

ANTI-CTHRC1 T CELL ENGAGERS AND METHODS OF USING THE SAME**CROSS-REFERENCE TO RELATED APPLICATIONS**

[0001] This application claims the benefit of U.S. Provisional Application No. 63/526,444, filed July 12, 2023, which is incorporated herein by reference in its entirety.

BACKGROUND

[0002] Collagen triple helix repeat containing 1 (CTHRC1) is a highly conserved protein that is found in both normal and cancerous tissues and is implicated in metabolism, arterial remodeling, bone formation and myelination of the peripheral nervous system (Liu, et al. 2023). While CTHRC1 has been associated with cancer, the exact mechanism of its function in cancer and even its form and location have been difficult to ascertain. In some studies, CTHRC1 has been found to be only in tumor stroma, which represents a primary barrier to anti-tumor immunity and therapeutic targeting, with stroma largely considered a major immunosuppressive component that facilitates resistance to immune checkpoint inhibitors. However, CTHRC1 has been found to exist in multiple different molecular weight forms with potentially different epitope mapping and it is possible that its association with certain cytoplasmic proteins may prevent certain epitopes from being exposed, and anti-CTHRC1 antibodies have been found to vary in specificity (Liu, et al. 2023).

[0003] The development of therapeutically effective T-cell engagers has also faced numerous challenges. On such challenge is antibody internalization, resulting in a reduced therapeutic window as the antibody has a low residence time on the cell surface. In the case of a bispecific antibody, upon internalization, the T-cell engager arm can no longer function to recruit T cells to the cell with the tumor antigen. The present invention addresses this and other unmet needs.

SUMMARY

[0004] As demonstrated herein, certain antibodies against the stromal target CTHRC1 can have varying properties with respect to cellular internalization. In the context of an anti-CTHRC1 T-cell engager, this lack of internalization can assist in therapeutic efficacy by avoiding internalization which otherwise reduces the ability of the T-cell engager to recruit T

cells to the cell. Given that certain antibodies can bind a CTHRC1 epitope on a cell without being internalized, this can provide an effective solution to the challenges in the prior art.

[0005] To that end, the present disclosure provides anti-CTHRC1 T-cell engagers and compositions thereof wherein the anti-CTHRC1 T-cell engager comprises at least one collagen triple helix repeat containing 1 (CTHRC) binding moiety that selectively binds to human CTHRC1 and a cell displaying a CTHRC1 epitope without being internalized, and at least one T cell engager moiety, preferably wherein the T cell engager moiety comprises an anti-CD3 antibody or fragment thereof. The present disclosure further provides methods for making the same and the use of same in the treatment of cancer.

[0006] In one aspect, an anti-CTHRC1 T-cell engager comprises at least one first domain comprising a CTHRC1 binding moiety that selectively binds to human CTHRC1 and a cell displaying a CTHRC1 epitope without being internalized, and at least one second domain comprising a T cell engager moiety. In embodiments, the T cell engager moiety comprises an anti-CD3 antibody or fragment thereof.

[0007] In embodiments, the CTHRC1 binding moiety comprises an anti-CTHRC1 antibody which binds to CTHRC1; preferably wherein the CTHRC1 antibody binds to CTHRC1 with a binding affinity of less than 10 nM. In embodiments, the anti-CTHRC1 T-cell engager binds to CTHRC1 with a binding affinity of less than 10 nM.

[0008] In embodiments, the CTHRC1 binding moiety comprises an anti-CTHRC1 antibody comprising a heavy chain complementarity determining region (HCDR) 1, HCDR2 and HCDR3 comprising SEQ ID NOs: 48, 53, and 58, respectively, and a light chain complementarity determining region (LCDR) 1, LCDR2 and LCDR3 of SEQ ID NOs: 63, 68 and 73, respectively.

[0009] In embodiments, the anti-CTHRC1 antibody comprises a heavy chain variable region (HCVR) comprising SEQ ID NO: 81 and a light chain variable region (LCVR) comprising SEQ ID NO: 82.

[0010] In embodiments, the anti-CTHRC1 antibody is a chimeric, humanized, or human antibody.

[0011] In embodiments, the anti-CTHRC1 antibody is a monoclonal antibody.

[0012] In embodiments, the anti-CTHRC1 antibody is an antibody fragment.

- [0013] In embodiments, the anti-CTHRC1 antibody comprises a single-chain antibody.
- [0014] In embodiments, the anti-CTHRC1 antibody is a heavy-chain only antibody (single domain antibody).
- [0015] In embodiments, the anti-CTHRC1 antibody fragments is selected from the group consisting of Fab, F(ab')₂, Fv, scFv, dsFv and a single domain antibody.
- [0016] In embodiments, the anti-CD3 antibody is a chimeric, humanized, or human antibody.
- [0017] In embodiments, the anti-CD3 antibody is a monoclonal antibody.
- [0018] In embodiments, the anti-CD3 antibody is an antibody fragment.
- [0019] In embodiments, the anti-CD3 antibody comprises a single-chain antibody.
- [0020] In embodiments, the anti-CD3 antibody is a heavy-chain only antibody (single domain antibody).
- [0021] In embodiments, the anti-CD3 antibody fragments is selected from the group consisting of Fab, F(ab')₂, Fv, scFv, dsFv and a single domain antibody.
- [0022] In embodiments, the anti-CTHRC1 T-cell engager comprises two first domains and/or two second domains. In embodiments, the anti-CTHRC1 T-cell engager has a ratio of 2:1, 1:2, or 2:2 of first domain to second domain. In embodiments, the anti-CTHRC1 T-cell engager has a ratio of 2:1 in cis configuration.
- [0023] In embodiments, a method for activating T cells in a tumor microenvironment comprises contacting a tumor with the anti-CTHRC1 T-cell engager or any of the foregoing embodiments.
- [0024] In embodiments, a method of inhibiting the growth of a cell displaying a CTHRC1 tumor epitope comprises contacting said cell with the anti-CTHRC1 T-cell engager of any of the foregoing embodiments.
- [0025] In embodiments, a method of treating a subject having cancer comprises administering the anti-CTHRC1 T-cell engager of any of the foregoing embodiments.
- [0026] In embodiments, the cancer is selected from the group consisting of breast cancer, lung cancer, colon cancer, ovarian cancer, melanoma cancer, bladder cancer, renal cancer, kidney

cancer, liver cancer, head and neck cancer, colorectal cancer, melanoma, pancreatic cancer, gastric carcinoma cancer, esophageal cancer, mesothelioma, prostate cancer, leukemia, lymphomas, and myelomas.

[0027] In embodiments, the foregoing methods can further comprise administering an allogeneic or autologous immune cell therapy in combination with the anti-CTHRC1 T-cell engager of any of the foregoing embodiments including T-cell therapies (*e.g.*, CAR-T and TiLs), NK cell therapies (*e.g.*, CAR-NK and LAK), and macrophage therapies (*e.g.* CAR-macrophage).

[0028] In embodiments, a pharmaceutical composition comprises the anti-CTHRC1 T-cell engager of any of the foregoing embodiments and a pharmaceutically acceptable carrier.

[0029] In embodiments, a use of the pharmaceutical composition of the foregoing embodiment can be in the preparation of a medicament for the treatment of a cell proliferative disorder, preferably cancer.

BRIEF DESCRIPTION OF THE DRAWINGS

[0030] Fig. 1A depicts a sample from a colorectal cancer patient stained with FAP showing tumor and stroma.

[0031] Fig. 1B depicts a sample from a colorectal cancer patient stained with anti-CTHRC1 antibody showing tumor and stroma.

[0032] Fig. 2A depicts FACS data for staining of cancer cell lines with anti-CTHRC1 antibodies.

[0033] Fig. 2B depicts FACS data for staining of cancer cell lines with anti-CTHRC1 antibodies.

[0034] Fig. 2C depicts FACS data for staining of SKOV3 cells with PAI-0323 (M23), PAI-0314 (M14), PAI-0305 (M5) and PAI-0303 (M3) with a pavilizumab control and secondary antibody only control.

[0035] Fig. 2D depicts MFI for fluorescent dye experiment data for PAI-0303 (M3) and PAI-0323 (M23) and a necitumumab control and isotype control to show lack of internalization for M3.

[0036] Fig. 2E depicts results for a bridging ELISA between CTHRC1 and CD3.

[0037] Figs. 3A-3B illustrate that CTHRC1 mRNA is a top ranked marker of cancer-associated fibroblasts (CAFs) in cancer-rich, immune-cold, tumor samples. Aggregated tumor samples were profiled across cancer scRNA studies, to create a large scRNA atlas. Samples were then grouped into T-cell rich, cancer poor samples (immune-hot), and cancer-rich, T-cell poor samples (immune-cold) (**FIG. 3A**). Genes expressed by CAFs were compared between the two groups, to determine which associate most with cancer rich samples (Wilcoxon Rank). These genes were then filtered down to those specifically expressed by CAFs in all samples (top 500; Wilcoxon rank). CTHRC1 was the 11th highest ranked gene in the analysis (**FIG. 3B**).

[0038] Fig. 4 illustrates that CTHRC1 is highly upregulated in cancer vs. normal adjacent tissues, as determined in bulk RNA measurements analyzed from The Cancer Genome Atlas. Across the set of indications profiled, the highest levels of CTHRC1 expression was seen in solid cancers, notably breast, lung, ovarian, pancreatic, sarcoma, melanoma, and uterine carcinosarcomas ($P < 0.001$ in all cases tested). Collectively this indicates that CTHRC1 expression is highest in more desmoplastic, stromal rich cancers, in line with the notion that it is principally a CAF secreted target.

[0039] Fig. 5 depicts a series of survival plots showing CTHRC1 survival curves for a number of solid tumors where CTHRC1 is associated with poor survival. Values were derived from The Cancer Genome Atlas. An online tool, GEPIA, was used to calculate and plot the survival curves.

[0040] Fig. 6 illustrates that CTHRC1 levels increase with stage in liver cancer (left) and colorectal cancer (right) alongside other indications (not shown). In both cases, $p < 0.05$; Student's T test between Stage I and Stage IV. Stage II and Stage III display intermediate CTHRC1 expression levels.

[0041] Figs. 7A-7C are histograms showing bulk-RNA expression level of known stromal targets FAP (**FIG. 7A**) and LRRC15 (**FIG. 7B**) alongside CTHRC1 (**FIG. 7C**) in pancreatic cancer samples (The Cancer Genome Atlas) vs. all normal tissue samples (GTEx). The histograms highlight that a significant therapeutic window exists for targeting CTHRC1 in pancreatic cancer, based on bulk-RNA measurements. This window is similar, if not larger, than that of the known/developed stromal targets FAP and LRRC15.

[0042] Fig. 8 is a dataset illustrating that very high levels of CTHRC1 expression is seen in CAFs across many solid cancers, as well as cancer epithelial expression in breast, pancreatic, lung, ovarian, and skin cancers. In contrast, minimal CTHRC1 expression is seen in normal tissue. The data was obtained based on previously generated large integrated single-cell RNA-sequence Atlas to enable probing of gene-expression at the single cell level across 60 toxic cancer and normal tissue samples (Swechha, 2021). The data highlights a potentially large therapeutic window for blocking CTHRC1 and value in using mAbs against CTHRC1 for targeting payloads, e.g., ADCs, to the tumor microenvironment.

[0043] Fig. 9 is a dataset using the same atlas discussed with regard to **FIG. 8**, performed for LRRC15, a known non-toxic stromal target, where antibodies ADCs have been engineered and shown to be safe in the clinic. Low levels of LRRC15 were seen in normal tissue as compared to CTHRC1. In cancerous single-cell RNA (scRNA) datasets it was observed that LRRC15 expression, unlike CTHRC1, is more selectively localized to specific CAFs in specific breast cancers, and low levels of expression are also seen on sarcoma cancer cells.

[0044] Fig. 10 is a graph illustrating quantitative ELISA of CTHRC1 in mono-culture and co-culture. Supernatants from a set of different mono-cultures (fibroblast or cancer cells) and co-cultures (fibroblasts and cancer cells) were profiled for CTHRC1 levels. CTHRC1 is expressed at low levels in fibroblast mono-cultures (BJ, CCD18-Co) and upregulated in co-culture, indicating that interactions between fibroblasts and cancer cells drives CTHRC1 expression.

[0045] Fig. 11 shows a series of images of tissue from three mouse models probed with CTHRC1 specific mAbs. Broad staining was observed within tumor regions, indicating CTHRC1 protein localizes to cancer regions *in vivo*.

[0046] Fig. 12 shows a series of images of tissue from three human cancers, showing variation in CTHRC1 expression patterns at the protein level, indicative of different expression dynamics. In Head and Neck and Melanoma cancer samples, CTHRC1 localizes to interfaces between cancer and stromal tissue. In pancreatic cancer, CTHRC1 is seen broadly expressed on regions dense in CAFs/stromal tissue.

[0047] Fig. 13 depicts different schematics for an exemplary anti-CTHRC1 T-cell engagers which includes a M3 Fab (shown in yellow) and a SP34-based scFv (shown in purple).

[0048] Figs. 14A-14C illustrate efficacy of CTHRC1 tested in the syngeneic mouse breast tumor model, EMT6. Results are shown for three different anti-CTHRC1 antibodies, specifically M5 (**FIG. 14A**), M23 (**FIG. 14B**), and M14 (**FIG. 14C**).

[0049] Figs. 15A-15B illustrate efficacy of anti-CTHRC1 (clone M5) in the PD-1 resistant Pan02 pancreatic cancer model.

[0050] Fig. 16 illustrates the effect of preconditioning of cells with anti-CTHRC1 antibody on infiltration of CD8 T cells.

[0051] Fig. 17A depicts a schematic representation of the anti-CTHRC1 T-cell engagers tested in Example 11.

[0052] Figs. 17B-17C depict the results of a cell killing assay using the anti-CTHRC1 T-cell engagers in Fig. 16A against control bispecific molecules. Fig. 16B depicts a visualization of caspase activation following treatment with the anti-CTHRC1 T-cell engagers (top is 1:1 and bottom is 2:1 cis) or control bispecific molecules for matched KP4 pancreatic tumors and CAFs. Fig. 16C depicts quantification of caspase activation across different treatment groups.

[0053] Fig. 18A depicts relative tumor cell viability for samples treated with an anti-CTHRC1 T-cell engager (Cis 2:1 configuration) or control dengue bispecific molecule for two donors.

[0054] Fig. 18B depicts the number of CD8 T cells for samples treated with an anti-CTHRC1 T-cell engager (Cis 2:1 configuration) or control dengue bispecific molecule for two donors.

[0055] Fig. 18C depicts the expression of 4-1BB and CD8 per mg of tumor for Donor 2 in Example 12.

[0056] Figs. 18D-F depict the supernatant characterization for Donor 2 samples for perforin (Fig. 18D), granzyme B (Fig. 18E), and IFN- γ (Fig. 18F).

[0057] Fig. 19A depicts the trial design for Example 13.

[0058] Fig. 19B depicts the average growth curve post-dose for both treatment groups in Example 13 with dots on the x-axis indicating dosing times. Data are shown as median +/- median absolute deviation.

[0059] Fig. 19C depicts the individual growth curves post-dose for both treatment groups in Example 13 with dots on the x-axis indicating dosing times.

DETAILED DESCRIPTION

[0060] The present disclosure provides anti-CTHRC1 T-cell engagers and compositions thereof, where the anti-CTHRC1 T-cell engager comprises a first domain comprising a collagen triple helix repeat containing 1 (CTHRC) binding moiety that selectively binds to human CTHRC1 and a cell displaying a CTHRC1 epitope without being internalized, and a second domain comprising a T cell engager, *e.g.*, an anti-CD3 antibody or fragment thereof. In embodiments, the T cell engager comprises an anti-CD3 antibody or fragment thereof. The present disclosure further provides methods for making the same and the use of the same in the treatment of cancer.

[0061] The practice of the present invention will employ, unless otherwise indicated, conventional techniques of molecular biology (including recombinant techniques), microbiology, cell biology, biochemistry, and immunology, which are within the skill of the art. Such techniques are explained fully in the literature, such as, “Molecular Cloning: A Laboratory Manual”, second edition (Sambrook et al., 1989); “Oligonucleotide Synthesis” (M. J. Gait, ed., 1984); “Animal Cell Culture” (R. I. Freshney, ed., 1987); “Methods in Enzymology” (Academic Press, Inc.); “Current Protocols in Molecular Biology” (F. M. Ausubel et al., eds., 1987, and periodic updates); “PCR: The Polymerase Chain Reaction”, (Mullis et al., ed., 1994); “A Practical Guide to Molecular Cloning” (Perbal Bernard V., 1988); “Phage Display: A Laboratory Manual” (Barbas et al., 2001).

[0062] One skilled in the art will recognize many methods and materials similar or equivalent to those described herein, which could be used in the practice of the present invention. Indeed, the present invention is in no way limited to the methods and materials described. For purposes of the present invention, the following terms are defined below.

Definitions

[0063] For purposes of interpreting this specification, the following definitions will apply and whenever appropriate, terms used in the singular will also include the plural and vice versa. In the event that any definition set forth conflicts with any document incorporated herein by reference, the definition set forth below shall control.

[0064] As used herein, the singular forms of “a,” “an,” and “the” include plural referents unless the context clearly dictates otherwise.

[0065] The use of the term “or” in the claims and the present disclosure is intended to mean “and/or” unless explicitly indicated to refer to alternatives only or the alternatives are mutually exclusive.

[0066] Use of the term “about,” when used with a numerical value, is intended to include +/- 10%. By way of example, but not limitation, if a number of amino acids is identified as “about 100,” this would include 90 to 110 (plus or minus 10%).

[0067] As used herein “comprising,” “including,” “containing,” “having” and the like are intended to be open-ended and inclusive such that they do not exclude additional, unrecited elements unless otherwise noted.

[0068] The term “Collagen Triple Helix Repeat Containing 1 (CTHRC1)”, as used herein, refers to any native CTHRC1 from any vertebrate source, including mammals such as primates (e.g., humans, primates, and rodents (e.g., mice and rats), unless otherwise indicated. The term encompasses several isoforms (see, e.g., SEQ ID NOs: 85-87). Human CTHRC1 is encoded by the nucleotide sequence corresponding to GenBank Accession No. NG031985.

[0069] The term “Collagen Triple Helix Repeat Containing 1” encompasses “full-length,” unprocessed CTHRC1 as well as any form of CTHRC1 that results from processing in the cell. The term encompasses naturally occurring variants of CTHRC1, e.g., splice variants, allelic variants and isoforms. The CTHRC1 polypeptides described herein may be isolated from a variety of sources, such as from human tissue types or from another source, or prepared by recombinant or synthetic methods. A “native sequence CTHRC1 polypeptide” comprises a polypeptide having the same amino acid sequence as the corresponding CTHRC1 polypeptide derived from nature. Such native sequence CTHRC1 polypeptides can be isolated from nature or can be produced by recombinant or synthetic means. The term “native sequence CTHRC1 polypeptide” specifically encompasses naturally-occurring truncated or secreted forms of the specific CTHRC1 polypeptide (e.g., an extracellular domain sequence), naturally-occurring variant forms (e.g., alternatively spliced forms) and naturally-occurring allelic variants of the polypeptide. In certain embodiments of the invention, the native sequence CTHRC1

polypeptides disclosed herein are mature or full-length native sequence polypeptides comprising the full-length amino acid sequences shown in the accompanying disclosure.

[0070] Percent “identity” between an amino acid sequence and a reference sequence, is defined as the percentage of amino acid residues in the polypeptide sequence that are identical to the amino acid residues in the reference sequence, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity. Alignment for purposes of determining percent amino acid sequence identity can be achieved in various ways that are within the skill in the art, for instance, using publicly available computer software such as BLAST, BLAST-2, ALIGN, MEGALIGN (DNASTAR), CLUSTALW, CLUSTAL OMEGA, or MUSCLE software. Those skilled in the art can determine appropriate parameters for aligning sequences, including any algorithms needed to achieve maximal alignment over the full length of the sequences being compared. In certain embodiments, default parameters are used.

[0071] A “modification” of an amino acid residue/position, as used herein, refers to a change of a primary amino acid sequence as compared to a starting amino acid sequence, wherein the change results from a sequence alteration involving said amino acid residue/positions. For example, typical modifications include substitution of the residue (or at said position) with another amino acid (e.g., a conservative or non-conservative substitution), insertion of one or more (generally fewer than 5 or 3) amino acids adjacent to said residue/position, and deletion of said residue/position. An “amino acid substitution”, or variation thereof, refers to the replacement of an existing amino acid residue in a predetermined (starting) amino acid sequence with a different amino acid residue. Generally, the modification results in alteration in at least one physicochemical activity of the variant polypeptide compared to a polypeptide comprising the starting (or “wild type”) amino acid sequence. For example, in the case of an antibody, a physicochemical activity that is altered can be binding affinity, binding capability and/or binding effect upon a target molecule.

[0072] The term “antibody” is used in the broadest sense and specifically covers, for example, single anti-CTHRC1 monoclonal antibodies (including agonist, antagonist, neutralizing antibodies, full length or intact monoclonal antibodies), anti-CTHRC1 antibody compositions with polypeptidic specificity, polyclonal antibodies, multivalent antibodies, multispecific antibodies (e.g., bispecific antibodies so long as they exhibit the desired biological activity),

formed from at least two intact antibodies, single chain anti-CTHRC1 antibodies, and fragments of anti-CTHRC1 antibodies (see below), including Fab, Fab', F(ab')₂ and Fv fragments, diabodies, single domain antibodies (sdAbs), as long as they exhibit the desired biological or immunological activity. Also included among anti-CTHRC1 antibodies, and among fragments in particular, are portions of anti-CTHRC1 antibodies (and combinations of portions of anti-CTHRC1 antibodies, for example, scFv) that may be used as targeting arms, directed to e.g., a CTHRC1 tumor epitope, in chimeric antigenic receptors of CAR-T cells, CAR-NK cells, or CAR-macrophages, and in the anti-CTHRC1 T-cell engagers provided herein. Such fragments are not necessarily proteolytic fragments but rather portions of polypeptide sequences that can confer affinity for target. The term "immunoglobulin" (Ig) is used interchangeably with antibody herein. An antibody can be, for example, human, humanized and/or affinity matured.

[0073] The terms "anti-CTHRC1 antibody", "CTHRC1 antibody", and "an antibody that binds to CTHRC1" are used interchangeably. Anti-CTHRC1 antibodies are preferably capable of binding with sufficient affinity such that the antibody is useful as a diagnostic and/or therapeutic agent, whether in isolation or as part of T-cell engager, cell, or cell composition.

[0074] In one embodiment, CTHRC1 antibody is used herein to specifically refer to an anti-CTHRC1 monoclonal antibody that (i) comprises the heavy chain variable domain of SEQ ID NO: 81; and/or the light chain variable domain of SEQ ID NO: 82, or (ii) comprises one, two, three, four, five, or six of the CDRs of SEQ ID NOs: 48, 53, 58, 63, 68 and 73.

[0075] An "isolated antibody" is one which has been identified and separated and/or recovered from a component of its natural environment. Contaminant components of its natural environment are materials which would interfere with therapeutic uses for the antibody, and may include enzymes, hormones, and other proteinaceous or non-proteinaceous solutes.

[0076] An "intact" antibody is one which comprises an antigen-binding site as well as a CL and at least heavy chain constant domains, CH1, CH2 and CH3. The constant domains may be native sequence constant domains (e.g., human native sequence constant domains) or amino acid sequence variant thereof. Preferably, the intact antibody has one or more effector functions.

[0077] The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical except for possible naturally occurring mutations that may be present

in minor amounts. Monoclonal antibodies are highly specific, being directed against a single antigenic site. Furthermore, in contrast to polyclonal antibody preparations which include different antibodies directed against different determinants (epitopes), each monoclonal antibody is directed against a single determinant on the antigen. In addition to their specificity, the monoclonal antibodies are advantageous in that they may be synthesized uncontaminated by other antibodies. The modifier “monoclonal” is not to be construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies useful in the present invention may be prepared by the hybridoma methodology first described by Kohler et al., *Nature*, 256: 495 (1975), or may be made using recombinant DNA methods in bacterial, eukaryotic animal or plant cells (e.g., 4,816,567). The “monoclonal antibodies” may also be isolated from phage antibody libraries using the techniques described in Clackson et al., *Nature*, 352: 624-8 (1991) and Marks et al., *J. Mol. Biol.*, 222: 581-97 (1991), for example.

[0078] The basic 4-chain antibody unit is a heterotetrameric glycoprotein composed of two identical light (L) chains and two identical heavy (H) chains. In the case of IgGs, the 4-chain unit is generally about 150,000 daltons. Each L chain is linked to a H chain by one covalent disulfide bond, while the two H chains are linked to each other by one or more disulfide bonds depending on the H chain isotype. Each H and L chain also has regularly spaced intrachain disulfide bridges. Each H chain has at the N-terminus, a variable domain (VH) followed by three constant domains (CH) for each of the α and γ chains and four CH domains for μ and ϵ isotypes. Each L chain has at the N-terminus, a variable domain (VL) followed by a constant domain (CL) at its other end. The VL is aligned with the VH and the CL is aligned with the first constant domain of the heavy chain (CH1). Particular amino acid residues are believed to form an interface between the light chain and heavy chain variable domains. The pairing of a VH and VL together forms a single antigen-binding site. For the structure and properties of the different classes of antibodies, see, e.g., *Basic and Clinical Immunology*, 8th edition, Daniel P. Stites, Abba I. Terr and Tristram G. Parslow (eds.), Appleton & Lange, Norwalk, CT, 1994, at page 71 and Chapter 6.

[0079] The L chain from any vertebrate species can be assigned to one of two clearly distinct types, called kappa and lambda, based on the amino acid sequences of their constant domains. Depending on the amino acid sequence of the constant domain of their heavy chains (CH), immunoglobulins can be assigned to different classes or isotypes. There are five classes of

immunoglobulins: IgA, IgD, IgE, IgG, and IgM, having heavy chains designated α , δ , ϵ , γ , and μ , respectively. The γ and α classes are further divided into subclasses on the basis of relatively minor differences in CH sequence and function, e.g., humans express the following subclasses: IgG1, IgG2, IgG3, IgG4, IgA1, and IgA2.

[0080] The “variable region” or “variable domain” of an antibody refers to the amino-terminal domains of the heavy or light chain of the antibody. The variable domain of the heavy chain may be referred to as “VH” or “V_H” The variable domain of the light chain may be referred to as “VL” or “V_L”. These domains are generally the most variable parts of an antibody and contain the antigen-binding sites.

[0081] The term “variable” refers to the fact that certain segments of the variable domains differ extensively in sequence among antibodies. The V domain mediates antigen binding and defines specificity of a particular antibody for its particular antigen. However, the variability is not evenly distributed across the 110-amino acid span of the variable domains. Instead, the V regions consist of relatively invariant stretches called framework regions (FRs) of 15-30 amino acids separated by shorter regions of extreme variability called “hypervariable regions” that are each 9-12 amino acids long. The variable domains of native heavy and light chains each comprise four FRs, largely adopting a β -sheet configuration, connected by three hypervariable regions, which form loops connecting, and in some cases forming part of, the β -sheet structure. The hypervariable regions in each chain are held together in close proximity by the FRs and, with the hypervariable regions from the other chain, contribute to the formation of the antigen-binding site of antibodies (see Kabat et al., Sequences of Proteins of Immunological Interest, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, MD. (1991)).

[0082] “Antibody fragments” comprise a portion of an intact antibody, preferably the antigen binding or one or more variable regions of the intact antibody. Examples of antibody fragments include Fab, Fab', F(ab')₂, and Fv fragments; diabodies; linear antibodies (see U.S. Patent No. 5,641,870, Example 2; Zapata et al., Protein Eng. 8(10): 1057-62 (1995)); single-chain antibody molecules; and multispecific antibodies formed from antibody fragments. In one embodiment, an antibody fragment comprises an antigen binding site of the intact antibody and thus retains the ability to bind antigen. Also included among anti-CTHRC1 antibody fragments are portions of anti-CTHRC1 antibodies (and combinations of portions of anti-CTHRC1 antibodies, for

example, scFv) that may be used as targeting arms, directed to e.g., a CTHRC1 tumor epitope, in chimeric antigenic receptors of CAR-T cells or CAR-NK cells, or CAR macrophages, and in the anti-CTHRC1 T-cell engagers provided herein. Such fragments are not necessarily proteolytic fragments but rather portions of polypeptide sequences that can confer affinity for target.

[0083] Papain digestion of antibodies produces two identical antigen-binding fragments, called “Fab” fragments, and a residual “Fc” fragment, a designation reflecting the ability to crystallize readily. The Fab fragment consists of an entire L chain along with the variable region domain of the H chain (VH), and the first constant domain of one heavy chain (CH1). Each Fab fragment is monovalent with respect to antigen binding, i.e., it has a single antigen-binding site. Pepsin treatment of an antibody yields a single large F(ab')₂ fragment which roughly corresponds to two disulfide linked Fab fragments having divalent antigen-binding activity and is still capable of cross-linking antigen. Fab' fragments differ from Fab fragments by having additional few residues at the carboxy terminus of the CH1 domain including one or more cysteines from the antibody hinge region. Fab'-SH is the designation herein for Fab' in which the cysteine residue(s) of the constant domains bear a free thiol group. F(ab')₂ antibody fragments originally were produced as pairs of Fab' fragments which have hinge cysteines between them. Other chemical couplings of antibody fragments are also known.

[0084] The Fc fragment comprises the carboxy-terminal portions of both H chains held together by disulfides. The effector functions of antibodies are determined by sequences in the Fc region, which region is also the part recognized by Fc receptors (FcR) found on certain types of cells.

[0085] “Fv” is the minimum antibody fragment which contains a complete antigen-recognition and -binding site. This fragment consists of a dimer of one heavy- and one light-chain variable region domain in tight, non-covalent association. In a single-chain Fv (scFv) species, one heavy- and one light-chain variable domain can be covalently linked by a flexible peptide linker such that the light and heavy chains can associate in a “dimeric” structure analogous to that in a two-chain Fv species. From the folding of these two domains emanate six hypervariable loops (3 loops each from the H and L chain) that contribute the amino acid residues for antigen binding and confer antigen binding specificity to the antibody. However, even a single variable domain

(or half of an Fv comprising only three CDRs specific for an antigen) has the ability to recognize and bind antigen, although at a lower affinity than the entire binding site.

[0086] “Single-chain Fv” also abbreviated as “sFv” or “scFv” are antibody fragments that comprise the VH and VL antibody domains connected into a single polypeptide chain. In some embodiments, the sFv polypeptide further comprises a polypeptide linker between the VH and VL domains which enables the sFv to form a desired structure for antigen binding. For a review of sFv, see, e.g., Pluckthun in *The Pharmacology of Monoclonal Antibodies*, vol. 113, Rosenberg and Moore eds., Springer-Verlag, New York, pp. 269-315 (1994); Borrebaeck 1995, *infra*. In one embodiment, an anti-CTHRC1 antibody derived scFv is used as the targeting arm of a CAR-T cell, a CAR-NK cell, or a CAR-macrophage disclosed herein.

[0087] The term “hypervariable region”, “HVR”, or “HV”, when used herein refers to the regions of an antibody variable domain which are hypervariable in sequence and/or form structurally defined loops. Generally, antibodies comprise six hypervariable regions; three in the VH (H1, H2, H3), and three in the VL (L1, L2, L3). A number of hypervariable region delineations are in use and are encompassed herein. The Kabat Complementarity Determining Regions (CDRs) are based on sequence variability and are the most commonly used (Kabat et al., *Sequences of Proteins of Immunological Interest*, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, MD. (1991)). Chothia refers instead to the location of the structural loops (Chothia and Lesk J. Mol. Biol. 196:901-917 (1987)). The end of the Chothia CDR-H1 loop when numbered using the Kabat numbering convention varies between H32 and H34 depending on the length of the loop (this is because the Kabat numbering scheme places the insertions at H35A and H35B; if neither 35A nor 35B is present, the loop ends at 32; if only 35A is present, the loop ends at 33; if both 35A and 35B are present, the loop ends at 34). The AbM hypervariable regions represent a compromise between the Kabat CDRs and Chothia structural loops, and are used by Oxford Molecular’s AbM antibody modeling software. The “contact” hypervariable regions are based on an analysis of the available complex crystal structures. The residues from each of these hypervariable regions are noted below.

Loop	Kabat	AbM	Chothia	Contact
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L1	L24-L34	L24-L34	L24-L34	L30-L36

L2	L50-L56	L50-L56	L50-L56	L46-L55
L3	L89-L97	L89-L97	L89-L97	L89-L96
H1	H31-H35B	H26-H35B	H26-H32...34	H30-H35B
(Kabat Numbering)				
H1	H31-H35	H26-H35	H26-H32	H30-H35
(Chothia Numbering)				
H2	H50-H65	H50-H58	H52-H56	H47-H58
H3	H95-H102	H95-H102	H95-H102	H93-H101

[0088] Hypervariable regions may comprise “extended hypervariable regions” as follows: 24-36 or 24-34 (L1), 46-56 or 50-56 (L2) and 89-97 (L3) in the VL and 26-35B (H1), 50-65, 47-65 or 49-65 (H2) and 93-102, 94-102 or 95-102 (H3) in the VH. The variable domain residues are numbered according to Kabat et al., *supra*, for each of these definitions.

[0089] “Framework” or “FR” residues are those variable domain residues other than the hypervariable region residues herein defined.

[0090] The term “variable domain residue numbering as in Kabat” or “amino acid position numbering as in Kabat”, and variations thereof, refers to the numbering system used for heavy chain variable domains or light chain variable domains of the compilation of antibodies in Kabat et al., *supra*. Using this numbering system, the actual linear amino acid sequence may contain fewer or additional amino acids corresponding to a shortening of, or insertion into, a FR or CDR of the variable domain. For example, a heavy chain variable domain may include a single amino acid insert (residue 52a according to Kabat) after residue 52 of H2 and inserted residues (e.g., residues 82a, 82b, and 82c, etc according to Kabat) after heavy chain FR residue 82. The Kabat numbering of residues may be determined for a given antibody by alignment at regions of homology of the sequence of the antibody with a “standard” Kabat numbered sequence.

[0091] The Kabat numbering system is generally used when referring to a residue in the variable domain (approximately residues 1-107 of the light chain and residues 1-113 of the heavy chain) (e.g., Kabat et al., *supra*). The “EU numbering system” or “EU index” is generally used when referring to a residue in an immunoglobulin heavy chain constant region (e.g., the EU index reported in Kabat et al., *supra*). The “EU index as in Kabat” refers to the residue numbering

of the human IgG1 EU antibody. Unless stated otherwise herein, references to residue numbers in the variable domain of antibodies means residue numbering by the Kabat numbering system.

[0092] An antibody that “binds” an antigen or epitope of interest is one that binds the antigen or epitope with sufficient affinity that is measurably different from a non-specific interaction. Specific binding can be measured, for example, by determining binding of a molecule compared to binding of a control molecule, which generally is a molecule of similar structure that does not have binding activity.

[0093] An antibody that inhibits the growth of tumor cells is one that results in measurable growth inhibition of cancer cells. In one embodiment, an anti-CTHRC1 antibody is capable of inhibiting the growth of cancer cells displaying a CTHRC1 tumor epitope. As referred to herein, a CTHRC1 tumor epitope comprises a CTHRC1 epitope capable of being bound by an anti-CTHRC1 antibody, or fragment thereof, as herein disclosed, or capable of being at least partially bound by an antibody or other molecule that competes with an anti-CTHRC1 antibody as herein disclosed for binding to said epitope. Preferred growth inhibitory anti-CTHRC1 antibodies inhibit growth of CTHRC1-displaying tumor cells by greater than 20%, preferably from about 20% to about 50%, and even more preferably, by greater than 50% (e.g., from about 50% to about 100%) as compared to the appropriate control, the control typically being tumor cells not treated with the antibody being tested.

[0094] Anti-CTHRC1 antibodies may (i) inhibit tumor metastasis in vivo; (ii) inhibit tumor growth in vivo; (iii) decrease tumor size in vivo; (iv) inhibit tumor vascularization in vivo; (v) exhibit cytotoxic activity on tumor cells and cancer associated fibroblasts displaying CTHRC1 in vivo; (vi) exhibit cytostatic activity on tumor cells or cancer associated fibroblasts displaying CTHRC1 in vivo; (vii) enhance infiltration of anti-tumor immune cells in vivo or (viii) prevent suppression of immune-cells in the tumor microenvironment in vivo.

[0095] The terms “cancer” and “cancerous” refer to or describe the physiological condition in mammals that is typically characterized by unregulated cell growth. A “tumor” comprises one or more cancerous cells. Examples of cancer include, but are not limited to, carcinoma, lymphoma, blastoma, sarcoma, and leukemia or lymphoid malignancies. More particular examples of such cancers include squamous cell cancer (e.g., epithelial squamous cell cancer), skin cancer, melanoma, lung cancer including small-cell lung cancer, non-small cell lung cancer

(“NSCLC”), adenocarcinoma of the lung and squamous carcinoma of the lung, cancer of the peritoneum, hepatocellular cancer, gastric or stomach cancer including gastrointestinal cancer, pancreatic cancer (e.g., pancreatic ductal adenocarcinoma), glioblastoma, cervical cancer, ovarian cancer (e.g., high grade serous ovarian carcinoma), liver cancer (e.g., hepatocellular carcinoma (HCC)), bladder cancer (e.g., urothelial bladder cancer), testicular (germ cell tumor) cancer, hepatoma, breast cancer, brain cancer (e.g., astrocytoma), colon cancer, rectal cancer, colorectal cancer, endometrial or uterine carcinoma, salivary gland carcinoma, kidney or renal cancer (e.g., renal cell carcinoma, nephroblastoma or Wilms’ tumor), prostate cancer, vulval cancer, thyroid cancer, hepatic carcinoma, anal carcinoma, penile carcinoma, as well as head and neck cancer. Additional examples of cancer include, without limitation, adrenocortical cancer, cholangiocarcinoma, colon adenocarcinoma, B-cell lymphoma, esophageal carcinoma, glioblastoma multiforme, kidney clear cell cancer, kidney papillary cell cancer, myeloid leukemia, lung adenocarcinoma, lung squamous cancer, prostate adenocarcinoma, rectal adenocarcinoma, sarcoma, stomach adenocarcinoma, thymoma, uterine corpus, and uterine carcinosarcoma.

[0096] The term “metastatic cancer” means the state of cancer where the cancer cells of a tissue of origin are transmitted from the original site to one or more sites elsewhere in the body, by the blood vessels or lymphatics, to form one or more secondary tumors in one or more organs besides the tissue of origin. A prominent example is metastatic breast cancer.

[0097] As used herein, an “CTHRC1-associated cancer” is a cancer that is associated with over-expression of a CTHRC1 gene or gene product and/or is associated with display of a CTHRC1 epitope. Suitable control cells can be, for example, cells from an individual who is not affected with cancer or non-cancerous cells from the subject who has cancer.

[0098] Anti-CTHRC1 T-cell engagers may (i) inhibit tumor metastasis in vivo; (ii) inhibit tumor growth in vivo; (iii) decrease tumor size in vivo; (iv) inhibit tumor vascularization in vivo; (v) exhibit cytotoxic activity activity on tumor cells and cancer associated fibroblasts displaying CTHRC1 in vivo; (vi) exhibit cytostatic activity on tumor cells or cancer associated fibroblasts displaying CTHRC1 in vivo; (vii) enhance infiltration of anti-tumor immune cells in vivo; or (viii) prevent suppression of immune-cells in the tumor microenvironment in vivo.

[0099] The terms “cell proliferative disorder” and “proliferative disorder” refer to disorders that are associated with some degree of abnormal cell proliferation. In one embodiment, the cell proliferative disorder is cancer.

[00100] “Tumor”, as used herein, refers to all neoplastic cell growth and proliferation, whether malignant or benign, and all pre-cancerous and cancerous cells and tissues.

[00101] The terms “patient,” “subject,” “individual,” and the like are used interchangeably herein, and refer to any animal amenable to the methods described herein. In certain non-limiting embodiments, the patient, subject or individual is a human.

[00102] The term “therapeutically effective amount”, or simply “effective amount” refers to the amount of an agent or composition (e.g., composition comprising an agent) that will elicit a biological or medical response of a tissue, system, or subject that is being sought by the researcher, veterinarian, medical doctor or other clinician. The term “therapeutically effective amount” includes that amount of an agent, or a composition comprising an agent, that, when administered, is sufficient to prevent development of, or alleviate to some extent, one or more of the signs or symptoms of the disorder or disease (e.g., hematological or solid tumor) being treated. The therapeutically effective amount will vary depending on the composition, the disease and its severity and the age, weight, etc., of the subject to be treated.

[00103] As used herein, the term “administration” means to provide or give a subject one or more agents, such as an agent that treats one or more signs or symptoms associated with a condition/disorder or disease including but not limited to cancer (e.g., lymphoma), viral infection, bacterial infection, etc., by any effective route. Exemplary routes of administration include, but are not limited to, injection (such as subcutaneous, intramuscular, intradermal, intraperitoneal, and intravenous), oral, sublingual, rectal, transdermal, intranasal, vaginal and inhalation routes. Administration “in combination with” one or more further therapeutic agents includes simultaneous (concurrent) and sequential administration in any order.

[00104] The term “pharmaceutically acceptable”, as used herein, refers to a material, including but not limited, to a salt, carrier or diluent, which does not abrogate the biological activity or properties of the compound, and is relatively non-toxic, i.e., the material may be administered to an individual without causing undesirable biological effects or interacting in a deleterious manner with any of the components of the composition in which it is contained.

The pharmaceutically acceptable carriers (vehicles) useful in this disclosure are conventional. Remington's Pharmaceutical Sciences, by E. W. Martin, Mack Publishing Co., Easton, Pa., 19th Edition (1995), describes compositions and formulations suitable for pharmaceutical delivery of one or more agents, such as one or more modulatory agents. In general, the nature of the carrier will depend on the particular mode of administration being employed. For instance, parenteral formulations can include injectable fluids that include pharmaceutically and physiologically acceptable fluids such as water, physiological saline, balanced salt solutions, aqueous dextrose, glycerol or the like as a vehicle. In addition to biologically-neutral carriers, pharmaceutical agents to be administered can contain minor amounts of non-toxic auxiliary substances, such as wetting or emulsifying agents, preservatives, and pH buffering agents and the like, for example sodium acetate or sorbitan monolaurate, sodium lactate, potassium chloride, calcium chloride, and triethanolamine oleate.

[00105] “Encoding” refers to the inherent property of specific sequences of nucleotides in a polynucleotide, such as a gene, a cDNA, or an mRNA, to serve as templates for synthesis of other polymers and macromolecules in biological processes having either a defined sequence of nucleotides (i.e., rRNA, tRNA and mRNA) or a defined sequence of amino acids and the biological properties resulting therefrom. Thus, a gene encodes a protein if transcription and translation of mRNA corresponding to that gene produces the protein in a cell or other biological system. Both the coding strand, the nucleotide sequence of which is identical to the mRNA sequence and is usually provided in sequence listings, and the non-coding strand, used as the template for transcription of a gene or cDNA, can be referred to as encoding the protein or other product of that gene or cDNA.

[00106] Unless otherwise specified, a “nucleotide sequence encoding an amino acid sequence” includes all nucleotide sequences that are degenerate versions of each other and that encode the same amino acid sequence. Nucleotide sequences that encode proteins and RNA may include introns.

[00107] In one aspect of the invention, the anti-CTHRC1 T-cell engager provides an activating signal to T-cells and NK-cells present in the tumor microenvironment.

[00108] In another aspect of the invention, the T-cell and NK-cell activating signal causes T and/or NK cell dependent cytotoxicity directed at cancer cells.

[00109] In another aspect, the anti-CTHRC1 T-cell engager brings immune cells including T-cells and/or NK cells into close proximity to CAFs or cancer cells in a CTHRC1 dependent manner.

[00110] In another aspect this enhances T and NK cell-mediated killing of CAFs and cancer cells.

[00111] In one aspect of the invention, the anti-CTHRC1 T-cell engager provides a pro-inflammatory signal to the broader set of immune-cells expressing the receptor that may include, macrophages, dendritic cells, monocytes, B-cells, plasma cells, neutrophils, mast cells, and other blood derived immune cells, and antigen presenting cells.

[00112] In another aspect of the invention, the immune activating signal induces a proinflammatory immune response in the tumor microenvironment, that will result in tumor cell killing.

[00113] In another aspect of the invention the anti-CTHRC1 T-cell engager brings inflammatory macrophages into close proximity to the CAF or cancer cells and induces direct macrophage mediated cell killing, for example, via complement system and complement receptor-mediated phagocytosis.

[00114] In another aspect of the invention the anti-CTHRC1 T-cell engager brings inflammatory macrophages, dendritic cells or other antigen presenting cells into close proximity to CAFs and cancer cells inducing enhanced display of antigens associated with the CAF or cancer cells and associated indirect adaptive immune response.

[00115] In another aspect of the invention the proximity of immune cells triggers a broad immune response that includes release of proinflammatory cytokines.

[00116] In embodiments, an anti-CTHRC1 T-cell engager is provided that can include at least one first domain comprising a CTHRC1 binding moiety that selectively binds to human CTHRC1 and a cell displaying a CTHRC1 epitope without being internalized and at least one second domain comprising a T cell engager. In embodiments, the T cell engager comprises an anti-CD3 antibody or fragment thereof. In embodiments, the anti-CTHRC1 T-cell engager comprises two first domains and/or two second domains. In embodiments, the anti-CTHRC1 T-cell engager has a ratio of 2:1, 1:2, or 2:2 of first domain to second domain. In embodiments

having more than one first and/or second domain, the CTHRC1 binding moieties and/or T cell engager moieties may be the same or different.

[00117] The presently disclosed subject matter encompasses multispecific antibodies, for example, bispecific antibodies. For example, proteins can be linked together either through chemical or genetic manipulation using methods known in the art.

[00118] In one example, the present disclosure encompasses an anti-CTHRC1 T-cell engager. In principle, an anti-CTHRC1 antibody as herein disclosed can be fused to any T cell engager moiety via the use of recombinant molecular biological techniques. In embodiments, anti-CTHRC1 T-cell engagers can comprise an anti-CTHRC1 antibody or fragment thereof and an anti-CD3 antibody or fragment thereof.

Multispecific Antibodies

[00119] In any aspect of the present disclosure, an anti-CTHRC1 T-cell engager provided herein is a multispecific antibody, for example, a bispecific antibody. Bispecific antibodies are antibodies that have binding specificities for at least two different epitopes. Exemplary bispecific antibodies may bind an epitope of a CTHRC1 protein as described herein and to an epitope on a T cell, *e.g.* CD3. Other such antibodies may combine a CTHRC1 binding site with a binding site for another protein present on T cells. In some examples, an anti-CTHRC1 arm may be combined with an arm which binds to a triggering molecule on a leukocyte such as a T-cell receptor molecule (*e.g.*, CD3), or Fc receptors for IgG (FcγR), such as FcγRI (CD64), FcγRII (CD32) and FcγRIII (CD16), so as to focus and localize cellular defense mechanisms to the CTHRC1-expressing cell. Bispecific antibodies can be prepared as full-length antibodies or antibody fragments (*e.g.*, F(ab')₂ bispecific antibodies), and in a variety of formats including, *e.g.*, BiTE, HLE-Bite, Duobody, TDB, common light chain, DART and DART-HLE.

[00120] Anti-CTHRC1 T-cell engagers of the present disclosure, in the form of bispecific antibodies, can be prepared in either *cis*- or *trans*- configurations. A *cis*- configuration refers to an engager where the first domain and the second domain are present on the same side of the bispecific antibody, *e.g.* at the end of each binding arm, while a *trans*- configuration refers to an engager where the first domain and the second domain are present on different ends, *e.g.* an N-terminal end and C-terminal end, of the engager. A description of *cis*- and *trans*- configurations

is provided in Santich, et al. (*Sci Transl Med.* 12(534):eaax1315 (2020)) which is incorporated herein by reference in its entirety.

[00121] A ratio of first domains to second domains or vice versa can also be used to describe the engagers referring to the number of first domains and second domains. For example, a 2:1 configuration can refer to an engager with two first domains and one second domain, where, if the configuration is cis, in the case of an antibody, there is one first binding domain present on a first binding arm, one first domain and one second domain present on the second binding arm. Exemplary configurations of such engagers are provided in Figs. 13 and 16A.

[00122] In addition, anti-CTHRC1 T-cell engagers of the present disclosure can be designed in a symmetric or asymmetric format whereby the first domain and the second domain are on the same binding arm or on different binding arms. Symmetric and asymmetric bispecific designs are disclosed in Madsen, et al. (*Front. Bioeng. Biotechnol.* 12 (2024)) which is incorporated herein by reference in its entirety.

[00123] Bispecific constructs in a *cis*- or *trans*- configuration can be synthesized via method known in the art and as described herein, including recombinant expression techniques.

[00124] In embodiments, the anti-CTHRC1 T-cell engager is a bispecific antibody in a *cis*-configuration. In embodiments, the anti-CTHRC1 T-cell engager is a bispecific antibody in a *trans*- configuration.

[00125] In embodiments, the anti-CTHRC1 T-cell engager has a ratio of 1:1, 2:1, 1:2, or 2:2 of first domain to second domain. In embodiments, the anti-CTHRC1 T-cell engager has a ratio of 2:1 in *cis* configuration.

[00126] In embodiments, the anti-CTHRC1 T-cell engager is asymmetric. In embodiments, the anti-CTHRC1 T-cell engager is symmetric.

[00127] Methods for making bispecific antibodies are known in the art. Traditional production of full-length bispecific antibodies is based on the co-expression of two immunoglobulin heavy chain-light chain pairs, where the two chains have different specificities (Millstein et al., *Nature* 305: 537-9 (1983)). Because of the random assortment of immunoglobulin heavy and light chains, these hybridomas (quadromas) produce a potential mixture of 10 different antibody molecules, of which only one has the correct bispecific structure. Purification of the correct molecule, which is

usually done by affinity chromatography steps, is rather cumbersome, and the product yields are low. Similar procedures are disclosed in WO 93/08829, and in Traunecker et al., EMBO J. 10:3655-3659 (1991).

[00128] Other approaches for making bispecific antibodies are known. One approach is the “knobs-into-holes” or “protuberance-into-cavity” approach (see, e.g., U.S. Pat. No. 5,731,168). In this approach, two immunoglobulin polypeptides (e.g., heavy chain polypeptides) each comprise an interface. An interface of one immunoglobulin polypeptide interacts with a corresponding interface on the other immunoglobulin polypeptide, thereby allowing the two immunoglobulin polypeptides to associate. These interfaces may be engineered such that a “knob” or “protuberance” (these terms may be used interchangeably herein) located in the interface of one immunoglobulin polypeptide corresponds with a “hole” or “cavity” (these terms may be used interchangeably herein) located in the interface of the other immunoglobulin polypeptide. In some embodiments, the hole is of identical or similar size to the knob and suitably positioned such that when the two interfaces interact, the knob of one interface is positionable in the corresponding hole of the other interface. Without wishing to be bound to theory, this is thought to stabilize the heteromultimer and favor formation of the heteromultimer over other species, for example homomultimers. In some embodiments, this approach may be used to promote the heteromultimerization of two different immunoglobulin polypeptides, creating a bispecific antibody comprising two immunoglobulin polypeptides with binding specificities for different epitopes.

[00129] Schematics of exemplary anti-CTHRC1 T-cell engagers are shown in FIGURE 13 where a M3 Fab (shown in yellow) and a SP34 scFv are attached to a Fc backbone in different ratios (1:1 and 2:1 (two alternative configurations)). Exemplary Fc backbones can include knob-into-hole designs which, for example, can include knob mutations S354C and T366W and hole mutations Y349C, T366S, L368A and Y407V. These include addition of disulfide bond to stabilize the heterodimeric Fc. In certain aspects, the hole side can be designed to always contain the anti-CTHRC1 moiety in order to avoid homodimerization of the “hole” chain. As discussed in more detail elsewhere in this disclosure, the constructs can include various linkers, including (G₄S)_n where n is 2, 3 or 4.

[00130] According to a different approach, antibody variable domains with the desired binding specificities (antibody-antigen combining sites) are fused to immunoglobulin constant domain sequences. The fusion preferably is with an immunoglobulin heavy chain constant domain, comprising at least part of the hinge, CH2, and CH3 regions. It is typical to have the first heavy-chain constant region (CH1) containing the site necessary for light chain binding, present in at least one of the fusions. DNAs encoding the immunoglobulin heavy chain fusions and, if desired, the immunoglobulin light chain, are inserted into separate expression vectors, and are co-transfected into a suitable host organism. This provides for great flexibility in adjusting the mutual proportions of the three polypeptide fragments in embodiments when unequal ratios of the three polypeptide chains used in the construction provide the optimum yields. It is, however, possible to insert the coding sequences for two or all three polypeptide chains in one expression vector when the expression of at least two polypeptide chains in equal ratios results in high yields or when the ratios are of no particular significance.

[00131] In one embodiment of this approach, the bispecific antibodies are composed of a hybrid immunoglobulin heavy chain with a first binding specificity in one arm, and a hybrid immunoglobulin heavy chain-light chain pair (providing a second binding specificity) in the other arm. It was found that this asymmetric structure facilitates the separation of the desired bispecific compound from unwanted immunoglobulin chain combinations, as the presence of an immunoglobulin light chain in only one half of the bispecific molecule provides for a facile way of separation. This approach is disclosed in WO 94/04690. For further details of generating bispecific antibodies see, for example, Suresh et al., *Methods in Enzymology*, 121:210 (1986).

[00132] According to another approach described in WO96/27011, the interface between a pair of antibody molecules can be engineered to maximize the percentage of heterodimers which are recovered from recombinant cell culture. One interface comprises at least a part of the C_H3 domain of an antibody constant domain. In this method, one or more small amino acid side chains from the interface of the first antibody molecule are replaced with larger side chains (e.g. tyrosine or tryptophan). Compensatory “cavities” of identical or similar size to the large side chain(s) are created on the interface of the second antibody molecule by replacing large amino acid side chains with smaller ones (e.g. alanine or threonine). This provides a mechanism for increasing the yield of the heterodimer over other unwanted end-products such as homodimers.

[00133] Bispecific antibodies include cross-linked or “heteroconjugate” antibodies. For example, one of the antibodies in the heteroconjugate can be coupled to avidin, the other to biotin. Such antibodies have, for example, been proposed to target immune system cells to unwanted cells (U.S. Pat. No. 4,676,980), and for treatment of HIV infection (WO 91/00360, WO 92/200373, and EP 03089). Heteroconjugate antibodies may be made using any convenient cross-linking methods. Suitable cross-linking agents are well known in the art, and are disclosed in U.S. Pat. No. 4,676,980, along with a number of cross-linking techniques.

[00134] Techniques for generating bispecific antibodies from antibody fragments have also been described in the literature. For example, bispecific antibodies can be prepared using chemical linkage. Brennan et al., *Science*, 229: 81 (1985) describe a procedure wherein intact antibodies are proteolytically cleaved to generate $F(ab')_2$ fragments. These fragments are reduced in the presence of the dithiol complexing agent sodium arsenite to stabilize vicinal dithiols and prevent intermolecular disulfide formation. The Fab' fragments generated are then converted to thionitrobenzoate (TNB) derivatives. One of the Fab' -TNB derivatives is then reconverted to the Fab' -thiol by reduction with mercaptoethylamine and is mixed with an equimolar amount of the other Fab' -TNB derivative to form the bispecific antibody. The bispecific antibodies produced can be used as agents for the selective immobilization of enzymes. Shalaby et al., *J. Exp. Med.*, 175: 217-225 (1992) describe the production of a fully humanized bispecific antibody $F(ab')_2$ molecule. Each Fab' fragment was separately secreted from *E. coli* and subjected to directed chemical coupling in vitro to form the bispecific antibody.

[00135] Various techniques for making and isolating bispecific antibody fragments directly from recombinant cell culture have also been described. For example, bispecific antibodies have been produced using leucine zippers. Kostelny et al., *J. Immunol.*, 148(5):1547-1553 (1992). The leucine zipper peptides from the Fos and Jun proteins were linked to the Fab' portions of two different antibodies by gene fusion. The antibody homodimers were reduced at the hinge region to form monomers and then re-oxidized to form the antibody heterodimers. This method can also be utilized for the production of antibody homodimers. The “diabody” technology described by Hollinger et al., *Proc. Natl. Acad. Sci. USA*, 90:6444-6448 (1993) has provided an alternative mechanism for making bispecific antibody fragments. The fragments comprise a heavy-chain variable domain (V_H) connected to a light-chain variable domain (V_L) by a linker which is too short to allow pairing between the two domains on the same chain. Accordingly, the V_H and

V_L domains of one fragment are forced to pair with the complementary V_L and V_H domains of another fragment, thereby forming two antigen-binding sites. Another strategy for making bispecific antibody fragments by the use of single-chain Fv (sFv) dimers has also been reported. See Gruber et al, *J. Immunol*, 152:5368 (1994).

[00136] Another technique for making bispecific antibody fragments is the “bispecific T cell engager” or BiTE® approach (see, e.g., WO2004/106381, WO2005/061547, WO2007/042261, and WO2008/119567). This approach utilizes two antibody variable domains arranged on a single polypeptide. For example, a single polypeptide chain includes two single chain Fv (scFv) fragments, each having a variable heavy chain (V_H) and a variable light chain (V_L) domain separated by a polypeptide linker of a length sufficient to allow intramolecular association between the two domains. This single polypeptide further includes a polypeptide spacer sequence between the two scFv fragments. Each scFv recognizes a different epitope, and these epitopes may be specific for different cell types, such that cells of two different cell types are brought into close proximity or tethered when each scFv is engaged with its cognate epitope. One particular embodiment of this approach includes a scFv recognizing a cell-surface antigen expressed by an immune cell, e.g., a CD3 polypeptide on a T cell, linked to another scFv that recognizes a cell-surface antigen expressed by a target cell, such as a malignant or tumor cell.

[00137] As it is a single polypeptide, the bispecific T cell engager may be expressed using any prokaryotic or eukaryotic cell expression system known in the art, e.g., a CHO cell line. However, specific purification techniques (see, e.g., EP1691833) may be necessary to separate monomeric bispecific T cell engagers from other multimeric species, which may have biological activities other than the intended activity of the monomer. In one exemplary purification scheme, a solution containing secreted polypeptides is first subjected to a metal affinity chromatography, and polypeptides are eluted with a gradient of imidazole concentrations. This eluate is further purified using anion exchange chromatography, and polypeptides are eluted using with a gradient of sodium chloride concentrations. Finally, this eluate is subjected to size exclusion chromatography to separate monomers from multimeric species.

[00138] Other relevant bispecific antibody fragment formats include but are not limited to dual-affinity re-targeting proteins (DARTs) and Tandem diabodies (TandAbs). A DART is composed of two Fv fragments, with two unique antigen-binding sites formed when two Fv fragments

heterodimerize (Holliger et al., *Proc. Natl. Acad. Sci. USA*. 90:6444–6448 (1993). Specifically, Fv1 consists of a VH from antibody “A” and a VL from antibody “B”, while Fv2 is made from a VH from antibody “B” and VL from antibody “A”. Unlike BiTE antibodies which are connected by a polypeptide linker, this combination allows DART to mimic natural interaction within an IgG molecule. Adding another cysteine residue to the end of each heavy-chain improves stability by forming a C-terminal disulfide bridge. TandAbs are tetravalent bispecific antibodies provide two binding sites for each antigen to maintain the avidity of a natural bivalent antibody. Moreover, TandAbs have a molecular weight (approximately 105 kDa) exceeding the first-pass renal clearance threshold, thus offering a longer half-life compared to smaller antibody constructs (Reusch et al., *Clin. Cancer Res. Off. J. Am. Assoc. Cancer Res.* 22:5829–5838 (2016); Reusch et al., *MAbs*. 6:728–739 (2014); Compte et al., *Oncoimmunology*. 3:e28810 (2014). For recent reviews of common formats of bispecific antibodies including BiTEs, scFv-based and full-length IgG-like asymmetric antibodies, including methods of production thereof, see Madsen et al., Design and engineering of bispecific antibodies: insights and practical considerations; *Front. Bioeng. Biotechnol.* 12 (2024) Santich, et al. (*Sci Transl Med.* 12(534):eaax1315 (2020)) and Wang et al., *Antibodies (Basel)*, 8(3): 43 (2019).

[00139] Additional teachings on exemplary bispecific formats and methods of making same can be found in Bacac et al., *Clin Cancer Res*; 24(19) October 1, 2018; Bacac et al., *Clin Cancer Res*; 22(13) July 1, 2016; Ravandi et al., *Blood advances*; 7(21) November 14, 2023; Engelberts et al., *EBioMedicine* 52, 2020; Giffin et al., *Clin Cancer Res* 2021;27:1526–37 and Chichili et al., *Science Transl Med* 27 May 2015 Vol 7 Issue 289; as well as in US_9914776_B2; US11124577; US10155815; WO_2022_262959_A1; US_11672858_B2; US9850320; US9856327; US10544220; US10294300; US9657102; and US20160200827; the disclosures of which are expressly incorporated by reference herein in their entireties.

T Cell Engager Moiety and Anti-CD3 Antibodies and Fragments Thereof

[00140] As described herein, the second domain of the anti-CTHRC1 T-cell engager comprises a T cell engager moiety. In embodiments, the anti-CTHRC1 T-cell engagers of the present disclosure include one or more second domains comprising a T cell engager moiety. In

embodiments, the anti-CTHRC1 T-cell engager has a ratio of 1:1, 2:1, 1:2, or 2:2 of first domain to second domain.

[00141] In any of the foregoing embodiments, the second domain comprises an anti-CD3 antibody or fragment thereof. In certain aspects, the anti-CD3 antibody is SP34 or a fragment thereof. In certain aspects, the anti-CD3 antibody is UCHT-1 or a fragment thereof. In certain aspects, the anti-CD3 antibody is BC-3 or a fragment thereof.

[00142] In any of the foregoing embodiments, the anti-CD3 antibody or fragment thereof can comprise a heavy chain complementarity determining region (HCDR) 1, HCDR2 and HCDR3 of SEQ ID NOs: 1, 2, and 3, respectively, and a light chain complementarity determining region (LCDR) 1, LCDR2, and LCDR3 of SEQ ID NOs: 4, 5, and 6, respectively.

[00143] In any of the foregoing embodiments, the anti-CD3 antibody or fragment thereof can comprise the variable heavy chain region (HCVR) of SEQ ID NO: 7 and a light chain variable chain region of SEQ ID NO: 8.

[00144] In embodiments, the T cell engager, *e.g.* anti-CD3 antibody or fragment thereof can include a heavy chain variable region (HCVR) having at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% sequence identity to SEQ ID NO: 7. In embodiments, the T cell engager, *e.g.* anti-CD3 antibody or fragment thereof can include a light chain variable region (LCVR) having at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% sequence identity to SEQ ID NO: 8.

[00145] In embodiments, the anti-CD3 antibody or fragment thereof can comprise the HCDR1, HCDR2 and HCDR3 of a HCVR having the sequence of any one of the following HCVRs:

Name	HCDR1	HCDR2	HCDR3
SP34 Parent	GFTFNTYA	IRSKYNNYAT	VRHGNGFGNSYVSWFAY
PAI-SP34	GFTFNTYA	IRSKYNNYAT	VRHGNGFGNSYVSWFAY
epcoritamab	GFTFNTYA	IRSKYNNYAT	VRHGNGFGNSYVSWFAY
duvortuxizumab	GFTFSTYA	IRSKYNNYAT	VRHGNGFGNSYVSWFAY
talquetamab	GFTFNTYA	IRSKYNNYAT	ARHGNGFGNSYVSWFAY
flotetuzumab	GFTFSTYA	IRSKYNNYAT	VRHGNGFGNSYVSWFAY
cibisatamab	GFTFSTYA	IRSKYNNYAT	VRHGNGFGNSYVSWFAY
vibecotamab	GFTFSTYA	IRSKYNNYAT	VRHGNGFGDSYVSWFAY
pavurutamab	GFTFNKYA	IRSKYNNYAT	VRHGNGFGNSYISYWAY
epcoritamabNo.7	GFTFNTYA	IRSKYNNYAT	VRHGNGFGNSYVSWFAY
epcoritamabNo.8	GFTFNTYA	IRSKYNNYAT	VRHGNGFGNSYVSWFAY

epcoritamabNo.9	GFTFNTYA	IRSKYNNYAT	VRHGNGNSYVSWFAY
blinatumomab	GYTFTRYT	INPSRGYT	ARYYDDHYCLDY
solitomab	GYTFTRYT	INPSRGYT	ARYYDDHYCLDY
visilizumab	GYTFISYT	INPSRGYT	ARSAYDYDGFAY
otelixizumab	GFTFSSFP	ISTSGGRT	AKFRQYSGGFDY
cevostamab	GFTFTSY	IYPENDNT	ARDGYSRYYFDY
tepoditamab	GFTFSSYG	IWYNARKQ	TRGTGYNWFDP
elranatamab	GFTFSDYY	IRNRARGYTS	ARDRPSYYVLDY
odronextamab	GFTFDDYT	ISWNSGSI	AKDNSGYGHHYYGMDV
muromonab	GYTFTRYT	INPSRGYT	ARYYDDHYCLDY

[00146] In embodiments, the anti-CD3 antibody or fragment thereof can comprise the LCDR1, LCDR2 and LCDR3 of a LCVR having the sequence of any one of the following LCVRs:

Name	LCDR1	LCDR2	LCDR3
SP34 Parent	TGAVTTSNY	GTN	ALWYSNLWV
PAI-SP34	TGAVTTSNY	GTN	ALWYSNLWV
epcoritamab	TGAVTTSNY	GTN	ALWYSNLWV
duvortuxizumab	TGAVTTSNY	GTN	ALWYSNLWV
talquetamab	TGAVTTSNY	GTN	ALWYSNLWV
flotetuzumab	TGAVTTSNY	GTN	ALWYSNLWV
cibisatamab	TGAVTTSNY	GTN	ALWYSNLWV
vibecotamab	TGAVTTSNY	GTN	ALWYSNHWV
pavurutamab	TGAVTSGNY	GTK	VLWYSNRWV
epcoritamab-No.11	TGAVTTSNY	GTN	ALWYSNLWV
epcoritamab-No.12	TGAVTTSNY	GTN	ALWYSNLWV
epcoritamab-No.30 (LKNH)	TGAVTTSNY	GTN	ALWYSNHWV
epcoritamab-No.31 (T41K)	TGAVTTSNY	GTN	ALWYSNLWV
blinatumomab	SSVSY	DTS	QQWSSNPLT
solitomab	QSVSY	DTS	QQWSSNPLT
visilizumab	SSVSY	DTS	QQWSSNPPT
otelixizumab	SGNIENNY	DDD	HSYVSSFNV
cevostamab	QSLNRSRTRKNY	WTS	KQSFILRT
tepoditamab	QSISSY	AAS	QQSYSTPPT
elranatamab	QSLFNVRSRKNY	WAS	KQSYDLFT
odronextamab	QSVSSN	GAS	QHYINWPLT
muromonab	SSVSY	DTS	QQWSSNPFT

[00147] In embodiments, the anti-CD3 antibody or fragment thereof can comprise the HCDR1, HCDR2, and HCDR3 of a HCVR and the LCDR1, LCDR2 and LCDR3 of a LCVR of any one of the following HCVR/LCVR pairs, including any combination of epcoritamab HCVR/LCVR and any combination of talquetamab HCVR/LC:

Name	vH	vL
SP34 Parent	EVKLLESGGGLVQPKGSLKLSAASGFT FNTYAMNWVRQAPGKGLEWVARIRSK YNNYATYYADSVKDRFTISRDDSQSILYL QMNNLKTEDTAMYYCVRHGNFGNSY VSWFAYWGQGLTVTVSA	QAVVTQESALTTSPGETVTLTCSRSTG AVTTSNYANWVQKPDHLFTGLIGGT NKRAPGVPARFSGSLIGDKAALTITGA QTEDEAIYFCALWYSNLWVFGGGTKL TVL
PAI-SP34	EVQLVESGGGLVQPGGSLRLSAAASGF TFNTYAMNWVRQAPGKGLEWVARIRSK KYNNTYATYYADSVKDRFTISRDDSKNTL YLQMNSLKAEDTAVYYCVRHGNFGNS YVSWFAYWGQGLTVTVSS	QAVVTQEPSLTVSPGGTVTLTCSSTG AVTTSNYANWVQKPGQAPRGLIGG TNKRAPGTPARFSGSLLGGKAALTLSG AQPEDEAEYYCALWYSNLWVFGGGT KLTVL
epcoritamab	EVKLLESGGGLVQPGGSLRLSAAASGF FNTYAMNWVRQAPGKGLEWVARIRSK YNNYATYYADSVKDRFTISRDDSKSSLYL QMNNLKTEDTAMYYCVRHGNFGNSY VSWFAYWGQGLTVTVSS	QAVVTQEPSFSVSPGGTVTLTCSRSTG AVTTSNYANWVQQTPGQAFRGLIGG TNKRAPGVPARFSGSLIGDKAALTITG AQADDESIYFCALWYSNLWVFGGGTK LTVL
duvortuxizumab	EVQLVESGGGLVQPGGSLRLSAAASGF TFSTYAMNWVRQAPGKGLEWVGRIIRS KYNNTYATYYADSVKGRFTISRDDSKNSL YLQMNSLKTEDTAVYYCVRHGNFGNSY VSWFAYWGQGLTVTVSS	QAVVTQEPSLTVSPGGTVTLTCSRSTG AVTTSNYANWVQKPGQAPRGLIGG TNKRAPWTPARFSGSLLGGKAALTITG AQAEDEADYYCALWYSNLWVFGGGT KLTVL
talquetamab	EVQLVESGGGLVQPGGSLRLSAAASGF TFNTYAMNWVRQAPGKGLEWVARIRSK KYNNTYATYYAASVKGRFTISRDDSKNSL YLQMNSLKTEDTAVYYCARHGNFGNSY VSWFAYWGQGLTVTVSS	QTVVTQEPSLTVSPGGTVTLTCSRSTG AVTTSNYANWVQKPGQAPRGLIGG TNKRAPGTPARFSGSLLGGKAALTLSG VQPEDEAEYYCALWYSNLWVFGGGT KLTVL
teclistamab (same as talquetamab)	EVQLVESGGGLVQPGGSLRLSAAASGF TFNTYAMNWVRQAPGKGLEWVARIRSK KYNNTYATYYAASVKGRFTISRDDSKNSL YLQMNSLKTEDTAVYYCARHGNFGNSY VSWFAYWGQGLTVTVSS	QTVVTQEPSLTVSPGGTVTLTCSRSTG AVTTSNYANWVQKPGQAPRGLIGG TNKRAPGTPARFSGSLLGGKAALTLSG VQPEDEAEYYCALWYSNLWVFGGGT KLTVL
flotetuzumab	EVQLVESGGGLVQPGGSLRLSAAASGF TFSTYAMNWVRQAPGKGLEWVGRIIRS KYNNTYATYYADSVKDRFTISRDDSKNSL YLQMNSLKTEDTAVYYCVRHGNFGNSY VSWFAYWGQGLTVTVSS	QAVVTQEPSLTVSPGGTVTLTCSRSTG AVTTSNYANWVQKPGQAPRGLIGG TNKRAPWTPARFSGSLLGGKAALTITG AQAEDEADYYCALWYSNLWVFGGGT KLTVL
obrindatamab	EVQLVESGGGLVQPGGSLRLSAAASGF TFSTYAMNWVRQAPGKGLEWVGRIIRS KYNNTYATYYADSVKDRFTISRDDSKNSL YLQMNSLKTEDTAVYYCVRHGNFGNSY VSWFAYWGQGLTVTVSS	QAVVTQEPSLTVSPGGTVTLTCSRSTG AVTTSNYANWVQKPGQAPRGLIGG TNKRAPWTPARFSGSLLGGKAALTITG AQAEDEADYYCALWYSNLWVFGGGT KLTVL

cibisatamab	EVQLLESGGGLVQPGGSLRLSCAASGFT FSTYAMNWVRQAPGKGLEWVSRIRSK YNNYATYYADSVKGRFTISRDDSKNTLY LQMNSLRAEDTAVYYCVRHGNFGNSY VSWFAYWGQGTLLTVSS	QAVVTQEPSLTVSPGGTVTLTCGSSTG AVTTSNYANWVQKPGQAFRGLIGGT NKRAPGTPARFSGSLLGGKAALTLSGA QPEDEAEYYCALWYSNLWVFGGGTKL TVL
alnuctamab	EVQLLESGGGLVQPGGSLRLSCAASGFT FSTYAMNWVRQAPGKGLEWVSRIRSK YNNYATYYADSVKGRFTISRDDSKNTLY LQMNSLRAEDTAVYYCVRHGNFGNSY VSWFAYWGQGTLLTVSS	QAVVTQEPSLTVSPGGTVTLTCGSSTG AVTTSNYANWVQKPGQAFRGLIGGT NKRAPGTPARFSGSLLGGKAALTLSGA QPEDEAEYYCALWYSNLWVFGGGTKL TVL
glofitamab	EVQLLESGGGLVQPGGSLRLSCAASGFT FSTYAMNWVRQAPGKGLEWVSRIRSK YNNYATYYADSVKGRFTISRDDSKNTLY LQMNSLRAEDTAVYYCVRHGNFGNSY VSWFAYWGQGTLLTVSS	QAVVTQEPSLTVSPGGTVTLTCGSSTG AVTTSNYANWVQKPGQAFRGLIGGT NKRAPGTPARFSGSLLGGKAALTLSGA QPEDEAEYYCALWYSNLWVFGGGTKL TVL
vibecotamab	EVQLVESGGGLVQPGGSLRLSCAASGF TFSTYAMNWVRQAPGKGLEWVGRIRS KYNMYATYYADSVKGRFTISRDDSKNTL YLQMNSLRAEDTAVYYCVRHGNFGDS YVSWFAYWGQGTLLTVSS	QAVVTQEPSLTVSPGGTVTLTCGSSTG AVTTSNYANWVQKPGKSPRGLIGGT NKRAPGVPARFSGSLLGGKAALTISGA QPEDEADYYCALWYSNHWVFGGGTK LTVL
tidutamab	EVQLVESGGGLVQPGGSLRLSCAASGF TFSTYAMNWVRQAPGKGLEWVGRIRS KYNMYATYYADSVKGRFTISRDDSKNTL YLQMNSLRAEDTAVYYCVRHGNFGDS YVSWFAYWGQGTLLTVSS	QAVVTQEPSLTVSPGGTVTLTCGSSTG AVTTSNYANWVQKPGKSPRGLIGGT NKRAPGVPARFSGSLLGGKAALTISGA QPEDEADYYCALWYSNHWVFGGGTK LTVL
plamotamab	EVQLVESGGGLVQPGGSLRLSCAASGF TFSTYAMNWVRQAPGKGLEWVGRIRS KYNMYATYYADSVKGRFTISRDDSKNTL YLQMNSLRAEDTAVYYCVRHGNFGDS YVSWFAYWGQGTLLTVSS	QAVVTQEPSLTVSPGGTVTLTCGSSTG AVTTSNYANWVQKPGKSPRGLIGGT NKRAPGVPARFSGSLLGGKAALTISGA QPEDEADYYCALWYSNHWVFGGGTK LTVL
pavurutamab	EVQLVESGGGLVQPGGSLKLSAASGF TFNKYAMNWVRQAPGKGLEWVARIRS KYNMYATYYADSVKDRFTISRDDSKNTA YLQMNNLKTEDTAVYYCVRHGNFGNS YISYWAYWGQGTLLTVSS	QTVVTQEPSLTVSPGGTVTLTCGSSTG AVTSGNYPNWVQKPGQAPRGLIGG TKFLAPGTPARFSGSLLGGKAALTLSGV QPEDEAEYYCVLWYSNRWVFGGGTK LTVL
gresonitamab	EVQLVESGGGLVQPGGSLKLSAASGF TFNKYAMNWVRQAPGKGLEWVARIRS KYNMYATYYADSVKDRFTISRDDSKNTA YLQMNNLKTEDTAVYYCVRHGNFGNS YISYWAYWGQGTLLTVSS	QTVVTQEPSLTVSPGGTVTLTCGSSTG AVTSGNYPNWVQKPGQAPRGLIGG TKFLAPGTPARFSGSLLGGKAALTLSGV QPEDEAEYYCVLWYSNRWVFGGGTK LTVL
tarlatamab	EVQLVESGGGLVQPGGSLKLSAASGF TFNKYAMNWVRQAPGKGLEWVARIRS KYNMYATYYADSVKDRFTISRDDSKNTA YLQMNNLKTEDTAVYYCVRHGNFGNS YISYWAYWGQGTLLTVSS	QTVVTQEPSLTVSPGGTVTLTCGSSTG AVTSGNYPNWVQKPGQAPRGLIGG TKFLAPGTPARFSGSLLGGKAALTLSGV QPEDEAEYYCVLWYSNRWVFGGGTK LTVL

etevritamab	EVQLVESGGGLVQPGGSLKLSAASGF TFNKYAMNWVRQAPGKGLEWVARIRS KYNMYATYYADSVKDRFTISRDDSKNTA YLQMNNLKTEDTAVYYCVRHGNFGNS YISYWAYWGQGTLLTVSS	QTVVTQEPSLTVSPGGTVTLTCSSTG AVTSGNYPNWVQQKPGQAPRGLIGG TKFLAPGTPARFSGSLLGGKAALTLGSV QPEDEAEYYCVLWYSNRWVFGGGTK LTVL
acapatamab	EVQLVESGGGLVQPGGSLKLSAASGF TFNKYAMNWVRQAPGKGLEWVARIRS KYNMYATYYADSVKDRFTISRDDSKNTA YLQMNNLKTEDTAVYYCVRHGNFGNS YISYWAYWGQGTLLTVSS	QTVVTQEPSLTVSPGGTVTLTCSSTG AVTSGNYPNWVQQKPGQAPRGLIGG TKFLAPGTPARFSGSLLGGKAALTLGSV QPEDEAEYYCVLWYSNRWVFGGGTK LTVL
pasotuxizumab	EVQLVESGGGLVQPGGSLKLSAASGF TFNKYAMNWVRQAPGKGLEWVARIRS KYNMYATYYADSVKDRFTISRDDSKNTA YLQMNNLKTEDTAVYYCVRHGNFGNS YISYWAYWGQGTLLTVSS	QTVVTQEPSLTVSPGGTVTLTCSSTG AVTSGNYPNWVQQKPGQAPRGLIGG TKFLAPGTPARFSGSLLGGKAALTLGSV QPEDEAEYYCVLWYSNRWVFGGGTK LTVL
pacanalotamab	EVQLVESGGGLVQPGGSLKLSAASGF TFNKYAMNWVRQAPGKGLEWVARIRS KYNMYATYYADSVKDRFTISRDDSKNTA YLQMNNLKTEDTAVYYCVRHGNFGNS YISYWAYWGQGTLLTVSS	QTVVTQEPSLTVSPGGTVTLTCSSTG AVTSGNYPNWVQQKPGQAPRGLIGG TKFLAPGTPARFSGSLLGGKAALTLGSV QPEDEAEYYCVLWYSNRWVFGGGTK LTVL
eluvixtamab	EVQLVESGGGLVQPGGSLKLSAASGF TFNKYAMNWVRQAPGKGLEWVARIRS KYNMYATYYADSVKDRFTISRDDSKNTA YLQMNNLKTEDTAVYYCVRHGNFGNS YISYWAYWGQGTLLTVSS	QTVVTQEPSLTVSPGGTVTLTCSSTG AVTSGNYPNWVQQKPGQAPRGLIGG TKFLAPGTPARFSGSLLGGKAALTLGSV QPEDEAEYYCVLWYSNRWVFGGGTK LTVL
emerfetamab	EVQLVESGGGLVQPGGSLKLSAASGF TFNKYAMNWVRQAPGKGLEWVARIRS KYNMYATYYADSVKDRFTISRDDSKNTA YLQMNNLKTEDTAVYYCVRHGNFGNS YISYWAYWGQGTLLTVSS	QTVVTQEPSLTVSPGGTVTLTCSSTG AVTSGNYPNWVQQKPGQAPRGLIGG TKFLAPGTPARFSGSLLGGKAALTLGSV QPEDEAEYYCVLWYSNRWVFGGGTK LTVL
epcoritamabNo.7	EVKLVESGGGLVKPGRSLRLSCAASGFT FNTYAMNWVRQAPGKGLEWVARIRSK YNNYATYYADSVKDRFTISRDDSKSILYL QMNNLKTEDTAMYYCVRHGNFGNSY VSWFAYWGQGTLLTVSS	NA
epcoritamabNo.8	EVKLVESGGGLVKPGRSLRLSCAASGFT FNTYAMNWVRQAPGKGLEWVARIRSK YNNYATYYADSVKDRFTISRDDSKSILYL QMNSLKTEDTAMYYCVRHGNFGNSYV SWFAYWGQGTLLTVSS	NA
epcoritamabNo.9	EVKLVESGGGLVKPGRSLRLSCAASGFT FNTYAMNWVRQAPGKGLEWVARIRSK YNNYATYYADSVKDRFTISRDDSKSILYL QMNSLKTEDTAMYYCVRHGNFGNSYV SWFAYWGQGTMTVTSS	NA

epcoritamabNo.11	NA	QAVVTQEPSFSVSPGGTVTLTCSRSTG AVTTSNYANWVQQTPGQAFRLIGG TNKRAPGVPARFSGSILGNKAALTITG AQADDESIYFCALWYSNLWVFGGGTK LTVL
epcoritamabNo.12	NA	QAVVTQEPSFSVSPGGTVTLTCSRSTG AVTTSNYANWVQQTPGQAFRLIGG TNKRAPGVPARFSGSILGNKAALTITG AQADDESDYFCALWYSNLWVFGGGT KLTVL
epcoritamabNo.30 (LKNH)	NA	QAVVTQEPSLSVSPGGTVTLTCSRSTG AVTTSNYANWVQQKPGQAFRLIGG TNNRAPGVPARFSGSLIGDKAALTITG AQADDESIYFCALWYSNLWVFGGGT KLTVL
epcoritamabNo.31 (T41K)	NA	QAVVTQEPSFSVSPGGTVTLTCSRSTG AVTTSNYANWVQQKPGQAFRLIGG TNKRAPGVPARFSGSLIGDKAALTITG AQADDESIYFCALWYSNLWVFGGGTK LTVL
blinatumomab	DIKLQQSGAELARPGASVKMSCKTSGY TFTRYTMHWWKQRPQGGLWIGYINP SRGYTNYNQKFKDKATLTDDKSSSTAY MQLSSLTSEDSAVYYCARYDDHYCLDY WGQGTTLTVSS	DIQLTQSPAIMSASPGEKVTMTCRASS SVSYMNWYQQKSGTSPKRWIYDTSK VASGVPYRFSGSGSGTSYSLTISSMEAE DAATYYCQWSSNPLTFGAGTKLELK
solitomab	DVQLVQSGAEVKKPGASVKVSKASGY TFTRYTMHVVQRAPQGGLWIGYINP SRGYTNYADSVKGRFTITDDKSTSTAYM ELSSLRSEDATVYYCARYDDHYCLDYW GQGTTVTVSS	DIVLTQSPATLSLSPGERATLSCRASQS VSYMNWYQQKPGKAPKRWIYDTSKV ASGVPARFSGSGSGTDYSLTINSLEAED AATYYCQWSSNPLTFGGGTKEIK
visilizumab	QVQLVQSGAEVKKPGASVKVSKASGY TFISYTMHVVQRAPQGGLWIMGYIN PRSGYTHYNQKLDKATLTADKSASTAY MELSSLRSEDATVYYCARSAYDYDGFA YWGQGTTLTVSS	DIQMTQSPSSLSASVGDRVITITCSASS VSYMNWYQQKPGKAPKRLIYDTSKLA SGVPSRFSGSGSGTDFTLTISLQPEDF ATYYCQWSSNPPTFGGGTKVEIK
otelixizumab	EVQLLESGGGLVQPGGSLRLSCAASGFT FSSFPMAWVRQAPGKGLWVSTISTSG GRYYRDSVKGRFTISRDNKNTLYLQM NSLRAEDTAVYYCAKFRQYSGGFDYWG QGTLTVTVSS	DIQLTQPNVSTSLGSTVKLSCTLSGSGN IENNYVHWYQLYEGRSPTTMIYDDDK RPDGVPDFRSGSIDRSSNSAFLTIHNV AIEDEAIYFCHSYVSSFNVFGGGTKLTV L
cevostamab	EVQLVQSGAEVKKPGASVKVSKASGF TFTSYIHVVQRAPQGGLWIGWIPE NDNTKYNEKFKDRVITADTSTSTAYLEL SSLRSEDATVYYCARDGYSRYFDYWG QGTLTVTVSS	DIVMTQSPDSLAVSLGERATINCKSSQ SLLNSRTRKNYLAWYQQKPGQSPKLLI YWTSTRKSGVPDRFSGSGSGTDFTLT SSLQAEDVAVYYCKQSFILRTFGQGTK VEIK

tepoditamab	QVQLVQSGGGVVPGRSLRLSCVASGF TFSSYGMHWVRQAPGKGLEWVAIIW YNARKQDYADSVKGRFTISRDNKNTLY LQMNSLRAEDTAVYYCTRGTYNWFD PWGQGTLTVSS	DIQMTQSPSSLSASVGDRTITCRASQ SISSYLNWYQQKPGKAPKLLIYAASSLQ SGVPSRFSGSGSGTDFTLTISSLQPEDF ATYYCQQSYSTPPTFGQGTKVEIK
elranatamab	EVQLVESGGGLVQPGGSLRLSCAASGF TFSDYYMTWVRQAPGKGLEWVAFIRN RARGYTS DHNPSVKGRFTISRDNKNSL YLQMNSLRAEDTAVYYCARDPSYYVL DYWGQGTTTVSS	DIVMTQSPDSLAVSLGERATINCKSSQ SLFNVRSRKNYLAWYQQKPGQPPKLLI SWASTRESGVPDRFSGSGSGTDFTLTI SSLQAEDVAVYYCKQSYDLFTFGSGTK LEIK
odronextamab	EVQLVESGGGLVQPGRSLRLSCAASGFT FDDYTMHWVRQAPGKGLEWVSGISW NSGSIGYADSVKGRFTISRDNKKSLLYL QMNSLRAEDTALYYCAKDNSGYGHYY GMDVWGQGTTTVAS	EIVMTQSPATLSVSPGERATLSCRASQ SVSSNLAWYQQKPGQAPRLLIYGAST RATGIPARFSGSGSGTEFTLTISLQSED FAVYYCQHYINWPLTFGGGTKVEIK
muromonab	QVQLQQSGAELARPGASVKMSCKASG YTFTRYTMHWVKRPGQGLEWIGYIN PSRGYTNYNQKFKDKATLTDDKSSSTAY MQLSSLTSEDSAVYYCARYDDHYCLDY WGQGTTTLTVSS	QIVLTQSPAISASPGKEVTMTCSASS SVSYMNWYQQKSGTSPKRWIYDTSKL ASGVPAHFRGSGSGTSYSLTISGMEAE DAATYYCQQWSSNPFTFGSGTKLEIN

[00148] In embodiments, the anti-CD3 antibody or fragment thereof can comprise a HCVR/LCVR pair of any one of the following HCVR/LCVR pairs, including any combination of epcoritamab HCVR/LCVR:

Name	vH	vL
SP34 Parent	EVKLVESGGGLVQPGSLRLSCAASGFT FNTYAMNWVRQAPGKGLEWVARIRSK YNNYATYYADSVKDRFTISRDDSQSILYL QMNNLKTEDTAMYYCVRHGNFGNSY VSWFAYWGQGTLTVSA	QAVVTQESALTTSPGETVTLTCSRSTG AVTTSNYANWVQKPDHFTGLIGGT NKRAPGVPARFSGSLIGDKAALTITGA QTEDEAIYFCALWYSNLWVFGGGTL TVL
PAI-SP34	EVQLVESGGGLVQPGGSLRLSCAASGF TFNTYAMNWVRQAPGKGLEWVARIRS KYNNTYATYYADSVKDRFTISRDDSKNTL YLQMNSLKAEDTAVYYCVRHGNFGNS YVSWFAYWGQGTLTVSS	QAVVTQEPSTVSPGGTVTLTCSSTG AVTTSNYANWVQQKPGQAPRGLIGG TNKRAPGTPARFSGSLGGKAALTLSG AQPEDEAEYFCALWYSNLWVFGGGT KLTVL
epcoritamab	EVKLVESGGGLVQPGGSLRLSCAASGFT FNTYAMNWVRQAPGKGLEWVARIRSK YNNYATYYADSVKDRFTISRDDSKSSLYL QMNNLKTEDTAMYYCVRHGNFGNSY VSWFAYWGQGTLTVSS	QAVVTQEPSFSVSPGGTVTLTCSRSTG AVTTSNYANWVQQTPGQAFRGLIGG TNKRAPGVPARFSGSLIGDKAALTITG AQADDESIFCALWYSNLWVFGGGTL LTVL
duvortuxizumab	EVQLVESGGGLVQPGGSLRLSCAASGF TFSTYAMNWVRQAPGKGLEWVGRIIRS KYNNTYATYYADSVKGRFTISRDDSKNSL	QAVVTQEPSTVSPGGTVTLTCSRSTG AVTTSNYANWVQQKPGQAPRGLIGG TNKRAPWTPARFSGSLGGKAALTITG

	YLQMNSLKTEDTAVYYCVRHGNFGNSY VSWFAYWGQGLTVTVSS	AQAEDEADYYCALWYSNLWVFGGGT KLTVL
talquetamab	EVQLVESGGGLVQPGGSLRLSCAASGF TFNTYAMNWVRQAPGKGLEWVARIRS KYNMYATYYAASVKGRFTISRDDSKNSL YLQMNSLKTEDTAVYYCARHGNFGNSY VSWFAYWGQGLTVTVSS	QTVVTQEPSLTVSPGGTVTLTCSRSTG AVTTSNYANWVQKPGQAPRGLIGG TNKRAPGTPARFSGSLLGGKAALTLSG VQPEDEAEYYCALWYSNLWVFGGGT KLTVL
teclistamab (same as talquetamab)	EVQLVESGGGLVQPGGSLRLSCAASGF TFNTYAMNWVRQAPGKGLEWVARIRS KYNMYATYYAASVKGRFTISRDDSKNSL YLQMNSLKTEDTAVYYCARHGNFGNSY VSWFAYWGQGLTVTVSS	QTVVTQEPSLTVSPGGTVTLTCSRSTG AVTTSNYANWVQKPGQAPRGLIGG TNKRAPGTPARFSGSLLGGKAALTLSG VQPEDEAEYYCALWYSNLWVFGGGT KLTVL
flotetuzumab	EVQLVESGGGLVQPGGSLRLSCAASGF TFSTYAMNWVRQAPGKLEWVGRIIRS KYNMYATYYADSVKDRFTISRDDSKNSL YLQMNSLKTEDTAVYYCVRHGNFGNSY VSWFAYWGQGLTVTVSS	QAVVTQEPSLTVSPGGTVTLTCSRSTG AVTTSNYANWVQKPGQAPRGLIGG TNKRAPWTPARFSGSLLGGKAALTITG AQAEDEADYYCALWYSNLWVFGGGT KLTVL
obrindatamab	EVQLVESGGGLVQPGGSLRLSCAASGF TFSTYAMNWVRQAPGKLEWVGRIIRS KYNMYATYYADSVKDRFTISRDDSKNSL YLQMNSLKTEDTAVYYCVRHGNFGNSY VSWFAYWGQGLTVTVSS	QAVVTQEPSLTVSPGGTVTLTCSRSTG AVTTSNYANWVQKPGQAPRGLIGG TNKRAPWTPARFSGSLLGGKAALTITG AQAEDEADYYCALWYSNLWVFGGGT KLTVL
cibisatamab	EVQLLESGGGLVQPGGSLRLSCAASGFT FSTYAMNWVRQAPGKLEWVSRIRSK YNNYATYYADSVKGRFTISRDDSKNTLY LQMNSLRAEDTAVYYCVRHGNFGNSY VSWFAYWGQGLTVTVSS	QAVVTQEPSLTVSPGGTVTLTCSSTG AVTTSNYANWVQKPGQAFRGLIGGT NKRAPGTPARFSGSLLGGKAALTLSGA QPEDEAEYYCALWYSNLWVFGGGTKL TVL
alnuctamab	EVQLLESGGGLVQPGGSLRLSCAASGFT FSTYAMNWVRQAPGKLEWVSRIRSK YNNYATYYADSVKGRFTISRDDSKNTLY LQMNSLRAEDTAVYYCVRHGNFGNSY VSWFAYWGQGLTVTVSS	QAVVTQEPSLTVSPGGTVTLTCSSTG AVTTSNYANWVQKPGQAFRGLIGGT NKRAPGTPARFSGSLLGGKAALTLSGA QPEDEAEYYCALWYSNLWVFGGGTKL TVL
glofitamab	EVQLLESGGGLVQPGGSLRLSCAASGFT FSTYAMNWVRQAPGKLEWVSRIRSK YNNYATYYADSVKGRFTISRDDSKNTLY LQMNSLRAEDTAVYYCVRHGNFGNSY VSWFAYWGQGLTVTVSS	QAVVTQEPSLTVSPGGTVTLTCSSTG AVTTSNYANWVQKPGQAFRGLIGGT NKRAPGTPARFSGSLLGGKAALTLSGA QPEDEAEYYCALWYSNLWVFGGGTKL TVL
vibecotamab	EVQLVESGGGLVQPGGSLRLSCAASGF TFSTYAMNWVRQAPGKLEWVGRIIRS KYNMYATYYADSVKGRFTISRDDSKNTL YLQMNSLRAEDTAVYYCVRHGNFGDS YVSWFAYWGQGLTVTVSS	QAVVTQEPSLTVSPGGTVTLTCSSTG AVTTSNYANWVQKPGKSPRGLIGGT NKRAPGVPARFSGSLLGGKAALTISGA QPEDEADYYCALWYSNHWVFGGGTK LTVL
tidutamab	EVQLVESGGGLVQPGGSLRLSCAASGF TFSTYAMNWVRQAPGKLEWVGRIIRS KYNMYATYYADSVKGRFTISRDDSKNTL	QAVVTQEPSLTVSPGGTVTLTCSSTG AVTTSNYANWVQKPGKSPRGLIGGT NKRAPGVPARFSGSLLGGKAALTISGA

	YLQMNSLRAEDTAVYYCVRHGNFGDS YVSWFAYWGQGLTVTVSS	QPEDEADYYCALWYSNHWVFGGGTK LTVL
plamotamab	EVQLVESGGGLVQPGGSLRLSCAASGF TFSTYAMNWVRQAPGKGLEWVGRIIRS KYNMYATYYADSVKGRFTISRDDSKNTL YLQMNSLRAEDTAVYYCVRHGNFGDS YVSWFAYWGQGLTVTVSS	QAVVTQEPSLTVSPGGTVTLTCSSTG AVTTSNYANWVQKPGKSPRGLIGGT NKRAPGVPARFSGSLLGGKAALTISGA QPEDEADYYCALWYSNHWVFGGGTK LTVL
pavurutamab	EVQLVESGGGLVQPGGSLKLSCAASGF TFNKYAMNWVRQAPGKGLEWVARIRS KYNMYATYYADSVKDRFTISRDDSKNTA YLQMNNLKTEDTAVYYCVRHGNFGNS YISYWAYWGQGLTVTVSS	QTVVTQEPSLTVSPGGTVTLTCSSTG AVTSGNYPNWVQKPGQAPRGLIGG TKFLAPGTPARFSGSLLGGKAALTLSGV QPEDEAEYYCVLWYSNRWVFGGGTK LTVL
gresonitamab	EVQLVESGGGLVQPGGSLKLSCAASGF TFNKYAMNWVRQAPGKGLEWVARIRS KYNMYATYYADSVKDRFTISRDDSKNTA YLQMNNLKTEDTAVYYCVRHGNFGNS YISYWAYWGQGLTVTVSS	QTVVTQEPSLTVSPGGTVTLTCSSTG AVTSGNYPNWVQKPGQAPRGLIGG TKFLAPGTPARFSGSLLGGKAALTLSGV QPEDEAEYYCVLWYSNRWVFGGGTK LTVL
tarlatamab	EVQLVESGGGLVQPGGSLKLSCAASGF TFNKYAMNWVRQAPGKGLEWVARIRS KYNMYATYYADSVKDRFTISRDDSKNTA YLQMNNLKTEDTAVYYCVRHGNFGNS YISYWAYWGQGLTVTVSS	QTVVTQEPSLTVSPGGTVTLTCSSTG AVTSGNYPNWVQKPGQAPRGLIGG TKFLAPGTPARFSGSLLGGKAALTLSGV QPEDEAEYYCVLWYSNRWVFGGGTK LTVL
etevritamab	EVQLVESGGGLVQPGGSLKLSCAASGF TFNKYAMNWVRQAPGKGLEWVARIRS KYNMYATYYADSVKDRFTISRDDSKNTA YLQMNNLKTEDTAVYYCVRHGNFGNS YISYWAYWGQGLTVTVSS	QTVVTQEPSLTVSPGGTVTLTCSSTG AVTSGNYPNWVQKPGQAPRGLIGG TKFLAPGTPARFSGSLLGGKAALTLSGV QPEDEAEYYCVLWYSNRWVFGGGTK LTVL
acapatamab	EVQLVESGGGLVQPGGSLKLSCAASGF TFNKYAMNWVRQAPGKGLEWVARIRS KYNMYATYYADSVKDRFTISRDDSKNTA YLQMNNLKTEDTAVYYCVRHGNFGNS YISYWAYWGQGLTVTVSS	QTVVTQEPSLTVSPGGTVTLTCSSTG AVTSGNYPNWVQKPGQAPRGLIGG TKFLAPGTPARFSGSLLGGKAALTLSGV QPEDEAEYYCVLWYSNRWVFGGGTK LTVL
pasotuxizumab	EVQLVESGGGLVQPGGSLKLSCAASGF TFNKYAMNWVRQAPGKGLEWVARIRS KYNMYATYYADSVKDRFTISRDDSKNTA YLQMNNLKTEDTAVYYCVRHGNFGNS YISYWAYWGQGLTVTVSS	QTVVTQEPSLTVSPGGTVTLTCSSTG AVTSGNYPNWVQKPGQAPRGLIGG TKFLAPGTPARFSGSLLGGKAALTLSGV QPEDEAEYYCVLWYSNRWVFGGGTK LTVL
pacanalotamab	EVQLVESGGGLVQPGGSLKLSCAASGF TFNKYAMNWVRQAPGKGLEWVARIRS KYNMYATYYADSVKDRFTISRDDSKNTA YLQMNNLKTEDTAVYYCVRHGNFGNS YISYWAYWGQGLTVTVSS	QTVVTQEPSLTVSPGGTVTLTCSSTG AVTSGNYPNWVQKPGQAPRGLIGG TKFLAPGTPARFSGSLLGGKAALTLSGV QPEDEAEYYCVLWYSNRWVFGGGTK LTVL
eluvixtamab	EVQLVESGGGLVQPGGSLKLSCAASGF TFNKYAMNWVRQAPGKGLEWVARIRS KYNMYATYYADSVKDRFTISRDDSKNTA	QTVVTQEPSLTVSPGGTVTLTCSSTG AVTSGNYPNWVQKPGQAPRGLIGG TKFLAPGTPARFSGSLLGGKAALTLSGV

	YLQMNNLKTEDTAVYYCVRHGNFGNS YISYWAYWGQGLTVTVSS	QPEDEAEYYCVLWYSNRWVFGGGTK LTVL
emerfetamab	EVQLVESGGGLVQPGGSLKLSCAASGF TFNKYAMNWVRQAPGKGLEWVARIRS KYNMYATYYADSVKDRFTISRDDSKNTA YLQMNNLKTEDTAVYYCVRHGNFGNS YISYWAYWGQGLTVTVSS	QTVVTQEPSLTVSPGGTVTLTCSSTG AVTSGNYPNWVQKPGQAPRGLIGG TKFLAPGTPARFSGSLLGGKAALTLSGV QPEDEAEYYCVLWYSNRWVFGGGTK LTVL
epcoritamabNo.7	EVKLVESGGGLVKPGRSLRLSCAASGFT FNTYAMNWVRQAPGKGLEWVARIRSK YNNYATYYADSVKDRFTISRDDSKSILYL QMNNLKTEDTAMYYCVRHGNFGNSY VSWFAYWGQGLTVTVSS	NA
epcoritamabNo.8	EVKLVESGGGLVKPGRSLRLSCAASGFT FNTYAMNWVRQAPGKGLEWVARIRSK YNNYATYYADSVKDRFTISRDDSKSILYL QMNSLKTEDTAMYYCVRHGNFGNSYV SWFAYWGQGLTVTVSS	NA
epcoritamabNo.9	EVKLVESGGGLVKPGRSLRLSCAASGFT FNTYAMNWVRQAPGKGLEWVARIRSK YNNYATYYADSVKDRFTISRDDSKSILYL QMNSLKTEDTAMYYCVRHGNFGNSYV SWFAYWGQGLTMVTVSS	NA
epcoritamabNo.11	NA	QAVVTQEPSFSVSPGGTVTLTCSRSTG AVTTSNYANWVQQTGQAFRGLIGG TNKRAPGVPARFSGSILGNKAALTITG AQADDESIYFCALWYSNLWVFGGGTK LTVL
epcoritamabNo.12	NA	QAVVTQEPSFSVSPGGTVTLTCSRSTG AVTTSNYANWVQQTGQAFRGLIGG TNKRAPGVPARFSGSILGNKAALTITG AQADDESDYYCALWYSNLWVFGGGT KLTVL
epcoritamabNo.30 (LKNH)	NA	QAVVTQEPSLSVSPGGTVTLTCSRSTG AVTTSNYANWVQKPGQAFRGLIGG TNNRAPGVPARFSGSLIGDKAALTITG AQADDESIYFCALWYSNHWVFGGGT KLTVL
epcoritamabNo.31 (T41K)	NA	QAVVTQEPSFSVSPGGTVTLTCSRSTG AVTTSNYANWVQKPGQAFRGLIGG TNKRAPGVPARFSGSLIGDKAALTITG AQADDESIYFCALWYSNLWVFGGGTK LTVL

blinatumomab	DIKLQQSGAELARPGASVKMSCKTSGY TFTRYTMHWVKQRPQGQLEWIGYINP SRGYTNYNQKFKDKATLTDDKSSSTAY MQLSSLTSEDSAVYYCARYYDDHYCLDY WGQGTTLVSS	DIQLTQSPAISASPGKEVTMTCRASS SVSYMNWYQQKSGTSPKRWIYDTSK VASGVPYRFSGSGSGTSYSLTSSMEAE DAATYYCQWSSNPLTFGAGTKLELK
solitomab	DVQLVQSGAEVKKPGASVKVSKASGY TFTRYTMHWVRQAPQGQLEWIGYINP SRGYTNYADSVKGRFTITDDKSTSTAYM ELSSLRSEDTATYYCARYYDDHYCLDYW GQGTTVTVSS	DIVLTQSPATLSLSPGERATLSCRASQS VSYMNWYQQKPGKAPKRWIYDTSKV ASGVPARFSGSGSGTDYSLTINSLEAED AATYYCQWSSNPLTFGGGKVEIK
visilizumab	QVQLVQSGAEVKKPGASVKVSKASGY TFISYTMHWVRQAPQGQLEWMGYIN PRSGYTHYNQKLKDKATLTADKSASTAY MELSSLRSEDTAVYYCARSAYYDYGFA YWGQGTLLTVSS	DIQMTQSPSSLSASVGDRTVITCSASS VSYMNWYQQKPGKAPKRLIYDTSKLA SGVPSRFSGSGSGTDFTLTISLQPEDF ATYYCQWSSNPFTFGGKVEIK
otelixizumab	EVQLLESGGGLVQPGGSLRLSCAASGFT FSSFPMWVRQAPGKQLEWVSTISTSG GRYYRDSVKGRFTISRDNKNTLYLQM NSLRAEDTAVYYCAKFRQYSGGFDYWG QGTLTVSS	DIQLTQPNVSTSLGSLVSLTSSGN IENNYVHWYQLYEGRSPTTMIYDDDK RPDGVPDFRSGSIDRSSNSAFLTIHNV AIEDEAIYFCHSYVSSFNVFGGKLT V L
cevostamab	EVQLVQSGAEVKKPGASVKVSKASGF TFTSYIHWVRQAPQGQLEWIGWIPE NDNTKYNEKFKDRVTITADTSTSTAYLEL SSLRSEDTAVYYCARDGYSRYFDYWG QGTLTVSS	DIVMTQSPDSLAVSLGERATINCKSSQ SLLNSRTRKNYLAWYQQKPGQSPKLLI YWTSTRKSGVPDRFSGSGSGTDFTLT SSLQAEDVAVYYCKQSFILRTFGQGT K VEIK
tepoditamab	QVQLVQSGGGVVPGRSLRLSCVASGF TFSSYGMHWVRQAPGKQLEWVAAIW YNARKQDYADSVKGRFTISRDNKNTLY LQMNSLRAEDTAVYYCTRGTGYNWFD PWGQGTLLTVSS	DIQMTQSPSSLSASVGDRTVITCRASQ SISSYLNWYQQKPGKAPKLLIYAASSLQ SGVPSRFSGSGSGTDFTLTISLQPEDF ATYYCQQSYSTPPTFGQGTKEIK
elranatamab	EVQLVESGGGLVQPGGSLRLSCAASGF TFSDYYMTWVRQAPGKQLEWVAFIRN RARGYTSDHNPVSKGRFTISRDNKNSL YLQMNSLRAEDTAVYYCARDRPSYYVL DYWGQGTTLTVSS	DIVMTQSPDSLAVSLGERATINCKSSQ SLFNVRSRKNYLAWYQQKPGQPPKLLI SWASTRESGVPDRFSGSGSGTDFTLT SSLQAEDVAVYYCKQSYDLFTFGSGT K LEIK
odronextamab	EVQLVESGGGLVQPGSLRLSCAASGFT FDDYTMHWVRQAPGKQLEWVSGISW NSGSGYADSVKGRFTISRDNKKSLLY QMNSLRAEDTALYYCAKDNSGYGHYY GMDVWGQGTTLTVAS	EIVMTQSPATLSVSPGERATLSCRASQ SVSSNLAWYQQKPGQAPRLLIYGAST RATGIPARFSGSGSGTEFTLTISLQSED FAVYYCQHYINWPLTFGGGKVEIK
muromonab	QVQLQQSGAELARPGASVKMSCKASG YTFTRYTMHWVKQRPQGQLEWIGYIN PSRGYTNYNQKFKDKATLTDDKSSSTAY MQLSSLTSEDSAVYYCARYYDDHYCLDY WGQGTTLVSS	QIVLTQSPAISASPGKEVTMTCSASS SVSYMNWYQQKSGTSPKRWIYDTSKL ASGVPAHFRGSGSGTSYSLTISGMEAE DAATYYCQWSSNPFTFGSGTKLEIN

[00149] In embodiments, the anti-CD3 antibody or fragment thereof can comprise a HCVR/LCVR pair having sequences having at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% sequence identity to the HCVR and LCVR of any one of the following HCVR/LCVR pairs, including any combination of epcoritamab HCVR/LCVR:

Name	vH	vL
SP34 Parent	EVKLLESGGGLVQPKGSLKLSAASGFT FNTYAMNWVRQAPGKGLEWVARIRSK YNNYATYYADSVKDRFTISRDDSQSILYL QMNNLKTEDTAMYYCVRHGNFGNSY VSWFAYWGQGLTVTVSA	QAVVTQESALTTSPGETVTLTCSRSTG AVTTSNYANWVQEKPDHLFTGLIGGT NKRAPGVPARFSGSLIGDKAALTITGA QTEDEAIYFCALWYSNLWVFGGGTKL TVL
PAI-SP34	EVQLVESGGGLVQPGGSLRLSAAASGF TFNTYAMNWVRQAPGKGLEWVARIRS KYNNTYATYYADSVKDRFTISRDDSKNTL YLQMNSLKAEDTAVYYCVRHGNFGNS YVSWFAYWGQGLTVTVSS	QAVVTQEPSLTVSPGGTVTLTCSRSTG AVTTSNYANWVQKPGQAPRGLIGG TNKRAPGTPARFSGSLLGGKAALTLSG AQPEDEAEYCALWYSNLWVFGGGT KLTVL
epcoritamab	EVKLLESGGGLVQPGGSLRLSAAASGF FNTYAMNWVRQAPGKGLEWVARIRSK YNNYATYYADSVKDRFTISRDDSKSSLYL QMNNLKTEDTAMYYCVRHGNFGNSY VSWFAYWGQGLTVTVSS	QAVVTQEPSFSVSPGGTVTLTCSRSTG AVTTSNYANWVQQTPGQAFRGLIGG TNKRAPGVPARFSGSLIGDKAALTITG AQADDESIYFCALWYSNLWVFGGGTK LTVL
duvortuxizumab	EVQLVESGGGLVQPGGSLRLSAAASGF TFNTYAMNWVRQAPGKGLEWVGRIIRS KYNNTYATYYADSVKGRFTISRDDSKNSL YLQMNSLKTEDTAVYYCVRHGNFGNSY VSWFAYWGQGLTVTVSS	QAVVTQEPSLTVSPGGTVTLTCSRSTG AVTTSNYANWVQKPGQAPRGLIGG TNKRAPWTPARFSGSLLGGKAALTITG AQAEDEADYCALWYSNLWVFGGGT KLTVL
talquetamab	EVQLVESGGGLVQPGGSLRLSAAASGF TFNTYAMNWVRQAPGKGLEWVARIRSK KYNNTYATYYAASVKGRFTISRDDSKNSL YLQMNSLKTEDTAVYYCARHGNFGNSY VSWFAYWGQGLTVTVSS	QTVVTQEPSLTVSPGGTVTLTCSRSTG AVTTSNYANWVQKPGQAPRGLIGG TNKRAPGTPARFSGSLLGGKAALTLSG VQPEDEAEYCALWYSNLWVFGGGT KLTVL
teclistamab (same as talquetamab)	EVQLVESGGGLVQPGGSLRLSAAASGF TFNTYAMNWVRQAPGKGLEWVARIRSK KYNNTYATYYAASVKGRFTISRDDSKNSL YLQMNSLKTEDTAVYYCARHGNFGNSY VSWFAYWGQGLTVTVSS	QTVVTQEPSLTVSPGGTVTLTCSRSTG AVTTSNYANWVQKPGQAPRGLIGG TNKRAPGTPARFSGSLLGGKAALTLSG VQPEDEAEYCALWYSNLWVFGGGT KLTVL
flotetuzumab	EVQLVESGGGLVQPGGSLRLSAAASGF TFNTYAMNWVRQAPGKGLEWVGRIIRS KYNNTYATYYADSVKDRFTISRDDSKNSL YLQMNSLKTEDTAVYYCVRHGNFGNSY VSWFAYWGQGLTVTVSS	QAVVTQEPSLTVSPGGTVTLTCSRSTG AVTTSNYANWVQKPGQAPRGLIGG TNKRAPWTPARFSGSLLGGKAALTITG AQAEDEADYCALWYSNLWVFGGGT KLTVL
obrindatamab	EVQLVESGGGLVQPGGSLRLSAAASGF TFNTYAMNWVRQAPGKGLEWVGRIIRS KYNNTYATYYADSVKDRFTISRDDSKNSL YLQMNSLKTEDTAVYYCVRHGNFGNSY VSWFAYWGQGLTVTVSS	QAVVTQEPSLTVSPGGTVTLTCSRSTG AVTTSNYANWVQKPGQAPRGLIGG TNKRAPWTPARFSGSLLGGKAALTITG AQAEDEADYCALWYSNLWVFGGGT KLTVL

cibisatamab	EVQLLESGGGLVQPGGSLRLSCAASGFT FSTYAMNWVRQAPGKGLEWVSRIRSK YNNYATYYADSVKGRFTISRDDSKNTLY LQMNSLRAEDTAVYYCVRHGNFGNSY VSWFAYWGQGLTVTVSS	QAVVTQEPSLTVSPGGTVTLTCGSSTG AVTTSNYANWVQKPGQAFRGLIGGT NKRAPGTPARFSGSLLGGKAALTLSGA QPEDEAEYYCALWYSNLWVFGGGTKL TVL
alnuctamab	EVQLLESGGGLVQPGGSLRLSCAASGFT FSTYAMNWVRQAPGKGLEWVSRIRSK YNNYATYYADSVKGRFTISRDDSKNTLY LQMNSLRAEDTAVYYCVRHGNFGNSY VSWFAYWGQGLTVTVSS	QAVVTQEPSLTVSPGGTVTLTCGSSTG AVTTSNYANWVQKPGQAFRGLIGGT NKRAPGTPARFSGSLLGGKAALTLSGA QPEDEAEYYCALWYSNLWVFGGGTKL TVL
glofitamab	EVQLLESGGGLVQPGGSLRLSCAASGFT FSTYAMNWVRQAPGKGLEWVSRIRSK YNNYATYYADSVKGRFTISRDDSKNTLY LQMNSLRAEDTAVYYCVRHGNFGNSY VSWFAYWGQGLTVTVSS	QAVVTQEPSLTVSPGGTVTLTCGSSTG AVTTSNYANWVQKPGQAFRGLIGGT NKRAPGTPARFSGSLLGGKAALTLSGA QPEDEAEYYCALWYSNLWVFGGGTKL TVL
vibecotamab	EVQLVESGGGLVQPGGSLRLSCAASGF TFSTYAMNWVRQAPGKGLEWVGRIRS KYNMYATYYADSVKGRFTISRDDSKNTL YLQMNSLRAEDTAVYYCVRHGNFGDS YVSWFAYWGQGLTVTVSS	QAVVTQEPSLTVSPGGTVTLTCGSSTG AVTTSNYANWVQKPGKSPRGLIGGT NKRAPGVPARFSGSLLGGKAALTISGA QPEDEADYYCALWYSNHWVFGGGTK LTVL
tidutamab	EVQLVESGGGLVQPGGSLRLSCAASGF TFSTYAMNWVRQAPGKGLEWVGRIRS KYNMYATYYADSVKGRFTISRDDSKNTL YLQMNSLRAEDTAVYYCVRHGNFGDS YVSWFAYWGQGLTVTVSS	QAVVTQEPSLTVSPGGTVTLTCGSSTG AVTTSNYANWVQKPGKSPRGLIGGT NKRAPGVPARFSGSLLGGKAALTISGA QPEDEADYYCALWYSNHWVFGGGTK LTVL
plamotamab	EVQLVESGGGLVQPGGSLRLSCAASGF TFSTYAMNWVRQAPGKGLEWVGRIRS KYNMYATYYADSVKGRFTISRDDSKNTL YLQMNSLRAEDTAVYYCVRHGNFGDS YVSWFAYWGQGLTVTVSS	QAVVTQEPSLTVSPGGTVTLTCGSSTG AVTTSNYANWVQKPGKSPRGLIGGT NKRAPGVPARFSGSLLGGKAALTISGA QPEDEADYYCALWYSNHWVFGGGTK LTVL
pavurutamab	EVQLVESGGGLVQPGGSLKLSAASGF TFNKYAMNWVRQAPGKGLEWVARIRS KYNMYATYYADSVKDRFTISRDDSKNTA YLQMNNLKTEDTAVYYCVRHGNFGNS YISYWAYWGQGLTVTVSS	QTVVTQEPSLTVSPGGTVTLTCGSSTG AVTSGNYPNWVQKPGQAPRGLIGG TKFLAPGTPARFSGSLLGGKAALTLSGV QPEDEAEYYCVLWYSNRWVFGGGTK LTVL
gresonitamab	EVQLVESGGGLVQPGGSLKLSAASGF TFNKYAMNWVRQAPGKGLEWVARIRS KYNMYATYYADSVKDRFTISRDDSKNTA YLQMNNLKTEDTAVYYCVRHGNFGNS YISYWAYWGQGLTVTVSS	QTVVTQEPSLTVSPGGTVTLTCGSSTG AVTSGNYPNWVQKPGQAPRGLIGG TKFLAPGTPARFSGSLLGGKAALTLSGV QPEDEAEYYCVLWYSNRWVFGGGTK LTVL
tarlatamab	EVQLVESGGGLVQPGGSLKLSAASGF TFNKYAMNWVRQAPGKGLEWVARIRS KYNMYATYYADSVKDRFTISRDDSKNTA YLQMNNLKTEDTAVYYCVRHGNFGNS YISYWAYWGQGLTVTVSS	QTVVTQEPSLTVSPGGTVTLTCGSSTG AVTSGNYPNWVQKPGQAPRGLIGG TKFLAPGTPARFSGSLLGGKAALTLSGV QPEDEAEYYCVLWYSNRWVFGGGTK LTVL

etevritamab	EVQLVESGGGLVQPGGSLKLSCAASGF TFNKYAMNWVRQAPGKGLEWVARIRS KYNMYATYYADSVKDRFTISRDDSKNTA YLQMNNLKTEDTAVYYCVRHGNFGNS YISYWAYWGQGLTVTVSS	QTVVTQEPSLTVSPGGTVTLTCSSTG AVTSGNYPNWVQQKPGQAPRGLIGG TKFLAPGTPARFSGSLLGGKAALTLGSV QPEDEAEYYCVLWYSNRWVFGGGTK LTVL
acapatamab	EVQLVESGGGLVQPGGSLKLSCAASGF TFNKYAMNWVRQAPGKGLEWVARIRS KYNMYATYYADSVKDRFTISRDDSKNTA YLQMNNLKTEDTAVYYCVRHGNFGNS YISYWAYWGQGLTVTVSS	QTVVTQEPSLTVSPGGTVTLTCSSTG AVTSGNYPNWVQQKPGQAPRGLIGG TKFLAPGTPARFSGSLLGGKAALTLGSV QPEDEAEYYCVLWYSNRWVFGGGTK LTVL
pasotuxizumab	EVQLVESGGGLVQPGGSLKLSCAASGF TFNKYAMNWVRQAPGKGLEWVARIRS KYNMYATYYADSVKDRFTISRDDSKNTA YLQMNNLKTEDTAVYYCVRHGNFGNS YISYWAYWGQGLTVTVSS	QTVVTQEPSLTVSPGGTVTLTCSSTG AVTSGNYPNWVQQKPGQAPRGLIGG TKFLAPGTPARFSGSLLGGKAALTLGSV QPEDEAEYYCVLWYSNRWVFGGGTK LTVL
pacanalotamab	EVQLVESGGGLVQPGGSLKLSCAASGF TFNKYAMNWVRQAPGKGLEWVARIRS KYNMYATYYADSVKDRFTISRDDSKNTA YLQMNNLKTEDTAVYYCVRHGNFGNS YISYWAYWGQGLTVTVSS	QTVVTQEPSLTVSPGGTVTLTCSSTG AVTSGNYPNWVQQKPGQAPRGLIGG TKFLAPGTPARFSGSLLGGKAALTLGSV QPEDEAEYYCVLWYSNRWVFGGGTK LTVL
eluvixtamab	EVQLVESGGGLVQPGGSLKLSCAASGF TFNKYAMNWVRQAPGKGLEWVARIRS KYNMYATYYADSVKDRFTISRDDSKNTA YLQMNNLKTEDTAVYYCVRHGNFGNS YISYWAYWGQGLTVTVSS	QTVVTQEPSLTVSPGGTVTLTCSSTG AVTSGNYPNWVQQKPGQAPRGLIGG TKFLAPGTPARFSGSLLGGKAALTLGSV QPEDEAEYYCVLWYSNRWVFGGGTK LTVL
emerfetamab	EVQLVESGGGLVQPGGSLKLSCAASGF TFNKYAMNWVRQAPGKGLEWVARIRS KYNMYATYYADSVKDRFTISRDDSKNTA YLQMNNLKTEDTAVYYCVRHGNFGNS YISYWAYWGQGLTVTVSS	QTVVTQEPSLTVSPGGTVTLTCSSTG AVTSGNYPNWVQQKPGQAPRGLIGG TKFLAPGTPARFSGSLLGGKAALTLGSV QPEDEAEYYCVLWYSNRWVFGGGTK LTVL
epcoritamabNo.7	EVKLVESGGGLVKPGRSLRLSCAASGFT FNTYAMNWVRQAPGKGLEWVARIRSK YNNYATYYADSVKDRFTISRDDSKSILYL QMNNLKTEDTAMYYCVRHGNFGNSY VSWFAYWGQGLTVTVSS	NA
epcoritamabNo.8	EVKLVESGGGLVKPGRSLRLSCAASGFT FNTYAMNWVRQAPGKGLEWVARIRSK YNNYATYYADSVKDRFTISRDDSKSILYL QMNSLKTEDTAMYYCVRHGNFGNSYV SWFAYWGQGLTVTVSS	NA
epcoritamabNo.9	EVKLVESGGGLVKPGRSLRLSCAASGFT FNTYAMNWVRQAPGKGLEWVARIRSK YNNYATYYADSVKDRFTISRDDSKSILYL QMNSLKTEDTAMYYCVRHGNFGNSYV SWFAYWGQGMTMTVTVSS	NA

epcoritamabNo.11	NA	QAVVTQEPSFSVSPGGTVTLTCSRSTG AVTTSNYANWVQQTPGQAFRLIGG TNKRAPGVPARFSGSILGNKAALTITG AQADDESIYFCALWYSNLWVFGGGTK LTVL
epcoritamabNo.12	NA	QAVVTQEPSFSVSPGGTVTLTCSRSTG AVTTSNYANWVQQTPGQAFRLIGG TNKRAPGVPARFSGSILGNKAALTITG AQADDESDYFCALWYSNLWVFGGGT KLTVL
epcoritamabNo.30 (LKNH)	NA	QAVVTQEPSLSVSPGGTVTLTCSRSTG AVTTSNYANWVQQKPGQAFRLIGG TNNRAPGVPARFSGSLIGDKAALTITG AQADDESIYFCALWYSNLWVFGGGT KLTVL
epcoritamabNo.31 (T41K)	NA	QAVVTQEPSFSVSPGGTVTLTCSRSTG AVTTSNYANWVQQKPGQAFRLIGG TNKRAPGVPARFSGSLIGDKAALTITG AQADDESIYFCALWYSNLWVFGGGTK LTVL
blinatumomab	DIKLQQSGAELARPGASVKMSCKTSGY TFTRYTMHWWKQRPQGGLWIGYINP SRGYTNYNQKFKDKATLTDDKSSSTAY MQLSSLTSEDSAVYYCARYYDDHYCLDY WGQGTTLTVSS	DIQLTQSPAIMSASPGEKVTMTCRASS SVSYMNWYQQKSGTSPKRWIYDTSK VASGVPYRFSGSGSGTSYSLTISSMEAE DAATYYCQWSSNPLTFGAGTKLELK
solitomab	DVQLVQSGAEVKKPGASVKVSKASGY TFTRYTMHVVQRAPQGGLWIGYINP SRGYTNYADSVKGRFTITDDKSTSTAYM ELSSLRSEDATYYCARYYDDHYCLDYW GQGTTVTVSS	DIVLTQSPATLSLSPGERATLSCRASQS VSYMNWYQQKPGKAPKRWIYDTSKV ASGVPARFSGSGSGTDYSLTINSLEAED AATYYCQWSSNPLTFGGGTKVEIK
visilizumab	QVQLVQSGAEVKKPGASVKVSKASGY TFISYTMHVVQRAPQGGLWIMGYIN PRSGYTHYNQKLKDKATLTADKSASTAY MELSSLRSEDATYYCARSAYYDYGFA YWGQGTTLTVSS	DIQMTQSPSSLSASVGDRTITCSASSS VSYMNWYQQKPGKAPKRLIYDTSKLA SGVPSRFSGSGSGTDFTLTISLQPEDF ATYYCQWSSNPPTFGGGTKVEIK
otelixizumab	EVQLLESGGGLVQPGGSLRLSCAASGFT FSSFPMAWVRQAPGKGLWVSTISTSG GRYYRDSVKGRFTISRDNKNTLYLQM NSLRAEDTAVYYCAKFRQYSGGFDYWG QGTLTVTVSS	DIQLTQPNVSTSLGSTVKLSCTLSGSGN IENNYVHWYQLYEGRSPTTMIYDDDK RPDGVPDFRSGSIDRSSNSAFLTIHNV AIEDEAIYFCHSYVSSFNVFGGGTKLTV L
cevostamab	EVQLVQSGAEVKKPGASVKVSKASGF TFTSYIHVVQRAPQGGLWIGWIPE NDNTKYNEKFKDRVITADTSTSTAYLEL SSLRSEDATYYCARDGYSRYFDYWG QGTLTVTVSS	DIVMTQSPDSLAVSLGERATINCKSSQ SLLNSRTRKNYLAWYQQKPGQSPKLLI YWTSTRKSGVPDRFSGSGSGTDFTLT SSLQAEDVAVYYCKQSFILRTFGQGTK VEIK

tepoditamab	QVQLVQSGGGVVPGRSLRLSCVASGF TFSSYGMHWVRQAPGKGLEWVAIIW YNARKQDYADSVKGRFTISRDNKNTLY LQMNSLRAEDTAVYYCTRGTYNWF PWGQGTTLTVSS	DIQMTQSPSSLSASVGRVTITCRASQ SISSYLNWYQQKPGKAPKLLIYAASSLQ SGVPSRFSGSGSGTDFTLTISSLQPEDF ATYYCQQSYSTPPTFGQGTKVEIK
elranatamab	EVQLVESGGGLVQPGGSLRLSCAASGF TFSDYYMTWVRQAPGKGLEWVAFIRN RARGYTS DHNPSVKGRFTISRDNKNSL YLQMNSLRAEDTAVYYCARDPSYYVL DYWGQGTTLTVSS	DIVMTQSPDSLAVSLGERATINCKSSQ SLFNVRSRKNYLAWYQQKPGQPPKLLI SWASTRESGVPDRFSGSGSGTDFTLTI SSLQAEDVAVYYCKQSYDLFTFGSGTK LEIK
odronextamab	EVQLVESGGGLVQPGRSLRLSCAASGFT FDDYTMHWVRQAPGKGLEWVSGISW NSGSIGYADSVKGRFTISRDNKSLYL QMNSLRAEDTALYYCAKDNSGYGHYY GMDVWGQGTTLTVAS	EIVMTQSPATLSVSPGERATLSCRASQ SVSSNLAWYQQKPGQAPRLLIYGAST RATGIPARFSGSGSGTEFTLTISLQSED FAVYYCQHYINWPLTFGGGTKVEIK
muromonab	QVQLQQSGAELARPGASVKMSCKASG YTFTRYTMHWVKRPGQGLEWIGYIN PSRGYTNYNQKFKDKATLTDDKSSSTAY MQLSSLTSEDSAVYYCARYDDHYCLDY WGQGTTLTVSS	QIVLTQSPAISASPGKVTMTCSASS SVSYMNWYQQKSGTSPKRWIYDTSKL ASGVPAHFRGSGSGTSYSLTISGMEAE DAATYYCQQWSSNPFTFGSGTKLEIN

[00150] In embodiments, where the T cell engager, *e.g.* anti-CD3, is an antibody, it can be a chimeric, humanized or human antibody. In embodiments, where the T cell engager is an antibody, it can be a fragment of a full-length antibody, single-chain antibody, a single domain antibody (*e.g.* a heavy chain only antibody), a single-chain variable fragment (scFv), or any other type of “antibody” defined herein. Exemplary antibodies include monoclonal, chimeric, humanized, and human antibodies.

[00151] In embodiments, the T cell engager can be a T cell engager, *e.g.* antibody or fragment thereof, can be as described in or derived from any of U.S. Patent Application Publication No. 2017/0355767, U.S. Patent No. 10,407,501, U.S. Patent No. 10,562,968, U.S. Patent Application Publication No. 2021/0253701, U.S. Patent No. 9,657,102, WO 2000/041474, and U.S. Patent Application Publication No. 2023/0002506, each of which is incorporated herein by reference in its entirety. Additional T cell engagers, including anti-CD3 antibodies and fragments thereof include those of alnuctamab, glofitamab, gresonitamab, acapatamab, pasotuxizumab, pacanalotamab, eluvixtamab, emerfetamab, solitomab, cevostamab, elranatamab, odronextamab, muromonab, huCLB-T3/4, blinatumomab, PAI-SP34, visilizumab, oteelixumab, epcoritamab, talquetamab, flotetuzumab, teclistamab, pavurutamab, cibistamab,

vibecotamab, pavurutamab, MGD007, tepoditamab, GBR1302, M802, plamotamab, GEM333, PF-06671008, AMV564, JNJ-63709178, ERY974, obrindatamab, tidutamab, IGM-2323, JNJ-63898081, duvortuxizumab, etevritamab, tarlatamab, OKT3, Teplizumab™ (MGA031, Eli Lilly), UCHT1, BC-3, NI0401, X35-3, VIT3, BMA030 (BW264/56), CLB-T3/3, CRIS7, YTH12.5, F111-409, CLB-T3.4.2, WT31, WT32, SPv-T3b, 11D8, XIII-141, XIII-46, XIII-87, 12F6, T3/RW2-8C8, T3/RW2-4B6, OKT3D, M-T301, SP34, SMC2 and F101.01. The anti-CD3 antibody or fragment thereof can include the six CDRs (HCDR1, HCDR2, HCDR3, LCDR1, LCDR2, and LCDR3) of the antibodies or fragments of any of these disclosures or antibodies and/or a heavy chain variable region (HCVR) and/or light chain variable region (LCVR) having at 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or 100% sequence identity to the sequences of such antibodies or fragments thereof.

[00152] In embodiments, the second domain comprises a means for binding to CD3 according to any of the foregoing embodiments.

CTHRC1 Binding Moiety

[00153] In embodiments of the present disclosure, the anti-CTHRC1 T-cell engagers of the present disclosure include one or more first domains comprising a CTHRC1 binding moiety. In embodiments, the anti-CTHRC1 T-cell engager has a ratio of 1:1, 2:1, 1:2, or 2:2 of first domain to second domain.

[00154] In embodiments, the CTHRC1 binding moiety can (i) selectively bind to CTHRC1 and/or (ii) block cell adhesion to CTHRC1. In embodiments, the CTHRC1 binding moiety can selectively bind to human CTHRC1 and a cell displaying a CTHRC1 epitope without being internalized.

[00155] In any of the foregoing embodiments, the CTHRC1 binding moiety can be an antibody, as defined herein, capable of binding to CTHRC1. In some embodiments, anti-CTHRC1 antibodies of the invention further comprise a human subgroup III heavy chain framework consensus sequence. In one embodiment of these antibodies, these antibodies further comprise a human κ I light chain framework consensus sequence. In one embodiment, an anti-CTHRC1 antibody inhibits or neutralizes one or more human CTHRC1 functions.

[00156] It should be understood that the CTHRC1 binding moiety can further include a peptide linker as described herein. By way of example, but not limitation, where the CTHRC1 binding moiety comprises a scFv, the VH and VL portions can be linked by any suitable linker, such as those described in the present disclosure.

[00157] Exemplary CDRs and antibody sequences are provided in the following tables:

Table 2. Complementarity Determining Regions, Heavy Variable Region (IMGT)

Name	Heavy Chain Variable Region CDRs		
	CDR1	CDR2	CDR3
AB987 (M5)	GFTFSTYS (SEQ ID NO: 45)	ISGSSGHI (SEQ ID NO: 50)	ASMHYYYYLDV (SEQ ID NO: 55)
AB988 (M23)	GYTFTSYD (SEQ ID NO: 46)	MNPNSGNT (SEQ ID NO: 51)	AITGTTAGTFDI (SEQ ID NO: 56)
AB989 (M11)	GFSLSTNVMC (SEQ ID NO: 47)	IDWDDDK (SEQ ID NO: 52)	ARTGITGTHYYYYMDV (SEQ ID NO: 57)
AB990 (M3)	GFTFSNCA (SEQ ID NO: 48)	ISGSGGGT (SEQ ID NO: 53)	AKANWNYAYFDF (SEQ ID NO: 58)
AB991 (M14)	RFTFSNYW (SEQ ID NO: 49)	IKQDGSEK (SEQ ID NO: 54)	AREGGYCSDTRCFFYMDV (SEQ ID NO: 59)

Table 3. Complementarity Determining Regions, Light Variable Region (IMGT)

Name	Light Chain Variable Region CDRs		
	CDR1	CDR2	CDR3
AB987	TGTVTTSNY (SEQ ID NO: 60)	ATN (SEQ ID NO: 65)	ALWYSNHWV (SEQ ID NO: 70)
AB988	QGISSY (SEQ ID NO: 61)	AAS (SEQ ID NO: 66)	QQHNSYPWT (SEQ ID NO: 71)
AB989	QGIRND (SEQ ID NO: 62)	AAS (SEQ ID NO: 67)	LQDYNYPWT (SEQ ID NO: 72)
AB990 (M3)	QRVSSN (SEQ ID NO: 63)	GAS (SEQ ID NO: 68)	QQYDIWPMYT (SEQ ID NO: 73)
AB991	QSIISW (SEQ ID NO: 64)	KAS (SEQ ID NO: 69)	QQYNSYSRT (SEQ ID NO: 74)

Table 4. Illustrative Immunoglobulin Sequences – Heavy Chain Variable Region

Name	SEQ ID	Heavy Chain Variable Region
AB987	75	EVQLVESGGGLVTPGGSRLRLSCAASGFTFSTYSMNWVRQAPGK GLEWVSSISGSSGHIYYADSVRGRFTISRDNKNSLYLQMNSLR AEDTAVYYCASMHYYYYLDVWGKGTTVTVSS

AB988	77	QVQPVQSGAEVKKPGASVKVSCKASGYTFTSYDINWVRQATG QGLEWMGWMNPNPNSGNTGYAQKFQGRVTMTRNTSISTAYMEL SSLRSEDNAVYYCAITGTTAGTFDIWGQGTMTVVSQDISMISS
AB989	79	QVTLRESGPALVKPTQTLTLCTFSGFSLSTNVMCVSWIRQPPG KALEWLALIDWDDDKYYSTSLKTRLTISKDTSKNQVVLMTN MDPVDATATYYCARTGITGTHYYYYYMDVWGKGTTVTVSS
AB990	81	EVQLVESGGGLVQPGGSLRLSCAASGFTFSNCAMNWVRQAPG KGLEWVSVISGSGGGTYYADSVKGRFTISRDN SKNTLYLQMNS LRAEDTAVYYCAKANWNYAYFDFWGQGTLLTVSS
AB991	83	EVQVVESGGGLVQPGGSLRLSCAASRFTFSNYWMSWVRQAPG RGLEWVANIKQDGSEKYYVDSVKGRFTISRDN AKNSLYLQMN SLRAEDTAVYYCAREGGYCSDTRCFFYMDVWGKGTTVTVSS

Table 5. Illustrative Immunoglobulin Sequences – Light Chain Variable Region

Name	SEQ ID	Light Chain Variable Region
AB987	76	QAVVTQESALTTSPGETVTLTCRSSTGTVTTSNYVTWVQEKPD HLFTGLIGATNNRAPGVPARFSGSLIGDKAALTITGAQTEDEAIY FCALWYSNHWFVGGGTKLTVL
AB988	78	DIQLTQSPSFLSASVGARVTTTCRASQGISSYLAWYEQKPGKAP KLLIYAASLTQSGVPSRFSGSGSGTEFTLTISLQPEDSATYYCQ QHNSYPWTFGQGTKVEIK
AB989	80	AIQMTQSPSSLSASVGDRVTTTCRASQGIRNDLGWYQQKPGKA PKLLIYAASSLQSGVPSRFSGSGFGTDFTLTISLQPEDFATYYCL QDYNYPWTFGQGTKVEIK
AB990	82	EIVMTQSPATLSVSPGERATLSCRASQRVSSNLAWYQQKPGQA PRLLIYGASTRATGIPARFSGSGSGTEFTLTINSLQSADFAVFFCQ QYDIWPMYTFGQGTKLEIK
AB991	84	DIQMTQSPSTLSASVGDRVTTTCRASQSIISWLAWYQQKPGKAP KLLIYKASSLEGGVPSRFSGSGSGTDFTLTISLQPDDEFATYYCQ QYNSYSRTFGQGTVEIK

[00158] AB990 (M3) has demonstrated unique properties in that it is not internalized when binding an epitope of CTHRC1 on a cell. This property is surprising and unexpected because it demonstrates variability in the internalization properties among antibodies to CTHRC1. Thus, in any of the foregoing embodiments, the CTHRC1 binding moiety can comprise an antibody portion that comprises HCDR1, HCDR2, and HCDR3 of AB 990 and/or LCDR1, LCDR2, and LCDR3 of AB990 (or, alternatively, if antibody internalization is acceptable any antibody from Tables 2 and 3). In any of the foregoing embodiments, the CTHRC1 binding moiety can comprise an antibody portion that comprises the VH and/or VL of any of AB990 (or, alternatively, if antibody internalization is acceptable any antibody from Tables 4 and 5).

[00159] Preferably, the CTHRC1 binding moiety of the invention is scFv, or scFab wherein the nucleic acid sequence of the scFv comprises the nucleic acid sequence(s) that encode for one or more light chain CDRs and one or more heavy chain CDRs disclosed herein for anti-CTHRC1 antibodies, and wherein the nucleic acid sequence of the scFab comprises the nucleic acid sequence(s) that encode for one or more light chain CDRs and one or more heavy chain CDRs disclosed herein for anti-CTHRC1 antibodies.

[00160] Preferably, the CTHRC1 binding moiety of the invention is an scFv, or scFab comprising an amino acid sequence selected from the group consisting of any one of SEQ ID Nos: 81-82.

[00161] Preferably, the CTHRC1 binding moiety of the invention is an scFv, or scFab comprising an amino acid sequence of SEQ ID No: 81.

[00162] Preferably, the CTHRC1 binding moiety of the invention is an scFv, or scFab comprising an amino acid sequence of SEQ ID No: 82.

[00163] Preferably, the CTHRC1 binding moiety of the invention is an scFv, or scFab comprising an amino acid sequence of SEQ ID No: 81, and of SEQ ID No: 82.

[00164] In a preferred embodiment, the CTHRC1 binding moiety comprises a HCDR1 of SEQ ID NO: 48, a HCDR2 of SEQ ID NO: 53, a HCDR 3 of SEQ ID NO: 58, a LCDR1 of SEQ ID NO: 63, a LCDR2 of SEQ ID NO: 68, and a LCDR3 of SEQ ID NO: 73.

[00165] In a preferred embodiment, the CTHRC1 binding moiety comprises a heavy chain variable domain comprising the sequence of SEQ ID NO: 81 and a light chain variable domain comprising the sequence of SEQ ID NO: 82.

[00166] In any of the foregoing embodiments, the anti-CTHRC1 binding moiety and/or anti-CTHRC1 T-cell engager can have a binding affinity for CTHRC1 of less than 10 nM, preferably less than 5 nM, more preferably less than 1 nM. The binding affinity of the anti-CTHRC1 T-cell engager can, for example, be determined by the Scatchard analysis described in Munson et al., Anal. Biochem. 107: 220 (1980).

[00167] In embodiments, where the CTHRC1 binding moiety is an antibody, it can be a chimeric, humanized or human antibody. In embodiments, where the CTHRC1 binding moiety is an antibody, it can be a fragment of a full-length antibody, single-chain antibody, a single

domain antibody (*e.g.* a heavy chain only antibody), a single-chain variable fragment (scFv), or any other type of “antibody” defined herein. Exemplary antibodies include monoclonal, chimeric, humanized, and human antibodies.

[00168] In embodiments, the CTHRC1 binding moiety can be a CTHRC1 binding moiety as described in any of WO 2010/047448, U.S. Patent Application Publication No. 2005/0147602, U.S. Patent Application Publication No. 2016/0000866, WO 2014/200134, U.S. Patent No. 9,050,296, U.S. Patent Application Publication No. 2013/0190357, U.S. Patent Application Publication No. 2018/0313846, U.S. Patent No. 9,718,878, U.S. Patent Application Publication No. 2022/0204599, WO 2021/063972, U.S. Patent Application Publication No. 2018/0221442, WO 2010/047448, CN110257389A, and JPWO2007123010, each of which is incorporated herein by reference in its entirety. Additional CTHRC1 binding moieties can include, by way of example but not limitation, 10G07 (Duarte et al., 2014 PLOS ONE, 9(6): e100449, incorporated herein by reference), 13D11, and 19C07, clone 13E09, anti-CTHRC1 antibody, H-213, incorporated herein by reference, anti-CTHRC1 antibody, T-19, which is incorporated herein by reference (Santa Cruz Biotechnology, Inc., Dallas, Tex.), anti-CTHRC1 antibodies: SAB1102667, HPA059806, SAB2107469, and SAB1402656, each of which is incorporated herein by reference (Sigma-Aldrich®, St. Louis, Mo.), and anti-CTHRC1 antibody PA5-38054, incorporated herein by reference (Thermo Scientific, Waltham, Mass.), Vli-55, an antibody or fragment thereof comprising HCDR1, HCDR2, and HCDR3 and LCDR1, LCDR2, and LCDR3 of SEQ ID Nos: 9-11 and 13-15, respectively of WO 2014/200134, and an antibody or fragment thereof comprising HCDR1, HCDR2, and HCDR2 and LCDR1, LCDR2, and LCDR3 of SEQ ID Nos: 1-3 or 11-13 (HCDRs) and 4-6 or 14-16 (LCDRs), respectively, and/or a heavy chain variable region of any of SEQ ID Nos: 7, 17, 21, or 25 and/or a light chain variable region of any of SEQ ID Nos: 9, 19, 23, or 27, including clones cCMAB45, cCMAB96, hCMAB45, and hCMAB96, 6C1 and 1D1 off U.S. Patent Application Publication No. 2022/0204599, each of which is incorporated herein by reference in its entirety.

[00169] In embodiments, the first domain comprises a means for binding to CTHRC1 according to any of the foregoing embodiments.

Monoclonal Antibodies

[00170] A monoclonal antibody (mAb) to an antigen of interest can be prepared by using any technique known in the art. These include, but are not limited to, the hybridoma technique originally described by Kohler and Milstein (1975, *Nature* 256, 495-497), the human B cell hybridoma technique (Kozbor et al., 1983, *Immunology Today* 4: 72), and the EBV-hybridoma technique (Cole et al., 1985, *Monoclonal Antibodies and Cancer Therapy*, Alan R. Liss, Inc., pp. 77-96). The Selected Lymphocyte Antibody Method (SLAM) (Babcook, J.S., et al., A novel strategy for generating monoclonal antibodies from single, isolated lymphocytes producing antibodies of defined specificities. *Proc Natl Acad Sci U S A*, 1996. 93 (15): p. 7843-8.) and (McLean G et al., 2005, *J Immunol.* 174(8): 4768-78. Such antibodies may be of any immunoglobulin class including IgG, IgM, IgE, IgA, and IgD and any subclass thereof. The hybridoma producing the mAbs of use in this invention may be cultivated in vitro or in vivo.

[00171] Monoclonal antibodies may be made using the hybridoma method first described by Kohler et al., *Nature*, 256: 495 (1975), or may be made by recombinant DNA methods (U.S. Pat. No. 4,816,567).

[00172] In the hybridoma method, a mouse or other appropriate host animal, such as a hamster, is immunized as described above to elicit lymphocytes that produce or are capable of producing antibodies that will specifically bind to the protein used for immunization. Alternatively, lymphocytes may be immunized in vitro. After immunization, lymphocytes are isolated and then fused with a myeloma cell line using a suitable fusing agent, such as polyethylene glycol, to form a hybridoma cell (Goding, *Monoclonal Antibodies: Principles and Practice*, pp. 59-103 (Academic Press, 1986)).

[00173] The hybridoma cells thus prepared are seeded and grown in a suitable culture medium which may contain one or more substances that inhibit the growth or survival of the unfused, parental myeloma cells (also referred to as fusion partner). For example, if the parental myeloma cells lack the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT or HPRT), the selective culture medium for the hybridomas typically will include hypoxanthine, aminopterin, and thymidine (HAT medium), which substances prevent the growth of HGPRT-deficient cells.

[00174] Preferred fusion partner myeloma cells are those that fuse efficiently, support stable high-level production of antibody by the selected antibody-producing cells, and are sensitive to a selective medium that selects against the unfused parental cells. Preferred myeloma cell lines

are murine myeloma lines, such as those derived from MOPC-21 and MPC-11 mouse tumors available from the Salk Institute Cell Distribution Center, San Diego, Calif. USA, and SP-2 and derivatives e.g., X63-Ag8-653 cells available from the American Type Culture Collection, Manassas, Va., USA. Human myeloma and mouse-human heteromyeloma cell lines also have been described for the production of human monoclonal antibodies (Kozbor, *J. Immunol.*, 133: 3001 (1984); and Brodeur et al., *Monoclonal Antibody Production Techniques and Applications*, pp. 51-63 (Marcel Dekker, Inc., New York, 1987)).

[00175] Culture medium in which hybridoma cells are growing is assayed for production of monoclonal antibodies directed against the antigen. Preferably, the binding specificity of monoclonal antibodies produced by hybridoma cells is determined by immunoprecipitation or by an in vitro binding assay, such as radioimmunoassay (RIA) or enzyme-linked immunosorbent assay (ELISA).

[00176] The binding affinity of the monoclonal antibody can, for example, be determined by the Scatchard analysis described in Munson et al., *Anal. Biochem.* 107: 220 (1980).

[00177] Once hybridoma cells that produce antibodies of the desired specificity, affinity, and/or activity are identified, the clones may be subcloned by limiting dilution procedures and grown by standard methods (Goding, *Monoclonal Antibodies: Principles and Practice*, pp. 59-103 (Academic Press, 1986)). Suitable culture media for this purpose include, for example, D-MEM or RPMI-1640 medium. In addition, the hybridoma cells may be grown in vivo as ascites tumors in an animal, e.g., by intraperitoneal injection of the cells into mice.

[00178] The monoclonal antibodies secreted by the subclones are suitably separated from the culture medium, ascites fluid, or serum by conventional antibody purification procedures such as, for example, affinity chromatography (e.g., using protein A or protein G-Sepharose) or ion-exchange chromatography, hydroxylapatite chromatography, gel electrophoresis, dialysis, etc.

[00179] DNA encoding the monoclonal antibodies is readily isolated and sequenced using conventional procedures (e.g., by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of murine antibodies). The hybridoma cells serve as a preferred source of such DNA. Once isolated, the DNA may be placed into expression vectors, which are then transfected into host cells such as *E. coli* cells, simian COS cells, Chinese Hamster Ovary (CHO) cells, or myeloma cells that do not otherwise produce

antibody protein, to obtain the synthesis of monoclonal antibodies in the recombinant host cells. Review articles on recombinant expression in bacteria of DNA encoding the antibody include Skerra et al., *Curr. Opinion in Immunol.* 5: 256-62 (1993) and Plückthun, *Immunol. Rev.* 130: 151-88 (1992).

[00180] In a further embodiment, monoclonal antibodies or antibody fragments can be isolated from antibody phage libraries generated using the techniques described in McCafferty et al., *Nature*, 348: 552-54 (1990). Clackson et al., *Nature*, 352: 624-28 (1991) and Marks et al., *J. Mol. Biol.*, 222: 581-97 (1991) describe the isolation of murine and human antibodies, respectively, using phage libraries. Subsequent publications describe the production of high affinity (nM range) human antibodies by chain shuffling (Marks et al., *Bio/Technology*, 10: 779-83 (1992)), as well as combinatorial infection and in vivo recombination as a strategy for constructing very large phage libraries (Waterhouse et al., *Nuc. Acids. Res.* 21: 2265-6 (1993)). Thus, these techniques are viable alternatives to traditional monoclonal antibody hybridoma techniques for isolation of monoclonal antibodies.

[00181] The DNA that encodes the antibody may be modified to produce chimeric or fusion antibody polypeptides, for example, by substituting human heavy chain and light chain constant domain (CH and CO sequences for the homologous murine sequences (U.S. Pat. No. 4,816,567; and Morrison, et al., *Proc. Natl. Acad. Sci. USA*, 81: 6851 (1984)), or by fusing the immunoglobulin coding sequence with all or part of the coding sequence for a non-immunoglobulin polypeptide (heterologous polypeptide). The non-immunoglobulin polypeptide sequences can substitute for the constant domains of an antibody, or they are substituted for the variable domains of one antigen-combining site of an antibody to create a chimeric bivalent antibody comprising one antigen-combining site having specificity for an antigen and another antigen-combining site having specificity for a different antigen.

Chimeric, Humanized and Human Antibodies

[00182] In some embodiments, the CTHRC1 binding moiety is a chimeric antibody. In some embodiments, the T cell engager moiety, e.g. anti-CD3 antibody or fragment thereof, is a chimeric antibody. Certain chimeric antibodies are described, e.g., in U.S. Pat. No. 4,816,567; and Morrison et al., *Proc. Natl. Acad. Sci. USA*, 81: 6851-5 (1984)). In one example, a chimeric antibody comprises a non-human variable region (e.g., a variable region derived from a mouse,

rat, hamster, rabbit, or non-human primate, such as a monkey) and a human constant region. In a further example, a chimeric antibody is a “class switched” antibody in which the class or subclass has been changed from that of the parent antibody. Chimeric antibodies include antigen-binding fragments thereof.

[00183] In some embodiments, a chimeric antibody is a humanized antibody. Typically, a non-human antibody is humanized to reduce immunogenicity to humans, while retaining the specificity and affinity of the parental non-human antibody. Generally, a humanized antibody comprises one or more variable domains in which HVRs, e.g., CDRs, (or portions thereof) are derived from a non-human antibody, and FRs (or portions thereof) are derived from human antibody sequences. A humanized antibody optionally will also comprise at least a portion of a human constant region. In some embodiments, some FR residues in a humanized antibody are substituted with corresponding residues from a non-human antibody (e.g., the antibody from which the CDR residues are derived), e.g., to restore or improve antibody specificity or affinity.

[00184] The anti-CTHRC1 antibodies and T cell engager moieties of the invention may comprise humanized antibodies or human antibodies. Humanized forms of non-human (e.g., murine or rabbit) antibodies are chimeric immunoglobulins, immunoglobulin chains or fragments thereof (such as Fv, Fab, Fab', F(ab')₂ or other antigen-binding subsequences of antibodies) which contain minimal sequence derived from non-human immunoglobulin. Humanized antibodies include human immunoglobulins (recipient antibody) in which residues from a complementary determining region (CDR) of the recipient are replaced by residues from a CDR of a non-human species (donor antibody) such as mouse, rat or rabbit having the desired specificity, affinity and capacity. In some instances, Fv framework residues of the human immunoglobulin are replaced by corresponding non-human residues. Humanized antibodies may also comprise residues which are found neither in the recipient antibody nor in the imported CDR or framework sequences. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the CDR regions correspond to those of a non-human immunoglobulin and all or substantially all of the FR regions are those of a human immunoglobulin consensus sequence. The humanized antibody optimally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin (Jones et al., *Nature*, 321: 522-5 (1986);

Riechmann et al., *Nature*, 332: 323-9 (1988); and Presta, *Curr. Op. Struct. Biol.*, 2: 593-6 (1992)).

[00185] A humanized antibody as a CTHRC1 binding moiety and/or T cell engager moiety of the invention may comprise one or more human and/or human consensus non-hypervariable region (e.g., framework) sequences in its heavy and/or light chain variable domain. In some embodiments, one or more additional modifications are present within the human and/or human consensus non-hypervariable region sequences. In one embodiment, the heavy chain variable domain of an antibody of the invention comprises a human consensus framework sequence, which in one embodiment is the subgroup III consensus framework sequence. In one embodiment, an antibody of the invention comprises a variant subgroup III consensus framework sequence modified at at least one amino acid position.

[00186] As is known in the art, and as described in greater detail herein, the amino acid position/boundary delineating a hypervariable region of an antibody can vary, depending on the context and the various definitions known in the art (as described below). Some positions within a variable domain may be viewed as hybrid hypervariable positions in that these positions can be deemed to be within a hypervariable region under one set of criteria while being deemed to be outside a hypervariable region under a different set of criteria. One or more of these positions can also be found in extended hypervariable regions (as further defined below). The invention provides antibodies comprising modifications in these hybrid hypervariable positions. In one embodiment, these hypervariable positions include one or more positions 26-30, 33-35B, 47-49, 57-65, 93, 94 and 101-102 in a heavy chain variable domain. In one embodiment, these hybrid hypervariable positions include one or more of positions 24-29, 35-36, 46-49, 56 and 97 in a light chain variable domain. In one embodiment, an antibody of the invention comprises a human variant human subgroup consensus framework sequence modified at one or more hybrid hypervariable positions.

[00187] A CTHRC1 binding moiety of the invention can comprise any suitable human or human consensus light chain framework sequences, provided the antibody exhibits the desired biological characteristics (e.g., a desired binding affinity). In one embodiment, an antibody of the invention comprises at least a portion (or all) of the framework sequence of human κ light

chain. In one embodiment, a CTHRC1 binding moiety of the invention comprises at least a portion (or all) of human κ subgroup I framework consensus sequence.

[00188] Methods for humanizing non-human antibodies are well known in the art. Generally, a humanized antibody has one or more amino acid residues introduced into it from a source which is non-human. These non-human amino acid residues are often referred to as “import” residues, which are typically taken from an “import” variable domain. Humanization can be essentially performed following the method of Winter and co-workers (Jones et al., *Nature*, 321:522-525 (1986); Riechmann et al., *Nature*, 332:323-327 (1988); Verhoeven et al., *Science*, 239:1534-1536 (1988)), by substituting rodent CDRs or CDR sequences for the corresponding sequences of a human antibody. Accordingly, such “humanized” antibodies are chimeric antibodies (U.S. Pat. No. 4,816,567), wherein substantially less than an intact human variable domain has been substituted by the corresponding sequence from a non-human species. In practice, humanized antibodies are typically human antibodies in which some CDR residues and possibly some FR residues are substituted by residues from analogous sites in rodent antibodies.

[00189] The choice of human variable domains, both light and heavy, to be used in making the humanized antibodies is very important to reduce antigenicity and HAMA response (human anti-mouse antibody) when the antibody is intended for human therapeutic use. Reduction or elimination of a HAMA response is a significant aspect of clinical development of suitable therapeutic agents (see, e.g., Khazzaeli et al., *J. Natl. Cancer Inst.* (1988), 80:937; Jaffers et al., *Transplantation* (1986), 41:572; Shawler et al., *J. Immunol.* (1985), 135:1530; Sears et al., *J. Biol. Response Mod.* (1984), 3:138; Miller et al., *Blood* (1983), 62:988; Hakimi et al., *J. Immunol.* (1991), 147:1352; Reichmann et al., *Nature* (1988), 332:323; Junghans et al., *Cancer Res.* (1990), 50: 1495). As described herein, the invention provides antibodies that are humanized such that HAMA response is reduced or eliminated. Variants of these antibodies can further be obtained using routine methods known in the art, some of which are further described below. According to the so-called “best-fit” method, the sequence of the variable domain of a rodent antibody is screened against the entire library of known human variable domain sequences. The human V domain sequence which is closest to that of the rodent is identified and the human framework region (FR) within it accepted for the humanized antibody (Sims et al., *J. Immunol.* 151: 2296 (1993); Chothia et al., *J. Mol. Biol.*, 196: 901 (1987)). Another method uses

a particular framework region derived from the consensus sequence of all human antibodies of a particular subgroup of light or heavy chains. The same framework may be used for several different humanized antibodies (Carter et al., *Proc. Natl. Acad. Sci. USA*, 89: 4285 (1992); Presta et al., *J. Immunol.* 151: 2623 (1993)).

[00190] For example, an amino acid sequence from an antibody as described herein can serve as a starting (parent) sequence for diversification of the framework and/or hypervariable sequence(s). A selected framework sequence to which a starting hypervariable sequence is linked is referred to herein as an acceptor human framework. While the acceptor human frameworks may be from, or derived from, a human immunoglobulin (the VL and/or VH regions thereof), preferably the acceptor human frameworks are from, or derived from, a human consensus framework sequence as such frameworks that have been demonstrated to have minimal, or no, immunogenicity in human patients.

[00191] Where the acceptor is derived from a human immunoglobulin, one may optionally select a human framework sequence that is selected based on its homology to the donor framework sequence by aligning the donor framework sequence with various human framework sequences in a collection of human framework sequences, and select the most homologous framework sequence as the acceptor.

[00192] In one embodiment, human consensus frameworks herein are from, or derived from, VH subgroup III and/or VL kappa subgroup I consensus framework sequences.

[00193] While the acceptor may be identical in sequence to the human framework sequence selected, whether that be from a human immunoglobulin or a human consensus framework, the present invention contemplates that the acceptor sequence may comprise pre-existing amino acid substitutions relative to the human immunoglobulin sequence or human consensus framework sequence. These pre-existing substitutions are preferably minimal; usually four, three, two or one amino acid differences only relative to the human immunoglobulin sequence or consensus framework sequence.

[00194] Hypervariable region residues of the non-human antibody are incorporated into the VL and/or VH acceptor human frameworks. For example, one may incorporate residues corresponding to the Kabat CDR residues, the Chothia hypervariable loop residues, the Abm residues, and/or contact residues. Optionally, the extended hypervariable region residues as

follows are incorporated: 24-34 (L1), 50-56 (L2) and 89-97 (L3), 26-35B (H1), 50-65, 47-65 or 49-65 (H2) and 93-102, 94-102, or 95-102 (H3).

[00195] While “incorporation” of hypervariable region residues is discussed herein, it will be appreciated that this can be achieved in various ways, for example, nucleic acid encoding the desired amino acid sequence can be generated by mutating nucleic acid encoding the mouse variable domain sequence so that the framework residues thereof are changed to acceptor human framework residues, or by mutating nucleic acid encoding the human variable domain sequence so that the hypervariable domain residues are changed to non-human residues, or by synthesizing nucleic acid encoding the desired sequence, etc.

[00196] As described herein, hypervariable region-grafted variants may be generated by Kunkel mutagenesis of nucleic acid encoding the human acceptor sequences, using a separate oligonucleotide for each hypervariable region. Kunkel et al., *Methods Enzymol.* 154:367-382 (1987). Appropriate changes can be introduced within the framework and/or hypervariable region, using routine techniques, to correct and re-establish proper hypervariable region-antigen interactions.

[00197] Phage(mid) display (also referred to herein as phage display in some contexts) can be used as a convenient and fast method for generating and screening many different potential variant antibodies in a library generated by sequence randomization. However, other methods for making and screening altered antibodies are available to the skilled person.

[00198] Phage(mid) display technology has provided a powerful tool for generating and selecting novel proteins which bind to a ligand, such as an antigen. Using the techniques of phage(mid) display allows the generation of large libraries of protein variants which can be rapidly sorted for those sequences that bind to a target molecule with high affinity. Nucleic acids encoding variant polypeptides are generally fused to a nucleic acid sequence encoding a viral coat protein, such as the gene III protein or the gene VIII protein. Monovalent phagemid display systems where the nucleic acid sequence encoding the protein or polypeptide is fused to a nucleic acid sequence encoding a portion of the gene III protein have been developed. (Bass, S., *Proteins*, 8:309 (1990); Lowman and Wells, *Methods: A Companion to Methods in Enzymology*, 3:205 (1991)). In a monovalent phagemid display system, the gene fusion is expressed at low levels and wild type gene III proteins are also expressed so that infectivity of the particles is retained.

Methods of generating peptide libraries and screening those libraries have been disclosed in many patents (e.g., U.S. Pat. No. 5,723,286, U.S. Pat. No. 5,432,018, U.S. Pat. No. 5,580,717, U.S. Pat. No. 5,427,908 and U.S. Pat. No. 5,498,530).

[00199] Libraries of antibodies or antigen binding polypeptides have been prepared in a number of ways including by altering a single gene by inserting random DNA sequences or by cloning a family of related genes. Methods for displaying antibodies or antigen binding fragments using phage(mid) display have been described in U.S. Pat. Nos. 5,750,373, 5,733,743, 5,837,242, 5,969,108, 6,172,197, 5,580,717, and 5,658,727. The library is then screened for expression of antibodies or antigen binding proteins with the desired characteristics.

[00200] Methods of substituting an amino acid of choice into a template nucleic acid are well established in the art, some of which are described herein. For example, hypervariable region residues can be substituted using the Kunkel method (e.g., Kunkel et al., Methods Enzymol. 154:367-382 (1987)).

[00201] The sequence of oligonucleotides includes one or more of the designed codon sets for the hypervariable region residues to be altered. A codon set is a set of different nucleotide triplet sequences used to encode desired variant amino acids. Codon sets can be represented using symbols to designate particular nucleotides or equimolar mixtures of nucleotides as shown in below according to the IUB code.

IUB Codes

G Guanine

A Adenine

T Thymine

C Cytosine

R (A or G)

Y (C or T)

M (A or C)

K (G or T)

S (C or G)

W (A or T)

H (A or C or T)

B (C or G or T)

V (A or C or G)

D (A or G or T) H

N (A or C or G or T)

[00202] For example, in the codon set DVK, D can be nucleotides A or G or T; V can be A or G or C; and K can be G or T. This codon set can present 18 different codons and can encode amino acids Ala, Trp, Tyr, Lys, Thr, Asn, Lys, Ser, Arg, Asp, Glu, Gly, and Cys.

[00203] Oligonucleotide or primer sets can be synthesized using standard methods. A set of oligonucleotides can be synthesized, for example, by solid phase synthesis, containing sequences that represent all possible combinations of nucleotide triplets provided by the codon set and that will encode the desired group of amino acids. Synthesis of oligonucleotides with selected nucleotide “degeneracy” at certain positions is well known in that art. Such sets of nucleotides having certain codon sets can be synthesized using commercial nucleic acid synthesizers (available from, for example, Applied Biosystems, Foster City, Calif.), or can be obtained commercially (for example, from Life Technologies, Rockville, Md.). Therefore, a set of oligonucleotides synthesized having a particular codon set will typically include a plurality of oligonucleotides with different sequences, the differences established by the codon set within the overall sequence. Oligonucleotides, as used according to the invention, have sequences that allow for hybridization to a variable domain nucleic acid template and also can include restriction enzyme sites for cloning purposes.

[00204] In one method, nucleic acid sequences encoding variant amino acids can be created by oligonucleotide-mediated mutagenesis. This technique is well known in the art as described by Zoller et al. *Nucleic Acids Res.* 10:6487-6504 (1987). Briefly, nucleic acid sequences encoding variant amino acids are created by hybridizing an oligonucleotide set encoding the desired codon sets to a DNA template, where the template is the single-stranded form of the plasmid containing a variable region nucleic acid template sequence. After hybridization, DNA polymerase is used to synthesize an entire second complementary strand of the template that will thus incorporate the oligonucleotide primer and will contain the codon sets as provided by the oligonucleotide set.

[00205] Generally, oligonucleotides of at least 25 nucleotides in length are used. An optimal oligonucleotide will have 12 to 15 nucleotides that are completely complementary to the template on either side of the nucleotide(s) coding for the mutation(s). This ensures that the oligonucleotide will hybridize properly to the single-stranded DNA template molecule. The

oligonucleotides are readily synthesized using techniques known in the art such as that described by Crea et al., Proc. Nat'l. Acad. Sci. USA, 75:5765 (1978).

[00206] The DNA template is generated by those vectors that are either derived from bacteriophage M13 vectors (the commercially available M13 mp 18 and M13 mp 19 vectors are suitable), or those vectors that contain a single-stranded phage origin of replication as described by Viera et al., Meth. Enzymol., 153:3 (1987). Thus, the DNA that is to be mutated can be inserted into one of these vectors in order to generate single-stranded template. Production of the single-stranded template is described in sections 4.21-4.41 of Sambrook et al., above.

[00207] To alter the native DNA sequence, the oligonucleotide is hybridized to the single stranded template under suitable hybridization conditions. A DNA polymerizing enzyme, usually T7 DNA polymerase or the Klenow fragment of DNA polymerase I, is then added to synthesize the complementary strand of the template using the oligonucleotide as a primer for synthesis. A heteroduplex molecule is thus formed such that one strand of DNA encodes the mutated form of gene 1, and the other strand (the original template) encodes the native, unaltered sequence of gene 1. This heteroduplex molecule is then transformed into a suitable host cell, usually a prokaryote such as *E. coli* JM101. After growing the cells, they are plated onto agarose plates and screened using the oligonucleotide primer radiolabelled with a ³²-Phosphate to identify the bacterial colonies that contain the mutated DNA.

[00208] The method described immediately above may be modified such that a homoduplex molecule is created wherein both strands of the plasmid contain the mutation(s). The modifications are as follows: The single stranded oligonucleotide is annealed to the single-stranded template as described above. A mixture of three deoxyribonucleotides, deoxyriboadenosine (dATP), deoxyriboguanosine (dGTP), and deoxyribothymidine (dTTP), is combined with a modified thiodeoxyribocytosine called dCTP-(aS) (which can be obtained from Amersham). This mixture is added to the template-oligonucleotide complex. Upon addition of DNA polymerase to this mixture, a strand of DNA identical to the template except for the mutated bases is generated. In addition, this new strand of DNA will contain dCTP-(aS) instead of dCTP, which serves to protect it from restriction endonuclease digestion. After the template strand of the double-stranded heteroduplex is nicked with an appropriate restriction enzyme, the template strand can be digested with ExoIII nuclease or another appropriate nuclease past the

region that contains the site(s) to be mutagenized. The reaction is then stopped to leave a molecule that is only partially single-stranded. A complete double-stranded DNA homoduplex is then formed using DNA polymerase in the presence of all four deoxyribonucleotide triphosphates, ATP, and DNA ligase. This homoduplex molecule can then be transformed into a suitable host cell.

[00209] As indicated previously the sequence of the oligonucleotide set is of sufficient length to hybridize to the template nucleic acid and may also, but does not necessarily, contain restriction sites. The DNA template can be generated by those vectors that are either derived from bacteriophage M13 vectors or vectors that contain a single-stranded phage origin of replication as described by Viera et al. *Meth. Enzymol.*, 153:3 (1987). Thus, the DNA that is to be mutated must be inserted into one of these vectors in order to generate single-stranded template. Production of the single-stranded template is described in sections 4.21-4.41 of Sambrook et al., *supra*.

[00210] According to another method, antigen binding may be restored during humanization of antibodies through the selection of repaired hypervariable regions (see, e.g., US application Ser. No. 11/061,841, filed Feb. 18, 2005). The method includes incorporating non-human hypervariable regions onto an acceptor framework and further introducing one or more amino acid substitutions in one or more hypervariable regions without modifying the acceptor framework sequence. Alternatively, the introduction of one or more amino acid substitutions may be accompanied by modifications in the acceptor framework sequence.

[00211] According to another method, a library can be generated by providing upstream and downstream oligonucleotide sets, each set having a plurality of oligonucleotides with different sequences, the different sequences established by the codon sets provided within the sequence of the oligonucleotides. The upstream and downstream oligonucleotide sets, along with a variable domain template nucleic acid sequence, can be used in a polymerase chain reaction to generate a “library” of PCR products. The PCR products can be referred to as “nucleic acid cassettes”, as they can be fused with other related or unrelated nucleic acid sequences, for example, viral coat proteins and dimerization domains, using established molecular biology techniques.

[00212] The sequence of the PCR primers includes one or more of the designed codon sets for the solvent accessible and highly diverse positions in a hypervariable region. As described above, a codon set is a set of different nucleotide triplet sequences used to encode desired variant amino acids.

[00213] Antibody selectants that meet the desired criteria, as selected through appropriate screening/selection steps can be isolated and cloned using standard recombinant techniques.

[00214] It is further important that antibodies be humanized with retention of high binding affinity for the antigen and other favorable biological properties. To achieve this goal, according to a preferred method, humanized antibodies are prepared by a process of analysis of the parental sequences and various conceptual humanized products using three-dimensional models of the parental and humanized sequences. Three-dimensional immunoglobulin models are commonly available and are familiar to those skilled in the art. Computer programs are available which illustrate and display probable three-dimensional conformational structures of selected candidate immunoglobulin sequences. Inspection of these displays permits analysis of the likely role of the residues in the functioning of the candidate immunoglobulin sequence, i.e., the analysis of residues that influence the ability of the candidate immunoglobulin to bind its antigen. In this way, FR residues can be selected and combined from the recipient and import sequences so that the desired antibody characteristic, such as increased affinity for the target antigen(s), is achieved. In general, the hypervariable region residues are directly and most substantially involved in influencing antigen binding.

[00215] Various forms of a humanized anti-CTHRC1 antibody are contemplated. For example, the humanized antibody may be an antibody fragment, such as a Fab. Alternatively, the humanized antibody may be an intact antibody, such as an intact IgG1 antibody.

[00216] As an alternative to humanization, human antibodies can be generated. For example, it is now possible to produce transgenic animals (e.g., mice) that are capable, upon immunization, of producing a full repertoire of human antibodies in the absence of endogenous immunoglobulin production. For example, it has been described that the homozygous deletion of the antibody heavy-chain joining region (JH) gene in chimeric and germ-line mutant mice results in complete inhibition of endogenous antibody production. Transfer of the human germ-line immunoglobulin gene array into such germ-line mutant mice will result in the production of human antibodies

upon antigen challenge (see, e.g., Jakobovits et al., *Proc. Natl. Acad. Sci. USA*, 90: 2551 (1993); Jakobovits et al., *Nature*, 362: 255-8 (1993); Bruggemann et al., *Year in Immuno.* 7: 33 (1993); U.S. Pat. Nos. 5,545,806, 5,569,825, 5,591,669; 5,545,807; and WO 97/17852).

[00217] Alternatively, phage display technology (McCafferty et al., *Nature* 348: 552-53 (1990)) can be used to produce human antibodies and antibody fragments in vitro, from immunoglobulin variable (V) domain gene repertoires from unimmunized donors. According to this technique, antibody V domain genes are cloned in-frame into either a major or minor coat protein gene of a filamentous bacteriophage, such as M13 or fd, and displayed as functional antibody fragments on the surface of the phage particle. Because the filamentous particle contains a single-stranded DNA copy of the phage genome, selections based on the functional properties of the antibody also result in selection of the gene encoding the antibody exhibiting those properties. Thus, the phage mimics some of the properties of the B-cell. Phage display can be performed in a variety of formats, reviewed in, e.g., Johnson, Kevin S, and Chiswell, David J., *Current Opinion in Structural Biology* 3:564-571 (1993). Several sources of V-gene segments can be used for phage display. Clackson et al., *Nature*, 352:624-628 (1991) isolated a diverse array of anti-oxazolone antibodies from a small random combinatorial library of V genes derived from the spleens of immunized mice. A repertoire of V genes from unimmunized human donors can be constructed and antibodies to a diverse array of antigens (including self-antigens) can be isolated essentially following the techniques described by Marks et al., *J. Mol. Biol.* 222:581-97 (1991), or Griffith et al., *EMBO J.* 12: 725-34 (1993) (see also, U.S. Pat. Nos. 5,565,332 and 5,573,905).

[00218] As discussed above, human antibodies may also be generated by in vitro activated B cells (see, e.g., U.S. Pat. Nos. 5,567,610 and 5,229,275).

[00219] In another embodiment, the anti-CTHRC1 T-cell engagers of this disclosure can include human monoclonal antibodies as the CTHRC1 binding moiety. Such human monoclonal antibodies directed against CTHRC1 can be generated using transgenic or transchromosomal mice carrying parts of the human immune system rather than the mouse system. These transgenic and transchromosomal mice include mice referred to herein as the HuMAb Mouse™ and KM Mouse™, respectively, and are collectively referred to herein as “human Ig mice.”

[00220] The HuMAb Mouse™ (Medarex, Inc.) contains human immunoglobulin gene miniloci that encode unrearranged human heavy (μ and γ) and κ light chain immunoglobulin sequences,

together with targeted mutations that inactivate the endogenous μ and κ chain loci (see e.g., Lonberg, et al. (1994) *Nature* 368(6474): 856-9). Accordingly, the mice exhibit reduced expression of mouse IgM or κ , and in response to immunization, the introduced human heavy and light chain transgenes undergo class switching and somatic mutation to generate high affinity human IgG κ monoclonal antibodies (Lonberg, N. et al. (1994), *supra*; reviewed in Lonberg, N. (1994) *Handbook of Experimental Pharmacology* 113: 49-101; Lonberg, N. and Huszar, D. (1995) *Intern. Rev. Immunol.* 13: 65-93, and Harding, F. and Lonberg, N. (1995) *Ann. N.Y. Acad. Sci.* 764: 536-46). Preparation and use of the HuMAb Mouse™, and the genomic modifications carried by such mice, is further described in Taylor, L. et al. (1992) *Nucleic Acids Research* 20:6287-6295; Chen, J. et al. (1993) *International Immunology* 5: 647-656; Tuaillon et al. (1993) *Proc. Natl. Acad. Sci. USA* 90: 3720-4; Choi et al. (1993) *Nature Genetics* 4:117-23; Chen, J. et al. (1993) *EMBO J.* 12: 21-830; Tuaillon et al., (1994) *J. Immunol.* 152: 2912-20; Taylor, L. et al. (1994) *International Immunology* 6: 579-91; and Fishwild, D. et al. (1996) *Nature Biotechnology* 14: 845-51, the contents of all of which are hereby specifically incorporated by reference in their entirety. See further, U.S. Pat. Nos. 5,545,806; 5,569,825; 5,625,126; 5,633,425; 5,789,650; 5,877,397; 5,661,016; 5,814,318; 5,874,299; and 5,770,429; U.S. Pat. No. 5,545,807; PCT Publication Nos. WO 92/03918, WO 93/12227, WO 94/25585, WO 97/13852, WO 98/24884 and WO 99/45962; and PCT Publication No. WO 01/14424.

[00221] In another embodiment, human antibodies of this disclosure can be raised using a mouse that carries human immunoglobulin sequences on transgenes and transchromosomes, such as a mouse that carries a human heavy chain transgene and a human light chain transchromosome. This mouse is referred to herein as a “KM Mouse™” and is described in detail in PCT Publication WO 02/43478.

[00222] Still further, alternative transgenic animal systems expressing human immunoglobulin genes are available in the art and can be used to raise anti-CTHRC1 antibodies of this disclosure. For example, an alternative transgenic system referred to as the Xenomouse (Abgenix, Inc.) can be used; such mice are described in, for example, U.S. Pat. Nos. 5,939,598; 6,075,181; 6,114,598; 6,150,584 and 6,162,963.

[00223] Moreover, alternative transchromosomal animal systems expressing human immunoglobulin genes are available in the art and can be used to raise anti-CTHRC1 antibodies

of this disclosure. For example, mice carrying both a human heavy chain transchromosome and a human light chain transchromosome, referred to as “TC mice” can be used; such mice are described in Tomizuka et al. (2000) *Proc. Natl. Acad. Sci. USA* 97: 722-7. As another example, cows carrying human heavy and light chain transchromosomes have been described in the art (e.g., Kuroiwa et al. (2002) *Nature Biotechnology* 20: 889-94 and PCT application No. WO 2002/092812) and can be used to raise anti-CTHRC1 antibodies of this disclosure. Additional examples of transgenic animals that can be used to produce anti-CTHCR1 antibodies include OmniRat™ and OmniMouse™ (see e.g., Osborn M., et al. (2013) *Journal of Immunology* 190: 1481-90; Ma B., et al. (2013) *Journal of Immunological Methods* 400-401: 78-86; Geurts A., et al. (2009) *Science* 325: 433, U.S. Pat. No. 8,907,157; European Pat. No. 2152880B1; European Pat. No. 2336329B1). Yet another example includes the use of VELOCIMMUNE® Technology (see, for example, U.S. Pat. No. 6,596,541, Regeneron Pharmaceuticals, VELOCIMMUNE®. Briefly, the VELOCIMMUNE® technology involves generation of a transgenic mouse having a genome comprising human heavy and light chain variable regions operably linked to endogenous mouse constant region loci such that the mouse produces an antigen-binding protein, e.g., antibody, comprising a human variable region and a mouse constant region in response to antigenic stimulation. The DNA encoding the variable regions of the heavy and light chains of the antibody are isolated and operably linked to DNA encoding the human heavy and light chain constant regions. The DNA is then expressed in a cell capable of expressing the fully human antibody.

Antibody Fragments

[00224] In certain circumstances there are advantages of using antibody fragments, rather than whole antibodies. The smaller size of the fragments allows for rapid clearance, and may lead to improved access to solid tumors.

[00225] Various techniques have been developed for the production of antibody fragments. Traditionally, these fragments were derived via proteolytic digestion of intact antibodies (see, e.g., Morimoto et al., *Journal of Biochemical and Biophysical Methods* 24:107-7 (1992); and Brennan et al., *Science*, 229: 81 (1985)). However, these fragments can now be produced directly by recombinant host cells. Fab, Fv and scFv antibody fragments can all be expressed in and secreted from *E. coli*, thus allowing the facile production of large amounts of these fragments.

Antibody fragments can be isolated from the antibody phage libraries discussed above. Alternatively, Fab'-SH fragments can be directly recovered from *E. coli* and chemically coupled to form F(ab')₂ fragments (Carter et al., *Bio/Technology* 10:163-7 (1992)). According to another approach, F(ab')₂ fragments can be isolated directly from recombinant host cell culture. Fab and F(ab')₂ fragment with increased in vivo half-life comprising a salvage receptor binding epitope residues are described in U.S. Pat. No. 5,869,046. Other techniques for the production of antibody fragments will be apparent to the skilled practitioner. In other embodiments, the antibody of choice is a single chain Fv fragment (scFv) (see WO 93/16185; U.S. Pat. No. 5,571,894; and U.S. Pat. No. 5,587,458). Fv and sFv are the only species with intact combining sites that are devoid of constant regions; thus, they are suitable for reduced nonspecific binding during in vivo use. sFv T-cell engager may be constructed to yield fusion of an effector protein at either the amino or the carboxy terminus of an sFv (see *Antibody Engineering*, ed. Borrebaeck, *supra*. The antibody fragment may also be a "linear antibody", e.g., as described in U.S. Pat. No. 5,641,870 for example.

Antibody Variants and Modifications

Substitution, Insertion and Deletion Variants

[00226] In addition to the anti-CTHRC1 antibodies and anti-CD3 antibodies described herein, it is contemplated that anti-CTHRC1 antibody and/or anti-CD3 antibody variants can be prepared. Anti-CTHRC1 antibody and/or anti-CD3 antibody variants can be prepared by introducing appropriate nucleotide changes into the encoding DNA, and/or by synthesis of the desired antibody or polypeptide. Those skilled in the art will appreciate that amino acid changes may alter post-translational processes of the anti-CTHRC1 antibody and/or anti-CD3 antibody, such as changing the number or position of glycosylation sites or altering the membrane anchoring characteristics.

[00227] Variations in the anti-CTHRC1 antibodies and/or anti-CD3 antibodies described herein, can be made, for example, using any of the techniques and guidelines for conservative and non-conservative mutations set forth, for instance, in U.S. Patent No. 5,364,934. Variations may be a substitution, deletion or insertion of one or more codons encoding the antibody or polypeptide that results in a change in the amino acid sequence as compared with the native sequence antibody or polypeptide. Optionally the variation is by substitution of at least one

amino acid with any other amino acid in one or more of the domains of the anti-CTHRC1 antibody and/or anti-CD3 antibody. Guidance in determining which amino acid residue may be inserted, substituted or deleted without adversely affecting the desired activity may be found by comparing the sequence of the anti-CTHRC1 antibody and/or anti-CD3 antibody with that of homologous known protein molecules and minimizing the number of amino acid sequence changes made in regions of high homology. Amino acid substitutions can be the result of replacing one amino acid with another amino acid having similar structural and/or chemical properties, such as the replacement of a leucine with a serine, i.e., conservative amino acid replacements. Insertions or deletions may optionally be in the range of about 1 to 5 amino acids. The variation allowed may be determined by systematically making insertions, deletions or substitutions of amino acids in the sequence and testing the resulting variants for activity exhibited by the full-length or mature native sequence.

[00228] Anti-CTHRC1 antibody fragments and anti-CD3 antibody fragments are provided herein. Such fragments may be truncated at the N-terminus or C-terminus, or may lack internal residues, for example, when compared with a full-length native antibody or protein. Certain fragments lack amino acid residues that are not essential for a desired biological activity of the anti-CTHRC1 antibody and/or anti-CD3 antibody.

[00229] Anti-CTHRC1 antibody fragments and anti-CD3 antibody fragments may be prepared by any of a number of conventional techniques. Desired peptide fragments may be chemically synthesized. An alternative approach involves generating antibody or polypeptide fragments by enzymatic digestion, e.g., by treating the protein with an enzyme known to cleave proteins at sites defined by particular amino acid residues, or by digesting the DNA with suitable restriction enzymes and isolating the desired fragment. Yet another suitable technique involves isolating and amplifying a DNA fragment encoding a desired antibody or polypeptide fragment, by polymerase chain reaction (PCR). Oligonucleotides that define the desired termini of the DNA fragment are employed at the 5' and 3' primers in the PCR. Preferably, anti-CTHRC1 antibody fragments share at least one biological and/or immunological activity with the native anti-CTHRC1 antibody disclosed herein, the same applying to the anti-CD3 antibody fragments and the at least one biological and/or immunological activity of the native anti-CD3 antibody.

[00230] In particular embodiments, conservative substitutions of interest are shown in Table 6 under the heading of preferred substitutions. If such substitutions result in a change in biological activity, then more substantial changes, denominated exemplary substitutions in Table 6, or as further described below in reference to amino acid classes, are introduced and the products screened.

Table 6

Original Residue	Exemplary Substitutions	Preferred Substitutions
Ala (A)	val; leu; ile	val
Arg (R)	lys; gln; asn	lys
Asn (N)	gln; his; lys; arg	gln
Asp (D)	glu	glu
Cys (C)	ser	ser
Gln (Q)	asn	asn
Glu (E)	asp	asp
Gly (G)	pro; ala	ala
His (H)	asn; gln; lys; arg	arg
Ile (I)	leu; val; met; ala; phe; norleucine	leu
Leu (L)	norleucine; ile; val; met; ala; phe	ile
Lys (K)	arg; gln; asn	arg
Met (M)	leu; phe; ile	leu
Phe (F)	leu; val; ile; ala; tyr	leu
Pro (P)	ala	ala
Ser (S)	thr	thr
Thr (T)	ser	ser
Trp (W)	tyr; phe	tyr
Tyr (Y)	trp; phe; thr; ser	phe
Val (V)	ile; leu; met; phe; ala; norleucine	leu

[00231] Substantial modifications in function or immunological identity of the anti-CTHRC1 antibody and anti-CD3 antibody are accomplished by selecting substitutions that differ significantly in their effect on maintaining (a) the structure of the polypeptide backbone in the area of the substitution, for example, as a sheet or helical conformation, (b) the charge or hydrophobicity of the molecule at the target site, or (c) the bulk of the side chain. Naturally occurring residues are divided into groups based on common side-chain properties:

- (1) hydrophobic: norleucine, met, ala, val, leu, ile;
- (2) neutral hydrophilic: cys, ser, thr;
- (3) acidic: asp, glu;
- (4) basic: asn, gln, his, lys, arg;
- (5) residues that influence chain orientation: gly, pro; and
- (6) aromatic: trp, tyr, phe.

[00232] Non-conservative substitutions will entail exchanging a member of one of these classes for another class. Such substituted residues also may be introduced into the conservative substitution sites or, more preferably, into the remaining (non-conserved) sites.

[00233] It should be understood that conservatively substituted versions of the anti-CTHRC1 T-cell engagers or the first domain or second domain of the anti-CTHRC1 T-cell engagers of the present disclosure are included within the scope of this disclosure.

[00234] The variations can be made using methods known in the art such as oligonucleotide-mediated (site-directed) mutagenesis, alanine scanning, and PCR mutagenesis. Site-directed mutagenesis (Carter et al., *Nucl. Acids Res.*, 13: 4331 (1986); Zoller et al., *Nucl. Acids Res.*, 10: 6487 (1987)), cassette mutagenesis (Wells et al., *Gene*, 34: 315 (1985)), restriction selection mutagenesis (Wells et al., *Philos. Trans. R. Soc. London SerA*, 317: 415 (1986)) or other known techniques can be performed on the cloned DNA to produce the anti-CTHRC1 antibody variant DNA.

[00235] Scanning amino acid analysis can also be employed to identify one or more amino acids along a contiguous sequence. Among the preferred scanning amino acids are relatively small, neutral amino acids. Such amino acids include alanine, glycine, serine, and cysteine. Alanine is typically a preferred scanning amino acid among this group because it eliminates the side-chain beyond the beta-carbon and is less likely to alter the main-chain conformation of the variant (Cunningham and Wells, *Science*, 244: 1081-5 (1989)). Alanine is also typically preferred because it is the most common amino acid. Further, it is frequently found in both buried and exposed positions (Creighton, *The Proteins*, (W.H. N.Y.); Chothia, *J. Mol. Biol.*, 150: 1 (1976)). If alanine substitution does not yield adequate amounts of variant, an isoteric amino acid can be used.

[00236] Any cysteine residue not involved in maintaining the proper conformation of the anti-CTHRC1 antibody and anti-CD3 antibody also may be substituted, generally with serine, to improve the oxidative stability of the molecule and prevent aberrant crosslinking. Conversely, cysteine bond(s) may be added to the anti-CTHRC1 antibody or anti-CD3 antibody to improve its stability (particularly where the antibody is an antibody fragment such as an Fv fragment).

[00237] A particularly preferred type of substitutional variant involves substituting one or more hypervariable region residues of a parent antibody (e.g., a humanized or human antibody). Generally, the resulting variant(s) selected for further development will have improved biological properties relative to the parent antibody from which they are generated. A convenient way for generating such substitutional variants involves affinity maturation using phage display. Briefly, several hypervariable region sites (e.g., 6-7 sites) are mutated to generate all possible amino substitutions at each site. The antibody variants thus generated are displayed in a monovalent fashion from filamentous phage particles as fusions to the gene III product of M13 packaged within each particle. The phage-displayed variants are then screened for their biological activity (e.g., binding affinity) as herein disclosed. In order to identify candidate hypervariable region sites for modification, alanine scanning mutagenesis can be performed to identify hypervariable region residues contributing significantly to antigen binding. Alternatively, or additionally, it may be beneficial to analyze a crystal structure of the antigen-antibody complex to identify contact points between the antibody and CTHRC1 polypeptide and/or the anti-CD3 antibody and CD3. Such contact residues and neighboring residues are candidates for substitution according to the techniques elaborated herein. Once such variants are generated, the panel of variants is subjected to screening as described herein and antibodies with superior properties in one or more relevant assays may be selected for further development.

[00238] Nucleic acid molecules encoding amino acid sequence variants of the anti-CTHRC1 antibody and/or anti-CD3 antibody are prepared by a variety of methods known in the art. These methods include, but are not limited to, isolation from a natural source (in the case of naturally occurring amino acid sequence variants) or preparation by oligonucleotide-mediated (or site-directed) mutagenesis, PCR mutagenesis, and cassette mutagenesis of an earlier prepared variant or a non-variant version of the anti-CTHRC1 antibody and/or anti-CD3 antibody.

Modifications

[00239] Covalent modifications of anti-CTHRC1 antibodies and anti-CD3 antibodies are included within the scope of this invention. One type of covalent modification includes reacting targeted amino acid residues of an anti-CTHRC1 antibody with an organic derivatizing agent that is capable of reacting with selected side chains or the N- or C- terminal residues of the anti-CTHRC1 antibody or anti-CD3 antibody. Derivatization with bifunctional agents is useful, for instance, for crosslinking anti-CTHRC1 antibody to a water-insoluble support matrix or surface for use in the method for purifying anti-CTHRC1 antibodies, and vice-versa. Commonly used crosslinking agents include, e.g., 1,1-bis(diazoacetyl)-2-phenylethane, glutaraldehyde, N-hydroxysuccinimide esters, for example, esters with 4-azidosalicylic acid, homobifunctional imidoesters, including disuccinimidyl esters such as 3,3'-dithiobis(succinimidyl)propionate, bifunctional maleimides such as bis-N-maleimido-1,8-octane and agents such as methyl-3-[(p-azidophenyl)dithio]propioimide.

[00240] Other modifications include deamidation of glutamyl and asparaginyl residues to the corresponding glutamyl and aspartyl residues, respectively, hydroxylation of proline and lysine, phosphorylation of hydroxyl groups of seryl or threonyl residues, methylation of the α -amino groups of lysine, arginine, and histidine side chains (T.E. Creighton, *Proteins: Structure and Molecular Properties*, W.H. Freeman & Co., San Francisco, pp. 79-86 (1983)), acetylation of the N-terminal amine, and amidation of any C-terminal carboxyl group.

[00241] Another type of covalent modification of the anti-CTHRC1 antibody or anti-CD antibody included within the scope of this invention comprises altering the native glycosylation pattern of the antibody or polypeptide. "Altering the native glycosylation pattern" is intended for purposes herein to mean deleting one or more carbohydrate moieties found in native sequence anti-CTHRC1 antibody (either by removing the underlying glycosylation site or by deleting the glycosylation by chemical and/or enzymatic means), and/or adding one or more glycosylation sites that are not present in the native sequence anti-CTHRC1 antibody. In addition, the phrase includes qualitative changes in the glycosylation of the native proteins, involving a change in the nature and proportions of the various carbohydrate moieties present.

[00242] Glycosylation of antibodies and other polypeptides is typically either N-linked or O-linked. N-linked refers to the attachment of the carbohydrate moiety to the side chain of an asparagine residue. The tripeptide sequences asparagine-X-serine and asparagine-X-threonine,

where X is any amino acid except proline, are the recognition sequences for enzymatic attachment of the carbohydrate moiety to the asparagine side chain. Thus, the presence of either of these tripeptide sequences in a polypeptide creates a potential glycosylation site. O-linked glycosylation refers to the attachment of one of the sugars N-acetylgalactosamine, galactose, or xylose to a hydroxyamino acid, most commonly serine or threonine, although 5-hydroxyproline or 5-hydroxylysine may also be used.

[00243] Addition of glycosylation sites to the anti-CTHRC1 antibody or anti-CD3 antibody is conveniently accomplished by altering the amino acid sequence such that it contains one or more of the above-described tripeptide sequences (for N-linked glycosylation sites). The alteration may also be made by the addition of, or substitution by, one or more serine or threonine residues to the sequence of the original anti-CTHRC1 antibody or anti-CD3 antibody (for O-linked glycosylation sites). The anti-CTHRC1 antibody or anti-CD3 antibody amino acid sequence may optionally be altered through changes at the DNA level, particularly by mutating the DNA encoding the anti-CTHRC1 antibody or anti-CD3 antibody at preselected bases such that codons are generated that will translate into the desired amino acids.

[00244] Another means of increasing the number of carbohydrate moieties on the anti-CTHRC1 antibody or anti-CD3 antibody is by chemical or enzymatic coupling of glycosides to the polypeptide. Such methods are described in the art, e.g., in WO 87/05330 published 11 September 1987, and in Aplin and Wriston, CRC Crit. Rev. Biochem., pp. 259-306 (1981).

[00245] Removal of carbohydrate moieties present on the anti-CTHRC1 antibody or anti-CD3 antibody may be accomplished chemically or enzymatically or by mutational substitution of codons encoding for amino acid residues that serve as targets for glycosylation. Chemical deglycosylation techniques are known in the art and described, for instance, by Hakimuddin, et al., Arch. Biochem. Biophys., 259:52 (1987) and by Edge et al., Anal. Biochem., 118:131 (1981). Enzymatic cleavage of carbohydrate moieties on polypeptides can be achieved by the use of a variety of endo- and exo-glycosidases as described by Thotakura et al., Meth. Enzymol., 138:350 (1987).

Fc Region Variants

[00246] It may be desirable to modify the antibody of the invention with respect to effector function, e.g., so as to enhance antigen-dependent cell-mediated cytotoxicity (ADCC) and/or

complement dependent cytotoxicity (CDC) of the antibody. This may be achieved by introducing one or more amino acid substitutions in an Fc region of the antibody. Alternatively or additionally, cysteine residue(s) may be introduced in the Fc region, thereby allowing interchain disulfide bond formation in this region. The homodimeric antibody thus generated may have improved internalization capability and/or increased complement-mediated cell killing and antibody-dependent cellular cytotoxicity (ADCC) (see Caron et al., *J. Exp Med.* 176: 1191-5 (1992); Shopes, B. *J. Immunol.* 148: 2918-22 (1992). Homodimeric antibodies with enhanced anti-tumor activity may also be prepared using heterobifunctional cross-linkers as described in Wolff et al., *Cancer Research* 53: 2560-5 (1993). Alternatively, an antibody can be engineered which has dual Fc regions and may thereby have enhanced complement lysis and ADCC capabilities. See Stevenson et al., *Anti-Cancer Drug Design* 3: 219-30 (1989). To increase the serum half life of the antibody, one may incorporate a salvage receptor binding epitope into the antibody (especially an antibody fragment) as described in U.S. Patent 5,739,277, for example. As used herein, the term “salvage receptor binding epitope” refers to an epitope of the Fc region of an IgG molecule (e.g., IgG1, IgG2, IgG3, or IgG4) that is responsible for increasing the in vivo serum half-life of the IgG molecule.

[00247] In some embodiments, the Fc portion of an anti-CTHRC1 T-cell engager can include a sequence having at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% sequence identity to any one of the following Fc sequences:

WT Fc	
IgG1_WT_(IGHG1*05)	ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPELGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK
IgG4_(IGHG4*01)_S228P	ASTKGPSVFPLAPCSRSTSESTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYTCNVDHKPSNTKVDKRVESKYGPPCPPCPAPEFLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSQEDPEVQFNWYVDGVEVHNAKTKPREEQFNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKGLPSSIEKISKAKGQPREPQVYTLPPSQEEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTPPVLDSDGSFFLYSRLTVDKSRWQEGNVFSCSVMHEALHNHYTQKSLSLSLGK
Fc effector reduced	
IgG1_LALA_(IGHG1*05)	ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLH

	QDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPS DIAVEWESNGQPENNYKTTTPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYT QKSLSLSPGK
IgG1_LALAPG (IGHG1*05)	ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTWNSGALTSKVHTFPFAVLQSSGLYSL SSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPEAAGGPSVFLFPPKPK KDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLH QDWLNGKEYKCKVSNKALGAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPS DIAVEWESNGQPENNYKTTTPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYT QKSLSLSPGK
IgG1_NNAS_(I GHG1*05)	ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTWNSGALTSKVHTFPFAVLQSSGLYSL SSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPK DTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNNASRVVSVLTVLH QDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPS DIAVEWESNGQPENNYKTTTPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYT QKSLSLSPGK
Fc effector enhanced	
IgG1_SDALIE_ (IGHG1*05)	ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTWNSGALTSKVHTFPFAVLQSSGLYSL SSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPELLGGPDVFLFPPKPK DTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQ DWLNGKEYKCKVSNKALPLPEEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDI AVEWESNGQPENNYKTTTPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQK LSLSPGK
IgG1_WT_(IG HG1*05)_	+ afucosylated glycan
IgG1_SAKA_(I GHG1*05)	ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTWNSGALTSKVHTFPFAVLQSSGLYSL SSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPK DTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNATYRVVSVLTVLHQ DWLNGKEYKCKVSNKALPAPIEATISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDI AVEWESNGQPENNYKTTTPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQK LSLSPGK
IgG1_LPLIL_(I GHG1*05)	ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTWNSGALTSKVHTFPFAVLQSSGLYSL SSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPK DTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPPEEQYNSTLRVVSILTTLHQD WLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIA VEWESNGQPENNYKTTPLVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKS LSLSPGK

or a sequence pair having at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% sequence identity to any one of the following Fc sequence pairs:

Fc heterodimerization	
IgG1_hole_(IG HG1*05)	ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTWNSGALTSKVHTFPFAVLQSSGLYSL SSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPK DTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQ DWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLSCAVKGFYPSDI

	AVEWESNGQPENNYKTPPVLDSDGSFFLVSKLTVDKSRWQQGNVFSCSVMEALHNHYTQK SLSLSPGK
IgG1_knob_(I GHG1*05)	ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSL SSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPK DTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQ DWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLWCLVKGFYPSD IAVEWESNGQPENNYKTPPVLDSDGSFFLVSKLTVDKSRWQQGNVFSCSVMEALHNHYTQ KLSLSPG
IgG1_hole- Cys_(IGHG1*0 5)	ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSL SSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPK DTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQ DWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVCTLPSPSRDELTKNQVSLCAVKGFYPSD IAVEWESNGQPENNYKTPPVLDSDGSFFLVSKLTVDKSRWQQGNVFSCSVMEALHNHYTQ KLSLSPG
IgG1_knob- Cys_(IGHG1*05)	ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSL SSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPK DTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQ DWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPCRDELTKNQVSLWCLVKGFYPS DIAVEWESNGQPENNYKTPPVLDSDGSFFLVSKLTVDKSRWQQGNVFSCSVMEALHNHYT QKLSLSPG

[00248] In some embodiments, the Fc portion of an anti-CTHRC1 T-cell engager can include a sequence having the sequence of any one of the following Fc sequences:

WT Fc	
IgG1_WT_(IG HG1*05)	ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSL SSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPK DTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQ DWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDI AVEWESNGQPENNYKTPPVLDSDGSFFLVSKLTVDKSRWQQGNVFSCSVMEALHNHYTQK SLSLSPGK
IgG4_(IGHG4* 01)_S228P	ASTKGPSVFPLAPCSRSTSESTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSL SVVTVPSSSLGTQTYTCNVDHKPSNTKVDKRVESKYGPPCPPCPAPEFLGGPSVFLFPPKPKDTL MISRTPEVTCVVVDVSDQEDPEVFQFNWYVDGVEVHNAKTKPREEQFNSTYRVVSVLTVLHQDW LNGKEYKCKVSNKGLPSSIEKTISKAKGQPREPQVYTLPPSQEEMTKNQVSLTCLVKGFYPSDIAV EWESNGQPENNYKTPPVLDSDGSFFLVSRITVDSRWQEGNVFSCSVMEALHNHYTQKSL LSLGK
Fc effector reduced	
IgG1_LALA_(I GHG1*05)	ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSL SSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPEAAGGPSVFLFPPKPK KDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLH QDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPS DIAVEWESNGQPENNYKTPPVLDSDGSFFLVSKLTVDKSRWQQGNVFSCSVMEALHNHYT QKLSLSPGK

IgG1_LALAPG_(IGHG1*05)	ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTWNSGALTSKVHTFPAYLQSSGLYSLSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALGAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK
IgG1_NNAS_(IGHG1*05)	ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTWNSGALTSKVHTFPAYLQSSGLYSLSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNNASRVVSVLTVLHQDWLNGKEYKCKVSNKALGAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK
Fc effector enhanced	
IgG1_SDALIE_(IGHG1*05)	ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTWNSGALTSKVHTFPAYLQSSGLYSLSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPELLGGPDVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPLPEEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK
IgG1_WT_(IGHG1*05)_	+ afucosylated glycan
IgG1_SAKA_(IGHG1*05)	ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTWNSGALTSKVHTFPAYLQSSGLYSLSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNATYRVVSVLTVLHQDWLNGKEYKCKVSNKALGAPIEATISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK
IgG1_LPLIL_(IGHG1*05)	ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTWNSGALTSKVHTFPAYLQSSGLYSLSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTLRVVSILTTLVHLDQDWLNGKEYKCKVSNKALGAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK

or sequence pair where each sequence has the sequence of any one of the following Fc sequence pairs:

Fc heterodimerization	
IgG1_hole_(IGHG1*05)	ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTWNSGALTSKVHTFPAYLQSSGLYSLSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALGAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK
IgG1_knob_(IGHG1*05)	ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTWNSGALTSKVHTFPAYLQSSGLYSLSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPK

	DTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQ DWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLWCLVKGFYPSD IAVEWESNGQPENNYKTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQ KSLSLSPG
IgG1_hole- Cys_(IGHG1*0 5)	ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTWNSGALTSGLVHTFPAVLQSSGLYSL SSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPK DTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQ DWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVCTLPSPRDELTKNQVSLSCAVKGFYPSD IAVEWESNGQPENNYKTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQ KSLSLSPG
IgG1_knob- Cys(IGHG1*05)	ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTWNSGALTSGLVHTFPAVLQSSGLYSL SSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPK DTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQ DWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPCRDELTKNQVSLWCLVKGFYPS DIAVEWESNGQPENNYKTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYT QKSLSLSPG

Cysteine Engineered Antibody Variants

[00249] In certain embodiments, it may be desirable to create cysteine engineered antibodies, e.g., “thioMAbs,” in which one or more residues of an antibody are substituted with cysteine residues. In particular embodiments, the substituted residues occur at accessible sites of the antibody. By substituting those residues with cysteine, reactive thiol groups are thereby positioned at accessible sites of the antibody and may be used to conjugate the antibody to other moieties, such as drug moieties or linker-drug moieties, to create an immunoconjugate, as described further herein. Cysteine engineered antibodies can be generated as described, e.g., in U.S. Patent No. 7,521,541.

Certain Methods of Making Antibodies

Screening for Anti-CTHRC1 Antibodies with Desired Properties

[00250] Techniques for generating antibodies that bind to CTHRC1 polypeptides have been described above. One may further select antibodies with certain biological characteristics, as desired, such as for lack of cell internalization.

[00251] The growth inhibitory effects of an anti-CTHRC1 antibody of the invention may be assessed by methods known in the art, e.g., using cells which express a CTHRC1 polypeptide either endogenously or following transfection with the CTHRC1 gene. For example, appropriate tumor cell lines and CTHRC1-transfected cells may be treated with an anti-CTHRC1

monoclonal antibody of the invention at various concentrations for a few days (e.g., 2-7) days and stained with crystal violet or MTT or analyzed by some other colorimetric assay. Another method of measuring proliferation would be by comparing ³H-thymidine uptake by the cells treated in the presence or absence an anti-CTHRC1 antibody of the invention. After treatment, the cells are harvested and the amount of radioactivity incorporated into the DNA quantitated in a scintillation counter. Appropriate positive controls include treatment of a selected cell line with a growth inhibitory antibody known to inhibit growth of that cell line. Growth inhibition of tumor cells in vivo can be determined in various ways known in the art. The tumor cell may be one that overexpresses and/or displays a CTHRC1 polypeptide. The anti-CTHRC1 antibody will inhibit cell proliferation of a CTHRC1-displaying tumor cell in vitro or in vivo by about 25-100% compared to the untreated tumor cell, more preferably, by about 30-100%, and even more preferably by about 50-100% or 70-100%, in one embodiment, at an antibody concentration of about 0.5 to 30 µg/mL. Growth inhibition can be measured at an antibody concentration of about 0.5 to 30 µg/mL or about 0.5 nM to 200 nM in cell culture, where the growth inhibition is determined 1-10 days after exposure of the tumor cells to the antibody. The antibody is growth inhibitory in vivo if administration of the anti-CTHRC1 antibody at about 1 µg/kg to about 100 mg/kg body weight results in reduction in tumor size or reduction of tumor cell proliferation within about 5 days to 3 months from the first administration of the antibody, preferably within about 5 to 30 days.

[00252] To select for an anti-CTHRC1 antibody which induces cell death, loss of membrane integrity as indicated by, e.g., propidium iodide (PI), trypan blue or 7AAD uptake may be assessed relative to control. A PI uptake assay can be performed in the absence of complement and immune effector cells. CTHRC1 polypeptide-displaying tumor cells are incubated with medium alone or medium containing the appropriate anti-CTHRC1 antibody (e.g., at about 10 µg/mL). The cells are incubated for a 3 day time period. Following each treatment, cells are washed and aliquoted into 35 mm strainer-capped 12 x 75 tubes (1 mL per tube, 3 tubes per treatment group) for removal of cell clumps. Tubes then receive PI (10 µg/mL). Samples may be analyzed using a FACSCAN® flow cytometer and FACSCONVERT® CellQuest software (Becton Dickinson). Those anti-CTHRC1 antibodies that induce statistically significant levels of cell death as determined by PI uptake may be selected as cell death-inducing anti-CTHRC1 antibodies.

[00253] To screen for antibodies which bind to an epitope on a CTHRC1 polypeptide bound by an antibody of interest, a routine cross-blocking assay such as that described in *Antibodies, A Laboratory Manual*, Cold Spring Harbor Laboratory, Ed Harlow and David Lane (1988), can be performed. This assay can be used to determine if a test antibody binds the same site or epitope as a known anti-CTHRC1 antibody. Alternatively, or additionally, epitope mapping can be performed by methods known in the art. For example, the antibody sequence can be mutagenized such as by alanine scanning, to identify contact residues. The mutant antibody is initially tested for binding with polyclonal antibody to ensure proper folding. In a different method, peptides corresponding to different regions of a CTHRC1 polypeptide can be used in competition assays with the test antibodies or with a test antibody and an antibody with a characterized or known epitope.

[00254] In addition, candidate antibodies may also be screened for function using one or more of the following: in vivo screening for inhibition of metastasis, inhibition of chemotaxis by an in vitro method (e.g., U.S. 2010/0061978, incorporated herein by reference in its entirety), inhibition of vascularization, inhibition of tumor growth, and decrease in tumor size.

Certain Library Screening Methods

[00255] Anti-CTHRC1 antibodies of the invention can be made by using combinatorial libraries to screen for antibodies with the desired activity or activities. For example, a variety of methods are known in the art for generating phage display libraries and screening such libraries for antibodies possessing the desired binding characteristics. Such methods are described generally in Hoogenboom et al. (2001) in *Methods in Molecular Biology* 178: 1-37 (O'Brien et al., ed., Human Press, Totowa, NJ), and in certain embodiments, in Lee et al. (2004) *J. Mol. Biol.* 340: 1073-93.

[00256] In principle, synthetic antibody clones are selected by screening phage libraries containing phage that display various fragments of antibody variable region (Fv) fused to phage coat protein. Such phage libraries are panned by affinity chromatography against the desired antigen. Clones expressing Fv fragments capable of binding to the desired antigen are adsorbed to the antigen and thus separated from the non-binding clones in the library. The binding clones are then eluted from the antigen, and can be further enriched by additional cycles of antigen adsorption/elution. Any of the anti-CTHRC1 antibodies of the invention can be obtained by

designing a suitable antigen screening procedure to select for the phage clone of interest followed by construction of a full length anti-CTHRC1 antibody clone using the Fv sequences from the phage clone of interest and suitable constant region (Fc) sequences described in Kabat et al., *Sequences of Proteins of Immunological Interest*, Fifth Edition, NIH Publication 91-3242, Bethesda MD (1991), vols. 1-3.

[00257] In certain embodiments, the antigen-binding domain of an antibody is formed from two variable (V) regions of about 110 amino acids, one each from the light (VL) and heavy (VH) chains, that both present three hypervariable loops (HVRs) or complementarity-determining regions (CDRs). Variable domains can be displayed functionally on phage, either as single-chain Fv (scFv) fragments, in which VH and VL are covalently linked through a short, flexible peptide, or as Fab fragments, in which they are each fused to a constant domain and interact non-covalently, as described in Winter et al., *Ann. Rev. Immunol.*, 12: 433-55 (1994). As used herein, scFv encoding phage clones and Fab encoding phage clones are collectively referred to as “Fv phage clones” or “Fv clones.”

[00258] Repertoires of VH and VL genes can be separately cloned by polymerase chain reaction (PCR) and recombined randomly in phage libraries, which can then be searched for antigen-binding clones as described in Winter et al., *Ann. Rev. Immunol.*, 12: 433-55 (1994). Libraries from immunized sources provide high-affinity antibodies to the immunogen without the requirement of constructing hybridomas. Alternatively, the naive repertoire can be cloned to provide a single source of human antibodies to a wide range of non-self and also self antigens without any immunization as described by Griffiths et al., *EMBO J*, 12: 725-34 (1993). Finally, naive libraries can also be made synthetically by cloning the unrearranged V-gene segments from stem cells and using PCR primers containing random sequence to encode the highly variable CDR3 regions and to accomplish rearrangement in vitro as described by Hoogenboom and Winter, *J. Mol. Biol.*, 227: 381-8 (1992).

[00259] In certain embodiments, filamentous phage is used to display antibody fragments by fusion to the minor coat protein pIII. The antibody fragments can be displayed as single chain Fv fragments, in which VH and VL domains are connected on the same polypeptide chain by a flexible polypeptide spacer, e.g., as described by Marks et al., *J. Mol. Biol.*, 222: 581-97 (1991), or as Fab fragments, in which one chain is fused to pIII and the other is secreted into the bacterial

host cell periplasm where assembly of a Fab-coat protein structure which becomes displayed on the phage surface by displacing some of the wild type coat proteins, e.g., as described in Hoogenboom et al., *Nucl. Acids Res.*, 19: 4133-7 (1991).

[00260] In general, nucleic acids encoding antibody gene fragments are obtained from immune cells harvested from humans or animals. If a library biased in favor of anti-CTHRC1 clones is desired, the subject is immunized with CTHRC1 to generate an antibody response, and spleen cells and/or circulating B cells other peripheral blood lymphocytes (PBLs) are recovered for library construction. In some embodiments, a human antibody gene fragment library biased in favor of anti-CTHRC1 clones is obtained by generating an anti-CTHRC1 antibody response in transgenic mice carrying a functional human immunoglobulin gene array (and lacking a functional endogenous antibody production system) such that CTHRC1 immunization gives rise to B cells producing human antibodies against CTHRC1. The generation of human antibody-producing transgenic mice is described below.

[00261] Additional enrichment for anti-CTHRC1 reactive cell populations can be obtained by using a suitable screening procedure to isolate B cells expressing CTHRC1-specific membrane bound antibody, e.g., by cell separation using CTHRC1 affinity chromatography or adsorption of cells to fluorochrome-labeled CTHRC1 followed by flow-activated cell sorting (FACS).

[00262] Alternatively, the use of spleen cells and/or B cells or other PBLs from an unimmunized donor provides a better representation of the possible antibody repertoire, and also permits the construction of an antibody library using any animal (human or non-human) species in which CTHRC1 is not antigenic. For libraries incorporating in vitro antibody gene construction, stem cells are harvested from the subject to provide nucleic acids encoding unrearranged antibody gene segments. The immune cells of interest can be obtained from a variety of animal species, such as human, mouse, rat, lagomorpha, luprine, canine, feline, porcine, bovine, equine, and avian species, etc.

[00263] Nucleic acid encoding antibody variable gene segments (including VH and VL segments) are recovered from the cells of interest and amplified. In the case of rearranged VH and VL gene libraries, the desired DNA can be obtained by isolating genomic DNA or mRNA from lymphocytes followed by polymerase chain reaction (PCR) with primers matching the 5'

and 3' ends of rearranged VH and VL genes as described in Orlandi et al., *Proc. Natl. Acad. Sci. (USA)*, 86: 3833-7 (1989), thereby making diverse V gene repertoires for expression.

[00264] The V genes can be amplified from cDNA and genomic DNA, with back primers at the 5' end of the exon encoding the mature V-domain and forward primers based within the J-segment as described in Orlandi et al. (1989) and in Ward et al., *Nature*, 341: 544-6 (1989). However, for amplifying from cDNA, back primers can also be based in the leader exon as described in Jones et al., *Biotechnol.*, 9: 88-9 (1991), and forward primers within the constant region as described in Sastry et al., *Proc. Natl. Acad. Sci. (USA)*, 86: 5728-32 (1989). To maximize complementarity, degeneracy can be incorporated in the primers as described in Orlandi et al. (1989) or Sastry et al. (1989). In certain embodiments, library diversity is maximized by using PCR primers targeted to each V-gene family in order to amplify all available VH and VL arrangements present in the immune cell nucleic acid sample, e.g., as described in the method of Marks et al., *J. Mol. Biol.*, 222: 581-97 (1991) or as described in the method of Orum et al., *Nucleic Acids Res.*, 21: 4491-98 (1993). For cloning of the amplified DNA into expression vectors, rare restriction sites can be introduced within the PCR primer as a tag at one end as described in Orlandi et al. (1989), or by further PCR amplification with a tagged primer as described in Clackson et al., *Nature*, 352: 624-628 (1991).

[00265] Repertoires of synthetically rearranged V genes can be derived in vitro from V gene segments. Most of the human VH-gene segments have been cloned and sequenced (reported in Tomlinson et al., *J. Mol. Biol.*, 227: 776-98 (1992)), and mapped (reported in Matsuda et al., *Nature Genet.*, 3: 88-94 (1993)); these cloned segments (including all the major conformations of the H1 and H2 loop) can be used to generate diverse VH gene repertoires with PCR primers encoding H3 loops of diverse sequence and length as described in Hoogenboom and Winter, *J. Mol. Biol.*, 227: 381-388 (1992). VH repertoires can also be made with all the sequence diversity focused in a long H3 loop of a single length as described in Barbas et al., *Proc. Natl. Acad. Sci. USA*, 89: 4457-61 (1992). Human V κ and V λ segments have been cloned and sequenced (reported in Williams and Winter, *Eur. J. Immunol.*, 23: 1456-61 (1993)) and can be used to make synthetic light chain repertoires. Synthetic V gene repertoires, based on a range of VH and VL folds, and L3 and H3 lengths, will encode antibodies of considerable structural diversity. Following amplification of V-gene encoding DNAs, germline V-gene segments can be

rearranged in vitro according to the methods of Hoogenboom and Winter, *J. Mol. Biol.*, 227: 381-8 (1992).

[00266] Repertoires of antibody fragments can be constructed by combining VH and VL gene repertoires together in several ways. Each repertoire can be created in different vectors, and the vectors recombined in vitro, e.g., as described in Hogrefe et al., *Gene*, 128: 119-26 (1993), or in vivo by combinatorial infection, e.g., the loxP system described in Waterhouse et al., *Nucl. Acids Res.*, 21: 2265-66 (1993). The in vivo recombination approach exploits the two-chain nature of Fab fragments to overcome the limit on library size imposed by *E. coli* transformation efficiency. Naive VH and VL repertoires are cloned separately, one into a phagemid and the other into a phage vector. The two libraries are then combined by phage infection of phagemid-containing bacteria so that each cell contains a different combination and the library size is limited only by the number of cells present (about 10¹² clones). Both vectors contain in vivo recombination signals so that the VH and VL genes are recombined onto a single replicon and are co-packaged into phage virions. These huge libraries provide large numbers of diverse antibodies of good affinity (K_d-1 of about 10⁻⁸ M).

[00267] Alternatively, the repertoires may be cloned sequentially into the same vector, e.g., as described in Barbas et al., *Proc. Natl. Acad. Sci. USA*, 88: 7978-7982 (1991), or assembled together by PCR and then cloned, e.g., as described in Clackson et al., *Nature*, 352: 624-628 (1991). PCR assembly can also be used to join VH and VL DNAs with DNA encoding a flexible peptide spacer to form single chain Fv (scFv) repertoires. In yet another technique, "in cell PCR assembly" is used to combine VH and VL genes within lymphocytes by PCR and then clone repertoires of linked genes as described in Embleton et al., *Nucl. Acids Res.*, 20: 3831-3837 (1992).

[00268] The antibodies produced by naive libraries (either natural or synthetic) can be of moderate affinity (K_d-1 of about 10⁶ to 10⁷ M⁻¹), but affinity maturation can also be mimicked in vitro by constructing and reselecting from secondary libraries as described in Winter et al. (1994), supra. For example, mutation can be introduced at random in vitro by using error-prone polymerase (reported in Leung et al., *Technique*, 1: 11-5 (1989)) in the method of Hawkins et al., *J. Mol. Biol.*, 226: 889-96 (1992) or in the method of Gram et al., *Proc. Natl. Acad. Sci USA*, 89: 3576-80 (1992). Additionally, affinity maturation can be performed by randomly mutating

one or more CDRs, e.g., using PCR with primers carrying random sequence spanning the CDR of interest, in selected individual Fv clones and screening for higher affinity clones. WO 9607754 described a method for inducing mutagenesis in a complementarity determining region of an immunoglobulin light chain to create a library of light chain genes. Another effective approach is to recombine the VH or VL domains selected by phage display with repertoires of naturally occurring V domain variants obtained from unimmunized donors and screen for higher affinity in several rounds of chain reshuffling as described in Marks et al., *Biotechnol.*, 10: 779-83 (1992). This technique allows the production of antibodies and antibody fragments with affinities of about 10^{-9} M or less.

[00269] Screening of the libraries can be accomplished by various techniques known in the art. For example, CTHRC1 can be used to coat the wells of adsorption plates, expressed on host cells affixed to adsorption plates or used in cell sorting, or conjugated to biotin for capture with streptavidin-coated beads, or used in any other method for panning phage display libraries.

[00270] The phage library samples are contacted with immobilized CTHRC1 under conditions suitable for binding at least a portion of the phage particles with the adsorbent. Normally, the conditions, including pH, ionic strength, temperature and the like are selected to mimic physiological conditions. The phages bound to the solid phase are washed and then eluted by acid, e.g., as described in Barbas et al., *Proc. Natl. Acad. Sci USA*, 88: 7978-82 (1991), or by alkali, e.g., as described in Marks et al., *J. Mol. Biol.*, 222: 581-97 (1991), or by CTHRC1 antigen competition, e.g., in a procedure similar to the antigen competition method of Clackson et al., *Nature*, 352: 624-8 (1991). Phages can be enriched 20 to 1,000-fold in a single round of selection. Moreover, the enriched phages can be grown in bacterial culture and subjected to further rounds of selection.

[00271] The efficiency of selection depends on many factors, including the kinetics of dissociation during washing, and whether multiple antibody fragments on a single phage can simultaneously engage with antigen. Antibodies with fast dissociation kinetics (and weak binding affinities) can be retained by use of short washes, multivalent phage display and high coating density of antigen in solid phase. The high density not only stabilizes the phage through multivalent interactions but favors rebinding of phage that has dissociated. The selection of antibodies with slow dissociation kinetics (and good binding affinities) can be promoted by use

of long washes and monovalent phage display as described in Bass et al., *Proteins*, 8: 309-314 (1990) and in WO 92/09690, and a low coating density of antigen as described in Marks et al., *Biotechnol.*, 10: 779-783 (1992).

[00272] It is possible to select between phage antibodies of different affinities, even with affinities that differ slightly, for CTHRC1. However, random mutation of a selected antibody (e.g., as performed in some affinity maturation techniques) is likely to give rise to many mutants, most binding to antigen, and a few with higher affinity. With limiting CTHRC1, rare high affinity phage could be competed out. To retain all higher affinity mutants, phages can be incubated with excess biotinylated CTHRC1, but with the biotinylated CTHRC1 at a concentration of lower molarity than the target molar affinity constant for CTHRC1. The high affinity-binding phages can then be captured by streptavidin-coated paramagnetic beads. Such “equilibrium capture” allows the antibodies to be selected according to their affinities of binding, with sensitivity that permits isolation of mutant clones with as little as two-fold higher affinity from a great excess of phages with lower affinity. Conditions used in washing phages bound to a solid phase can also be manipulated to discriminate on the basis of dissociation kinetics.

[00273] Anti-CTHRC1 clones may be selected based on activity. In certain embodiments, the invention provides anti-CTHRC1 antibodies that bind to living cells that naturally express CTHRC1. In one embodiment, the invention provides anti-CTHRC1 antibodies that block the binding between a CTHRC1 ligand and CTHRC1, but do not block the binding between a CTHRC1 ligand and a second protein. Fv clones corresponding to such anti-CTHRC1 antibodies can be selected by (1) isolating anti-CTHRC1 clones from a phage library as described above, and optionally amplifying the isolated population of phage clones by growing up the population in a suitable bacterial host; (2) selecting CTHRC1 and a second protein against which blocking and non-blocking activity, respectively, is desired; (3) adsorbing the anti-CTHRC1 phage clones to immobilized CTHRC1; (4) using an excess of the second protein to elute any undesired clones that recognize CTHRC1-binding determinants which overlap or are shared with the binding determinants of the second protein; and (5) eluting the clones which remain adsorbed following step (4). Optionally, clones with the desired blocking/non-blocking properties can be further enriched by repeating the selection procedures described herein one or more times.

[00274] DNA encoding hybridoma-derived monoclonal antibodies or phage display Fv clones of the invention is readily isolated and sequenced using conventional procedures (e.g., by using oligonucleotide primers designed to specifically amplify the heavy and light chain coding regions of interest from hybridoma or phage DNA template). Once isolated, the DNA can be placed into expression vectors, which are then transfected into host cells such as *E. coli* cells, simian COS cells, Chinese hamster ovary (CHO) cells, or myeloma cells that do not otherwise produce immunoglobulin protein, to obtain the synthesis of the desired monoclonal antibodies in the recombinant host cells. Review articles on recombinant expression in bacteria of antibody-encoding DNA include Skerra et al., *Curr. Opinion in Immunol.* 5: 256 (1993) and Pluckthun, *Immunol. Rev.* 130: 151 (1992).

[00275] DNA encoding the Fv clones of the invention can be combined with known DNA sequences encoding heavy chain and/or light chain constant regions (e.g., the appropriate DNA sequences can be obtained from Kabat et al., *supra*) to form clones encoding full or partial length heavy and/or light chains. It will be appreciated that constant regions of any isotype can be used for this purpose, including IgG, IgM, IgA, IgD, and IgE constant regions, and that such constant regions can be obtained from any human or animal species. An Fv clone derived from the variable domain DNA of one animal (such as human) species and then fused to constant region DNA of another animal species to form coding sequence(s) for “hybrid,” full length heavy chain and/or light chain is included in the definition of “chimeric” and “hybrid” antibody as used herein. In certain embodiments, an Fv clone derived from human variable DNA is fused to human constant region DNA to form coding sequence(s) for full- or partial-length human heavy and/or light chains.

[00276] DNA encoding anti-CTHRC1 antibody derived from a hybridoma can also be modified, for example, by substituting the coding sequence for human heavy- and light-chain constant domains in place of homologous murine sequences derived from the hybridoma clone (e.g., as in the method of Morrison et al., *Proc. Natl. Acad. Sci. USA*, 81: 6851-5 (1984)). DNA encoding a hybridoma- or Fv clone-derived antibody or fragment can be further modified by covalently joining to the immunoglobulin coding sequence all or part of the coding sequence for a non-immunoglobulin polypeptide. In this manner, “chimeric” or “hybrid” antibodies are prepared that have the binding specificity of the Fv clone or hybridoma clone-derived antibodies of the invention.

Generation of Antibodies Using CAR-T Cells

[00277] Anti-CTHRC1 antibodies of the invention can be made by using CAR T-cell platforms to screen for antibodies with the desired activity or activities. Chimeric antigen receptors (CARs) are composed of an extracellular antigen recognition domain (usually a single-chain variable fragment (scFv) antibody) attached to transmembrane and cytoplasmic signaling domains. Alvarez-Vallina, L, *Curr Gene Ther* 1: 385–97 (2001). CAR-mediated recognition converts tumor-associated antigens (TAA) expressed on the cell surface into recruitment points of effector functions, addressing the goal of major histocompatibility complex-independent activation of effector cells. First-generation CARs were constructed through the fusion of a scFv-based TAA-binding domain to a cytoplasmic signaling domain typically derived either from the ζ chain of the T cell receptor (TCR)/CD3 complex or from the γ chain associated with some Fc receptors (Gross, G. et al., *Proc Natl Acad Sci USA* 86: 10024-8 (1989)). Second-generation CARs (CARv2) comprising the signaling region of the TCR ζ in series with the signaling domain derived from the T-cell co-stimulatory receptors CD28, 4-1BB (CD137) or OX40 (CD134) have also been developed (Sanz, L. et al., *Trends Immunol* 25: 85-91 (2004)). Third-generation CARs further combine the signaling potential of two costimulatory domains (e.g., both CD28 and 4-1BB) (Subklewe, M., et al., *Transfus Med Hemother* 46(1): 15-24 (2019)).

[00278] Upon encountering antigen, the interaction of a genetically transferred CAR triggers effector functions and can mediate cytolysis of tumor cells. The utility and effectiveness of the CAR approach have been demonstrated in a variety of animal models, and ongoing clinical trials using CAR-based genetically engineered T lymphocytes for the treatment of cancer patients. Lipowska-Bhalla, G. et al., *Cancer Immunol Immunother* 61: 953-62 (2012). CARs enable targeting of effector cells toward any native extracellular antigen for which a suitable antibody exists. Engineered cells can be targeted not only to proteins but also to structures such as carbohydrate and glycolipid tumor antigens (Mezzanzanica, D. et al., *Cancer Gene Ther* 5: 401-7 (1998); Kershaw, MH. et al., *Nat Rev Immunol* 5: 928-40 (2005)).

[00279] Current methods for the generation of recombinant antibodies are mainly based on the use of purified proteins. Hoogenboom, H.R. et al., *Nat Biotechnol* 23: 1105–1116 (2005). However, a mammalian cell-based antibody display platform has recently been described, which takes advantage of the functional capabilities of T lymphocytes. Alonso-Camino et al, *Molecular*

Therapy Nucleic Acids (2013) 2, e93. The display of antibodies on the surface of T lymphocytes, as a part of a CAR-mediating signaling, may ideally link the antigen-antibody interaction to a demonstrable change in cell phenotype, due to the surface expression of activation markers. Alonso-Camino, V. et al., *PLoS ONE* 4: e7174 (2009). By using a scFv-based CAR that recognizes a TAA, it has been demonstrated that combining CAR-mediated activation with fluorescence-activated cell sorting (FACS) of CD69+ T cells makes it possible to isolate binders to surface TAA, with an enrichment factor of at least 10^3 -fold after two rounds, resulting in a homogeneous population of T cells expressing TAA-specific CAR. Alonso-Camino, V, et al., *PLoS ONE* 4: e7174 (2009).

Preparation of Anti-CTHRC1 Antibodies

[00280] The description below relates primarily to production of anti-CTHRC1 antibodies by culturing cells transformed or transfected with a vector containing anti-CTHRC1 antibody-encoding nucleic acid. It is, of course, contemplated that alternative methods, which are well known in the art, may be employed to prepare anti-CTHRC1 antibodies. For instance, the appropriate amino acid sequence, or portions thereof, may be produced by direct peptide synthesis using solid-phase techniques (e.g., Stewart et al., *Solid-Phase Peptide Synthesis*, W.H. Freeman Co., San Francisco, CA (1969); Merrifield, J. *Am. Chem. Soc.*, 85:2149-2154 (1963)). In vitro protein synthesis may be performed using manual techniques or by automation. Automated synthesis may be accomplished, for instance, using an Applied Biosystems Peptide Synthesizer (Foster City, CA) using manufacturer's instructions. Various portions of the anti-CTHRC1 antibody may be chemically synthesized separately and combined using chemical or enzymatic methods to produce the desired anti-CTHRC1 antibody.

Isolation of DNA Encoding Anti-CTHRC1 Antibody

[00281] DNA encoding anti-CTHRC1 antibody may be obtained from a cDNA library prepared from tissue believed to possess the anti-CTHRC1 antibody mRNA and to express it at a detectable level. Accordingly, human anti-CTHRC1 antibody DNA can be conveniently obtained from a cDNA library prepared from human tissue. The anti-CTHRC1 antibody-encoding gene may also be obtained from a genomic library or by known synthetic procedures (e.g., automated nucleic acid synthesis).

[00282] Libraries can be screened with probes (such as oligonucleotides of at least about 20-80 bases) designed to identify the gene of interest or the protein encoded by it. Screening the cDNA or genomic library with the selected probe may be conducted using standard procedures, such as described in Sambrook et al., *Molecular Cloning: A Laboratory Manual* (New York: Cold Spring Harbor Laboratory Press, 1989). An alternative means to isolate the gene encoding anti-CTHRC1 antibody is to use PCR methodology (Sambrook et al., *supra*; Dieffenbach et al., *PCR Primer: A Laboratory Manual* (Cold Spring Harbor Laboratory Press, 1995)).

[00283] Techniques for screening a cDNA library are well known in the art. The oligonucleotide sequences selected as probes should be of sufficient length and sufficiently unambiguous that false positives are minimized. The oligonucleotide is preferably labeled such that it can be detected upon hybridization to DNA in the library being screened. Methods of labeling are well known in the art, and include the use of radiolabels like ³²P-labeled ATP, biotinylation or enzyme labeling. Hybridization conditions, including moderate stringency and high stringency, are provided in Sambrook et al., *supra*.

[00284] Sequences identified in such library screening methods can be compared and aligned to other known sequences deposited and available in public databases such as GenBank or other private sequence databases. Sequence identity (at either the amino acid or nucleotide level) within defined regions of the molecule or across the full-length sequence can be determined using methods known in the art and as described herein.

[00285] Nucleic acid having protein coding sequence may be obtained by screening selected cDNA or genomic libraries using the deduced amino acid sequence disclosed herein for the first time, and, if necessary, using conventional primer extension procedures as described in Sambrook et al., *supra*, to detect precursors and processing intermediates of mRNA that may not have been reverse-transcribed into cDNA.

Selection and Transformation of Host Cells

[00286] Host cells are transfected or transformed with expression or cloning vectors described herein for anti-CTHRC1 antibody production and cultured in conventional nutrient media modified as appropriate for inducing promoters, selecting transformants, or amplifying the genes encoding the desired sequences. The culture conditions, such as media, temperature, pH and the like, can be selected by the skilled artisan without undue experimentation. In general, principles,

protocols, and practical techniques for maximizing the productivity of cell cultures can be found in *Mammalian Cell Biotechnology: a Practical Approach*, M. Butler, ed. (IRL Press, 1991) and Sambrook et al., *supra*.

Linkers

[00287] In any of the foregoing embodiments, the anti-CTHRC1 T-cell engager can further comprise a peptide linker positioned between the first domain and the second domain. In certain aspects, the peptide linker directly links the first domain to the second domain. It should be understood that the first domain and the second domain can be positioned in at either end of the peptide linker. For example, the anti-CTHRC1 T-cell engager can comprise (First domain-Linker-Second Domain) or (Second domain-Linker-First Domain).

[00288] In any of the foregoing embodiments, the peptide linker can be any suitable linker. By way of example, but not limitation, a peptide linker can include $(G_4S)_n$, $(GSG)_n$, $(SG_4)_n$, $G_4(SG_4)_n$ peptide linkers where n is from 1 to 4. Preferably, the linker has the amino acid sequence of SEQ ID NO: 100 $((G_4S)_3)$.

[00289] Additional linkers are also described in U.S. Patent No. 11,053,293 and U.S. Patent Application Publication No. 2019/0016771, each of which is incorporated herein by reference in its entirety. Further exemplary linkers can include GGGGSGGGGS (SEQ ID NO: 109), SGGGSGGGG (SEQ ID NO: 110), GGGGSGGGSGGGG or $G_4(SG_4)_2$ (SEQ ID NO: 111), and $(G_4S)_4$ or GGGGSGGGSGGGSGGGGS (SEQ ID NO: 112), GSPGSSSSGS (SEQ ID NO: 113), GSGSGSGS (SEQ ID NO: 114), GSGSGNGS (SEQ ID NO: 115), GSGSGSGS (SEQ ID NO: 116), GGSGSG (SEQ ID NO: 117), GGSG (SEQ ID NO: 118), GGSGNGSG (SEQ ID NO: 119), GGNGSGSG (SEQ ID NO: 120) and GGNGSG (SEQ ID NO: 121).

[00290] Alternative methods and designs for constructing anti-CTHRC1 T-cell engagers of the present disclosure are provided in U.S. Patent No. 10,392,445, which is incorporated herein by reference.

Pharmaceutical Compositions

[00291] In embodiments, the anti-CTHRC1 T-cell engager of the present disclosure can be formulated into a pharmaceutically acceptable composition that can include the anti-CTHRC1 T-cell engager and a pharmaceutically acceptable carrier.

[00292] The anti-CTHRC1 T-cell engager and compositions thereof of the invention may be administered by any route appropriate to the condition to be treated. The T-cell engager will typically be administered parenterally, i.e. infusion, subcutaneous, intramuscular, intravenous, intradermal, intrathecal and epidural.

[00293] For treating these cancers, in one embodiment, the T-cell engager is administered via intravenous infusion. The dosage administered via infusion is in the range of about 0.001 mg/kg to about 100 mg/kg per dose by the subject's body weight, generally one dose per week for a total of one, two, three or four doses. Alternatively, the dosage range is of about 0.01 mg/kg to about 100 mg/kg, about 0.1 mg/kg to about 100 mg/kg, about 1 mg/kg to about 100 mg/kg, about 0.001 mg/kg to about 50 mg/kg, about 0.01 mg/kg to about 50 mg/kg, about 0.1 mg/kg to about 50 mg/kg, about 1 mg/kg to about 50 mg/kg, about 0.001 mg/kg to about 10 mg/kg, about 0.01 mg/kg to about 10 mg/kg, about 0.1 mg/kg to about 10 mg/kg, about 1 mg/kg to about 10 mg/kg, about 0.001 mg/kg to about 5 mg/kg, about 0.01 mg/kg to about 5 mg/kg, about 0.1 mg/kg to about 5 mg/kg, about 1 mg/kg to about 5 mg/kg, about 0.001 mg/kg to about 1 mg/kg, about 0.01 mg/kg to about 1 mg/kg, and about 0.1 mg/kg to about 1 mg/kg. The dose may be administered once per day, once per week, multiple times per week, but less than once per day, multiple times per month but less than once per day, multiple times per month but less than once per week, once per month or intermittently to relieve or alleviate symptoms of the disease. Administration may continue at any of the disclosed intervals until remission of the tumor or symptoms of the cancer being treated. Administration may continue after remission or relief of symptoms is achieved where such remission or relief is prolonged by such continued administration.

[00294] Therapeutic formulations comprising an anti-CTHRC1 T-cell engager used in accordance with the present invention are prepared for storage by mixing the anti-CTHRC1 T-cell engager having the desired degree of purity with optional pharmaceutically acceptable carriers, excipients or stabilizers (Remington's Pharmaceutical Sciences 16th edition, Osol, A. Ed. (1980)), in the form of lyophilized formulations or aqueous solutions. Acceptable carriers, excipients, or stabilizers are nontoxic to recipients at the dosages and concentrations employed, and include buffers such as acetate, Tris, phosphate, citrate, and other organic acids; antioxidants including ascorbic acid and methionine; preservatives (such as octadecyldimethylbenzyl ammonium chloride; hexamethonium chloride; benzalkonium chloride, benzethonium chloride; phenol, butyl or benzyl alcohol; alkyl parabens such as methyl or propyl paraben; catechol;

resorcinol; cyclohexanol; 3-pentanol; and m-cresol); low molecular weight (less than about 10 residues) polypeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, histidine, arginine, or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrans; chelating agents such as EDTA; tonicifiers such as trehalose and sodium chloride; sugars such as sucrose, mannitol, trehalose or sorbitol; surfactant such as polysorbate; salt-forming counter-ions such as sodium; metal complexes (e.g., Zn-protein complexes); and/or non-ionic surfactants such as TWEEN®, PLURONICS® or polyethylene glycol (PEG). Pharmaceutical formulations to be used for in vivo administration are generally sterile. This is readily accomplished by filtration through sterile filtration membranes.

[00295] The active ingredients may also be entrapped in microcapsules prepared, for example, by coacervation techniques or by interfacial polymerization, for example, hydroxymethylcellulose or gelatin-microcapsules and poly-(methylmethacrylate) microcapsules, respectively, in colloidal drug delivery systems (for example, liposomes, albumin microspheres, microemulsions, nano-particles and nanocapsules) or in macroemulsions. Such techniques are disclosed in Remington's Pharmaceutical Sciences, 16th edition, Osol, A. Ed. (1980).

[00296] Sustained-release preparations may be prepared. Suitable examples of sustained-release preparations include semi-permeable matrices of solid hydrophobic polymers containing the antibody, which matrices are in the form of shaped articles, e.g., films, or microcapsules. Examples of sustained-release matrices include polyesters, hydrogels (for example, poly(2-hydroxyethyl-methacrylate), or poly(vinylalcohol)), polylactides (U.S. Pat. No. 3,773,919), copolymers of L-glutamic acid and γ ethyl-L-glutamate, non-degradable ethylene-vinyl acetate, degradable lactic acid-glycolic acid copolymers such as the LUPRON DEPOT® (injectable microspheres composed of lactic acid-glycolic acid copolymer and leuprolide acetate), and poly-D-(-)-3-hydroxybutyric acid. While polymers such as ethylene-vinyl acetate and lactic acid-glycolic acid enable release of molecules for over 100 days, certain hydrogels release proteins for shorter time periods. When encapsulated immunoglobulins remain in the body for a long time, they may denature or aggregate as a result of exposure to moisture at 37°C, resulting in a loss of biological activity and possible changes in immunogenicity. Rational strategies can be devised for stabilization depending on the mechanism involved. For example, if the aggregation

mechanism is discovered to be intermolecular S-S bond formation through thio-disulfide interchange, stabilization may be achieved by modifying sulfhydryl residues, lyophilizing from acidic solutions, controlling moisture content, using appropriate additives, and developing specific polymer matrix compositions.

[00297] An anti-CTHRC1 T-cell engager of the present disclosure may be formulated in any suitable form for delivery to a target cell/tissue. For example, anti-CTHRC1 T-cell engagers may be formulated as immunoliposomes. A “liposome” is a small vesicle composed of various types of lipids, phospholipids and/or surfactant which is useful for delivery of a drug to a mammal. The components of the liposome are commonly arranged in a bilayer formation, similar to the lipid arrangement of biological membranes. Liposomes containing the antibody are prepared by methods known in the art, such as described in Epstein et al., Proc. Natl. Acad. Sci. USA 82:3688 (1985); Hwang et al., Proc. Natl. Acad. Sci. USA 77:4030 (1980); U.S. Pat. Nos. 4,485,045 and 4,544,545; and WO97/38731 published October 23, 1997. Liposomes with enhanced circulation time are disclosed in U.S. Patent No. 5,013,556.

[00298] Particularly useful liposomes can be generated by the reverse phase evaporation method with a lipid composition comprising phosphatidylcholine, cholesterol and PEG-derivatized phosphatidylethanolamine (PEG-PE). Liposomes are extruded through filters of defined pore size to yield liposomes with the desired diameter. Fab' fragments of the antibody of the present invention can be conjugated to the liposomes as described in Martin et al., J. Biol. Chem. 257: 286-8 (1982) via a disulfide interchange reaction. A chemotherapeutic agent is optionally contained within the liposome (See Gabizon et al., J. National Cancer Inst. 81(19): 1484 (1989)).

[00299] The formulations to be used for in vivo administration must be sterile. This is readily accomplished by filtration through sterile filtration membranes.

Methods of Manufacture

[00300] Conventional methods for the production of T-cell engager are known in the art.

[00301] Methods of eukaryotic cell transfection and prokaryotic cell transformation, which means introduction of DNA into the host so that the DNA is replicable, either as an extrachromosomal or by chromosomal integrant, are known to the ordinarily skilled artisan, for example, CaCl₂, CaPO₄, liposome-mediated, polyethylene-glycol/DMSO and electroporation.

Depending on the host cell used, transformation is performed using standard techniques appropriate to such cells. The calcium treatment employing calcium chloride, as described in Sambrook et al., *supra*, or electroporation is generally used for prokaryotes. Infection with *Agrobacterium tumefaciens* is used for transformation of certain plant cells, as described by Shaw et al., *Gene*, 23:315 (1983) and WO 89/05859 published 29 June 1989. For mammalian cells without such cell walls, the calcium phosphate precipitation method of Graham and van der Eb, *Virology*, 52:456-457 (1978) can be employed. General aspects of mammalian cell host system transfections have been described in U.S. Patent No. 4,399,216. Transformations into yeast are typically carried out according to the method of Van Solingen et al., *J. Bact.*, 130:946 (1977) and Hsiao et al., *Proc. Natl. Acad. Sci. (USA)*, 76:3829 (1979). However, other methods for introducing DNA into cells, such as by nuclear microinjection, electroporation, bacterial protoplast fusion with intact cells, or polycations, e.g., polybrene, polyornithine, may also be used. For various techniques for transforming mammalian cells, see Keown et al., *Methods in Enzymology*, 185:527-537 (1990) and Mansour et al., *Nature*, 336:348-352 (1988).

[00302] In some embodiments, a polynucleotide is provided that encodes an anti-CTHRC1 T-cell engager of the present disclosure. In some embodiments, the polynucleotide encoding the anti-CTHRC1 T-cell engager can be incorporated into a vector suitable for expressing the anti-CTHRC1 T-cell engager in host cells.

[00303] The vector may, for example, be in the form of a plasmid, cosmid, viral particle, or phage. The appropriate nucleic acid sequence may be inserted into the vector by a variety of procedures. In general, DNA is inserted into an appropriate restriction endonuclease site(s) using techniques known in the art. Vector components generally include, but are not limited to, one or more of a signal sequence, an origin of replication, one or more marker genes, an enhancer element, a promoter, and a transcription termination sequence. Construction of suitable vectors containing one or more of these components employs standard ligation techniques which are known to the skilled artisan.

[00304] The anti-CTHRC1 T-cell engager may be produced recombinantly and can further include heterologous polypeptide, which may be a signal sequence or other polypeptide having a specific cleavage site at the N-terminus of the mature protein or polypeptide, or can be an affinity tag such as a 6xHis tag. In general, the signal sequence may be a component of the vector,

or it may be a part of the anti-CTHRC1 T-cell engager -encoding DNA that is inserted into the vector. The signal sequence may be a prokaryotic signal sequence selected, for example, from the group of the alkaline phosphatase, penicillinase, lpp, or heat-stable enterotoxin II leaders. For yeast secretion the signal sequence may be, e.g., the yeast invertase leader, alpha factor leader (including *Saccharomyces* and *Kluyveromyces* α -factor leaders, the latter described in U.S. Patent No. 5,010,182), or acid phosphatase leader, the *C. albicans* glucoamylase leader (EP 362,179 published 4 April 1990), or the signal described in WO 90/13646 published 15 November 1990. In mammalian cell expression, mammalian signal sequences may be used to direct secretion of the protein, such as signal sequences from secreted polypeptides of the same or related species, as well as viral secretory leaders.

[00305] For recombinant production of a T-cell engager of the invention, the nucleic acid (e.g., cDNA or genomic DNA) encoding it is isolated and inserted into a replicable vector for further cloning (amplification of the DNA) or for expression. DNA encoding the T-cell engager is readily isolated and sequenced using conventional procedures (e.g., by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of the antibody). Many vectors are available. The choice of vector depends in part on the host cell to be used. Generally, preferred host cells are of either prokaryotic or eukaryotic (generally mammalian) origin. Suitable host cells for cloning or expressing the DNA in the vectors herein include prokaryote, yeast, or higher eukaryote cells.

[00306] The vector may, for example, be in the form of a plasmid, cosmid, viral particle, or phage. The appropriate nucleic acid sequence may be inserted into the vector by a variety of procedures. In general, DNA is inserted into an appropriate restriction endonuclease site(s) using techniques known in the art. Vector components generally include, but are not limited to, one or more of a signal sequence, an origin of replication, one or more marker genes, an enhancer element, a promoter, and a transcription termination sequence. Construction of suitable vectors containing one or more of these components employs standard ligation techniques which are known to the skilled artisan.

[00307] An anti-CTHRC1 T-cell engager may be produced recombinantly not only directly, but also as a fusion polypeptide with a heterologous polypeptide, which may be a signal sequence or other polypeptide having a specific cleavage site at the N-terminus of the mature protein or

polypeptide. In general, the signal sequence may be a component of the vector, or it may be a part of the anti-CTHRC1 T-cell engager -encoding DNA that is inserted into the vector. The signal sequence may be a prokaryotic signal sequence selected, for example, from the group of the alkaline phosphatase, penicillinase, lpp, or heat-stable enterotoxin II leaders. For yeast secretion the signal sequence may be, e.g., the yeast invertase leader, alpha factor leader (including *Saccharomyces* and *Kluyveromyces* α -factor leaders, the latter described in U.S. Patent No. 5,010,182), or acid phosphatase leader, the *C. albicans* glucoamylase leader (EP 362,179 published 4 April 1990), or the signal described in WO 90/13646 published 15 November 1990. In mammalian cell expression, mammalian signal sequences may be used to direct secretion of the protein, such as signal sequences from secreted polypeptides of the same or related species, as well as viral secretory leaders. In embodiments, the anti-CTHRC1 T-cell engager can be produced with a tag, such as a 6x His tag.

Vectors

[00308] The nucleic acid sequences coding for the desired molecules can be obtained using recombinant methods known in the art, such as, for example by screening libraries from cells expressing the gene, by deriving the gene from a vector known to include the same, or by isolating directly from cells and tissues containing the same, using standard techniques. Alternatively, the gene of interest can be produced synthetically, rather than cloned.

[00309] The present invention also provides vectors in which a DNA of the present invention is inserted. Vectors derived from retroviruses such as the lentivirus are suitable tools to achieve long-term gene transfer since they allow long-term, stable integration of a transgene and its propagation in daughter cells. Lentiviral vectors have the added advantage over vectors derived from onco-retroviruses such as murine leukemia viruses in that they can transduce non-proliferating cells, such as hepatocytes. They also have the added advantage of low immunogenicity.

[00310] In brief summary, the expression of natural or synthetic nucleic acids encoding CARs is typically achieved by operably linking a nucleic acid encoding the anti-CTHRC1 T-cell engager or portions thereof to a promoter and incorporating the construct into an expression vector. The vectors can be suitable for replication and integration eukaryotes. Typical cloning

vectors contain transcription and translation terminators, initiation sequences, and promoters useful for regulation of the expression of the desired nucleic acid sequence.

[00311] In addition to the methods described above, the following methods may be used.

[00312] The expression constructs of the present invention may also be used for nucleic acid immunization and gene therapy, using standard gene delivery protocols. Methods for gene delivery are known in the art (e.g., U.S. Pat. Nos. 5,399,346, 5,580,859, 5,589,466, incorporated by reference herein in their entireties). In another embodiment, the invention provides a gene therapy vector.

[00313] The nucleic acid can be cloned into a number of types of vectors. For example, the nucleic acid can be cloned into a vector including, but not limited to a plasmid, a phagemid, a phage derivative, an animal virus, and a cosmid. Vectors of particular interest include expression vectors, replication vectors, probe generation vectors, and sequencing vectors.

[00314] Further, the expression vector may be provided to a cell in the form of a viral vector. Viral vector technology is well known in the art and is described, for example, in Sambrook et al. (2001, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, New York), and in other virology and molecular biology manuals. Viruses, which are useful as vectors include, but are not limited to, retroviruses, adenoviruses, adeno-associated viruses, herpes viruses, and lentiviruses. In general, a suitable vector contains an origin of replication functional in at least one organism, a promoter sequence, convenient restriction endonuclease sites, and one or more selectable markers, (e.g., WO 01/96584; WO 01/29058; and U.S. Pat. No. 6,326,193).

[00315] A number of viral based systems have been developed for gene transfer into mammalian cells. For example, retroviruses provide a convenient platform for gene delivery systems. A selected gene can be inserted into a vector and packaged in retroviral particles using techniques known in the art. The recombinant virus can then be isolated and delivered to cells of the subject either in vivo or ex vivo. A number of retroviral systems are known in the art. In some embodiments, adenovirus vectors are used. A number of adenovirus vectors are known in the art. In one embodiment, lentivirus vectors are used.

[00316] Additional promoter elements, e.g., enhancers, regulate the frequency of transcriptional initiation. Typically, these are located in the region 30-110 bp upstream of the start site, although

a number of promoters have recently been shown to contain functional elements downstream of the start site as well. The spacing between promoter elements frequently is flexible, so that promoter function is preserved when elements are inverted or moved relative to one another. In the thymidine kinase (tk) promoter, the spacing between promoter elements can be increased to 50 bp apart before activity begins to decline. Depending on the promoter, it appears that individual elements can function either cooperatively or independently to activate transcription.

[00317] One example of a suitable promoter is the immediate early cytomegalovirus (CMV) promoter sequence. This promoter sequence is a strong constitutive promoter sequence capable of driving high levels of expression of any polynucleotide sequence operatively linked thereto. Another example of a suitable promoter is Elongation Growth Factor-1 α (EF-1 α). However, other constitutive promoter sequences may also be used, including, but not limited to the simian virus 40 (SV40) early promoter, mouse mammary tumor virus (MMTV), human immunodeficiency virus (HIV) long terminal repeat (LTR) promoter, MoMuLV promoter, an avian leukemia virus promoter, an Epstein-Barr virus immediate early promoter, a Rous sarcoma virus promoter, as well as human gene promoters such as, but not limited to, the actin promoter, the myosin promoter, the hemoglobin promoter, and the creatine kinase promoter. Further, the invention should not be limited to the use of constitutive promoters. Inducible promoters are also contemplated as part of the invention. The use of an inducible promoter provides a molecular switch capable of turning on expression of the polynucleotide sequence which it is operatively linked when such expression is desired or turning off the expression when expression is not desired. Examples of inducible promoters include, but are not limited to a metallothioneine promoter, a glucocorticoid promoter, a progesterone promoter, and a tetracycline promoter.

[00318] In order to assess the expression of an anti-CTHRC1 T-cell engager or portions thereof, the expression vector to be introduced into a cell can also contain either a selectable marker gene or a reporter gene or both to facilitate identification and selection of expressing cells from the population of cells sought to be transfected or infected through viral vectors. In other aspects, the selectable marker may be carried on a separate piece of DNA and used in a co-transfection procedure. Both selectable markers and reporter genes may be flanked with appropriate regulatory sequences to enable expression in the host cells. Useful selectable markers include, for example, antibiotic-resistance genes, such as neo and the like.

[00319] Reporter genes are used for identifying potentially transfected cells and for evaluating the functionality of regulatory sequences. In general, a reporter gene is a gene that is not present in or expressed by the recipient organism or tissue and that encodes a polypeptide whose expression is manifested by some easily detectable property, e.g., enzymatic activity. Expression of the reporter gene is assayed at a suitable time after the DNA has been introduced into the recipient cells. Suitable reporter genes may include genes encoding luciferase, beta-galactosidase, chloramphenicol acetyl transferase, secreted alkaline phosphatase, or the green fluorescent protein gene (e.g., Ui-Tei et al., 2000 *FEBS Letters* 479: 79-82). Suitable expression systems are well known and may be prepared using known techniques or obtained commercially. In general, the construct with the minimal 5' flanking region showing the highest level of expression of reporter gene is identified as the promoter. Such promoter regions may be linked to a reporter gene and used to evaluate agents for the ability to modulate promoter-driven transcription.

[00320] Methods of introducing and expressing genes into a cell are known in the art. In the context of an expression vector, the vector can be readily introduced into a host cell, e.g., mammalian, bacterial, yeast, or insect cell by any method in the art. For example, the expression vector can be transferred into a host cell by physical, chemical, or biological means.

[00321] Physical methods for introducing a polynucleotide into a host cell include calcium phosphate precipitation, lipofection, particle bombardment, microinjection, electroporation, and the like. Methods for producing cells comprising vectors and/or exogenous nucleic acids are well-known in the art. See, for example, Sambrook et al. (2001, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, New York). One method for the introduction of a polynucleotide into a host cell is calcium phosphate transfection.

[00322] Biological methods for introducing a polynucleotide of interest into a host cell include the use of DNA and RNA vectors. Viral vectors, and especially retroviral vectors, have become the most widely used method for inserting genes into mammalian, e.g., human cells. Other viral vectors can be derived from lentivirus, poxviruses, herpes simplex virus I, adenoviruses and adeno-associated viruses, and the like. See, for example, U.S. Pat. Nos. 5,350,674 and 5,585,362.

[00323] Chemical means for introducing a polynucleotide into a host cell include colloidal dispersion systems, such as macromolecule complexes, nanocapsules, microspheres, beads, and

lipid-based systems including oil-in-water emulsions, micelles, mixed micelles, and liposomes. An exemplary colloidal system for use as a delivery vehicle in vitro and in vivo is a liposome (e.g., an artificial membrane vesicle). In the case where a non-viral delivery system is utilized, an exemplary delivery vehicle is a liposome. The use of lipid formulations is contemplated for the introduction of the nucleic acids into a host cell (in vitro, ex vivo or in vivo). In another aspect, the nucleic acid may be associated with a lipid. The nucleic acid associated with a lipid may be encapsulated in the aqueous interior of a liposome, interspersed within the lipid bilayer of a liposome, attached to a liposome via a linking molecule that is associated with both the liposome and the oligonucleotide, entrapped in a liposome, complexed with a liposome, dispersed in a solution containing a lipid, mixed with a lipid, combined with a lipid, contained as a suspension in a lipid, contained or complexed with a micelle, or otherwise associated with a lipid. Lipid, lipid/DNA or lipid/expression vector associated compositions are not limited to any particular structure in solution. For example, they may be present in a bilayer structure, as micelles, or with a “collapsed” structure. They may also simply be interspersed in a solution, possibly forming aggregates that are not uniform in size or shape. Lipids are fatty substances which may be naturally occurring or synthetic lipids. For example, lipids include the fatty droplets that naturally occur in the cytoplasm as well as the class of compounds which contain long-chain aliphatic hydrocarbons and their derivatives, such as fatty acids, alcohols, amines, amino alcohols, and aldehydes.

[00324] Lipids suitable for use can be obtained from commercial sources. For example, dimyristyl phosphatidylcholine (“DMPC”) can be obtained from Sigma, St. Louis, Mo.; dicetyl phosphate (“DCP”) can be obtained from K & K Laboratories (Plainview, N.Y.); cholesterol (“Choi”) can be obtained from Calbiochem-Behring; dimyristyl phosphatidylglycerol (“DMPG”) and other lipids may be obtained from Avanti Polar Lipids, Inc. (Birmingham, AL). Stock solutions of lipids in chloroform or chloroform/methanol can be stored at about -20.degree. C. Chloroform is used as the only solvent since it is more readily evaporated than methanol. “Liposome” is a generic term encompassing a variety of single and multilamellar lipid vehicles formed by the generation of enclosed lipid bilayers or aggregates. Liposomes can be characterized as having vesicular structures with a phospholipid bilayer membrane and an inner aqueous medium. Multilamellar liposomes have multiple lipid layers separated by aqueous medium. They form spontaneously when phospholipids are suspended in an excess of aqueous

solution. The lipid components undergo self-rearrangement before the formation of closed structures and entrap water and dissolved solutes between the lipid bilayers (Ghosh et al., 1991 *Glycobiology* 5: 505-10). However, compositions that have different structures in solution than the normal vesicular structure are also encompassed. For example, the lipids may assume a micellar structure or merely exist as nonuniform aggregates of lipid molecules. Also contemplated are lipofectamine-nucleic acid complexes.

[00325] Regardless of the method used to introduce exogenous nucleic acids into a host cell or otherwise expose a cell to the inhibitor of the present invention, in order to confirm the presence of the recombinant DNA sequence in the host cell, a variety of assays may be performed. Such assays include, for example, “molecular biological” assays well known to those of skill in the art, such as Southern and Northern blotting, RT-PCR and PCR; “biochemical” assays, such as detecting the presence or absence of a particular peptide, e.g., by immunological means (ELISAs and Western blots) or by assays described herein to identify agents falling within the scope of the invention.

Prokaryotic Host Cells

[00326] Polynucleotide sequences encoding polypeptide components of the antibody of the invention can be obtained using standard recombinant techniques. Desired polynucleotide sequences may be isolated and sequenced from antibody producing cells such as hybridoma cells. Alternatively, polynucleotides can be synthesized using nucleotide synthesizer or PCR techniques. Once obtained, sequences encoding the polypeptides are inserted into a recombinant vector capable of replicating and expressing heterologous polynucleotides in prokaryotic hosts. Many vectors that are available and known in the art can be used for the purpose of the present invention. Selection of an appropriate vector will depend mainly on the size of the nucleic acids to be inserted into the vector and the particular host cell to be transformed with the vector. Each vector contains various components, depending on its function (amplification or expression of heterologous polynucleotide, or both) and its compatibility with the particular host cell in which it resides.

[00327] In general, plasmid vectors containing replicon and control sequences which are derived from species compatible with the host cell are used in connection with these hosts. Both expression and cloning vectors contain a nucleic acid sequence that enables the vector to

replicate in one or more selected host cells, as well as marking sequences which are capable of providing phenotypic selection in transformed cells. Such sequences are well known for a variety of bacteria, yeast, and viruses. The origin of replication from the plasmid pBR322, which contains genes encoding ampicillin (Amp) and tetracycline (Tet) resistance and thus provides easy means for identifying transformed cells, is suitable for most Gram-negative bacteria, the 2 μ plasmid origin is suitable for yeast, and various viral origins (SV40, polyoma, adenovirus, VSV or BPV) are useful for cloning vectors in mammalian cells. pBR322, its derivatives, or other microbial plasmids or bacteriophage may also contain, or be modified to contain, promoters which can be used by the microbial organism for expression of endogenous proteins. Examples of pBR322 derivatives used for expression of particular antibodies are described in detail in Carter et al., U.S. Patent No. 5,648,237.

[00328] In addition, phage vectors containing replicon and control sequences that are compatible with the host microorganism can be used as transforming vectors in connection with these hosts. For example, bacteriophage such as λ GEM™-11 may be utilized in making a recombinant vector which can be used to transform susceptible host cells such as *E. coli* LE392.

[00329] The expression vector of the invention may comprise two or more promoter-cistron pairs, encoding each of the polypeptide components. A promoter is an untranslated regulatory sequence located upstream (5') to a cistron that modulates its expression. Prokaryotic promoters typically fall into two classes, inducible and constitutive. Inducible promoter is a promoter that initiates increased levels of transcription of the cistron under its control in response to changes in the culture condition, e.g., the presence or absence of a nutrient or a change in temperature.

[00330] A large number of promoters recognized by a variety of potential host cells are well known. The selected promoter can be operably linked to cistron DNA encoding the light or heavy chain by removing the promoter from the source DNA via restriction enzyme digestion and inserting the isolated promoter sequence into the vector of the invention. Both the native promoter sequence and many heterologous promoters may be used to direct amplification and/or expression of the target genes. In some embodiments, heterologous promoters are utilized, as they generally permit greater transcription and higher yields of expressed target gene as compared to the native target polypeptide promoter.

[00331] Promoters recognized by a variety of potential host cells are well known. Promoters suitable for use with prokaryotic hosts include the PhoA promoter, the β -galactamase and lactose promoter systems (Chang et al., *Nature*, 275:615 (1978); Goeddel et al., *Nature*, 281:544 (1979)), alkaline phosphatase, a tryptophan (trp) promoter system (Goeddel, *Nucleic Acids Res.*, 8:4057 (1980); EP 36,776) and hybrid promoters such as the tac (deBoer et al., *Proc. Natl. Acad. Sci. USA*, 80:21-25 (1983)) or the trc promoter. Promoters for use in bacterial systems also will contain a Shine-Dalgarno (S.D.) sequence operably linked to the DNA encoding anti-CTHRC1 antibody. However, other promoters that are functional in bacteria (such as other known bacterial or phage promoters) are suitable as well. Their nucleotide sequences have been published, thereby enabling a skilled worker operably to ligate them to cistrons encoding the target light and heavy chains (Siebenlist et al. (1980) *Cell* 20: 269) using linkers or adaptors to supply any required restriction sites.

[00332] In one aspect of the invention, each cistron within the recombinant vector comprises a secretion signal sequence component that directs translocation of the expressed polypeptides across a membrane. In general, the signal sequence may be a component of the vector, or it may be a part of the target polypeptide DNA that is inserted into the vector. The signal sequence selected for the purpose of this invention should be one that is recognized and processed (i.e. cleaved by a signal peptidase) by the host cell. For prokaryotic host cells that do not recognize and process the signal sequences native to the heterologous polypeptides, the signal sequence is substituted by a prokaryotic signal sequence selected, for example, from the group consisting of the alkaline phosphatase, penicillinase, Ipp, or heat-stable enterotoxin II (STII) leaders, LamB, PhoE, PelB, OmpA and MBP. In one embodiment of the invention, the signal sequences used in both cistrons of the expression system are STII signal sequences or variants thereof.

[00333] In another aspect, the production of the immunoglobulins according to the invention can occur in the cytoplasm of the host cell, and therefore does not require the presence of secretion signal sequences within each cistron. In that regard, immunoglobulin light and heavy chains are expressed, folded and assembled to form functional immunoglobulins within the cytoplasm. Certain host strains (e.g., the *E. coli* trxB- strains) provide cytoplasm conditions that are favorable for disulfide bond formation, thereby permitting proper folding and assembly of expressed protein subunits. Proba and Pluckthun *Gene*, 159:203 (1995).

[00334] The present invention provides an expression system in which the quantitative ratio of expressed polypeptide components can be modulated in order to maximize the yield of secreted and properly assembled T-cell engagers of the invention. Such modulation is accomplished at least in part by simultaneously modulating translational strengths for the polypeptide components.

[00335] One technique for modulating translational strength is disclosed in Simmons et al., U.S. Pat. No. 5,840,523. It utilizes variants of the translational initiation region (TIR) within a cistron. For a given TIR, a series of amino acid or nucleic acid sequence variants can be created with a range of translational strengths, thereby providing a convenient means by which to adjust this factor for the desired expression level of the specific chain. TIR variants can be generated by conventional mutagenesis techniques that result in codon changes which can alter the amino acid sequence, although silent changes in the nucleotide sequence are preferred. Alterations in the TIR can include, for example, alterations in the number or spacing of Shine-Dalgarno sequences, along with alterations in the signal sequence. One method for generating mutant signal sequences is the generation of a "codon bank" at the beginning of a coding sequence that does not change the amino acid sequence of the signal sequence (i.e., the changes are silent). This can be accomplished by changing the third nucleotide position of each codon; additionally, some amino acids, such as leucine, serine, and arginine, have multiple first and second positions that can add complexity in making the bank. This method of mutagenesis is described in detail in Yansura et al. (1992) METHODS: A Companion to Methods in Enzymol. 4:151-158.

[00336] Preferably, a set of vectors is generated with a range of TIR strengths for each cistron therein. This limited set provides a comparison of expression levels of each chain as well as the yield of the desired antibody products under various TIR strength combinations. TIR strengths can be determined by quantifying the expression level of a reporter gene as described in detail in Simmons et al. U.S. Pat. No. 5, 840,523. Based on the translational strength comparison, the desired individual TIRs are selected to be combined in the expression vector constructs of the invention.

[00337] Suitable prokaryotes include but are not limited to archaeobacteria and eubacteria, such as Gram-negative or Gram-positive organisms, for example, Enterobacteriaceae such as E. coli. Various E. coli strains are publicly available, such as K12 strain MM294 (ATCC 31,446); X1776

(ATCC 31,537); W3110 (ATCC 27,325) and K5 772 (ATCC 53,635). Other suitable prokaryotic host cells include Enterobacteriaceae such as *Escherichia*, e.g., *E. coli*, *Enterobacter*, *Erwinia*, *Klebsiella*, *Proteus*, *Salmonella*, e.g., *Salmonella typhimurium*, *Serratia*, e.g., *Serratia marcescans*, and *Shigella*, as well as Bacilli such as *B. subtilis* and *B. licheniformis* (e.g., *B. licheniformis* 41P disclosed in DD 266,710 published 12 April 1989), *Pseudomonas* such as *P. aeruginosa*, *Rhizobia*, *Vitreoscilla*, *Paracoccus* and *Streptomyces*. These examples are illustrative rather than limiting. *E. coli* strain W3110 is one particularly preferred host or parent host because it is a common host strain for recombinant DNA product fermentations. Preferably, the host cell secretes minimal amounts of proteolytic enzymes. For example, strain W3110 (Bachmann, Cellular and Molecular Biology, vol. 2 (Washington, D.C.: American Society for Microbiology, 1987), pp. 1190-1219; ATCC Deposit No. 27,325) may be modified to effect a genetic mutation in the genes encoding proteins endogenous to the host, with examples of such hosts including *E. coli* W3110 strain 1A2, which has the complete genotype tonA ; *E. coli* W3110 strain 9E4, which has the complete genotype tonA ptr3; *E. coli* W3110 strain 27C7 (ATCC 55,244), which has the complete genotype tonA ptr3 phoA E15 (argF-lac)¹⁶⁹ degP ompT kanr; *E. coli* W3110 strain 37D6, which has the complete genotype tonA ptr3 phoA E15 (argF-lac)¹⁶⁹ degP ompT rbs7 ilvG kanr; *E. coli* W3110 strain 40B4, which is strain 37D6 with a non-kanamycin resistant degP deletion mutation; *E. coli* W3110 strain 33D3 having genotype W3110 ΔfhuA (ΔtonA) ptr3 lac Iq lacL8 ΔompTΔ(nmpc-fepE) degP41 kanR (U.S. Pat. No. 5,639,635) and an *E. coli* strain having mutant periplasmic protease disclosed in U.S. Patent No. 4,946,783 issued 7 August 1990. Other strains and derivatives thereof, such as *E. coli* 294 (ATCC 31,446), *E. coli* B, *E. coli* λ 1776 (ATCC 31,537) and *E. coli* RV308 (ATCC 31,608) are also suitable. These examples are illustrative rather than limiting. Methods for constructing derivatives of any of the above-mentioned bacteria having defined genotypes are known in the art and described in, for example, Bass et al., Proteins, 8:309-314 (1990). It is generally necessary to select the appropriate bacteria taking into consideration replicability of the replicon in the cells of a bacterium. For example, *E. coli*, *Serratia*, or *Salmonella* species can be suitably used as the host when well known plasmids such as pBR322, pBR325, pACYC177, or pKN410 are used to supply the replicon. Typically the host cell should secrete minimal amounts of proteolytic enzymes, and additional protease inhibitors may desirably be incorporated in the cell culture.

Alternatively, in vitro methods of cloning, e.g., PCR or other nucleic acid polymerase reactions, are suitable.

[00338] Antibody T-cell engager can be produced in bacteria, in particular when glycosylation and Fc effector function are not needed. Full length antibodies have greater half life in circulation. Production in *E. coli* is faster and more cost efficient. For expression of antibody fragments and polypeptides in bacteria, see, e.g., U.S. 5,648,237; U.S. 5,789,199 and U.S. 5,840,523, which describe translation initiation region (TIR) and signal sequences for optimizing expression and secretion, these patents incorporated herein by reference. After expression, the T-cell engager is isolated from the *E. coli* cell paste in a soluble fraction and can be purified through, e.g., a protein A or G column depending on the isotype. Final purification can be carried out similar to the process for purifying antibody expressed e.g., in CHO cells.

Eukaryotic Host Cells

[00339] In addition to prokaryotes, eukaryotic microbes such as filamentous fungi or yeast are suitable cloning or expression hosts for anti-CTHRC1 T-cell engager-encoding vectors. *Saccharomyces cerevisiae* is a commonly used lower eukaryotic host microorganism. Others include *Schizosaccharomyces pombe* (Beach and Nurse, *Nature*, 290: 140 (1981); EP 139,383 published 2 May 1985); *Kluyveromyces* hosts (U.S. Patent No. 4,943,529; Fleer et al., *Bio/Technology*, 9: 968-75 (1991)) such as, e.g., *K. lactis* (MW98-8C, CBS683, CBS4574; Louvencourt et al., *J. Bacteriol.*, 154(2):737-742 (1983)), *K. fragilis* (ATCC 12,424), *K. bulgaricus* (ATCC 16,045), *K. wickerhamii* (ATCC 24,178), *K. waltii* (ATCC 56,500), *K. drosophilum* (ATCC 36,906; Van den Berg et al., *Bio/Technology*, 8:135 (1990)), *K. thermotolerans*, and *K. marxianus*; *Yarrowia* (EP 402,226); *Pichia pastoris* (EP 183,070; Sreekrishna et al., *J. Basic Microbiol.*, 28:265-278 (1988)); *Candida*; *Trichoderma reesei* (EP 244,234); *Neurospora crassa* (Case et al., *Proc. Natl. Acad. Sci. USA*, 76:5259-5263 (1979)); *Schwanniomyces* such as *Schwanniomyces occidentalis* (EP 394,538 published 31 October 1990); and filamentous fungi such as, e.g., *Neurospora*, *Penicillium*, *Tolypocladium* (WO 91/00357 published 10 January 1991), and *Aspergillus* hosts such as *A. nidulans* (Ballance et al., *Biochem. Biophys. Res. Commun.*, 112:284-289 (1983); Tilburn et al., *Gene*, 26:205-221 (1983); Yelton et al., *Proc. Natl. Acad. Sci. USA*, 81: 1470-1474 (1984)) and *A. niger* (Kelly and Hynes, *EMBO J.*, 4:475-479 (1985)). Methylotrophic yeasts are suitable herein and include,

but are not limited to, yeast capable of growth on methanol selected from the genera consisting of *Hansenula*, *Candida*, *Kloeckera*, *Pichia*, *Saccharomyces*, *Torulopsis*, and *Rhodotorula*. A list of specific species that are exemplary of this class of yeasts may be found in C. Anthony, *The Biochemistry of Methylotrophs*, 269 (1982).

[00340] Suitable host cells for the expression of glycosylated T-cell engager are derived from multicellular organisms. Examples of invertebrate cells include insect cells such as *Drosophila* S2 and *Spodoptera* Sf9, as well as plant cells, such as cell cultures of cotton, corn, potato, soybean, petunia, tomato, and tobacco. Numerous baculoviral strains and variants and corresponding permissive insect host cells from hosts such as *Spodoptera frugiperda* (caterpillar), *Aedes aegypti* (mosquito), *Aedes albopictus* (mosquito), *Drosophila melanogaster* (fruitfly), and *Bombyx mori* have been identified. A variety of viral strains for transfection are publicly available, e.g., the L-1 variant of *Autographa californica* NPV and the Bm-5 strain of *Bombyx mori* NPV, and such viruses may be used as the virus herein according to the present invention, particularly for transfection of *Spodoptera frugiperda* cells.

[00341] However, interest has been greatest in vertebrate cells, and propagation of vertebrate cells in culture (tissue culture) has become a routine procedure. Examples of useful mammalian host cell lines are monkey kidney CV1 line transformed by SV40 (COS-7, ATCC CRL 1651); human embryonic kidney line (293 or 293 cells subcloned for growth in suspension culture, Graham et al., *J. Gen Virol.* 36:59 (1977)); baby hamster kidney cells (BHK, ATCC CCL 10); Chinese hamster ovary cells/-DHFR (CHO, Urlaub et al., *Proc. Natl. Acad. Sci. USA* 77:4216 (1980)); mouse sertoli cells (TM4, Mather, *Biol. Reprod.* 23:243-251 (1980)); monkey kidney cells (CV1 ATCC CCL 70); African green monkey kidney cells (VERO-76, ATCC CRL-1587); human cervical carcinoma cells (HELA, ATCC CCL 2); canine kidney cells (MDCK, ATCC CCL 34); buffalo rat liver cells (BRL 3A, ATCC CRL 1442); human lung cells (W138, ATCC CCL 75); human liver cells (Hep G2, HB 8065); mouse mammary tumor (MMT 060562, ATCC CCL51); TRI cells (Mather et al., *Annals N.Y. Acad. Sci.* 383:44-68 (1982)); MRC 5 cells; FS4 cells; and a human hepatoma line (Hep G2).

[00342] The vector components generally include, but are not limited to, one or more of the following: a signal sequence, an origin of replication, one or more marker genes, an enhancer element, a promoter, and a transcription termination sequence.

Signal Sequence Component

[00343] A vector for use in a eukaryotic host cell may also contain a signal sequence or other polypeptide having a specific cleavage site at the N-terminus of the mature protein or polypeptide of interest. The heterologous signal sequence selected preferably is one that is recognized and processed (i.e., cleaved by a signal peptidase) by the host cell. In mammalian cell expression, mammalian signal sequences as well as viral secretory leaders, for example, the herpes simplex gD signal, are available.

[00344] The DNA for such precursor region is ligated in reading frame to DNA encoding the T-cell engager.

Origin of Replication

[00345] Generally, an origin of replication component is not needed for mammalian expression vectors. For example, the SV40 origin may typically be used only because it contains the early promoter.

Selection Gene Component

[00346] Expression and cloning vectors will typically contain a selection gene, also termed a selectable marker. Typical selection genes encode proteins that (a) confer resistance to antibiotics or other toxins, e.g., ampicillin, neomycin, methotrexate, or tetracycline, (b) complement auxotrophic deficiencies, or (c) supply critical nutrients not available from complex media, e.g., the gene encoding D-alanine racemase for Bacilli.

[00347] One example of a selection scheme utilizes a drug to arrest growth of a host cell. Those cells that are successfully transformed with a heterologous gene produce a protein conferring drug resistance and thus survive the selection regimen. Examples of such dominant selection use the drugs neomycin, mycophenolic acid and hygromycin.

[00348] An example of suitable selectable markers for mammalian cells are those that enable the identification of cells competent to take up the anti-CTHRC1 T-cell engager-encoding nucleic acid, such as DHFR or thymidine kinase, metallothionein-I and -II, preferably primate metallothionein genes, adenosine deaminase, ornithine decarboxylase, etc. An appropriate host cell when wild-type DHFR is employed is the CHO cell line deficient in DHFR activity (e.g., ATCC CRL-9096), prepared and propagated as described by Urlaub et al., Proc. Natl. Acad. Sci.

USA, 77:4216 (1980). For example, cells transformed with the DHFR selection gene are first identified by culturing all of the transformants in a culture medium that contains methotrexate (Mtx), a competitive antagonist of DHFR. Alternatively, host cells (particularly wild-type hosts that contain endogenous DHFR) transformed or co-transformed with DNA sequences encoding an anti-CTHRC1 T-cell engager, wild-type DHFR protein, and another selectable marker such as aminoglycoside 3'-phosphotransferase (APH) can be selected by cell growth in medium containing a selection agent for the selectable marker such as an aminoglycosidic antibiotic, e.g., kanamycin, neomycin, or G418. See U.S. Patent No. 4,965,199.

[00349] A suitable selection gene for use in yeast is the *trp1* gene present in the yeast plasmid YRp7 (Stinchcomb et al., *Nature*, 282:39 (1979); Kingsman et al., *Gene*, 7:141 (1979); Tschemper et al., *Gene*, 10:157 (1980)). The *trp1* gene provides a selection marker for a mutant strain of yeast lacking the ability to grow in tryptophan, for example, ATCC No. 44076 or PEP4-1 (Jones, *Genetics*, 85:12 (1977)).

Promoter Component

[00350] Expression and cloning vectors usually contain a promoter operably linked to the anti-CTHRC1 T-cell engager-encoding nucleic acid sequence to direct mRNA synthesis. Promoters recognized by a variety of potential host cells are well known.

[00351] Virtually all eukaryotic genes have an AT-rich region located approximately 25 to 30 bases upstream from the site where transcription is initiated. Another sequence found 70 to 80 bases upstream from the start of transcription of many genes is a CNCAAT region where N may be any nucleotide. At the 3' end of most eukaryotic genes is an AATAAA sequence that may be the signal for addition of the poly A tail to the 3' end of the coding sequence. All of these sequences are suitably inserted into eukaryotic expression vectors.

[00352] Examples of suitable promoting sequences for use with yeast hosts include the promoters for 3-phosphoglycerate kinase (Hitzeman et al., *J. Biol. Chem.*, 255:2073 (1980)) or other glycolytic enzymes (Hess et al., *J. Adv. Enzyme Reg.*, 7:149 (1968); Holland, *Biochemistry*, 17:4900 (1978)), such as enolase, glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase, and glucokinase.

[00353] Other yeast promoters, which are inducible promoters having the additional advantage of transcription controlled by growth conditions, are the promoter regions for alcohol dehydrogenase 2, isocytochrome C, acid phosphatase, degradative enzymes associated with nitrogen metabolism, metallothionein, glyceraldehyde-3-phosphate dehydrogenase, and enzymes responsible for maltose and galactose utilization. Suitable vectors and promoters for use in yeast expression are further described in EP 73,657.

[00354] Anti-CTHRC1 T-cell engager transcription from vectors in mammalian host cells is controlled, for example, by promoters obtained from the genomes of viruses such as polyoma virus, fowlpox virus (UK 2,211,504 published 5 July 1989), adenovirus (such as Adenovirus 2), bovine papilloma virus, avian sarcoma virus, cytomegalovirus, a retrovirus, hepatitis-B virus and Simian Virus 40 (SV40), from heterologous mammalian promoters, e.g., the actin promoter or an immunoglobulin promoter, and from heat-shock promoters, provided such promoters are compatible with the host cell systems.

[00355] The early and late promoters of the SV40 virus are conveniently obtained as an SV40 restriction fragment that also contains the SV40 viral origin of replication. The immediate early promoter of the human cytomegalovirus is conveniently obtained as a HindIII E restriction fragment. A system for expressing DNA in mammalian hosts using the bovine papilloma virus as a vector is disclosed in U.S. Patent No. 4,419,446. A modification of this system is described in U.S. Patent No. 4,601,978. See also Reyes et al., Nature 297:598-601 (1982) on expression of human β -interferon cDNA in mouse cells under the control of a thymidine kinase promoter from herpes simplex virus. Alternatively, the Rous Sarcoma Virus long terminal repeat can be used as the promoter.

Enhancer Element Component

[00356] Transcription of a DNA encoding the anti-CTHRC1 T-cell engager by higher eukaryotes may be increased by inserting an enhancer sequence into the vector. Enhancers are cis-acting elements of DNA, usually about from 10 to 300 bp, that act on a promoter to increase its transcription. Many enhancer sequences are now known from mammalian genes (globin, elastase, albumin, α -fetoprotein, and insulin). Typically, however, one will use an enhancer from a eukaryotic cell virus. Examples include the SV40 enhancer on the late side of the replication origin (bp 100-270), the cytomegalovirus early promoter enhancer, the polyoma enhancer on the

late side of the replication origin, and adenovirus enhancers. See also Yaniv, Nature 297:17-18 (1982) on enhancing elements for activation of eukaryotic promoters. The enhancer may be spliced into the vector at a position 5' or 3' to the anti-CTHRC1 T-cell engager coding sequence, but is preferably located at a site 5' from the promoter.

Transcription Termination Component

[00357] Expression vectors used in eukaryotic host cells (yeast, fungi, insect, plant, animal, human, or nucleated cells from other multicellular organisms) will also contain sequences necessary for the termination of transcription and for stabilizing the mRNA. Such sequences are commonly available from the 5' and, occasionally 3', untranslated regions of eukaryotic or viral DNAs or cDNAs. These regions contain nucleotide segments transcribed as polyadenylated fragments in the untranslated portion of the mRNA encoding anti-CTHRC1 T-cell engager. One useful transcription termination component is the bovine growth hormone polyadenylation region. See WO94/11026 and the expression vector disclosed therein.

[00358] Still other methods, vectors, and host cells suitable for adaptation to the synthesis of anti-CTHRC1 T-cell engager in recombinant vertebrate cell culture are described in Gething et al., Nature, 293:620-625 (1981); Mantei et al., Nature, 281:40-46 (1979); EP 117,060; and EP 117,058.

[00359] Host cells are transformed with the above-described expression or cloning vectors for anti-CTHRC1 T-cell engager production and cultured in conventional nutrient media modified as appropriate for inducing promoters, selecting transformants, or amplifying the genes encoding the desired sequences.

Culturing the Host Cells

[00360] The host cells used to produce the anti-CTHRC1 T-cell engager of this invention may be cultured in a variety of media.

Prokaryotic Host Cells

[00361] Prokaryotic cells used to produce the polypeptides of the invention are grown in media known in the art and suitable for culture of the selected host cells. Examples of suitable media include luria broth (LB) plus necessary nutrient supplements. In some embodiments, the media also contains a selection agent, chosen based on the construction of the expression vector, to

selectively permit growth of prokaryotic cells containing the expression vector. For example, ampicillin is added to media for growth of cells expressing ampicillin resistant gene.

[00362] Any necessary supplements besides carbon, nitrogen, and inorganic phosphate sources may also be included at appropriate concentrations introduced alone or as a mixture with another supplement or medium such as a complex nitrogen source. Optionally the culture medium may contain one or more reducing agents selected from the group consisting of glutathione, cysteine, cystamine, thioglycollate, dithioerythritol and dithiothreitol.

[00363] The prokaryotic host cells are cultured at suitable temperatures. For *E. coli* growth, for example, the preferred temperature ranges from about 20°C to about 39°C, more preferably from about 25°C to about 37°C, even more preferably at about 30°C. The pH of the medium may be any pH ranging from about 5 to about 9, depending mainly on the host organism. For *E. coli*, the pH is preferably from about 6.8 to about 7.4, and more preferably about 7.0.

[00364] If an inducible promoter is used in the expression vector of the invention, protein expression is induced under conditions suitable for the activation of the promoter. In one aspect of the invention, PhoA promoters are used for controlling transcription of the polypeptides. Accordingly, the transformed host cells are cultured in a phosphate-limiting medium for induction. In some embodiments, the phosphate-limiting medium is the C.R.A.P medium (see, e.g., Simmons et al., *J. Immunol. Methods* (2002), 263: 133-47). A variety of other inducers may be used, according to the vector construct employed, as is known in the art.

[00365] In one embodiment, the expressed polypeptides of the present invention are secreted into and recovered from the periplasm of the host cells. Protein recovery typically involves disrupting the microorganism, generally by such means as osmotic shock, sonication or lysis. Once cells are disrupted, cell debris or whole cells may be removed by centrifugation or filtration. The proteins may be further purified, for example, by affinity resin chromatography. Alternatively, proteins can be transported into the culture media and isolated therein. Cells may be removed from the culture and the culture supernatant being filtered and concentrated for further purification of the proteins produced. The expressed polypeptides can be further isolated and identified using commonly known methods such as polyacrylamide gel electrophoresis (PAGE) and Western blot assay.

[00366] In one aspect of the invention, T-cell engager production is conducted in large quantity by a fermentation process. Various large-scale fed-batch fermentation procedures are available for production of recombinant proteins. Large-scale fermentations have at least 1000 liters of capacity, preferably about 1,000 to 100,000 liters of capacity. These fermentors use agitator impellers to distribute oxygen and nutrients, especially glucose (the preferred carbon/energy source). Small scale fermentation refers generally to fermentation in a fermentor that is no more than approximately 100 liters in volumetric capacity, and can range from about 1 liter to about 100 liters.

[00367] In a fermentation process, induction of protein expression is typically initiated after the cells have been grown under suitable conditions to a desired density, e.g., an OD₅₅₀ of about 180-220, at which stage the cells are in the early stationary phase. A variety of inducers may be used, according to the vector construct employed, as is known in the art and described above. Cells may be grown for shorter periods prior to induction. Cells are usually induced for about 12-50 hours, although longer or shorter induction time may be used.

[00368] To improve the production yield and quality of the polypeptides of the invention, various fermentation conditions can be modified. For example, to improve the proper assembly and folding of the secreted T-cell engager polypeptides, additional vectors overexpressing chaperone proteins, such as Dsb proteins (DsbA, DsbB, DsbC, DsbD and or DsbG) or FkpA (a peptidylprolyl cis,trans-isomerase with chaperone activity) can be used to co-transform the host prokaryotic cells. The chaperone proteins have been demonstrated to facilitate the proper folding and solubility of heterologous proteins produced in bacterial host cells. Chen et al. (1999) *J Bio Chem* 274: 19601-5; U.S. Patent No. 6,083,715; U.S. Patent No. 6,027,888; Bothmann and Pluckthun (2000) *J. Biol. Chem.* 275:17100-5; Ramm and Pluckthun (2000) *J. Biol. Chem.* 275:17106-13; Arie et al. (2001) *Mol. Microbiol.* 39:199-210.

[00369] To minimize proteolysis of expressed heterologous proteins (especially those that are proteolytically sensitive), certain host strains deficient for proteolytic enzymes can be used for the present invention. For example, host cell strains may be modified to effect genetic mutation(s) in the genes encoding known bacterial proteases such as Protease III, OmpT, DegP, Tsp, Protease I, Protease Mi, Protease V, Protease VI and combinations thereof. Some *E. coli* protease-deficient strains are available and described in, for example, Joly et al. (1998), supra;

U.S. Patent No. 5,264,365; U.S. Patent No. 5,508,192; Hara et al., *Microbial Drug Resistance*, 2:63-72 (1996).

[00370] In one embodiment, *E. coli* strains deficient for proteolytic enzymes and transformed with plasmids overexpressing one or more chaperone proteins are used as host cells in the expression system of the invention.

Eukaryotic Host Cells

[00371] Commercially available media such as Ham's F10 (Sigma), Minimal Essential Medium ((MEM), (Sigma), RPMI-1640 (Sigma), and Dulbecco's Modified Eagle Medium ((DMEM), Sigma) are suitable for culturing the host cells. In addition, any of the media described in Ham et al., *Meth. Enz.* 58: 44 (1979), Barnes et al., *Anal. Biochem.* 102: 255 (1980), U.S. Pat. Nos. 4,767,704; 4,657,866; 4,927,762; 4,560,655; or 5,122,469; WO 90/03430; WO 87/00195; or U.S. Patent Re. 30,985 may be used as culture media for the host cells. Any of these media may be supplemented as necessary with hormones and/or other growth factors (such as insulin, transferrin, or epidermal growth factor), salts (such as sodium chloride, calcium, magnesium, and phosphate), buffers (such as HEPES), nucleotides (such as adenosine and thymidine), antibiotics (such as GENTAMYCIN™ drug), trace elements (defined as inorganic compounds usually present at final concentrations in the micromolar range), and glucose or an equivalent energy source. Any other necessary supplements may also be included at appropriate concentrations that would be known to those skilled in the art. The culture conditions, such as temperature, pH, and the like, are those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan.

Detecting Gene Amplification/Expression

[00372] Gene amplification and/or expression may be measured in a sample directly, for example, by conventional Southern blotting, Northern blotting to quantitate the transcription of mRNA (Thomas, *Proc. Natl. Acad. Sci. USA*, 77: 5201-5 (1980)), dot blotting (DNA analysis), or in situ hybridization, using an appropriately labeled probe, based on the sequences provided herein. Alternatively, antibodies may be employed that can recognize specific duplexes, including DNA duplexes, RNA duplexes, and DNA-RNA hybrid duplexes or DNA-protein duplexes. The antibodies in turn may be labeled and the assay may be carried out where the

duplex is bound to a surface, so that upon the formation of duplex on the surface, the presence of antibody bound to the duplex can be detected.

[00373] Gene expression, alternatively, may be measured by immunological methods, such as immunohistochemical staining of cells or tissue sections and assay of cell culture or body fluids, to quantitate directly the expression of gene product. Antibodies useful for immunohistochemical staining and/or assay of sample fluids may be either monoclonal or polyclonal, and may be prepared in any mammal. Conveniently, the antibodies may be prepared against a native sequence CTHRC1 polypeptide or against a synthetic peptide based on the DNA sequences provided herein or against exogenous sequence fused to CTHRC1 DNA and encoding a specific antibody epitope.

Purification of anti-CTHRC1 T-cell engager

[00374] Forms of anti-CTHRC1 T-cell engager may be recovered from culture medium or from host cell lysates. If membrane-bound, it can be released from the membrane using a suitable detergent solution (e.g., Triton-X 100) or by enzymatic cleavage. Cells employed in expression of anti-CTHRC1 T-cell engager can be disrupted by various physical or chemical means, such as freeze-thaw cycling, sonication, mechanical disruption, or cell lysing agents.

[00375] It may be desired to purify anti-CTHRC1 T-cell engager from recombinant cell proteins or polypeptides. The following procedures are exemplary of suitable purification procedures: by fractionation on an ion-exchange column; ethanol precipitation; reverse phase HPLC; chromatography on silica or on a cation-exchange resin such as DEAE; chromatofocusing; SDS-PAGE; ammonium sulfate precipitation; gel filtration using, for example, Sephadex G-75; protein A Sepharose columns to remove contaminants such as IgG; and metal chelating columns to bind epitope-tagged forms of the anti-CTHRC1 T-cell engager. Various methods of protein purification may be employed and such methods are known in the art and described for example in Deutscher, *Methods in Enzymology*, 182 (1990); *Scopes, Protein Purification: Principles and Practice*, Springer-Verlag, New York (1982). The purification step(s) selected will depend, for example, on the nature of the production process used and the particular anti-CTHRC1 T-cell engager produced.

[00376] When using recombinant techniques, the T-cell engager can be produced intracellularly, in the periplasmic space, or directly secreted into the medium. If the T-cell

engager is produced intracellularly, as a first step, the particulate debris, either host cells or lysed fragments, are removed, for example, by centrifugation or ultrafiltration. Carter et al., *Bio/Technology* 10: 163-7 (1992) describe a procedure for isolating antibodies which are secreted to the periplasmic space of *E. coli*. Briefly, cell paste is thawed in the presence of sodium acetate (pH 3.5), EDTA, and phenylmethylsulfonylfluoride (PMSF) over about 30 min. Cell debris can be removed by centrifugation. Where the T-cell engager is secreted into the medium, supernatants from such expression systems are generally first concentrated using a commercially available protein concentration filter, for example, an Amicon or Millipore Pellicon ultrafiltration unit. A protease inhibitor such as PMSF may be included in any of the foregoing steps to inhibit proteolysis and antibiotics may be included to prevent the growth of adventitious contaminants.

[00377] The T-cell engager composition prepared from the cells can be purified using, for example, hydroxylapatite chromatography, gel electrophoresis, dialysis, and affinity chromatography, with affinity chromatography being the preferred purification technique. The suitability of protein A as an affinity ligand depends on the species and isotype of any immunoglobulin Fc domain that is present in the T-cell engager. Protein A can be used to purify antibodies that are based on human $\gamma 1$, $\gamma 2$ or $\gamma 4$ heavy chains (Lindmark et al., *J. Immunol. Meth.* 62: 1-13 (1983)). Protein G is recommended for all mouse isotypes and for human $\gamma 3$ (Guss et al., *EMBO J.* 5: 1567-1575 (1986)). The matrix to which the affinity ligand is attached is most often agarose, but other matrices are available. Mechanically stable matrices such as controlled pore glass or poly(styrenedivinyl)benzene allow for faster flow rates and shorter processing times than can be achieved with agarose. Where the CTHRC1 binding moiety comprises a CH3 domain, the Bakerbond ABX™ resin (J. T. Baker, Phillipsburg, NJ) is useful for purification. Other techniques for protein purification such as fractionation on an ion-exchange column, ethanol precipitation, Reverse Phase HPLC, chromatography on silica, chromatography on heparin SEPHAROSE™ chromatography on an anion or cation exchange resin (such as a polyaspartic acid column), chromatofocusing, SDS-PAGE, and ammonium sulfate precipitation are also available depending on the T-cell engager to be recovered.

[00378] Following any preliminary purification step(s), the mixture comprising the T-cell engager of interest and contaminants may be subjected to low pH hydrophobic interaction

chromatography using an elution buffer at a pH between about 2.5-4.5, and generally at low salt concentrations (e.g., from about 0-0.25M salt).

Assays

Activity Assays

[00379] In one aspect, assays are provided for identifying anti-CTHRC1 antibodies thereof having biological activity. Biological activity may include, e.g., the ability to inhibit cell growth or proliferation (e.g., “cell killing” activity), or the ability to induce cell death, including programmed cell death (apoptosis). Antibodies having such biological activity in vivo and/or in vitro are also provided.

[00380] In certain embodiments, an anti-CTHRC1 antibody is tested for its ability to inhibit cell growth or proliferation in vitro. Assays for inhibition of cell growth or proliferation are well known in the art. Certain assays for cell proliferation, exemplified by the “cell killing” assays described herein, measure cell viability. One such assay is the CellTiter-Glo™ Luminescent Cell Viability Assay, which is commercially available from Promega (Madison, WI). That assay determines the number of viable cells in culture based on quantitation of ATP present, which is an indication of metabolically active cells. See Crouch et al (1993) *J. Immunol. Meth.* 160: 81-8, US Pat. No. 6602677. The assay may be conducted in 96- or 384-well format, making it amenable to automated high-throughput screening (HTS) (see Cree et al (1995) *AntiCancer Drugs* 6: 398-404). The assay procedure involves adding a single reagent (CellTiter-Glo® Reagent) directly to cultured cells. This results in cell lysis and generation of a luminescent signal produced by a luciferase reaction. The luminescent signal is proportional to the amount of ATP present, which is directly proportional to the number of viable cells present in culture. Data can be recorded by luminometer or CCD camera imaging device. The luminescence output is expressed as relative light units (RLU).

[00381] Another assay for cell proliferation is the “MTT” assay, a colorimetric assay that measures the oxidation of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to formazan by mitochondrial reductase. Like the CellTiter-Glo™ assay, this assay indicates the number of metabolically active cells present in a cell culture (see, e.g., Mosmann (1983) *J. Immunol. Meth.* 65:55-63, and Zhang et al. (2005) *Cancer Res.* 65: 3877-82).

[00382] In one aspect, an anti-CTHRC1 antibody is tested for its ability to induce cell death in vitro. Assays for induction of cell death are well known in the art. In some embodiments, such assays measure, e.g., loss of membrane integrity as indicated by uptake of propidium iodide (PI), trypan blue (see Moore et al. *Cytotechnology*, 17: 1-11 (1995)), or 7AAD. In an exemplary PI uptake assay, cells are cultured in Dulbecco's Modified Eagle Medium (D-MEM):Ham's F-12 (50:50) supplemented with 10% heat-inactivated FBS (Hyclone) and 2 mM L-glutamine. Thus, the assay is performed in the absence of complement and 118mmunee effector cells. Cells are seeded at a density of 3×10^6 per dish in 100 x 20 mm dishes and allowed to attach overnight. The medium is removed and replaced with fresh medium alone or medium containing various concentrations of the antibody. The cells are incubated for a 3-day time period. Following treatment, monolayers are washed with PBS and detached by trypsinization. Cells are then centrifuged at 1200 rpm for 5 minutes at 4 °C, the pellet resuspended in 3 mL cold Ca²⁺ binding buffer (10 mM Hepes, pH 7.4, 140 mM NaCl, 2.5 mM CaCl₂) and aliquoted into 35 mm strainer-capped 12 x 75 mm tubes (1 mL per tube, 3 tubes per treatment group) for removal of cell clumps. Tubes then receive PI (10 µg/mL). Samples are analyzed using a FACSCAN™ flow cytometer and FACSCONVERT™ CellQuest software (Becton Dickinson). Antibodies which induce statistically significant levels of cell death as determined by PI uptake are thus identified.

[00383] In one aspect, an anti-CTHRC1 antibody is tested for its ability to induce apoptosis (programmed cell death) in vitro. An exemplary assay for antibodies that induce apoptosis is an annexin binding assay. In an exemplary annexin binding assay, cells are cultured and seeded in dishes as discussed in the preceding paragraph. The medium is removed and replaced with fresh medium alone or medium containing 0.001 to 10 µg/mL of the antibody. Following a three-day incubation period, monolayers are washed with PBS and detached by trypsinization. Cells are then centrifuged, resuspended in Ca²⁺ binding buffer, and aliquoted into tubes as discussed in the preceding paragraph. Tubes then receive labeled annexin (e.g., annexin V-FITC) (1 µg/mL). Samples are analyzed using a FACSCAN™ flow cytometer and FACSCONVERT™ CellQuest software (BD Biosciences). Antibodies that induce statistically significant levels of annexin binding relative to control are thus identified. Another exemplary assay for antibodies that induce apoptosis is a histone DNA ELISA colorimetric assay for detecting internucleosomal degradation of genomic DNA. Such an assay can be performed using, e.g., the Cell Death Detection ELISA kit (Roche, Palo Alto, CA).

[00384] Cells for use in any of the above in vitro assays include cells or cell lines that naturally express CTHRC1 or that have been engineered to express CTHRC1. Such cells include tumor cells that overexpress CTHRC1 relative to normal cells of the same tissue origin. Such cells also include cell lines (including tumor cell lines) that express CTHRC1 and cell lines that do not normally express CTHRC1 but have been transfected with nucleic acid encoding CTHRC1.

[00385] In one aspect, an anti-CTHRC1 antibody thereof is tested for its ability to inhibit cell growth or proliferation in vivo. In certain embodiments, an anti-CTHRC1 antibody thereof is tested for its ability to inhibit tumor growth in vivo. In vivo model systems, such as xenograft models, can be used for such testing. In an exemplary xenograft system, human tumor cells are introduced into a suitably immunocompromised non-human animal, e.g., a SCID mouse. An antibody of the invention is administered to the animal. The ability of the antibody to inhibit or decrease tumor growth is measured. In certain embodiments of the above xenograft system, the human tumor cells are tumor cells from a human patient. In certain embodiments, the human tumor cells are introduced into a suitably immunocompromised non-human animal by subcutaneous injection or by transplantation into a suitable site, such as a mammary fat pad.

Binding Assays and Other Assays

[00386] In one aspect, an anti-CTHRC1 antibody is tested for its antigen binding activity. For example, in certain embodiments, an anti-CTHRC1 antibody is tested for its ability to bind to CTHRC1 expressed on the surface of a cell. A FACS assay may be used for such testing.

[00387] In one aspect, competition assays may be used to identify a monoclonal antibody that competes with a monoclonal antibody comprising the variable domains of any one of SEQ ID NOs: 81-82 or a chimeric antibody comprising the variable domain of the monoclonal antibody comprising the sequences of Table 2 and Table 3, and constant domains from IgG1 or IgG4 for binding to CTHRC1. In certain embodiments, such a competing antibody binds to the same epitope (e.g., a linear or a conformational epitope) that is bound by a monoclonal antibody comprising the variable domains of any one of SEQ ID NOs: 81-82 or a chimeric antibody comprising the variable domain of the monoclonal antibody comprising the sequences of Table 2 and Table 3, and constant domains from IgG1 or IgG4. Exemplary competition assays include, but are not limited to, routine assays such as those provided in Harlow and Lane (1988) *Antibodies: A Laboratory Manual* ch.14 (Cold Spring Harbor Laboratory, Cold Spring Harbor,

NY). Detailed exemplary methods for mapping an epitope to which an antibody binds are provided in Morris (1996) "Epitope Mapping Protocols," in Methods in Molecular Biology vol. 66 (Humana Press, Totowa, NJ). Two antibodies are said to bind to the same epitope if each blocks binding of the other by 50% or more.

[00388] In one aspect, purified anti-CTHRC1 antibodies can be further characterized by a series of assays including, but not limited to, N-terminal sequencing, amino acid analysis, non-denaturing size exclusion high pressure liquid chromatography (HPLC), mass spectrometry, ion exchange chromatography and papain digestion.

Methods of Treatment

[00389] An anti-CTHRC1 T-cell engager of the invention may be used in, for example, in vitro, ex vivo, and in vivo therapeutic methods. In one aspect, the invention provides methods for inhibiting cell growth or proliferation, either in vivo or in vitro, the method comprising exposing a cell to anti-CTHRC1 T-cell engager or composition thereof of the present disclosure under conditions permissive for binding of the T-cell engager to CTHRC1. "Inhibiting cell growth or proliferation" means decreasing a cell's growth or proliferation by at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, or 100%, and includes inducing cell death. In certain embodiments, the cell is a tumor cell. The anti-CTHRC1 T-cell engager or composition thereof may additionally or alternatively (i) inhibit tumor metastasis in vivo; (ii) inhibit tumor growth in vivo; (iii) decrease tumor size in vivo; (iv) inhibit tumor vascularization in vivo; (v) exhibit cytotoxic activity on tumor cells and cancer associated fibroblasts expressing and/or displaying CTHRC1 in vivo; (vi) exhibit cytostatic activity on tumor cells or cancer associated fibroblasts expressing and/or displaying CTHRC1 in vivo; (vii) enhance infiltration of anti-tumor immune cells in vivo; or (viii) prevent suppression of immune-cells in the tumor microenvironment in vivo.

[00390] Cancers that may be treated include tumors that are not vascularized, or not yet substantially vascularized, as well as vascularized tumors. The cancers may comprise non-solid tumors (such as hematological tumors, for example, leukemias and lymphomas) or may comprise solid tumors. Types of cancers to be treated with the T-cell engagers of the invention include, but are not limited to, carcinoma, blastoma, and sarcoma, and certain leukemia or lymphoid malignancies, benign and malignant tumors, and malignancies e.g., sarcomas, carcinomas, and

melanomas. Adult tumors/cancers and pediatric tumors/cancers are also included. In certain embodiments, CAR T cells can be used therapeutically for patients suffering from non-hematological tumors such as solid tumors arising from breast, CNS, and skin malignancies.

[00391] Hematologic cancers are cancers of the blood or bone marrow. Examples of hematological (or hematogenous) cancers include leukemias, including acute leukemias (such as acute lymphocytic leukemia, acute myelocytic leukemia, acute myelogenous leukemia and myeloblastic, promyelocytic, myelomonocytic, monocytic and erythroleukemia), chronic leukemias (such as chronic myelocytic (granulocytic) leukemia, chronic myelogenous leukemia, and chronic lymphocytic leukemia), polycythemia vera, lymphoma, Hodgkin's disease, non-Hodgkin's lymphoma (indolent and high grade forms), multiple myeloma, Waldenstrom's macroglobulinemia, heavy chain disease, myelodysplastic syndrome, hairy cell leukemia and myelodysplasia.

[00392] Solid tumors are abnormal masses of tissue that usually do not contain cysts or liquid areas. Solid tumors can be benign or malignant. Different types of solid tumors are named for the type of cells that form them (such as sarcomas, carcinomas, and lymphomas). Examples of solid tumors, such as sarcomas and carcinomas, include fibrosarcoma, myxosarcoma, liposarcoma, chondrosarcoma, osteosarcoma, and other sarcomas, synovioma, mesothelioma, Ewing's tumor, leiomyosarcoma, rhabdomyosarcoma, colon carcinoma, lymphoid malignancy, pancreatic cancer, breast cancer, lung cancers, ovarian cancer, prostate cancer, hepatocellular carcinoma, squamous cell carcinoma, basal cell carcinoma, adenocarcinoma, sweat gland carcinoma, medullary thyroid carcinoma, papillary thyroid carcinoma, pheochromocytomas, sebaceous gland carcinoma, papillary carcinoma, papillary adenocarcinomas, medullary carcinoma, bronchogenic carcinoma, renal cell carcinoma, hepatoma, bile duct carcinoma, choriocarcinoma, Wilms' tumor, cervical cancer, testicular tumor, seminoma, bladder carcinoma, melanoma, and CNS tumors (such as a glioma (such as brainstem glioma and mixed gliomas), glioblastoma (also known as glioblastoma multiforme) astrocytoma, CNS lymphoma, germinoma, medulloblastoma, Schwannoma, craniopharyngioma, ependymoma, pinealoma, hemangioblastoma, acoustic neuroma, oligodendroglioma, meningioma, neuroblastoma, retinoblastoma and brain metastases).

[00393] In embodiments, the subject anti-CTHRC1 T-cell engagers find advantageous use in the treatment of breast, lung, ovarian, pancreatic, and colorectal cancers, including MSI and MSS colorectal cancer. In embodiments, the subject anti-CTHRC1 T-cell engagers find advantageous use in the treatment of breast, colon, pancreas, lung, stomach and liver cancers. See, e.g. Liu et al. CTHRC1, a novel gene with multiple functions in physiology, disease and solid tumors (Review), *Oncol Lett.* 2023 Jun; 25(6): 266

[00394] In some embodiments, a method of treating a cell proliferative disorder can include a step of administering to a subject a therapeutically effective amount of an anti-CTHRC1 T-cell engager or pharmaceutical composition thereof of any of the foregoing embodiments. In certain embodiments, the cell proliferative disorder is associated with increased expression, display and/or activity of CTHRC1. For example, in certain embodiments, the cell proliferative disorder is associated with increased expression and/or display of CTHRC1 on the surface of a cell, either directly or in a complex. In certain embodiments, the cell proliferative disorder is a tumor or a cancer. In certain embodiments, the T-cell engager can be administered at a dose of 0.001 mg/kg to about 100 mg/kg based on the patient's body weight.

[00395] In some embodiments, a method of treating cancer can include a step of administering to a subject a therapeutically effective amount of an anti-CTHRC1 T-cell engager or pharmaceutical composition thereof of any of the foregoing embodiments. By way of example, but not limitation, the cancer can be selected from the group consisting of breast cancer, lung cancer, colon cancer, ovarian cancer, melanoma cancer, bladder cancer, renal cancer, kidney cancer, liver cancer, head and neck cancer, colorectal cancer, melanoma, pancreatic cancer, gastric carcinoma cancer, esophageal cancer, mesothelioma, prostate cancer, leukemia, lymphomas, and myelomas. In certain embodiments, the cell proliferative disorder is a tumor or a cancer. In certain embodiments, the T-cell engager can be administered at a dose of 0.001 mg/kg to about 100 mg/kg based on the patient's body weight.

[00396] In some embodiments, the subject anti-CTHRC1 T-cell engagers may be used to treat an immune disease, such as an autoimmune disease. Inflammatory diseases, including autoimmune diseases are also a class of diseases associated with B- cell disorders. Examples of immune diseases or conditions, including autoimmune conditions, include: rheumatoid arthritis, rheumatic fever, multiple sclerosis, experimental autoimmune encephalomyelitis, psoriasis,

uveitis, diabetes mellitus, lupus, systemic lupus erythematosus (SLE), lupus nephritis, eczema, scleroderma, polymyositis/scleroderma, polymyositis/dermatomyositis, ulcerative proctitis, ulcerative colitis, severe combined immunodeficiency (SCID), DiGeorge syndrome, ataxia-telangiectasia, seasonal allergies, perennial allergies, food allergies, anaphylaxis, mastocytosis, allergic rhinitis, atopic dermatitis, Parkinson's, Alzheimer's, hypersplenism, leukocyte adhesion deficiency, X-linked lymphoproliferative disease, X-linked agammaglobulinemia, selective immunoglobulin A deficiency, hyper IgM syndrome, HIV, autoimmune lymphoproliferative syndrome, Wiskott-Aldrich syndrome, chronic granulomatous disease, common variable immunodeficiency (CVID), hyperimmunoglobulin E syndrome, Hashimoto's thyroiditis, acute idiopathic thrombocytopenic purpura, chronic idiopathic thrombocytopenia purpura, dermatomyositis, Sydenham's chorea, myasthenia gravis, polyglandular syndromes, bullous pemphigoid, Henoch-Schonlein purpura, poststreptococcal nephritis, erythema nodosum, erythema multiforme, glomerulonephropathy, Takayasu's arteritis, Addison's disease, sarcoidosis, ulcerative colitis, polyarteritis nodosa, ankylosing spondylitis, Goodpasture's syndrome, thromboangitis obliterans, Sjogren's syndrome, primary biliary cirrhosis, Hashimoto's thyroiditis, thyrotoxicosis, chronic active hepatitis, polychondritis, pemphigus vulgaris, Wegener's granulomatosis, membranous nephropathy, amyotrophic lateral sclerosis, tabes dorsalis, giant cell arteritis, polymyalgia, pernicious anemia, rapidly progressive glomerulonephritis, psoriasis, fibrosing alveolitis, and cancer.

[00397] Pharmaceutical compositions of the present invention may be administered in a manner appropriate to the disease to be treated (or prevented). The quantity and frequency of administration will be determined by such factors as the condition of the patient, and the type and severity of the patient's disease, although appropriate dosages may be determined by clinical trials.

[00398] When "an immunologically effective amount", "an anti-tumor effective amount", "an tumor-inhibiting effective amount", or "therapeutic amount" is indicated, the precise amount of the compositions of the present invention to be administered can be determined by a physician with consideration of individual differences in age, weight, tumor size, extent of infection or metastasis, and condition of the patient (subject).

[00399] In any of the foregoing embodiments, the step of administering the anti-CTHRC1 T-cell engager or pharmaceutical composition thereof (and any additional therapeutic agent or adjuvant) can be performed by any suitable means, including parenteral, subcutaneous, intraperitoneal, intrapulmonary, and intranasal, and, if desired for local treatment, intralesional administration. Parenteral infusions include intramuscular, intravenous, intraarterial, intraperitoneal, or subcutaneous administration. By way of further example, but not limitation, the administration can be performed by known methods, such as intravenous administration, e.g., as a bolus or by continuous infusion over a period of time, by intramuscular, intraperitoneal, intracerebrospinal, subcutaneous, intra-articular, intrasynovial, intrathecal, oral, topical, or inhalation routes. In addition, the anti-CTHRC1 T-cell engager is suitably administered by pulse infusion, particularly with declining doses of the anti-CTHRC1 T-cell engager. Dosing can be by any suitable route, e.g., by injections, such as intravenous or subcutaneous injections, depending in part on whether the administration is brief or chronic. In some embodiments, intravenous or subcutaneous administration of the anti-CTHRC1 T-cell engager is preferred.

[00400] The administration of the subject compositions may be carried out in any convenient manner, including by aerosol inhalation, injection, ingestion, transfusion, implantation or transplantation. The compositions described herein may be administered to a patient subcutaneously, intradermally, intratumorally, intranodally, intramedullary, intramuscularly, by intravenous (i.v.) injection, or intraperitoneally.

[00401] As discussed, the anti-CTHRC1 T-cell engagers are administered to a human patient, in accordance with known methods, such as intravenous administration, e.g., as a bolus or by continuous infusion over a period of time, by intramuscular, intraperitoneal, intracerebrospinal, subcutaneous, intra-articular, intrasynovial, intrathecal, oral, topical, or inhalation routes. In some embodiments, intravenous or subcutaneous administration of the anti-CTHRC1 T-cell engager is preferred.

[00402] The anti-CTHRC1 T-cell engager and/or composition thereof of the invention will be formulated, dosed, and administered in a fashion consistent with good medical practice. Factors for consideration in this context include the particular disorder being treated, the particular mammal being treated, the clinical condition of the individual patient, the cause of the disorder,

the site of delivery of the agent, the method of administration, the scheduling of administration, and other factors known to medical practitioners.

[00403] The CTHRC1 binding moieties of the invention can be in the different forms encompassed by the definition of “antibody” herein. Thus, the antibodies include full length or intact antibody, antibody fragments, native sequence antibody or amino acid variants, humanized, chimeric or fusion antibodies, and functional fragments thereof. In fusion antibodies an antibody sequence is fused to a heterologous polypeptide sequence. The antibodies can be modified in the Fc region to provide desired effector functions. As discussed in more detail in the sections herein, with the appropriate Fc regions, the naked antibody bound on the cell surface can induce cytotoxicity, e.g., via antibody-dependent cellular cytotoxicity (ADCC) or by recruiting complement in complement dependent cytotoxicity, or some other mechanism. Alternatively, where it is desirable to eliminate or reduce effector function, so as to minimize side effects or therapeutic complications, certain other Fc regions may be used.

[00404] In one embodiment, the antibody (i) competes for binding to the same epitope, and/or (ii) binds substantially to the same epitope, as the antibodies of the invention.

[00405] Methods of producing the above T-cell engagers are described in detail herein.

[00406] The present anti-CTHRC1 T-cell engager are useful for treating a CTHRC1-displaying cancer or alleviating one or more symptoms of the cancer in a mammal. The cancers encompass metastatic cancers of any of the cancers described herein. The T-cell engager is able to bind to at least a portion of the cancer cells that display CTHRC1 directly or in a complex in the mammal. In a preferred embodiment, the T-cell engager is effective to destroy or kill CTHRC1-displaying tumor cells or inhibit the growth of such tumor cells, in vitro or in vivo, upon binding to CTHRC1 epitope on the cell. In other preferred embodiments, the T-cell engagers are effective to i) inhibit tumor metastasis in vivo; (ii) inhibit tumor growth in vivo; (iii) decrease tumor size in vivo; (iv) inhibit tumor vascularization in vivo; (v) exhibit cytotoxic activity on tumor cells and cancer associated fibroblasts expressing and/or displaying CTHRC1 in vivo; (vi) exhibit cytostatic activity on a tumor cells or cancer associated fibroblasts expressing and/or displaying CTHRC1 in vivo; (vii) enhance infiltration of anti-tumor immune cells in vivo; or (viii) prevent suppression of immune-cells in the tumor microenvironment in vivo.

[00407] The invention provides a composition comprising an anti-CTHRC1 T-cell engager of the invention, and a carrier. The invention also provides formulations comprising an anti-CTHRC1 T-cell engager of the invention, and a carrier. In one embodiment, the formulation is a therapeutic formulation comprising a pharmaceutically acceptable carrier.

[00408] Another aspect of the invention is isolated nucleic acids encoding the anti-CTHRC1 T-cell engagers. For example, nucleic acids encoding both the H and L chains and especially the hypervariable region residues, chains which encode the native sequence CTHRC1 binding moiety as well as variants, modifications and humanized versions of the antibody, are encompassed.

[00409] The invention also provides methods useful for treating a CTHRC1 polypeptide-displaying cancer or alleviating one or more symptoms of the cancer in a mammal, comprising administering a therapeutically effective amount of an anti-CTHRC1 T-cell engager to the mammal. The antibody therapeutic compositions can be administered short term (acute) or chronic, or intermittent as directed by physician. Also provided are methods of inhibiting the growth of, and killing a CTHRC1 polypeptide-displaying cell.

[00410] For the prevention or treatment of disease, the dosage and mode of administration will be chosen by the physician according to known criteria. The appropriate dosage of anti-CTHRC1 T-cell engager will depend on the type of disease to be treated, as defined above, the severity and course of the disease, whether the anti-CTHRC1 T-cell engager is administered for preventive or therapeutic purposes, previous therapy, the patient's clinical history and response to the antibody, and the discretion of the attending physician. The anti-CTHRC1 T-cell engager is suitably administered to the patient at one time or over a series of treatments. Preferably, the anti-CTHRC1 T-cell engager or pharmaceutical composition thereof is administered by intravenous infusion or by subcutaneous injections. Depending on the type and severity of the disease, about 1 µg/kg to about 100 mg/kg body weight (e.g., about 0.1-30 mg/kg/dose) of anti-CTHRC1 T-cell engager can be an initial candidate dosage for administration to the patient, whether, for example, by one or more separate administrations, or by continuous infusion. A dosing regimen can comprise administering an initial loading dose of about 4 mg/kg, followed by a weekly maintenance dose of about 2 mg/kg of the anti-CTHRC1 T-cell engager. However, other dosage regimens may be useful. A typical daily dosage might range from about 1 µg/kg to

1000 mg/kg or more, depending on the factors mentioned above. For repeated administrations over several days or longer, depending on the condition, the treatment is sustained until a desired suppression of disease symptoms occurs. The progress of this therapy can be readily monitored by conventional methods and assays and based on criteria known to the physician or other persons of skill in the art.

[00411] In embodiments, the anti- anti-CTHRC1 T-cell engagers of the subject invention may be advantageously administered in conjunction with adoptive cell therapies (ACT) (including allogeneic and autologous hematopoietic stem cell transplantation (HSCT) and recombinant cell (i.e., CAR T) therapies) is the treatment of choice for many malignant disorders (for reviews of HSCT and adoptive cell therapy approaches, see, Rager & Porter, *Ther Adv Hematol* (2011) 2(6) 409-428; Roddie & Peggs, *Expert Opin. Biol. Ther.* (2011) 11(4):473-487; Wang et al. *Int. J. Cancer*: (2015)136, 1751-1768; and Chang, Y. J. and X. J. Huang, *Blood Rev*, 2013, 27(1): 55-62). Such adoptive cell therapies include, but are not limited to, allogeneic and autologous hematopoietic stem cell transplantation, donor leukocyte (or lymphocyte) infusion (DLI), adoptive transfer of tumor infiltrating lymphocytes, or adoptive transfer of T cells or NK cells (including recombinant cells, i.e., CAR T, CAR NK). Beyond the necessity for donor-derived cells to reconstitute hematopoiesis after radiation and chemotherapy, immunologic reconstitution from transferred cells is important for the elimination of residual tumor cells. The efficacy of ACT as a curative option for malignancies is influenced by a number of factors including the origin, composition and phenotype (lymphocyte subset, activation status) of the donor cells, the underlying disease, the pre-transplant conditioning regimen and post-transplant immune support (i.e., IL-2 therapy) and the graft-versus-tumor (GVT) effect mediated by donor cells within the graft. Additionally, these factors must be balanced against transplant-related mortality, typically arising from the conditioning regimen and/or excessive immune activity of donor cells within the host (i.e., graft-versus-host disease, cytokine release syndrome, etc.).

[00412] Methods for preparing and administering adoptive cell therapies, *i.e.* cell-based therapies, are known in the art and can be performed by standard practices in the art.

[00413] In any of the foregoing embodiments, the method of treatment can further include a cell-based therapy. In any of the foregoing embodiments, the cell-based therapy can include

administering allogenic or autologous T cell therapies, CAR-T cell therapies, macrophage therapies, such as CAR macrophages, and/or NK cell therapies, including but not limited to CAR NK therapies. In certain aspects, the cell-based therapy can include administering allogenic or autologous T cell therapy.

[00414] In any of the foregoing embodiments, the amount of the cell-based therapy can be an effective amount. One of skill in the art can determine an effective amount using ordinary skill in the art.

[00415] In any of the foregoing embodiments, the subject can be a human or a non-human mammal.

[00416] In certain aspects, a pharmaceutical composition of the present disclosure can be used in the preparation of a medicament for the treatment of a cell proliferative disorder, preferably cancer.

Articles of Manufacture and Kits

[00417] Another embodiment of the invention is an article of manufacture containing materials useful for the treatment, prevention and/or diagnosis of CTHRC1-displaying cancer. The article of manufacture comprises a container and a label or package insert on or associated with the container. Suitable containers include, for example, bottles, vials, syringes, etc. The containers may be formed from a variety of materials such as glass or plastic. The container holds a composition which is effective for treating, preventing and/or diagnosing the cancer condition and may have a sterile access port (for example the container may be an intravenous solution bag or a vial having a stopper pierceable by a hypodermic injection needle). At least one active agent in the composition is an anti-CTHRC1 T-cell engager of the invention, or a CAR-modified immune cell of the invention, or a nucleic acid of the invention. Optionally, a composition further comprises a carrier, for example a pharmaceutically acceptable carrier. The label or package insert indicates that the composition is used for treating cancer. The label or package insert will further comprise instructions for administering the antibody composition to the cancer patient. Additionally, the article of manufacture may further comprise a second container comprising a pharmaceutically-acceptable buffer, such as bacteriostatic water for injection (BWFI), phosphate-buffered saline, Ringer's solution and dextrose solution. It may further include other

materials desirable from a commercial and user standpoint, including other buffers, diluents, filters, needles, and syringes.

[00418] Kits are also provided that are useful for various purposes, e.g., for CTHRC1-displaying cell killing assays, for purification or immunoprecipitation of CTHRC1 polypeptide from cells. For isolation and purification of CTHRC1 polypeptide, the kit can contain an anti-CTHRC1 antibody coupled to beads (e.g., sepharose beads). Kits can be provided which contain the antibodies for detection and quantitation of CTHRC1 polypeptide in vitro, e.g., in an ELISA or a Western blot. As with the article of manufacture, the kit comprises a container and a label or package insert on or associated with the container. The container holds a composition comprising at least one anti-CTHRC1 T-cell engager of the invention. Additional containers may be included that contain, e.g., diluents and buffers, control antibodies. The label or package insert may provide a description of the composition as well as instructions for the intended in vitro or detection use.

[00419] For example, a kit can comprise a first container comprising a composition comprising one or more CTHRC1 T-cell engagers or CAR modified immune cells, such as CAR-T or CAR-NK cells, or CAR macrophages, of the invention; and a second container comprising a buffer. The buffer may be pharmaceutically acceptable.

[00420] The following examples are offered for illustrative purposes only and are not intended to limit the scope of the present invention in any way.

[00421] All patent, patent application, and literature references cited in the present specification are hereby incorporated by reference in their entirety.

EXAMPLES

Example 1: CTHRC1 Expression on Tumor and Stroma in Human Colorectal Cancer

Samples by IHC

[00422] A tissue sample from a colorectal cancer patient was stained with FAP as shown in FIGURE 1A. A separate tissue sample for a colorectal cancer patient was assessed by IHC using an anti-CTHRC1, rabbit polyclonal antibody (Abcam, ab 85739) as shown in FIGURE 1B. Tissue slides from a subject with stage IV colorectal cancer were obtained from commercial sources and stained with the indicated antibody at the recommended dilution for immunohistochemistry. After incubation with primary antibody, slides were washed and bound

mAb detected by common chromogenic methods. As shown in FIGURE 1A, tumor stroma is stained with FAP while tumor nests (blue) are devoid of FAP staining. However, as shown FIGURE 1B, CTHRC1 localizes in both tumor stroma and tumor nests which comports with scRNA data (not shown).

Example 2: Membrane Binding of CTHRC1 Antibodies

[00423] Staining of tumor cell lines by anti-CTHRC1 mAbs was assessed by flow cytometry. Human SKOV3 ovarian cancer, KP4 PDAC, and HCT115 colorectal cancer lines as well as the mouse EMT6 breast cancer line were selected. Cell lines were incubated with 0.25ug/mL recombinant CTHRC1 to form complexes on the cell surface. Following incubation, cells were washed and anti-CTHRC1 mAbs were added at 10ug/mL and incubated for 30 minutes. Cells were then washed and incubated with an anti-mouse secondary antibody diluted 1:250 in FACS buffer for 20 minutes. Cells were washed again and resuspended in FACS buffer for analysis on a Sony Cell Analyzer. Results are shown in FIGURES 2A-2B which confirm that anti-CTHRC1 antibodies can bind to the surface of tumor cells.

[00424] For FIGURE 2C, The Cancer Cell Line Encyclopedia (sites.broadinstitute.org/ccle/datasets) was used to determine the expression of CTHRC1 across human cancer cell lines. High CTHRC1 expression was seen in several cell lines, including SKOV3 ovarian cancer. For these experiments, SKOV3 cells were incubated with 1 ug/mL recombinant CTHRC1 to form complexes on the cell surface. Following incubation, cells were washed and anti-CTHRC1 mAbs were added at 1 ug/mL and incubated for 30 minutes. Cells were then washed and incubated with an anti-mouse secondary antibody diluted 1:250 in FACS buffer for 20 minutes. Cells were washed again and resuspending in FACS buffer containing DAPI for analysis on a Sony Cell Analyzer. This analysis identified that CTHRC1S-M3 (PAI-0303) and CTHRC1S-M23 (PAI-0323) bind to SKOV3 ovarian cancer cells (FIG. 2C). Data for other cell lines are also shown in FIG. 2C.

[00425] To assess whether CTHRC1S-M3 (PAI-0303) was internalized by cancer cells, antibodies were first labeled with pHrodo, a pH sensitive dye that covalently binds free lysines present within an antibody. pHrodo is non fluorescent outside of the cell (neutral pH) and fluoresces in the acidic environment of phagosomes and endosomes once the antibody has been internalized. Following pHrodo antibody labelling, SKOV3 ovarian cancer cell lines were

treated for 24 hours with two concentrations (0.1 and 1 ug/mL) of pHrodo labeled anti-CTHRC1 antibody, as well as a pHrodo labeled isotype control (negative control) and pHrodo labeled Necitumumab (positive control). The following day, cells were detached using a cell dissociation buffer, washed and the levels of internalization were measured by flow cytometry using a Sony Cell Analyzer. CTHRC1S-M3 (PAI-0303) was not internalized by human cancer cell lines, whereas CTHRC1S-M23 (PAI-0323) showed maximal levels of internalization by 24 hours (FIG. 2D).

[00426] A bridging ELISA was performed to determine the relative affinities of each CTHRC1-targeted immune engager. Recombinant human CTHRC1 protein was coated on maxisorp plates overnight at 4 °C. The following day, plates were washed once with PBST then blocked with 1% BSA blocking buffer for 90 min at RT with shaking after which an additional wash with PBST was performed. An 8-point titration of each CTHRC1-targeted immune engager was prepared, added to the plates and allowed to bind for 45 minutes at RT. Plates were then washed three times with PBST followed by incubation with biotinylated antigen (either human CD3ε or human 4-1BB) for 45 minutes at RT. Plates were then washed three times with PBST followed by incubation with an HRP conjugated streptavidin secondary antibody for 45 minutes at RT. Finally, plates were washed six times with PBST and developed using TMB substrate and 0.5M Phosphoric Acid. In PAI-03023-hu41BBL_bsIgG (1:1 KiH), the orientation of the bridging ELISA was reversed (capture with biotinylated human 4-1BB and His-tagged human CTHRC1 as detection antigen). The an anti-CTHRC1 T-cell (CD3) engager based on antibody M3 (including SEQ ID NOs: 81 and 82) included a T cell engager moiety including SEQ ID NOs: 7 and 8 (PAI-SP34) with the results shown in FIGURE 2E. In particular, antibody M3 is shown to bind to the cell surface and is not internalized.

Example 3: In vivo efficacy: combination with cell therapies

[00427] NK cells and CD8 T cells

[00428] Run in cell-line derived xenografts

[00429] In vivo efficacy is assessed in common cell line derived xenograft tumor bearing mice (SCID/nude). Naïve mice are inoculated with tumor cells s.c. and monitored for tumor growth. When tumors reach ~150mm³, mice are randomized to treatment group. Mice are infused with CAR-T or CAR-NK cells and treated with anti-CTHRC1 T-cell engagers or a relevant control

protein. Tumor growth inhibition and survival are assessed for the duration of the study. Anti-CTHRC1 T-cell engagers should enhance anti-tumor activity of CAR-T and CAR-NK cells.

Example 4: CTHRC1 highly selectively localizes to cancer, is associated with poor outcome, and is most upregulated on cancer-associated fibroblasts (CAFs) in immune-cold tumor microenvironments

[00430] Without being bound to theory, in the context of cancer, it is expected that inhibiting CTHRC1 may confer therapeutic benefit through blocking CAF and Autocrine pro-survival signalling to cancer cells alongside disrupting immune suppression mediated by CTHRC1. Targeting CTHRC1 with antibodies bound to toxins or that engage immune cells may further drive anti-tumor activity. Data was gathered which supports the idea that CTHRC1 is both selectively upregulated in cancer and contributes to cancer progression.

[00431] Binding data (from bio-layer interferometry) for exemplary anti-CTHRC1 antibodies are provided in the table below. anti-CTHRC1 antibodies were screened in the cell adhesion assay to assess for functional activity. Of the 12 clones identified to be selective CTHRC1 binders by ELISA, three clones (CTHRC1S-M5, CTHRC1S-M11 & CTHRC1S-M23) showed functional activity.

Clone	mAb ID	BLI Epitope Bin	Affinity (nM)	huCTHRC1 EC50 (nM)	muCTHRC1 EC50 (nM)	Rat CTHRC1 EC50 (nM)
CTHRC1S-M5	AB987	B	3.18	1.85	0.26	1.73
CTHRC1S-M23	AB988	D	0.23	0.18	0.08	0.18
CTHRC1S-M11	AB989	C	2.00	9.76	n/a	n/a
CTHRC1S-M14	AB991	unknown	<0.001	0.05	0.94	0.05

CTHRC1 mRNA is a top ranked marker of CAFs in cancer-rich, immune-cold, tumour samples

[00432] In this example it is demonstrated that CTHRC1 is upregulated specifically in CAFs in cancer rich, immune-cold, samples vs. CAFs in T-cell rich, immune-hot, samples (Figures 3A-3B). Cancer rich, T-cell poor tumours, i.e., Immune excluded or Immune deserts, are also associated with poor outcome, treatment resistance, and immune-suppression (Gooden et al. *British J. of Cancer*, 2011), thus, therapeutic targeting of CTHRC1 represents an opportunity to target these challenging tumor types. Cancer cells transform fibroblasts into CAFs, which in turn promote cancer progression, treatment resistance and immune suppression (Sahai et al. *Nat. Rev. Cancer*, 2020). Targets that associate closely with CAFs in cancer rich vs. T-cell rich samples are therefore an opportunity for therapeutic intervention in these cancers. To show CTHRC1 is associated with CAFs in these tumor types samples were grouped in the scRNA atlas described in Swechha et al. (*bioRxiv* 2021) into those that comprise at least 50% cancer cells, and less than 25% T-cells (immune-cold) and those that have over 50% T-cells in the sample and less than 25% cancer cells (immune-hot) (Figure 3A). Four cancers were included in this analysis, namely, Pancreatic, Lung, Breast, and Colorectal, and are representative of the solid tumours in the scRNA atlas (Swechha et al., *bioRxiv*, 2021). CAF specific gene expression levels in the Cancer-cell rich, T-cell poor samples were then ranked by the number of samples where expression is significantly greater ($P < 0.05$) than in the T-cell rich, Cancer-cell poor sample; as well as the overall P-value of this finding (Wilcoxon Rank; Navon, Roy, et al., *PLoS One*, 2009). Finally, targets were filtered down to those upregulated by CAFs but not in other cell types, in all or the majority of samples (Top 500 CAF genes as measured by Wilcoxon Rank). This result demonstrates CTHRC1 is ranked as a target highly localized to CAFs in Cancer-rich, T-cell poor tumour samples (Figure 3B), and thus an opportunity for therapeutic intervention.

CTHRC1 mRNA is upregulated in cancer vs. adjacent tissue and correlates with disease progression

[00433] In this Example it is demonstrated that CTHRC1 expression is highly upregulated in many solid tumors. Specifically, an analysis of bulk-scRNA data taken from the cancer genome atlas (TCGA) demonstrates that CTHRC1 is highly upregulated in cancerous tissue samples vs. normal adjacent tissue samples across numerous solid cancers including Breast, Lung, Ovarian, Pancreatic, Sarcoma, Melanoma, and Uterine Carcinosarcomas (Figure 4). This indicates that

within these organs CTHRC1 selectively localizes to cancerous regions. It is also demonstrated that CTHRC1 is a prognostic indicator of survival in many solid cancers based on analysis of TCGA data with the GEPIA online tool (Tang, Z. et al. *Nucleic Acids Res*, 2017) (Figure 5). Notably poor survival is seen in patients high for CTHRC1 in liver cancer, stomach cancer, and sarcomas. These cancers are all stromal rich, fibrotic cancers, in line with the above example that shows CTHRC1 localizes to CAFs in cancer-rich immune-poor tumor samples. It is also demonstrated that CTHRC1 expression increases with cancer stage in colorectal cancer and liver cancer (Figure 6); this shows that targeting CTHRC1 may be valuable in patients with late-stage aggressive cancers and where prognosis is poor.

CTHRC mRNA has a favorable expression profile in normal tissue

[00434] As well as being upregulated in cancer vs. adjacent tissues, this Example also demonstrates that CTHRC1 expression is highly selective to cancer tissue and is expressed at comparatively very low levels in normal healthy tissues in the body. This indicates CTHRC1 targeting is accompanied with a significant therapeutic window and can be used to target payloads to the tumor microenvironment. For example, comparing CTHRC1 bulk-RNA expression in pancreatic cancer samples (TCGA) to CTHRC1 expression in normal tissue samples (GTEx data; both reanalyzed by the UCSC Xena project, Goldman et al., *Nat. Biotech*, 2020) highlights a significant therapeutic window in almost all pancreatic samples analyzed (Figures 7A-7C). At the single-cell level it was seen that CTHRC1 expression localizes to CAFs and epithelial cancer cells in cancer specimens and is not seen in any cell-types in tissue (Figure 8). This level of localization is comparable if not better than previous mAb targets that have been used to target Antibody Drug Conjugates (ADCs) to the tumour microenvironment and shown safe and non-toxic in clinical trials, such as, LRRC15 (Figure 9). Of note, in Breast, Ovarian, Pancreatic, and Lung cancers as well as Melanoma we see cancer epithelial cells displaying CTHRC1. Expression is not seen in normal epithelial cells; this indicates a mesenchymal program is switched on in cancer and further implicates CTHRC1 as playing a pro-vs. anti-tumor role, since there is significant evolutionary pressure for cancer cells to down-regulate anti-tumor targets and mechanisms. Overall, this shows CTHRC1 expression is selective enough to cancer vs. normal tissue that mAbs could be used to target payloads to cancer, and that CTHRC1 expression by cancer cells indicates a pro-tumor role for this protein in humans. Collectively,

this example therefore shows the value of CTHRC1 mAbs as a method of treating cancer in humans.

CTHRC1 protein is expressed in cancer-fibroblast co-cultures, and in mouse tumours

[00435] This example also demonstrates that CTHRC1 is upregulated under experimental conditions where fibroblasts are co-cultured with cancer cells versus monocultures of the same cells (Figure 10), indicating induction of CTHRC1 is dependent on fibroblast-cancer cell interactions, showing specificity to cancer tissue. It was also found *in vivo* that CTHRC1 protein is selectively expressed in tumor sections using mAbs that selectively bind CTHRC1 (Figure 11). Finally it was seen that CTHRC1 is expressed on human cancer samples (Figure 12), either on cancer cells at stromal interfaces (Melanoma and Head and Neck Cancer) or in regions rich in CAFs (Pancreatic Cancer). Overall this analysis shows that at the protein level CTHRC1 expression is seen in cancers similarly to CTHRC1 mRNA.

Example 5: T Cell Proliferation

[00436] Naïve CD8 T cells will be isolated from PBMCs obtained from normal healthy donors by negative selection using commercial magnetic isolation kits (StemCell Technologies). After isolation, CD8 T cells will be labeled with CFSE and incubated for 72 hours on plates coated with a dose-response of anti-CTHRC1 T-cell (CD3) engager. After 72 hours, T cell proliferation will be quantified by flow cytometry by measuring CFSE dilution.

Example 6: Tumor Cell Killing

[00437] Naïve CD8 T cells will be isolated from PBMCs obtained from normal healthy donors by negative selection using commercial magnetic isolation kits (StemCell Technologies). After isolation, CD8 T cells will be incubated with CTHRC1 expressing tumor cells (SKOV3, etc) and a dose-response of either anti-CTHRC1 T-cell (CD3) or isotype targeted CD3 engager for a period of 6-24 hours. After incubation, killing of tumor cells will be assessed by flow cytometry and by measuring LDH release in culture supernatant.

Example 7: In vivo Experiments

[00438] Anti-tumor efficacy will be assessed in the CT26 syngeneic model. Balb/c transgenic mice expressing human CD3 ϵ chains will be inoculated s.c. with CT26 tumor cells. One week after inoculation, mice will be randomized to treatment groups with an average tumor volume of 120mm³. Treatment with anti-CTHRC1 T-cell (CD3) engagers will start at the day of randomization, with dosing continuing for 2 weeks. Tumor volume will be measured every 3 days with tumor growth and survival reported as endpoints.

Example 8: In vivo models

[00439] The efficacy of anti-CTHRC1 was tested in syngeneic mouse breast tumor model, EMT6. Briefly, 100,000 EMT6 cells were injected into the mammary fat pad (MFP) of female Balb/c mice. Mice were grouped out according to tumor volume once size reached 120-250 mm³ range, 10 days post inoculation. Following group out, mice were dosed with 2.5mg/kg anti-CTHRC1 or Isotype control, 5mg/kg aPD-1, and/or 10mg/kg a-TGF β (SR) according to group treatment. Tumor volume was assessed twice weekly following caliper measurement and calculated as $(\text{length} \times \text{width}^2)/2$. Initial dose was given intravenous (iv), and remaining doses were administered intraperitoneal (ip) three times a week for three weeks. Mice were euthanized when tumor size exceeds 1500 mm³ or due to tumor ulceration. Figure 14 illustrates the tumor-growth curves obtained from measurements for 31 days post-tumor cell inoculation. As shown in Figure 14A, anti-CTHRC1 clone M5 in combination with anti-PD-1 resulted in tumor growth inhibition comparable to that obtained with anti-PD-1 in combination with anti-TGF β . Similar results were obtained for anti-CTHRC1 clone M23 (Figure 14B) whereas only modest combination activity was observed with anti-CTHRC1 clone M14 in combination with anti-PD-1 (Figure 14C). Control data are identical for Figures 14A-C. Data are plotted as mean $\pm$ standard deviation for each data point across 9 mice per group.

Example 9: In vivo model

[00440] Female C57BL/6J mice were inoculated s.c. with Pan02 cells in Matrigel. Tumors were measured, and mice were randomized to treatment group when tumors reached an average volume of 100mm³. Treatment with 10mg/kg of isotype control or anti-CTHRC1 mAb (clone M5) began 24 hours post-randomization and continued for 3 doses/week for the indicated treatment duration. Mice were monitored for tumor growth (Figure 15A) and overall survival (Figure 15B). Tumor growth inhibition was deemed significantly significant by ANOVA. As

shown in Figure 15A, anti-CTHRC1 clone M5 resulted in tumor growth inhibition relative to isotype control. Data are plotted as mean tumor volume $\pm$ standard deviation for each data point across 9 mice per group. As shown in Figure 15B, anti-CTHRC1 clone M5 resulted in improved survival relative to isotype control.

Example 10: Anti-CTHRC1 Pretreatment Results in Recruitment of CD8 T cells in the tumor microenvironment

[00441] Balb/c mice were inoculated orthotopically with EMT6 tumor cells in Matrigel. When tumors reached an average volume of 200 mm³, animals were assigned to treatment group. Mice were treated with isotype or anti-CTHRC1 mAb (M5 clone) at 10 mg/kg for 1 week (3 doses). Following dosing, tumors were isolated and processed to slides. Slides were stained with an anti-CD8 antibody, and level of CD8 infiltration into tumor nests was quantified by HALO image analysis software. Data were plotted as number of infiltrating CD8 T cells versus distance from tumor margin (FIGURE 16). As shown in FIGURE 16, pretreatment of cells with an anti-CTHRC1 antibody resulted in improved CD8 T cell recruitment in the tumor microenvironment. These results suggest that preconditioning treatment with an anti-CTHRC1 antibody of the invention can result in CD8 T cell recruitment that could be advantageous for cellular immunotherapies such as CAR-T cell therapy.

Example 11: Cell-killing Assay

[00442] A co-culture of pancreatic cancer associated fibroblast (CAF) cells and indication matched KP4 tumor cells was established by plating both cell types at a pre-determined ratio 24 hours prior to drug administration. The following day, the adherent co-culture wells or singular cell type control culture wells were treated with CTHRC1-T-cell engager molecules in either a 1:1 or 2:1 cis orientation or matched bispecific control molecules in the same format at a singular concentration along with PBMCs as effector cells. Controls were identical to the CTHRC1xCD3 engagers except that the anti-CTHRC1 variable domain sequences were replaced with anti-Dengue variable domain sequences derived from US9073981B2. Treated culture plates were placed into a Incucyte S3 live cell imaging instrument (Sartorius) for 72 hours where cell killing waws assessed through detection and quantification of Caspase-3/7 Dye for Apoptosis (Sartorius Cat. #4440) fluorescent signal at the terminal endpoint of the assay.

[00443] The anti-CTHRC1 T-cell engagers tested are schematically depicted in Fig. 17A and the corresponding abridged and annotated sequences are provided below.

[00444] 1:1 anti-CTHRC1 x anti-CD3 engager [abridged]

Chain 1	EVQLVESGGGLVQPGGSLRLSCAASGFTFSNCAMNWVRQAPGKGLEWVS VISGSGGGTTYADSVKGRFTISRDN SKNTLYLQMNSLRAEDTAVYYCAKA NWNAYYDFWGGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVK DYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVTVPSSSLGTQTYI CNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPEAAGGPSVFLFPPKPKDT LMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTY RVVSVLTVLHQDWLNGKEYKCKVSNKALGAPIEKTISKAKGQPREPQVCT LPPSRDELTKNQVSLSCAVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSD GSFFLVSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK
Chain 2	EVQLVESGGGLVQPGGSLRLSCAASGFTFNTYAMNWVRQAPGKGLEWVA RIRSKYNNYATYYADSVKDRFTISRDDSKNTLYLQMNSLKAEDTAVYYCV RHGNFGNSYVSWFAYWGQGTTLVTVSSGGGGSGGGGSGGGGSGGGGSQA VVTQEPSLTVSPGGTVTLTCGSSTGAVTTSNYANWVQQKPGQAPRGLIGG TNKRAPGTPARFSGSLLGGKAALTLGAQPEDEAEYYCALWYSNLWVFGG GTKLTVLGGGGSDKTHTCPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVT CVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVL HQDWLNGKEYKCKVSNKALGAPIEKTISKAKGQPREPQVYTLPPCRDEL TKNQVSLWCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLVSKL TVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK
Chain 3	EIVMTQSPATLSVSPGERATLSCRASQRVSSNLAWYQQKPGQAPRLLIYGA STRATGIPARFSGSGSGTEFTLTINSLSQADFVFFCQQYDIWPMYTFGQGT KLEIKRTVAAPS FIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNA LQSGNSQESVTEQDSKSTYSLSSLTLSKADYEKHKVYACEVTHQGLSSP VTKSFNRGEC

[00445] 1:1 anti-CTHRC1 x anti-CD3 engager [annotated]

Chain 1	anti-CTHRC1 M3 heavy variable	EVQLVESGGGLVQPGGSLRLSCAASGFTFSNCAMNWVRQAPGKGL LEWVSVISGSGGGTTYADSVKGRFTISRDN SKNTLYLQMNSLRAE DTAVYYCAKANWNAYYDFWGGTLVTVSS
	Human IgG1 hole	ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGA LTSGVHTFPAVLQSSGLYSLSSVTVPSSSLGTQTYICNVNHKPSN TKVDKKVEPKSCDKTHTCPPCPAPEAAGGPSVFLFPPKPKDTLMIS RTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNS TYRVVSVLTVLHQDWLNGKEYKCKVSNKALGAPIEKTISKAKGQ PREPQVCTLPPSRDELTKNQVSLSCAVKGFYPSDIAVEWESNGQPE

		NNYKTPPVLDSDGSFFLVSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK
Chain 2	Anti-CD3e variable heavy	EVQLVESGGGLVQPGGSLRLSCAASGFTFNTYAMNWVRQAPGKGLEWVARIRSKYNNYATYYADSVKDRFTISRDDSKNTLYLQMNSLKAEDTAVYYCVRHGNFGNSYVSWFAYWGQGTLLTVSS
	Linker	GGGGSGGGGSGGGGSGGGGS
	Anti-CD3e variable light	QAVVTQEPSTVSPGGTVTLTCGSSTGAVTTSNYANWVQKPGQAPRGLIGGTNKRAPGTPARFSGSLLGGKAALTLGAQPEDEAEYYCALWYSNLWVFGGGTKLTVL
	Linker	GGGGG
	Human IgG1 knob	DKTHTCPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALGAPIEKISKAKGQPREPQVYTLPPCRDELTKNQVSLWCLVKGFYPSDIAVEWESNGQPENNYKTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK
Chain 3	anti-CTHRC1 M3 light variable	EIVMTQSPATLSVSPGERATLSCRASQRVSSNLAWYQQKPGQAPRLLIYGASTRATGIPARFSGSGSGTEFTLTINSLQSADFVFFCQQYDIWPMYTFGQGTKLEIK
	Human kappa constant	RTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKIDSTYLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC

[00446] 2:1 anti-CTHRC1 x anti-CD3 engager [abridged]

Chain 1	EVQLVESGGGLVQPGGSLRLSCAASGFTFSNCAMNWVRQAPGKGLEWVSVISGSGGGTYYADSVKGRFTISRDNKNTLYLQMNSLRAEDTAVYYCAKANWNYAYFDFWGQGTLLTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALGAPIEKISKAKGQPREPQVCTLPSPRDELTKNQVSLSCAVKGFYPSDIAVEWESNGQPENNYKTPPVLDSDGSFFLVSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK
Chain 2	EVQLVESGGGLVQPGGSLRLSCAASGFTFSNCAMNWVRQAPGKGLEWVSVISGSGGGTYYADSVKGRFTISRDNKNTLYLQMNSLRAEDTAVYYCAKANWNYAYFDFWGQGTLLTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCGGGGSGGGGSEVQLVESGGGLVQPGGSLRLSCAASGFTFNTYAMNWVRQAPGKGLEWVARIRSKYNNYATYYADSVKDRFTISRDDSKNTLYLQMNSLKAEDTAVYYCVRHGNFGNSYVSWFAYWG

	QGTLVTVSSGGGGSGGGGSGGGGSGGGGSGQAVVTQEPSLTVSPGGTVTLT CGSSTGAVTTSNYANWVQQKPGQAPRGLIGGTNKRAPGTPARFSGSLLGG KAALTLGAQPEDEAEYYCALWYSNLWVFGGGTKLTVLGGGGSGGGGSK THTCPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVDVSHEDPEVK FNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCK VSNKALGAPIEKTISKAKGQPREPQVYTLPPCRDELTKNQVSLWCLVKGFY PSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFS CSVMHEALHNHYTQKSLSLSPGK
Chain 3	EIVMTQSPATLSVSPGERATLSCRASQVRSSNLAWYQQKPGQAPRLLIYGA STRATGIPARFSGSGSGTEFTLTINSLSQADFAVFFCQQYDIWPMYTFGQGT KLEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNA LQSGNSQESVTEQDSKSTYLSSTLTLSKADYEKHKVYACEVTHQGLSSP VTKSFNRGEC

[00447] 2:1 anti-CTHRC1 x anti-CD3 engager [annotated]

Chain 1	anti-CTHRC1 M3 heavy variable	EVQLVESGGGLVQPGGSLRLSCAASGFTFSNCAMNWVRQAPGKG LEWVSVISGSGGGTYADSVKGRFTISRDNKNTLYLQMNSLRAE DTAVYYCAKANWNYAYFDFWQGQGLTVTVSS
	Human IgG1 hole	ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGAL TSGVHTFPAVLQSSGLYSLSSVTVPSSSLGTQTYICNVNHKPSNTK VDKKVEPKSCDKTHTCPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALGAPIEKTISKAKGQPREPQVCTLPSSRDELTKNQVSLSCAVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLVSKLTVDKSRWQQGNVFSFCSVMHEALHNHYTQKSLSLSPGK
Chain 2	anti-CTHRC1 M3 heavy variable	EVQLVESGGGLVQPGGSLRLSCAASGFTFSNCAMNWVRQAPGKG LEWVSVISGSGGGTYADSVKGRFTISRDNKNTLYLQMNSLRAE DTAVYYCAKANWNYAYFDFWQGQGLTVTVSS
	Human CH1+hi nge	ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGAL TSGVHTFPAVLQSSGLYSLSSVTVPSSSLGTQTYICNVNHKPSNTK VDKKVEPKSC
	Linker	GGGGSGGGGS
	Anti-CD3e variable heavy	EVQLVESGGGLVQPGGSLRLSCAASGFTFNTYAMNWVRQAPGKG LEWVARIRSKYNNYATYYADSVKDRFTISRDDSKNTLYLQMNSLK AEDTAVYYCVRHGNFGNSYVSWFAYWGQGLTVTVSS
	Linker	GGGGSGGGGSGGGGSGGGGS

	Anti-CD3e variable light	QAVVTQEPSLTVSPGGTVTLTCGSSTGAVTTSNYANWVQQKPGQA PRGLIGGTNKRAPGTPARFSGSLLGGKAALTLGAQPEDEAEYYCA LWYSNLWVFGGGTKLTVL
	Linker	GGGSGGGG
	Human IgG1 knob	ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGAL TSGVHTFPAVLQSSGLYSLSSVTVPSSSLGTQTYICNVNHKPSNTK VDKKVEPKSCDKTHTCPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVT CVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQD WLNGKEYKCKVSNKALGAPIEKTISKAKGQPREPQVCTLPSSRDELTKNQVSL SCAVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLVSKLTVDKSRWQ QGNVFSCSVMHEALHNHYTQKSLSLSPGK
Chain 3	anti-CTHRC1 M3 light variable	EIVMTQSPATLSVSPGERATLSCRASQRVSSNLAWYQQKPGQAPRL LIYGASTRATGIPARFSGSGSGTEFTLTINSLQSADFAVFFCQQYDIW PMYTFGQGTKLEIK
	Human kappa constant	RTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDN ALQSGNSQESVTEQDSKDYSLSSSTLTLSKADYEKHKVYACEVTH QGLSSPVTKSFNRGEC

[00448] Caspase activation results, both visual and quantitative, are shown in Figs. 17B-17C. CTHRC1 indicates treatment with the anti-CTHRC1 T-cell engager.

[00449] These results demonstrate that the anti-CTHRC1 T-cell engagers induce robust killing of KP4 tumor cells in co-culture with CAFs.

Example 12: Ex vivo Effect on Tumor Biopsies

[00450] Human CRC tumor biopsies obtained from surgical resection were used to establish *ex vivo* histocultures. Tumor tissue was first embedded in 2% agarose for slicing using a vibratome to obtain ~200um slices. Each tumor piece was weighed and randomized into treatment groups containing four to five tumor pieces. Tumor pieces were transferred to a cell culture insert within a well of a 24-well tissue culture plate containing media to generate an air-liquid interface and then treated with anti-CTHRC1 T-cell engager in a Cis 2:1 configuration or Dengue targeted CD3 bispecific control at a singular concentration for 48 hours. After 48 hours, tumor pieces were pooled from each treatment group, dissociated to obtain single cells and flow cytometry analysis was performed to quantify tumor cell viability, number of CD8 T cells present and CD8 T cells activation as assessed by 4-1BB expression. In parallel, culture supernatants were

collected for MSD cytokine analysis to assess changes in Granzyme B, Perforin and IFN γ post-treatment.

[00451] Figs. 18A-18F show the results for two human colorectal cancer samples that were treated. Donor 1 was a 73 year old male CRC patient with microsatellite instability; Donor 2 was a 69 year old male CRC patient who is microsatellite stable. Fig. 18A shows quantification of tumor cell viability while Fig. 18B shows the number of CD8 T cells relative to control CD3 engager treatment for both donors. Figs. 18C-18F show the levels of 4-1BB expression and profiling of culture supernatants for perforin, granzyme B and IFN- γ for Donor 2. CTHRC1 indicates treatment with the anti-CTHRC1 T-cell engager.

[00452] These results demonstrate that the anti-CTHRC1 T-cell engager demonstrated tumor cell killing, CD8 T cell expansion, activation, and cytokine release in human tumors treated *ex vivo*.

Example 13: In vivo Mouse Tumor Model

[00453] 6–8-week-old female C56BL/6 mice were obtained from the Jackson Laboratories and implanted subcutaneously on the right flank with 2.5×10^5 MC38-hCTHRC1 cells mixed 1:1 with matrigel under isoflurane anesthesia. When tumor volumes reached approximately 100mm³, mice were randomized by tumor volume and assigned to treatment groups of 10 animals per group. Mice were injected intraperitoneally on Day 0 (day of randomization) and Day 7 with 60 μ g (approximately 3mg/kg) of anti-CTHRC1 T-cell engager or Dengue targeted CD3 bispecific control prepared in sterile PBS. Tumor growth and animal body weights were monitored at least twice weekly. Mice were sacrificed when tumor volumes exceeded 2000mm³, tumors became ulcerated, body weight decreased by at least 20% or other signs of clinical distress were noted, in accordance with IACUC protocols.

[00454] Fig. 19A depicts the trial design for this example. Fig. 19B depicts the average growth curve post-dose for both treatment groups with dots on the x-axis indicating dosing times. Fig. 19C depicts the individual growth curves post-dose for both treatment groups with dots on the x-axis indicating dosing times.

[00455] These data show that the anti-CTHRC1 T-cell engager resulted in robust anti-tumor activity in the MC38-CTHRC1 colon cancer model including 7/10 mice tumor free in the anti-CTHRC1 T-cell engager-treated group.

While the foregoing invention has been described in some detail for purposes of clarity and understanding, it will be clear to one skilled in the art from a reading of this disclosure that various changes in form and detail can be made without departing from the true scope of the invention. For example, all the techniques and apparatus described above can be used in various combinations. All publications, patents, patent applications, and/or other documents cited in this application are incorporated by reference in their entirety for all purposes to the same extent as if each individual publication, patent, patent application, and/or other document were individually indicated to be incorporated by reference for all purposes.

SEQUENCE LISTING

SEQ ID NO	SEQUENCE
1 (SP34 HCDR1)	GFTFNTYA
2 (SP34 HCDR2)	IRSKYNNYAT
3 (SP34 HCDR3)	VRHGNFGNSYVSWFAY
4 (SP34 LCDR1)	TGAVTTSNY
5 (SP34 LCDR2)	GTN
6 (SP34 LCDR3)	ALWYSNLWV
7 (PAI-SP34 VH)	EVQLVESGGGLVQPGGSLRLSCAASGFTFNTYAMNWVRQAPGKG LEWVARIRSKYNNYATYYADSVKDRFTISRDDSKNTLYLQMNSL KAEDTAVYYCVRHGNFGNSYVSWFAYWGQGTLVTVSS

8 (PAI-SP34 VL)	QAVVTQEPSLTVSPGGTVTLTCGSSTGAVTTSNYANWVQKPGQ APRGLIGGTNKRAPGTPARFSGSLLGGKAALTLGAQPEDEAEYY CALWYSNLWVFGGGTKLTVL
85 (Human Collagen triple helix repeat-containing protein 1 Isoform 1 Gene (CTHRC1), Uniprot Accession number Q96CG8- 1)	MRPQGPAASPQRLRGLLLLLLLQLPAPSSASEIPKGKQKAQLRQRE VVDLYNGMCLQGPAAGVPGRDGSPGANGIPGTPGIPGRDGFKEK GECLRESFEESWTPNYKQCSWSSLNYGIDLGKIAECTFTKMRSNS ALRVLFSGSLRLKCRNACCQRWYFTFNGAECSGPLPIEAIYLDQG SPEMNSTINIHRTSSVEGLCEGIGAGLVDVAIWVGTCSDYPKGDAS TGWNSVSRIIIIEELPK
86 (Human Collagen triple helix repeat-containing protein 1 Isoform 2 Gene (CTHRC1), Uniprot Accession number Q96CG8- 2)	MWPPGRSITVKLREKTVSRKLEMNGPSAFQGLICGKYNGMCLQG PAGVPGRDGSPGANGIPGTPGIPGRDGFKEKGECLRESFEESWTP NYKQCSWSSLNYGIDLGKIAECTFTKMRSNSALRVLFSGSLRLKC RNACCQRWYFTFNGAECSGPLPIEAIYLDQGSPEMNSTINIHRTSS VEGLCEGIGAGLVDVAIWVGTCSDYPKGDASTGWNSVSRIIIIEELP IYML
87 (Human Collagen triple helix repeat-containing protein 1 Isoform 3 Gene (CTHRC1) Uniprot Accession number Q96CG8-3)	MWPPGRSITVKLREKTVSRKLEMNGPSAFQGLICGKYNGMCLQG PAGVPGRDGSPGANGIPGTPGIPGRDGFKEKGECLRESFEESWTP NYKQCSWSSLNYGIDLGKIAECTFTKMRSNSALRVLFSGSLRLKC RNACCQRWYFTFNGAECSGPLPIEAIYLDQGSPEMNSTINIHRTSS VEGLCEGIGAGLVDVAIWVGTCSDYPKGDASTGWNSVSRIIIIEELP K
88 (AB987 heavy	GAGGTGCAGTTGGTAGAAAGCGGCGGTGGACTCGTGACCCCTG GTGGGTCTCTTCGCTTATCTTGCGCTGCTTCCGGCTTCACTTTTT

chain polynucleotide)	CTACGTACAGTATGAATTGGGTTCGACAGGCACCCGGCAAGGG CCTGGAATGGGTGTCCAGTATCTCGGGATCCTCAGGACATATTT ACTACGCTGATAGCGTGAGGGGGCGGTTACAAATCAGCCGTGA TAATGCCAAGAACTCTCTCTATCTACAAATGAACAGTCTGAGA GCAGAGGACACAGCCGTCTACTACTGTGCCTCAATGCACTACT ATTATTATCTGGACGTCTGGGGGAAAGGTACTACCGTTACCGT GAGCTCC
89 (AB987 light chain polynucleotide)	CAGGCAGTGGTGACTCAGGAATCCGCTCTTACAACCTCCCCCG GCGAAACCGTAACATTAACCTGCAGATCAAGTACCGGAACCGT GACTACATCTAACTATGTCACCTGGGTCCAAGAGAAGCCTGAT CACCTGTTACACGGCTTGATTGGAGCCACAAATAACCGGGCCC CGGGTGTTCCAGCGAGGTTACGCGGATCTCTCATCGGTGACAA AGCTGCCCTCACAATCACCGGGGCTCAGACTGAGGACGAGGCA ATATACTTTTGTGCCCTGTGGTACAGCAATCATTGGGTTTTTGG TGGGGGCACTAAGCTGACGGTGCTA
90 (AB988 heavy chain polynucleotide)	CAGGTCCAGCCTGTGCAATCAGGAGCCGAAGTTAAGAAGCCCG GGGCTAGCGTTAAAGTGAGTTGTAAAGCTTCCGGTTACACTTTT ACATCTTATGATATCAACTGGGTGCGCCAGGCGACTGGCCAGG GACTCGAGTGGATGGGCTGGATGAATCCAAATTCCGGTAACAC CGGCTACGCCCAAAAGTTCCAGGGACGGGTGACCATGACCAGG AACACATCCATTTGACCGCATACATGGAGCTGAGCAGTTTGA GATCCGAAGATACCGCTGTATATTATTGCGCAATTACAGGCAC CACTGCCGGGACGTTTCGACATCTGGGGGCAGGGTACTATGGTG ACAGTCTCTCAGGACATATCAATGATCAGCTCT
91 (AB988 light chain polynucleotide)	GACATCCAGCTCACTCAATCGCCTTCCTTTCTGTCAGCTTCTGT CGGAGCCAGGGTGACTACCACCTGTCCGGCTAGTCAGGGAATA AGCTCTTACTTGGCATGGTACGAGCAGAAACCAGGGAAGGCAC CAAAGCTACTGATCTATGCGGCCTCCACTCTGCAGTCAGGTGT GCCTAGCAGATTCTCTGGCAGCGGGTCTGGCACGGAGTTTACA CTTACAATTTCCAGCCTCCAACCCGAAGATAGTGCCACATACT

	ATTGCCAGCAGCACAACTCCTATCCCTGGACCTTCGGCCAGGG TACCAAGGTTGAAATTAAA
92 (AB989 heavy chain polynucleotide)	CAGGTGACATTGAGAGAATCCGGCCCCGCACTGGTCAAGCCCA CCCAGACTCTAACATTAACCTGCACTTTTTCTGGGTTCAGTCTG TCGACCAACGTGATGTGCGTCTCATGGATAAGGCAACCTCCAG GTAAAGCTCTGGAGTGGCTCGCCCTTATTGACTGGGATGATGA CAAATATTACAGCACCAGCCTGAAGACGCGGCTCACCATCTCA AAGGACACTAGTAAAAACCAGGTGGTTTTTGACGATGACCAATA TGGATCCTGTAGACACAGCCACTTACTATTGTGCTCGCACAGG AATCACAGGGACTCACTACTACTATTACTATATGGATGTGTGG GGCAAGGGAACAACCGTGACCGTTTCTTCC
93 (AB989 light chain polynucleotide)	GCCATCCAGATGACACAAAGCCCATCCAGTCTGTCTGCCAGCG TTGGAGACCGGGTGACTATAACCTGTAGAGCATCACAGGGCAT CCGCAACGATCTGGGGTGGTATCAGCAGAAGCCAGGTAAAGC ACCCAAGTTGCTCATTTATGCCGCTTCGTCTCTTCAGTCTGGAG TCCCTTCCAGGTTTTCCGGCAGTGGTTTCGGCACAGACTTTACT CTAACCATTTCAAGCCTGCAACCCGAAGATTTTCGTACCTACTA CTGCCTCCAGGACTATAATTACCCTTGGACGTTTCGGACAGGGG ACAAAGGTGGAGATCAAA
94 (AB990 heavy chain polynucleotide)	GAGGTGCAGCTGGTTGAATCTGGCGGAGGGCTGGTCCAACCCG GAGGCTCCCTGCGCCTCAGTTGCGCTGCATCCGGCTTTACTTTC TCTAACTGTGCTATGAACTGGGTGAGACAGGCCCTGGTAAAG GTCTCGAATGGGTGAGCGTGATTAGCGGCAGCGGCGGAGGTAC ATATTATGCCGATTCCGTAAAAGGGCGATTACCATCTCACGG GACAATTCAAAGAATACCTTGTACCTTCAGATGAACTCTCTAA GGGCAGAGGACACAGCCGTGTACTATTGCGCCAAGGCTAATTG GAACTACGCGTACTTTGATTTCTGGGGACAGGGGACTTTAGTC ACCGTTAGTTTCG
95 (AB990 light)	GAAATCGTGATGACTCAGTCTCCAGCCACTCTCTGTTCACC CGGAGAGCGTGCTACACTGTCCTGCAGAGCATCCCAACGAGTG TCCAGCAATCTAGCCTGGTACCAGCAGAAGCCAGGGCAGGCGC

chain polynucleotide)	CTCGCCTGCTTATCTATGGGGCTTCTACACGGGCTACCGGTATT CCCGCAAGGTTTCAGCGGCTCAGGAAGTGGCACCGAGTTTACAC TGACCATAAACAGTTTGCAGAGCGCCGACTTTGCCGTCTTCTTT TGTCAGCAGTATGATATCTGGCCTATGTACACGTTTCGGCCAAG GTACCAAACCTCGAAATTAAG
96 (AB991 heavy chain polynucleotide)	GAGGTGCAGGTTGTCGAATCGGGAGGCGGGCTGGTGCAGCCCG GTGGGAGTCTGAGACTCTCCTGCGCTGCATCAAGGTTACCTTT AGCAATTACTGGATGTCCTGGGTGCGTCAAGCTCCTGGTAGGG GGCTGGAATGGGTCGCCAATATTAAGCAGGATGGTAGCGAGA AGTACTATGTCGACTCTGTAAAAGGCCGTTTACAATCTCCCG AGACAACGCCAAAAACAGTCTTTATCTACAGATGAACAGCTTG CGCGCAGAGGACACTGCCGTGTACTACTGTGCGCGGGAGGGAG GCTATTGCTCTGATACCAGATGTTTCTTCTATATGGATGTGTGG GGAAAGGGCACCCTGTGACAGTTTCTTCA
97 (AB991 light chain polynucleotide)	GACATTCAAATGACTCAATCTCCATCCACTCTATCCGCATCAGT GGGCGATCGGGTGACCATCACATGTAGAGCTTCCCAGAGCATT ATATCTTGTTGGCGTGGTACCAGCAGAAACCTGGCAAGGCTC CCAAGCTTCTGATCTATAAGGCCTCATCTCTCGAAGGTGGAGTC CCCAGCCGCTTTAGCGGGTCCGGGAGTGGTACCGACTTTACGC TGACAATCAGTTCGCTGCAGCCTGATGACTTCGCCACCTATTAC TGCCAGCAGTACAACAGCTATTCTAGGACCTTCGGACAGGGCA CAACTGTTGAGATCAAA
98 (Linker)	GGGS
99 (Linker)	GSG
100 (Linker)	GGGSGGGSGGGGS
102 (M23 scFv)	QVQPVQSGAEVKKPGASVKVSCKASGYTFTSYDINWVRQATGQG LEWMGWMNPNSGNTGYAQKFQGRVTMTRNTSISTAYMELSSLR

	SEDTAVYYCAITGTTAGTFDIWGQGTMTVTSSGGGGSGGGSGG GGS DIQLTQSPSFLSASVGARVTTTCRASQGISSYLAWYEQKPGKAPKL LIYAASLTQSGVPSRFSGSGSGTEFTLTISLQPEDSATYYCQQHNS YPWTFGQGTKVEIK
104 (M23 alternate scFv)	DIQLTQSPSFLSASVGARVTTTCRASQGISSYLAWYEQKPGKAPKL LIYAASLTQSGVPSRFSGSGSGTEFTLTISLQPEDSATYYCQQHNS YPWTFGQGTKVEIKGGGGSGGGSGGGGSQVQPVQSGAEVKKP GASVKVSCKASGYTFTSYDINWVRQATGQGLEWMGWMNPNSG NTGYAQKFQGRVTMTRNTSISTAYMELSSLRSEDNAVYYCAITGT TAGTFDIWGQGTMTVTSS
106 (M5 scFv)	EVQLVESGGGLVTPGGSLRLSCAASGFTFSTYSMNWVRQAPGKG LEWVSSISGSSGHIYYADSVRGRFTISRDNKNSLYLQMNSLRAED TAVYYCASMHHYYYLDVWGKGTTVTVSSGGGGSGGGSGGGG S QAVVTQESALTTSPGETVTLTCSRSTGTVTTSNYVTWVQEKPDHL FTGLIGATNNRAPGVPARFSGSLIGDKAALTITGAQTEDEAIYFCA LWYSNHWVFGGGTKLTVL
108 (M5 alternate scFv)	QAVVTQESALTTSPGETVTLTCSRSTGTVTTSNYVTWVQEKPDHL FTGLIGATNNRAPGVPARFSGSLIGDKAALTITGAQTEDEAIYFCA LWYSNHWVFGGGTKLTVL GGGGSGGGSGGGGSEVQLVESGGGLVTPGGSLRLSCAASGFTFS TYSMNWVRQAPGKGLEWVSSISGSSGHIYYADSVRGRFTISRDN KNSLYLQMNSLRAEDTAVYYCASMHHYYYLDVWGKGTTVTVSS

CLAIMS

1. An anti-CTHRC1 T-cell engager comprising at least one first domain comprising a CTHRC1 binding moiety that selectively binds to human CTHRC1 and a cell displaying a CTHRC1 epitope without being internalized, and at least one second domain comprising a T cell engager moiety.

2. The anti-CTHRC1 T-cell engager of claim 1, wherein the CTHRC1 binding moiety comprises an anti-CTHRC1 antibody comprising a heavy chain complementarity determining region (HCDR) 1, HCDR2 and HCDR3 of SEQ ID NOs: 48, 53, and 58, respectively, and a light chain complementarity determining region (LCDR) 1, LCDR2 and LCDR3 of SEQ ID NOs: 63, 68 and 73, respectively.

3. The anti-CTHRC1 T-cell engager of claim 1 or 2, wherein the T cell engager moiety comprises an anti-CD3 antibody or fragment thereof.

4. The anti-CTHRC1 T-cell engager of claim 2, wherein the CTHRC1 binding moiety comprises a heavy chain variable region (HCVR) comprising SEQ ID NO: 81 and a light chain variable region (LCVR) comprising SEQ ID NO: 82.

5. The anti-CTHRC1 T-cell engager of any preceding claim, wherein the anti-CD3 antibody or fragment thereof comprises a heavy chain complementarity determining region (HCDR) 1, HCDR2 and HCDR 3 of SEQ ID NOs: 1, 2, and 3, respectively, and a light chain complementarity determining region (LCDR) 1, LCDR2 and LCDR3 of SEQ ID NOs: 4, 5, and 6, respectively.

6. The anti-CTHRC1 T-cell engager of any preceding claim, wherein the anti-CD3 antibody or fragment thereof comprises a heavy chain variable region (HCVR) sequence having at least 80% sequence identity to the sequence of SEQ ID NO: 7 and/or a light chain variable region (LCVR) sequence having at least 80% sequence identity the sequence of SEQ ID NO: 8.

7. The anti-CTHRC1 T-cell engager of any preceding claim, wherein the anti-CD3 antibody or fragment thereof comprises a heavy chain variable region (HCVR) sequence having the sequence of SEQ ID NO: 7 and/or a light chain variable region (LCVR) sequence having the sequence of SEQ ID NO: 8.

8. The anti-CTHRC1 T-cell engager of any preceding claim, wherein the CTHRC1 binding moiety comprises an anti-CTHRC1 antibody which binds to CTHRC1; preferably wherein the CTHRC1 antibody binds to CTHRC1 with a binding affinity of less than 10 nM.

9. The anti-CTHRC1 T-cell engager of claim 8, wherein the anti-CTHRC1 antibody is a chimeric, humanized or human antibody.
10. The anti-CTHRC1 T-cell engager of claim 8, wherein the anti-CTHRC1 antibody is a monoclonal antibody.
11. The anti-CTHRC1 T-cell engager of claim 8, wherein the anti-CTHRC1 antibody comprises an anti-CTHRC1 antibody fragment; preferably wherein the antibody fragment is selected from the group consisting of Fab, F(ab')₂, Fv, scFv, dsFv and a single domain antibody.
12. The anti-CTHRC1 T-cell engager of claim 11, wherein the anti-CTHRC1 antibody comprises a single-chain antibody.
13. The anti-CTHRC1 T-cell engager of claim 11, wherein the anti-CTHRC1 antibody comprises a heavy-chain only antibody (single domain antibody).
14. The anti-CTHRC1 T-cell engager of any preceding claim comprising two first domains and/or two second domains; wherein the anti-CTHRC1 T-cell engager has a ratio of 2:1, 1:2, or 2:2 of first domain to second domain.
15. The anti-CTHRC1 T-cell engager of claim 14, wherein the anti-CTHRC1 T-cell engager has a ratio of 2:1 in cis configuration.
16. A method for activating T cells in a tumor microenvironment, comprising contacting a tumor with the anti-CTHRC1 T-cell engager of any one of claims 1-15.
17. A method for inhibiting the growth of a cell displaying a CTHRC1 tumor epitope in a patient in need thereof, comprising administering to said patient a therapeutically effective amount of an anti-CTHRC1 T-cell engager of any one of claims 1-15.
18. The method of claim 16 or 17, wherein the cancer is selected from the group consisting of breast cancer, lung cancer, colon cancer, ovarian cancer, melanoma cancer, bladder cancer, renal cancer, kidney cancer, liver cancer, head and neck cancer, colorectal cancer, melanoma, pancreatic cancer, gastric carcinoma cancer, esophageal cancer, mesothelioma, prostate cancer, leukemia, lymphomas, and myelomas.
19. The method of any one of claims 16-18, further comprising administering an allogenic or autologous T cell therapy in combination with a cell-based therapy.
20. The method of claim 19, wherein the cell-based therapy is selected from the group consisting of administering an allogenic or autologous T cell therapy, NK cell therapy, or macrophage therapy.

21. The method of any one of claims 16-18, further comprising administering an allogenic or autologous T cell therapy in combination with the anti-CTHRC1 T-cell engager.

22. The method of any one of claims 16-21, wherein the subject is a human.

23. A pharmaceutical composition comprising the anti-CTHRC1 T-cell engager of any one of claims 1-15 and a pharmaceutically acceptable carrier.

24. Use of the pharmaceutical composition of claim 23 in the preparation of a medicament for the treatment of a cell proliferative disorder, preferably cancer, or a fibrotic disease.