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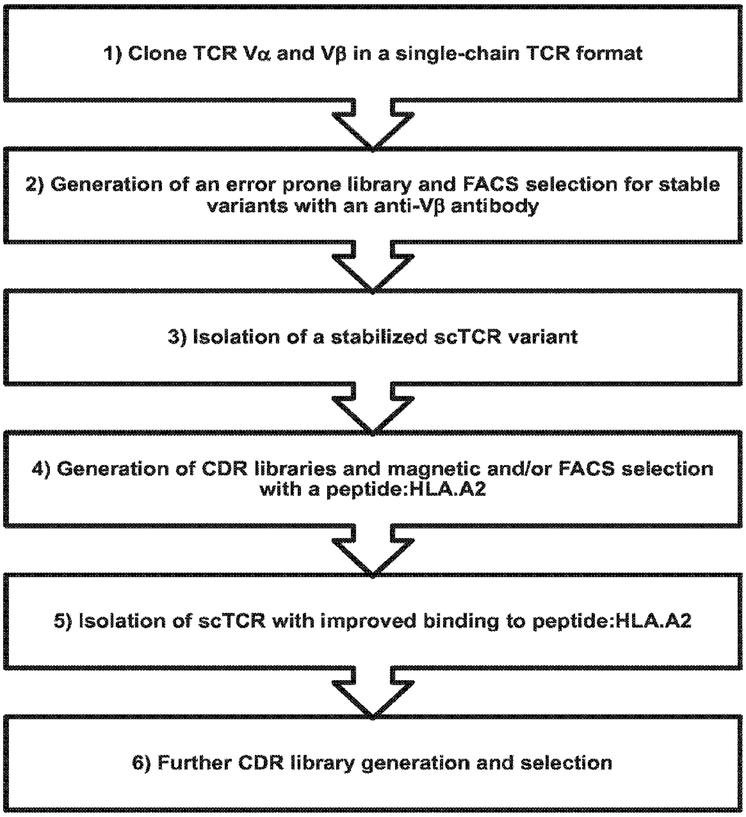
(54) **Titre :** RECEPTEURS MODIFIES DE HAUTE AFFINITE POUR LES LYMPHOCYTES T HUMAINS
(54) **Title:** ENGINEERED HIGH-AFFINITY HUMAN T CELL RECEPTORS



(57) **Abrégé/Abstract:**

T cell receptors (TCRs) that have higher affinity for the Survivin antigen are provided. The high affinity TCRs were engineered through the generation of mutational libraries of TCRs in a single-chain format, followed by selection for improved stability and affinity on the surface of yeast (i.e. directed evolution). In embodiments, the engineered TCRs can be used in soluble form for targeted delivery in vivo, or as genes introduced into T cells in an adoptive T cell setting.

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— *as to applicant's entitlement to apply for and be granted a patent (Rule 4.17(ii))*

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[Continued on next page]

(54) **Title**: ENGINEERED HIGH-AFFINITY HUMAN T CELL RECEPTORS

(57) **Abstract**: T cell receptors (TCRs) that have higher affinity for the Survivin antigen are provided. The high affinity TCRs were engineered through the generation of mutational libraries of TCRs in a single-chain format, followed by selection for improved stability and affinity on the surface of yeast (i.e. directed evolution). In embodiments, the engineered TCRs can be used in soluble form for targeted delivery in vivo, or as genes introduced into T cells in an adoptive T cell setting.

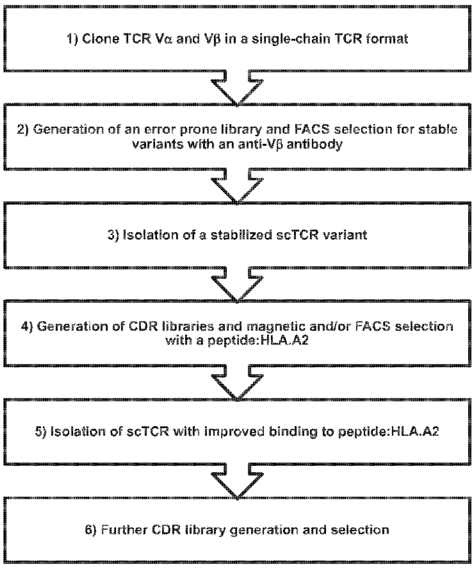

FIG. 1

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ENGINEERED HIGH-AFFINITY HUMAN T CELL RECEPTORS

[0001]

STATEMENT REGUARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

[0002] This disclosure was made with U.S. Government support under Grant numbers R01 GM55767 and T32 GM070421, awarded by the National Institutes of Health.

STATEMENT REGARDING SEQUENCE LISTING

[0003] The Sequence Listing associated with this application is provided in text format in lieu of a paper copy,

The name of the text file containing the Sequence Listing is IMMU_003_01WO_ST25.txt. The text file is 12 KB, was created on November 21, 2014 and is being submitted electronically via EFS-Web.

FIELD OF THE INVENTION

[0004] The disclosure relates to high-affinity T cell receptors (TCR), engineered by in vitro techniques, against the Survivin antigen, as well as methods of producing modified TCRs and single-chain TCRs and the corresponding uses of the TCRs for therapeutic, diagnostic, and imaging methods.

BACKGROUND

[0005] T cell receptors (TCRs) and antibodies are molecules that have evolved to recognize different classes of antigens (ligands)((Murphy (2012), xix, 868 p.)). TCRs are antigen-specific molecules that are responsible for recognizing antigenic peptides presented in the context of a product of the major histocompatibility complex (MHC) on the surface of antigen presenting cells (APCs) or any nucleated cell (*e.g.,* all human cells in the body, except red blood cells). In contrast, antibodies typically recognize soluble or cell-surface antigens, and do not require presentation of the antigen by an MHC. This system endows T cells, via their TCRs, with the potential ability to recognize the entire array of intracellular antigens expressed by a cell (including virus proteins) that are processed intracellularly into short peptides, bound to an intracellular MHC molecule, and delivered to the surface as a peptide-MHC complex (pepMHC). This system allows virtually any foreign protein (*e.g.,* mutated cancer antigen or virus protein) or aberrantly expressed protein to serve a target for T cells (reviewed in Davis and Bjorkman (1988) Nature, 334, 395-402; Davis et al. (1998) Annu Rev Immunol, 16, 523-544; Murphy (2012), xix, 868 p.).

[0006] The interaction of a TCR and a pepMHC can drive the T cell into various states of activation, depending on the affinity (or dissociation rate) of binding. The TCR recognition process allows a T cell to discriminate between a normal, healthy cell and, *e.g.*, one that has become transformed via a virus or malignancy, by providing a diverse repertoire of TCRs, wherein there is a high probability that one or more TCRs will be present with a binding affinity for the foreign peptide bound to an MHC molecule that is above the threshold for stimulating T cell activity (Manning and Kranz (1999) Immunology Today, 20, 417-422).

[0007] To date, wild type TCRs isolated from either human or mouse T cell clones that were identified by *in vitro* culturing have been shown to have relatively low binding affinities (K_d = 1 – 300 μM) (Davis et al. (1998) Annu Rev Immunol, 16, 523-544). Part of the explanation for this seems to be that T cells that develop in the thymus are negatively selected (tolerance induction) on self-pepMHC ligands, such that T cells with too high of an affinity are deleted (Starr et al. (2003) Annu Rev Immunol, 21, 139-76). To compensate for these relatively low affinities, T cells have evolved a co-receptor system in which the cell surface molecules CD4 and CD8 bind to the MHC molecules (class II and class I, respectively) and synergize with the TCR in mediating signaling activity. CD8 is particularly effective in this process, allowing TCRs with very low affinity (*e.g.,* K_d =300 μM) to mediate potent antigen-specific activity.

[0008] In vitro, directed evolution has been used to generate TCRs with higher affinity for a specific pepMHC. The three different display methods that have been used are yeast display (Holler et al. (2003) Nat Immunol, 4, 55-62; Holler et al.

(2000) Proc Natl Acad Sci U S A, 97, 5387-92), phage display (Li et al. (2005) Nat Biotechnol, 23, 349-54), and T cell display (Chervin et al. (2008) J Immunol Methods, 339, 175-84) . In all three approaches, the process involves engineering, or modifying, a TCR that exhibits the normal, low affinity of the wild-type TCR, so that affinity of mutants of the TCR have increased affinity for the cognate pepMHC (the original antigen that the T cells were specific for). Thus, the wild-type TCR was used as a template for producing mutagenized libraries in one or more of the CDRs, and mutants with higher affinity were selected by binding to the cognate peptide-MHC antigen.

[0009] In the present disclosure, high affinity T cell receptors specific for a Survivin cancer antigen engineered by yeast display are disclosed. The Survivin protein promotes oncogenesis by inhibiting signaling that leads to normal apoptosis (Dohi et al. (2004) Journal of Clinical Investigation 114, 1117-1127). Survivin is upregulated in cancerous tissue (Ambrosini et al. (1997) Nat Med 3, 917-921). It has been the target of vaccine efforts, and various adoptive T cell approaches using T cells with wild-type T cell receptors.

[0010] Survivin peptide antigen has been ranked number 21 in a prioritization list of the top 75 cancer antigens by the National Cancer Institute (Cheever et al. (2009) Clin Cancer Res, 15, 5323-5337). Accordingly, there is a need to identify agents, e.g., therapeutic agents, that specifically target this cancer antigen. The present invention provides in vitro engineered, higher affinity TCRs that can be used, e.g., in soluble form for targeted delivery in vivo or as genes introduced into T cells in an adoptive T cell setting.

SUMMARY OF THE INVENTION

[0011] The present invention relates to in vitro engineered T cell receptors (TCR) that bind to the Survivin antigen with improved affinity. More specifically, the present disclosure relates to stabilizing and affinity mutations selected through the display of libraries on the surface of yeast, phage, or mammalian cells; to TCR proteins selected from these libraries for binding to an antigen with increased affinity; and to the use of *in vitro* selected TCR derivatives for therapeutic, diagnostic, or imaging applications.

[0012] One aspect of the invention relates to a modified T cell receptor, or antigen binding fragment thereof, comprising a Vα and a Vβ derived from a wild type T cell receptor, wherein the Vα, the Vβ, or both, comprise a mutation in one or more complementarity determining regions (CDRs) relative to the wild type T cell receptor, wherein the modified T cell receptor binds to the peptide/MHC antigen known as Survivin/HLA-A2 (the Survivin peptide LMLGEFLKL (SEQ ID NO:5), bound to the MHC product known as HLA-A2).

[0013] In one embodiment, the modified T cell receptor comprises a modified Vα comprising an amino acid sequence having at least 80% identity to the Vα amino acid sequence set forth in SEQ ID NO:3, wherein the modified T cell receptor binds to Survivin/HLA-A2 with an affinity (K_A value) of 10^6 M higher.

[0014] In another embodiment, the modified T cell receptor comprises a modified Vα comprising an amino acid sequence having at least 80% identity to the Vα amino acid sequence set forth in SEQ ID NO:4, wherein the modified T cell receptor binds to Survivin/HLA-A2 with an affinity (K_A value) of 10^6 M higher.

[0015] In another embodiment, the T cell receptor is a single-chain T cell receptor comprising the amino acid sequence set forth in SEQ ID NO:6.

[0016] In another embodiment, the T cell receptor is a single-chain T cell receptor comprising the amino acid sequence set forth in SEQ ID NO:7.

[0017] In another embodiment, the T cell receptor contains at least one of the mutations in CDR3α selected from N92S, N100K, A101G, R102Y, and L103K of the amino acid sequence set forth in SEQ ID NO:3.

[0018] In another embodiment, the T cell receptor contains at least one of the mutations in CDR3α selected from N92H, N100G, A101W, R102Y, and L103T of the amino acid sequence set forth in SEQ ID NO:4.

[0019] In one embodiment, the modified T cell receptor is generated by in vitro selection of a yeast display library of mutant T cell receptors.

[0020] In another embodiment, the modified T cell receptor is expressed as a soluble protein that binds to its target antigen.

[0021] In another embodiment, the modified T cell receptor is expressed on the surface of T cells in order to mediate the activity of either $CD4^+$ or $CD8^+$ T cells.

[0022] One aspect of the invention relates to a therapeutic agent that targets cancer cells that express the survivin antigen, wherein the therapeutic agent comprises a modified T cell receptor described herein. In one embodiment, a therapeutic agent that targets cancer cells that express the survivin antigen, wherein the therapeutic agent comprises a human T cell that expresses a modified T cell receptor described herein.

[0023] One embodiment provides a method of treating a subject having a cancer that expresses the survivin antigen comprising administering a therapeutic agent described herein.

BRIEF DESCRIPTION OF THE DRAWINGS

[0024] Figure 1 is a diagram that shows a method for engineering single chain TCRs for improved affinity against a peptide:HLA.A2. The general process used to engineer high affinity TCRs is shown.

[0025] Figure 2A is a 3-dimensional diagram that shows a side view of the TCR:pepMHC complex (A6; PDB:1AO7). The variable (V) and constant (C) regions of the α-chain and β-chain are indicated. The structure shown does not include the Cα region of the TCR. HLA-A2 (α1, α2, α3, and β2m) is shown in gray, and the Tax peptide (LLFGYPVYV, SEQ ID NO:6) is shown in black. A6 and Survivin TCRs examined in the present invention all use the Vα2 segment (also referred to as TRAV12 based on IMGT nomenclature).

[0026] Figure 2B is a 3-dimensional diagram that shows the top down view of the TCR (CDR) footprint over the peptide-MHC (Tax/HLA-A2). Although no crystal structures have been described for the Survivin TCR used in the present disclosure, this diagonal docking orientation, with the Vα region positioned over the α2 MHC helix and the N-terminal end of the peptide, and the Vβ region positioned over the α1 MHC helix and C-terminal end of the peptide, has been observed in virtually all complexes to date.

[0027] Figure 3 is a schematic of the yeast-display system for engineering single-chain T cell receptor fragments (Vα-linker Vβ or Vβ-linker-Vα).

[0028] Figures 4A and 4B show flow cytometry histograms of the Survivin error prone library after sorting with an antibody that recognizes a conformation epitope of Vβ20. The Survivin error prone library was sorted sequentially with a 1:10 dilution of BC hVβ20 FITC IgG, followed by AlexaFluor® 488 goat anti-mouse IgG (1:100) secondary antibody, for a total of 3 sorts. Aliquots of yeast cells after each sort were incubated with a 1:10 dilution of BC hVβ20 (Figure 4A). Gray indicates yeast cells stained with secondary antibody only. The stable clones K2 stained with a 1:20 dilution of hVβ20 FITC IgG, followed by AlexaFlour 647 goat anti-mouse IgG (1:100) secondary antibody (Figure 4B).

[0029] Figures 5A and 5B show flow cytometry histograms of the Survivin CDR3α library after sorting with BC hVβ20 and SurvT2M:HLA-A2, and the binding of two high-affinity TCRs to SurvT2M:HLA-A2. The Survivin CDR3α library was sorted first with BC hVβ20 (1:10), followed by MB anti-mouse IgG MicroBeads (1:25) secondary antibody, using magnetic columns. The Survivin CDR3α libraries was then sorted with 100 nM SurvT2M:HLA-A2 dimer (DimerX; obtained from BD Pharmingen), followed by MB anti-mouse IgG MicroBeads (1:25) secondary antibody, for a total of three magnetic sorts. Isolated yeast were subsequently sorted using fluorescence-activated cell sorting (FACS) with 100 nM SurvT2M:HLA-A2 dimer (DimerX; obtained from BD Pharmingen), followed by AlexaFluor® 647 goat anti-mouse IgG (1:100) secondary antibody. Aliquots of yeast cells after each sort were then incubated with 100 nM SurvT2M:HLA-A2 dimer (DimerX; obtained from BD Pharmingen), followed by AlexaFluor® 647 goat anti-mouse IgG (1:100) secondary antibody(Figure 5A). Gray indicates yeast cells stained with secondary antibody only. The improved binding clones K2.4.1 (Figure 5B, left panel) and K2.4.6 (Figure 5B, right panel), isolated after 4th sort using FACS, are stained with 100 nM SurvT2M:HLA-A2 dimer (DimerX; obtained from BD Pharmingen), followed by AlexaFluor® 647 goat anti-mouse IgG (1:100) secondary antibody (Figure 5B).

[0030] Figures 6A and 6B show the binding properties of a high affinity TCR, K2.4.1, for SurvT2M:HLA-A2 monomers. Figure 6A is a flow cytometry histogram showing the high affinity scTCR K2.4.1 stained with various concentrations of SurvT2M:HLA-A2 monomer, followed by SA-PE (1:100) secondary antibody. Figure 6B is a line graph showing mean fluorescence intensity (MFI) values of histograms in Figure 6A plotted versus SurvT2M:HLA-A2 monomer concentration.

[0031] Figure 7 depicts the sequences of the Survivin-specific (K2.4.1 and K2.4.6) high-affinity TCRs. High-affinity single-chain variants were isolated from CDR libraries that were then screened for affinity maturation. Mutations isolated from stability libraries are underlined and bolded; mutations isolated from affinity maturation libraries are boxed and bolded. The wild-type V regions sequence with the "stabilizing" mutations in the K2 yeast displayed clone are also shown. The amino acid sequences shown for the Vβ chain correspond to SEQ ID NO:12, and the linker sequence depicted is SEQ ID NO:7. The amino acid sequences shown for the Vα chain correspond to SEQ ID NOs:13, 1 and 2, from top to bottom.

[0032] Figures 8A-8C show the results of a T cell assay in which T cells were transduced with the K2.4.1 TCR. T cells were isolated from AAD transgenic mice (these are mice that have a hybrid class I gene consisting of the α1 and α2 domains of HLA-A2 and the α3 domain of the mouse D^b; these AAD mice are available from Jackson Laboratories). The cells were activated with beads coupled with anti-CD3/anti-CD28 beads for 24 hours. T cells were retrovirally transduced using the pMP71 vector containing the Vα and β domains of the K2.4.1 TCR linked to the Cα and Cβ domains of the murine 2C TCR (Figure 8A). Mock (Gray) and K2.4.1 transduced (Black line) T cells were then stained with SurvT2M:HLA-A2 tetramer at a concentration of 20 nM (Figure 8B). T cells were then incubated for at a 1:1 E:T with human T2 cells that express HLA-A2, and various concentrations of survivin peptide for 24 hours. Supernatants were collected and IFN-γ release was measured using an ELISA (Figure 8C).

[0033] Figures 9A and 9B are diagrams that illustrate exemplary therapeutic applications of the high-affinity, single-chain TCRs isolated from the scaffold libraries. Figure 9A depicts five examples of TCR formats for use as soluble therapeutic products: 1) single-chain TCR in either a Vα-Vβ orientation or Vβ-Vα orientation (mutated high-affinity V domains are shown with an asterisk); 2) single-chain TCR fused in frame with the constant region domains of an antibody; 3) in-frame immunoglobulin fusion to either the constant region of the light chain or the heavy chain; 4) single-chain TCR (or the immunoglobulin fusions shown in 2 and 3) directly coupled to a drug; and 5) single-chain TCR linked in-frame with a single-chain Fv (VL-linker-VH) to generate a bispecific agent. Figure 9B depicts two examples of cellular based therapies that would use the high-affinity variable domains (V) isolated by yeast display, cloned into mammalian cell vectors, for expression by T cells in adoptive T cell therapy as: 1) single-chain receptors in chimeric antigen receptors (CAR) and 2) full length α and β TCRs.

BRIEF DESCRIPTION OF THE SEQUENCES

[0034] SEQ ID NO:1 is the amino acid sequence of a modified Vα region of the TCR (Survivin-K2.4.1) that binds with high-affinity to Survivin/HLA-A2.

[0035] SEQ ID NO:2 is the amino acid sequence of another modified Vα region of the TCR (Survivin-K2.4.6) that binds with high-affinity to Survivin/HLA-A2.

[0036] SEQ ID NO:3 is the amino acid sequence of a single-chain TCR (Survivin-K2.4.1) that binds with high-affinity to Survivin/HLA-A2.

[0037] SEQ ID NO:4 is the amino acid sequence of another single-chain TCR (Survivin-K2.4.6) that binds with high-affinity to Survivin/HLA-A2.

[0038] SEQ ID NO:5 is the amino acid sequence of the Survivin antigen.

[0039] SEQ ID NO:6 is the amino acid sequence of the Tax antigen.

[0040] SEQ ID NO:7 is the amino acid sequence of the linker.

[0041] SEQ ID NO:8 is the is the polynucleotide sequence of the primer Splice 4L.

[0042] SEQ ID NO:9 is the is the polynucleotide sequence of the primer T7.

[0043] SEQ ID NO:10 is the is the polynucleotide sequence of the reverse primer used to generate the PreSOE #1 of the Surv CDR3α library.

[0044] SEQ ID NO:11 is the is the polynucleotide sequence of the forward primer used to generate the PreSOE #2 of the Surv CDR3α library.

[0045] SEQ ID NO:12 is the amino acid sequence of the Vb region of the TCR (Survivin-K2) that binds to Survivin/HLA-A2.

[0046] SEQ ID NO:13 is the amino acid sequence of the Va region of the TCR (Survivin-K2) that binds to Survivin/HLA-A2.

[0047] SEQ ID NO:14 is the amino acid sequence of the WT1 antigen.

[0048] SEQ ID NO:15 is the amino acid sequence of an influenza A peptide.

[0049] SEQ ID NO:16 is the amino acid sequence of a variant influenza A peptide.

DETAILED DESCRIPTION

[0050] The following description is intended to facilitate understanding of the disclosure but is not intended to be limiting.

[0051] In general, the terms and phrases used herein have their art-recognized meaning, which can be found by reference to standard texts, journal references and contexts known to those skilled in the art. The following definitions are provided to clarify their specific use in the context of the disclosure.

[0052] As used herein, “linked” refers to an association between two groups, which can be a covalent or non-covalent association. Groups may be linked using a variable length peptide chain, a non-amino acid chemical group or other means as known in the art. A linker region can be an amino acid sequence that operably links two functional or structural domains of a protein or peptide.

[0053] As used herein, the term “chemotherapeutic agent” refers to any substance capable of reducing or preventing the growth, proliferation, or spread of a cancer cell, a population of cancer cells, tumor, or other malignant tissue. The term is intended also to encompass any antitumor or anticancer agent.

[0054] As used herein, the term “effective amount” is intended to encompass contexts such as a pharmaceutically effective amount or therapeutically effective amount. For example, in certain embodiments, the effective amount is capable of achieving a beneficial state, beneficial outcome, functional activity in a screening assay, or improvement of a clinical condition.

[0055] As used herein, the term “cancer cell” is intended to encompass definitions as broadly understood in the art. In one embodiment, the term refers to an abnormally regulated cell that can contribute to a clinical condition of cancer in a human or animal. In one embodiment, the term can refer to a cultured cell line or a cell within or derived from a human or animal body. A cancer cell can be of a wide variety of differentiated cell, tissue, or organ types as is understood in the art. Particular examples of cancer cells include breast cancer, colon cancer, skin cancer, ovarian cancer, leukemia, lung cancer, liver cancer, testicular cancer, esophageal cancer, and other types of cancer.

[0056] As used herein, “treating” or “treatment” refers to an approach for obtaining beneficial or desired results, including and preferably clinical results. Treatment can refer to either the amelioration of symptoms of the disease or condition, or the delaying of the progression of the disease or condition.

[0057] As used herein, "prevention" or “preventing” refers to an approach for preventing, inhibiting, or reducing the likelihood of, the onset or recurrence of a disease or condition. It also refers to preventing, inhibiting, or reducing the likelihood of, the occurrence or recurrence of the symptoms of a disease or condition, and it also includes reducing the intensity, effect, symptoms and/or burden of a disease or condition prior to onset or recurrence of the disease or condition.

[0058] As used herein, “inhibiting cell growth” or “inhibiting proliferation of cells” refers to reducing or halting the growth rate of cells. For example, by inhibiting the growth of tumor cells, the rate of increase in size of the tumor may slow. In other embodiments, the tumor may stay the same size or decrease in size, i.e., regress. In particular embodiments, the rate of cell growth or cell proliferation is inhibited by at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, or at least 90%.

[0059] The terms “wild type” and “wt” are used interchangeably herein and are used in reference to a TCR having an amino acid sequence or a polynucleotide encoding the variable regions isolated from a naturally occurring or non-modified TCR, e.g., the original or parent T cell clone, with specificity for the antigen.

[0060] In the figures and tables that present amino acid sequences, the wild type is designated “wt”. In the sequences presented below the top sequence, a dash indicates the amino acid is the same as that present in the wt or top sequence of the alignment. A letter indicates a substitution has been made in that position from the top sequence.

[0061] As used herein, the terms “modified”, “variant”, “mutant”, “mutated” and “derived” T cell receptor refer to TCR sequences of the variable regions having one or more mutations compared to the original or wild type T cell clone. Examples of modified TCRs include higher affinity TCRs.

[0062] A coding sequence is the part of a gene or cDNA which codes for the amino acid sequence of a protein, or for a functional RNA such as a tRNA or rRNA.

[0063] Complement or complementary sequence means a sequence of nucleotides that forms a hydrogen-bonded duplex with another sequence of nucleotides according to Watson-Crick base-pairing rules.

[0064] Downstream refers to a relative position in DNA or RNA and is the region toward the 3' end of a strand.

[0065] Expression refers to the transcription of a gene into structural RNA (rRNA, tRNA) or messenger RNA (mRNA) and subsequent translation of an mRNA into a protein.

[0066] Two nucleic acid sequences are heterologous to one another if the sequences are derived from separate organisms, whether or not such organisms are of different species, as long as the sequences do not naturally occur together in the same arrangement in the same organism.

[0067] Homology refers to the extent of identity between two nucleotide or amino acid sequences.

[0068] An amino acid sequence that is functionally equivalent to a specifically exemplified TCR sequence is an amino acid sequence that has been modified by single or multiple amino acid substitutions, by addition and/or deletion of amino acids, or where one or more amino acids have been chemically modified, but which nevertheless retains the binding specificity and high affinity binding activity of a cell bound or a soluble TCR protein of the present disclosure. Functionally equivalent nucleotide sequences are those that encode polypeptides having substantially the same biological activity as a specifically exemplified cell-bound or soluble TCR protein. In the context of the present disclosure, a soluble TCR protein lacks the portions of a native cell-bound TCR and is stable in solution (i.e., it does not generally aggregate in solution when handled as described herein and under standard conditions for protein solutions).

[0069] The term “isolated” refers to a composition, compound, substance, or molecule altered by the hand of man from the natural state. For example, a composition or substance that occurs in nature is isolated if it has been changed or removed from its original environment, or both. For example, a polynucleotide or a polypeptide naturally present in a living animal is not isolated, but the same polynucleotide or polypeptide separated from the coexisting materials of its natural state is isolated, as the term is employed herein.

[0070] A nucleic acid construct is a nucleic acid molecule which is isolated from a naturally occurring gene or which has been modified to contain segments of nucleic acid which are combined and juxtaposed in a manner which would not otherwise exist in nature.

[0071] Nucleic acid molecule means a single- or double-stranded linear polynucleotide containing either deoxyribonucleotides or ribonucleotides that are linked by 3'-5'-phosphodiester bonds.

[0072] Two DNA sequences are operably linked if the nature of the linkage does not interfere with the ability of the sequences to affect their normal functions relative to each other. For instance, a promoter region would be operably linked to a coding sequence if the promoter were capable of effecting transcription of that coding sequence.

[0073] A polypeptide is a linear polymer of amino acids that are linked by peptide bonds.

[0074] The term “promoter” refers to a cis-acting DNA sequence, generally 80-120 base pairs long and located upstream of the initiation site of a gene, to which RNA polymerase may bind and initiate correct transcription. There can be associated additional transcription regulatory sequences which provide on/off regulation of transcription and/or which enhance (increase) expression of the downstream coding sequence.

[0075] A recombinant nucleic acid molecule, for instance a recombinant DNA molecule, is a novel nucleic acid sequence formed *in vitro* through the ligation of two or more nonhomologous DNA molecules (for example a recombinant plasmid containing one or more inserts of foreign DNA cloned into at least one cloning site).

[0076] The terms “transformation” and “transfection” refer to the directed modification of the genome of a cell by the external application of purified recombinant DNA from another cell of different genotype, leading to its uptake and integration into the subject cell’s genome. In bacteria, the recombinant DNA is not typically integrated into the bacterial chromosome, but instead replicates autonomously as a plasmid. The terms “transformed” and “transfected” are used interchangeably herein. For example, a T cell may be transfected with a DNA sequence encoding a modified or high affinity TCR described herein prior to adoptive T cell treatment.

[0077] Upstream means on the 5' side of any site in DNA or RNA.

[0078] A vector is a nucleic acid molecule that is able to replicate autonomously in a host cell and can accept foreign DNA. A vector carries its own origin of replication, one or more unique recognition sites for restriction endonucleases which can be used for the insertion of foreign DNA, and usually selectable markers such as genes coding for antibiotic resistance, and often recognition sequences (e.g., promoter) for the expression of the inserted DNA. Common vectors include plasmid vectors and phage vectors.

[0079] A high affinity T cell receptor (TCR) is an engineered TCR with stronger binding to a target ligand than the wild type TCR. Some examples of high affinity include an equilibrium binding constant for a target ligand of between about 10^{-6} M and 10^{-12} M and all individual values and ranges therein. This range encompasses affinities between those reported to be wild type affinities (10^{-4} to 10^{-6} M), and those which have been isolated by directed evolution (about 10^{-12} M).

[0080] A cytokine is a protein, peptide or glycoprotein made by cells that affect other cells.

[0081] Mammal includes both human and non-human mammals.

[0082] It will be appreciated by those of skill in the art that, due to the degeneracy of the genetic code, numerous functionally equivalent nucleotide sequences encode the same amino acid sequence.

T Cell Receptors

[0083] The T cell receptor (TCR) is composed of two chains (αβ or γδ) that pair on the surface of the T cell to form a heterodimeric receptor. The αβ TCR is expressed on most T cells in the body and is known to be involved in the recognition of MHC-restricted antigens. The molecular genetics, structure, and biochemistry of αβ TCRs have now been studied thoroughly. Each α and β chain is composed of two domains: Constant domains (C) that anchor the protein in the cell membrane and that associate with invariant subunits of the CD3 signaling apparatus, and Variable domains (V) that confer antigen recognition through six loops, called complementarity determining regions (CDR). Each of the V domains has three CDRs. These CDRs interact with a complex between an antigenic peptide bound to a protein encoded by the major histocompatibility complex (pepMHC) (Davis and Bjorkman (1988) Nature, 334, 395-402; Davis et al. (1998) Annu Rev Immunol, 16, 523-544; Murphy (2012), xix, 868 p.).

[0084] The molecular genetics of the TCR have revealed a process of genetic recombination between multiple genes that combine to form the coding region of the V domains. The process is analogous to antibody development in which the heavy and light chain genes rearrange to generate the tremendous diversity exhibited by B cell-derived antibodies (Tonegawa (1988) In Vitro Cell Dev Biol, 24, 253-65). In the case of T cells, the α chain V domain is formed by the rearrangement of one V region (among about 75 in humans) to one Joining (J) gene segment (among about 61 in humans) (Figure 5.8, Janeway, 8th edition). The β chain V domain is formed by the rearrangement of one V region (among about 52 in humans) to one Diversity (D) gene (among 2 in humans) to one Joining (J) gene segment (among 13 in humans)

(Figure 5.8, (Murphy (2012), xix, 868 p.)). The junctions of the VαJα and VβDβJβ gene rearrangements encode the CDR3 loops of each chain, and they contribute to the tremendous diversity of the αβ TCR, with a theoretical limit of over 10^{15} different TCRs (Davis and Bjorkman (1988) Nature, 334, 395-402), well above the achievable diversity in a human because there are only about 10^{11} T cells total (Mason (1998) Immunol Today, 19, 395-404). The possible CDR1 and CDR2 diversity of each chain is represented by the number of V genes, as these loops are encoded within the V gene, and TCRs do not undergo somatic mutation in vivo. Although the diversity of CDR1 and CDR2 loops are relatively limited compared to CDR3 loops, there have been a number of examples shown where there has been selection for particular V regions based on the peptide antigen and/or MHC product.

[0085] Class I MHC products bind to peptides of 8 to 10 amino acids in length and they are expressed on all nucleated cells in the body (reviewed by (Rock and Goldberg (1999) Annu Rev Immunol, 17, 739-79)). Whereas all the binding energy of an antibody-antigen interaction is focused on the foreign antigen, a substantial fraction of the binding energy of the TCR-peptide:MHC is directed at the self-MHC molecule (Manning and Kranz (1999) Immunology Today, 20, 417-422). In fact, more recent studies have suggested that particular residues of the CDR1 and/or CDR2 loops have evolved to interact with particular residues on the MHC helices, thereby providing a basal affinity for MHC, accounting for the process of MHC-restriction (Garcia et al. (2009) Nat Immunol, 10, 143-7; Marrack et al. (2008) Annu Rev Immunol, 26, 171-203).

[0086] There has been interest in using TCRs that have affinities for a peptide-MHC antigen (class I) above the normal range (so called higher affinity TCRs) in order to: 1) drive the activity of CD4 helper T cells (which lack the CD8 coreceptor) or 2) develop soluble TCRs that could be used for direct targeting of a cell, by attaching an "effector" molecule (e.g., antibody Fc regions, a toxic drug, or an antibody scFv such as an anti-CD3 antibody, to form a bispecific protein)((Ashfield and Jakobsen (2006) IDrugs, 9, 554-9; Foote and Eisen (2000) Proc Natl Acad Sci USA, 97, 10679-81; Holler et al. (2000) Proc Natl Acad Sci U S A, 97, 5387-92; Molloy et al. (2005) Curr Opin Pharmacol, 5, 438-43; Richman and Kranz (2007) Biomol Eng, 24, 361-73). This approach also could overcome a problem faced by some cancer patients, whereby their T cells do not express TCRs with adequate specificity and binding affinity to potential tumor antigens (in part due to the thymic and peripheral processes of tolerance). For example, over 300 MHC-restricted, T cell-defined tumor antigens have now been identified (cancerimmunity.org/peptide/) (Boon and Old (1997) Curr Opin Immunol, 9, 681-3; Cheever et al. (2009) Clin Cancer Res, 15, 5323-37). These tumor antigens include mutated peptides, differentiation antigens, and overexpressed antigens, all of which could serve as targets for therapies. Because the majority of the cancer antigens described to date were derived from intracellular proteins that can only be targeted at the cell surface in the context of an MHC molecule, TCRs make the ideal candidate for therapeutics as they have evolved to recognize this class of antigen.

[0087] Similarly, TCRs can detect peptides derived from viral proteins that have been naturally processed in infected cells and displayed by an MHC molecule on the cell surface. Many viral antigen targets have been identified over the past 25 years, including peptides derived from viral genomes in HIV and HTLV (e.g., Addo et al. (2007) PLoS ONE, 2, e321; Tsomides et al. (1994) J Exp Med, 180, 1283-93; Utz et al. (1996) J Virol, 70, 843-51). However, patients with these diseases may lack the optimal TCRs for binding and destruction of the infected cells. Finally, it is possible that TCRs could be used as receptor antagonists of autoimmune targets, or as delivery agents to immunosuppress the local immune cell response, in a process that would be highly specific, thereby avoiding general immune suppression ((Molloy et al. (2005) Curr Opin Pharmacol, 5, 438-43; Stone et al. (2012) Protein Engineering)).

Modified T Cell Receptors

[0088] Directed evolution has been used to generate TCRs with higher affinity for a specific pepMHC. The three different display methods that have been used are yeast display (Holler et al. (2003) Nat Immunol, 4, 55-62; Holler et al. (2000) Proc Natl Acad Sci U S A, 97, 5387-92), phage display (Li et al. (2005) Nat Biotechnol, 23, 349-54), and T cell display (Chervin et al. (2008) J Immunol Methods, 339, 175-84). In all three approaches, the process involves the engineering of a TCR that exhibits the normal, low affinity of the wild-type TCR, so mutants of the TCR had increased affinity for the specific pepMHC (i.e., for the original antigen that the T cells were specific for). Thus, the wild-type TCR was used as a template for producing mutagenized libraries in one or more of the CDRs, followed by selection of mutants with higher affinity, by binding to the cognate peptide-MHC antigen. It is well known in the art that such *in vitro*, directed evolution, is necessary in order to engineer affinities that are more than just a few fold above the wild type affinity.

[0089] Yeast display allows for the protein of interest to be expressed on the surface as an Aga2-fusion (Boder and Wittrup (1997) Nat. Biotech., 15, 553-557; Boder and Wittrup (2000) Methods Enzymol, 328, 430-44). This system has been used successfully in the engineering of higher affinity TCRs, single-chain antibodies, fibronectin, and other proteins. In the yeast display system, the TCR has been displayed as a stabilized single-chain protein, in Vβ-linker-Vα or Vα-linker-Vβ forms (Aggen et al. (2011) Protein Engineering, Design, & Selection, 24, 361-72; Holler et al. (2000) Proc Natl Acad Sci U S A, 97, 5387-92; Kieke et al. (1999) Proc Natl Acad Sci U S A, 96, 5651-6; Richman et al. (2009) Mol Immunol, 46, 902-16; Weber et al. (2005) Proc Natl Acad Sci U S A, 102, 19033-8), or as a two-chain heterodimer (Aggen et al. (2011) Protein Engineering, Design, & Selection, 24, 361-72; Richman et al. (2009) Mol Immunol, 46, 902-16). Two mouse TCRs have been engineered for higher affinity using this system: 2C (MHC class-I restricted) and 3.L2 (MHC class-II restricted) (Holler et al. (2000) Proc Natl Acad Sci U S A, 97, 5387-92; Weber et al. (2005) Proc Natl Acad Sci U S A, 102, 19033-8). Human TCR single-chain VαVβ fragments (called scTv or scTCR) have also recently been developed by taking advantage of the exceptional stability of the human Vα region called Vα2, also known as TCRA12 by IMGT nomenclature (Aggen et al. (2011) Protein Engineering, Design, & Selection, 24, 361-72). In this case, in vitro engineered, high-affinity T cell receptors in a single-chain format were used to isolate human stabilized scTv fragments (Vβ-linker-Vα), which could be expressed as stable proteins, both on the surface of yeast and in soluble form from *E. coli*. The TCRs included two stabilized, human scTv fragments, the A6 scTv that is specific for a peptide derived from the human T cell lymphotrophic virus Tax protein and the 868 scTv that is specific for a peptide derived from the human immunodeficiency virus Gag protein (peptide: SL977-85). Both of these TCRs used the Vα2 gene (IMGT: TRAV12 family), but they had CDR3α, CDR1β, CDR2β, and CDR3β residues derived from the original T cell clone from which the TCRs were isolated. Thus, the higher affinity mutants of these scTCRs were each derived from their original (parental) TCR against their cognate peptide-MHC antigens.

[0090] In a second system, phage display, the protein of interest is fused to the N-terminus of a viral coat protein (Scott and Smith (1990) Science, 249, 386-90). Various TCRs, including those called A6, 868, and 1G4 (MHC class-I restricted), have been engineered for higher affinity using this method (Li et al. (2005) Nat Biotechnol, 23, 349-54; Sami et al. (2007) Protein Eng Des Sel, 20, 397-403; Varela-Rohena et al. (2008) Nat Med, 14, 1390-5). Phage display of these TCRs was enabled by introduction of a non-native disulfide bond between the two C domains in order to promote pairing of the α and β chains. This system thus uses full-length (VαCα/VβCβ) heterodimeric proteins derived from the original T cell clones for engineering against their cognate peptide-MHC.

[0091] A third system that has been reported for the engineering of TCRs is mammalian cell display (Chervin et al. (2008) J Immunol Methods, 339, 175-84; Kessels et al. (2000) Proc Natl Acad Sci U S A, 97, 14578-83). This system uses a retroviral vector to introduce the TCR α and β-chains into a TCR-negative T cell hybridoma. In one study (Kessels et al. (2000) Proc Natl Acad Sci U S A, 97, 14578-83), the selected mutant TCR was shown to bind to a peptide that was structurally very similar to the cognate peptide (ASNENMDAM versus ASNENMETM, SEQ ID NOs:15 and 16, respectively). In the other study, the affinity of the mutant TCR was shown to be increased for the cognate pepMHC (Chervin et al. (2008) J Immunol Methods, 339, 175-84). It has been shown in many studies that such higher affinity TCRs also exhibit higher affinities against structurally similar variants of the cognate peptide (e.g.,(Holler et al. (2003) Nat Immunol, 4, 55-62)). In the mammalian cell display system, introduced TCRs were expressed on the surface in its native conformation, in complex with CD3 subunits, allowing for a fully functional T cell (signaling competent). Full-length, heterodimeric TCRs in their native host were thus engineered using this method.

High-Affinity TCRs that Bind to Survivin/HLA-A2

[0092] The present invention provides for high-affinity TCRs against the well-known cancer antigen Survivin/HLA-A2. In certain embodiments, the engineered TCRs can be used in soluble form for targeted delivery in vivo, or as recombinantly expressed by T cells in an adoptive transfer method or treatment. In a particular embodiment, a single-chain VαVβ form of the TCR (scTCR) scaffold can be prepared and used with a payload such as a cytokine, toxin, radioisotope, chemotherapeutic agent, or drug (similar to antibody-drug conjugates) to deliver the effector molecule to the location where the TCR binds (e.g., tumor). The TCR can also be used in cell therapies, such as adoptive transfer of CD4+ T cells, CD8+ T cells, and/or natural killer (NK) cells, to mediate a response against cancer cells that express Survivin. The scTCR scaffolds provided herein can also be used for diagnosis of, e.g., malignant or viral-infected cells through identification of, e.g., neoplastic or viral-associated cell-surface antigens by covalent linkage, for example through amine-reactive or sulfhydryl-reactive amino acid side chains of the TCR, to a detectable group, such as a radioisotope or fluorescent moiety.

[0093] In one embodiment, the scTCR proteins described herein are displayable on the surface of yeast, phage, or mammalian cells and can be used to engineer TCRs with even higher affinity to the Survivin antigen. In one embodiment, the scTCR proteins described herein can be expressed in a prokaryotic cell, such as *Escherichia coli, Aspergillus niger, Aspergillus ficuum, Aspergillus awamori, Aspergillus oryzae, Trichoderma reesei, Mucor miehei, Kluyveromyces lactis, Pichia pastoris, Saccharomyces cerevisiae, Bacillus subtilis* or *Bacillus licheniformis*, insect cells (e.g., *Drosophila melanogaster*), mammalian cells including cell lines such as Chinese hamster ovary cell lines (CHO), or plant species (e.g., canola, soybean, corn, potato, barley, rye, wheat) for example, or other art-known protein expression sources and produced in large quantities. The TCR can also be used, for example and by way of example only, to detect the specific peptide/MHC on the surface of a cell. In one embodiment, the scTCR genes disclosed can be linked by use of suitable peptide sequences, encoded within the DNA construct, to the genes for signaling domains and introduced into T cells that can eliminate the targeted cells. These constructs have been termed chimeric antigen receptors (CARs), which are now widely used in the field, including the use of CARs that contain a scTCR.

[0094] In the single-chain VαVβ TCR proteins provided, the variable alpha and variable beta chains are connected using any suitable peptide linker, including those known in the art such as with antibody single-chain Fv linkages (Bird et al. (1988) Science, 242, 423-426; Holliger et al. (1993) Proc Natl Acad Sci U S A, 90, 6444-8;

Hoogenboom (2005) Nat Biotechnol, 23, 1105-16; Turner et al. (1997) J Immunol Methods, 205, 43-54). In one embodiment, a soluble human single-chain TCR having the structure: Vα-L-Vβ or Vβ-L-Vα, wherein L is a linker peptide that links Vβ with Vα, Vβ is a TCR variable β region, and Vα is a TCR variable α region is provided.

[0095] In one embodiment, the VβVα TCR is called Survivin K2.4.1 where Vβ is a TCR variable β region of group 20, and Vα2 is a TCR variable α region of group 2 (Utz, U., et al., 1996)(Aggen, D.A., et al., 2011). In one embodiment, the VβVα TCR is called Survivin K2.4.6 where Vβ is a TCR variable β region of group 20, and Vα2 is a TCR variable α region of group 2.

[0096] In one embodiment, the linker peptide contains more than 5 lysine residues. In one embodiment, the linker peptide contains between 5 and 30 amino acids. In one embodiment, the linker peptide has an amino acid sequence of GSADDAKKDAAKKDGKS (SEQ ID NO:7). In one embodiment, the sc VβVα TCR provided does not contain a constant region. When the terminology sc VβVα TCR is used herein, it is understood that sc VβVα TCR is also included as the terminology is understood and used in the art. Thus, the Vβ and Vα chains can be connected to each other in any configuration through the linker.

[0097] In an aspect of the disclosure, the VβVα TCR of the disclosure binds specifically to a ligand with an equilibrium binding constant K_D of between about 10^{-6} M and 10^{-12} M. In one embodiment of this aspect of the disclosure, the ligand is a peptide/MHC ligand. In one embodiment, the VβVα TCR of the disclosure has enhanced affinity toward a ligand compared to the affinities of normal, wild type TCRs.

Biologically Active Groups

[0098] Also provided are VβVα TCR proteins as described herein which includes a biologically active group. As used herein, "biologically active group" is a group that causes a measurable or detectable effect in a biological system. In one embodiment, the biologically active group is selected from: an anti-tumor agent such as, but not limited to, angiogenesis inhibitors, enzyme inhibitors, microtubule inhibitors, DNA intercalators or cross-linkers, DNA synthesis inhibitors; a cytokine such as, but not limited to IL-2, IL-15, GM-CSF, IL-12, TNF-α, IFN-γ or LT-α (Schrama et al. (2006) Nat Rev Drug Discov, 5, 147-59; Wong et al. (2011) Protein Eng Des Sel, 24, 373-83); an anti-inflammatory group such as, but not limited to, TGF-β, IL-37, IL-10 (Nold et al. (2010) Nat Immunol, 11, 1014-22; Stone et al. (2012) Protein Engineering), a radioisotope such as, but not limited to, ^{90}Y or ^{131}I (Reichert and Valge-Archer (2007) Nat Rev Drug Discov, 6, 349-56); a toxin such as, but not limited to, Pseudomonas exotoxin A, diphtheria toxin, or the A chain of ricin (Pastan et al. (2006) Nat Rev Cancer, 6, 559-65; Schrama et al. (2006) Nat Rev Drug Discov, 5, 147-59); a drug, or an antibody such as a single-chain Fv.

[0099] In one embodiment of this aspect of the disclosure, the biologically active group is a cytotoxic molecule, sometimes referred to as a drug (e.g., in the term "antibody drug conjugate"). As used herein, "cytotoxic" means toxic to cells. Examples of cytotoxic molecules include, but are not limited to, doxorubicin, methotrexate, mitomycin, 5-fluorouracil, duocarmycin, auristatins, maytansines, calicheamicins and analogs of the above molecules (Jarvis (2012) Chemical and Engineering News, 90, 12-18; Litvak-Greenfeld and Benhar (2012) Adv Drug Deliv Rev; Ricart and Tolcher (2007) Nat Clin Pract Oncol, 4, 245-55). Cytotoxic molecules do not need to cause complete cell death, but rather, a measurable or detectable inhibition of growth or decrease in cell activity.

[00100] In one embodiment, a TCR described herein is linked to an enzyme capable of converting a prodrug into a drug. This is useful, for example, by allowing the active form of the drug to be created at the location targeted by the TCR (e.g., at the site of a tumor).

[00101] In one embodiment, the biologically active group is bound to the single-chain TCR through a linker, which may be accomplished through standard chemical reactions such as with free amine groups or sulfhydryl groups of the TCR.

[00102] In another embodiment, the TCR is attached to a single-chain antibody fragment (scFv) to generate a bispecific agent. Bispecific antibodies that contain one scFv against a tumor antigen, and one against the CD3 molecule of the T cell have now been used successfully in the clinic (Bargou et al. (2008) Science, 321, 974-7). In addition, a bispecific agent containing a TCR and a scFv against CD3 has also been reported (Liddy et al. (2012) Nat Med, 18, 980-7).

[00103] Also provided is a single-chain VβVα TCR as described herein which includes a detectable group. In one embodiment, the detectable group is one that can be detected by spectroscopic or enzyme-based methods. In one embodiment, the detectable group is a fluorescent group, such as, but not limited to fluorescein, R-phycoerythrin (PE), PE-Cy5, PE-Cy7, Texas red, or allophycocyanin (APC); a radiolabeled group such as, but not limited to, 125I, 32P, 99mTc; an absorbing group, or an enzyme with properties that generate detectable products such as, but not limited to, horseradish peroxidase, or alkaline phosphatase.

[00104] As known in the art, a biologically active group, detectable group or other group attached to the TCR can be attached using a flexible peptide linker or by chemical conjugation, and can be covalently or noncovalently attached to the TCR.

[00105] Also provided herein is a human TCR for use in a method of treating or preventing cancer in a mammal, comprising administering an effective amount of a modified TCR linked to a therapeutically effective molecule to a mammal. In a particular embodiment, the mammal is human. In another embodiment, the mammal is a companion animal (e.g., a dog, cat, rabbit, rodent, horse) or a livestock animal (e.g., a cow, horse, pig).

[00106] Also provided is an isolated single-chain TCR (scTCR) as described herein, and a method for producing the single-chain TCR in *E. coli*. Also provided is a pharmaceutical composition comprising a scTCR as described herein and a pharmaceutically acceptable carrier.

[00107] Also provided is the sc VαVβ TCRs described herein which have been linked to signaling domains that yields an active TCR on the surface of a T cell. In one embodiment, this scTCR can be used in a method of treating cancer in a mammal, comprising: cloning the TCR into a vector, introducing the vector into T cells of a patient, and adoptive transferring of the T cells back into a patient.

Modified TCR Polypeptides and Polynucleotides

[00108] The disclosure contemplates a DNA vector that includes at least one DNA segment encoding a single-chain T cell receptor (scTCR).

[00109] Those of skill in the art, through standard mutagenesis techniques, conjunction with the assays described herein, can obtain altered TCR sequences and test them for particular binding affinity and/or specificity. Useful mutagenesis

techniques known in the art include, without limitation, de novo gene synthesis, oligonucleotide-directed mutagenesis, region-specific mutagenesis, linker-scanning mutagenesis, and site-directed mutagenesis by PCR (see e.g., Sambrook et al. (1989) and Ausubel et al. (1999)).

[00110] In obtaining modified TCR coding sequences, those of ordinary skill in the art will recognize that TCR-derived proteins may be modified by certain amino acid substitutions, additions, deletions, and post-translational modifications, without loss or reduction of biological activity. In particular, it is well known that conservative amino acid substitutions, that is, substitution of one amino acid for another amino acid of similar size, charge, polarity and conformation, are unlikely to significantly alter protein function. The 20 standard amino acids that are the constituents of proteins can be broadly categorized into four groups of conservative amino acids as follows: the nonpolar (hydrophobic) group includes alanine, isoleucine, leucine, methionine, phenylalanine, proline, tryptophan and valine; the polar (uncharged, neutral) group includes asparagine, cysteine, glutamine, glycine, serine, threonine and tyrosine; the positively charged (basic) group contains arginine, histidine and lysine; and the negatively charged (acidic) group contains aspartic acid and glutamic acid. Substitution in a protein of one amino acid for another within the same group is unlikely to have an adverse effect on the biological activity of the protein.

[00111] In one embodiment, a scTCR of the disclosure may contain additional mutations in any region or regions of the variable domain that results in a stabilized protein. In one embodiment, one or more additional mutations is in one or more of CDR1, CDR2, HV4, CDR3, FR2, and FR3. The regions used for mutagenesis can be determined by directed evolution, where crystal structures or molecular models are used to generate regions of the TCR which interact with the ligand of interest (antigen, for example). In other examples, the variable region can be reshaped, by adding or deleting amino acids to engineer a desired interaction between the scTCR and the ligand.

[00112] Polypeptides of the invention include modified TCRs, and antigen binding fragments thereof (e.g., scTCR), and chimeric antigen receptors (CARs). The terms "polypeptide" "protein" and "peptide" and "glycoprotein" are used interchangeably and mean a polymer of amino acids not limited to any particular length. The term does not exclude modifications such as myristylation, sulfation, glycosylation, phosphorylation and addition or deletion of signal sequences. The terms

"polypeptide" or "protein" means one or more chains of amino acids, wherein each chain comprises amino acids covalently linked by peptide bonds, and wherein said polypeptide or protein can comprise a plurality of chains non-covalently and/or covalently linked together by peptide bonds, having the sequence of native proteins, that is, proteins produced by naturally-occurring and specifically non-recombinant cells, or genetically-engineered or recombinant cells, and comprise molecules having the amino acid sequence of the native protein, or molecules having deletions from, additions to, and/or substitutions of one or more amino acids of the native sequence. The terms "polypeptide" and "protein" specifically encompass the modified TCRs, or antigen-binding fragments thereof, of the present disclosure, or sequences that have deletions from, additions to, and/or substitutions of one or more amino acid of a modified TCR, or antigen binding fragment thereof. Thus, a "polypeptide" or a "protein" can comprise one (termed "a monomer") or a plurality (termed "a multimer") of amino acid chains.

[00113] The term "isolated protein" referred to herein means that a subject protein (1) is free of at least some other proteins with which it would typically be found in nature, (2) is essentially free of other proteins from the same source, e.g., from the same species, (3) is expressed by a cell from a different species, (4) has been separated from at least about 50 percent of polynucleotides, lipids, carbohydrates, or other materials with which it is associated in nature, (5) is not associated (by covalent or noncovalent interaction) with portions of a protein with which the "isolated protein" is associated in nature, (6) is operably associated (by covalent or noncovalent interaction) with a polypeptide with which it is not associated in nature, or (7) does not occur in nature. Such an isolated protein can be encoded by genomic DNA, cDNA, mRNA or other RNA, of may be of synthetic origin, or any combination thereof. In certain embodiments, the isolated protein is substantially free from proteins or polypeptides or other contaminants that are found in its natural environment that would interfere with its use (therapeutic, diagnostic, prophylactic, research or otherwise).

[00114] In particular embodiments, a subject modified TCR may have: a) a TCR alpha chain variable region having an amino acid sequence that is at least 80% identical, at least 85% identical, at least 90%, at least 95% or at least 98% or 99% identical, to the alpha chain variable region of a modified TCR described herein; and b) a beta chain variable region having an amino acid sequence that is at least 80%

identical, at least 85%, at least 90%, at least 95% or at least 98% or 99% identical, to the beta chain variable region of a modified TCR described herein.

[00115] In particular embodiments, the modified TCR may comprise: a) a TCR alpha chain variable region comprising: i. a CDR1 region that is identical in amino acid sequence to the alpha chain CDR1 region of a selected TCR described herein; ii. a CDR2 region that is identical in amino acid sequence to the alpha chain CDR2 region of the selected TCR; and iii. a CDR3 region that is identical in amino acid sequence to the alpha chain CDR3 region of the selected TCR; and b) a beta chain variable region comprising: i. a CDR1 region that is identical in amino acid sequence to the beta chain CDR1 region of the selected TCR; ii. a CDR2 region that is identical in amino acid sequence to the beta chain CDR2 region of the selected TCR; and iii. a CDR3 region that is identical in amino acid sequence to the beta chain CDR3 region of the selected TCR; wherein the TCR specifically binds a selected non-cognate antigen. In a further embodiment, the modified TCR, or antigen-binding fragment thereof, is a variant modified TCR wherein the variant comprises an alpha chain and a beta chain identical to the selected modified TCR except for up to 8, 9, 10, 11, 12, 13, 14, 15, or more amino acid substitutions in the CDR regions of the V alpha and V beta regions. In this regard, there may be 1, 2, 3, 4, 5, 6, 7, 8, or in certain embodiments, 9, 10, 11, 12, 13, 14, 15 more amino acid substitutions in the CDR regions of the selected variant modified TCR. Substitutions may be in CDRs either in the V alpha and/or the V beta regions. (See e.g., Muller, 1998, Structure 6:1153-1167).

[00116] In one embodiment, a polynucleotide encoding a modified TCR, or an antigen-binding fragment thereof, is provided. In other related embodiments, the polynucleotide may be a variant of a polynucleotide encoding the modified TCR. Polynucleotide variants may have substantial identity to a polynucleotide sequence encoding a modified TCR described herein. For example, a polynucleotide may be a polynucleotide comprising at least 70% sequence identity, preferably at least 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% or higher, sequence identity compared to a reference polynucleotide sequence such as a sequence encoding an TCR described herein, using the methods described herein, (e.g., BLAST analysis using standard parameters, as described below). One skilled in this art will recognize that these values can be appropriately adjusted to determine corresponding identity

of proteins encoded by two nucleotide sequences by taking into account codon degeneracy, amino acid similarity, reading frame positioning and the like.

[00117] Typically, polynucleotide variants will contain one or more substitutions, additions, deletions and/or insertions, preferably such that the binding affinity of the TCR encoded by the variant polynucleotide is not substantially diminished relative to an antibody encoded by a polynucleotide sequence specifically set forth herein.

[00118] When comparing polynucleotide sequences, two sequences are said to be "identical" if the sequence of nucleotides in the two sequences is the same when aligned for maximum correspondence, as described below. Comparisons between two sequences are typically performed by comparing the sequences over a comparison window to identify and compare local regions of sequence similarity. A "comparison window" as used herein, refers to a segment of at least about 20 contiguous positions, usually 30 to about 75, 40 to about 50, in which a sequence may be compared to a reference sequence of the same number of contiguous positions after the two sequences are optimally aligned.

[00119] Optimal alignment of sequences for comparison may be conducted using the Megalign program in the Lasergene suite of bioinformatics software (DNASTAR, Inc., Madison, WI), using default parameters. This program embodies several alignment schemes described in the following references: Dayhoff, M.O. (1978) A model of evolutionary change in proteins – Matrices for detecting distant relationships. In Dayhoff, M.O. (ed.) Atlas of Protein Sequence and Structure, National Biomedical Research Foundation, Washington DC Vol. 5, Suppl. 3, pp. 345-358; Hein J., Unified Approach to Alignment and Phylogenes, pp. 626-645 (1990); Methods in Enzymology vol. 183, Academic Press, Inc., San Diego, CA; Higgins, D.G. and Sharp, P.M., CABIOS 5:151-153 (1989); Myers, E.W. and Muller W., CABIOS 4:11-17 (1988); Robinson, E.D., Comb. Theor 11:105 (1971); Santou, N. Nes, M., Mol. Biol. Evol. 4:406-425 (1987); Sneath, P.H.A. and Sokal, R.R., Numerical Taxonomy – the Principles and Practice of Numerical Taxonomy, Freeman Press, San Francisco, CA (1973); Wilbur, W.J. and Lipman, D.J., Proc. Natl. Acad., Sci. USA 80:726-730 (1983).

[00120] Alternatively, optimal alignment of sequences for comparison may be conducted by the local identity algorithm of Smith and Waterman, Add. APL. Math 2:482 (1981), by the identity alignment algorithm of Needleman and Wunsch, J. Mol. Biol. 48:443 (1970), by the search for similarity methods of Pearson and Lipman,

Proc. Natl. Acad. Sci. USA 85: 2444 (1988), by computerized implementations of these algorithms (GAP, BESTFIT, BLAST, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group (GCG), 575 Science Dr., Madison, WI), or by inspection.

[00121] One preferred example of algorithms that are suitable for determining percent sequence identity and sequence similarity are the BLAST and BLAST 2.0 algorithms, which are described in Altschul et al., Nucl. Acids Res. 25:3389-3402 (1977), and Altschul et al., J. Mol. Biol. 215:403-410 (1990), respectively. BLAST and BLAST 2.0 can be used, for example with the parameters described herein, to determine percent sequence identity among two or more the polynucleotides. Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information. In one illustrative example, cumulative scores can be calculated using, for nucleotide sequences, the parameters M (reward score for a pair of matching residues; always >0) and N (penalty score for mismatching residues; always <0). Extension of the word hits in each direction are halted when: the cumulative alignment score falls off by the quantity X from its maximum achieved value; the cumulative score goes to zero or below, due to the accumulation of one or more negative-scoring residue alignments; or the end of either sequence is reached. The BLAST algorithm parameters W, T and X determine the sensitivity and speed of the alignment. The BLASTN program (for nucleotide sequences) uses as defaults a wordlength (W) of 11, and expectation (E) of 10, and the BLOSUM62 scoring matrix (see Henikoff and Henikoff, Proc. Natl. Acad. Sci. USA 89:10915 (1989)) alignments, (B) of 50, expectation (E) of 10, M=5, N=-4 and a comparison of both strands.

[00122] In certain embodiments, the “percentage of sequence identity” is determined by comparing two optimally aligned sequences over a window of comparison of at least 20 positions, wherein the portion of the polynucleotide sequence in the comparison window may comprise additions or deletions (i.e., gaps) of 20 percent or less, usually 5 to 15 percent, or 10 to 12 percent, as compared to the reference sequences (which does not comprise additions or deletions) for optimal alignment of the two sequences. The percentage is calculated by determining the number of positions at which the identical nucleic acid bases occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the reference sequence (i.e.,

the window size) and multiplying the results by 100 to yield the percentage of sequence identity.

[00123] It will be appreciated by those of ordinary skill in the art that, as a result of the degeneracy of the genetic code, there are many nucleotide sequences that encode an TCR as described herein. Some of these polynucleotides bear minimal sequence identity to the nucleotide sequence of the native or original polynucleotide sequence that encode modified TCRs that bind to, e.g., the same antigen. Nonetheless, polynucleotides that vary due to differences in codon usage are expressly contemplated by the present disclosure. In certain embodiments, sequences that have been codon-optimized for mammalian expression are specifically contemplated.

[00124] Standard techniques for cloning, DNA isolation, amplification and purification, for enzymatic reactions involving DNA ligase, DNA polymerase, restriction endonucleases and the like, and various separation techniques are those known and commonly employed by those skilled in the art. A number of standard techniques are described in Sambrook et al. (1989) Molecular Cloning, Second Edition, Cold Spring Harbor Laboratory, Plainview, New York; Maniatis et al. (1982) Molecular Cloning, Cold Spring Harbor Laboratory, Plainview, New York; Wu (ed.) (1993) Meth. Enzymol. 218, Part I; Wu (ed.) (1979) Meth Enzymol. 68; Wu et al. (eds.) (1983) Meth. Enzymol. 100 and 101; Grossman and Moldave (eds.) Meth. Enzymol. 65; Miller (ed.) (1972) Experiments in Molecular Genetics, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York; Old and Primrose (1981) Principles of Gene Manipulation, University of California Press, Berkeley; Schleif and Wensink (1982) Practical Methods in Molecular Biology; Glover (ed.) (1985) DNA Cloning Vol. I and II, IRL Press, Oxford, UK; Hames and Higgins (eds.) (1985) Nucleic Acid Hybridization, IRL Press, Oxford, UK; and Setlow and Hollaender (1979) Genetic Engineering: Principles and Methods, Vols. 1-4, Plenum Press, New York. Abbreviations and nomenclature, where employed, are deemed standard in the field and commonly used in professional journals such as those cited herein.

[00125] Homology between nucleotide sequences can be determined by DNA hybridization analysis, wherein the stability of the double-stranded DNA hybrid is dependent on the extent of base pairing that occurs. Conditions of high temperature and/or low salt content reduce the stability of the hybrid, and can be varied to prevent annealing of sequences having less than a selected degree of homology.

For instance, for sequences with about 55% G - C content, hybridization and wash conditions of 40 - 50°C, 6 X SSC (sodium chloride/sodium citrate buffer) and 0.1% SDS (sodium dodecyl sulfate) indicate about 60 - 70% homology, hybridization and wash conditions of 50 - 65°C, 1 X SSC and 0.1% SDS indicate about 82 - 97% homology, and hybridization and wash conditions of 52°C, 0.1 X SSC and 0.1% SDS indicate about 99 - 100% homology. A wide range of computer programs for comparing nucleotide and amino acid sequences (and measuring the degree of homology) are also available, and a list providing sources of both commercially available and free software is found in Ausubel et al. (1999). Readily available sequence comparison and multiple sequence alignment algorithms are, respectively, the Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1997) and ClustalW programs. BLAST is available on the Internet at ncbi.nlm.nih.gov and a version of ClustalW is available at www2.ebi.ac.uk.

[00126] Industrial strains of microorganisms (e.g., *Aspergillus niger*, *Aspergillus ficuum*, *Aspergillus awamori*, *Aspergillus oryzae*, *Trichoderma reesei*, *Mucor miehei*, *Kluyveromyces lactis*, *Pichia pastoris*, *Saccharomyces cerevisiae*, *Escherichia coli*, *Bacillus subtilis* or *Bacillus licheniformis*), insect (Drosophila), mammalian (e.g.,Chinese hamster ovary cell lines, CHO), or plant species (e.g., canola, soybean, corn, potato, barley, rye, wheat) may be used as host cells for the recombinant production of the TCR proteins. In certain embodiments, the first step in the heterologous expression of a high affinity TCR protein or soluble protein, an expression construct is assembled to include the TCR or soluble TCR coding sequence and control sequences such as promoters, enhancers and terminators. Other sequences such as signal sequences and selectable markers may also be included. To achieve extracellular expression of the TCR, the expression construct may include a secretory signal sequence. In embodiments, the signal sequence is not included on the expression construct if cytoplasmic expression is desired. In embodiments, the promoter and signal sequence are functional in the host cell and provide for expression and secretion of the TCR or soluble TCR protein. Transcriptional terminators may be included to ensure efficient transcription. Ancillary sequences enhancing expression or protein purification may also be included in the expression construct.

[00127] Various promoters (transcriptional initiation regulatory region) may be used according to the disclosure. The selection of the appropriate promoter may be

dependent upon the proposed expression host. Promoters from heterologous sources may be used as long as they are functional in the chosen host.

[00128] Promoter selection is also dependent upon the desired efficiency and level of peptide or protein production. Inducible promoters such as tac are often employed in order to dramatically increase the level of protein expression in *E. coli*. Overexpression of proteins may be harmful to the host cells. Consequently, host cell growth may be limited. The use of inducible promoter systems allows the host cells to be cultivated to acceptable densities prior to induction of gene expression, thereby facilitating higher product yields.

[00129] Various signal sequences may be used according to the disclosure. A signal sequence which is homologous to the TCR coding sequence may be used. Alternatively, a signal sequence which has been selected or designed for efficient secretion and processing in the expression host may also be used. For example, suitable signal sequence/host cell pairs include the *B. subtilis* sacB signal sequence for secretion in *B. subtilis*, and the *Saccharomyces cerevisiae* α-mating factor or *P. pastoris* acid phosphatase phol signal sequences for *P. pastoris* secretion. The signal sequence may be joined directly through the sequence encoding the signal peptidase cleavage site to the protein coding sequence, or through a short nucleotide bridge consisting of usually fewer than ten codons, where the bridge ensures correct reading frame of the downstream TCR sequence.

[00130] Elements for enhancing transcription and translation have been identified for eukaryotic protein expression systems. For example, positioning the cauliflower mosaic virus (CaMV) promoter 1000 bp on either side of a heterologous promoter may elevate transcriptional levels by 10- to 400-fold in plant cells. The expression construct should also include the appropriate translational initiation sequences. Modification of the expression construct to include a Kozak consensus sequence for proper translational initiation may increase the level of translation by 10 fold.

[00131] A selective marker is often employed, which may be part of the expression construct or separate from it (e.g., carried by the expression vector), so that the marker may integrate at a site different from the gene of interest. Examples include markers that confer resistance to antibiotics (e.g., bla confers resistance to ampicillin for *E. coli* host cells, nptII confers kanamycin resistance to a wide variety of prokaryotic and eukaryotic cells) or that permit the host to grow on minimal medium (e.g., HIS4 enables *P. pastoris* or His- *S. cerevisiae* to grow in the absence of

histidine). The selectable marker has its own transcriptional and translational initiation and termination regulatory regions to allow for independent expression of the marker. If antibiotic resistance is employed as a marker, the concentration of the antibiotic for selection will vary depending upon the antibiotic, generally ranging from 10 to 600 μg of the antibiotic/mL of medium.

[00132] The expression construct is assembled by employing known recombinant DNA techniques (Sambrook et al., 1989; Ausubel et al., 1999). Restriction enzyme digestion and ligation are the basic steps employed to join two fragments of DNA. The ends of the DNA fragment may require modification prior to ligation, and this may be accomplished by filling in overhangs, deleting terminal portions of the fragment(s) with nucleases (e.g., ExoIII), site directed mutagenesis, or by adding new base pairs by PCR. Polylinkers and adaptors may be employed to facilitate joining of selected fragments. The expression construct is typically assembled in stages employing rounds of restriction, ligation, and transformation of *E. coli*. Numerous cloning vectors suitable for construction of the expression construct are known in the art (λZAP and pBLUESCRIPT SK-1, Stratagene, LaJolla, CA; pET, Novagen Inc., Madison, WI - cited in Ausubel et al., 1999) and the particular choice is not critical to the disclosure. The selection of cloning vector will be influenced by the gene transfer system selected for introduction of the expression construct into the host cell. At the end of each stage, the resulting construct may be analyzed by restriction, DNA sequence, hybridization and PCR analyses.

[00133] The expression construct may be transformed into the host as the cloning vector construct, either linear or circular, or may be removed from the cloning vector and used as is or introduced onto a delivery vector. The delivery vector facilitates the introduction and maintenance of the expression construct in the selected host cell type. The expression construct is introduced into the host cells by any of a number of known gene transfer systems (e.g., natural competence, chemically mediated transformation, protoplast transformation, electroporation, biolistic transformation, transfection, or conjugation) (Ausubel et al., 1999; Sambrook et al., 1989). The gene transfer system selected depends upon the host cells and vector systems used.

[00134] For instance, the expression construct can be introduced into *S. cerevisiae* cells by protoplast transformation or electroporation. Electroporation of *S. cerevisiae*

is readily accomplished, and yields transformation efficiencies comparable to spheroplast transformation.

[00135] Monoclonal or polyclonal antibodies, preferably monoclonal, specifically reacting with a TCR protein at a site other than the ligand binding site may be made by methods known in the art, and many are commercially available. See, e.g., Harlow and Lane (1988) Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratories; Goding (1986) Monoclonal Antibodies: Principles and Practice, 2d ed., Academic Press, New York; and Ausubel et al. (1999) Current Protocols in Molecular Biology, John Wiley & Sons, Inc., New York.

[00136] TCRs in cell-bound or soluble form which are specific for a particular target ligand are useful, for example, as diagnostic probes for screening biological samples (such as cells, tissue samples, biopsy material, bodily fluids and the like) or for detecting the presence of the target ligand in a test sample. Frequently, the TCRs are labeled by joining, either covalently or noncovalently, a substance which provides a detectable signal. Suitable labels include but are not limited to radionuclides, enzymes, substrates, cofactors, inhibitors, fluorescent agents, chemiluminescent agents, magnetic particles and the like. Additionally the TCR can be coupled to a ligand for a second binding molecules: for example, the TCR can be biotinylated. Detection of the TCR bound to a target cell or molecule can then be effected by binding of a detectable streptavidin (a streptavidin to which a fluorescent, radioactive, chemiluminescent, or other detectable molecule is attached or to which an enzyme for which there is a chromophoric substrate available). United States Patents describing the use of such labels and/or toxic compounds to be covalently bound to the scTCR include but are not limited to Nos. 3,817,837; 3,850,752; 3,927,193; 3,939,350; 3,996,345; 4,277,437; 4,275,149; 4,331,647; 4,348,376; 4,361,544; 4,468,457; 4,444,744; 4,640,561; 4,366,241; RE 35,500; 5,299,253; 5,101,827; 5,059,413.

[00137] Labeled TCRs can be detected using a monitoring device or method appropriate to the label used. Fluorescence microscopy or fluorescence activated cell sorting can be used where the label is a fluorescent moiety, and where the label is a radionuclide, gamma counting, autoradiography or liquid scintillation counting, for example, can be used with the proviso that the method is appropriate to the sample being analyzed and the radionuclide used. In addition, there can be secondary detection molecules or particle employed where there is a detectable

molecule or particle which recognized the portion of the TCR which is not part of the binding site for the target ligand in the absence of a MHC component as noted herein. The art knows useful compounds for diagnostic imaging in situ; see, e.g., U.S. Patent No. 5,101,827; 5,059,413. Radionuclides useful for therapy and/or imaging in vivo include ^{111}Indium, ^{97}Rubidium, ^{125}Iodine, ^{131}Iodine, ^{123}Iodine, ^{67}Gallium, ^{99}Technetium. Toxins include diphtheria toxin, ricin and castor bean toxin, among others, with the proviso that once the TCR-toxin complex is bound to the cell, the toxic moiety is internalized so that it can exert its cytotoxic effect. Immunotoxin technology is well known to the art, and suitable toxic molecules include, without limitation, chemotherapeutic drugs such as vindesine, antifolates, e.g., methotrexate, cisplatin, mitomycin, anthrocyclines such as daunomycin, daunorubicin or adriamycin, and cytotoxic proteins such as ribosome inactivating proteins (e.g., diphtheria toxin, pokeweed antiviral protein, abrin, ricin, pseudomonas exotoxin A or their recombinant derivatives. See, generally, e.g., Olsnes and Pihl (1982) Pharmac. Ther. 25:355-381 and Monoclonal Antibodies for Cancer Detection and Therapy, Eds. Baldwin and Byers, pp. 159-179, Academic Press, 1985.

[00138] The general structure of TCR molecules and methods of making and using, including binding to a peptide:Major Histocompatibility Complex have been disclosed. See, for example PCT/US98/04274; PCT/US98/20263; WO99/60120.

Pharmaceutical Compositions and Therapeutic Agents

[00139] TCRs specific for a particular target ligand are useful in treating animals and mammals, including humans believed to be suffering from a disease associated with the particular antigen, e.g., a neoplastic disease or disorder, such as cancer. Examples of types of cancers that may be treated according to the methods described herein include, but are not limited to, Wilm's tumor, bladder cancer, breast cancer, colon cancer, colorectal cancer, esophageal carcinomas, gastric cancer, hepatocellular carcinoma, kidney cancer, leukemia, liver cancer, lung cancer, lymphoma, melanoma, neuroblastoma, non-small cell lung carcinoma, oral cancer, osteosarcoma, ovarian cancer, pancreatic cancer, prostate cancer, renal cancer, skin cancer, small cell lung carcinoma, and testicular cancer.

[00140] Therapeutic products can be made using the materials shown herein. Effective amounts of therapeutic products are the minimum dose that produces a

measurable effect in a subject. Therapeutic products are easily prepared by one of ordinary skill in the art. In one embodiment, a TCR of the disclosure is administered directly to a patient. In one embodiment, a TCR of the disclosure is linked to PEG or to immunoglobulin constant regions, as known in the art. This embodiment lengthens the serum clearance. In one embodiment, the TCR is linked to a chemotherapeutic agent or drug in order to deliver the drug to a target cell such as a cancer cell. In one embodiment, the scTCR is linked to a biologic effector molecule such as a cytokine (Tayal and Kalra (2008) Eur J Pharmacol, 579, 1-12). In one embodiment, the TCR is linked to a cytokine with anti-tumor activity, such as IL-2, IL-12, or TNFα (Wong et al. (2011) Protein Eng Des Sel, 24, 373-83). In one embodiment, the TCR is linked to an immune-inhibitory cytokine, such as IL-10 or IL-13 (Stone et al. (2012) Protein Engineering). In one embodiment, the TCR is linked to another antigen binding molecule to form a bispecific agent (Miller et al. (2010) Protein Eng Des Sel, 23, 549-57; Thakur and Lum (2010) Curr Opin Mol Ther, 12, 340-9). In one embodiment, the bispecific molecule is comprised of a TCR linked to a single chain Fv, such as an anti-CD3 ((Bargou et al. (2008) Science, 321, 974-7; Liddy et al. (2012) Nat Med, 18, 980-7), to crosslink T cells and diseased cells. In one embodiment, the TCR is linked to TCR signaling domains, such as CD3, to form a chimeric antigen receptor ((Porter et al. (2011) N Engl J Med, 365, 725-33; Sadelain et al. (2009) Curr Opin Immunol, 21, 215-23; Stroncek et al. (2012) J Transl Med, 10, 48). These methods and other methods of administering, such as intravenously, are known in the art. Useful dosages can be determined by one of ordinary skill in the art.

[00141] The TCR compositions can be formulated by any of the means known in the art. They can be typically prepared as injectables, especially for intravenous, intraperitoneal or synovial administration (with the route determined by the particular disease) or as formulations for intranasal or oral administration, either as liquid solutions or suspensions. Solid forms suitable for solution in, or suspension in, liquid prior to injection or other administration may also be prepared. The preparation may also, for example, be emulsified, or the protein(s)/peptide(s) encapsulated in liposomes.

[00142] The active ingredients are often mixed with optional pharmaceutical additives such as excipients or carriers which are pharmaceutically acceptable and compatible with the active ingredient. Suitable excipients include but are not limited to water, saline, dextrose, glycerol, ethanol, or the like and combinations thereof.

The concentration of the scTCR in injectable, aerosol or nasal formulations is usually in the range of 0.05 to 5 mg/ml. The selection of the particular effective dosages is known and performed without undue experimentation by one of ordinary skill in the art. Similar dosages can be administered to other mucosal surfaces.

[00143] In addition, if desired, vaccines that could include a scTCR may contain minor amounts of pharmaceutical additives such as auxiliary substances such as wetting or emulsifying agents, pH buffering agents, and/or adjuvants which enhance the effectiveness of the vaccine. Examples of adjuvants which may be effective include but are not limited to: aluminum hydroxide; N-acetyl-muramyl-L-threonyl-Disoglutamine (thr-MDP); N-acetyl-nor-muramyl-L-alanyl-D-isoglutamine (CGP 11637, referred to as nor-MDP); N-acetylmuramyl-L-alanyl-D-isoglutaminyl-L-alanine-2-(1'-2'-dipalmitoyl-sn-glycero-3hydroxyphosphoryloxy)-ethylamine (CGP 19835A, referred to as MTP-PE); and RIBI, which contains three components extracted from bacteria: monophosphoryl lipid A, trehalose dimycolate and cell wall skeleton (MPL+TDM+CWS) in a 2% squalene/Tween® 80 emulsion. Such additional formulations and modes of administration as are known in the art may also be used.

[00144] The TCRs of the present disclosure and/or binding fragments having primary structure similar (more than 90% identity) to the TCR variable regions and which maintain the high affinity for the target ligand may be formulated into vaccines as neutral or salt forms. Pharmaceutically acceptable salts include but are not limited to the acid addition salts (formed with free amino groups of the peptide) which are formed with inorganic acids, e.g., hydrochloric acid or phosphoric acids; and organic acids, e.g., acetic, oxalic, tartaric, or maleic acid. Salts formed with the free carboxyl groups may also be derived from inorganic bases, e.g., sodium, potassium, ammonium, calcium, or ferric hydroxides, and organic bases, e.g., isopropylamine, trimethylamine, 2-ethylamino-ethanol, histidine, and procaine.

[00145] TCRs for therapeutic use are administered in a manner compatible with the dosage formulation, and in such amount and manner as are prophylactically and/or therapeutically effective, according to what is known to the art. The quantity to be administered, which is generally in the range of about 100 to 20,000 μg of protein per dose, more generally in the range of about 1000 to 10,000 μg of protein per dose. Similar compositions can be administered in similar ways using labeled TCRs for use in imaging, for example, to detect cells to which a target ligand is bound.

Precise amounts of the active ingredient required to be administered may depend on the judgment of the physician or veterinarian and may be peculiar to each individual, but such a determination is within the skill of such a practitioner.

[00146] The TCR product may be given in a single dose; two dose schedule, for example two to eight weeks apart; or a multiple dose schedule. A multiple dose schedule is one in which a primary course of treatment may include 1 to 10 or more separate doses, followed by other doses administered at subsequent time intervals as required to maintain and/or reinforce the response.

[00147] Every formulation or combination of components described or exemplified can be used to practice the disclosure, unless otherwise stated. Specific names of substances are intended to be exemplary, as it is known that one of ordinary skill in the art can name the same substances differently. When a compound is described herein such that a particular isomer or enantiomer of the compound is not specified, for example, in a formula or in a chemical name, that description is intended to include each isomers and enantiomer of the compound described individual or in any combination. One of ordinary skill in the art will appreciate that methods, target ligands, biologically active groups, starting materials, and synthetic methods other than those specifically exemplified can be employed in the practice of the disclosure without resort to undue experimentation. All art-known functional equivalents, of any such methods, target ligands, biologically active groups, starting materials, and synthetic methods are intended to be included in this disclosure. Whenever a range is given in the specification, for example, a temperature range, a time range, or a composition range, all intermediate ranges and subranges, as well as all individual values included in the ranges given are intended to be included in the disclosure.

[00148] The exact formulation, route of administration and dosage can be chosen by the individual physician in view of the patient's condition (see e.g., Fingl et. al., in The Pharmacological Basis of Therapeutics, 1975, Ch. 1 p. 1).

[00149] It should be noted that the attending physician would know how to and when to terminate, interrupt, or adjust administration due to toxicity, or to organ dysfunctions. Conversely, the attending physician would also know to adjust treatment to higher levels if the clinical response were not adequate (precluding toxicity). The magnitude of an administered dose in the management of the disorder of interest will vary with the severity of the condition to be treated and to the route of administration. The severity of the condition may, for example, be evaluated, in part,

by standard prognostic evaluation methods. Further, the dose and perhaps dose frequency, will also vary according to the age, body weight, and response of the individual patient. A program comparable to that discussed above also may be used in veterinary medicine.

[00150] Depending on the specific conditions being treated and the targeting method selected, such agents may be formulated and administered systemically or locally. Techniques for formulation and administration may be found in Alfonso and Gennaro (1995). Suitable routes may include, for example, oral, rectal, transdermal, vaginal, transmucosal, or intestinal administration; parenteral delivery, including intramuscular, subcutaneous, or intramedullary injections, as well as intrathecal, intravenous, or intraperitoneal injections.

[00151] For injection, the agents of the disclosure may be formulated in aqueous solutions, preferably in physiologically compatible buffers such as Hanks' solution, Ringer's solution, or physiological saline buffer. For transmucosal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art.

[00152] Use of pharmaceutically acceptable carriers to formulate the compounds herein disclosed for the practice of the disclosure into dosages suitable for systemic administration is within the scope of the disclosure. With proper choice of carrier and suitable manufacturing practice, the compositions of the present disclosure, in particular those formulated as solutions, may be administered parenterally, such as by intravenous injection. Appropriate compounds can be formulated readily using pharmaceutically acceptable carriers well known in the art into dosages suitable for oral administration. Such carriers enable the compounds of the disclosure to be formulated as tablets, pills, capsules, liquids, gels, syrups, slurries, suspensions and the like, for oral ingestion by a patient to be treated.

[00153] Agents intended to be administered intracellularly may be administered using techniques well known to those of ordinary skill in the art. For example, such agents may be encapsulated into liposomes, and then administered as described above. Liposomes are spherical lipid bilayers with aqueous interiors. All molecules present in an aqueous solution at the time of liposome formation are incorporated into the aqueous interior. The liposomal contents are both protected from the external microenvironment and, because liposomes fuse with cell membranes, are

efficiently delivered into the cell cytoplasm. Additionally, due to their hydrophobicity, small organic molecules may be directly administered intracellularly.

[00154] Pharmaceutical compositions suitable for use in the present disclosure include compositions wherein the active ingredients are contained in an effective amount to achieve the intended purpose. Determination of the effective amounts is well within the capability of those skilled in the art, especially in light of the detailed disclosure provided herein.

[00155] In addition to the active ingredients, these pharmaceutical compositions may contain suitable pharmaceutically acceptable carriers comprising excipients and auxiliaries which facilitate processing of the active compounds into preparations which can be used pharmaceutically. The preparations formulated for oral administration may be in the form of tablets, dragees, capsules, or solutions, including those formulated for delayed release or only to be released when the pharmaceutical reaches the small or large intestine.

[00156] The pharmaceutical compositions of the present disclosure may be manufactured in a manner that is itself known, e.g., by means of conventional mixing, dissolving, granulating, dragee-making, levitating, emulsifying, encapsulating, entrapping or lyophilizing processes.

[00157] Pharmaceutical formulations for parenteral administration include aqueous solutions of the active compounds in water-soluble form. Additionally, suspensions of the active compounds may be prepared as appropriate oily injection suspensions. Suitable lipophilic solvents or vehicles include fatty oils such as sesame oil, or synthetic fatty acid esters, such as ethyl oleate or triglycerides, or liposomes. Aqueous injection suspensions may contain substances which increase the viscosity of the suspension, such as sodium carboxymethyl cellulose, sorbitol, or dextran. Optionally, the suspension may also contain suitable stabilizers or agents which increase the solubility of the compounds to allow for the preparation of highly concentrated solutions.

[00158] Pharmaceutical preparations for oral use can be obtained by combining the active compounds with solid excipient, optionally grinding a resulting mixture, and processing the mixture of granules, after adding suitable auxiliaries, if desired, to obtain tablets or dragee cores. Suitable excipients are, in particular, fillers such as sugars, including lactose, sucrose, mannitol, or sorbitol; cellulose preparations such as, for example, maize starch, wheat starch, rice starch, potato starch, gelatin, gum

tragacanth, methyl cellulose, hydroxypropylmethyl-cellulose, sodium carboxymethylcellulose, and/or polyvinylpyrrolidone (PVP). If desired, disintegrating agents may be added, such as the cross-linked polyvinyl pyrrolidone, agar, or alginic acid or a salt thereof such as sodium alginate.

[00159] Dragee cores are provided with suitable coatings. For this purpose, concentrated sugar solutions may be used, which may optionally contain gum arabic, talc, polyvinyl pyrrolidone, carbopol gel, polyethylene glycol, and/or titanium dioxide, lacquer solutions, and suitable organic solvents or solvent mixtures. Dyestuffs or pigments may be added to the tablets or dragee coatings for identification or to characterize different combinations of active compound doses.

[00160] Pharmaceutical preparations which can be used orally include push-fit capsules made of gelatin, as well as soft, sealed capsules made of gelatin and a plasticizer, such as glycerol or sorbitol. The push-fit capsules can contain the active ingredients in admixture with filler such as lactose, binders such as starches, and/or lubricants such as talc or magnesium stearate and, optionally, stabilizers. In soft capsules, the active compounds may be dissolved or suspended in suitable liquids, such as fatty oils, liquid paraffin, or liquid polyethylene glycols. In addition, stabilizers may be added.

Methods of Treatment

[00161] The high affinity TCRs and pharmaceutical compositions comprising a high affinity TCR may be used, for example, to treat a patient having a cancer, tumor, malignancy, or neoplastic disease or disorder. In one embodiment, a method of treating a patient having cancer comprises administering a high affinity TCR described herein. In another embodiment, the high affinity TCR is specific for Survivin. In one embodiment, the TCR comprises a Vα comprising the amino acid sequence set forth in SEQ ID NO:1. In another embodiment, the TCR comprises a Vα comprising the amino acid sequence set forth in SEQ ID NO:2. In one embodiment, the high affinity TCR is a single chain TCR comprising the amino acid sequence set forth in SEQ ID NO:3. In another embodiment, the high affinity TCR is a single chain TCR comprising the amino acid sequence set forth in SEQ ID NO:4. In another embodiment, the high affinity TCR is administered in combination with a

therapeutic agent, e.g., a chemotherapeutic agent. In yet another embodiment, the high affinity TCR is conjugated to a biologically active group.

[00162] Another aspect of the invention provides a method for the adoptive transfer of T cells to a patient in need thereof, comprising administering T cells that express a high affinity TCR described herein. In one embodiment, the T cells have been transfected with a polynucleotide that encodes a high affinity TCR that is specific for Survivin. In one embodiment, the TCR comprises a Vα comprising the amino acid sequence set forth in SEQ ID NO:1. In another embodiment, the TCR comprises a Vα comprising the amino acid sequence set forth in SEQ ID NO:2. In one embodiment, the high affinity TCR is a single chain TCR comprising the amino acid sequence set forth in SEQ ID NO:3. In one embodiment, the high affinity TCR is a single chain TCR comprising the amino acid sequence set forth in SEQ ID NO:4.

EXAMPLES

[00163] The following examples further describe non-limiting examples of the disclosure.

EXAMPLE 1

ENGINEERING TCRS FOR HIGHER AFFINITY FOR PEPTIDE/HLA-A2 ANTIGENS

[00164] The general strategy used to discover, or generate single-chain TCRs for improved affinity and stability is shown in Figure 1. The process involves six steps, as illustrated:

[00165] 1) Cloning the Vα and Vβ TCR genes from a T cell clone which recognizes a MHC-restricted antigenic peptide of interest into a single chain TCR format for display. In the present invention, the TCR genes from one human T cell clone that was reactive with the Survivin antigen (from Delores Schendel, Thomas Blankenstein, and Wolfgang Uckert; see, e.g., Leisegang et al. (2010) J Clin Invest. 120(11), 3869) were cloned as a single-chain format (Vβ-linker-Vα) and introduced into a yeast display vector for expression on the surface of yeast. Further description of the wild type TCR reactive with the Survivin antigen, see US 2012/0128704.

[00166] 2) Generation of an error prone library and FACs or magnetic bead selection for stabilized variants with an anti-Vβ antibody. Because the single-chain

Vα and Vβ TCRs are often unstable due to loss of the stabilizing constant regions, error-prone mutagenesis libraries are generated to select for stabilizing mutations that allow for stable expression on the surface of yeast, although other display formats including but not limited to phage and mammalian display may be used. Phage display vectors and cloning have yielded library sizes of 10^{11}, whereas yeast display vectors and homologous recombination steps have yielded library sizes of 10^{10} ((Benatuil et al. (2010) Protein Eng Des Sel, 23, 155-9). Various methods have been used for selection of variants, including affinity-based binding to immobilized ligands (phage display) or magnetic particle selections with antigens (yeast display), or fluorescent activated cell sorting with labeled-peptide-MHC antigens (yeast display). Utilizing antibodies against the TCR Vβ that recognize folded epitopes, fluorescent activated cell sorting (FACS) or magnetic bead selection are used to isolate variants with improved antibody binding in the present example.

[00167] 3) scTCR clones isolated from the selection of the error prone library are assessed for thermal stability and a stabilize variant is chosen for a template for affinity maturation, and sequenced. Typically, single-site mutations are identified that contribute to increased surface levels on yeast, and greater stability in solution.

[00168] 4) The stabilized scTCR sequences are used as a template for the generation of CDR libraries, usually in the CDR1α, CDR3α, CDR3β, although other regions including but not limited to the CDR1β, CDR2α, CDR2β, and HV4 can also be used. In the present disclosure, yeast displayed variants are selected for improved binding to peptide:MHC, from the CDR libraries, by using magnetic bead selections and/or fluorescence activated cell sorting (FACS), although selections utilizing other methods including but not limited to panning with phage display or magnetic selections or FACS with mammalian display may be used.

[00169] 5) scTCR clones isolated from the selection of the CDR libraries are assessed for specific binding to the peptide:MHC against which they were engineered. Plasmids are rescued from the yeast clones, and sequenced.

[00170] 6) If further improvements of affinity required, the scTCR clone selected in step 5 can be used as a template for the generation of additional libraries in other loops or regions that did not select mutations such as CDR1α, CDR3α, CDR3β, although other regions including but not limited to the CDR1β, CDR2α, CDR2β, and HV4 can also be used. Examples of each of these steps are described further below.

EXAMPLE 2

ANALYSIS OF THE HUMAN TCR A6, WHICH USES THE Vα2, IN COMPLEX WITH TAX:HLA.A2

[00171] TCRs all adopt a similar Ig-fold and docking angle, and TCR recognition of pepMHC is mediated entirely by specific residues on CDR loops (Garcia et al. (2009) Nat Immunol, 10, 143-7; Marrack et al. (2008) Annu Rev Immunol, 26, 171-203; Rudolph et al. (2006) Annu Rev Immunol, 24, 419-66)). Although crystal structures for Survivin TCRs are not available at the time of the present disclosure, the structure of the A6:Tax peptide:HLA-A2 complex (PDB: 1AO7) (Garboczi et al. (1996) Nature, 384, 134-141), which used the same Vα2 domain as the Survivin TCR, is shown. The side view of the complex showed that the ends of the variable domains that contained the six CDRs docked onto the Tax:HLA.A2 molecule, with the central region of the binding site positioned over the peptide Tax (Figure 2A). The crystal structure does not include the constant region α, although the constant regions help stabilize the full length construct. Stabilizing mutations selected in step 2 described above are often selected in framework regions, such as the Vα/Vβ interphase or where the junctions of the Cα/Vα or Cβ/Vβ interphase occurs in the full length TCR.

[00172] The top down view of the Tax:HLA.A2 complex, with the TCR “removed”, except for the six CDR loops is shown (Figure 2B). This view shows that the TCR adopts a diagonal position over the peptide-MHC, a finding which has now been observed for all TCR:peptide-MHC structures. In this orientation, the two CDR3 loops are positioned over the peptide, while there are various residues from CDR1 and CDR2 loops that interact predominantly with the helices of the MHC molecule. For purposes of affinity maturation in steps 4 and 6, these loops are often the targeted for the generation of affinity maturations libraries, although other regions may be used.

EXAMPLE 3

YEAST DISPLAY OF SURVIVIN TCRS

[00173] In order to perform selections for improved stability (step 2) or improved affinity (step 5), it is necessary to use a display system in which a library of TCR mutants can be screened for binding to an antibody which recognizes a conformation epitope or a peptide:MHC ligand, respectively. Three display systems have been

used for engineering TCRs for higher affinity, and could be used for this process: yeast display, phage display, and T cell (mammalian cell) display. Alternative display methods, such as ribosome, RNA, DNA, and CIS display, may also be suitable for this process. In all of these cases, the wild type TCR with low affinity for the antigen was cloned into the system, and used as a template for engineering TCRs with enhanced stability and affinity against the peptide:MHC ligand. Any of these systems could be applied to the approach described here, in which a single TCR is used as a template for libraries and the selection of TCRs with enhanced binding properties.

[00174] In the present example, yeast display was used as the platform (Figure 3). The Survivin TCR was used as the template for stabilizing mutations via error prone mutagenesis, and stabilized clones isolated from the selections were used as templates for affinity maturation.

EXAMPLE 4

ERROR-PRONE LIBRARY CONSTRUCTION AND SELECTION OF A STABILIZED SURVIVIN TCR, SURV-K2

[00175] The Survivin error-prone library was generated as previously described (Richman et al. (2009) Methods Mol Biol, 504, 323-350) utilizing a Survivin-reactive TCR obtained from a collaborator called Survivin 71 as a template. The human Survivin error-prone library was introduced into the yeast display vector by combining the linearized pCT302 vector, Survivin error-prone PCR product, and competent EBY 100 yeast cells. The resultant library was judged by plating limiting dilution aliquots of yeast after electroporation and contained approximately 8.25 x 10^6 independent clones. The library was selected for binding to an antibody that recognizes human Vβ20, anti-hVβ20 FITC IgG (Beckman Coulter), via FACS according to Table 4.

Table 4. Sorting Conditions

Sort	Conditions
1	BC hVβ20 FITC (1:10); AlexaFluor® 488 goat anti-mouse IgG (1:100)
2	BC hVβ20 FITC (1:10); AlexaFluor® 488 goat anti-mouse IgG (1:100)
3	BC hVβ20 FITC (1:10); AlexaFluor® 488 goat anti-mouse IgG (1:100)

[00176] Using thermal denaturation studies, we identified this antibody to recognize folded epitopes on Vβ20 (data not shown). Signals were amplified using AlexaFluor® 488 goat anti-mouse IgG (Life Technologies) secondary antibody. During 3 iterative sorts, a Vβ20-positiviely staining population emerged (Figure 4A). Following the 3rd sort, a clone called Surv-K2 was isolated for improved Vβ20 fluorescence (Figure 4B). The SurvK2 clone was used as a template for affinity maturation.

EXAMPLE 5

CDR3α LIBRARY CONSTRUCTION AND SELECTION OF TWO SURVIVIN TCRS WITH ENHANCED BINDING TO SURVIVIN:HLA.A2, SURV-K2.4.1 AND SURV-K2.4.6

[00177] The stabilized Surv-K2 clone isolated from selection of error-prone PCR libraries was used as a template for generation of a CDR3α library spanning 5 residues via splicing by overlap extension (SOE). The human Surv-K2 CDR3α scTCR library was thus introduced into the yeast display vector by combining the linearized pCT302 vector, Surv-K2 CDR3β library PCR product, and competent EBY100 yeast cells. The resultant library was judged by plating limiting dilution aliquots of yeast after electroporation and contained 2.98 x 10^7 independent clones. The Surv-K2 CDR3α library was sorted three consecutive times using magnetic columns and once using FACS according to Table 5.

Table 5. Sorting Conditions

Sort	Conditions
1	BC hVβ20 FITC (1:20) MB Anti-Mouse IgG MicroBeads (1:25)
2	100 nM SurvT2M:HLA.A2 dimer MB Anti-Mouse IgG MicroBeads (1:25)
3	100 nM SurvT2M:HLA.A2 dimer MB Anti-Mouse IgG MicroBeads (1:25)
4	100 nM SurvT2M:HLA.A2 dimer AlexaFluor® 647 Goat Anti-Mouse IgG (1:100)

[00178] After two sorts using magnetic beads a modestly positively staining population began to emerge (Figure 5A). Clones Surv-K2.4.1 and Surv-K2.4.6 were isolated following the fourth sort. Surv-K2.4.1 and SurvK2.4.6 showed enhanced binding to SurvT2M (LMLGEFLKL, SEQ ID NO:5)/HLA-A2 (Figure 5B).

EXAMPLE 6

BINDING ANALYSIS OF HIGH AFFINITY SURVIVIN TCR, SURV-K2.4.1

[00179] In order to assess the binding of the Surv-K2.4.1 clone isolated from selections of CDR3α libraries, yeast displaying Surv-K2.4.1 were titrated with SurvT2M (LMLGEFLKL, SEQ ID NO:5)/HLA-A2 monomers at 6.4 nM, 32 nM, 160 nM, 800 nM and 4 μM and analyzed by flow cytometry (Figure 6A). Values were normalized using nonlinear regression analysis and an $K_{D,\, app}$ of 279 ± 44.5 nM was determined (Figure 6B).

EXAMPLE 7

SEQUENCE ANALYSIS OF THE ISOLATED TCRS FOR IMPROVED AFFINITY AGAINST THE SURVIVIN ANTIGEN

[00180] Sequences of the stabilized scTCR clone K2 and the survivin-specific (K2.4.1 and K2.4.6) high-affinity single-chain variants isolated from affinity maturation libraries were determined. As shown in Figure 7, there were mutations in CDR regions of the two high-affinity clones derived from the yeast display libraries. The underlined positions in Figure 7 indicate mutations that arose from error-prone library selections for stabilizing mutations. The positions in boxes show the affinity enhancing mutations that were selected from CDR libraries.

EXAMPLE 8

IN VITRO ACTIVITY OF THE K2.4.1 TCR IN T CELLS

[00181] To assess the activity of the K2.4.1 TCR in T cells, CD8 T cells were isolated from AAD transgenic mice. These T cells were then activated with anti-CD3/anti-CD28 beads for 24 hours. T cells were retrovirally transduced with pMP71 vector containing the Vα and β domains of the K2.4.1 TCR linked to the Cα and Cβ domains of the murine 2C TCR (Figure 8A). To confirm expression of the K2.4.1

TCR, T cells were stained 48 hours post-transduction with SurvT2M:HLA-A2 tetramer at a concentration of 20 nM (Figure 8B). K2.4.1 transduced T cells (Black) showed increased binding of SurvT2M:HLA-A2 over mock transduced T cells (Gray), confirming surface expression of the high-affinity TCR. T cells were then incubated at a 1:1 E:T with T2 cells exogenously loaded with titrating concentrations of survivin peptide. T cells expressing the K2.4.1 TCR activated in the presence of SurvT2M peptide and not when presented with a control peptide called WT1 (RMFPNAPYL, SEQ ID NO:14), suggesting that this TCR is active and specific in CD8 T cells.

EXAMPLE 9

THERAPEUTIC FORMATS OF THE SURVIVIN, SURV-K2.4.1 AND SURV-K2.4.6, TCRS

[00182] It is now well known that higher affinity TCRs can be used in various formats for targeting cells that express the corresponding antigen. Thus, it is clear that the TCRs generated from the engineering strategies shown above can be used either in soluble form or in TCR gene therapy for adoptive T cell therapies, as illustrated in Figure 9.

Materials and methods

Antibodies, peptide:HLA-A2, MACS, and Flow Cytometry Reagents

[00183] Antibodies used to detect yeast surface expression included: anti-HA epitope tag (Clone HA.11; Covance), anti-hVβ3 FITC antibody (Clone CH92; Beckman-Coulter), anti-hVβ3.1 FITC antibody (Clone 8F10; Thermo Scientific), anti-hVβ20 antibody (Clone ELL1.4; Beckman-Coulter), anti-Vα2 monoclonal antibody generated in our laboratory (data not shown), Goat-anti-mouse IgM APC (Life Technologies), Goat-anti-mouse IgG F(ab')$_2$ AlexaFluor® 647 secondary antibody (Invitrogen), Streptavidin-phycoerythrin (SA:PE, BD Pharmingen), and MACS microbeads (Miltenyl Biotec).

[00184] Peptides that bind to HLA-A2 SurvT2M: LMLGEFLKL (SEQ ID NO:5) anchor residue 2 modified from T to M for improved HLA-A2 binding (Andersen et al, 2001, Cancer Research 61, 5964-5968) were synthesized by standard F-moc (N-(9-fluorenyl)methoxycarbonyl) chemistry at the Macromolecular Core Facility at Penn State University College of Medicine (Hershey, PA, USA). For FACS and flow

cytometry analysis, recombinant soluble dimeric HLA-A2:Ig fusion protein (BD™ DimerX) was used. Additionally, a monomeric HLA.A2-biotin reagent generated by the exchange of a UV-cleavable peptide for another HLA.A2-restricted peptide in the presence of UV light was utilized for flow cytometry and MACS selections (Rodenko et al. (2006) Nat Protoc, 1, 1120-1132; Toebes et al. (2006) Nat Med, 12, 246-251).

Cloning and expression of scTv in yeast display vectors

[00185] TCR variable region fragments (scTv) were expressed in yeast display plasmid pCT302 (Vβ-L-Vα) (Boder and Wittrup (2000) Methods Enzymol, 328, 430-444), which contains a galactose-inducable AGA2 fusion allowing for growth in Trp media. Induction of the scTv gene involves growth of the transformed EBY100 yeast cells to stationary phase in selection media followed by transfer to galactose-containing media. The template Survivin single-chain TCR genes was synthesized by Genscript (Piscataway, NJ, USA) with a F49S mutation in the Vα2-domain of the construct (Aggen et al. (2011) Protein Eng Des Sel, 24, 361-372).

[00186] The Survivin specific TCR genes were isolated from CTL clones (TCR genes against Survivin from Delores Schendel, Thomas Blankenstein, and Wolfgang Uckert; e.g. Leisegang et al. (2010) J Clin Invest. 120(11), 3869), the genes were synthesized by Genscript, cloned as a single-chain format (Vβ-linker-Vα), introduced into a yeast display vector for expression on the surface of yeast. The scTvs consisted of the variable contains attached by the linker region GSADDAKKDAAKKDGKS (SEQ ID NO:7) (Hoo et al. (1992) Proc Natl Acad Sci USA, 89, 4759-4763; Weber et al. (2005) Proc Natl Acad Sci USA, 102, 19033-19038; Aggen et al. (2011) Protein Eng Des Sel, 24, 361-372). The scTv was introduced into the NheI and XhoI restrictions sites of pCT302.

Generation, display, and selection of mutated scTv yeast display libraries

[00187] Error-prone PCR was used to generate random mutations, as previously described (Richman et al. (2009) Mol Immunol, 46, 902-916). CDR1 and 3 libraries were generated using Splicing by overlap extension (SOE) PCR spanning 4-5 adjacent codons at a time (Horton et al. (1990) Biotechniques, 8, 528-535).
[00188] For the Surv CDR3α library, pre-SOE PCR products were generated utilizing the following primer pairs: 5’ – GGC AGC CCC ATA AAC ACA CAG TAT -3’

(Splice 4L) (SEQ ID NO:8) and 5' – CAC AGC GCA CAG ATA GGT AGC -3' (SEQ ID NO:10) and 5' – CTG ATT CAG CTA CCT ATC TGT GCG CTG TGN NSN NSN NSN NSN NSA TGT TTG GCG ATG GTA CTC AGC TGG TTG TG -3' (SEQ ID NO:11) and 5' – TAA TAC GAC TCA CTA TAG GG -3' (T7) (SEQ ID NO:9). SOE PCR was performed with each corresponding Pre-SOE along with T7 and Splice 4L.

[00189] Yeast libraries were made by homologous recombination in EBY100 yeast by electroporating error prone or SOE PCR products along with NheI and XhoI digested pCT302 (Horton et al. (1990) Biotechniques, 8, 528-535). The libraries were induced in galactose-containing media (SG-CAA) for 48 h, washed with 1 mL 1% PBS/BSA, and stained with antibodies or peptide:MHC reagents at the concentrations indicated in Figures 4A, 5A, 6A, 8A, and 9A. Cells were washed (1 ml, 1% PBS/BSA), and the most fluorescent cells were selected using a FACS Aria (BD Bioscience) high-speed sorter or via MACS LS columns on a QuadroMACS™ Separator (Miltenyl Biotec). In order to test thermal stability of isolated clones, yeast were incubated at elevated temperature for 30 min prior to the staining protocol (data not shown).

Isolation and staining of high affinity clones

[00190] Following selections, library clones were isolated by plating limiting dilutions. Colonies were expanded and induced in galactose-containing media (SG-CAA) for 48 hours, washed with 1 mL 1% PBS/BSA, and stained with various concentrations of peptide/HLA.A2 DimerX, goat-anti-mouse IgG F(ab')$_2$ AlexaFluor® 647 secondary antibody, or various concentrations of UV-exchanged peptide/HLA.A2, SA-PE. Cells were washed (1 ml, 1% PBS/BSA) and analyzed on an Accuri C6 flow cytometer.

[00191] Plasmids were recovered using Zymoprep™ Yeast Plasmid Miniprep II (Zymo Research) and introduced back into E. coli via heat shock transformation into Subcloning Efficiency™ DH5α™ Competent Cells (Invitrogen). E. coli cells were expanded and plasmids were isolated using QIAprep™ Spin Miniprep Kit (Qiagen). Sequences of individual clones were determined by Sanger sequencing

[00192]

[00193]

[00194] All patents and publications mentioned in the specification are indicative of the levels of skill of those skilled in the art to which the disclosure pertains. References cited herein to indicate the state of the art, in some cases as of their filing date, and it is intended that this information can be employed herein, if needed, to exclude (for example, to disclaim) specific embodiments that are in the prior art or to use methods or materials that are in the state of the art without the specific inclusion of the methods or materials in the disclosure herein. For example, when a compound is claimed, it should be understood that compounds known in the prior art, including certain compounds disclosed in the references disclosed herein (particularly in referenced patent documents), are not intended to be included in the claim.

[00195] When a Markush group or other grouping is used herein, all individual members of the group and all combinations and subcombinations possible of the group are intended to be individually included in the disclosure.

[00196] Where the terms “comprise”, “comprises”, “comprised”, or “comprising” are used herein, they are to be interpreted as specifying the presence of the stated features, integers, steps, or components referred to, but not to preclude the presence or addition of one or more other feature, integer, step, component, or group thereof. Separate embodiments of the disclosure are also intended to be encompassed wherein the terms “comprising” or “comprise(s)” or “comprised” are optionally replaced with the terms, analogous in grammar, e.g.; “consisting/consist(s)” or “consisting essentially of/consist(s) essentially of” to thereby describe further embodiments that are not necessarily coextensive. For clarification, as used herein “comprising” is synonymous with “having,” "including," "containing," or "characterized by," and is inclusive or open-ended and does not exclude additional, unrecited elements or method steps. As used herein, "consisting of" excludes any element, step, component, or ingredient not specified in the claim

element. As used herein, "consisting essentially of" does not exclude materials or steps that do not materially affect the basic and novel characteristics of the claim (e.g., not affecting an active ingredient). In each instance herein any of the terms "comprising", "consisting essentially of" and "consisting of" may be replaced with either of the other two terms. The disclosure illustratively described herein suitably may be practiced in the absence of any element or elements, limitation or limitations which is not specifically disclosed herein.

[00197] The disclosure has been described with reference to various specific and preferred embodiments and techniques. However, it should be understood that many variations and modifications may be made while remaining within the spirit and scope of the disclosure. It will be appreciated by one of ordinary skill in the art that compositions, methods, devices, device elements, materials, optional features, procedures and techniques other than those specifically described herein can be applied to the practice of the disclosure as broadly disclosed herein without resort to undue experimentation. All art-known functional equivalents of compositions, methods, devices, device elements, materials, procedures and techniques described herein; and portions thereof; are intended to be encompassed by this disclosure. Whenever a range is disclosed, all subranges and individual values are intended to be encompassed. This disclosure is not to be limited by the embodiments disclosed, including any shown in the drawings or exemplified in the specification, which are given by way of example or illustration and not of limitation. Some references provide details concerning additional starting materials, additional methods of synthesis, and additional methods of analysis and additional uses of the disclosure.

[00198] One skilled in the art would readily appreciate that the present disclosure is well adapted to carry out the objects and obtain the ends and advantages mentioned, as well as those inherent therein. The compositions and methods and accessory methods described herein as presently representative of preferred embodiments are exemplary and are not intended as limitations on the scope of the disclosure. Changes therein and other uses will occur to those skilled in the art, which are encompassed within the spirit of the disclosure.

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<u>U.S. Patents</u>

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8,143,376; Filed 18-May-05; Issued 27-Mar-12; Immunocore Limited; High affinity NY-ESO T cell receptor.

8,088,379; Filed 26-Sep-07; Issued 3-Jan-12; Immunocore Limited; Modified T cell receptors and related materials and methods.

8,017,730; Filed 19-May-06; Issued 13-Sep-11; Immunocore Limited; T cell receptors which bind to VYGFVRACL-HLA-A24.

7,763,718; Filed 29-Oct-07; Issued 27-Jul-10; Immunocore Limited; Soluble T cell receptors.

7,666,604; Filed 9-Jul-03; Issued 23-Feb-10; Immunocore Limited; Modified soluble T cell receptor.

7,608,410; Filed 7-Oct-08; Issued 27-Oct-09; Immunocore Limited; Method of improving T cell receptors.

7,569,664; Filed 3-Oct-03; Issued 4-Aug-09; Immunocore Limited; Single chain recombinant T cell receptors.

8,105,830; Filed 5-Nov-02; Issued 31-Jan-12; Altor Bioscience Corporation; Polyspecific binding molecules and uses therof.

6,534,633; Filed 21-Oct-99; 18-Mar-03; Altor Bioscience Corporation; Polyspecific binding molecules and uses therof.

CLAIMS

What is claimed is:

1. A modified T cell receptor, or antigen-binding fragment thereof, comprising a Vα and a Vβ derived from a wild type T cell receptor, wherein the Vα or the Vβ, or both, comprise a mutation in one or more complementarity determining regions (CDRs) relative to the wild type T cell receptor, wherein the Vα comprises an amino acid sequence selected from the group consisting of SEQ ID NO: 1 and SEQ ID NO: 2, and wherein the Vβ comprises the amino acid sequence set forth in SEQ ID NO: 12, and wherein the modified T cell receptor binds to a complex of a peptide Survivin and a Human Leukocyte Antigen A2 (HLA-A2) molecule with a K_D value of 10^{-6}M to 10^{-12}M.

2. The modified T cell receptor of claim 1, wherein the modified T cell receptor comprises a single-chain T cell receptor with the amino acid sequence set forth in SEQ ID NO: 3.

3. The modified T cell receptor of claim 1, wherein the modified T cell receptor comprises a single-chain T cell receptor with the amino acid sequence set forth in SEQ ID NO: 4.

4. The modified T cell receptor of claim 1 that is in soluble form.

5. A therapeutic agent that targets cancer cells that express a Survivin antigen, wherein the therapeutic agent comprises the modified T cell receptor of claim 4.

6. A therapeutic agent that targets cancer cells that express a Survivin antigen, wherein the therapeutic agent comprises a human T cell that expresses the modified T cell receptor of claim 1.

7. Use of the therapeutic agent of claim 5 or 6 in the manufacture of a medicament to treat cancer, wherein the cancer expresses a Survivin antigen.

8. The therapeutic agent of claim 5 or 6 for use in treating cancer, wherein the cancer expresses a Survivin antigen.

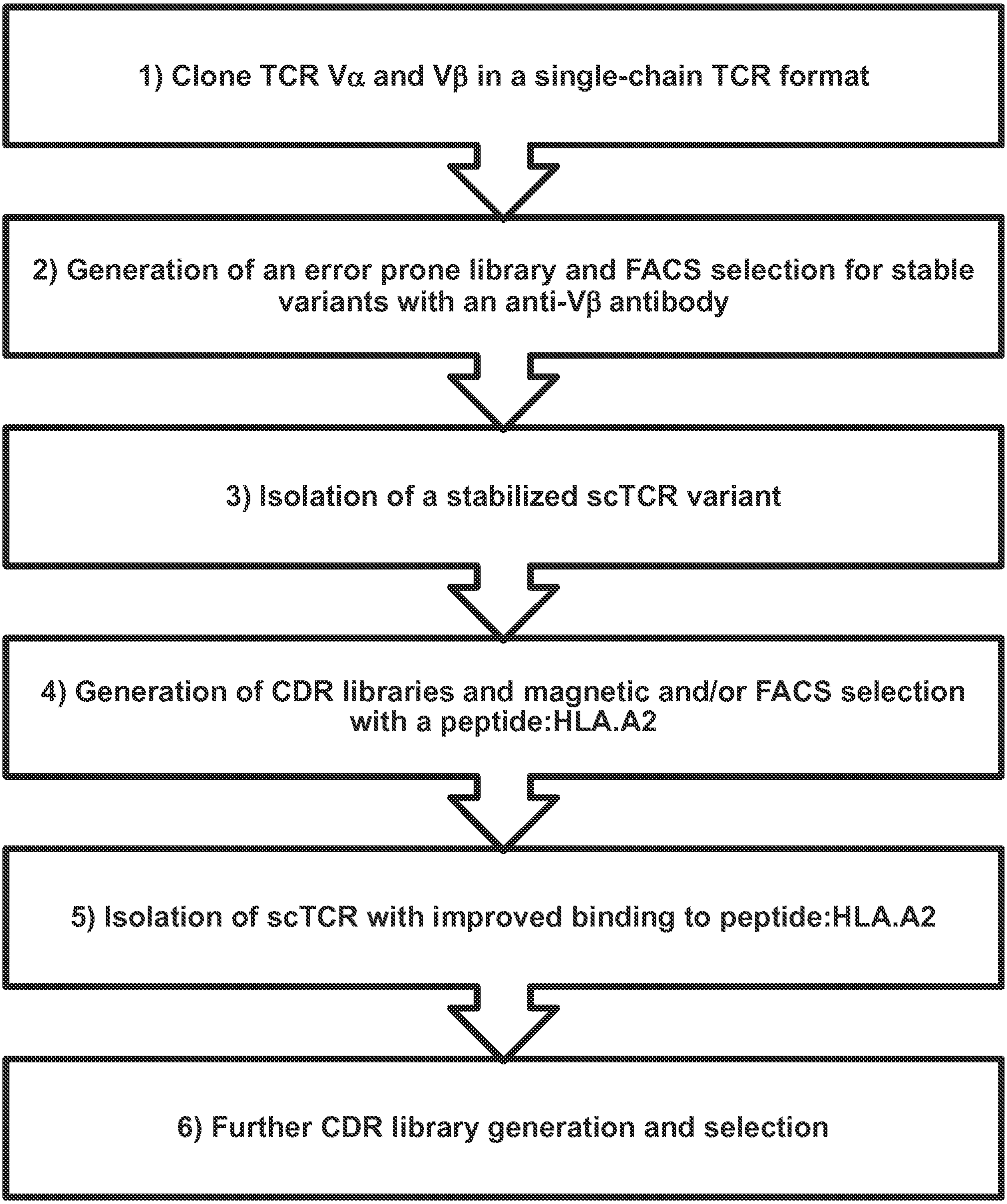

1) Clone TCR Vα and Vβ in a single-chain TCR format
2) Generation of an error prone library and FACS selection for stable variants with an anti-Vβ antibody
3) Isolation of a stabilized scTCR variant
4) Generation of CDR libraries and magnetic and/or FACS selection with a peptide:HLA.A2
5) Isolation of scTCR with improved binding to peptide:HLA.A2
6) Further CDR library generation and selection

FIG. 1

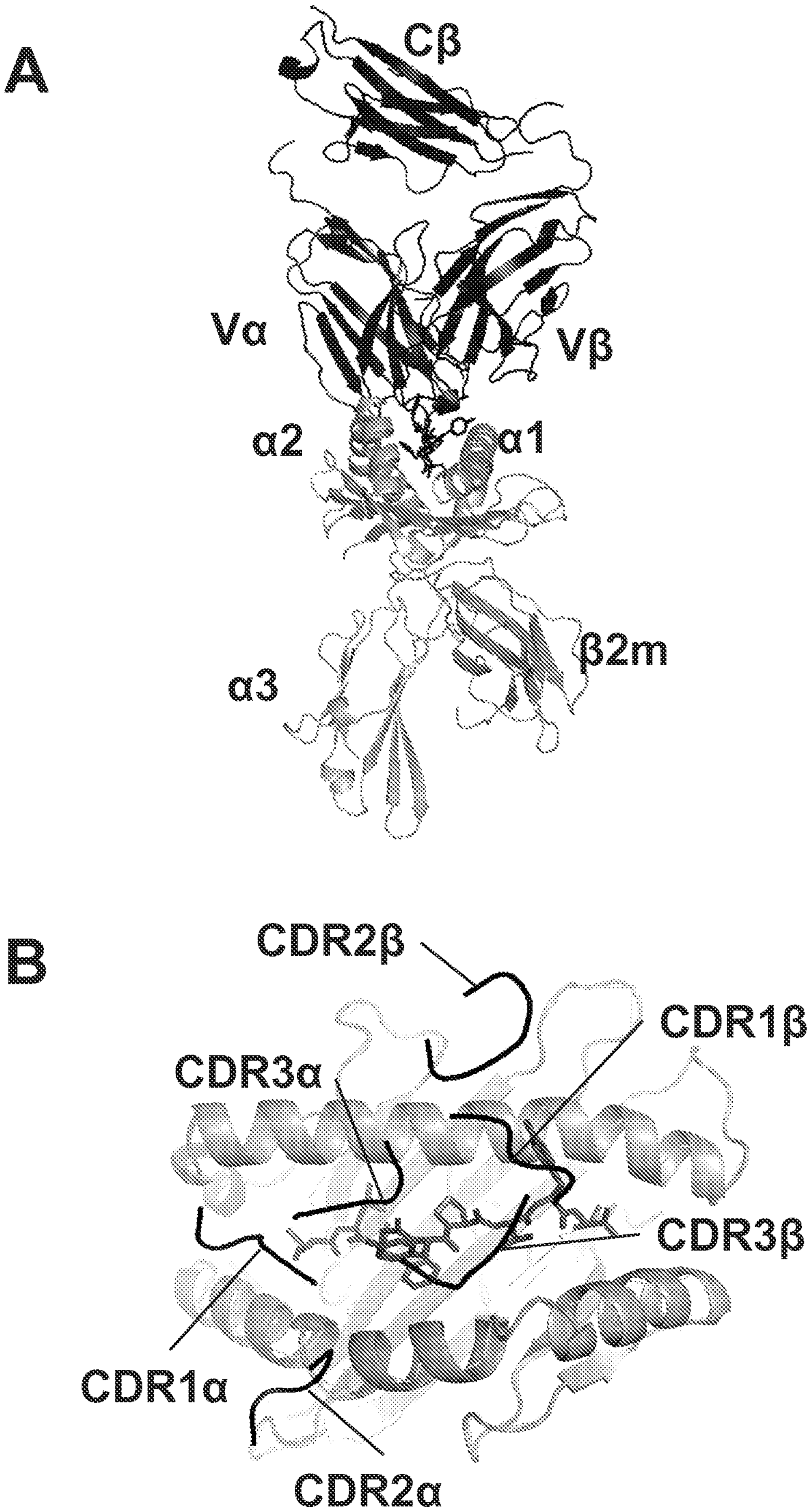


FIG. 2

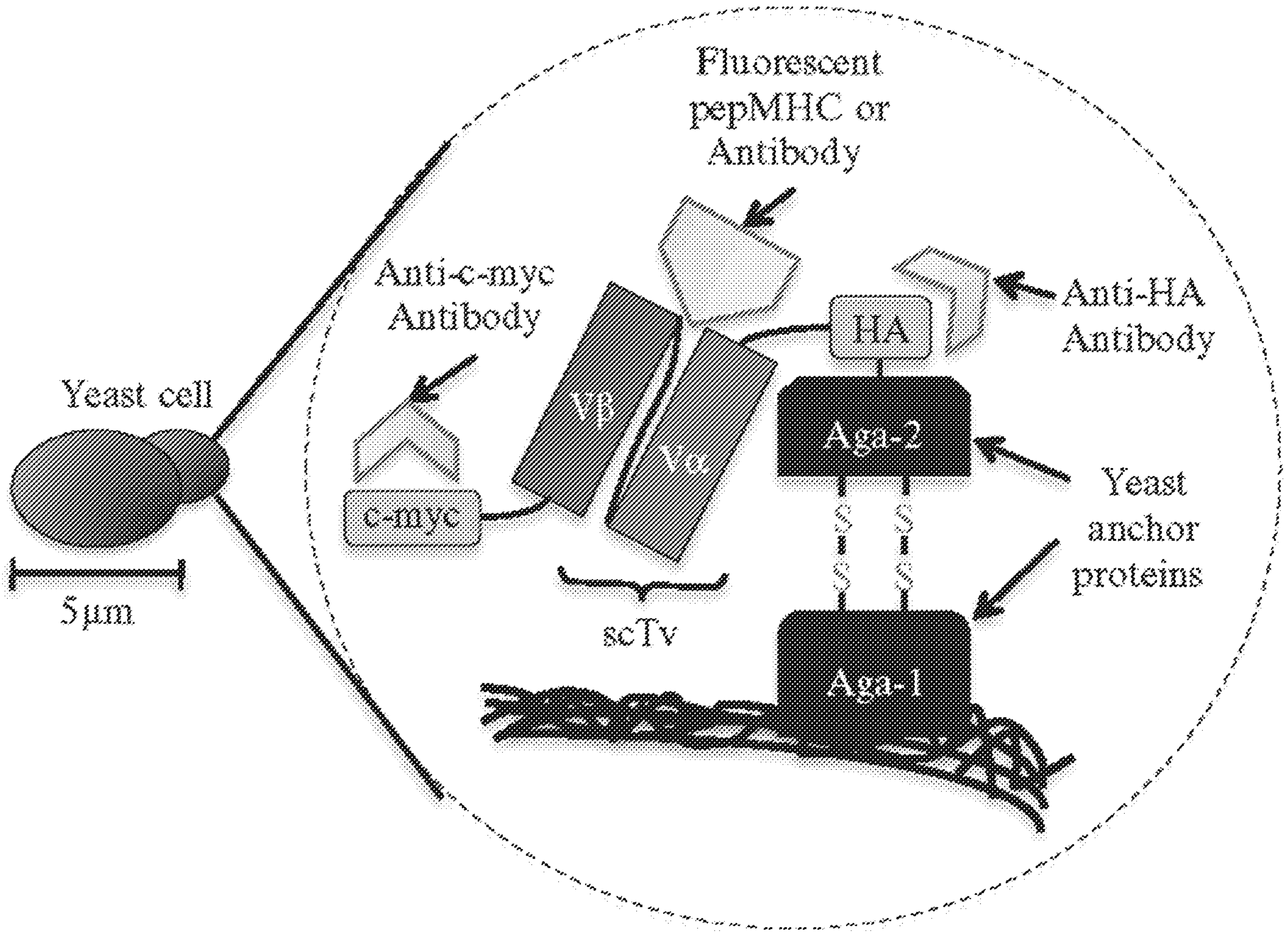

Yeast cell
5μm
Anti-c-myc Antibody
c-myc
Fluorescent pepMHC or Antibody
Vβ
Vα
scTv
HA
Anti-HA Antibody
Aga-2
S S
S S
Aga-1
Yeast anchor proteins

FIG. 3

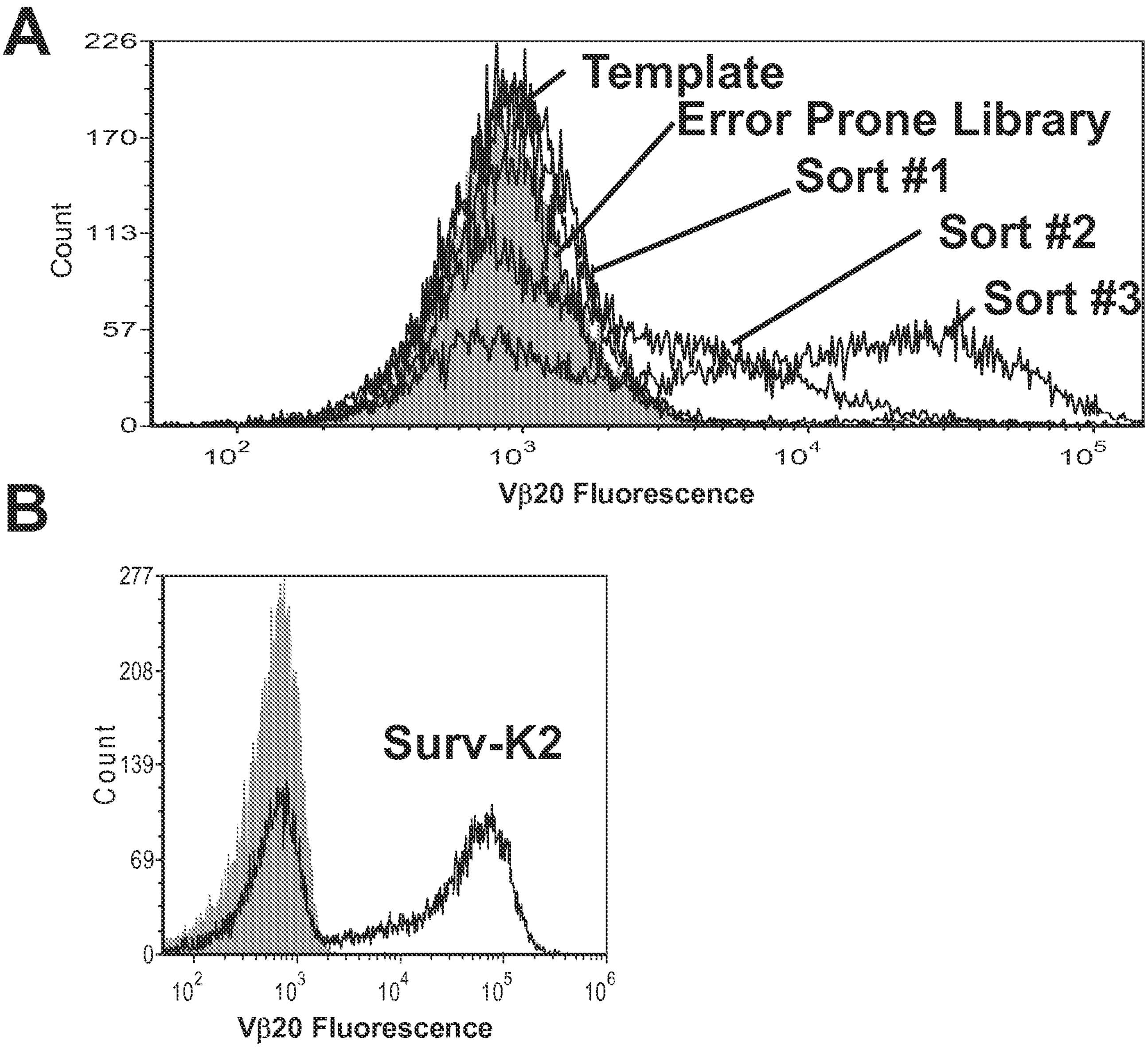

A
226
170
113
57
0
Count
Template
Error Prone Library
Sort #1
Sort #2
Sort #3
10^2
10^3
10^4
10^5
Vβ20 Fluorescence
B
277
208
139
69
0
Count
Surv-K2
10^2
10^3
10^4
10^5
10^6
Vβ20 Fluorescence

FIG. 4

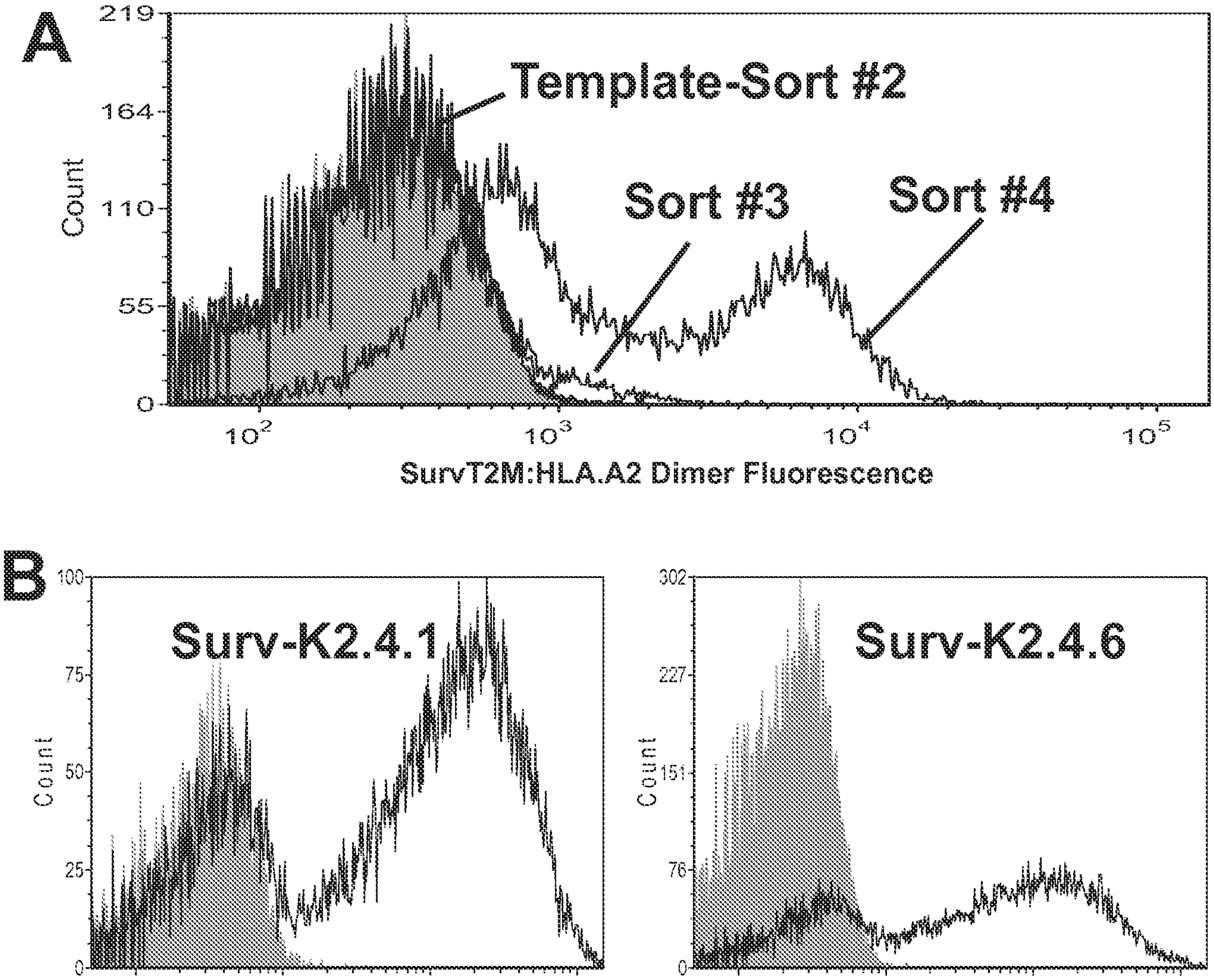

A
219
164
110
55
0
Count
Template-Sort #2
Sort #3
Sort #4
10^2
10^3
10^4
10^5
SurvT2M:HLA.A2 Dimer Fluorescence
B
Surv-K2.4.1
100
75
50
25
0
Count
10^2
10^3
10^4
10^5
SurvT2M:HLA.A2 Dimer Fluorescence
Surv-K2.4.6
302
227
151
76
0
Count
10^2
10^3
10^4
10^5
SurvT2M:HLA.A2 Dimer Fluorescence

FIG. 5

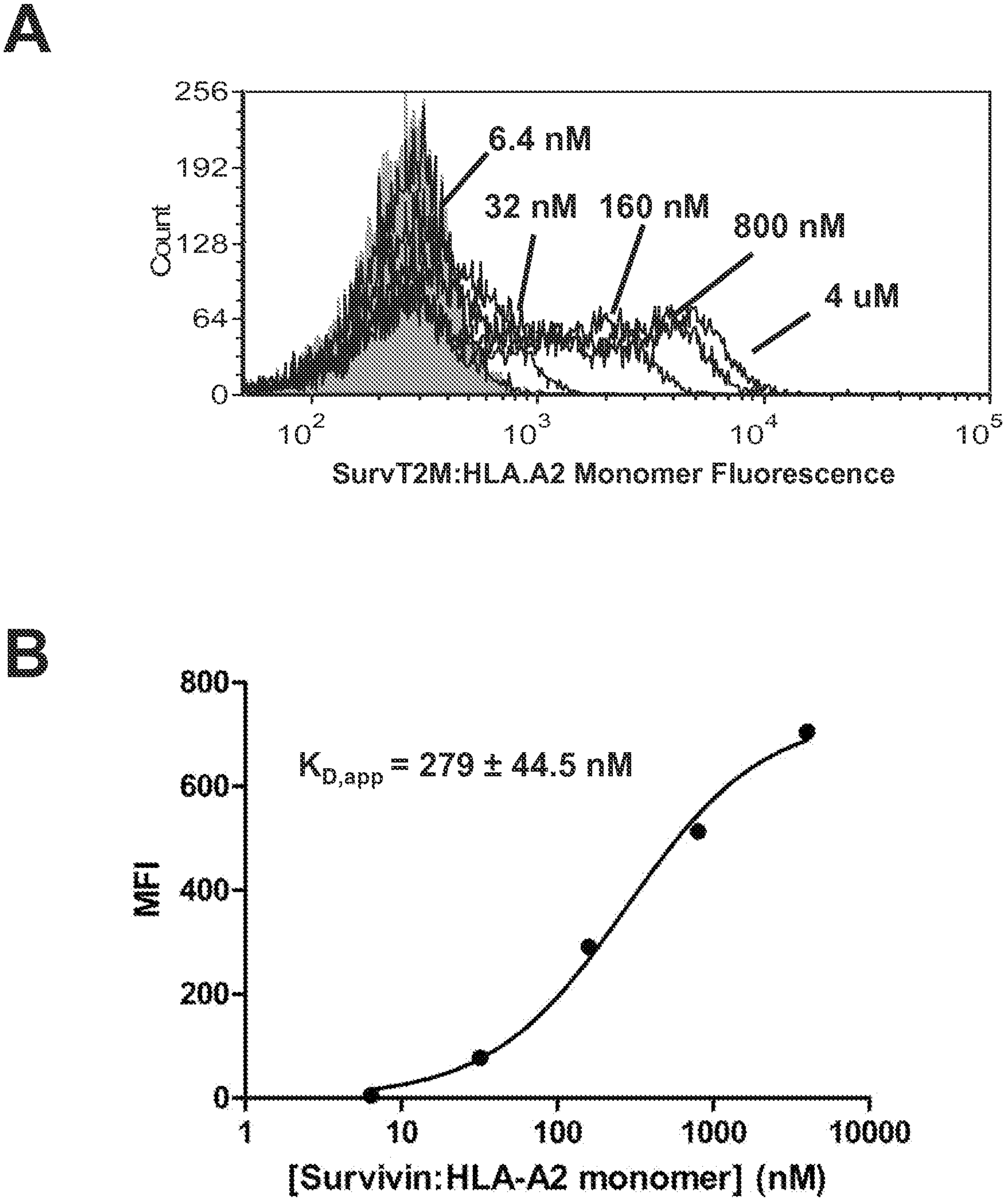

A
256
192
128
64
0
Count
6.4 nM
32 nM
160 nM
800 nM
4 uM
10^2
10^3
10^4
10^5
SurvT2M:HLA.A2 Monomer Fluorescence
B
800
600
400
200
0
MFI
$K_{D,app}$ = 279 ± 44.5 nM
1
10
100
1000
10000
[Survivin:HLA-A2 monomer] (nM)

FIG. 6

Vβ Chain

																											CDR 1																						CDR 2										
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59
Survivin K2	S	Q	T	I	H	Q	W	P	A	T	L	V	Q	P	V	G	S	P	L	S	L	E	C	T	V	E	G	T	S	N	P	N	L	Y	W	Y	R	Q	A	A	G	R	G	L	**E**	L	L	F	Y	S	V	G	I	G	_	_	Q	I	S
Survivin K2.4.1	S	Q	T	I	H	Q	W	P	A	T	L	V	Q	P	V	G	S	P	L	S	L	E	C	T	V	E	G	T	S	N	P	N	L	Y	W	Y	R	Q	A	A	G	R	G	L	**E**	L	L	F	Y	S	V	G	I	G	_	_	Q	I	S
Survivin K2.4.6	S	Q	T	I	H	Q	W	P	A	T	L	V	Q	P	V	G	S	P	L	S	L	E	C	T	V	E	G	T	S	N	P	N	L	Y	W	Y	R	Q	A	A	G	R	G	L	**E**	L	L	F	Y	S	V	G	I	G	_	_	Q	I	S

Vβ (Continued)

																																			CDR 3																												
	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90	91	92	93	94	95	96	97	98	99	100	101	102	103	104	105	106	107	108	109	110	111	112	113	114	115	116	117	118	119	120	121	112
Survivin K2	S	E	V	P	Q	N	L	**F**	A	S	R	P	Q	D	R	Q	F	I	L	S	S	K	K	L	L	L	S	D	S	G	F	Y	L	C	A	W	S	I	_	_	_	_	_	G	A	E	Q	F	F	G	P	G	T	R	L	T	V	L	E	D	L	K	N
Survivin K2.4.1	S	E	V	P	Q	N	L	**F**	A	S	R	P	Q	D	R	Q	F	I	L	S	S	K	K	L	L	L	S	D	S	G	F	Y	L	C	A	W	S	I	_	_	_	_	_	G	A	E	Q	F	F	G	P	G	T	R	L	T	V	L	E	D	L	K	N
Survivin K2.4.6	S	E	V	P	Q	N	L	**F**	A	S	R	P	Q	D	R	Q	F	I	L	S	S	K	K	L	L	L	S	D	S	G	F	Y	L	C	A	W	S	I	_	_	_	_	_	G	A	E	Q	F	F	G	P	G	T	R	L	T	V	L	E	D	L	K	N

Linker

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
Survivin K2	G	S	A	D	D	A	K	K	D	A	A	K	K	D	G	K	S
Survivin K2.4.1	G	S	A	D	D	A	K	K	D	A	A	K	K	D	G	K	S
Survivin K2.4.6	G	S	A	D	D	A	K	K	D	A	A	K	K	D	G	K	S

Vα Chain

																									CDR 1																									CDR2						
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56
Survivin K2	Q	K	E	V	E	Q	N	S	G	P	L	S	V	P	E	G	A	I	A	S	L	N	C	T	Y	S	D	R	G	S	Q	S	F	F	W	Y	R	Q	Y	S	G	K	S	P	E	L	I	M	S	I	Y	S	N	G	D	K
Survivin K2.4.1	Q	K	E	V	E	Q	N	S	G	P	L	S	V	P	E	G	A	I	A	S	L	N	C	T	Y	S	D	R	G	S	Q	S	F	F	W	Y	R	Q	Y	S	G	K	S	P	E	L	I	M	S	I	Y	S	N	G	D	K
Survivin K2.4.6	Q	K	E	V	E	Q	N	S	G	P	L	S	V	P	E	G	A	I	A	S	L	N	C	T	Y	S	D	R	G	S	G	S	F	F	W	Y	R	Q	Y	S	G	K	S	P	E	L	I	M	S	I	Y	S	N	G	D	K

Vα (Continued)

																																			CDR 3																										
	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90	91	92	93	94	95	96	97	98	99	100	101	102	103	104	105	106	107	108	109	110	111	112	113	114	115	116	117
Survivin K2	E	D	G	R	F	T	A	Q	L	N	K	A	S	Q	Y	V	S	L	L	I	R	D	S	Q	P	S	D	S	A	T	Y	L	C	A	A	N	_	_	_	_	_	_	_	N	A	R	L	M	F	G	D	G	T	Q	L	V	V	K	P	N	I
Survivin K2.4.1	E	D	G	R	F	T	A	Q	L	N	K	A	S	Q	Y	V	S	L	L	I	R	D	S	Q	P	S	D	S	A	T	Y	L	C	A	V	**S**	_	_	_	_	_	_	_	**K**	**G**	**Y**	**K**	M	F	G	D	G	T	Q	L	V	V	K	P	N	I
Survivin K2.4.6	E	D	G	R	F	T	A	Q	L	N	K	A	S	Q	Y	V	S	L	L	I	R	D	S	Q	P	S	D	S	A	T	Y	L	C	A	V	**H**	_	_	_	_	_	_	_	**G**	**W**	**Y**	**T**	M	F	G	D	G	T	Q	L	V	V	K	P	N	I

FIG. 7

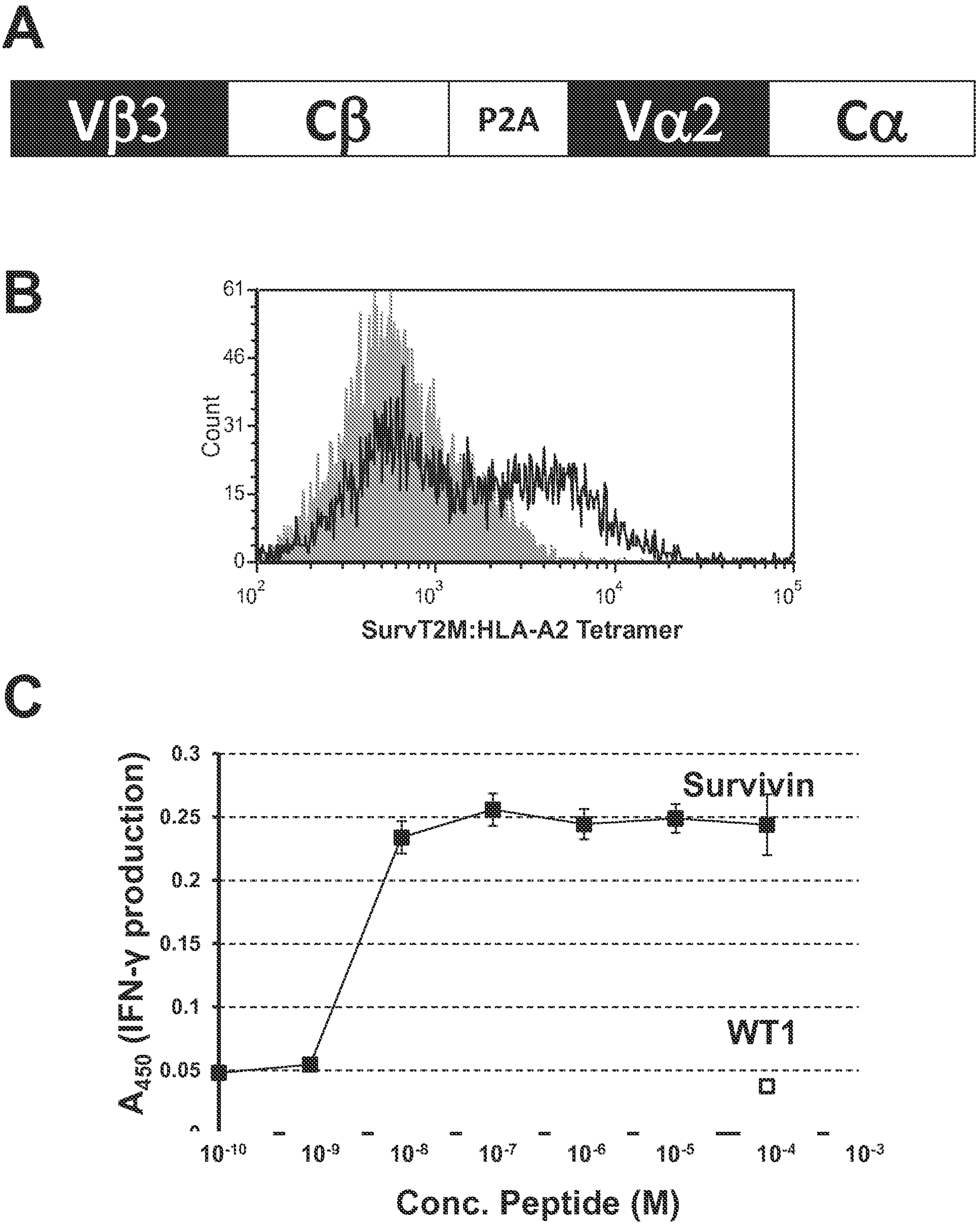

A
Vβ3
Cβ
P2A
Vα2
Cα
B
61
46
31
15
0
Count
10^2
10^3
10^4
10^5
SurvT2M:HLA-A2 Tetramer
C
0.3
0.25
0.2
0.15
0.1
0.05
0
A450 (IFN-γ production)
Survivin
WT1
10^-10
10^-9
10^-8
10^-7
10^-6
10^-5
10^-4
10^-3
Conc. Peptide (M)

FIG. 8

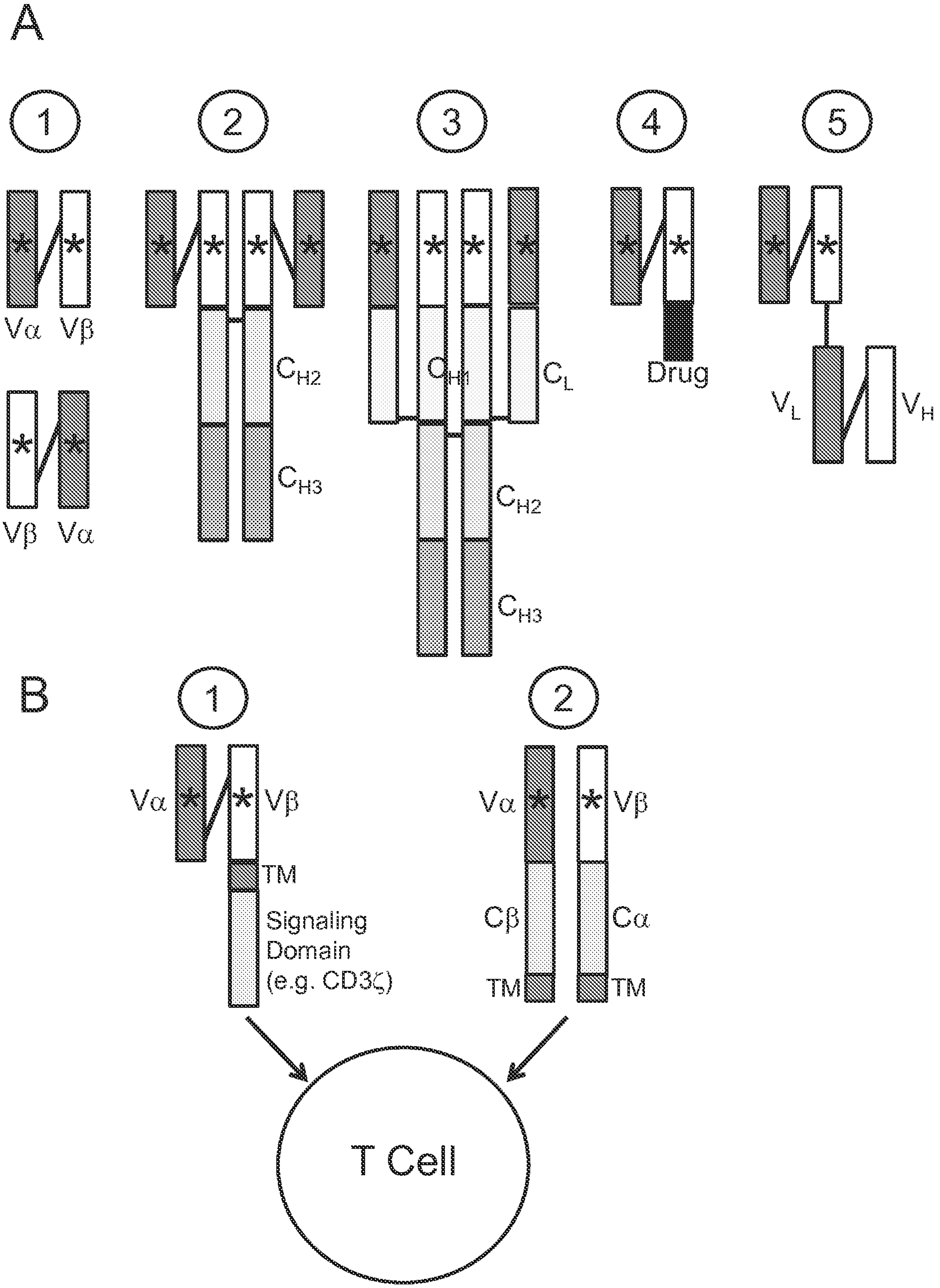
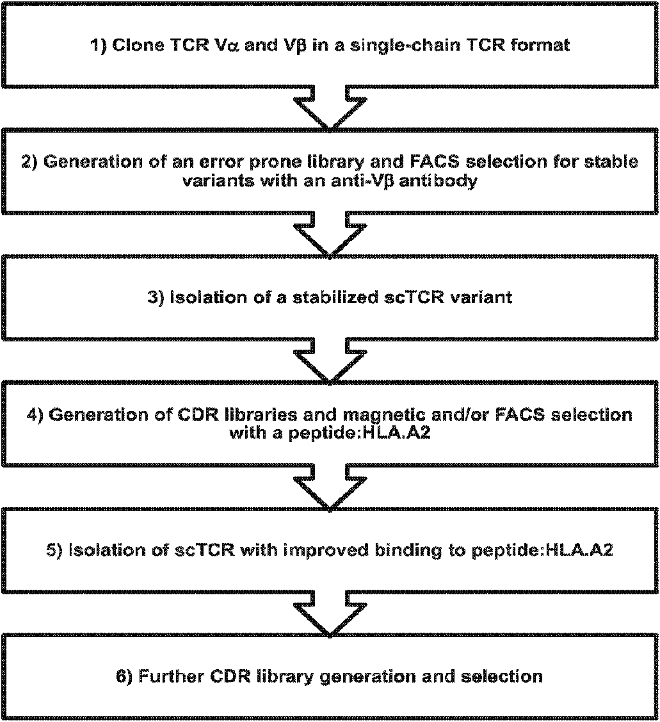

A
①
②
③
④
⑤
Vα
Vβ
Vβ
Vα
C_{H2}
C_{H3}
C_{H1}
C_L
C_{H2}
C_{H3}
Drug
V_L
V_H
B
①
②
Vα
Vβ
TM
Signaling
Domain
(e.g. CD3ζ)
Vα
Vβ
Cβ
Cα
TM
TM
T Cell

FIG. 9


1) Clone TCR Vα and Vβ in a single-chain TCR format
2) Generation of an error prone library and FACS selection for stable variants with an anti-Vβ antibody
3) Isolation of a stabilized scTCR variant
4) Generation of CDR libraries and magnetic and/or FACS selection with a peptide:HLA.A2
5) Isolation of scTCR with improved binding to peptide:HLA.A2
6) Further CDR library generation and selection