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(71) Applicant: **FRED HUTCHINSON CANCER RESEARCH CENTER** [US/US]; 1100 Fairview Avenue North, Seattle, Washington 98109 (US).

(72) Inventors; and
(71) Applicants : **SCHMITT, Thomas, M.** [US/US]; 4426 1st Avenue Northwest, Seattle, Washington 98107 (US). **GREENBERG, Philip, D.** [US/US]; 6510 82nd Avenue Southeast, Mercer Island, Washington 98040 (US). **NGUYEN, Hieu** [US/US]; 32616 7th Avenue Southwest, Federal Way, Washington 98023 (US).

(74) Agents: **PEPE, Jeffrey, C.** et al.; Seed Intellectual Property Law Group PLLC, Suite 5400, 701 Fifth Avenue, Seattle, Washington 98104-7064 (US).

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(54) Title: T CELL IMMUNOTHERAPY SPECIFIC FOR WT-1

(57) Abstract: The present disclosure provides high affinity and enhanced affinity T cell receptors specific for human Wilms tumor protein 1 (WT 1) epitopes for use in treating diseases or disorders, such as cancer cells that overexpress WT-1.

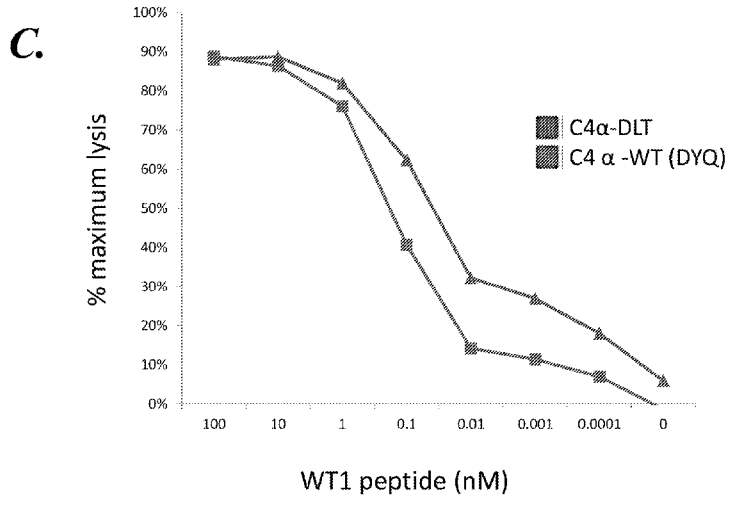

Fig. 4C

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T CELL IMMUNOTHERAPY SPECIFIC FOR WT-1

STATEMENT OF GOVERNMENT INTEREST

This invention was made with government support under Grant No. P01 CA18029 awarded by the National Institutes of Health / National Cancer Institute. The government has certain rights in this invention.

CROSS REFERENCE TO RELATED APPLICATIONS

This application claims the benefit under 35 U.S.C. § 119(e) to U.S. Provisional Application No. 62/033,045 filed 04 August 2014 and U.S. Provisional Application No. 62/164,783 filed 21 May 2015, which applications are incorporated by reference herein in their entireties.

STATEMENT REGARDING SEQUENCE LISTING

The Sequence Listing associated with this application is provided in text format in lieu of a paper copy, and is hereby incorporated by reference into the specification. The name of the text file containing the Sequence Listing is 360056_427WO_SEQUENCE_LISTING.txt. The text file is 138 KB, was created on July 29, 2015, and is being submitted electronically via EFS-Web.

BACKGROUND

Technical Field

The present disclosure relates generally to high affinity or enhanced affinity T cell receptors (TCRs) specific for antigens associated with a hyperproliferative disease. More specifically, the present disclosure relates to TCRs with high or enhanced affinity against a human Wilms tumor protein 1 (WT-1) epitope, T cells expressing such WT-1-specific TCRs, nucleic acids encoding the same, and compositions for use in treating diseases or disorders in which cells overexpress WT-1, such as in cancer.

Description of the Related Art

T cell receptor (TCR) gene therapy is an emerging treatment approach designed to overcome obstacles associated with conventional T cell adoptive immunotherapy, such as the extensive time and labor required to isolate, characterize, and expand tumor antigen-specific T cells (Schmitt *et al.*, *Hum. Gene Ther. 20:*1240, 2009). Another hurdle is that most identified tumor antigens that can be targeted by conventional T cell immunotherapy are over-expressed self-proteins, so high affinity T cells specific for these antigens are generally eliminated during thymic selection, and are rare or non-existent in the peripheral repertoire.

Strategies are being developed to enhance the affinity of TCRs intended for use in TCR gene therapy (Udyavar *et al.*, *J. Immunol. 182*:4439, 2009; Zhao *et al.*, *J. Immunol. 179*:5845, 2007; Richman and Kranz, *Biomol. Eng. 24*:361, 2007). These approaches generally entail generating libraries of mutated TCR genes and subsequent screening for mutations that confer higher affinity for the complex of target peptide with major histocompatibility complex (MHC) ligand. Mutations are usually targeted to the complementarity determining regions (CDRs) known to interact with the peptide (CDR3) and/or MHC (CDR1/2) (Wucherpfennig *et al.*, *Cold Spring Harb. Perspect. Biol. 2*:a005140, 2010). But, changes to MHC contact residues may create a risk in the clinical setting since this can increase the affinity for MHC independent of peptide or increase the likelihood of cross-reactivity (off-target effects). This notion has been highlighted by the results of a trial, in which T cells expressing a TCR containing CDR2 mutations were infused into patients and mediated rapid and fatal toxicity from unpredicted cross-reactivity with a disparate self-antigen expressed in the heart (Cameron *et al.*, *Sci. Transl. Med. 5*:197ra103, 2013; Linette *et al.*, *Blood 122*:863, 2013). Certain available methodologies used to target specific CDR residues for amino acid substitution limit the diversity of the generated libraries, as these are generally constrained by the length of the parental CDR sequence. In contrast, the natural process generally produces greater diversity in the thymus, where the V(D)J recombination machinery active during T cell development results in TCR gene rearrangements that generate highly diverse CDRs, particularly CDR3s, that vary in both length and amino acid composition.

A strategy for targeted T-cell therapy achieving a maximal clinical effect that would be accompanied by minimal immunological toxicity involves identifying disease associated antigens with high expression in and presentation by, for example, a malignant cell compartment, but without significant expression in normal tissue. For example, several acute myeloid leukemia (AML) associated antigens have been described, and Wilms tumor protein 1 (WT-1) has been shown to be expressed in the leukemia stem cell (LSC) compartment of the majority of AML patients at levels significantly higher than in physiological hematopoietic stem cells (HSCs). WT-1 is being targeted in clinical trials both with adoptive T-cell transfer and peptide vaccination (*see*, *e.g.*, U.S. Pat. Nos. 7,342,092; 7,608,685; 7,622,119). In addition, WT-1 expression has been reported to be a marker of minimal residual disease because increased transcript levels in patients with AML in morphologic remission were predictive of overt clinical relapse (Inoue *et al.*, *Blood 84*:3071, 1994; Ogawa *et al.*, *Blood 101*:1698, 2003).

Since WT-1 is an intracellular (usually nuclear) protein, immunotherapies targeting WT-1 generally use cellular approaches aimed at generating WT-1-specific CD8+ cytotoxic T lymphocyte (CTL) responses that recognize peptides presented on the cell surface by MHC class I molecules. For induction of a CTL response, intracellular proteins are usually degraded by the proteasome or endo/lysosomes, with the resulting peptide fragments binding to MHC class I or class II molecules. These peptide-MHC complexes are displayed on the cell surface where they are bound by T cells via the peptide-MHC-TCR interaction. Peptides derived from the WT-1 protein can be used in a vaccine in humans to induce human leukocyte antigen (HLA)-restricted cytotoxic CD8+ T cells that are capable of killing tumor cells. However, because WT-1 is a self-protein, such immunization may only elicit responses by T cells with low affinity TCRs. In addition, antibodies against WT-1 are detectable in patients with hematopoietic malignancies and solid tumors, which show that WT-1 can be a highly immunogenic antigen (Gaiger *et al.*, *Clin. Cancer Res. 7 (Suppl. 3)*:761, 2001).

Clearly there is a need for alternative TCR gene therapies for use as highly specific, WT-1 targeted immunotherapies directed against various cancers, such as

leukemia and tumors. Presently disclosed embodiments address this need and provide other related advantages.

BRIEF SUMMARY

The present disclosure provides, according to certain embodiments, a binding protein (*e.g.*, an immunoglobulin superfamily binding protein, TCR or the like) having (a) an α-chain variable (V_α) domain having a CDR1 amino acid sequence shown in SEQ ID NO.:23, a CDR2 amino acid sequence shown in SEQ ID NO.:24 and a CDR3 amino acid sequence shown in any one of SEQ ID NOS.:25, 26, 32, 38, 44, 50 and 51, and/or a β-chain variable (V_β) domain; or (b) a V_α domain of (a) and a V_β domain having a CDR1 amino acid sequence shown in SEQ ID NO.:27, a CDR2 amino acid sequence shown in SEQ ID NO.:28 and/or a CDR3 amino acid sequence shown in SEQ ID NO.:29; wherein the binding protein is capable of binding to a WT1-derived peptide:human leukocyte antigen (HLA) complex with a high affinity, such as a RMFPNAPYL (SEQ ID NO.:16):human leukocyte antigen (HLA) complex, for example, with a K_d less than or equal to about 8 nM.

In certain embodiments the immunoglobulin superfamily binding protein, comprises (a) an α-chain variable (V_α) domain having at least 90% sequence identity to an amino acid sequence as set forth in any one of SEQ ID NO.:1 or 2, and/or a β-chain variable (V_β) domain; or (b) a V_α domain, and a V_β domain having at least 90% sequence identity to an amino acid sequence as set forth in SEQ ID NO.:9; or (c) a V_α domain of (a) and/or a V_β domain of (b); wherein the binding protein is capable of binding to a RMFPNAPYL (SEQ ID NO.:16):HLA complex with a K_d less than or equal to about 5 nM.

In another aspect there is provided a high affinity recombinant T cell receptor (TCR), comprising an α-chain and a β-chain, wherein the α-chain comprises a V_α domain having at least 90% sequence identity to an amino acid sequence as set forth in SEQ ID NO.:1 or 2, wherein the TCR binds to a RMFPNAPYL (SEQ ID NO.:16)::HLA-A*201 complex independent or in the absence of CD8.

In a further aspect there is provided a method for treating a hyperproliferative disorder, comprising administering to human subject in need thereof a composition

comprising any of the aforementioned binding proteins or high affinity recombinant TCRs specific for human Wilms tumor protein 1 (WT-1). In yet another aspect there is provided an adoptive immunotherapy method for treating a condition characterized by WT-1 overexpression in cells of a subject having a hyperproliferative disorder, comprising administering to the subject an effective amount of a recombinant host cell expressing any of the aforementioned binding proteins or high affinity recombinant TCRs.

In certain embodiments the methods provided are for treating a hyperproliferative disorder that is a hematological malignancy or a solid cancer. For example, the hematological malignancy to be treated may be acute lymphoblastic leukemia (ALL), acute myeloid leukemia (AML), chronic myelogenous leukemia (CML), chronic eosinophilic leukemia (CEL), myelodysplastic syndrome (MDS), non-Hodgkin's lymphoma (NHL), or multiple myeloma (MM). Exemplary solid cancer to be treated may be biliary cancer, bladder cancer, bone and soft tissue carcinoma, brain tumor, breast cancer, cervical cancer, colon cancer, colorectal adenocarcinoma, colorectal cancer, desmoid tumor, embryonal cancer, endometrial cancer, esophageal cancer, gastric cancer, gastric adenocarcinoma, glioblastoma multiforme, gynecological tumor, head and neck squamous cell carcinoma, hepatic cancer, lung cancer, malignant melanoma, osteosarcoma, ovarian cancer, pancreatic cancer, pancreatic ductal adenocarcinoma, primary astrocytic tumor, primary thyroid cancer, prostate cancer, renal cancer, renal cell carcinoma, rhabdomyosarcoma, skin cancer, soft tissue sarcoma, testicular germ-cell tumor, urothelial cancer, uterine sarcoma, or uterine cancer.

These and other aspects and embodiments of the herein described invention will be evident upon reference to the following detailed description and attached drawings. All of the U.S. patents, U.S. patent application publications, U.S. patent applications, foreign patents, foreign patent applications and non-patent publications referred to in this specification and/or listed in the Application Data Sheet are incorporated herein by reference in their entirety, as if each was incorporated individually. Aspects and embodiments of the invention can be modified, if necessary, to employ concepts of the various patents, applications and publications to provide yet further embodiments.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows equilibrium binding curves from a titration of WT-1 specific TCRs binding to WT-1 peptide:HLA-A tetramers. T cell clones specific for WT-$1^{126-134}$ (having amino acid sequence RMFPNAPYL, as set forth in SEQ ID NO.:16) were generated from the peripheral repertoire of more than 50 donors, and several candidate high affinity T cell clones were analyzed to determine their relative affinity. Each WT-1-specific T cell clone was stained with WT-$1^{126-134}$ peptide/MHC tetramers ("WT1 tetramers") and analyzed by flow cytometry, and mean fluorescence intensity of tetramer staining was determined using FlowJo software (Treestar). K_D measurements were performed using 2-fold dilutions of PE-conjugated tetramers at a range of concentrations (1–33 nM). Apparent K_D values were determined from binding curves by non-linear regression, as the concentration of ligand that yielded half-maximal binding (Bmax).

Figures 2A and 2B show that assessed high affinity TCR clones could efficiently outcompete endogenous TCR chains and bind WT-$1^{126-134}$ tetramers independently of CD8. Codon-optimized TCRα-P2A-β constructs were generated containing the TCRα and TCRβ chains from the three TCRs generated from the peripheral repertoires having the highest affinity for this WT-1 epitope (C4, P1, P22). (A) These constructs were transduced into PBMCs and the percentage of WT-$1^{126-134}$ tetramer staining cells within the transduced cell population was assessed by flow cytometry, with tetramer staining represented on the Y-axis and the respective transgenic β-chain staining represented on the X-axis. The transduced population was calculated as the total percentage of cells expressing the transgenic TCRβ chain minus the percentage of T cells endogenously expressing that TCRβ chain in an untransduced culture of the same PBMCs. (B) Tetramer binding by the different TCRs in the absence of CD8 was assessed by measuring WT-1 tetramer staining on transduced $CD4^+$ cells (CD8 negative, $CD8^-$) versus $CD8^+$ cells within the transduced population of PBMCs. One of the TCR clones, C4, exhibited the highest degree of tetramer binding on $CD4^+CD8^-$ cells

Figures 3A-3C show a comparison of TCR surface expression for various different C4-derived TCR constructs. Three different C4-derived TCR constructs, each

with a 2A element from the porcine teschovirus (P2A) linking the α and β chains, have the following features: (1) C4α-P2A-β (C4αβWT), (2) a codon-optimized version of C4α-P2A-β (C4αβ CO), and (3) a variant of the codon optimized TCR in which the C4β rather than C4α precedes the P2A element (C4βα CO). (A) Surface expression was detected as a measure of WT-1:HLA-A tetramer binding. (B) Differences in TCR expression between the C4α-P2A-β CO and C4β-P2A-α CO constructs over time were examined and observed to be more apparent towards the end of the T cell's cycle, when endogenous TCRs were expressed at higher levels. (C) Shown is a schematic drawing of a C4 TCRβα construct.

Figures 4A-4C show detection and analysis of an enhanced affinity variant of C4 TCR identified by saturation mutagenesis. (A) PBMCs were transduced to express either the wildtype C4α-P2A-C4β construct or the enhanced affinity C4α-P2A-C4-β(DLT) construct; transduced cells were isolated by cell sorting, expanded, and analyzed for relative WT-$1^{126-134}$ tetramer staining on cells expressing equivalent levels of the introduced TCR (as measured by Vβ17 staining). (B) Sorted PBMCs expressing either the wildtype C4α-P2A-C4β construct or the enhanced affinity C4α-P2A-C4-β(DLT) were stained with WT-1 peptide/MHC tetramer, analyzed by flow cytometry, and mean fluorescence intensity of tetramer staining was determined using FlowJo software (Treestar). K_D measurements were performed using 2-fold dilutions of PE-conjugated tetramers at a range of concentrations. Apparent K_D values were determined from binding curves by non-linear regression, as the concentration of ligand that yielded half-maximal binding. (C) Sorted PBMCs expressing either the wildtype C4α-P2A-C4β construct or the enhanced affinity C4α-P2A-C4-β(DLT) were incubated with ^{51}Chromium loaded target cells pulsed with decreasing concentrations of WT-$1^{126-134}$ peptide as indicated, and specific T cell mediated killing was measured as a percent of maximum chromium release.

Figure 5 shows PBMCs transduced with the C4αβ(DLT) TCR show enhanced killing of tumor cells naturally presenting WT-1 antigen. The HLA-A2 negative K562 tumor cell line or K562 cells transduced to express HLA-A2 were used as target cells for PBMCs expressing either the wildtype C4α-P2A-C4β construct or the enhanced

affinity C4α-P2A-C4-β(DLT) TCR. Tumor killing was determined by measuring cleaved caspase-3 in tumor cells by flow cytometry.

Figures 6A-6C show results from the generation and screening of human agonist-selected TCRβ libraries. CD34+ HPCs were purified from umbilical cord blood, lentivirally transduced with either C4α-IRES-GFP or C4αβ and co-cultured with the OP9-A2-DL1 cell line in the presence of 1 μg/mL WT-1 peptide. (A) Cultures were analyzed on day 31 for expression of CD3 and CD27. (B) On day 34, cultures were analyzed for expression of CD27 and Vβ17, and about 2.5 x 10^5 Vβ17+CD27+ cells were sorted for TCRβ library generation. (C) Vβ17-Cβ1 and Vβ17-Cβ2 libraries were generated, transduced into the H9.CD8–C4α cell line, followed by sorting for transduced C4α-GFP+ Vβ17+ cells. Cells were then sorted twice with WT-1-tetramer and analyzed for tetramer and Vβ17 staining by flow cytometry.

Figure 7 shows the persistence in 9 leukemia patients of donor-derived virus-specific CD8 T cells transduced to express the C4βα TCR construct . Donor T cells were stimulated with a peptide from either EBV or CMV to specifically activate a population of virus-specific T cells with a central memory phenotype, and to target lentiviral transduction preferentially to these activated and, thus, rapidly dividing cells. Cells were transduced with the C4βα TCR construct and sorted for T cells that stained positive for both HLA-A2/WT-1 and HLA-A2/viral peptide-specific tetramers by flow cytometric cell sorting. Sorted cells were expanded and infused into leukemia patients at the time-points indicated with a down-facing arrow.

Figures 8A-8C show results demonstrating cell surface stability of functional WT-1-specific TCR. (A) Donor PBMCs were stimulated with dendritic cells (DCs) presenting EBV peptide GLCTLVAML (SEQ ID NO.:127), transduced with the C4 TCR construct 24 hours later, and sorted on WT_1 tetramer$^+$, EBV tetramer$^+$, or double positive populations on day 12 as indicated. (B) Sorted populations were analyzed immediately post-sorting, or following 12 days of additional *in vitro* culture. (C) Donor PBMCs were stimulated with DCs presenting EBV peptide GLCTLVAML (SEQ ID NO.:127) and then sorted for EBV tetramer$^+$ T cells after 12 days of culture. Sorted cells were then re-stimulated with or without C4 TCR transduction on day 1 post stimulation and analyzed by flow cytometry on day 6.

DETAILED DESCRIPTION

In one aspect, the present disclosure provides T cell receptors (TCRs) having high affinity for WT-1 peptide antigen associated with a major histocompatibility complex (MHC) (*e.g.*, human leukocyte antigen, HLA) for use in, for example, adoptive immunotherapy to treat cancer. By way of background, most tumor targets for T cell-based immunotherapies are self-antigens since tumors arise from previously normal tissue. For example, such tumor-associated antigens (TAAs) may be expressed at high levels in a cancer cell, but may not be expressed or may be minimally expressed in other cells. During T cell development in the thymus, T cells that bind weakly to self-antigens are allowed to survive in the thymus, which can undergo further development to increase specificity against foreign invaders, while T cells that bind strongly to self-antigens are eliminated by the immune system since such cells would mount an undesirable autoimmune response. Hence, T cells are sorted by their relative ability to bind to antigens to prepare the immune system to respond against a foreign invader (*i.e.*, recognition of non-self-antigen) while at the same time preventing an autoimmune response (*i.e.*, recognition of self-antigen). This tolerance mechanism limits naturally occurring T cells that can recognize tumor (self) antigens with high affinity and, therefore, eliminates the T cells that would effectively eliminate tumor cells. Consequently, isolating T cells having high affinity TCRs specific for tumor antigens is difficult because such cells are essentially eliminated by the immune system.

An advantage of the instant disclosure is to provide a high affinity or an enhanced affinity TCR specific for a WT-1 peptide, wherein a cell expressing such a TCR is capable of binding to a WT-1::HLA complex independent or in the absence of CD8, is capable of more efficiently associating with a CD3 protein as compared to endogenous TCR, or both. In certain embodiments, an enhanced affinity TCR specific for a WT-1 peptide comprises a T cell receptor (TCR) α-chain as set forth in SEQ ID NO.:7 or 8, and a TCR β-chain variable (V_β) domain as set forth in SEQ ID NO.:12 or 13, wherein the enhanced affinity TCR is capable of binding to a RMFPNAPYL (SEQ ID NO.:16):HLA complex with a K_d less than or equal to about 3 nM, or wherein the enhanced affinity TCR dissociates from a RMFPNAPYL (SEQ ID NO.:16):HLA

complex at a reduced k_{off} rate as compared to a TCR composed of an α-chain of SEQ ID NO.:5 or 6 and a β-chain of SEQ ID NO.:12 or 13.

The compositions and methods described herein will in certain embodiments have therapeutic utility for the treatment of diseases and conditions associated with WT-1 overexpression (*e.g.,* detectable WT-1 expression at a level that is greater in magnitude, in a statistically significant manner, than the level of WT-1 expression that is detectable in a normal or disease-free cell). Such diseases include various forms of hyperproliferative disorders, such as hematological malignancies and solid cancers. Non-limiting examples of these and related uses are described herein and include *in vitro*, *ex vivo* and *in vivo* stimulation of WT-1 antigen-specific T-cell responses, such as by the use of recombinant T cells expressing an enhanced affinity TCR specific for a WT-1 peptide (*e.g.*, RMFPNAPYL, SEQ ID NO.:16).

Prior to setting forth this disclosure in more detail, it may be helpful to an understanding thereof to provide definitions of certain terms to be used herein. Additional definitions are set forth throughout this disclosure.

In the present description, any concentration range, percentage range, ratio range, or integer range is to be understood to include the value of any integer within the recited range and, when appropriate, fractions thereof (such as one tenth and one hundredth of an integer), unless otherwise indicated. Also, any number range recited herein relating to any physical feature, such as polymer subunits, size or thickness, are to be understood to include any integer within the recited range, unless otherwise indicated. As used herein, the term "about" means ± 20% of the indicated range, value, or structure, unless otherwise indicated. It should be understood that the terms "a" and "an" as used herein refer to "one or more" of the enumerated components. The use of the alternative (*e.g.*, "or") should be understood to mean either one, both, or any combination thereof of the alternatives. As used herein, the terms "include," "have" and "comprise" are used synonymously, which terms and variants thereof are intended to be construed as non-limiting.

In addition, it should be understood that the individual compounds, or groups of compounds, derived from the various combinations of the structures and substituents described herein, are disclosed by the present application to the same extent as if each

compound or group of compounds was set forth individually. Thus, selection of particular structures or particular substituents is within the scope of the present disclosure.

The term "consisting essentially of" limits the scope of a claim to the specified materials or steps, or to those that do not materially affect the basic characteristics of a claimed invention. For example, a protein domain, region, or module (*e.g.*, a binding domain, hinge region, linker module) or a protein (which may have one or more domains, regions, or modules) "consists essentially of" a particular amino acid sequence when the amino acid sequence of a domain, region, module, or protein includes extensions, deletions, mutations, or a combination thereof (*e.g.*, amino acids at the amino- or carboxy-terminus or between domains) that, in combination, contribute to at most 20% (*e.g.*, at most 15%, 10%, 8%, 6%, 5%, 4%, 3%, 2% or 1%) of the length of a domain, region, module, or protein and do not substantially affect (*i.e.*, do not reduce the activity by more than 50%, such as no more than 40%, 30%, 25%, 20%, 15%, 10%, 5%, or 1%) the activity of the domain(s), region(s), module(s), or protein (*e.g.*, the target binding affinity of a binding protein).

As used herein, an "immune system cell" means any cell of the immune system that originates from a hematopoietic stem cell in the bone marrow, which gives rise to two major lineages, a myeloid progenitor cell (which give rise to myeloid cells such as monocytes, macrophages, dendritic cells, meagakaryocytes and granulocytes) and a lymphoid progenitor cell (which give rise to lymphoid cells such as T cells, B cells and natural killer (NK) cells). Exemplary immune system cells include a CD4+ T cell, a CD8+ T cell, a CD4- CD8- double negative T cell, a γδ T cell, a regulatory T cell, a natural killer cell, and a dendritic cell. Macrophages and dendritic cells may be referred to as "antigen presenting cells" or "APCs," which are specialized cells that can activate T cells when a major histocompatibility complex (MHC) receptor on the surface of the APC complexed with a peptide interacts with a TCR on the surface of a T cell.

"Major histocompatibility complex" (MHC) refers to glycoproteins that deliver peptide antigens to a cell surface. MHC class I molecules are heterodimers having a membrane spanning α chain (with three α domains) and a non-covalently associated β2 microglobulin. MHC class II molecules are composed of two transmembrane

glycoproteins, α and β, both of which span the membrane. Each chain has two domains. MHC class I molecules deliver peptides originating in the cytosol to the cell surface, where a peptide:MHC complex is recognized by $CD8^+$ T cells. MHC class II molecules deliver peptides originating in the vesicular system to the cell surface, where they are recognized by $CD4^+$ T cells. Human MHC is referred to as human leukocyte antigen (HLA).

A "T cell" is an immune system cell that matures in the thymus and produces T cell receptors (TCRs). T cells can be naïve (not exposed to antigen; increased expression of CD62L, CCR7, CD28, CD3, CD127, and CD45RA, and decreased expression of CD45RO as compared to T_{CM}), memory T cells (T_M) (antigen-experienced and long-lived), and effector cells (antigen-experienced, cytotoxic). T_M can be further divided into subsets of central memory T cells (T_{CM}, increased expression of CD62L, CCR7, CD28, CD127, CD45RO, and CD95, and decreased expression of CD54RA as compared to naïve T cells) and effector memory T cells (T_{EM}, decreased expression of CD62L, CCR7, CD28, CD45RA, and increased expression of CD127 as compared to naïve T cells or T_{CM}). Effector T cells (T_E) refers to antigen-experienced CD8+ cytotoxic T lymphocytes that have decreased expression of CD62L ,CCR7, CD28, and are positive for granzyme and perforin as compared to T_{CM}. Other exemplary T cells include regulatory T cells, such as CD4+ CD25+ (Foxp3+) regulatory T cells and Treg17 cells, as well as Tr1, Th3, CD8+CD28-, and Qa-1 restricted T cells.

"T cell receptor" (TCR) refers to an immunoglobulin superfamily member (having a variable binding domain, a constant domain, a transmembrane region, and a short cytoplasmic tail; *see, e.g.*, Janeway *et al.*, *Immunobiology: The Immune System in Health and Disease*, 3rd Ed., Current Biology Publications, p. 4:33, 1997) capable of specifically binding to an antigen peptide bound to a MHC receptor. A TCR can be found on the surface of a cell or in soluble form and generally is comprised of a heterodimer having α and β chains (also known as TCRα and TCRβ, respectively), or γ and δ chains (also known as TCRγ and TCRδ, respectively). Like immunoglobulins, the extracellular portion of TCR chains (*e.g.*, α-chain, β-chain) contain two immunoglobulin domains, a variable domain (*e.g.*, α-chain variable domain or V_α, β-

chain variable domain or V_β; typically amino acids 1 to 116 based on Kabat numbering Kabat *et al.*, "Sequences of Proteins of Immunological Interest, US Dept. Health and Human Services, Public Health Service National Institutes of Health, 1991, 5th ed.) at the N-terminus, and one constant domain (*e.g.*, α-chain constant domain or C_α, typically amino acids 117 to 259 based on Kabat, β-chain constant domain or C_β, typically amino acids 117 to 295 based on Kabat) adjacent to the cell membrane. Also like immunoglobulins, the variable domains contain complementary determining regions (CDRs) separated by framework regions (FRs) (*see*, *e.g.*, Jores *et al.*, *Proc. Nat'l Acad. Sci. U.S.A. 87*:9138, 1990; Chothia *et al.*, *EMBO J. 7*:3745, 1988; *see also* Lefranc *et al.*, *Dev. Comp. Immunol. 27*:55, 2003). In certain embodiments, a TCR is found on the surface of T cells (or T lymphocytes) and associates with the CD3 complex. The source of a TCR as used in the present disclosure may be from various animal species, such as a human, mouse, rat, rabbit or other mammal.

"CD3" is known in the art as a multi-protein complex of six chains (*see*, Abbas and Lichtman, 2003; Janeway *et al.*, p172 and 178, 1999). In mammals, the complex comprises a CD3γ chain, a CD3δ chain, two CD3ε chains, and a homodimer of CD3ζ chains. The CD3γ, CD3δ, and CD3ε chains are highly related cell surface proteins of the immunoglobulin superfamily containing a single immunoglobulin domain. The transmembrane regions of the CD3γ, CD3δ, and CD3ε chains are negatively charged, which is a characteristic that allows these chains to associate with the positively charged T cell receptor chains. The intracellular tails of the CD3γ, CD3δ, and CD3ε chains each contain a single conserved motif known as an immunoreceptor tyrosine-based activation motif or ITAM, whereas each CD3ζ chain has three. Without wishing to be bound by theory, it is believed the ITAMs are important for the signaling capacity of a TCR compelx. CD3 as used in the present disclosure may be from various animal species, including human, mouse, rat, or other mammals.

As used herein, "TCR complex" refers to a complex formed by the association of CD3 with TCR. For example, a TCR complex can be composed of a CD3γ chain, a CD3δ chain, two CD3ε chains, a homodimer of CD3ζ chains, a TCRα chain, and a TCRβ chain. Alternatively, a TCR complex can be composed of a CD3γ chain, a CD3δ

chain, two CD3ε chains, a homodimer of CD3ζ chains, a TCRγ chain, and a TCRδ chain.

A "component of a TCR complex," as used herein, refers to a TCR chain (*i.e.*, TCRα, TCRβ, TCRγ or TCRδ), a CD3 chain (*i.e.*, CD3γ, CD3δ, CD3ε or CD3ζ), or a complex formed by two or more TCR chains or CD3 chains (*e.g.*, a complex of TCRα and TCRβ, a complex of TCRγ and TCRδ, a complex of CD3ε and CD3δ, a complex of CD3γ and CD3ε, or a sub-TCR complex of TCRα, TCRβ, CD3γ, CD3δ, and two CD3ε chains).

A "binding domain" (also referred to as a "binding region" or "binding moiety"), as used herein, refers to a molecule or portion thereof (*e.g.*, peptide, oligopeptide, polypeptide, protein) that possesses the ability to specifically and non-covalently associate, unite, or combine with a target (*e.g.*, WT-1 or WT-1 peptide:MHC complex). A binding domain includes any naturally occurring, synthetic, semi-synthetic, or recombinantly produced binding partner for a biological molecule, a molecular complex (*i.e.*, complex comprising two or more biological molecules), or other target of interest. Exemplary binding domains include single chain immunoglobulin variable regions (*e.g.*, scTCR, scFv), receptor ectodomains, ligands (*e.g.*, cytokines, chemokines), or synthetic polypeptides selected for their specific ability to bind to a biological molecule, a molecular complex or other target of interest.

As used herein, "specifically binds" or "specific for" refers to an association or union of a binding protein (*e.g.*, TCR receptor) or a binding domain (or fusion protein thereof) to a target molecule with an affinity or K_a (*i.e.*, an equilibrium association constant of a particular binding interaction with units of 1/M) equal to or greater than 10^5 M^{-1} (which equals the ratio of the on-rate [k_{on}] to the off-rate [k_{off}] for this association reaction), while not significantly associating or uniting with any other molecules or components in a sample. Binding proteins or binding domains (or fusion proteins thereof) may be classified as "high affinity" binding proteins or binding domains (or fusion proteins thereof) or as "low affinity" binding proteins or binding domains (or fusion proteins thereof). "High affinity" binding proteins or binding domains refer to those binding proteins or binding domains having a K_a of at least 10^7 M^{-1}, at least 10^8 M^{-1}, at least 10^9 M^{-1}, at least 10^{10} M^{-1}, at least 10^{11} M^{-1}, at least 10^{12} M^{-}

^{1}, or at least 10^{13} M^{-1}. "Low affinity" binding proteins or binding domains refer to those binding proteins or binding domains having a K_a of up to 10^{7} M^{-1}, up to 10^{6} M^{-1}, up to 10^{5} M^{-1}. Alternatively, affinity may be defined as an equilibrium dissociation constant (K_d) of a particular binding interaction with units of M (*e.g.*, 10^{-5} M to 10^{-13} M).

In certain embodiments, a receptor or binding domain may have "enhanced affinity," which refers to selected or engineered receptors or binding domains with stronger binding to a target antigen than a wild type (or parent) binding domain. For example, enhanced affinity may be due to a K_a (equilibrium association constant) for the target antigen that is higher than the wild type binding domain, due to a K_d (dissociation constant) for the target antigen that is less than that of the wild type binding domain, due to an off-rate (k_{off}) for the target antigen that is less than that of the wild type binding domain, or a combination thereof. In certain embodiments, enhanced affinity TCRs may be codon optimized to enhance expression in a particular host cell, such as T cells (Scholten *et al.*, *Clin. Immunol. 119*:135, 2006).

A variety of assays are known for identifying binding domains of the present disclosure that specifically bind a particular target, as well as determining binding domain or fusion protein affinities, such as Western blot, ELISA, analytical ultracentrifugation, spectroscopy and surface plasmon resonance (Biacore®) analysis (*see*, *e.g.*, Scatchard *et al.*, *Ann. N.Y. Acad. Sci. 51*:660, 1949; Wilson, *Science 295*:2103, 2002; Wolff *et al.*, *Cancer Res. 53*:2560, 1993; and U.S. Patent Nos. 5,283,173, 5,468,614, or the equivalent).

The term "WT-1-specific binding protein" refers to a protein or polypeptide that specifically binds to WT-1 or peptide thereof. In some embodiments, a protein or polypeptide binds to WT-1 or a peptide thereof, such as a WT-1 peptide in complexed with an MHC or HLA molecule, *e.g.*, on a cell surface, with at or at least about a particular affinity. In certain embodiments, a WT-1-specific binding protein binds a WT-1-derived peptide:HLA complex (or WT-1-derived peptide:MHC complex) with a K_d of less than about 10^{-8} M, less than about 10^{-9} M, less than about 10^{-10} M, less than about 10^{-11} M, less than about 10^{-12} M, or less than about 10^{-13} M, or with an affinity that is about the same as, at least about the same as, or is greater than at or about the affinity exhibited by an exemplary WT-1 specific binding protein provided herein, such

as any of the WT-1-specific TCRs provided herein, for example, as measured by the same assay. Assays for assessing affinity or apparent affinity or relative affinity are known. In certain examples, apparent affinity for a TCR is measured by assessing binding to various concentrations of tetramers, for example, by flow cytometry using labeled tetramers. In some examples, apparent K_D of a TCR is measured using 2-fold dilutions of labeled tetramers at a range of concentrations, followed by determination of binding curves by non-linear regression, apparent K_D being determined as the concentration of ligand that yielded half-maximal binding. In certain embodiments, a WT-1-specific binding protein comprises a WT-1-specific immunoglobulin superfamily binding protein or binding portion thereof.

The term "WT-1 binding domain" or "WT-1 binding fragment" refer to a domain or portion of a WT-1-specific binding protein responsible for the specific WT-1 binding. A WT-1-specific binding domain alone (*i.e.*, without any other portion of a WT-1-specific binding protein) can be soluble and can bind to WT-1 with a K_d of less than about 10^{-8} M, less than about 10^{-9} M, less than about 10^{-10} M, less than about 10^{-11} M, less than about 10^{-12} M, or less than about 10^{-13} M. Exemplary WT-1-specific binding domains include WT-1-specific scTCR (*e.g.*, single chain αβTCR proteins such as Vα-L-Vβ, Vβ-L-Vα, Vα-Cα-L-Vα, or Vα-L-Vβ-Cβ, wherein Vα and Vβ are TCRα and β variable domains respectively, Cα and Cβ are TCRα and β constant domains, respectively, and L is a linker) and scFv fragments as described herein, which can be derived from an anti-WT-1 TCR or antibody.

"WT-1 antigen" or "WT-1 peptide antigen" refer to a naturally or synthetically produced portion of a WT-1 protein ranging in length from about 7 amino acids to about 15 amino acids, which can form a complex with a MHC (*e.g.*, HLA) molecule and such a complex can bind with a TCR specific for a WT-1 peptide:MHC (*e.g.*, HLA) complex. Principles of antigen processing by antigen presenting cells (APC) (such as dendritic cells, macrophages, lymphocytes or other cell types), and of antigen presentation by APC to T cells, including major histocompatibility complex (MHC)-restricted presentation between immunocompatible (*e.g.,* sharing at least one allelic form of an MHC gene that is relevant for antigen presentation) APC and T cells, are well established (*see, e.g.,* Murphy, Janeway's Immunobiology (8^{th} Ed.) 2011 Garland

Science, NY; chapters 6, 9 and 16). For example, processed antigen peptides originating in the cytosol (*e.g.*, tumor antigen, intrcellular pathogen) are generally from about 7 amino acids to about 11 amino acids in length and will associate with class I MHC molecules, whereas peptides processed in the vesicular system (*e.g.*, bacterial, viral) will vary in length from about 10 amino acids to about 25 amino acids and associate with class II MHC molecules. Since WT-1 is an internal host protein, WT-1 antigen peptides will be presented in the context of class I MHC. In particular embodiments, a WT-1 peptide is RMFPNAPYL (SEQ ID NO.:16), which is known to associate with human class I HLA (and, more specifically, associates with allele HLA-A*201).

A "linker" refers to an amino acid sequence that connects two proteins, polypeptides, peptides, domains, regions, or motifs and may provide a spacer function compatible with interaction of the two sub-binding domains so that the resulting polypeptide retains a specific binding affinity (*e.g.*, scTCR) to a target molecule or retains signaling activity (*e.g.*, TCR complex). In certain embodiments, a linker is comprised of about two to about 35 amino acids, for instance, or about four to about 20 amino acids or about eight to about 15 amino acids or about 15 to about 25 amino acids.

"Junction amino acids" or "junction amino acid residues" refer to one or more (*e.g.*, about 2-10) amino acid residues between two adjacent motifs, regions or domains of a polypeptide, such as between a binding domain and an adjacent constant domain or between a TCR chain and an adjacent self-cleaving peptide. Junction amino acids may result from the construct design of a fusion protein (e.g., amino acid residues resulting from the use of a restriction enzyme site during the construction of a nucleic acid molecule encoding a fusion protein).

An "altered domain" or "altered protein" refers to a motif, region, domain, peptide, polypeptide, or protein with a non-identical sequence identity to a wild type motif, region, domain, peptide, polypeptide, or protein (*e.g.*, a wild type TCRα chain, TCRβ chain, TCRα constant domain, TCRβ constant domain) of at least 85% (*e.g.*, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.1%, 99.2%, 99.3%, 99.4%, 99.5%, 99.6%, 99.7%, 99.8%, 99.9%).

As used herein, "nucleic acid" or "nucleic acid molecule" refers to any of deoxyribonucleic acid (DNA), ribonucleic acid (RNA), oligonucleotides, fragments generated, for example, by the polymerase chain reaction (PCR) or by *in vitro* translation, and fragments generated by any of ligation, scission, endonuclease action, or exonuclease action. In certain embodiments, the nucleic acids of the present disclosure are produced by PCR. Nucleic acids may be composed of monomers that are naturally occurring nucleotides (such as deoxyribonucleotides and ribonucleotides), analogs of naturally occurring nucleotides (*e.g.,* α-enantiomeric forms of naturally-occurring nucleotides), or a combination of both. Modified nucleotides can have modifications in or replacement of sugar moieties, or pyrimidine or purine base moieties. Nucleic acid monomers can be linked by phosphodiester bonds or analogs of such linkages. Analogs of phosphodiester linkages include phosphorothioate, phosphorodithioate, phosphoroselenoate, phosphorodiselenoate, phosphoroanilothioate, phosphoranilidate, phosphoramidate, and the like. Nucleic acid molecules can be either single stranded or double stranded.

The term "isolated" means that the material is removed from its original environment (*e.g.*, the natural environment if it is naturally occurring). For example, a naturally occurring nucleic acid or polypeptide present in a living animal is not isolated, but the same nucleic acid or polypeptide, separated from some or all of the co-existing materials in the natural system, is isolated. Such nucleic acid could be part of a vector and/or such nucleic acid or polypeptide could be part of a composition (*e.g.,* a cell lysate), and still be isolated in that such vector or composition is not part of the natural environment for the nucleic acid or polypeptide. The term "gene" means the segment of DNA involved in producing a polypeptide chain; it includes regions preceding and following the coding region "leader and trailer" as well as intervening sequences (introns) between individual coding segments (exons).

As used herein, the term "recombinant" refers to a cell, microorganism, nucleic acid molecule, or vector that has been modified by introduction of an exogenous nucleic acid molecule, or refers to a cell or microorganism that has been altered such that expression of an endogenous nucleic acid molecule or gene is controlled, deregulated or constitutive, where such alterations or modifications may be introduced by genetic

engineering. Genetic alterations may include, for example, modifications introducing nucleic acid molecules (which may include an expression control element, such as a promoter) encoding one or more proteins or enzymes, or other nucleic acid molecule additions, deletions, substitutions, or other functional disruption of or addition to a cell's genetic material. Exemplary modifications include those in coding regions or functional fragments thereof of heterologous or homologous polypeptides from a reference or parent molecule.

As used herein, "mutation" refers to a change in the sequence of a nucleic acid molecule or polypeptide molecule as compared to a reference or wild-type nucleic acid molecule or polypeptide molecule, respectively. A mutation can result in several different types of change in sequence, including substitution, insertion or deletion of nucleotide(s) or amino acid(s). In certain embodiments, a mutation is a substitution of one or three codons or amino acids, a deletion of one to about 5 codons or amino acids, or a combination thereof.

A "conservative substitution" is recognized in the art as a substitution of one amino acid for another amino acid that has similar properties. Exemplary conservative substitutions are well known in the art (*see*, *e.g.*, WO 97/09433 at page 10; Lehninger, Biochemistry, 2nd Edition; Worth Publishers, Inc. NY, NY, pp.71-77, 1975; Lewin, Genes IV, Oxford University Press, NY and Cell Press, Cambridge, MA, p. 8, 1990).

The term "construct" refers to any polynucleotide that contains a recombinant nucleic acid molecule. A construct may be present in a vector (*e.g.*, a bacterial vector, a viral vector) or may be integrated into a genome. A "vector" is a nucleic acid molecule that is capable of transporting another nucleic acid molecule. Vectors may be, for example, plasmids, cosmids, viruses, a RNA vector or a linear or circular DNA or RNA molecule that may include chromosomal, non-chromosomal, semi-synthetic or synthetic nucleic acid molecules. Exemplary vectors are those capable of autonomous replication (episomal vector) or expression of nucleic acid molecules to which they are linked (expression vectors).

Viral vectors include retrovirus, adenovirus, parvovirus (*e.g.*, adeno-associated viruses), coronavirus, negative strand RNA viruses such as ortho-myxovirus (*e.g.*, influenza virus), rhabdovirus (*e.g.*, rabies and vesicular stomatitis virus), paramyxovirus

(*e.g.*, measles and Sendai), positive strand RNA viruses such as picornavirus and alphavirus, and double-stranded DNA viruses including adenovirus, herpesvirus (*e.g.*, Herpes Simplex virus types 1 and 2, Epstein-Barr virus, cytomegalovirus), and poxvirus (*e.g.*, vaccinia, fowlpox and canarypox). Other viruses include Norwalk virus, togavirus, flavivirus, reoviruses, papovavirus, hepadnavirus, and hepatitis virus, for example. Examples of retroviruses include avian leukosis-sarcoma, mammalian C-type, B-type viruses, D type viruses, HTLV-BLV group, lentivirus, spumavirus (Coffin, J. M., Retroviridae: The viruses and their replication, In Fundamental Virology, Third Edition, B. N. Fields *et al.*, Eds., Lippincott-Raven Publishers, Philadelphia, 1996).

"Lentiviral vector," as used herein, means HIV-based lentiviral vectors for gene delivery, which can be integrative or non-integrative, have relatively large packaging capacity, and can transduce a range of different cell types. Lentiviral vectors are usually generated following transient transfection of three (packaging, envelope and transfer) or more plasmids into producer cells. Like HIV, lentiviral vectors enter the target cell through the interaction of viral surface glycoproteins with receptors on the cell surface. On entry, the viral RNA undergoes reverse transcription, which is mediated by the viral reverse transcriptase complex. The product of reverse transcription is a double-stranded linear viral DNA, which is the substrate for viral integration into the DNA of infected cells.

The term "operably-linked" refers to the association of two or more nucleic acid molecules on a single nucleic acid fragment so that the function of one is affected by the other. For example, a promoter is operably-linked with a coding sequence when it is capable of affecting the expression of that coding sequence (*i.e.*, the coding sequence is under the transcriptional control of the promoter). "Unlinked" means that the associated genetic elements are not closely associated with one another and the function of one does not affect the other.

As used herein, "expression vector" refers to a DNA construct containing a nucleic acid molecule that is operably-linked to a suitable control sequence capable of effecting the expression of the nucleic acid molecule in a suitable host. Such control sequences include a promoter to effect transcription, an optional operator sequence to control such transcription, a sequence encoding suitable mRNA ribosome binding sites,

and sequences which control termination of transcription and translation. The vector may be a plasmid, a phage particle, a virus, or simply a potential genomic insert. Once transformed into a suitable host, the vector may replicate and function independently of the host genome, or may, in some instances, integrate into the genome itself. In the present specification, "plasmid," "expression plasmid," "virus" and "vector" are often used interchangeably.

The term "expression", as used herein, refers to the process by which a polypeptide is produced based on the encoding sequence of a nucleic acid molecule, such as a gene. The process may include transcription, post-transcriptional control, post-transcriptional modification, translation, post-translational control, post-translational modification, or any combination thereof.

The term "introduced" in the context of inserting a nucleic acid molecule into a cell, means "transfection", or 'transformation" or "transduction" and includes reference to the incorporation of a nucleic acid molecule into a eukaryotic or prokaryotic cell wherein the nucleic acid molecule may be incorporated into the genome of a cell (*e.g.*, chromosome, plasmid, plastid, or mitochondrial DNA), converted into an autonomous replicon, or transiently expressed (*e.g.*, transfected mRNA).

As used herein, "heterologous" or "exogenous" nucleic acid molecule, construct or sequence refers to a nucleic acid molecule or portion of a nucleic acid molecule that is not native to a host cell, but may be homologous to a nucleic acid molecule or portion of a nucleic acid molecule from the host cell. The source of the heterologous or exogenous nucleic acid molecule, construct or sequence may be from a different genus or species. In certain embodiments, a heterologous or exogenous nucleic acid molecule is added (*i.e.*, not endogenous or native) to a host cell or host genome by, for example, conjugation, transformation, transfection, electroporation, or the like, wherein the added molecule may integrate into the host genome or exist as extra-chromosomal genetic material (*e.g.*, as a plasmid or other form of self-replicating vector), and may be present in multiple copies. In addition, "heterologous" refers to a non-native enzyme, protein or other activity encoded by an exogenous nucleic acid molecule introduced into the host cell, even if the host cell encodes a homologous protein or activity.

As described herein, more than one heterologous or exogenous nucleic acid molecule can be introduced into a host cell as separate nucleic acid molecules, as a plurality of individually controlled genes, as a polycistronic nucleic acid molecule, as a single nucleic acid molecule encoding a fusion protein, or any combination thereof. For example, as disclosed herein, a host cell can be modified to express two or more heterologous or exogenous nucleic acid molecules encoding desired TCR specific for a WT-1 antigen peptide (*e.g.*, TCRα and TCRβ). When two or more exogenous nucleic acid molecules are introduced into a host cell, it is understood that the two or more exogenous nucleic acid molecules can be introduced as a single nucleic acid molecule (*e.g.*, on a single vector), on separate vectors, integrated into the host chromosome at a single site or multiple sites, or any combination thereof. The number of referenced heterologous nucleic acid molecules or protein activities refers to the number of encoding nucleic acid molecules or the number of protein activities, not the number of separate nucleic acid molecules introduced into a host cell.

As used herein, the term "endogenous" or "native" refers to a gene, protein, or activity that is normally present in a host cell. Moreover, a gene, protein or activity that is mutated, overexpressed, shuffled, duplicated or otherwise altered as compared to a parent gene, protein or activity is still considered to be endogenous or native to that particular host cell. For example, an endogenous control sequence from a first gene (*e.g.*, promoter, translational attenuation sequences) may be used to alter or regulate expression of a second native gene or nucleic acid molecule, wherein the expression or regulation of the second native gene or nucleic acid molecule differs from normal expression or regulation in a parent cell.

The term "homologous" or "homolog" refers to a molecule or activity found in or derived from a host cell, species or strain. For example, a heterologous or exogenous nucleic acid molecule may be homologous to a native host cell gene, and may optionally have an altered expression level, a different sequence, an altered activity, or any combination thereof.

"Sequence identity," as used herein, refers to the percentage of amino acid residues in one sequence that are identical with the amino acid residues in another reference polypeptide sequence after aligning the sequences and introducing gaps, if

necessary, to achieve the maximum percent sequence identity, and not considering any conservative substitutions as part of the sequence identity. The percentage sequence identity values can be generated using the NCBI BLAST2.0 software as defined by Altschul *et al.* (1997) "Gapped BLAST and PSI-BLAST: a new generation of protein database search programs", Nucleic Acids Res. 25:3389-3402, with the parameters set to default values.

As used herein, a "hematopoietic progenitor cell" is a cell that can be derived from hematopoietic stem cells or fetal tissue and is capable of further differentiation into mature cells types (*e.g.*, immune system cells). Exemplary hematopoietic progenitor cells include those with a $CD24^{Lo}$ Lin^{-} $CD117^{+}$ phenotype or those found in the thymus (referred to as progenitor thymocytes).

As used herein, the term "host" refers to a cell (*e.g.*, T cell) or microorganism targeted for genetic modification with a heterologous or exogenous nucleic acid molecule to produce a polypeptide of interest (*e.g.*, high or enhanced affinity anti-WT-1 TCR). In certain embodiments, a host cell may optionally already possess or be modified to include other genetic modifications that confer desired properties related or unrelated to biosynthesis of the heterologous or exogenous protein (*e.g.*, inclusion of a detectable marker; deleted, altered or truncated endogenous TCR; increased co-stimulatory factor expression). In certain embodiments, a host cell is a human hematopoietic progenitor cell transduced with a heterologous or exogenous nucleic acid molecule encoding a TCRα chain specific for a WT-1 antigen peptide.

As used herein, "hyperproliferative disorder" refers to excessive growth or proliferation as compared to a normal or undiseased cell. Exemplary hyperproliferative disorders include tumors, cancers, neoplastic tissue, carcinoma, sarcoma, malignant cells, pre-malignant cells, as well as non-neoplastic or non-malignant hyperproliferative disorders (*e.g.*, adenoma, fibroma, lipoma, leiomyoma, hemangioma, fibrosis, restenosis, as well as autoimmune diseases such as rheumatoid arthritis, osteoarthritis, psoriasis, inflammatory bowel disease, or the like).

Binding Proteins Specific for WT-1 Antigen Peptides

In certain aspects, the instant disclosure provides a binding protein (*e.g.*, an immunoglobulin superfamily binding protein or portion thereof), comprising (a) a T cell

receptor (TCR) α-chain variable (V_α) domain having a CDR1 amino acid sequence shown in SEQ ID NO.:23, a CDR2 amino acid sequence shown in SEQ ID NO.:24 and a CDR3 amino acid sequence shown in any one of SEQ ID NOS.:25, 26, 32, 38, 44, 50 and 51, and a TCR β-chain variable (V_β) domain; or (b) a V_α domain of (a) and a V_β domain having a CDR1 amino acid sequence shown in SEQ ID NO.:27, a CDR2 amino acid sequence shown in SEQ ID NO.:28 and a CDR3 amino acid sequence shown in SEQ ID NO.:29. Such a binding protein is capable of binding with a high affinity to a WT1-derived peptide:human leukocyte antigen (HLA) complex. In particular embodiments, the binding protein binds to a RMFPNAPYL (SEQ ID NO.:16):human leukocyte antigen (HLA) complex with a K_d less than or equal to about 8 nM.

In certain embodiments, a binding protein (*e.g.*, an immunoglobulin superfamily binding protein or portion thereof) or high affinity recombinant T cell receptor (TCR) specific for WT-1 as described herein includes variant polypeptide species that have one or more amino acid substitutions, insertions, or deletions in the amino acid sequence relative to the sequences of SEQ ID NOS:1-15, 21 and 22 as presented herein, provided that the binding protein retains or substantially retains its specific binding function. Conservative substitutions of amino acids are well known and may occur naturally or may be introduced when the binding protein or TCR is recombinantly produced. Amino acid substitutions, deletions, and additions may be introduced into a protein using mutagenesis methods known in the art (*see, e.g.,* Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual*, 3d ed., Cold Spring Harbor Laboratory Press, NY, 2001). Oligonucleotide-directed site-specific (or segment specific) mutagenesis procedures may be employed to provide an altered polynucleotide that has particular codons altered according to the substitution, deletion, or insertion desired. Alternatively, random or saturation mutagenesis techniques, such as alanine scanning mutagenesis, error prone polymerase chain reaction mutagenesis, and oligonucleotide-directed mutagenesis may be used to prepare immunogen polypeptide variants (*see, e.g.,* Sambrook *et al.*, *supra*).

Species (or variants) of a particular immunoglobulin superfamily binding protein or high affinity recombinant T cell receptor (TCR) specific for WT-1 may include a protein that has at least 85%, 90%, 95%, or 99% amino acid sequence identity

to any of the exemplary amino acid sequences disclosed herein (*e.g.*, SEQ ID NOS:1-15, 21 and 22), provided that (a) at least three or four of the CDRs have no mutations, (b) the CDRs that do have mutations have only up to two amino acid substitutions, up to a contiguous five amino acid deletion, or a combination thereof, and (c) the binding protein retains its ability to bind to a RMFPNAPYL (SEQ ID NO.:16):HLA complex with a K_d less than or equal to about 8 nM.

In other aspects, the present disclosure provides an immunoglobulin superfamily binding protein, comprising (a) a T cell receptor (TCR) α-chain variable (V_α) domain having at least 90% sequence identity to an amino acid sequence as set forth in SEQ ID NO.:1 or 2, and a TCR β-chain variable (V_β) domain; or (b) a V_α domain, and a V_β domain having at least 90% sequence identity to an amino acid sequence as set forth in SEQ ID NO.:9; or (c) a V_α domain of (a) and a V_β domain of (b); wherein the binding protein is capable of binding to a RMFPNAPYL (SEQ ID NO.:16):HLA complex with a K_d less than or equal to about 5 nM. In certain embodiments, the V_α domain comprises or consists of an amino acid sequence as set forth in SEQ ID NO.:1 or 2, the V_β domain comprises or consists of an amino acid sequence as set forth in SEQ ID NO.:9, or a combination thereof.

In further aspects, the present disclosure provides a high affinity recombinant T cell receptor (TCR), comprising an α-chain and a β-chain, wherein the α-chain comprises a V_α domain having at least 90% sequence identity to an amino acid sequence as set forth in SEQ ID NO.:1 or 2, wherein the TCR binds to a RMFPNAPYL (SEQ ID NO.:16)::HLA-A*201 complex on a cell surface independent or in the absence of CD8. In certain embodiments, the V_α domain comprises or consists of an amino acid sequence as set forth in SEQ ID NO.:1 or 2, the V_β domain comprises or consists of an amino acid sequence as set forth in SEQ ID NO.:9, or a combination thereof. In certain embodiments, a V_β chain is a V_β17 allele.

A variety of criteria known to persons skilled in the art indicate whether an amino acid that is substituted at a particular position in a peptide or polypeptide is conservative (or similar). For example, a similar amino acid or a conservative amino acid substitution is one in which an amino acid residue is replaced with an amino acid residue having a similar side chain. Similar amino acids may be included in the

following categories: amino acids with basic side chains (*e.g.*, lysine, arginine, histidine); amino acids with acidic side chains (*e.g.*, aspartic acid, glutamic acid); amino acids with uncharged polar side chains (*e.g.*, glycine, asparagine, glutamine, serine, threonine, tyrosine, cysteine, histidine); amino acids with nonpolar side chains (*e.g.*, alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan); amino acids with beta-branched side chains (*e.g.*, threonine, valine, isoleucine), and amino acids with aromatic side chains (*e.g.*, tyrosine, phenylalanine, tryptophan). Proline, which is considered more difficult to classify, shares properties with amino acids that have aliphatic side chains (*e.g.,* leucine, valine, isoleucine, and alanine). In certain circumstances, substitution of glutamine for glutamic acid or asparagine for aspartic acid may be considered a similar substitution in that glutamine and asparagine are amide derivatives of glutamic acid and aspartic acid, respectively. As understood in the art "similarity" between two polypeptides is determined by comparing the amino acid sequence and conserved amino acid substitutes thereto of the polypeptide to the sequence of a second polypeptide (*e.g.,* using GENEWORKS, Align, the BLAST algorithm, or other algorithms described herein and practiced in the art).

In certain embodiments, a WT-1 specific binding protein or TCR comprises a V_α domain that is at least about 90% identical to an amino acid sequence as set forth in SEQ ID NO.:21 or 22, and comprises a V_β domain that is at least about 90% identical to the amino acid sequence as set forth in SEQ ID NO:9, provided that (a) at least three or four of the CDRs have no mutations and (b) the CDRs that do have mutations have only up to two amino acid substitutions, up to a contiguous five amino acid deletion, or a combination thereof. In further embodiments, a WT-1 specific binding protein or TCR comprises a V_α domain that is at least about 95% identical to an amino acid sequence as set forth in SEQ ID NO.:1 or 2, and comprises a V_β domain that is at least about 95% identical to the amino acid sequence as set forth in SEQ ID NO.:9, provided that the binding protein is capable of binding to a RMFPNAPYL (SEQ ID NO.:16):HLA complex with a K_d less than or equal to about 5 nM.

In any of the aforementioned embodiments, a WT-1 specific binding protein or TCR is capable of (a) specifically binding to a RMFPNAPYL (SEQ ID NO.:16):HLA complex on a cell surface independent or in the absence of CD8, (b) specifically

binding to a RMFPNAPYL (SEQ ID NO.:16):HLA-A*201 complex, (c) binding to the RMFPNAPYL (SEQ ID NO.:16):HLA-A*201 complex with a K_d less than or equal to about 3 nM, or (d) any combination thereof.

In certain embodiments, the V_α domain of a WT-1 specific binding protein or TCR comprises or consists of an amino acid sequence as set forth in SEQ ID NO.:1 or 2. In other embodiments, the V_β domain comprises or consists of an amino acid sequence as set forth in SEQ ID NO.:9.

In still further embodiments, a WT-1 specific binding protein or TCR comprises an α-chain constant domain having at least 90% sequence identity to an amino acid sequence as set forth in SEQ ID NO.:3 or 4, comprises a β-chain constant domain having at least 90% sequence identity to an amino acid sequence as set forth in SEQ ID NO.:10 or 11, or any combination thereof. In certain embodiments, a V_β chain is a V_β17 allele.

In certain embodiments, a WT-1 specific binding protein is a T cell receptor (TCR), a chimeric antigen receptor or an antigen-binding fragment of a TCR, any of which can be chimeric, humanized or human. In further embodiments, an antigen-binding fragment of the TCR comprises a single chain TCR (scTCR) or a chimeric antigen receptor (CAR). In certain embodiments, a WT-1 specific binding protein is a TCR. In related embodiments, a WT-1 specific binding protein (a) comprises a TCR α-chain having an amino acid sequence as set forth in any one of SEQ ID NOS.:5-8, and comprises a TCR β-chain having an amino acid sequence as set forth in SEQ ID NO.:12 or 13; (b) has a TCR α-chain that comprises or consists of an amino acid sequence as set forth in SEQ ID NO.:5, and the TCR β-chain comprises or consists of an amino acid sequence as set forth in SEQ ID NO.:12; (c) has a TCR α-chain comprises or consists of an amino acid sequence as set forth in SEQ ID NO.:7, and the TCR β-chain comprises or consists of an amino acid sequence as set forth in SEQ ID NO.:12; (d) has a TCR α-chain comprises or consists of an amino acid sequence as set forth in SEQ ID NO.:6, and the TCR β-chain comprises or consists of an amino acid sequence as set forth in SEQ ID NO.:13; or (e) has a TCR α-chain comprises or consists of an amino acid sequence as set forth in SEQ ID NO.:8, and the TCR β-chain comprises or consists of an amino acid sequence as set forth in SEQ ID NO.:13.

In certain embodiments, there is provided a composition comprising a WT-specific binding protein or high affinity recombinant TCR according to any one of the aforementioned embodiments and a pharmaceutically acceptable carrier, diluent, or excipient.

Methods useful for isolating and purifying recombinantly produced soluble TCR, by way of example, may include obtaining supernatants from suitable host cell/vector systems that secrete the recombinant soluble TCR into culture media and then concentrating the media using a commercially available filter. Following concentration, the concentrate may be applied to a single suitable purification matrix or to a series of suitable matrices, such as an affinity matrix or an ion exchange resin. One or more reverse phase HPLC steps may be employed to further purify a recombinant polypeptide. These purification methods may also be employed when isolating an immunogen from its natural environment. Methods for large scale production of one or more of the isolated/recombinant soluble TCR described herein include batch cell culture, which is monitored and controlled to maintain appropriate culture conditions. Purification of the soluble TCR may be performed according to methods described herein and known in the art and that comport with laws and guidelines of domestic and foreign regulatory agencies.

In certain embodiments, nucleic acid molecules encoding an immunoglobulin superfamily binding protein or enhanced affinity TCR specific for WT-1 are used to transfect/transduce a host cell (*e.g.*, T cells) for use in adoptive transfer therapy. Advances in TCR sequencing have been described (*e.g.*, Robins et al., 2009 *Blood* 114:4099; Robins et al., 2010 *Sci. Translat. Med.* 2:47ra64, PMID: 20811043; Robins et al. 2011 (Sept. 10) *J. Imm. Meth.* Epub ahead of print, PMID: 21945395; Warren et al., 2011 *Genome Res.* 21:790) and may be employed in the course of practicing the embodiments according to the present disclosure. Similarly, methods for transfecting/transducing T-cells with desired nucleic acids have been described (*e.g.*, US 2004/0087025) as have adoptive transfer procedures using T-cells of desired antigen-specificity (*e.g.*, Schmitt *et al.*, *Hum. Gen. 20*:1240, 2009; Dossett *et al.*, *Mol. Ther. 17*:742, 2009; Till *et al.*, *Blood 112*:2261, 2008; Wang *et al.*, *Hum. Gene Ther. 18*:712, 2007; Kuball *et al.*, *Blood 109*:2331, 2007; US 2011/0243972;

US2011/0189141; Leen *et al.*, *Ann. Rev. Immunol. 25*:243, 2007), such that adaptation of these methodologies to the presently disclosed embodiments is contemplated, based on the teachings herein, including those directed to enhanced affinity TCRs specific for WT-1 peptide antigen RMFPNAPYL (SEQ ID NO.:16) complexed with an HLA receptor.

The WT-1-specific binding proteins or domains as described herein (*e.g.*, SEQ ID NOS:1-15 and 21-31, and variants thereof), may be functionally characterized according to any of a large number of art accepted methodologies for assaying T cell activity, including determination of T cell binding, activation or induction and also including determination of T cell responses that are antigen-specific. Examples include determination of T cell proliferation, T cell cytokine release, antigen-specific T cell stimulation, MHC restricted T cell stimulation, CTL activity (*e.g.*, by detecting ^{51}Cr release from pre-loaded target cells), changes in T cell phenotypic marker expression, and other measures of T-cell functions. Procedures for performing these and similar assays are may be found, for example, in Lefkovits (*Immunology Methods Manual: The Comprehensive Sourcebook of Techniques*, 1998). *See also Current Protocols in Immunology*; Weir, *Handbook of Experimental Immunology*, Blackwell Scientific, Boston, MA (1986); Mishell and Shigii (eds.) *Selected Methods in Cellular Immunology*, Freeman Publishing, San Francisco, CA (1979); Green and Reed, *Science* 281:1309 (1998) and references cited therein).

"MHC-peptide tetramer staining" refers to an assay used to detect antigen-specific T cells, which features a tetramer of MHC molecules, each comprising an identical peptide having an amino acid sequence that is cognate (*e.g.*, identical or related to) at least one antigen (*e.g.*, WT-1), wherein the complex is capable of binding T cell receptors specific for the cognate antigen. Each of the MHC molecules may be tagged with a biotin molecule. Biotinylated MHC/peptides are tetramerized by the addition of streptavidin, which can be fluorescently labeled. The tetramer may be detected by flow cytometry via the fluorescent label. In certain embodiments, an MHC-peptide tetramer assay is used to detect or select enhanced affinity TCRs of the instant disclosure.

Levels of cytokines may be determined according to methods described herein and practiced in the art, including for example, ELISA, ELISPOT, intracellular cytokine staining, and flow cytometry and combinations thereof (*e.g.,* intracellular cytokine staining and flow cytometry). Immune cell proliferation and clonal expansion resulting from an antigen-specific elicitation or stimulation of an immune response may be determined by isolating lymphocytes, such as circulating lymphocytes in samples of peripheral blood cells or cells from lymph nodes, stimulating the cells with antigen, and measuring cytokine production, cell proliferation and/or cell viability, such as by incorporation of tritiated thymidine or non-radioactive assays, such as MTT assays and the like. The effect of an immunogen described herein on the balance between a Th1 immune response and a Th2 immune response may be examined, for example, by determining levels of Th1 cytokines, such as IFN-γ, IL-12, IL-2, and TNF-β, and Type 2 cytokines, such as IL-4, IL-5, IL-9, IL-10, and IL-13.

Polynucleotides Encoding Binding Proteins Specific for WT-1 Antigen Peptides

Isolated or recombinant nucleic acid molecules encoding immunoglobulin superfamily binding protein or high affinity recombinant T cell receptor (TCR) specific for WT-1 as described herein may be produced and prepared according to various methods and techniques of the molecular biology or polypeptide purification arts. Construction of an expression vector that is used for recombinantly producing an immunoglobulin superfamily binding protein or high affinity recombinant TCR specific for WT-1 of interest can be accomplished by using any suitable molecular biology engineering techniques known in the art, including the use of restriction endonuclease digestion, ligation, transformation, plasmid purification, and DNA sequencing, for example as described in Sambrook *et al.* (1989 and 2001 editions; *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, NY) and Ausubel et al. (Current Protocols in Molecular Biology (2003)). To obtain efficient transcription and translation, a polynucleotide in each recombinant expression construct includes at least one appropriate expression control sequence (also called a regulatory sequence), such as a leader sequence and particularly a promoter operably (*i.e.*, operatively) linked to the nucleotide sequence encoding the immunogen. In certain embodiments, a polynucleotide is codon optimized for efficient expression in a target host cell.

Certain embodiments relate to nucleic acids that encode the polypeptides contemplated herein, for instance, immunoglobulin superfamily binding proteins or high affinity recombinant TCRs specific for WT-1. As one of skill in the art will recognize, a nucleic acid may refer to a single- or a double-stranded DNA, cDNA or RNA in any form, and may include a positive and a negative strand of the nucleic acid which complement each other, including anti-sense DNA, cDNA and RNA. Also included are siRNA, microRNA, RNA—DNA hybrids, ribozymes, and other various naturally occurring or synthetic forms of DNA or RNA.

Standard techniques may be used for recombinant DNA, peptide and oligonucleotide synthesis, immunoassays and tissue culture and transformation (*e.g.*, electroporation, lipofection). Enzymatic reactions and purification techniques may be performed according to manufacturer's specifications or as commonly accomplished in the art or as described herein. These and related techniques and procedures may be generally performed according to conventional methods well-known in the art and as described in various general and more specific references in microbiology, molecular biology, biochemistry, molecular genetics, cell biology, virology and immunology techniques that are cited and discussed throughout the present specification. See, *e.g.*, Sambrook, et al., *Molecular Cloning: A Laboratory Manual*, 3d ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.; *Current Protocols in Molecular Biology* (John Wiley and Sons, updated July 2008); *Short Protocols in Molecular Biology: A Compendium of Methods from Current Protocols in Molecular Biology*, Greene Pub. Associates and Wiley-Interscience; Glover, *DNA Cloning: A Practical Approach*, vol. I & II (IRL Press, Oxford Univ. Press USA, 1985); *Current Protocols in Immunology* (Edited by: John E. Coligan, Ada M. Kruisbeek, David H. Margulies, Ethan M. Shevach, Warren Strober 2001 John Wiley & Sons, NY, NY); *Real-Time PCR: Current Technology and Applications*, Edited by Julie Logan, Kirstin Edwards and Nick Saunders, 2009, Caister Academic Press, Norfolk, UK; Anand, *Techniques for the Analysis of Complex Genomes*, (Academic Press, New York, 1992); Guthrie and Fink, *Guide to Yeast Genetics and Molecular Biology* (Academic Press, New York, 1991); *Oligonucleotide Synthesis* (N. Gait, Ed., 1984); *Nucleic Acid Hybridization* (B. Hames & S. Higgins, Eds., 1985); *Transcription and Translation* (B. Hames & S.

Higgins, Eds., 1984); *Animal Cell Culture* (R. Freshney, Ed., 1986); Perbal, *A Practical Guide to Molecular Cloning* (1984); *Next-Generation Genome Sequencing* (Janitz, 2008 Wiley-VCH); *PCR Protocols (Methods in Molecular Biology)* (Park, Ed., 3rd Edition, 2010 Humana Press); *Immobilized Cells And Enzymes* (IRL Press, 1986); the treatise, *Methods In Enzymology* (Academic Press, Inc., N.Y.); *Gene Transfer Vectors For Mammalian Cells* (J. H. Miller and M. P. Calos eds., 1987, Cold Spring Harbor Laboratory); Harlow and Lane, *Antibodies*, (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1998); *Immunochemical Methods In Cell And Molecular Biology* (Mayer and Walker, eds., Academic Press, London, 1987); *Handbook Of Experimental Immunology*, Volumes I-IV (D. M. Weir andCC Blackwell, eds., 1986); Roitt, *Essential Immunology,* 6th Edition, (Blackwell Scientific Publications, Oxford, 1988); *Embryonic Stem Cells: Methods and Protocols* (Methods in Molecular Biology) (Kurstad Turksen, Ed., 2002); *Embryonic Stem Cell Protocols: Volume I: Isolation and Characterization* (Methods in Molecular Biology) (Kurstad Turksen, Ed., 2006); *Embryonic Stem Cell Protocols: Volume II: Differentiation Models* (Methods in Molecular Biology) (Kurstad Turksen, Ed., 2006); *Human Embryonic Stem Cell Protocols* (Methods in Molecular Biology) (Kursad Turksen Ed., 2006); *Mesenchymal Stem Cells: Methods and Protocols* (Methods in Molecular Biology) (Darwin J. Prockop, Donald G. Phinney, and Bruce A. Bunnell Eds., 2008); *Hematopoietic Stem Cell Protocols* (Methods in Molecular Medicine) (Christopher A. Klug, and Craig T. Jordan Eds., 2001); *Hematopoietic Stem Cell Protocols* (Methods in Molecular Biology) (Kevin D. Bunting Ed., 2008) *Neural Stem Cells: Methods and Protocols* (Methods in Molecular Biology) (Leslie P. Weiner Ed., 2008).

Certain embodiments include nucleic acids contained in a vector. One of skill in the art can readily ascertain suitable vectors for use with certain embodiments disclosed herein. A typical vector may comprise a nucleic acid molecule capable of transporting another nucleic acid to which it has been linked, or which is capable of replication in a host organism. Some examples of vectors include plasmids, viral vectors, cosmids, and others. Some vectors may be capable of autonomous replication in a host cell into which they are introduced (*e.g.* bacterial vectors having a bacterial origin of replication and episomal mammalian vectors), whereas other vectors may be integrated into the

genome of a host cell upon introduction into the host cell and thereby replicate along with the host genome. Additionally, some vectors are capable of directing the expression of genes to which they are operatively linked (these vectors may be referred to as "expression vectors"). According to related embodiments, it is further understood that, if one or more agents (*e.g.*, polynucleotides encoding immunoglobulin superfamily binding proteins or high affinity recombinant TCRs specific for WT-1, or variants thereof, as described herein) is co-administered to a subject, that each agent may reside in separate or the same vectors, and multiple vectors (each containing a different agent the same agent) may be introduced to a cell or cell population or administered to a subject.

In certain embodiments, the nucleic acid encoding immunoglobulin superfamily binding proteins or high affinity recombinant TCRs specific for WT-1, may be operatively linked to certain elements of a vector. For example, polynucleotide sequences that are needed to effect the expression and processing of coding sequences to which they are ligated may be operatively linked. Expression control sequences may include appropriate transcription initiation, termination, promoter and enhancer sequences; efficient RNA processing signals such as splicing and polyadenylation signals; sequences that stabilize cytoplasmic mRNA; sequences that enhance translation efficiency (*i.e.* Kozak consensus sequences); sequences that enhance protein stability; and possibly sequences that enhance protein secretion. Expression control sequences may be operatively linked if they are contiguous with the gene of interest and expression control sequences that act in trans or at a distance to control the gene of interest.

In particular embodiments, the recombinant expression vector is delivered to an appropriate cell, for example, a T cell or an antigen-presenting cell, *i.e.*, a cell that displays a peptide/MHC complex on its cell surface (*e.g.*, a dendritic cell) and lacks CD8. The recombinant expression vectors may therefore also include, for example, lymphoid tissue-specific transcriptional regulatory elements (TRE) such as a B lymphocyte, T lymphocyte, or dendritic cell specific TRE. Lymphoid tissue specific TRE are known in the art (*see, e.g.,* Thompson *et al.*, *Mol. Cell. Biol. 12*:1043, 1992); Todd *et al.*, *J. Exp. Med. 177*:1663, 1993); Penix *et al.*, *J. Exp. Med. 178*:1483, 1993).

In addition to vectors, certain embodiments relate to host cells that comprise the vectors that are presently disclosed. One of skill in the art readily understands that many suitable host cells are available in the art. A host cell may include any individual cell or cell culture which may receive a vector or the incorporation of nucleic acids and/or proteins, as well as any progeny cells. The term also encompasses progeny of the host cell, whether genetically or phenotypically the same or different. Suitable host cells may depend on the vector and may include mammalian cells, animal cells, human cells, simian cells, insect cells, yeast cells, and bacterial cells. These cells may be induced to incorporate the vector or other material by use of a viral vector, transformation via calcium phosphate precipitation, DEAE-dextran, electroporation, microinjection, or other methods. For example, see Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual* 2d ed. (Cold Spring Harbor Laboratory, 1989).

Methods of Treatment

In certain aspects, the instant disclosure is directed to methods for treating a hyperproliferative disorder or a condition characterized by WT-1 overexpression by administering to human subject in need thereof a composition comprising a binding protein or high affinity recombinant TCR specific for human Wilms tumor protein 1 (WT-1) according to any the aforementioned binding proteins or TCRs.

The presence of a hyperproliferative disorder or malignant condition in a subject refers to the presence of dysplastic, cancerous and/or transformed cells in the subject, including, for example neoplastic, tumor, non-contact inhibited or oncogenically transformed cells, or the like (*e.g.,* solid cancers; hematologic cancers including lymphomas and leukemias, such as acute myeloid leukemia, chronic myeloid leukemia, etc.), which are known in the art and for which criteria for diagnosis and classification are established (*e.g.,* Hanahan and Weinberg, 2011 *Cell* 144:646; Hanahan and Weinberg 2000 *Cell* 100:57; Cavallo et al., 2011 *Canc. Immunol. Immunother.* 60:319; Kyrigideis et al., 2010 *J. Carcinog.* 9:3). In certain embodiments, such cancer cells may be cells of acute myeloid leukemia, B-cell lymphoblastic leukemia, T-cell lymphoblastic leukemia, or myeloma, including cancer stem cells that are capable of initiating and serially transplanting any of these types of cancer (*see*, *e.g.,* Park et al. 2009 *Molec. Therap.* 17:219).

In certain embodiments, there are provided methods for treating a hyperproliferative disorder, such as a hematological malignancy or a solid cancer. Exemplary hematological malignancies include acute lymphoblastic leukemia (ALL), acute myeloid leukemia (AML), chronic myelogenous leukemia (CML), chronic eosinophilic leukemia (CEL), myelodysplastic syndrome (MDS), non-Hodgkin's lymphoma (NHL), or multiple myeloma (MM).

In further embodiments, there are provided methods for treating a hyperproliferative disorder, such as a solid cancer is selected from biliary cancer, bladder cancer, bone and soft tissue carcinoma, brain tumor, breast cancer, cervical cancer, colon cancer, colorectal adenocarcinoma, colorectal cancer, desmoid tumor, embryonal cancer, endometrial cancer, esophageal cancer, gastric cancer, gastric adenocarcinoma, glioblastoma multiforme, gynecological tumor, head and neck squamous cell carcinoma, hepatic cancer, lung cancer, malignant melanoma, osteosarcoma, ovarian cancer, pancreatic cancer, pancreatic ductal adenocarcinoma, primary astrocytic tumor, primary thyroid cancer, prostate cancer, renal cancer, renal cell carcinoma, rhabdomyosarcoma, skin cancer, soft tissue sarcoma, testicular germ-cell tumor, urothelial cancer, uterine sarcoma, or uterine cancer.

As understood by a person skilled in the medical art, the terms, "treat" and "treatment," refer to medical management of a disease, disorder, or condition of a subject (*i.e.,* patient, host, who may be a human or non-human animal) (*see, e.g.,* Stedman's Medical Dictionary). In general, an appropriate dose and treatment regimen provide one or more of a binding protein or high affinity recombinant TCR specific for human WT-1 (*e.g.*, SEQ ID NOS:1-15 and 21-31, and variants thereof) or a host cell expressing the same, and optionally an adjunctive therapy (e.g., a cytokine such as IL-2, IL-15, IL-21 or any combination thereof), in an amount sufficient to provide therapeutic or prophylactic benefit. Therapeutic or prophylactic benefit resulting from therapeutic treatment or prophylactic or preventative methods include, for example an improved clinical outcome, wherein the object is to prevent or retard or otherwise reduce (*e.g.,* decrease in a statistically significant manner relative to an untreated control) an undesired physiological change or disorder, or to prevent, retard or otherwise reduce the expansion or severity of such a disease or disorder. Beneficial or desired clinical results

from treating a subject include abatement, lessening, or alleviation of symptoms that result from or are associated the disease or disorder to be treated; decreased occurrence of symptoms; improved quality of life; longer disease-free status (*i.e.,* decreasing the likelihood or the propensity that a subject will present symptoms on the basis of which a diagnosis of a disease is made); diminishment of extent of disease; stabilized (*i.e.,* not worsening) state of disease; delay or slowing of disease progression; amelioration or palliation of the disease state; and remission (whether partial or total), whether detectable or undetectable; or overall survival.

"Treatment" can also mean prolonging survival when compared to expected survival if a subject were not receiving treatment. Subjects in need of the methods and compositions described herein include those who already have the disease or disorder, as well as subjects prone to have or at risk of developing the disease or disorder. Subjects in need of prophylactic treatment include subjects in whom the disease, condition, or disorder is to be prevented (*i.e.,* decreasing the likelihood of occurrence or recurrence of the disease or disorder). The clinical benefit provided by the compositions (and preparations comprising the compositions) and methods described herein can be evaluated by design and execution of *in vitro* assays, preclinical studies, and clinical studies in subjects to whom administration of the compositions is intended to benefit, as described in the examples.

Cells expressing the binding protein or high affinity recombinant TCR specific for human WT-1 as described herein may be administered to a subject in a pharmaceutically or physiologically acceptable or suitable excipient or carrier. Pharmaceutically acceptable excipients are biologically compatible vehicles, *e.g.*, physiological saline, which are described in greater detail herein, that are suitable for administration to a human or other non-human mammalian subject.

A therapeutically effective dose is an amount of host cells (expressing a binding protein or high affinity recombinant TCR specific for human WT-1) used in adoptive transfer that is capable of producing a clinically desirable result (*i.e.,* a sufficient amount to induce or enhance a specific T cell immune response against cells overexpressing WT-1 (*e.g.*, a cytotoxic T cell response) in a statistically significant manner) in a treated human or non-human mammal. As is well known in the medical

arts, the dosage for any one patient depends upon many factors, including the patient's size, weight, body surface area, age, the particular therapy to be administered, sex, time and route of administration, general health, and other drugs being administered concurrently. Doses will vary, but a preferred dose for administration of a host cell comprising a recombinant expression vector as described herein is about 10^7 cells/m^2, about 5 x 10^7 cells/m^2, about 10^8 cells/m^2, about 5 x 10^8 cells/m^2, about 10^9 cells/m^2, about 5 x 10^9 cells/m^2, about 10^{10} cells/m^2, about 5 x 10^{10} cells/m^2, or about 10^{11} cells/m^2.

Pharmaceutical compositions may be administered in a manner appropriate to the disease or condition to be treated (or prevented) as determined by persons skilled in the medical art. An appropriate dose and a suitable duration and frequency of administration of the compositions will be determined by such factors as the health condition of the patient, size of the patient (*i.e.,* weight, mass, or body area), the type and severity of the patient's disease, the particular form of the active ingredient, and the method of administration. In general, an appropriate dose and treatment regimen provide the composition(s) in an amount sufficient to provide therapeutic and/or prophylactic benefit (such as described herein, including an improved clinical outcome, such as more frequent complete or partial remissions, or longer disease-free and/or overall survival, or a lessening of symptom severity). For prophylactic use, a dose should be sufficient to prevent, delay the onset of, or diminish the severity of a disease associated with disease or disorder. Prophylactic benefit of the immunogenic compositions administered according to the methods described herein can be determined by performing pre-clinical (including *in vitro* and *in vivo* animal studies) and clinical studies and analyzing data obtained therefrom by appropriate statistical, biological, and clinical methods and techniques, all of which can readily be practiced by a person skilled in the art.

A condition associated with WT-1 overexpression includes any disorder or condition in which underactivity, overactivity or improper activity of a WT-1 cellular or molecular event is present, and typically results from unusually high (with statistical significance) levels of WT-1 expression in afflicted cells (*e.g.*, leukemic cells), relative to normal cells. A subject having such a disorder or condition would benefit from

treatment with a composition or method of the presently described embodiments. Some conditions associated with WT-1 overexpression thus may include acute as well as chronic disorders and diseases, such as those pathological conditions that predispose the subject to a particular disorder.

Some examples of conditions associated with WT-1 overexpression include hyperproliferative disorders, which refer to states of activated and/or proliferating cells (which may also be transcriptionally overactive) in a subject including tumors, neoplasms, cancer, malignancy, etc. In addition to activated or proliferating cells, the hyperproliferative disorder may also include an aberration or dysregulation of cell death processes, whether by necrosis or apoptosis. Such aberration of cell death processes may be associated with a variety of conditions, including cancer (including primary, secondary malignancies as well as metastasis), or other conditions.

According to certain embodiments, virtually any type of cancer that is characterized by WT-1 overexpression may be treated through the use of compositions and methods disclosed herein, including hematological cancers (*e.g.,* leukemia including acute myeloid leukemia (AML), T or B cell lymphomas, myeloma, and others). Furthermore, "cancer" may refer to any accelerated proliferation of cells, including solid tumors, ascites tumors, blood or lymph or other malignancies; connective tissue malignancies; metastatic disease; minimal residual disease following transplantation of organs or stem cells; multi-drug resistant cancers, primary or secondary malignancies, angiogenesis related to malignancy, or other forms of cancer. Also contemplated within the presently disclosed embodiments are specific embodiments wherein only one of the above types of disease is included, or where specific conditions may be excluded regardless of whether or not they are characterized by WT-1 overexpression.

Certain methods of treatment or prevention contemplated herein include administering a host cell (which may be autologous, allogeneic or syngeneic) comprising a desired nucleic acid molecule as described herein that is stably integrated into the chromosome of the cell. For example, such a cellular composition may be generated *ex vivo* using autologous, allogeneic or syngeneic immune system cells (*e.g.,* T cells,

antigen-presenting cells, natural killer cells) in order to administer a desired, WT-1-targeted T-cell composition to a subject as an adoptive immunotherapy.

As used herein, administration of a composition or therapy refers to delivering the same to a subject, regardless of the route or mode of delivery. Administration may be effected continuously or intermittently, and parenterally. Administration may be for treating a subject already confirmed as having a recognized condition, disease or disease state, or for treating a subject susceptible to or at risk of developing such a condition, disease or disease state. Co-administration with an adjunctive therapy may include simultaneous and/or sequential delivery of multiple agents in any order and on any dosing schedule (*e.g.*, WT-1 specific recombinant host cells with one or more cytokines; immunosuppressive therapy such as calcineurin inhibitors, corticosteroids, microtubule inhibitors, low dose of a mycophenolic acid prodrug, or any combination thereof).

In certain embodiments, a plurality of doses of a recombinant host cell as described herein is administered to the subject, which may be administered at intervals between administrations of about two to about four weeks. In further embodiments, a cytokine is administered sequentially, provided that the subject was administered the recombinant host cell at least three or four times before cytokine administration. In certain embodiments, the cytokine is administered subcutaneously (*e.g.*, IL-2, IL-15, IL-21). In still further embodiments, the subject being treated is further receiving immunosuppressive therapy, such as calcineurin inhibitors, corticosteroids, microtubule inhibitors, low dose of a mycophenolic acid prodrug, or any combination thereof. In yet further embodiments, the subject being treated has received a non-myeloablative or a myeloablative hematopoietic cell transplant, wherein the treatment may be administered at least two to at least three months after the non-myeloablative hematopoietic cell transplant.

An effective amount of a therapeutic or pharmaceutical composition refers to an amount sufficient, at dosages and for periods of time needed, to achieve the desired clinical results or beneficial treatment, as described herein. An effective amount may be delivered in one or more administrations. If the administration is to a subject already known or confirmed to have a disease or disease-state, the term "therapeutic amount"

may be used in reference to treatment, whereas "prophylactically effective amount" may be used to describe administrating an effective amount to a subject that is susceptible or at risk of developing a disease or disease-state (*e.g.*, recurrence) as a preventative course.

The level of a CTL immune response may be determined by any one of numerous immunological methods described herein and routinely practiced in the art. The level of a CTL immune response may be determined prior to and following administration of any one of the herein described WT-1-specific binding proteins expressed by, for example, a T cell. Cytotoxicity assays for determining CTL activity may be performed using any one of several techniques and methods routinely practiced in the art (*see, e.g.,* Henkart et al., "Cytotoxic T-Lymphocytes" *in Fundamental Immunology*, Paul (ed.) (2003 Lippincott Williams & Wilkins, Philadelphia, PA), pages 1127-50, and references cited therein).

Antigen-specific T cell responses are typically determined by comparisons of observed T cell responses according to any of the herein described T cell functional parameters (*e.g.,* proliferation, cytokine release, CTL activity, altered cell surface marker phenotype, etc.) that may be made between T cells that are exposed to a cognate antigen in an appropriate context (*e.g.*, the antigen used to prime or activate the T cells, when presented by immunocompatible antigen-presenting cells) and T cells from the same source population that are exposed instead to a structurally distinct or irrelevant control antigen. A response to the cognate antigen that is greater, with statistical significance, than the response to the control antigen signifies antigen-specificity.

A biological sample may be obtained from a subject for determining the presence and level of an immune response to a WT-1-derived antigen peptide as described herein. A "biological sample" as used herein may be a blood sample (from which serum or plasma may be prepared), biopsy specimen, body fluids (*e.g.,* lung lavage, ascites, mucosal washings, synovial fluid), bone marrow, lymph nodes, tissue explant, organ culture, or any other tissue or cell preparation from the subject or a biological source. Biological samples may also be obtained from the subject prior to receiving any immunogenic composition, which biological sample is useful as a control for establishing baseline (*i.e.,* pre-immunization) data.

The pharmaceutical compositions described herein may be presented in unit-dose or multi-dose containers, such as sealed ampoules or vials. Such containers may be frozen to preserve the stability of the formulation until. In certain embodiments, a unit dose comprises a recombinant host cell as described herein at a dose of about 10^7 cells/m^2 to about 10^{11} cells/m^2. The development of suitable dosing and treatment regimens for using the particular compositions described herein in a variety of treatment regimens, including *e.g.,* parenteral or intravenous administration or formulation.

If the subject composition is administered parenterally, the composition may also include sterile aqueous or oleaginous solution or suspension. Suitable non-toxic parenterally acceptable diluents or solvents include water, Ringer's solution, isotonic salt solution, 1,3-butanediol, ethanol, propylene glycol or polythethylene glycols in mixtures with water. Aqueous solutions or suspensions may further comprise one or more buffering agents, such as sodium acetate, sodium citrate, sodium borate or sodium tartrate. Of course, any material used in preparing any dosage unit formulation should be pharmaceutically pure and substantially non-toxic in the amounts employed. In addition, the active compounds may be incorporated into sustained-release preparation and formulations. Dosage unit form, as used herein, refers to physically discrete units suited as unitary dosages for the subject to be treated; each unit may contain a predetermined quantity of recombinant cells or active compound calculated to produce the desired therapeutic effect in association with an appropriate pharmaceutical carrier.

In general, an appropriate dosage and treatment regimen provides the active molecules or cells in an amount sufficient to provide therapeutic or prophylactic benefit. Such a response can be monitored by establishing an improved clinical outcome (*e.g.*, more frequent remissions, complete or partial, or longer disease-free survival) in treated subjects as compared to non-treated subjects. Increases in preexisting immune responses to a tumor protein generally correlate with an improved clinical outcome. Such immune responses may generally be evaluated using standard proliferation, cytotoxicity or cytokine assays, which are routine in the art and may be performed using samples obtained from a subject before and after treatment.

EXAMPLES

EXAMPLE 1

METHODS

Lentiviral Constructs

Various TCR expression constructs were generated containing codon-optimized TCRα and TCRβ genes, derived from an HLA-A2-restricted $CD8^+$ T cell clone (C4), encoding a high affinity TCR specific for a WT-1 peptide RMFPNAPYL (SEQ ID NO.:16) complexed with an HLA receptor. The TCRα- and TCRβ-encoding nucleic acid molecules were linked by a 2A element from the porcine teschovirus (P2A) to ensure coordinated expression under the control of a murine stem cell virus (MSCV) U3 promoter. In certain embodiments, the portions of the nucleic acid molecules encoding the constant domains of the C4 TCRα and TCRβ were modified to express complementary cysteine residues at positions 48 (Thr to Cys) and 57 (Ser to Cys), respectively, to promote inter-chain pairing of the C4 TCR chains and to minimize mispairing of the exogenous C4 TCR chains with endogenous TCR chains.

The vector pRRLSIN-C4α-P2A-C4β contained the TCR expression construct ligated into the pRRLSIN.cPPT.MSCV/GFP.WPRE lentiviral vector between the *Asc*I and *Sal*I restriction endonuclease sites, replacing GFP. The pRRLSIN.cPPT.MSCV/GFP.WPRE plasmid is a third-generation, self-inactivating lentiviral vector (*see* Yang *et al.*, *J. Immunother. 31*:830, 2008).

Saturation Mutagenesis Libraries

Two saturation mutagenesis libraries were constructed to generate and identify mutations within the C4α CDR regions (particularly the CDR3) that resulted in a higher or enhanced affinity for HLA-A2/WT-1 complex. The CDR3 region of C4α is comprised of the following amino acids: CAATEDYQLIW (SEQ ID NO.:25). Two randomized libraries were constructed encompassing residues ATE and DYQ using the Quikchange II site-directed mutagenesis kit (Agilent), using the lentiviral vector pRRLSIN-C4α-P2A-β as a template. Mutagenesis primers were designed according to the modified Quikchange protocol described by Zheng *et al.* (*Nucleic Acids Res.*

32:e115, 2004), and incorporated randomized nucleotides NNK (where N = A, C, G, or T, and K = G or T) for each amino acid position to be randomized. This yielded 32 different codons, encoding all 20 amino acids, and one stop codon. High transformation efficiency (greater than 1 x 10^{10}) Electromax DH10B T1 cells were transformed with the mutagenesis reaction composition, and the number of independent clones was determined by titrating and culturing a fraction of the transformation reaction on LB-Ampicillin plates. After determining the total number of clones, the transformation mix was plated on LB-Ampicillin plates at about 5,000 clones per plate. After 18 hours of culture at 37°C, 0.5-1 mL LB was added to the library plates and all colonies were harvested together, centrifuged, and high quality plasmid library DNA was isolated using the Endofree plasmid Maxi kit (Qiagen).

The library size was estimated to include about 100,000 independent clones, which was estimated to result in a library that was about 95% complete. To measure the efficiency of the mutagenesis reaction and the diversity of the library, the combined library plasmid DNA was sequenced, and the proportion of each nucleotide at each of the randomized positions was determined to be equivalent by comparing the relative signal for each nucleotide on a sequencing chromatogram (data not shown).

Screening of Saturation Mutagenesis Libraries

For each library, lentivirus was generated by transducing three plates of 293T cells (about 7 x 10^6 cells/plate) with the high quality plasmid library, concomitantly with the three packaging vectors pMDLg/pRRE, pMD2-G, and pRSV-REV. After 2 days, supernatant from the three plates were combined and aliquots were frozen for future use.

The lentiviral supernatant was titrated to determine the optimal concentration to utilize for transductions in order to minimize the probability that target cells are transduced with more than one library-derived TCR. Total transduction efficiency was determined by analyzing the percentage of cells expressing the transgenic beta chain (Vβ17). A dilution that yielded about a 20% transduction rate was chosen, and used to transduce about 2-5 x 10^7 J.RT3 cells. Library transduced cells were sorted by flow cytometry for high levels of WT-1 tetramer staining in the presence or absence of 1 μg/ml of competing anti-MHC class I antibody and expanded in culture multiple

times. Sorted populations that bound WT-1 tetramer at higher levels than J.RT3 cells transduced with the parental C4α-P2A-C4β construct were lysed, and genomic DNA was isolated using a DNeasy kit (Qiagen). The isolated DNA was used for PCR amplification of the lentiviral insert using primers that flank the TCR expression construct and that yielded a single band of the corresponding size. The PCR product was cloned into pENTR-D-Topo (Invitrogen), and clones were characterized by DNA sequence analysis.

Following sequence analysis of the isolated clones, a 750bp *Asc*I-*BamH*I fragment containing the C4α CDR3 region was excised from candidate clones and ligated into the parental pRRLSIN-C4α-P2A-C4β vector. Candidate mutants were then transduced into J.RT3 cells and PBMCs alongside the parental C4 construct, and mutants were assessed for binding affinity to HLA-A2/WT-$1^{126-134}$ (RMFPNAPYL, SEQ ID NO.:16).

Relative Affinity by Tetramer Titration

T cell clones were stained with 2-fold serial dilutions of WT-1 tetramer and analyzed by flow cytometry. Statistical analysis was performed in Graphpad Prism. KD values were extrapolated using a non-linear regression algorithm to a saturation binding curve with the formula $Y=B_{max}*X/[K_D+X]$.

EXAMPLE 2

IDENTIFICATION AND CLONING OF HIGH AFFINITY WT-1-SPECIFIC TCRS

In order to identify high affinity HLA-A2-restricted WT-$1^{126-134}$-specific T cell clones, T cell clones were generated from the peripheral repertoire of more than 50 donors. The top ten clones that exhibited the highest apparent affinity by tetramer staining were further assessed by staining each clone with titrated concentrations of WT-1 tetramer and fitting the resulting mean fluorescence intensity data to a saturation binding curve (Figure 1). TCRα and TCRβ gene sequences were identified by RACE PCR and sequencing of the four clones with the highest relative affinity was performed (C4, P1, P20, and P22).

To further characterize the WT-$1^{126-134}$-specific TCRs from these candidate T cell clones, codon-optimized expression constructs were generated for each TCRα and TCRβ chain pair. For each construct, the α and β chains were separated by a P2A element to promote coordinated expression of the TCRα and TCRβ chains (*see, e.g.*, Szymczak *et al.*, *Nat. Biotechnol. 22*:589, 2004; Dossett *et al.*, *Mol. Ther. 17*:742,2009). In addition, point mutations to create a second pair of cysteine residues in the external membrane-proximal regions of TCRα and TCRβ constant domains were introduced to promote preferential pairing of introduced TCR chains (Kuball *et al.*, *Blood 109*:2331, 2007). Finally, these codon-optimized, cysteine-modified constructs were cloned into the lentiviral vector pRRLSIN.cPPT-MSCV.WPRE (*see* Figure 3C).

Next, the ability of each of the introduced TCRαβ pairs to out-compete endogenous TCR chains for association with CD3 components and expression on the T cell surface was examined (*see, e.g.*, Heemskerk *et al.*, *Blood 109*:235, 2007). The codon-optimized, cysteine-modified TCR_{C4}, TCR_{P20} and TCR_{P22} were transduced into PBMCs and the percentage of tetramer positive cells within the transduced $CD8^+$ T cell population was determined (Figure 2A). The total transduced population was determined by subtracting the percentage of endogenous Vβ chain expression in the untransduced control from the percentage of TCR Vβ-specific T cells in the transduced populations (Figure 2A).

To determine the ability of each TCR to bind tetramer independently of CD8, which is associated with high affinity for peptide/MHC, transduced PBMCs were gated on $CD4^+$ T cells and WT-1 tetramer staining was assessed (Figure 2B). Both the TCR_{C4} and TCR_{P1} clones bound tetramer independently of CD8. Furthermore, TCR_{C4} exhibited the highest levels of CD8-independent tetramer staining, and PBMCs transduced with the TCR_{C4} construct exhibited the highest percentage of WT-1 tetramer positive T cells. The codon-optimized, cysteine-modified TCR_{C4} clone, which also exhibited the highest relative affinity among all the clones studied, was selected for modification and functional studies.

EXAMPLE 3

IMPROVING THE HIGH AFFINITY WT-1-SPECIFIC TCR_{C4} CONSTRUCT

As described in Example 2, the wild-type (WT) TCR_{C4} expression construct (C4αβ WT) was generated from full-length TCRα and TCRβ produced by 5'-RACE PCR from the TCR_{C4} clone. This construct included the nucleic acid encoding the C4 TCRα chain in the 5'-position, followed by a P2A element, and then the nucleic acid encoding the C4 TCRβ chain. Although T cells expressing this construct expressed similar levels of transgenic Vβ17 chain on the cell surface, WT-1 tetramer staining was essentially undetectable, indicating that despite the cysteine modification, this construct did not result in sufficient TCR gene expression to out-compete the endogenous TCR. As described in Example 2, the next step was to generate a codon optimized TCR_{C4} construct (*see* Scholten *et al.*, *Clin. Immunol. 119*:135, 2006), which showed a substantial increase in tetramer staining (*see* Figures 2A and 3A).

C4αβ and C4βα constructs were examined to determine whether a positional effect of the variable chains might influence surface expression of TCR_{C4} (*see* Leisegang *et al.*, *J. Mol. Med. 86*:573, 2008). Figures 3A and 3B show a clear increase in tetramer staining when the C4 TCRβ chain was positioned 5' of the P2A element, and this effect was more pronounced at later time-points post-stimulation, indicating that the C4αβ TCR construct was relatively less efficient than the C4βα TCR construct at competing for surface expression with the endogenous TCR, which is down-regulated following the initial T cell stimulation, and gradually increases with time.

These data indicate that (1) the C4βα TCR construct was more efficient at competing for surface expression with the endogenous TCR than the C4αβ TCR construct, and (2) the 21 P2A amino acids affected the TCRα protein function more than the TCRβ protein when located in the 5' position of the P2A-linked TCR_{C4} construct.

EXAMPLE 4

CELL SURFACE STABILITY OF FUNCTIONAL WT-1-SPECIFIC TCR_{C4}

In order to study the stability of functional TCR_{C4} expression on the surface of transduced T cells, $CD8^+$ T cells from an A2+ donor were stimulated with EBV peptide GLCTLVAML (SEQ ID NO.:127) to generate a population of EBV-specific T cells for which endogenous TCR expression could be monitored by EBV tetramer staining. T cells were transduced with the TCR_{C4} expression construct 24 hours following EBV peptide stimulation, resulting in preferential transduction of EBV-specific T cells. This approach resulted in a population of EBV+ T cells, a readily detectable population of TCR_{C4} transduced WT-1 $tetramer^+$/EBV $tetramer^+$ double positive (DP) T cells, and a population of TCR_{C4}-transduced WT-1 $tetramer^+$ and EBV $tetramer^-$ T cells that either were not reactive to EBV or that had lost EBV TCR surface expression due to competition with TCR_{C4} (Figure 8A).

Each of these populations was then sorted and expanded to directly assess the stability of functional TCR_{C4} expression on cells co-expressing an EBV-reactive TCR (Figure 8B). After 12 days, cultures were analyzed for WT-1 and EBV tetramer staining. T cell populations that initially bound only one of the tetramers remained almost exclusively single positive (SP). However, T cells that were uniformly DP for both tetramers following cell sorting, preferentially became single positive for WT-1 tetramer expression following 12 days of *in vitro* culture, while very few cells lost WT-1-tetramer staining to become EBV SP (Figure 8B). These results indicate that TCR_{C4} can readily outcompete endogenous TCRs for surface expression.

To determine whether the WT-1 tetramer SP population contains primarily cells that had out-competed the endogenous TCR for surface expression, EBV $tetramer^+$ T cells were sorted, and this purified population was then either transduced with the TCR_{C4} construct following restimulation with anti-CD3/CD28, or restimulated without further manipulation (Figure 8C). EBV-specific T cells that were transduced with the TCR_{C4} construct almost exclusively bound the WT-1-tetramer, indicating that the TCR_{C4} is capable of outcompeting most endogenous TCRs for expression on the surface of T cells.

EXAMPLE 5

GENERATION OF VARIANT HIGH AFFINITY WT-1-SPECIFIC TCRS

Even the highest affinity WT-1-specific T cell clones identified from the peripheral T cell repertoire generally will have an attenuated affinity compared to T cells specific for non-self antigens (for example, virus antigens), due to the influence of negative selection during T cell development, which promotes self-tolerance and protects against autoimmunity. Accordingly, saturation mutagenesis techniques were used to generate and identify high affinity TCRs having enhanced affinity *in vitro*. Two saturation mutagenesis libraries were generated that span the CDR3 region of the C4 TCRα chain (CAATEDYQLIW, SEQ ID NO.:25), as described in Example 1. Both libraries were screened for variants that had an enhanced affinity for the WT-1 epitope following transduction into J.RT3 cells followed by cell sorting based on high level binding of HLA-A2/WT-1 tetramer.

Candidate HLA-A2/WT-1 tetramer-binding variants were isolated from both libraries, and one variant having a DYQ to DLT mutation (C4-DLT) exhibited higher levels of HLA-A2/WT-1 tetramer binding compared to the unmutated C4 TCR (C4-WT), and was found to have enhanced HLA-A2/WT-1 tetramer equilibrium binding kinetics (Figure 4A). It should be noted that these experiments were done in the presence of CD8, which contributes significantly to TCR-peptide/MHC interactions, which thus may decrease apparent differences in the relative affinity between TCRs.

When transferred into CD8+ T cells and assessed for the ability to kill target cells pulsed with decreasing concentrations of peptide, C4-DLT showed a 5-10 fold increase in antigen sensitivity compared to C4-WT (Figure 4B), and a similar increase in antigen sensitivity was observed when cytokine production (IFNγ) in response to limiting peptide concentrations was assayed (Figure 4C). Likewise, T cells expressing C4-DLT exhibited enhanced killing (through caspase-3 activation) compared to T cells expressing C4-WT when targeting an HLA-2 expressing version of the leukemia cell line K562, which expresses WT-1, and can process and present the WT-1 peptide RMFPNAPYL (SEQ ID NO.:16) epitope to C4-WT and C4-DLT when transduced with HLA-A2 (Figure 5).

EXAMPLE 6

KINETIC AND CYTOLYTIC ACTIVITY OF TCR C4α MUTANT AS COMPARED TO WILD-TYPE TCR C4

Cord blood-derived $CD34^+$ HPCs were transduced to express the TCRα chain of a high affinity HLA-A2-restricted WT-1-specific TCR (TCR_{C4}) studied in clinical trials as described in Example 7. The transduced cells were cultured on OP9-DL1 cells expressing HLA-A2 (OP9-A2-DL1) in the presence of WT1 peptide. As a positive control, cord blood HSCs were also transduced with both the TCRα and TCRβ chains of TCR_{C4} (C4αβ). The majority of human γδ-T cell progenitors express CD4 and CD8 during development on OP9-DL1 (Van Coppernolle *et al.*, *Leukemia 26*:127, 2011), but since phenotypically mature γδ T cells (similar to murine DN $CD24^-$ cells) express high levels of CD27 (Van Coppernolle *et al.*, *J. Immunol. 183*:4859, 2009), expression of CD27 and the parental Vβ17 TCRβ chain were used to enrich for agonist-selected T cells. The relative proportion of $CD3^+CD27^+$ cells was analyzed for untransduced, C4α-transduced, and C4αβ-transduced cells after 31 days of *in vitro* culture. The majority of cells in the C4αβ-transduced cultures were $CD3^+CD27^+$. The C4α-transduced cultures had an increased percentage of $CD3^+CD27^+$ cells compared to untransduced controls, and a 5-fold increase in the percentage of $CD3^+$ cells expressing the parental Vβ17 (Figure 6A). Only $V\beta17^+CD27^+$ cells were collected for the TCRβ library construction on day 34 of culture (Figure 6B).

Vβ17-Cβ1 and Vβ17-Cβ2 libraries were transduced into H9.CD8-C4α cells and sorted for WT-1 $tetramer^+$ cells within the transduced population. After a single WT-1 MHC tetramer sort, cells transduced with Vβ17-Cβ1 library exhibited a range of tetramer reactivity, indicating that multiple TCRβ chains present in the Vβ17-sorted population could confer WT-1 antigen specificity. Cells exhibiting the highest level of WT-1 MHC tetramer staining were isolated by a second WT-1 MHC tetramer sort, and compared to H9.CD8-C4α cells expressing the parental C4β chain. While both transduced cell populations expressed similar levels of Vβ17, substantially higher tetramer staining was observed for the $tetramer^{hi}$ cells enriched from the Vβ17-Cβ1 library (Figure 6C). In some embodiments, these high affinity, Cβ1 library-derived

TCRβ chains can have utility as second generation WT-1-specific receptors in TCR gene therapy trials.

The retroviral packaging line PlatE was obtained from Cell Biolabs (San Diego, CA). The OP9-K^bD^bDL1 cell line was generated by transducing the OP9 cell line (Riken, Japan) with a retroviral construct containing the Dll-1 gene followed by an IRES and H-$2D^b$ (to generate OP9-K^bDL1 cells), and separately transduced with H-$2K^b$. The OP9-K^bD^bDL1-WT1 cell line was further transduced to express murine WT1. The OP9-A2-DL1 cell line was generated by transducing OP9-K^bDL1 cells with a retroviral construct encoding HLA-A2-IRES-human β2M. The OP9 cells and a retroviral construct containing the Dll1 gene followed by IRES-GFP were obtained from the lab of Juan Carlos Zúñiga-Pflücker. The $58^{-/-}$3D-PYYα cell line was generated by retrovirally tranducing the TCRα/TCRβ-deficient cell line 58-/- with Mig2-3D-PYYα. The H9.CD8-C4α cell line was generated by lentivirally transducing the human T cell line H9 with a CD8α-P2A-β construct followed by lentiviral transduction to express C4α-IRES-GFP.

EXAMPLE 7

DONOR-DERIVED, VIRUS SPECIFIC CD8+ T CELLS EXPRESSING A WT-1-SPECIFIC T CELL RECEPTOR PROVIDES ANTI-LEUKEMIC RELAPSE ACTIVITY IN AML PATIENTS

Relapse is the leading cause of death following allogeneic hematopoietic cell transplant (HCT) for hematological malignancies. Although evidence suggests that the beneficial donor T cell-mediated graft versus leukemia (GVL) effect can reduce post-HCT relapse rates, this effect is often mitigated by morbidity and mortality associated with the accompanying graft versus host disease (GVHD). Thus, providing antigen-specific T cells that selectively target leukemia associated antigen (LAA) can provide a distinct opportunity to promote GVL activity without inducing GVHD. Wilms Tumor protein 1 (WT-1) is a non-polymorphic zinc finger transcription factor that plays a key role in cell growth and differentiation. WT-1 has very limited expression in normal adult tissues, is expressed 10-1000 fold more in leukemia cells as compared to normal

$CD34^+$ cells, and has been shown to contribute to leukemogenesis. Furthermore, the magnitude of expression of WT-1 in leukemic cells correlates with prognosis and clinical aggressiveness. WT-1 is immunogenic and, thus, constitutes an attractive candidate immunotherapeutic target for induced $CD8^+$ cytotoxic T-cells (CTL) responses (Cheever *et al.*, *Clin. Cancer Res. 15*:5323, 2009). Transferred donor derived WT-1-reactive CD8+ CTL clones can persist in post-transplant patients and mediate anti-leukemic activity (Chapuis *et al.*, *Sci. Transl. Med. 5*:174ra27, 2013).

In this study, escalating doses of donor-derived virus specific CD8+ T cells that had been transduced to express a high affinity T cell receptor specific for the HLA A*02:01-restricted $WT1_{126\text{-}134}$ (RMFPNAPYL, SEQ ID NO.:16) epitope were administered to high-risk acute myeloid leukemia (AML) patients after allogeneic HCT, with escalating doses withheld if a previous dose persisted at a frequency of >3% of peripheral blood CD8+ T cells. At one observation point, at which nine patients had been treated in the study and received a total of 22 infusions, three (3) patients had completed the four T cell infusions, with the last infusion followed by a two week course of IL-2. CTC Grade ≥ 3 Adverse Events had been transient hypotension and a febrile reaction, and transient leukopenia, lymphopenia and thrombocytopenia. No end-organ toxicities attributed to the infused T cells had been observed. One patient had experienced exacerbation of acute GVHD after T cell infusion, and one patient developed chronic GVHD, although there was no evidence the GVHD in either patient reflected activity of the infused T cells.

Three patients who were treated with T cells after a second allogeneic HCT for relapsed AML (two of whom had persistent/relapse disease after second transplant) were alive with no evidence of disease 14, 8 and 7 months after initiation of T cell infusion (16, 26, and 9 months after second transplant accordingly) with no additional anti-leukemic therapy after completion of study treatment. One patient with high risk AML who was treated prophylactically after allogeneic HCT for AML in second complete remission (CR2) was alive and with no evidence of disease 13 months after initiation of study treatment (15 months after transplant) (Table 1).

Persistence of the transduced CTL *in vivo* was observed as being variable, with transferred CTL detectable between 4 to at least 290 days after T cell infusions (Table 1, Figure 7).

Table1. Clinical Outcomes

Patient	Diagnosis	Disease Status prior to Study Treatment	Disease Burden during T cell Infusion	Number of Infusions	CTL Persistence (Days after last Infusion)	Outcome*	Survival*
1	AML	Relapse 5 years after allogeneic HCT (medullary and extramedullary disease)	Present	3	14+	Progressive disease	Alive
2	AML	Relapse 10 years after first allogeneic HCT. MRD early after second HCT	Present	4(+IL2)	290+	Remission 16 mos. after transplant	Alive
3	AML	HCT at CR2. No evidence of disease after HCT	Absent	4(+IL2)	20	Remission 15 mos. after transplant	Alive
4	AML	Relapse with extramedulary disease one year after second allogeneic HCT	Absent	1	210+	Remission 26 mos. after transplant (8 mos. after treatment)	Alive
5	MDS → AML	Persistent disease after HCT	Present	1	5+	Progressive disease	Dead
6	AML	Second HCT for relapse 4 years after first HCT	Absent	4(+IL2)	4	Remission 9 mos. after transplant	Alive
7	AML	Persistence disease after HCT	Present	1	30+	Progressive disease	Dead
8	AML	HCT in CR2. MRD early after transplant	Present	1	50+	Ongoing treatment	Alive
9	AML	HCT in CR2. Relapse early after transplant	Absent	3	14+	Ongoing treatment	Alive

* as of July 2014

\+ persisting T cells detected at most recent analysis as of the assessment

These preliminary results of this study indicate that transfer of donor-derived virus specific CD8+ T cells transduced to express a WT-1-specific T cell receptor of provided embodiments(α-chain SEQ ID NO.:5 or 6 and β-chain SEQ ID NO.:12 or 13,

respectively) could be accomplished without significant toxicity and that such therapy could provide anti-leukemic activity.

The various embodiments described above can be combined to provide further embodiments. All of the U.S. patents, U.S. patent application publications, U.S. patent applications, foreign patents, foreign patent applications and non-patent publications referred to in this specification and/or listed in the Application Data Sheet are incorporated herein by reference, in their entirety. Aspects of the embodiments can be modified, if necessary to employ concepts of the various patents, applications and publications to provide yet further embodiments.

These and other changes can be made to the embodiments in light of the above-detailed description. In general, in the following claims, the terms used should not be construed to limit the claims to the specific embodiments disclosed in the specification and the claims, but should be construed to include all possible embodiments along with the full scope of equivalents to which such claims are entitled. Accordingly, the claims are not limited by the disclosure.

CLAIMS

What is claimed is:

1. A binding protein, comprising:

(a) a T cell receptor (TCR) α-chain variable (V_α) domain having a CDR1 amino acid sequence shown in SEQ ID NO.:23, a CDR2 amino acid sequence shown in SEQ ID NO.:24 and a CDR3 amino acid sequence shown in any one of SEQ ID NOS.:25, 26, 32, 38, 44, 50 and 51, and a TCR β-chain variable (V_β) domain; or

(b) a V_α domain of (a) and a V_β domain having a CDR1 amino acid sequence shown in SEQ ID NO.:27, a CDR2 amino acid sequence shown in SEQ ID NO.:28 and a CDR3 amino acid sequence shown in SEQ ID NO.:29.

2. The binding protein according to claim 1, wherein the binding protein is capable of binding to a RMFPNAPYL (SEQ ID NO.:16):human leukocyte antigen (HLA) complex with a K_d less than or equal to about 8 nM.

3. The binding protein according to claim 1 or claim 2, wherein the binding protein comprises a V_α domain that is at least about 90% identical to an amino acid sequence as set forth in SEQ ID NO.:21 or 22, and comprises a V_β domain that is at least about 90% identical to the amino acid sequence as set forth in SEQ ID NO:9, provided that (a) at least three or four of the CDRs have no mutations and (b) the CDRs that do have mutations have only up to two amino acid substitutions, up to a contiguous five amino acid deletion, or a combination thereof.

4. The binding protein according to claim 1 or claim 2, wherein the binding protein comprises a V_α domain that is at least about 95% identical to an amino acid sequence as set forth in SEQ ID NO.:1 or 2, and comprises a V_β domain that is at least about 95% identical to the amino acid sequence as set forth in SEQ ID NO.:9, provided that the binding protein is capable of binding to a RMFPNAPYL (SEQ ID NO.:16):HLA complex with a K_d less than or equal to about 5 nM.

5. The binding protein according to any one of claims 1-4, wherein the binding protein is capable of specifically binding to a RMFPNAPYL (SEQ ID NO.:16):HLA complex on a cell surface independent or in the absence of CD8.

6. The binding protein according to any one of claims 1-5, wherein the binding protein is capable of specifically binding to a RMFPNAPYL (SEQ ID NO.:16):HLA-A*201 complex.

7. The binding protein according to claim 6, wherein the binding protein is capable of binding to the RMFPNAPYL (SEQ ID NO.:16):HLA-A*201 complex with a K_d less than or equal to about 3 nM.

8. The binding protein according to any one of claims 1-7, wherein the V_α domain comprises or consists of an amino acid sequence as set forth in SEQ ID NO.:1.

9. The binding protein according to any one of claims 1-7, wherein the V_α domain comprises or consists of an amino acid sequence as set forth in SEQ ID NO.:2.

10. The binding protein according to any one of claims 1-9, wherein the V_β domain comprises or consists of an amino acid sequence as set forth in SEQ ID NO.:9.

11. The binding protein according to any one of claims 1-10, wherein the V_α domain comprises an α-chain constant domain having at least 90% sequence identity to an amino acid sequence as set forth in SEQ ID NO.:3 or 4.

12. The binding protein according to any one of claims 1-11, wherein the V_β domain comprises a β-chain constant domain having at least 90% sequence identity to an amino acid sequence as set forth in SEQ ID NO.:10 or 11.

13. The binding protein according to any one of claims 1-13, wherein the binding protein is a T cell receptor (TCR), a chimeric antigen receptor or an antigen-binding fragment of a TCR.

14. The binding protein according to claim 13, wherein the TCR, the chimeric antigen receptor or the antigen-binding fragment of the TCR is chimeric, humanized or human.

15. The binding protein according to claim 13 or 14, wherein the antigen-binding fragment of the TCR comprises a single chain TCR (scTCR).

16. The binding protein according to any one of claims 13-15, wherein the binding protein is a chimeric antigen receptor.

17. The binding protein according to any one of claims 13-15, wherein the binding protein is a TCR.

18. The binding protein according to any one of claims 1-17, wherein the binding protein comprises a TCR α-chain having an amino acid sequence as set forth in any one of SEQ ID NOS.:5-8, and a TCR β-chain having an amino acid sequence as set forth in SEQ ID NO.:12 or 13.

19. The binding protein according to claim 18, wherein the TCR α-chain comprises or consists of an amino acid sequence as set forth in SEQ ID NO.:5, and the TCR β-chain comprises or consists of an amino acid sequence as set forth in SEQ ID NO.:12.

20. The binding protein according to claim 18, wherein the TCR α-chain comprises or consists of an amino acid sequence as set forth in SEQ ID NO.:7, and the TCR β-chain comprises or consists of an amino acid sequence as set forth in SEQ ID NO.:12.

21. The binding protein according to claim 18, wherein the TCR α-chain comprises or consists of an amino acid sequence as set forth in SEQ ID NO.:6, and the TCR β-chain comprises or consists of an amino acid sequence as set forth in SEQ ID NO.:13.

22. The binding protein according to claim 18, wherein the TCR α-chain comprises or consists of an amino acid sequence as set forth in SEQ ID NO.:8, and the TCR β-chain comprises or consists of an amino acid sequence as set forth in SEQ ID NO.:13.

23. A binding protein, comprising:

(a) a T cell receptor (TCR) α-chain variable (V_α) domain having at least 90% sequence identity to an amino acid sequence as set forth in SEQ ID NO.:1 or 2, and a TCR β-chain variable (V_β) domain; or

(b) a V_α domain, and a V_β domain having at least 90% sequence identity to an amino acid sequence as set forth in SEQ ID NO.:9; or

(c) a V_α domain of (a) and a V_β domain of (b);

wherein the binding protein is capable of binding to a RMFPNAPYL (SEQ ID NO.:16):HLA complex with a K_d less than or equal to about 5 nM.

24. The binding protein according to claim 23, wherein the V_α domain comprises or consists of an amino acid sequence as set forth in SEQ ID NO.:1.

25. The binding protein according to claim 23, wherein the V_α domain comprises or consists of an amino acid sequence as set forth in SEQ ID NO.:2.

26. The binding protein according to any one of claims 23-25, wherein the V_β domain comprises or consists of an amino acid sequence as set forth in SEQ ID NO.:9.

27. The binding protein according to any one of claims 23-26, wherein the V_α domain comprises an α-chain constant domain having at least 90% sequence identity to an amino acid sequence as set forth in SEQ ID NO.:3 or 4.

28. The binding protein according to any one of claims 23-27, wherein the V_β domain comprises a β-chain constant domain having at least 90% sequence identity to an amino acid sequence as set forth in SEQ ID NO.:10 or 11.

29. The binding protein according to any one of claims 23-28, wherein the binding protein is a T cell receptor (TCR), a chimeric antigen receptor or an antigen-binding fragment of a TCR.

30. The binding protein according to claim 29, wherein the TCR, the chimeric antigen receptor or the antigen-binding fragment of the TCR is chimeric, humanized or human.

31. The binding protein according to claim 29 or 30, wherein the antigen-binding fragment of the TCR comprises a single chain TCR (scTCR).

32. The binding protein according to any one of claims 29-31, wherein the binding protein is a chimeric antigen receptor.

33. The binding protein according to any one of claims 29-31, wherein the binding protein is a TCR.

34. The binding protein according to any one of claims 23-33, wherein the binding protein comprises a TCR α-chain having an amino acid sequence as set forth in any one of SEQ ID NOS.:5-8, and a TCR β-chain having an amino acid sequence as set forth in SEQ ID NO.:12 or 13.

35. The binding protein according to claim 34, wherein the TCR α-chain comprises or consists of an amino acid sequence as set forth in SEQ ID NO.:5, and the TCR β-chain comprises or consists of an amino acid sequence as set forth in SEQ ID NO.:12.

36. The binding protein according to claim 34, wherein the TCR α-chain comprises or consists of an amino acid sequence as set forth in SEQ ID NO.:7, and the TCR β-chain comprises or consists of an amino acid sequence as set forth in SEQ ID NO.:12.

37. The binding protein according to claim 34, wherein the TCR α-chain comprises or consists of an amino acid sequence as set forth in SEQ ID NO.:6, and the TCR β-chain comprises or consists of an amino acid sequence as set forth in SEQ ID NO.:13.

38. The binding protein according to claim 34, wherein the TCR α-chain comprises or consists of an amino acid sequence as set forth in SEQ ID NO.:8, and the TCR β-chain comprises or consists of an amino acid sequence as set forth in SEQ ID NO.:13.

39. The binding protein according to any one of claims 23-38, wherein the binding protein is capable of specifically binding to a RMFPNAPYL (SEQ ID NO.:16):HLA complex on a cell surface independent or in the absence of CD8.

40. The binding protein according to any one of claims 23-39, wherein the binding protein is capable of specifically binding to a RMFPNAPYL (SEQ ID NO.:16):HLA-A*201 complex.

41. The binding protein according to claim 40, wherein the binding protein is capable of binding to a RMFPNAPYL (SEQ ID NO.:16):HLA-A*201 complex with a K_d less than or equal to about 3 nM.

42. A high affinity recombinant T cell receptor (TCR), comprising an α-chain and a β-chain, wherein the α-chain comprises a V_α domain having at least 90% sequence identity to an amino acid sequence as set forth in SEQ ID NO.:1 or 2, wherein the TCR binds to a RMFPNAPYL (SEQ ID NO.:16)::HLA-A*201 complex on a cell surface independent or in the absence of CD8.

43. The high affinity recombinant TCR according to claim 42, wherein the V_α domain comprises or consists of an amino acid sequence as set forth in SEQ ID NO.:1.

44. The high affinity recombinant TCR according to claim 42, wherein the V_α domain comprises or consists of an amino acid sequence as set forth in SEQ ID NO.:2.

45. The high affinity recombinant TCR according to any one of claims 42-44, wherein the β-chain comprises a V_β domain having at least 90% sequence identity to an amino acid sequence as set forth in SEQ ID NO.:9.

46. The high affinity recombinant TCR according to claim 45, wherein the V_β domain comprises or consists of an amino acid sequence as set forth in SEQ ID NO.:9.

47. The high affinity recombinant TCR according to any one of claims 42-46, wherein the TCR α-chain comprises an α-chain constant domain having at least 90% sequence identity to an amino acid sequence as set forth in SEQ ID NO.:3 or 4.

48. The high affinity recombinant TCR according to any one of claims 42-47, wherein the TCR β-chain comprises a β-chain constant domain having at least 90% sequence identity to an amino acid sequence as set forth in SEQ ID NO.:10 or 11.

49. The high affinity recombinant TCR according to any one of claims 42-48, wherein the TCR α-chain comprises or consists of an amino acid sequence as set forth in any one of SEQ ID NOS.:5-8, and the TCR β-chain comprises or consists of an amino acid sequence as set forth in SEQ ID NO.:12 or 13.

50. The high affinity recombinant TCR according to claim 49, wherein the TCR α-chain comprises or consists of an amino acid sequence as set forth in SEQ ID NO.:5, and the TCR β-chain comprises or consists of an amino acid sequence as set forth in SEQ ID NO.:12.

51. The high affinity recombinant TCR according to claim 49, wherein the TCR α-chain comprises or consists of an amino acid sequence as set forth in SEQ ID NO.:7, and the TCR β-chain comprises or consists of an amino acid sequence as set forth in SEQ ID NO.:12.

52. The high affinity recombinant TCR according to claim 49, wherein the TCR α-chain comprises or consists of an amino acid sequence as set forth in SEQ ID NO.:6, and the TCR β-chain comprises or consists of an amino acid sequence as set forth in SEQ ID NO.:13.

53. The high affinity recombinant TCR according to claim 49, wherein the TCR α-chain comprises or consists of an amino acid sequence as set forth in SEQ ID NO.:8, and the TCR β-chain comprises or consists of an amino acid sequence as set forth in SEQ ID NO.:13.

54. The high affinity recombinant TCR according to any one of claims 42-53, wherein the TCR binds to a RMFPNAPYL (SEQ ID NO.:16):HLA-A*201 complex with a K_d less than or equal to 3 nM.

55. A composition comprising a binding protein or high affinity recombinant TCR according to any one of claims 1-54 and a pharmaceutically acceptable carrier, diluent, or excipient.

56. An isolated polynucleotide encoding a binding protein or high affinity recombinant TCR according to any one of claims 1-54.

57. The polynucleotide according to claim 56, wherein the polynucleotide encoding a binding protein or high affinity recombinant TCR is codon optimized.

58. An expression vector, comprising a polynucleotide of claim 56 or 57 operably linked to an expression control sequence.

59. The expression vector according to claim 58, wherein the vector is capable of delivering the polynucleotide to a host cell.

60. The expression vector according to claim 59, wherein the host cell is a hematopoietic progenitor cell or a human immune system cell.

61. The expression vector according to claim 60, wherein the immune system cell is a CD4+ T cell, a CD8+ T cell, a CD4- CD8- double negative T cell, a γδ T cell, a natural killer cell, a dendritic cell, or any combination thereof.

62. The expression vector according to claim 61, wherein the T cell is a naïve T cell, a central memory T cell, an effector memory T cell, or any combination thereof.

63. The expression vector according to any one of claims 58-62, wherein the vector is a viral vector.

64. The expression vector according to claim 63, wherein the viral vector is a lentiviral vector or a γ-retroviral vector.

65. A recombinant host cell, comprising a polynucleotide according to claim 56 or 57 or an expression vector according to any one of claims 58-64, wherein the host cell expresses on its cell surface a binding protein or high affinity recombinant TCR encoded by the polynucleotide.

66. The recombinant host cell according to claim 65, wherein a polynucleotide encodes a V_α domain that is at least about 80% identical to a nucleotide sequence as set forth in any one of SEQ ID NOS.:32-35, and a polynucleotide encodes a V_β domain that is at least about 80% identical to a nucleotide sequence as set forth in SEQ ID NO.:88 or 89.

67. The recombinant host cell according to claim 65 or 66, wherein a polynucleotide encodes a V_α domain comprising or consisting of a nucleotide sequence as set forth in SEQ ID NO.:77 or 78.

68. The recombinant host cell according to any one of claims 65-67, wherein a polynucleotide encodes a V_α domain comprising or consisting of a nucleotide sequence as set forth in SEQ ID NO.:79 or 80.

69. The recombinant host cell according to any one of claims 65-68, wherein a polynucleotide encodes a V_β domain comprising or consisting of a nucleotide sequence as set forth in SEQ ID NO.:88 or 89.

70. The recombinant host cell according to any one of claims 65-69, wherein a V_α domain encoding polynucleotide comprises a nucleotide sequence encoding an α-chain constant domain that is at least about 80% identical to a nucleotide sequence as set forth in any one of SEQ ID NOS.:81-84.

71. The recombinant host cell according to any one of claims 65-70, wherein a V_β domain encoding polynucleotide comprises a nucleotide sequence encoding a β-chain constant domain that is at least about 80% identical to a nucleotide sequence as set forth in any one of SEQ ID NOS.:90-93.

72. The recombinant host cell according to any one of claims 65-71, wherein a polynucleotide encoding a TCR α-chain comprises a nucleotide sequence as set forth in SEQ ID NO.:85, 86 or 87, and a polynucleotide encoding a TCR β-chain comprises a nucleotide sequence as set forth in SEQ ID NO.:94 or 95.

73. The recombinant host cell according to claim 72, wherein the TCR α-chain encoding polynucleotide comprises or consists of a nucleotide sequence as set forth in SEQ ID NO.:85, and the TCR β-chain encoding polynucleotide comprises or consists of a nucleotide sequence as set forth in SEQ ID NO.:94.

74. The recombinant host cell according to claim 72, wherein the TCR α-chain encoding polynucleotide comprises or consists of a nucleotide sequence as set forth in SEQ ID NO.:86, and the TCR β-chain encoding polynucleotide comprises or consists of a nucleotide sequence as set forth in SEQ ID NO.:95.

75. The recombinant host cell according to claim 72, wherein the TCR α-chain encoding polynucleotide comprises or consists of a nucleotide sequence as set forth in SEQ ID NO.:87, and the TCR β-chain encoding polynucleotide comprises or consists of a nucleotide sequence as set forth in SEQ ID NO.:95.

76. The recombinant host cell according to any one of claims 65-75, wherein a polynucleotide encodes a self-cleaving peptide disposed between a TCR α-chain encoding polynucleotide and a TCR β-chain encoding polynucleotide.

77. The recombinant host cell according to claim 76, wherein the polynucleotide encoding a self-cleaving peptide comprises or consists of a nucleotide sequence as set forth in any one of SEQ ID NOS.:98-101.

78. The recombinant host cell according to claim 76, wherein the polynucleotide encodes a self-cleaving peptide comprising or consisting of an amino acid sequence as set forth in any one of SEQ ID NOS.:17-20.

79. The recombinant host cell according to claim 76, wherein the polynucleotide encoding a TCR α-chain, self-cleaving peptide, and TCR β-chain comprises or consists of a nucleotide sequence as set forth in SEQ ID NO.:96 or 97.

80. The recombinant host cell according to any one of claims 65-79, wherein the host cell is allogeneic, syngeneic, or autologous.

81. The recombinant host cell according to any one of claims 65-80, wherein the host cell is a hematopoietic progenitor cell or a human immune system cell.

82. The recombinant host cell according to claim 81, wherein the immune system cell is a CD4+ T cell, a CD8+ T cell, a CD4- CD8- double negative T cell, a γδ T cell, a natural killer cell, a dendritic cell, or any combination thereof.

83. The recombinant host cell according to claim 81, wherein the immune system cell is a T cell.

84. The recombinant host cell according to claim 83, wherein the T cell is a naïve T cell, a central memory T cell, an effector memory T cell, or any combination thereof.

85. The recombinant host cell according to claim 83 or 84, wherein the binding protein or high affinity recombinant TCR expressed by the T cell is capable of more efficiently associating with a CD3 protein as compared to endogenous TCR.

86. The recombinant host cell according to any one of claims 83-85, wherein the binding protein or high affinity recombinant TCR has higher T cell surface expression as compared to endogenous TCR.

87. A method for treating a hyperproliferative disorder, comprising administering to human subject in need thereof a composition comprising a binding protein or high affinity recombinant TCR specific for human Wilms tumor protein 1 (WT-1) according to any one of claims 1-54.

88. The method according to claim 87, wherein the hyperproliferative disorder is a hematological malignancy or a solid cancer.

89. The method according to claim 88, wherein the hematological malignancy is selected from acute lymphoblastic leukemia (ALL), acute myeloid leukemia (AML), chronic myelogenous leukemia (CML), chronic eosinophilic leukemia (CEL), myelodysplastic syndrome (MDS), non-Hodgkin's lymphoma (NHL), or multiple myeloma (MM).

90. The method according to claim 88, wherein the solid cancer is selected from biliary cancer, bladder cancer, bone and soft tissue carcinoma, brain tumor, breast cancer, cervical cancer, colon cancer, colorectal adenocarcinoma, colorectal cancer, desmoid tumor, embryonal cancer, endometrial cancer, esophageal cancer, gastric cancer, gastric adenocarcinoma, glioblastoma multiforme, gynecological tumor, head and neck squamous cell carcinoma, hepatic cancer, lung cancer, malignant melanoma, osteosarcoma, ovarian cancer, pancreatic cancer, pancreatic ductal adenocarcinoma, primary astrocytic tumor, primary thyroid cancer, prostate cancer, renal cancer, renal cell carcinoma, rhabdomyosarcoma, skin cancer, soft tissue sarcoma, testicular germ-cell tumor, urothelial cancer, uterine sarcoma, or uterine cancer.

91. The method according to any one of claims 87-90, wherein the binding protein is capable of promoting an antigen-specific T cell response against a human WT-1 in a class I HLA-restricted manner.

92. The method according to claim 91, wherein the class I HLA-restricted response is transporter-associated with antigen processing (TAP)-independent.

93. The method according to claim 91 or 92, wherein the antigen-specific T cell response comprises at least one of a $CD4^+$ helper T lymphocyte (Th) response and a CD8+ cytotoxic T lymphocyte (CTL) response.

94. The method according to claim 93, wherein the CTL response is directed against a WT-1-overexpressing cell.

95. The method according to any one of claims 87-94, wherein the composition comprises a host cell according to any one of claims 65-86.

96. An adoptive immunotherapy method for treating a condition characterized by WT-1 overexpression in cells of a subject having a hyperproliferative disorder, comprising

administering to the subject an effective amount of a recombinant host cell according to any one of claims 65-86.

97. The method according to claim 96, wherein the recombinant host cell is modified *ex vivo*.

98. The method according to claim 96 or 97, wherein the host cell is an allogeneic cell, a syngeneic cell, or an autologous cell.

99. The method according to any one of claims 96-98, wherein the host cell is a hematopoietic progenitor cell or a human immune system cell.

100. The method according to claim 99, wherein the immune system cell is a CD4+ T cell, a CD8+ T cell, a CD4- CD8- double negative T cell, a γδ T cell, a natural killer cell, a dendritic cell, or any combination thereof.

101. The method according to claim 100, wherein the T cell is a naïve T cell, a central memory T cell, an effector memory T cell, or any combination thereof.

102. The method according to any one of claims 96-101, wherein the hyperproliferative disorder is a hematological malignancy or a solid cancer.

103. The method according to claim 102, wherein the hematological malignancy is selected from acute lymphoblastic leukemia (ALL), acute myeloid leukemia (AML), chronic myelogenous leukemia (CML), chronic eosinophilic leukemia (CEL), myelodysplastic syndrome (MDS), non-Hodgkin's lymphoma (NHL), or multiple myeloma (MM).

104. The method according to claim 102, wherein the solid cancer is selected from biliary cancer, bladder cancer, bone and soft tissue carcinoma, brain tumor, breast cancer, cervical cancer, colon cancer, colorectal adenocarcinoma, colorectal cancer, desmoid tumor, embryonal cancer, endometrial cancer, esophageal cancer, gastric cancer, gastric

adenocarcinoma, glioblastoma multiforme, gynecological tumor, head and neck squamous cell carcinoma, hepatic cancer, lung cancer, malignant melanoma, osteosarcoma, ovarian cancer, pancreatic cancer, pancreatic ductal adenocarcinoma, primary astrocytic tumor, primary thyroid cancer, prostate cancer, renal cancer, renal cell carcinoma, rhabdomyosarcoma, skin cancer, soft tissue sarcoma, testicular germ-cell tumor, urothelial cancer, uterine sarcoma, or uterine cancer.

105. The method according to any one of claims 96-104, wherein the recombinant host cell is administered parenterally.

106. The method according to any one of claims 96-105, wherein the method comprises administering a plurality of doses of the recombinant host cell to the subject.

107. The method according to claim 106, wherein the plurality of doses are administered at intervals between administrations of about two to about four weeks.

108. The method according to any one of claims 96-107, wherein the recombinant host cell is administered to the subject at a dose of about 10^7 cells/m^2 to about 10^{11} cells/m^2.

109. The method according to any one of claims 96-108, wherein the method further comprises administering a cytokine.

110. The method according to claim 109, wherein the cytokine is IL-2, IL-15, IL-21 or any combination thereof.

111. The method according to claim 110, wherein the cytokine is IL-2 and is administered concurrently or sequentially with the recombinant host cell.

112. The method according to claim 111, wherein the cytokine is administered sequentially, provided that the subject was administered the recombinant host cell at least three or four times before cytokine administration.

113. The method according to any one of claims 110-112, wherein the cytokine is IL-2 and is administered subcutaneously.

114. The method according to any one of claims 96-113, wherein the subject is further receiving immunosuppressive therapy.

115. The method according to claim 114, wherein the immunosuppressive therapy is selected from calcineurin inhibitors, corticosteroids, microtubule inhibitors, low dose of a mycophenolic acid prodrug, or any combination thereof.

116. The method according to any one of claims 96-115, wherein the subject has received a non-myeloablative or a myeloablative hematopoietic cell transplant.

117. The method according to claim 116, wherein the subject is administered the recombinant host cell at least three months after the non-myeloablative hematopoietic cell transplant.

118. The method according to claim 116, wherein the subject is administered the recombinant host cell at least two months after the myeloablative hematopoietic cell transplant.

119. A unit dose form comprising a recombinant host cell according to any one of claims 65-86.

120. The unit dose form according to claim 119, wherein the recombinant host cell is at a dose of about 10^7 cells/m^2 to about 10^{11} cells/m^2.

121. A binding protein or high affinity recombinant TCR according to any one of claims 1-54 for use in the treatment of a hyperproliferative disorder.

122. The binding protein or high affinity recombinant TCR according to claim 121, wherein the hyperproliferative disorder is a hematological malignancy or a solid cancer.

123. The binding protein or high affinity recombinant TCR according to claim 122, wherein the hematological malignancy is selected from acute lymphoblastic leukemia (ALL), acute myeloid leukemia (AML), chronic myelogenous leukemia (CML), chronic eosinophilic leukemia (CEL), myelodysplastic syndrome (MDS), non-Hodgkin's lymphoma (NHL), or multiple myeloma (MM).

124. The binding protein or high affinity recombinant TCR according to claim 122, wherein the solid cancer is selected from biliary cancer, bladder cancer, bone and soft tissue carcinoma, brain tumor, breast cancer, cervical cancer, colon cancer, colorectal adenocarcinoma, colorectal cancer, desmoid tumor, embryonal cancer, endometrial cancer, esophageal cancer, gastric cancer, gastric adenocarcinoma, glioblastoma multiforme, gynecological tumor, head and neck squamous cell carcinoma, hepatic cancer, lung cancer, malignant melanoma, osteosarcoma, ovarian cancer, pancreatic cancer, pancreatic ductal adenocarcinoma, primary astrocytic tumor, primary thyroid cancer, prostate cancer, renal cancer, renal cell carcinoma, rhabdomyosarcoma, skin cancer, soft tissue sarcoma, testicular germ-cell tumor, urothelial cancer, uterine sarcoma, or uterine cancer.

125. A recombinant host cell according to any one of claims 65-86 for use in adoptive immunotherapy of a hyperproliferative disorder.

126. The recombinant host cell according to claim 125, wherein the hyperproliferative disorder is a hematological malignancy or a solid cancer.

127. The recombinant host cell according to claim 126, wherein the hematological malignancy is selected from acute lymphoblastic leukemia (ALL), acute myeloid leukemia (AML), chronic myelogenous leukemia (CML), chronic eosinophilic leukemia (CEL), myelodysplastic syndrome (MDS), non-Hodgkin's lymphoma (NHL), or multiple myeloma (MM).

128. The recombinant host cell according to claim 126, wherein the solid cancer is selected from biliary cancer, bladder cancer, bone and soft tissue carcinoma, brain tumor, breast cancer, cervical cancer, colon cancer, colorectal adenocarcinoma, colorectal cancer, desmoid tumor, embryonal cancer, endometrial cancer, esophageal cancer, gastric cancer, gastric adenocarcinoma, glioblastoma multiforme, gynecological tumor, head and neck squamous cell carcinoma, hepatic cancer, lung cancer, malignant melanoma, osteosarcoma, ovarian cancer, pancreatic cancer, pancreatic ductal adenocarcinoma, primary astrocytic tumor, primary thyroid cancer, prostate cancer, renal cancer, renal cell carcinoma, rhabdomyosarcoma, skin cancer, soft tissue sarcoma, testicular germ-cell tumor, urothelial cancer, uterine sarcoma, or uterine cancer.

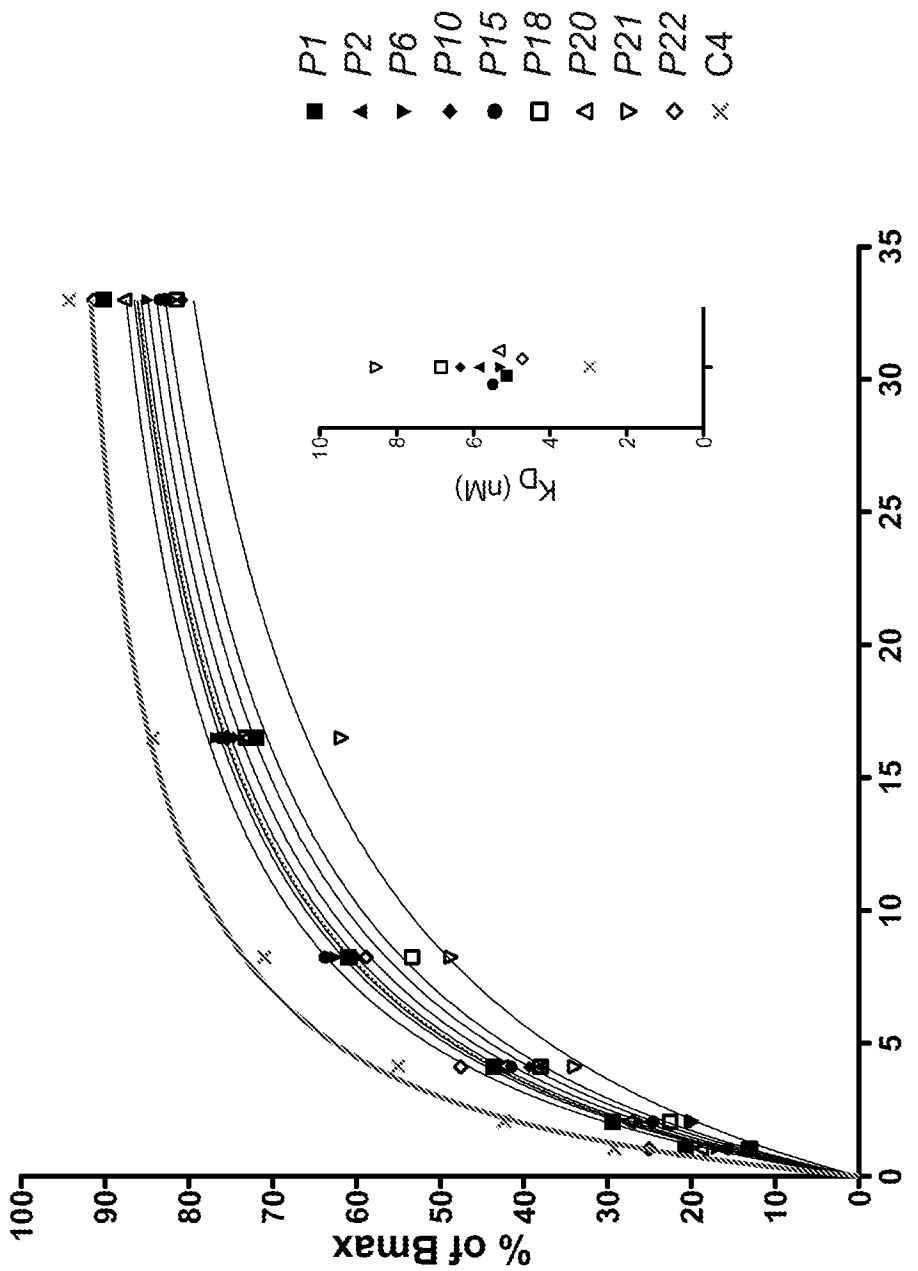
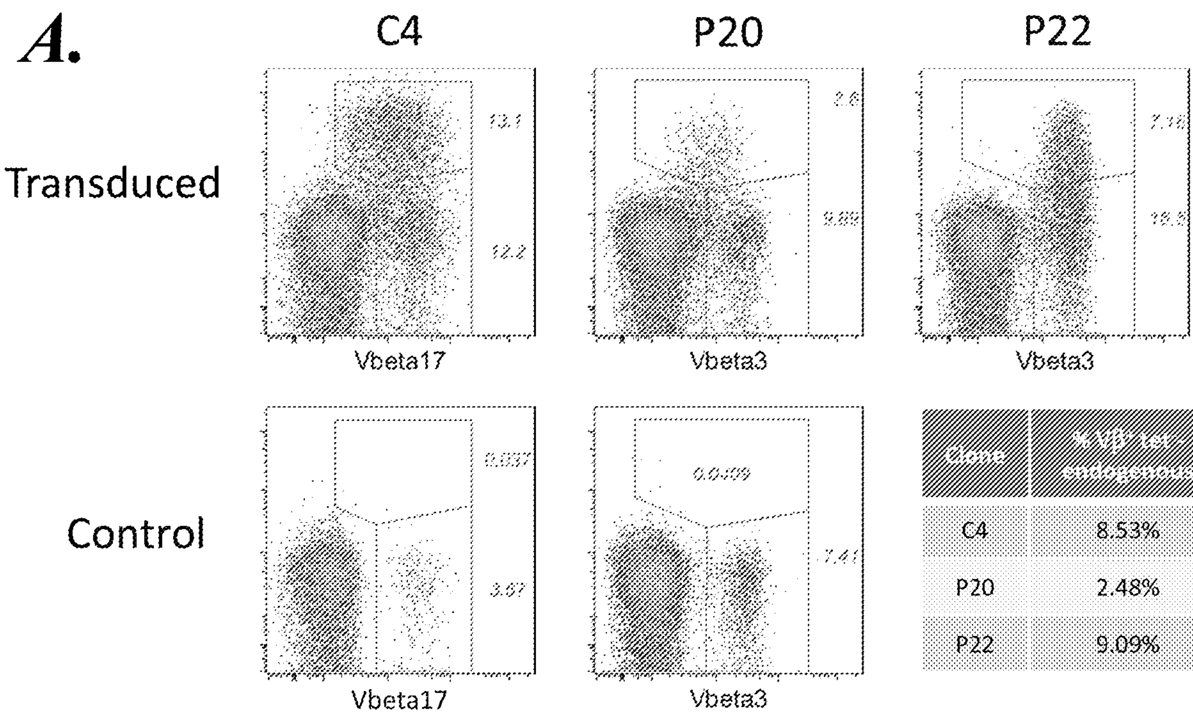


Fig. 1



Clone	% Vβ+ tet- endogenous	% Vβ+ tet+	% transduced Vβ+ tet+
C4	8.53%	13.1%	60.6%
P20	2.48%	2.6%	51.2%
P22	9.09%	7.16%	44.1%

Fig. 2A

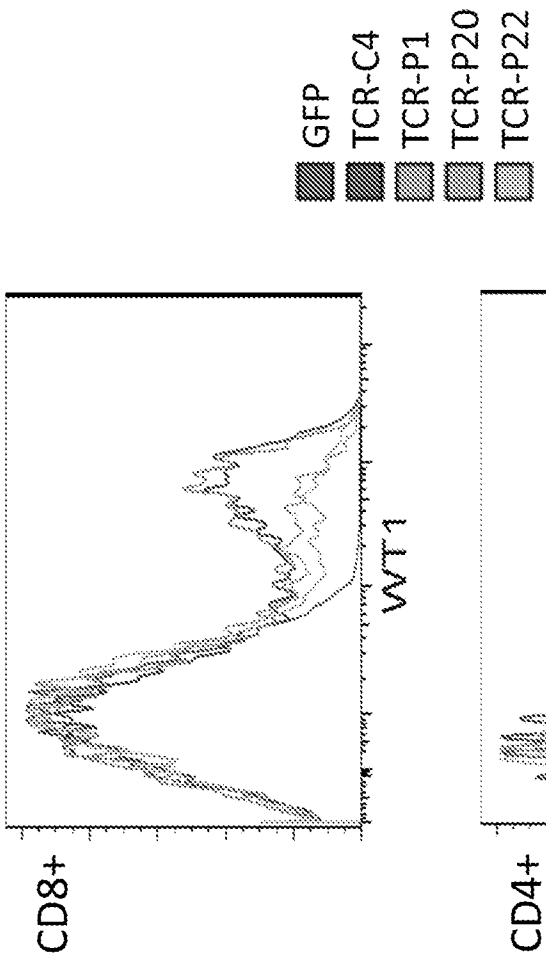


Fig. 2B

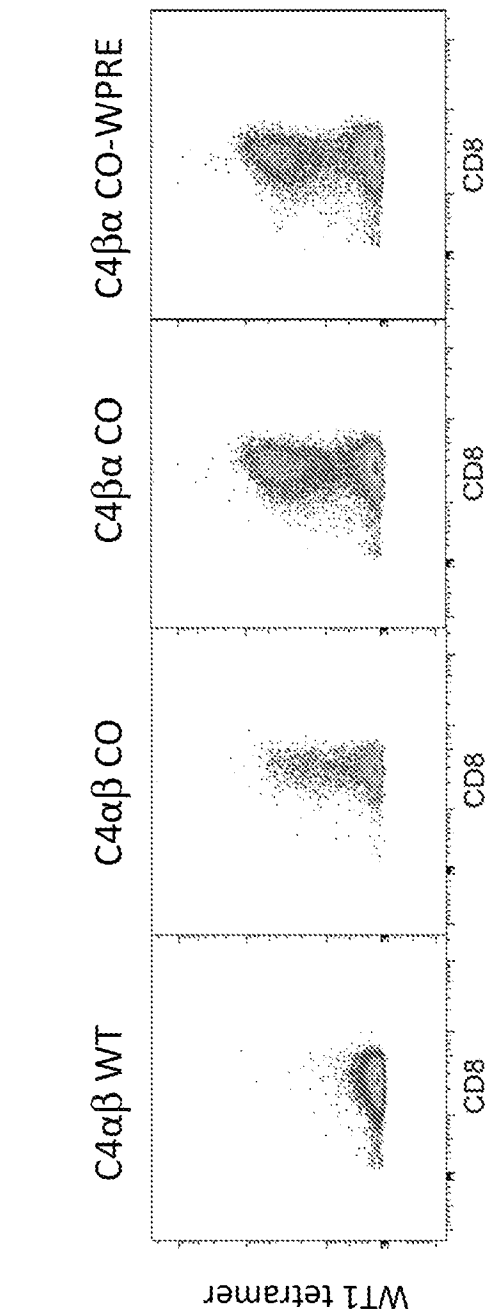

A.
WT1 tetramer
C4αβ WT
C4αβ CO
C4βα CO
C4βα CO-WPRE
CD8
CD8
CD8
CD8

Fig. 3A

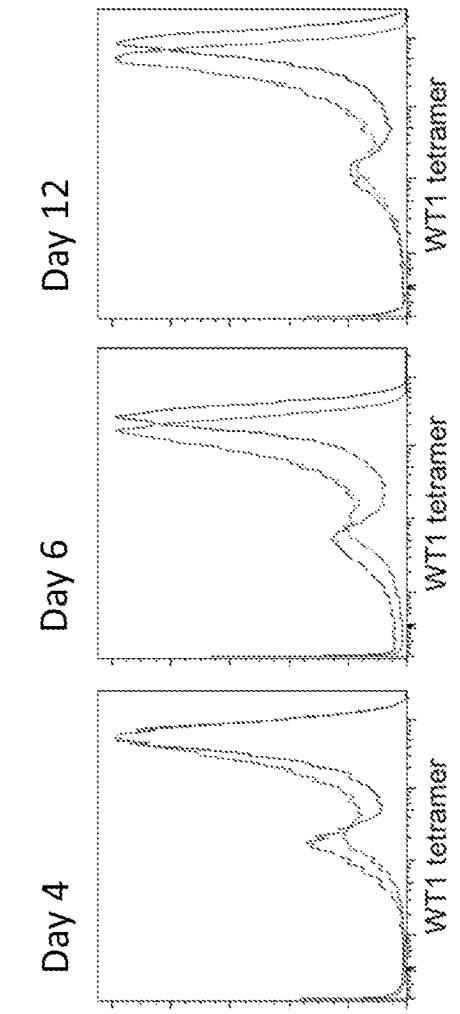

B.
Day 4
Day 6
Day 12
WT1 tetramer
WT1 tetramer
WT1 tetramer
C4αβ
C4βα

Fig. 3B

C.

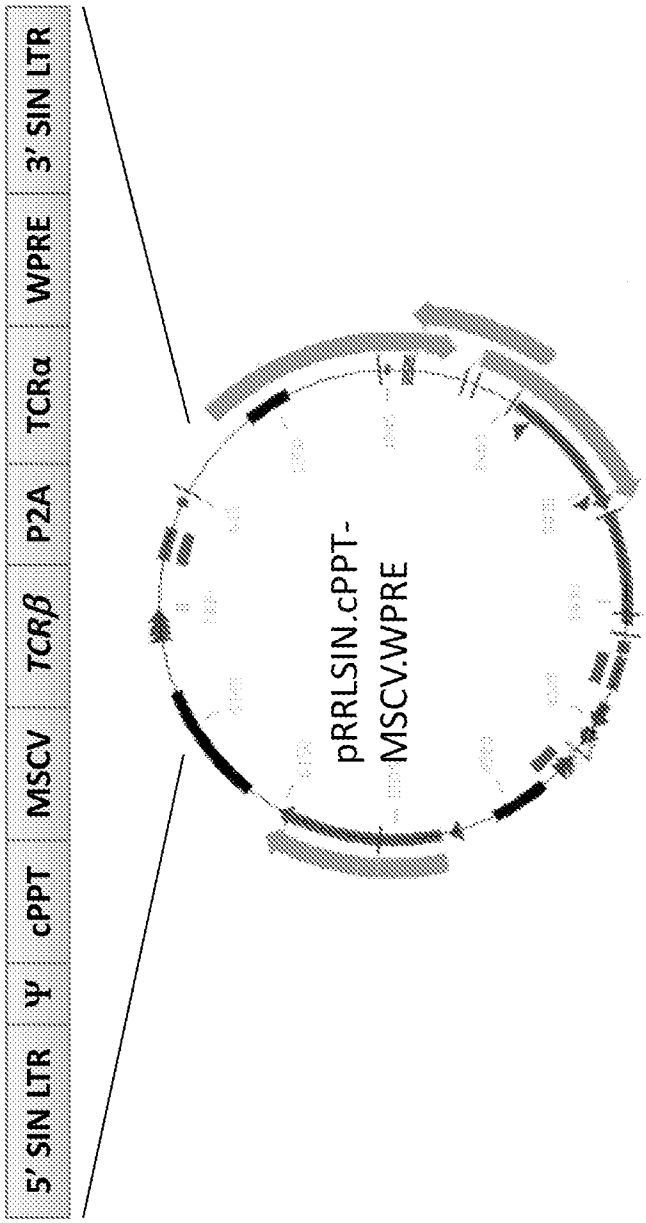

5' SIN LTR
Ψ
cPPT
MSCV
TCRβ
P2A
TCRα
WPRE
3' SIN LTR
pRRLSIN.cPPT-MSCV.WPRE

Fig. 3C

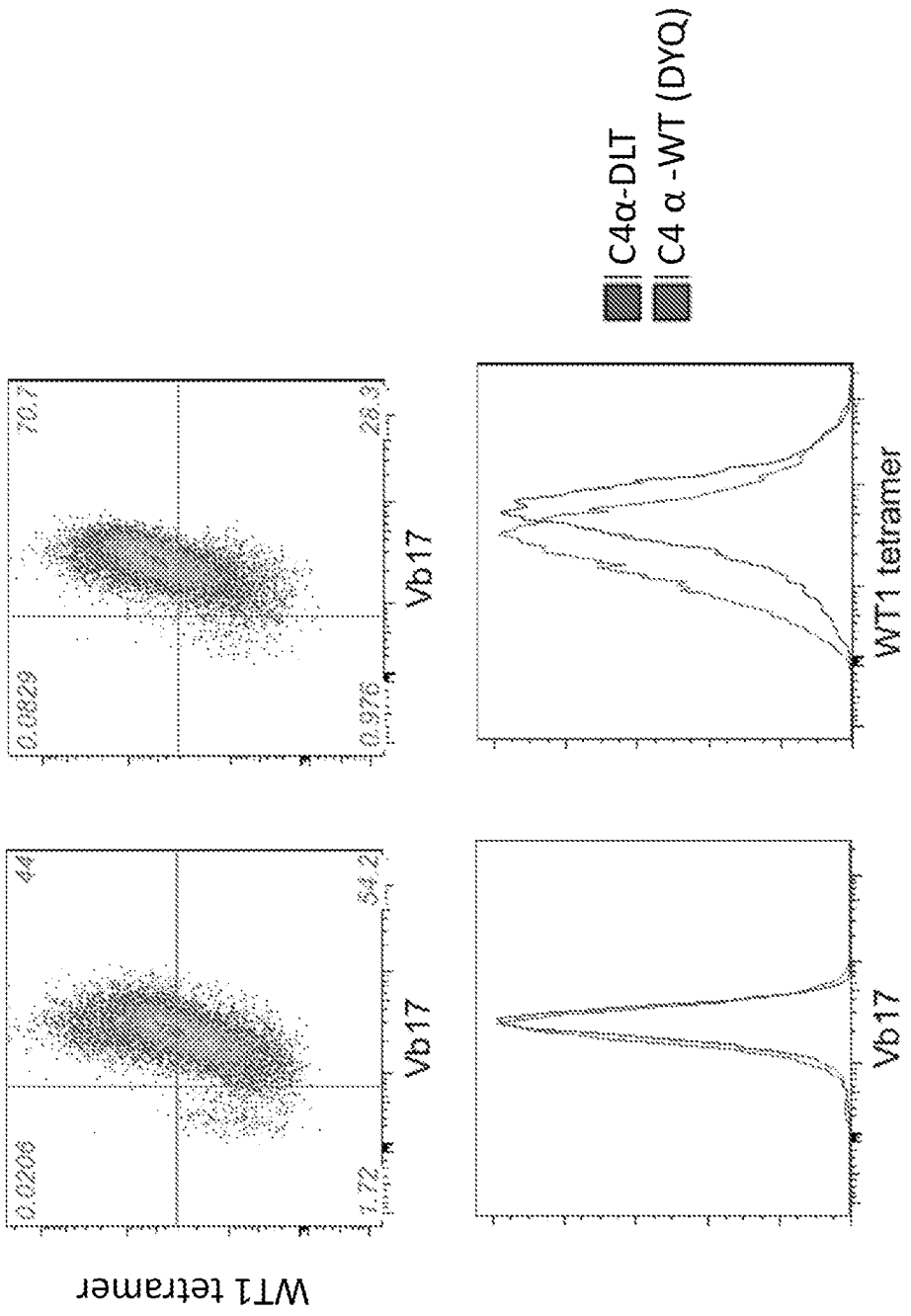

A.
WT1 tetramer
44
0.0206
1.72
54.2
Vb17
70.7
0.0829
0.976
28.9
Vb17
Vb17
WT1 tetramer
C4α-DLT
C4 α -WT (DYQ)

Fig. 4A

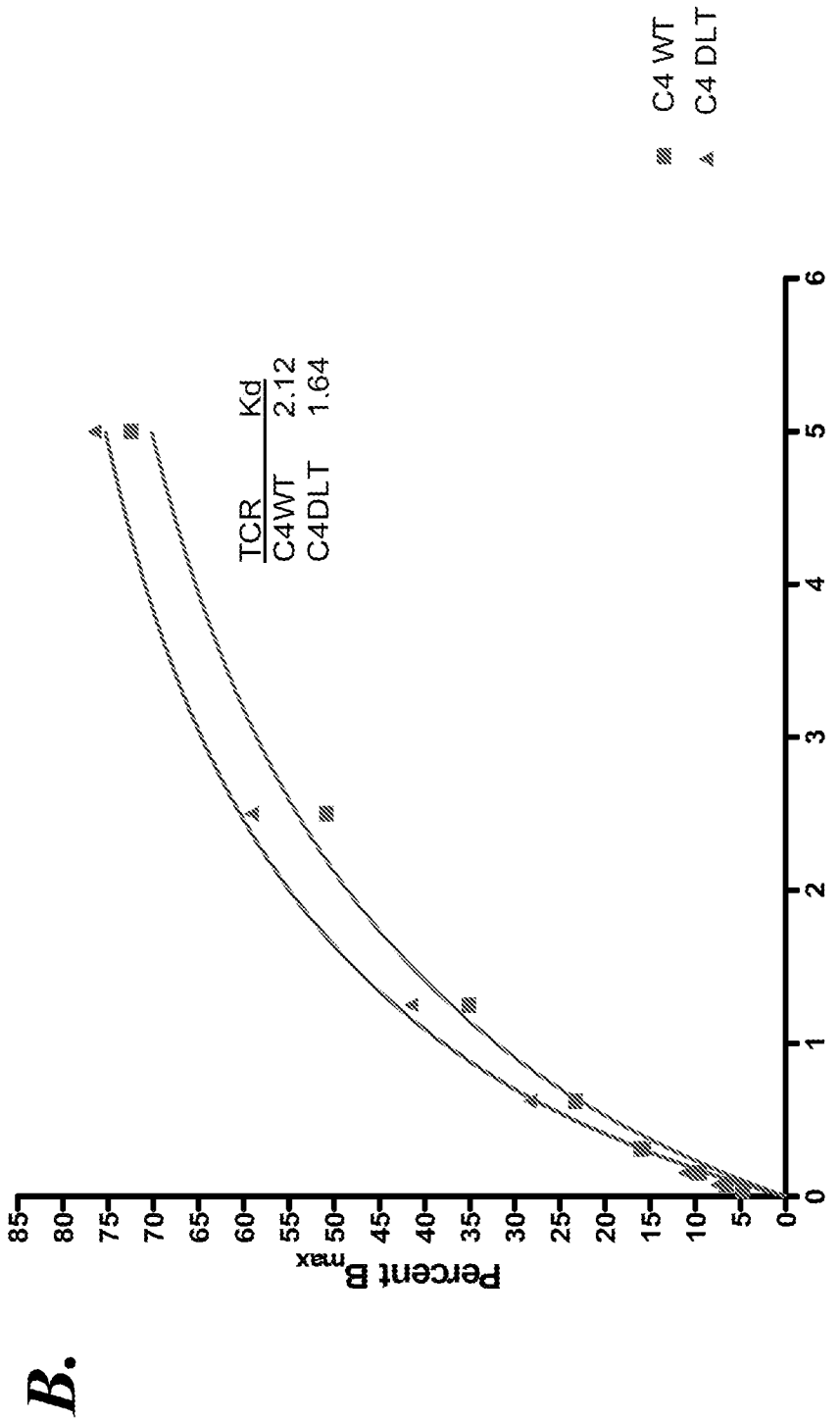

B.
Percent B max
ul WT1 tetramer
C4 WT
C4 DLT
TCR Kd
C4WT 2.12
C4DLT 1.64
0
5
10
15
20
25
30
35
40
45
50
55
60
65
70
75
80
85
0
1
2
3
4
5
6

Fig. 4B

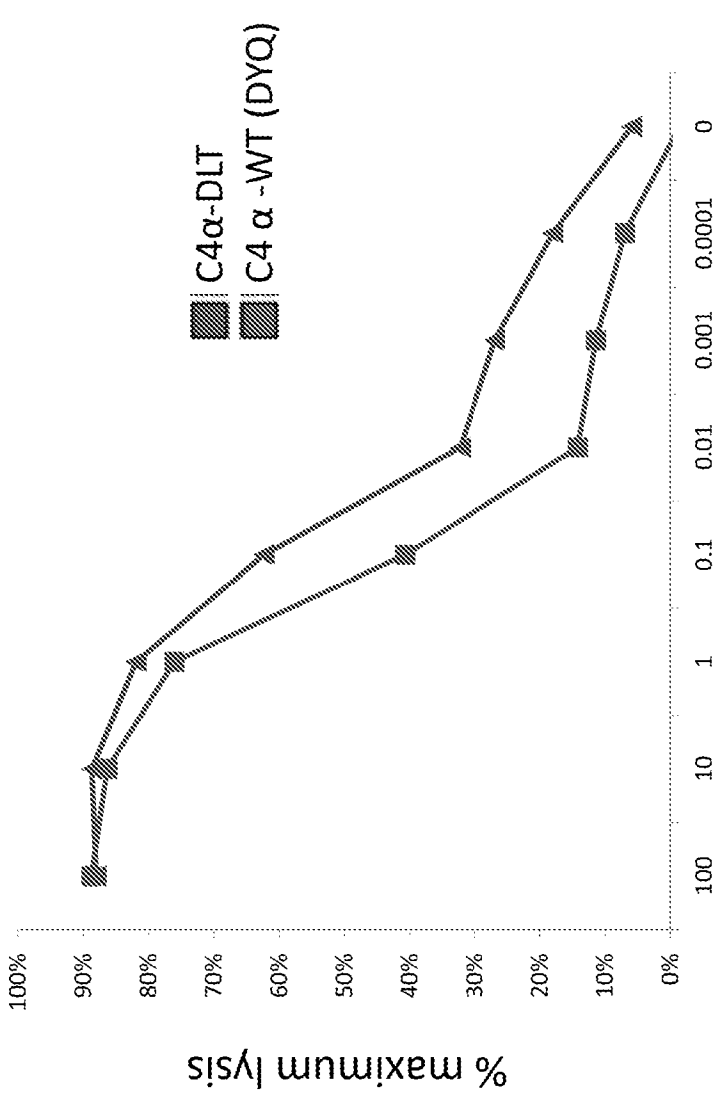

C.
% maximum lysis
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
100
10
1
0.1
0.01
0.001
0.0001
0
WT1 peptide (nM)
C4α-DLT
C4 α -WT (DYQ)

Fig. 4C

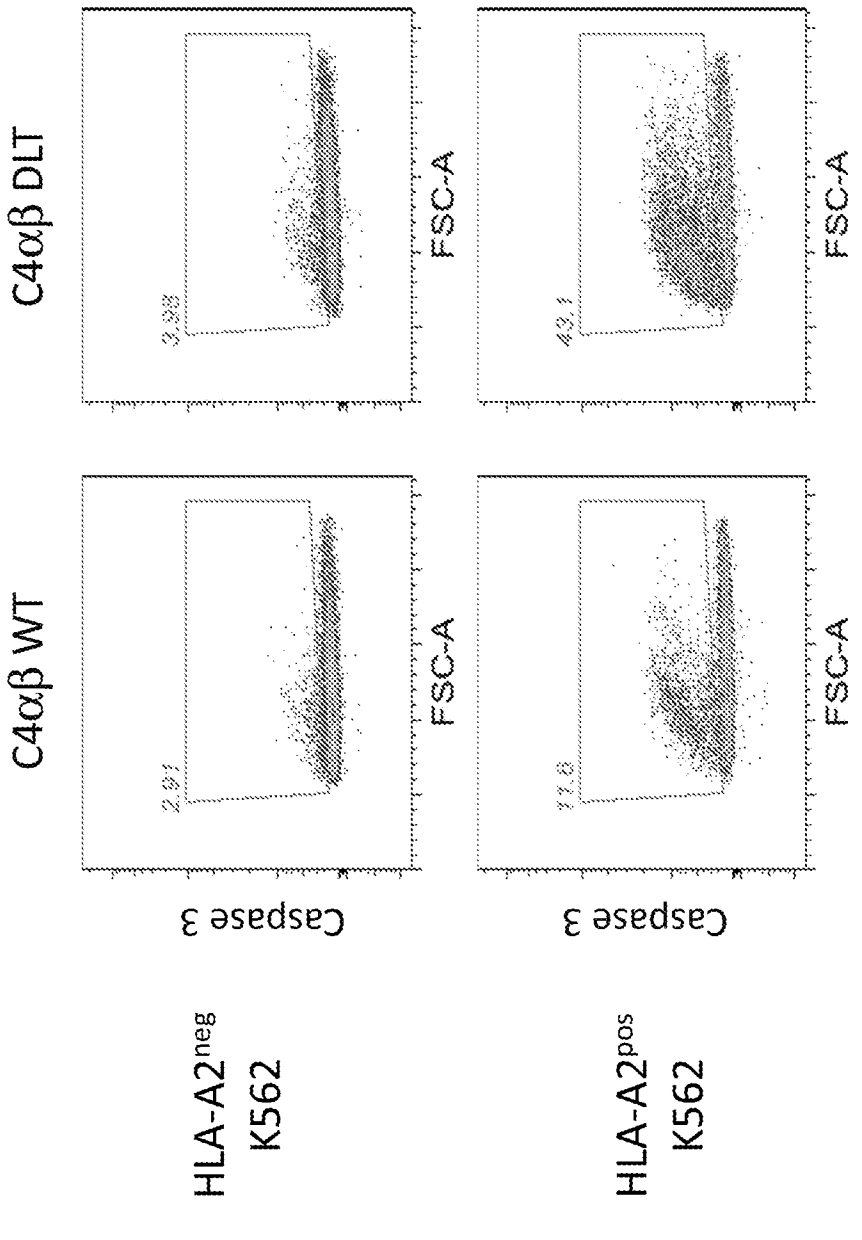

C4αβ WT
C4αβ DLT
HLA-A2neg K562
HLA-A2pos K562
Caspase 3
FSC-A
2.91
3.38
11.8
43.1

Fig. 5

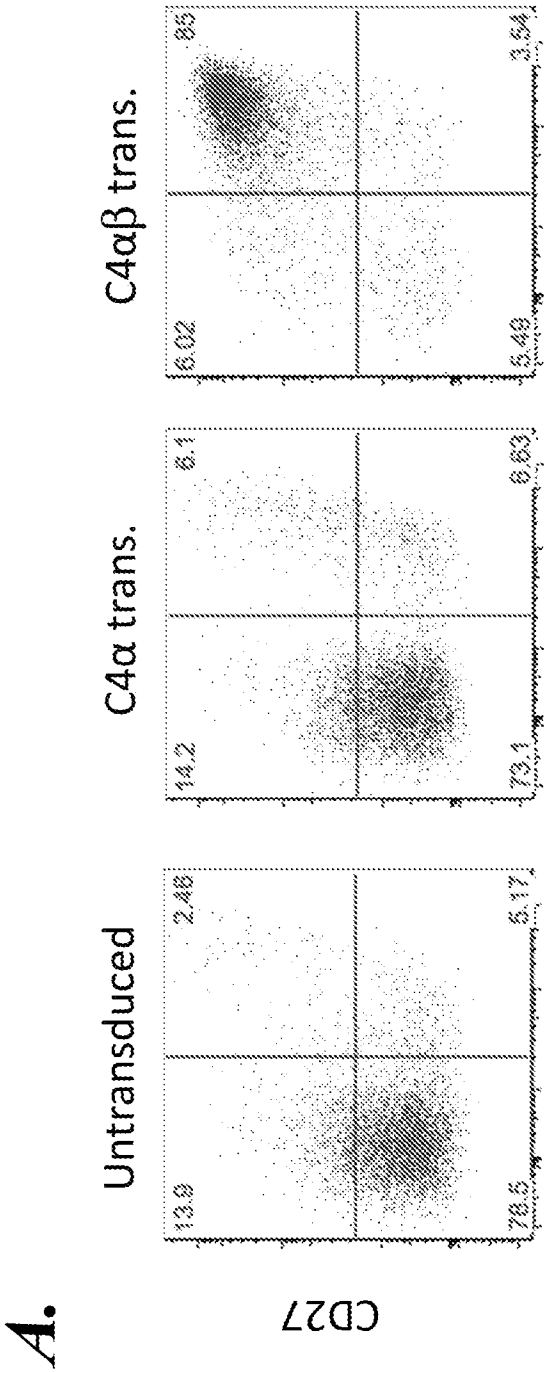

A.
CD27
CD3
Untransduced
C4α trans.
C4αβ trans.
13.9
2.46
78.5
5.17
14.2
6.1
73.1
6.63
6.02
85
5.49
3.64

Fig. 6A

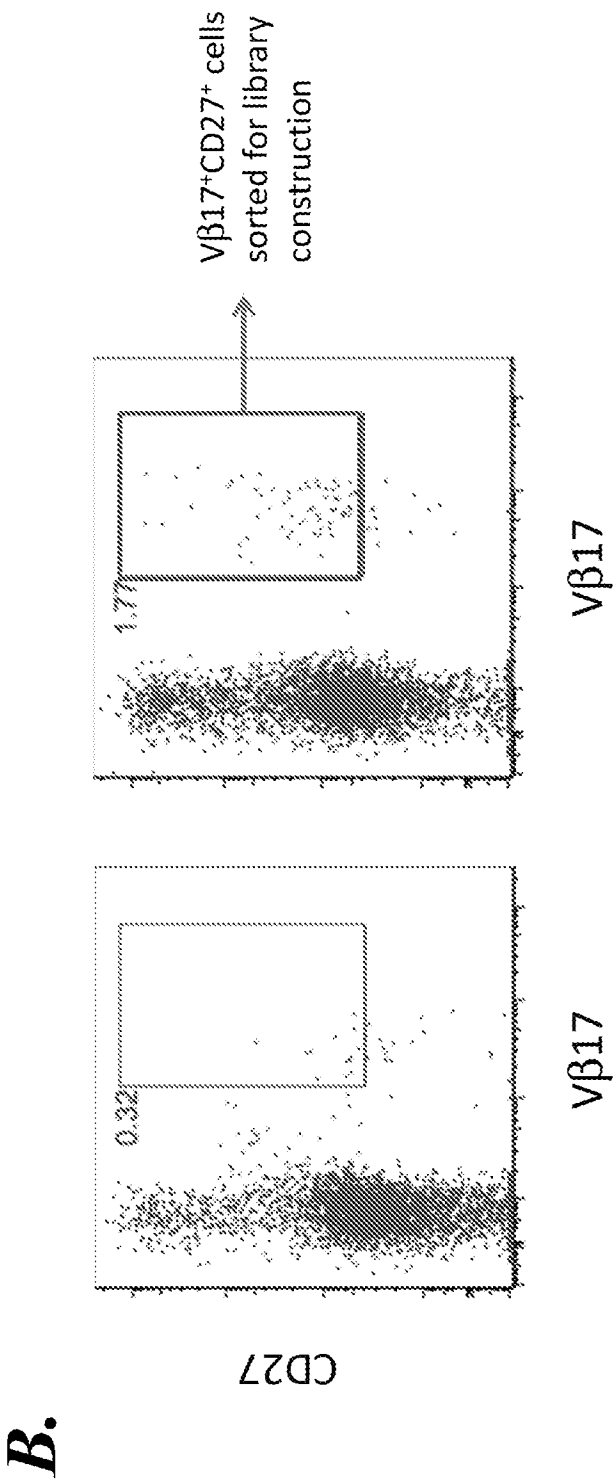

B.
CD27
Vβ17
0.32
Vβ17
1.77
Vβ17+CD27+ cells sorted for library construction

Fig. 6B

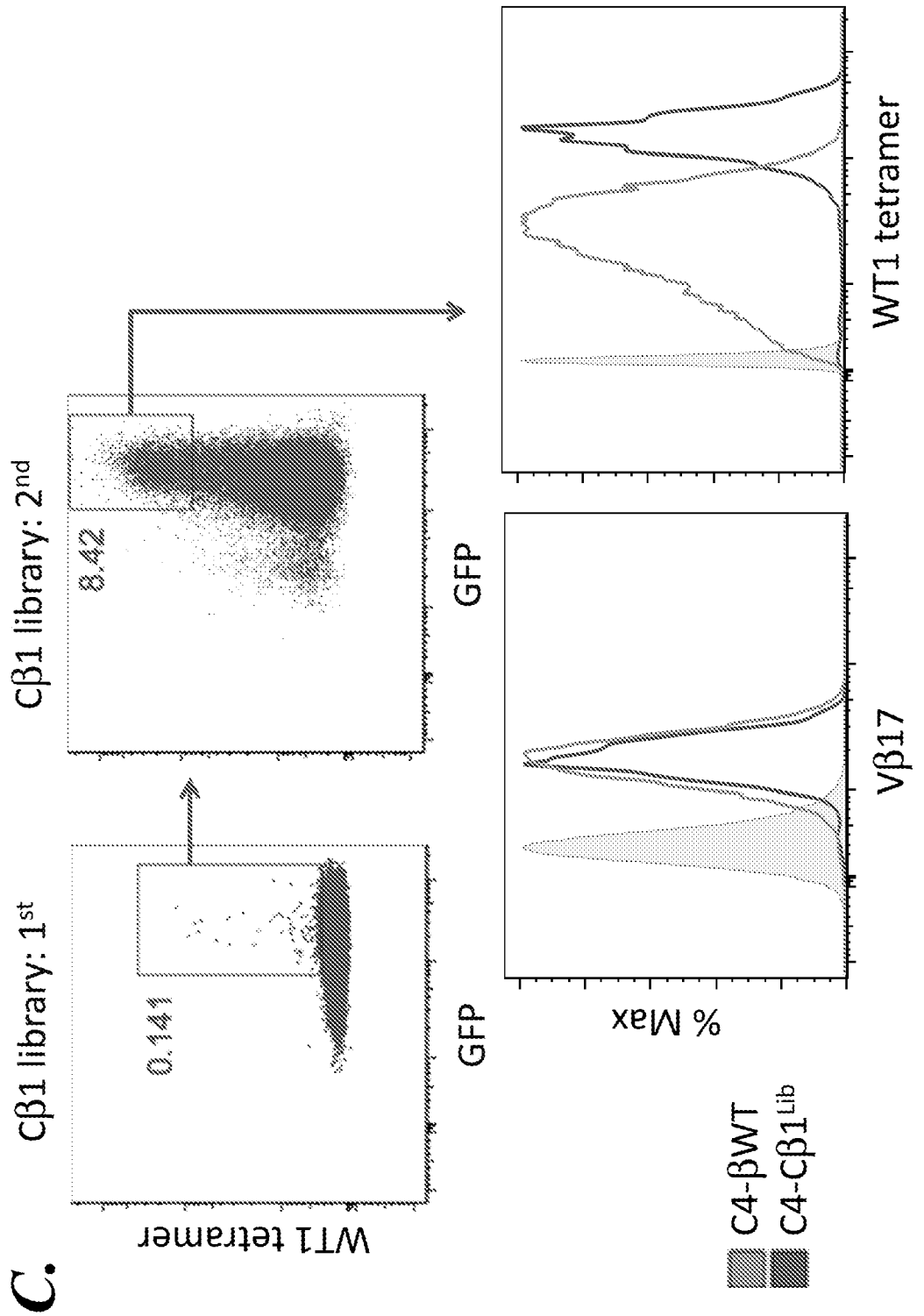

C.
WT1 tetramer
Cβ1 library: 1st
0.141
GFP
Cβ1 library: 2nd
8.42
GFP
% Max
Vβ17
WT1 tetramer
C4-βWT
C4-Cβ1Lib

Fig. 6C

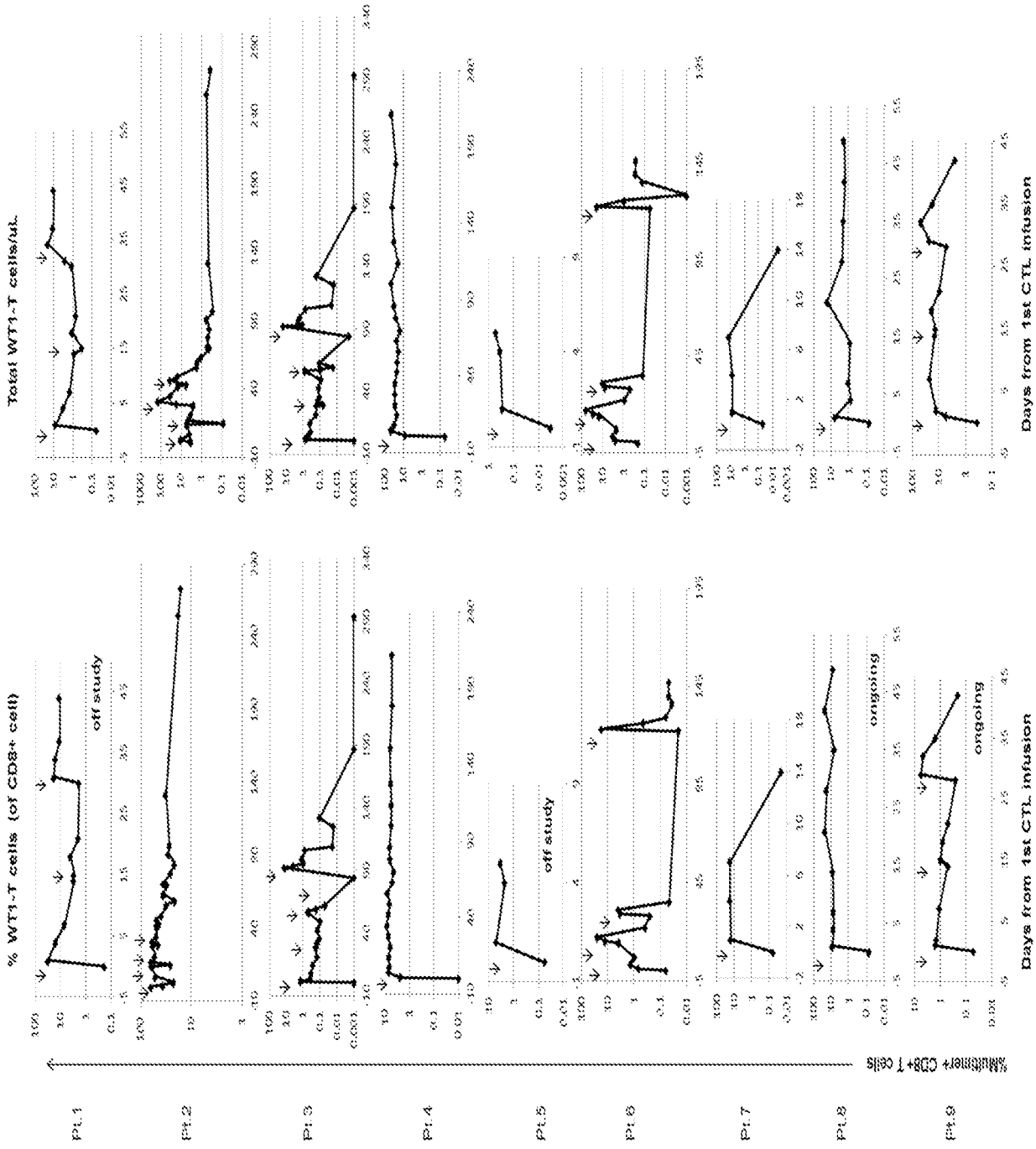

% WT1-T cells (of CD8+ cell)
Total WT1-T cells/uL
Multimer+ CD8+ T cells
Days from 1st CTL infusion
off study
ongoing
Pt.1
Pt.2
Pt.3
Pt.4
Pt.5
Pt.6
Pt.7
Pt.8
Pt.9

Fig. 7

A.

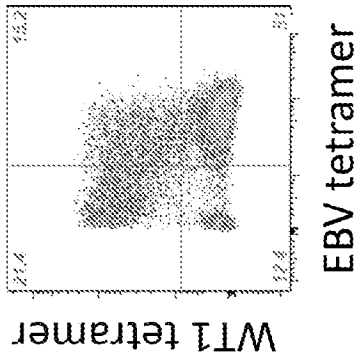

Day 0:
Stim with EBV peptide
Day 1:
Transduce with C4 TCR
12 days
WT1 tetramer
EBV tetramer

Fig. 8A

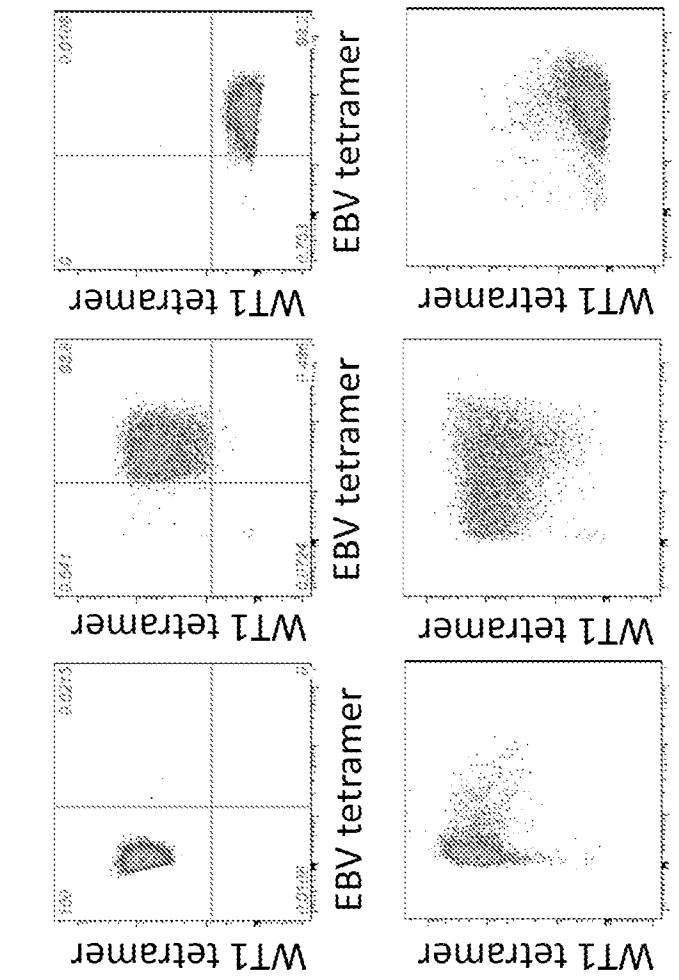


Fig. 8B

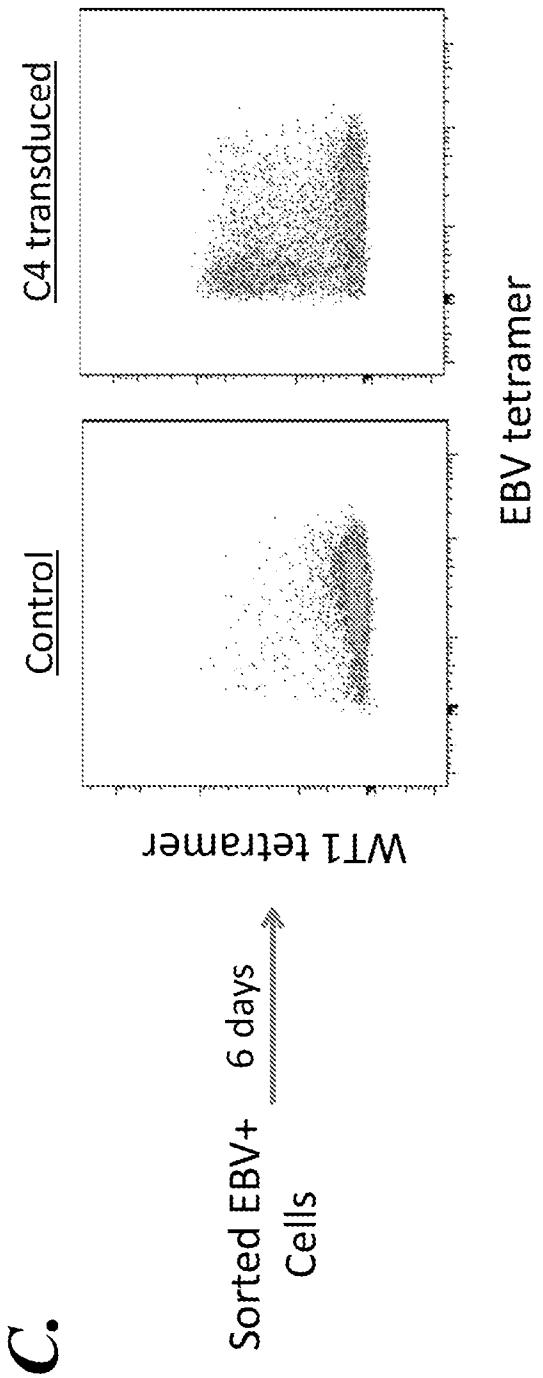

C.
Sorted EBV+ Cells
6 days
WT1 tetramer
Control
C4 transduced
EBV tetramer

Fig. 8C

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2015/042986

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K38/17 A61K39/00 C07K14/725
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K C07K

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, BIOSIS, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2013/166321 A1 (HUTCHINSON FRED CANCER RES [US]) 7 November 2013 (2013-11-07) page 36, line 4 - line 14 -----	1-128
X	Ingunn M Stromnes ET AL: "Re-adapting T cells for cancer therapy: from mouse models to clinical trials Authors' addresses", , vol. 257, no. 1 1 January 2014 (2014-01-01), pages 145-164, XP055215685, Retrieved from the Internet: URL:http://onlinelibrary.wiley.com/doi/10.1111/imr.12141/abstract;jsessionid=A40FBB72CB716E50B4627F47F14E7EE0.f02t01 [retrieved on 2015-09-23] page 153, left-hand column -----	1,23,42

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[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

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"P" document published prior to the international filing date but later than the priority date claimed

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"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
5 October 2015	13/10/2015

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Lanzrein, Markus

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INTERNATIONAL SEARCH REPORT

International application No
PCT/US2015/042986

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

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A	WO 2014/018863 A1 (UNIV ILLINOIS [US]) 30 January 2014 (2014-01-30) paragraphs [0008], [0055]; figure 1; sequence 9 figure 17E -----	1-128
A	JÜRGEN KUBALL ET AL: "Facilitating matched pairing and expression of TCR chains introduced into human T cells", BLOOD, AMERICAN SOCIETY OF HEMATOLOGY, US, vol. 109, 15 March 2007 (2007-03-15), pages 2331-2338, XP007906412, ISSN: 0006-4971, DOI: 10.1182/BLOOD-2006-05-023069 [retrieved on 2006-11-02] abstract -----	1-128
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A	WO 2012/038055 A1 (UNICELL GMBH [DE]; UNIVERSITAETSMEDIZIN DER JOHANNES GUTENBERG UNI MAI) 29 March 2012 (2012-03-29) sequences 80, 95 -----	1-128

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/US2015/042986

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		EP 2618835 A1	31-07-2013
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		US 2013273647 A1	17-10-2013
		WO 2012038055 A1	29-03-2012