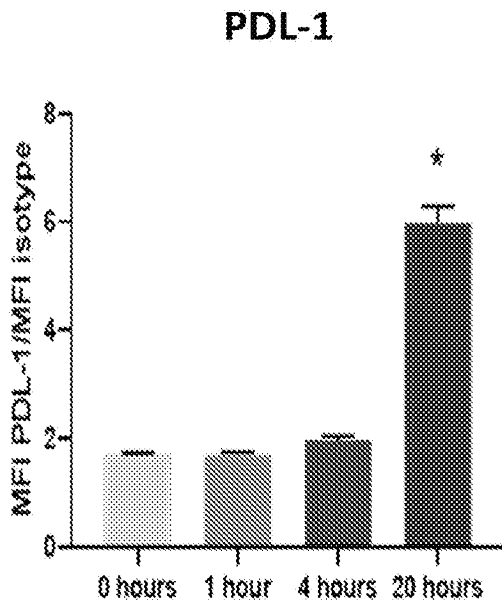


FIG. 9B

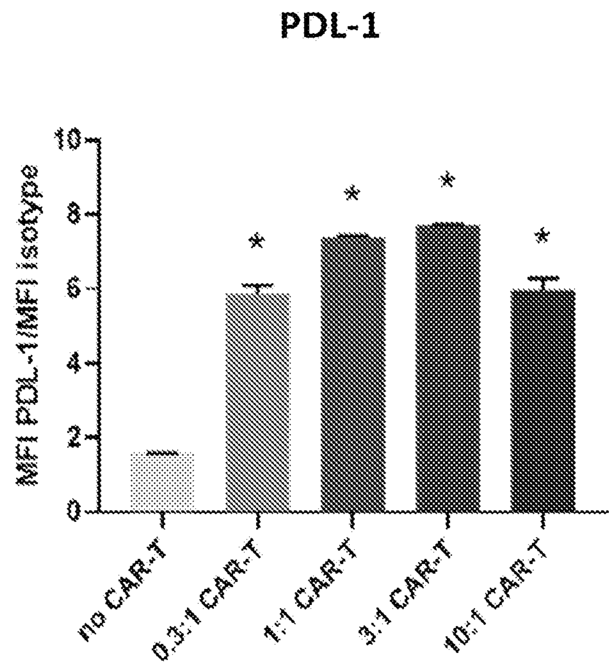


FIG. 9C

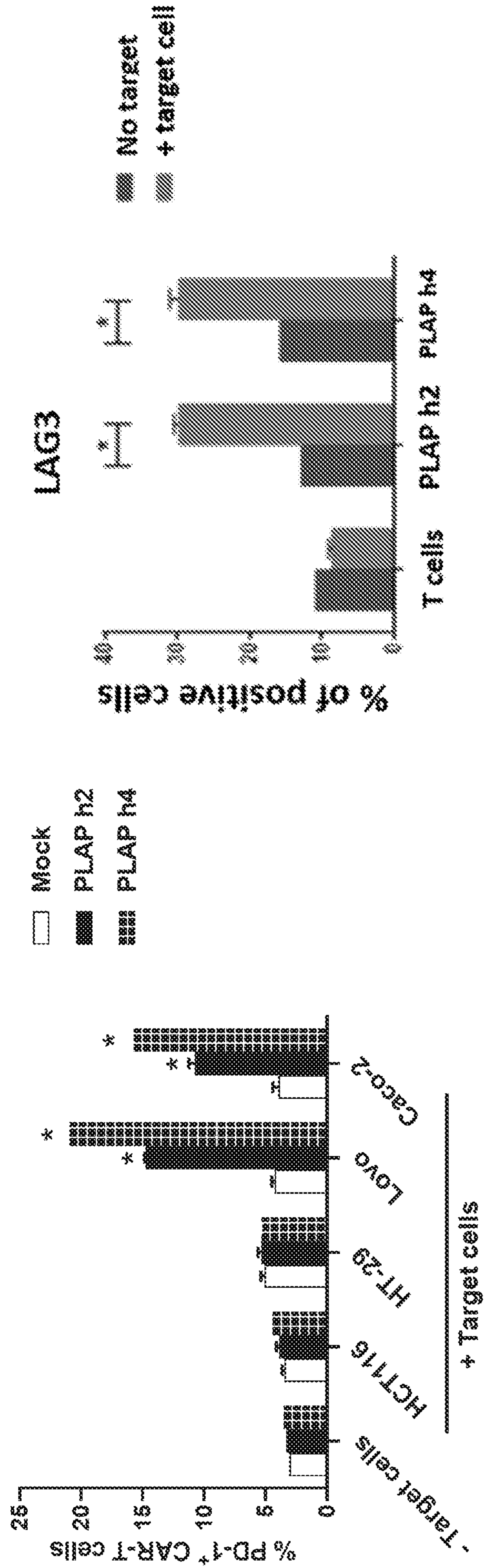


FIG. 9D

FIG. 9E

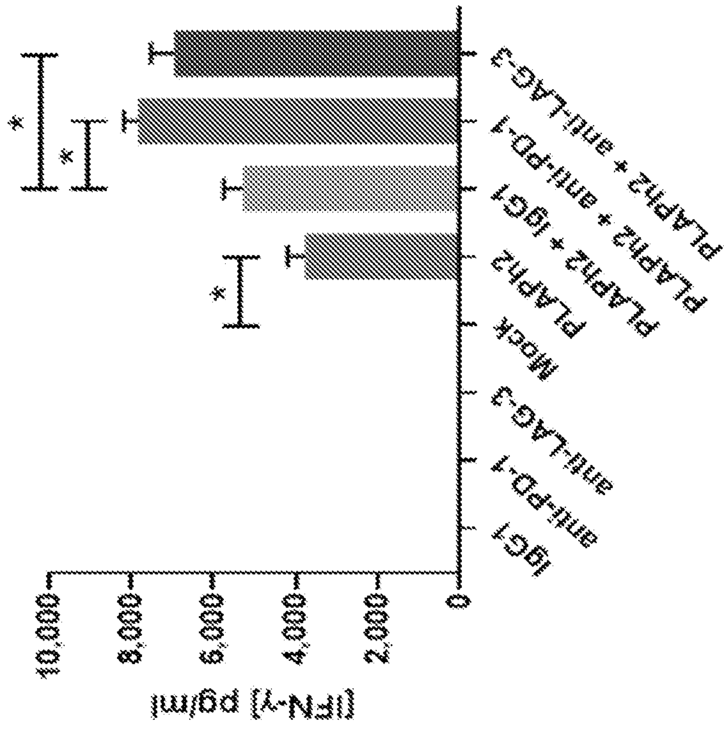


FIG. 9G

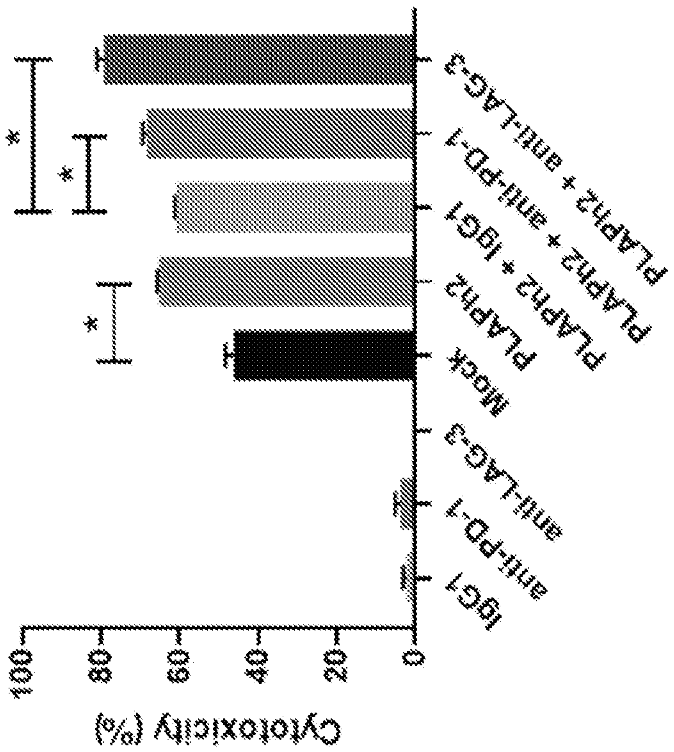


FIG. 9F

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2019/033953

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - A61K 39/00; A61K 39/395; C07K 14/705 (2019.01)
CPC - A61K 39/0011; C07K 14/7051; C07K 14/70521; C07K 2317/622; C07K 2319/03 (2019.08)

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
USPC - 424/134.1; 514/19.3 (keyword delimited)

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.
X -- Y	US 2015/0307623 A1 (ANTHROGENESIS CORPORATION) 29 October 2015 (29.10.2015) entire document	1, 7, 8, 10-12 ---------------- 13
Y	US 2018/0021378 A1 (ANTHROGENESIS CORPORATION) 25 January 2018 (25.01.2018) entire document	13
P, X	US 2018/0273640 A1 (CELGENE CORPORATION) 27 September 2018 (27.09.2018) entire document	1-14
P, X	WO 2018/200713 A1 (DANA-FARBER CANCER INSTITUTE, INC.) 01 November 2018 (01.11.2018) entire document	1-14
A	EP 0429242 B1 (UNILEVER PLC et al) 14 June 1995 (14.06.1995) entire document	1-14

☐ 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
"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
09 August 2019	03 SEP 2019

Name and mailing address of the ISA/US	Authorized officer
Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, VA 22313-1450 Facsimile No. 571-273-8300	Blaine R. Copenheaver PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2019/033953

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. [X] forming part of the international application as filed:
      - [X] 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:

SEQ ID NOs: 5, 6, 8, 15, 18, 20-23, 25-27, 29-31, and 33-35 were searched.