T CELL RECEPTOR-LIKE ANTIBODIES SPECIFIC FOR A WT1 PEPTIDE PRESENTED BY HLA-A2

Cross-Reference to Related Applications

[0001] This application claims priority from U.S. Provisional Application No. 61/470,635, filed April 1, 2011, and U.S. Provisional Application No. 61/491,392 filed May 31, 2011. These Provisional Applications are hereby incorporated by reference in their entirety into the present disclosure.

Statement of Rights Under Federally-Sponsored Research

[0002] This invention was made with government support under grants P01CA23766 and R01CA55349 awarded by the U.S. National Institutes of Health. The government has certain rights in the invention.

Sequence Listing

[0003] This application contains a Sequence Listing, created on March 29, 2012; the file, in ASCII format, is designated 3314013AWO_Sequence Listing_ST25.txt . The file is hereby incorporated by reference in its entirety into the application

Technical Field

[0004] The present invention relates generally to antibodies against cytosolic proteins. More particularly, the invention relates to antibodies against Wilm's tumor oncogene protein (WT1), specifically antibodies that recognize a WT1 peptide in conjunction with a major histocompatability antigen.

Background of the Invention

[0005] The Wilms' tumor oncogene protein (WT1) is an attractive target for immunotherapy for most leukemias and a wide range of cancers. WT1 is a zinc finger transcription factor that is normally expressed in mesodermal tissues during embryogenesis. In normal adult tissue, WT1 expression is limited to low levels in $CD34^+$ hematopoietic stem cells but is over-expressed in leukemias of multiple lineages and a wide range of solid tumors (1-2). More recently, WT1 expression has been reported to be a marker of minimal residual disease. Increasing transcript

levels in patients with acute myeloid leukemia (AML) in morphologic remission have been predictive of overt clinical relapse (3, 4). Furthermore, antibodies to WT1 are detected in patients with hematopoietic malignancies and solid tumors, indicating that WT1 is a highly immunogenic antigen (7).

[0006] For the most part, clinically approved therapeutic monoclonal antibodies (mAbs) recognize structures of cell surface proteins. WT1, however, is a nuclear protein and, therefore, is inaccessible to classical antibody therapy. Up until now, immunotherapy targeting WT1 has been limited to cellular approaches, exclusively aimed at generating WT1-specific cytotoxic CD8 T cell (CTL) responses that recognize peptides presented on the cell surface by MHC class I molecules.

[0007] For induction of CTL responses, intracellular proteins are usually degraded by the proteasome or endo/lysosomes, and the resulting peptide fragments bind to MHC class I or II molecules. These peptide-MHC complexes are displayed at the cell surface where they provide targets for T cell recognition via a peptide-MHC (pMHC)-T cell receptor (TCR) interaction (8, 9). Vaccinations with peptides derived from the WT1 protein induce HLA-restricted cytotoxic CD8 T cells, which are capable of killing tumor cells.

[0008] To improve efficacy, cancer antigens can be targeted with monoclonal antibody therapy. Monoclonal antibody (mAb) therapy has been shown to exert powerful antitumor effects by multiple mechanisms, including complement-dependent cytotoxicity (CDC), antibody-dependent cellular cytotoxicity (ADCC) and direct cell inhibition or apoptosis-inducing effects on tumor cells that over-express the target molecules. Furthermore, mAb can be used as carriers to specifically deliver a cytotoxic moiety such as a radionuclide, cytotoxic drug or toxin to the tumor cells (18).

[0009] A tremendous benefit would exist if, in addition to a cellular immunotherapy approach, a humoral immunotherapy approach was available to target non-cell surface tumor antigens. Therefore, a monoclonal antibody (mAb) that mimics a T cell receptor in that it is specific for a target comprising a fragment of an intracellular protein in conjunction with an MHC molecule, for example, a WT1 peptide/HLA-A2 complex, would be a novel and effective therapeutic agent alone or as a vehicle

capable of delivering potent anti-cancer reagents, such as drugs, toxins and radioactive elements. Such mAbs would also be useful as diagnostic or prognostic tools.

Summary of the Invention

[0010] The present disclosure identifies and characterizes antigen-binding proteins, such as antibodies, that are able to target cytosolic/intracellular proteins, for example, the WT1 oncoprotein. The disclosed antibodies target a peptide/MHC complex as it would typically appear on the surface of a cell following antigen processing of WT1 protein and presentation by the cell. In that regard, the antibodies mimic T-cell receptors in that the antibodies have the ability to specifically recognize and bind to a peptide in an MHC-restricted fashion, that is, when the peptide is bound to an MHC antigen. The peptide/MHC complex recapitulates the antigen as it would typically appear on the surface of a cell following antigen processing and presentation of the WT1 protein to a T-cell.

[0011] The antibodies disclosed specifically recognize and bind to epitopes of a peptide/HLA-A2 complex, particularly a WT1/HLA-A0201 complex. Examples of peptides that are recognized by the antigen-binding proteins of the invention as part of an HLA-peptide complex include, but are not limited to, those shown in Table 7, for example, a peptide with the amino acid sequence RMFPNAPYL (SEQ ID NO: 1.)

[0012] In one aspect, therefore, the invention relates to an isolated antibody, or antigen-binding fragment thereof, that binds to a peptide with the amino acid sequence, RMFPNAPYL, when said peptide is bound to an MHC antigen, such as HLA-A2.

[0013] In another aspect, the invention relates to an isolated antigen-binding protein, antibody, or antigen-binding fragment thereof, comprising (A) (i) a heavy chain (HC) variable region comprising HC-CDR1, HC-CDR2 and HC-CDR3 respectively, comprising amino acid sequences SEQ ID NOS: 2, 3, and 4; and a light chain (LC) variable region comprising LC-CDR1, LC-CDR2 and LC-CDR3 respectively, comprising amino acid sequences SEQ ID NOS: 8, 9 and 10; (ii) a heavy chain (HC) variable region comprising HC-CDR1, HC-CDR2 and HC-CDR3 respectively, comprising amino acid sequences SEQ ID NOS: 20, 21and 22; and a

light chain (LC) variable region comprising LC-CDR1, LC-CDR2 and LC-CDR3 respectively, comprising amino acid sequences SEQ ID NOS: 26, 27 and 28; (iii) a heavy chain (HC) variable region comprising HC-CDR1, HC-CDR2 and HC-CDR3 respectively, comprising amino acid sequences SEQ ID NOS: 38, 39 and 40; and a light chain (LC) variable region comprising LC-CDR1, LC-CDR2 and LC-CDR3 respectively, comprising amino acid sequences selected from SEQ ID NOS: 44, 45 and 46; (iv) a heavy chain (HC) variable region comprising HC-CDR1, HC-CDR2 and HC-CDR3 respectively, comprising amino acid sequences SEQ ID NOS: 56, 57 and 58; and a light chain (LC) variable region comprising LC-CDR1, LC-CDR2 and LC-CDR3 respectively, comprising amino acid sequences SEQ ID NOS: 62, 63 and 64; (v) a heavy chain (HC) variable region comprising HC-CDR1, HC-CDR2 and HC-CDR3 respectively, comprising amino acid sequences SEQ ID NOS: 74, 75 and 76; and a light chain (LC) variable region comprising LC-CDR1, LC-CDR2 and LC-CDR3 respectively, comprising amino acid sequences SEQ ID NOS: 80, 81 and 82; or (vi) a heavy chain (HC) variable region comprising HC-CDR1, HC-CDR2 and HC-CDR3 respectively, comprising amino acid sequences SEQ ID NOS: 92, 93 and 94; and a light chain (LC) variable region comprising LC-CDR1, LC-CDR2 and LC-CDR3 respectively, comprising amino acid sequences SEQ ID NOS: 98, 99 and 100.

[0014] In another aspect, the invention relates to an isolated antigen-binding protein, antibody, or antigen-binding fragment thereof, comprising a V_H and V_L comprising first and second amino acid sequences, respectively, selected from SEQ ID NOS: 14 and 16; 32 and 34; 50 and 52; 68 and 70; 86 and 88; and 104 and 106.

[0015] In yet another aspect, the invention relates to an isolated antigen-binding protein, antibody, or antigen-binding fragment thereof,comprising an amino acid sequence selected from SEQ ID NOS: 18, 36, 54, 72, 90, and108.

[0016] In a related aspect, the isolated antigen-binding protein comprises an antigen-binding region as disclosed in any of Tables 1-8. The antigen-binding protein may be a fusion protein.

[0017] In another aspect, the invention relates to an immunoconjugate comprising a first component which is an antigen-binding protein, antibody or antigen-binding fragment thereof as disclosed herein. The immunoconjugate comprises a second

component that is a cytotoxin, a detectable label, a radioisotope, a therapeutic agent, a binding protein or a molecule having a second amino acid sequence. Where the second component is a binding protein or second antibody, the binding protein or second antibody has binding specificity for a target that is different from the HLA-peptide complex for which the first is specific.

[0018] In a related aspect, therefore, the present invention relates to bispecific antibody comprising an antigen-binding protein or functional fragment thereof as described herein.

[0019] In yet another aspect, the invention relates to nucleic acids that encode antigen binding proteins, including antibodies and chimeric antigen receptors specific for a WT1 peptide/HLA complex, in particular the complex of WT1 peptide RMFPNAPYL/HLA-A0201.

[0020] In another related aspect, the invention relates to cells comprising the nucleic acids or antigen binding proteins disclosed herein, including recombinant immune effector cells, such as, T-cells genetically modified to express a chimeric antigen receptor comprising an antigen binding region in accordance with the present disclosure. Cells which have been engineered to produce antibodies in accordance with the disclosure are also encompassed by the invention.

[0021] In a related aspect, the invention relates to vectors comprising the nucleic acids to encode the antigen binding proteins disclosed herein, including vectors to facilitate expression and/or secretion of an antigen binding protein such as an antibody or chimeric antigen receptor in accordance with the present disclosure.

[0022] In a related aspect, the invention relates to pharmaceutical compositions comprising the antigen binding proteins, antibodies, nucleic acids, vectors or cells comprising the nucleic acids or antigen binding proteins disclosed herein, together with a pharmaceutically acceptable carrier.

[0023] In another aspect, the invention relates to a method for detecting WT1 on the surface of cells or tissues using WT1 antibodies of the invention.

[0024] In yet another aspect, the invention relates to methods for treatment of a subject having a WT1-positive disease, comprising administering to the subject a therapeutically effective amount of an antigen binding protein, antibody or antigen binding fragment thereof, nucleic acid encoding the antigen binding protein or antibody or a cell comprising the nucleic acids or proteins as disclosed herein. The WT1-positive disease is a chronic leukemia, acute leukemia or $WT1^+$ cancer selected from the group consisting of chronic myelocytic leukemia, multiple myeloma (MM), acute lymphoblastic leukemia (ALL), acute myeloid/myelogenous leukemia (AML), myelodysplastic syndrome (MDS), mesothelioma, ovarian cancer, gastrointestinal cancers, breast cancer, prostate cancer and glioblastoma. In some embodiments, the antigen binding protein or antibody is a conjugate thereof having a cytotoxic moiety linked thereto.

Brief Description of the Drawings

[0025] Figure 1 shows the amino acid sequence of Wilms tumor protein, (GenBank Accession No. P19544) with some HLA-restricted peptides bolded. The 121-140 peptide further encompasses a 9-mer (underlined), RMFPNAPYL (SEQ ID NO: 1), which, in addition to analogs thereof, has been shown to induce WT1-specific cytotoxic T-cell activity.

[0026] Figure 2 is a graph showing that vaccination with WT1peptides induces cytotoxic T cells against $WT1^+$ leukemia cells.

[0027] Figure 3 shows the results of a phage ELISA for specific binding of WT1/A2 (WA) versus PBS control or R3/HLAA0201 (R3).

[0028] Figure 4 shows specific binding of only WT1 phage antibodies that bind to T2 cells pulsed with WT1A peptide were selected.

[0029] Figure 5 shows the binding affinity of a full-length IgG1 of a WT1 antibody to RMF/A0201 complex tested by titration of the antibody at various concentrations. Results are shown for T2 cells pulsed with 50ug/ml RMF (upper panel). Control antibody is shown in the lower panel.

[0030] Figure 6 shows the dependence on density of RMF/HLA-A0201 complex recognized by WT1 antibody on T2 cells pulsed with RMF (upper panel) or control, RHAMM-R3 (lower panel).

[0031] Figure 7 shows an expression vector for expression of human antibodies.

[0032] Figure 8 shows the results of SDS-PAGE analysis of WT1/A2 antibodies under reducing and non-reducing conditions.

[0033] Figure 9 shows the results of kinetic binding analysis of an WT1/A2 antibody demonstrating affinity of the antibody toward WT1/A2.

[0034] Figure 10 shows the affinity (K_D) of antibody binding to WT1/A2 complex.

[0035] Figure 11 shows the mean fluorescence intensity (MFI) by flow cytometry of peptide titration on binding of some embodiments, mAb clone 5 (upper panel), clone 15 (middle panel) and control (lower panel) to live T2 cells pulsed with varying concentrations of peptide, WT1-A, WT1-A1 or control.

[0036] Figure 12 shows the results of peptide titration on binding of a WT1 antibody, mAb 5 (upper panel), mAb 15 (lower panel) to live T2 cells pulsed with varying concentrations of WT1A peptide.

[0037] Figure 13 shows the binding specificity of one embodiment, mAb 5, at different concentrations (50 µg/ml upper; 25 µg/ml middle; and 12.5 µg/ml lower) of peptide (R3, WT1-A1, WT1-A or no peptide.)

[0038] Figure 14 shows the binding specificity of one embodiment, mAb 15, at different concentrations (50 µg/ml upper; 25 µg/ml lower) of peptide (R3, WT1-A1, WT1-A or no peptide).

[0039] Figure 15 shows dose-dependent binding of mAbs 5 (upper panel) and 15 (lower panel) to T2 cells pulsed with WT1-A, WT1-A1, or RHAMM-R3 peptide.

[0040] Figure 16 shows binding of mAbs 5 and 15 to U266, a myeloma cell line.

[0041] Figure 17 shows binding of mAb 15 to BV173, a cell line derived from an individual with (Ph1)-positive acute leukemia.

[0042] Figure 18 shows the specific binding of ESK1 (#13) to WT1/A2 complex on the surface of T2 cells pulsed with WT1 peptide.

[0043] Figure 19 and 20 show that WT1 antibody is able to recognize RMF peptide in which substitution of different positions of the RMF peptide with alanine is made (see also Table 10) and that the loss of binding seen with substitution of position 1 by either alanine (WT1-A1-B) or tyrosine (WT1-A1), was not due to the reduction of peptide binding affinity to the HLA-A2 molecule, as both peptides showed the strongest binding in T2 stabilization assay using the mAb specific for the HLA-A2 molecule, clone BB7.

[0044] Figure 21 shows recognition by WT1 antibody of naturally presented RMF/HLA-A0201 complex on the cell surface of human mesothelioma cell lines, JMN ($WT1^+/A0201^+$) but not MSTO ($WT1^+/HLA\text{-}A0201^-$).

[0045] Figure 22 shows binding of WT1 antibodies to human CML-derived cell line BV173.

[0046] Figure 23 is a Scatchard analysis based on binding of WT1 antibody to JMN cells and shows an avidity constant of about 0.2nM.

[0047] Figure 24 shows WT1 antibody binding to a panel of mesothelioma and leukemia cells.

[0048] Figure 25 shows the results of flow cytometric analyses gated on CD33 and CD34 double positive AML blast cells from an HLA-A2 positive patient. ESK1 binds to the leukemia blasts.

[0049] Figure 26 shows the results of flow cytometric analyses gated on CD33 and CD34 double positive AML blast cells from an HLA-A2 negative patient. WT1mAb ESK1 did not bind to the blasts.

[0050] Figure 27 shows WT1mAb ESK1 mediated ADCC against T2 cells pulsed with RMF peptide.

[0051] Figure 28 shows the ability of WT1 antibody to mediate ADCC with human effectors in JMN and leukemia cell line BV173 (lower panel) but not MSTO cells.

[0052] Figure 29 shows that WT1 mAbs are effective against human leukemia cell line BV173 but not HL60 cells, which are not HLA-$A2^+$.

[0053] Figure 30 shows that WT1 antibody induces ADCC against primary AML blasts from an HLA-A2 positive patient.

[0054] Figure 31shows the results of treatment of human BV173 in NSG mice using antibodies of the invention.

[0055] Figure 32 shows that at later time points, mice treated with WT1 antibody only began to relapse, while antibody with effectors cured 2 of 5 mice.

[0056] Figure 33 shows that WT1 antibody significantly reduces tumor burden in a dose-dependent manner.

[0057] Figure 34 shows that antibody with altered carbohydrate in Fc (MAGE) is more active in ADCC than original antibody.

Detailed Description of the Invention

[0058] All publications, patents and other references cited herein are incorporated by reference in their entirety into the present disclosure.

[0059] In practicing the present invention, many conventional techniques in molecular biology, microbiology, cell biology, biochemistry, and immunology are used, which are within the skill of the art. These techniques are described in greater detail in, for example, Molecular Cloning: a Laboratory Manual 3rd edition, J.F. Sambrook and D.W. Russell, ed. Cold Spring Harbor Laboratory Press 2001; Recombinant Antibodies for Immunotherapy, Melvyn Little, ed. Cambridge University Press 2009; "Oligonucleotide Synthesis" (M. J. Gait, ed., 1984); "Animal Cell Culture" (R. I. Freshney, ed., 1987); "Methods in Enzymology" (Academic Press, Inc.); "Current Protocols in Molecular Biology" (F. M. Ausubel et al., eds., 1987, and periodic updates); "PCR: The Polymerase Chain Reaction", (Mullis et al., ed., 1994); "A Practical Guide to Molecular Cloning" (Perbal Bernard V., 1988); "Phage Display: A Laboratory Manual" (Barbas et al., 2001). The contents of these references and other references containing standard protocols, widely known to and relied upon by those of skill in the art, including manufacturers' instructions are hereby incorporated

by reference as part of the present disclosure. The following abbreviations are used throughout the application:

[0060] **Ab:** Antibody

[0061] **ADCC:** Antibody-dependent cellular cytotoxicity

[0062] **ALL:** Acute lymphocytic leukemia

[0063] **AML**: Acute myeloid leukemia

[0064] **APC:** Antigen presenting cell

[0065] **β2M:** Beta-2-microglobulin

[0066] **BiTE:** Bi-specific T cell engaging antibody

[0067] **CAR:** Chimeric antigen receptor

[0068] **CDC:** Complement dependent cytotoxicity

[0069] **CMC:** Complement mediated cytotoxicity

[0070] **CDR:** Complementarity determining region (see also HVR below)

[0071] **C_L:** Constant domain of the light chain

[0072] **CH_1:** 1st constant domain of the heavy chain

[0073] **$CH_{1, 2, 3}$:** 1st, 2nd and 3rd constant domains of the heavy chain

[0074] **$CH_{2, 3}$:** 2nd and 3rd constant domains of the heavy chain

[0075] **CHO:** Chinese hamster ovary

[0076] **CTL:** Cytotoxic T cell

[0077] **E:T Ratio:** Effector:Target ratio

[0078] **Fab:** Antibody binding fragment

[0079] **FACS:** Flow assisted cytometric cell sorting

[0080] **FBS:** Fetal bovine serum

[0081] **FR:** Framework region

[0082] **HC:** Heavy chain

[0083] **HLA:** Human leukocyte antigen

[0084] **HVR-H**: Hypervariable region-heavy chain (see also CDR)

[0085] **HVR-L**: Hypervariable region-light chain (see also CDR)

[0086] **Ig:** Immunoglobulin

[0087] **IRES:** Internal ribosome entry site

[0088] K_D: *Dissociation constant*

[0089] k_{off}: Dissociation rate

[0090] k_{on}: Association rate

[0091] **MHC:** Major histocompatibility complex

[0092] **MM:** Multiple myeloma

[0093] **scFv:** Single-chain variable fragment

[0094] **TCR:** T cell receptor

[0095] V_H: Variable heavy chain includes heavy chain hypervariable region and heavy chain variable framework region

[0096] V_L: Variable light chain includes light chain hypervariable region and light chain variable framework region

[0097] **WT1:** Wilms tumor protein 1

[0098] In the description that follows, certain conventions will be followed as regards the usage of terminology. Generally, terms used herein are intended to be

interpreted consistently with the meaning of those terms as they are known to those of skill in the art.

[0099] An “antigen-binding protein” is a protein or polypeptide that comprises an antigen-binding region or antigen-binding portion, that is, has a strong affinity to another molecule to which it binds. Antigen-binding proteins encompass antibodies, chimeric antigen receptors (CARs) and fusion proteins.

[00100] "Antibody" and "antibodies" as those terms are known in the art refer to antigen binding proteins of the immune system. The term "antibody" as referred to herein includes whole, full length antibodies having an antigen-binding region, and any fragment thereof in which the "antigen-binding portion" or "antigen-binding region" is retained, or single chains, for example, single chain variable fragment (scFv), thereof. A naturally occurring "antibody" is a glycoprotein comprising at least two heavy (H) chains and two light (L) chains inter-connected by disulfide bonds. Each heavy chain is comprised of a heavy chain variable region (abbreviated herein as V_H) and a heavy chain constant (CH) region. The heavy chain constant region is comprised of three domains, CH1, CH2 and CH3. Each light chain is comprised of a light chain variable region (abbreviated herein as V_L) and a light chain constant C_L region. The light chain constant region is comprised of one domain, C_L. The V_H and V_L regions can be further subdivided into regions of hypervariability, termed complementarity determining regions (CDR), interspersed with regions that are more conserved, termed framework regions (FR). Each V_H and V_L is composed of three CDRs and four FRs arranged from amino-terminus to carboxy-terminus in the following order: FR1, CDR1, FR2, CDR2, FR3, CDR3, FR4. The variable regions of the heavy and light chains contain a binding domain that interacts with an antigen. The constant regions of the antibodies may mediate the binding of the immunoglobulin to host tissues or factors, including various cells of the immune system (e.g., effector cells) and the first component (C1q) of the classical complement system.

[00101] The term "antigen-binding portion" or "antigen-binding region" of an antibody, as used herein, refers to that region or portion of the antibody that binds to the antigen and which confers antigen specificity to the antibody; fragments of antigen-binding proteins, for example, antibodies includes one or more fragments of

an antibody that retain the ability to specifically bind to an antigen (e.g., an peptide/HLA complex). It has been shown that the antigen-binding function of an antibody can be performed by fragments of a full-length antibody. Examples of antigen-binding fragments encompassed within the term "antibody fragments" of an antibody include a Fab fragment, a monovalent fragment consisting of the V_L, V_H, C_L and CH1 domains; a $F(ab)_2$ fragment, a bivalent fragment comprising two Fab fragments linked by a disulfide bridge at the hinge region; a Fd fragment consisting of the V_H and CH1 domains; a Fv fragment consisting of the V_L and V_H domains of a single arm of an antibody; a dAb fragment (Ward et al., 1989 Nature 341:544-546), which consists of a V_H domain; and an isolated complementarity determining region (CDR).

[00102] Furthermore, although the two domains of the Fv fragment, V_L and V_H, are coded for by separate genes, they can be joined, using recombinant methods, by a synthetic linker that enables them to be made as a single protein chain in which the V_L and V_H regions pair to form monovalent molecules. These are known as single chain Fv (scFv); see e.g., Bird et al., 1988 Science 242:423-426; and Huston et al., 1988 Proc. Natl. Acad. Sci. 85:5879-5883. These antibody fragments are obtained using conventional techniques known to those of skill in the art, and the fragments are screened for utility in the same manner as are intact antibodies.

[00103] An "isolated antibody" or “isolated antigen-binding protein” is one which has been identified and separated and/or recovered from a component of its natural environment. "Synthetic antibodies" or "recombinant antibodies" are generally generated using recombinant technology or using peptide synthetic techniques known to those of skill in the art.

[00104] Traditionally, the MHC-peptide complex could only be recognized by a T-cell receptor (TCR), limiting our ability to detect an epitope of interest using T cell-based readout assays. In the present disclosure, antigen binding proteins, including antibodies, having an antigen-binding region based on scFvs that are selected from human scFv phage display libraries using recombinant HLA-peptide complexes are described. These molecules demonstrated exquisite specificity, for example as shown with anti-WT1 antibodies that recognize only HLA-A2-RMFPNAPYL complexes. In addition, along with their inability to bind to HLA-complexes

containing other peptides, the molecules were also unable to bind to the peptides themselves, further demonstrating their TCR-like specificity.

[00105] The scFvs of the disclosure selected by phage display were initially tested for their ability to bind to peptide presented on the surface of HLA-positive cells. After T2 cells were incubated in the presence of peptide, fluorescently labeled antibodies could be used to selectively recognize the antigen pulsed cells using flow cytometry.

[00106] In some embodiments, the invention includes antibodies that have the scFv sequence fused to one or more constant domains of the heavy to form an antibody with an Fc region of a human immunoglobulin to yield a bivalent protein, increasing the overall avidity and stability of the antibody. In addition, the Fc portion allows the direct conjugation of other molecules, including but not limited to fluorescent dyes, cytotoxins, radioisotopes etc. to the antibody for example, for use in antigen quantitation studies, to immobilize the antibody for affinity measurements, for targeted delivery of a therapeutic agent, to test for Fc-mediated cytotoxicity using immune effector cells and many other applications.

[00107] The results presented here highlight the specificity, sensitivity and utility of the antibodies of the invention in targeting MHC-peptide complexes.

[00108] The molecules of the invention are based on the identification and selection of single chain variable fragments (scFv) using phage display, the amino acid sequence of which confers the molecules' specificity for the MHC restricted peptide of interest and forms the basis of all antigen binding proteins of the disclosure. The scFv, therefore, can be used to design a diverse array of "antibody" molecules, including, for example, full length antibodies, fragments thereof, such as Fab and $F(ab')_2$, minibodies, fusion proteins, including scFv-Fc fusions, multivalent antibodies, that is, antibodies that have more than one specificity for the same antigen or different antigens, for example, bispecific T-cell engaging antibodies (BiTe), tribodies, etc. (see Cuesta et al., Multivalent antibodies: when design surpasses evolution. *Trends in Biotechnology* 28:355-362 2010).

[00109] In an embodiment in which the antigen-binding protein is a full length antibody, the heavy and light chains of an antibody of the invention may be full-

length (e.g., an antibody can include at least one, and preferably two, complete heavy chains, and at least one, and preferably two, complete light chains) or may include an antigen-binding portion (a Fab, $F(ab')_2$, Fv or a single chain Fv fragment ("scFv")). In other embodiments, the antibody heavy chain constant region is chosen from, e.g., IgG1, IgG2, IgG3, IgG4, IgM, IgA1, IgA2, IgD, and IgE. In some embodiments, the immunoglobulin isotype is selected from IgG1, IgG2, IgG3, and IgG4, more particularly, IgG1 (e.g., human IgG1). The choice of antibody type will depend on the immune effector function that the antibody is designed to elicit.

[00110] In constructing a recombinant immunoglobulin, appropriate amino acid sequences for constant regions of various immunoglobulin isotypes and methods for the production of a wide array of antibodies are known to those of skill in the art.

[00111] In one embodiment, the antibody or other antigen binding protein is an anti-WT1/HLA-A2 scFv or or antigen-binding fragment thereof having an antigen binding region that comprises the amino acid sequence of SEQ ID NO: 18 and specifically binds to a peptide with the amino acid sequence RMFPNAPYL (SEQ ID NO: 1) in conjunction with HLA-A0201. In some embodiments, the anti-WT1 antibody is a scFv-Fc fusion protein or full length human IgG with VH and VL regions or CDRs selected from Table 1.

Table 1

Antigen	WT1 (Ext002 #3)		
Peptide	RMFPNAPYL (SEQ ID NO: 1)		
CDRs:	1	2	3
VH	GGTFSSYAIS (SEQ ID NO: 2)	GIIPIFGTANYAQKFQG (SEQ ID NO: 3)	RIPPYYGMDV (SEQ ID NO: 4)
DNA	ggaggcaccttcagcagctatgctatcagc (SEQ ID NO: 5)	gggatcatccctatctttggtacagcaaactacgcacagaagttccagggc (SEQ ID NO: 6)	cggattcccccgtactacggtatggacgtc (SEQ ID NO: 7)
VL	SGSSSNIGSNYVY (SEQ ID NO: 8)	RSNQRPS (SEQ ID NO: 9)	AAWDDSLNGVV (SEQ ID NO: 10)
DNA	tctggaagcagctccaacatcggaagtaattatgtatac (SEQ ID NO: 11)	aggagtaatcagcggccctca (SEQ ID NO: 12)	gcagcatgggatgacagcctgaatggtgtggta (SEQ ID NO: 13)

Antigen	WT1 (Ext002 #3)
Peptide	RMFPNAPYL (SEQ ID NO: 1)
Full VH	QVQLVQSGAEVKKPGSSVKVSCKASGGTFSSYAISWVRQAPGQGLE WMGGIIPIFGTANYAQKFQGRVTITADESTSTAYMELSSLRSEDTAVYY CARRIPPYYGMDVWGQGTTVTVSS (SEQ ID NO: 14)
DNA	caggtgcagctggtgcagtctggggctgaggtgaagaagcctgggtcctcggtgaaggtctcctgc aaggcttctggaggcaccttcagcagctatgctatcagctgggtgcgacaggcccctggacaagg gcttgagtggatgggagggatcatccctatctttggtacagcaaactacgcacagaagttccaggg cagagtcacgattaccgcggacgaatccacgagcacagcctacatggagctgagcagcctgag atctgaggacacggccgtgtattactgtgcgagacggattcccccgtactacggtatggacgtctgg ggccaagggaccacggtcaccgtctcctca (SEQ ID NO: 15)
Full VL	QTVVTQPPSASGTPGQRVTISCSGSSSNIGSNYVYWYQQLPGTAPKL LIYRSNQRPSGVPDRFSGSKSGTSASLAISGPRSVDEADYYCAAWDD SLNGVVFGGGTKLTVLG (SEQ ID NO: 16)
DNA	cagactgtggtgactcagccaccctcagcgtctgggacccccgggcagagggtcaccatctcttgtt ctggaagcagctccaacatcggaagtaattatgtatactggtaccaacagctcccaggaacggcc cccaaactcctcatctataggagtaatcagcggccctcaggggtccctgaccgattctctggctcca agtctggcacctcagcctccctggccatcagtgggccccggtccgtggatgaggctgattattactgt gcagcatgggatgacagcctgaatggtgtggtattcggcggagggaccaagctgaccgtcctagg t (SEQ ID NO: 17)
scFv	QTVVTQPPSASGTPGQRVTISCSGSSSNIGSNYVYWYQQLPGTAPKL LIYRSNQRPSGVPDRFSGSKSGTSASLAISGPRSVDEADYYCAAWDD SLNGVVFGGGTKLTVLG**SRGGGGSGGGGSGGGSLEMA**QVQLVQSG AEVKKPGSSVKVSCKASGGTFSSYAISWVRQAPGQGLEWMGGIIPIFG TANYAQKFQGRVTITADESTSTAYMELSSLRSEDTAVYYCARRIPPYY GMDVWGQGTTVTVSS (SEQ ID NO: 18)
DNA	cagactgtggtgactcagccaccctcagcgtctgggacccccgggcagagggtcaccatctcttgtt ctggaagcagctccaacatcggaagtaattatgtatactggtaccaacagctcccaggaacggcc cccaaactcctcatctataggagtaatcagcggccctcaggggtccctgaccgattctctggctcca agtctggcacctcagcctccctggccatcagtgggccccggtccgtggatgaggctgattattactgt gcagcatgggatgacagcctgaatggtgtggtattcggcggagggaccaagctgaccgtcctagg t**tctagaggtggtggtggtagcggcggcggcggctctggtggtggatccctcgagatggc c**caggtgcagctggtgcagtctggggctgaggtgaagaagcctgggtcctcggtgaaggtctcctg caaggcttctggaggcaccttcagcagctatgctatcagctgggtgcgacaggcccctggacaag ggcttgagtggatgggagggatcatccctatctttggtacagcaaactacgcacagaagttccagg gcagagtcacgattaccgcggacgaatccacgagcacagcctacatggagctgagcagcctga gatctgaggacacggccgtgtattactgtgcgagacggattcccccgtactacggtatggacgtctg gggccaagggaccacggtcaccgtctcctca (SEQ ID NO: 19)

[00112] In another embodiment, the antibody or antigen binding protein is an anti-WT1 scFv or antigen-binding fragment thereof that has an antigen binding region that comprises the amino acid sequence of SEQ ID NO: 36 and specifically binds to a peptide with the amino acid sequence RMFPNAPYL (SEQ ID NO: 1) in conjunction with HLA-A0201. In other embodiments, the anti-WT-1 antibody is a scFv-Fc fusion

protein or full length human IgG with VH and VL regions or CDRs selected from Table 2.

Table 2

Antigen	WT1 (Ext002 #5)		
Peptide	RMFPNAPYL (SEQ ID NO: 1)		
CDRs:	1	2	3
VH	GDSVSSNSAAWN (SEQ ID NO: 20)	RTYYGSKWYNDYAVSVKS (SEQ ID NO: 21)	GRLGDAFDI (SEQ ID NO: 22)
DNA	ggggacagtgtctctagcaacagtgctgcttggaac (SEQ ID NO: 23)	aggacatactacgggtccaaggtggtataatgattatgcagtatctgtgaaaagt (SEQ ID NO: 24)	ggtcgcttaggggatgcttttgatatc (SEQ ID NO: 25)
VL	RASQSISSYLN (SEQ ID NO: 26)	AASSLQS (SEQ ID NO: 27)	QQSYSTPLT (SEQ ID NO: 28)
DNA	cgggcaagtcagagcattagcagctatttaaat (SEQ ID NO: 29)	gctgcatccagtttgcaaagt (SEQ ID NO: 30)	caacagagttacagtacccctctcact (SEQ ID NO: 31)
Full VH	QVQLQQSGPGLVKPSQTLSLTCAISGDSVSSNSAAWNWIRQSPSRGLEWLGRTYYGSKWYNDYAVSVKSRITINPDTSKNQFSLQLNSVTPEDTAVYYCARGRLGDAFDIWGQGTMVTVSS (SEQ ID NO: 32)		
DNA	caggtacagctgcagcagtcaggtccaggactggtgaagccctcgcagaccctctcactcacctgtgccatctccggggacagtgtctctagcaacagtgctgcttggaactggatcaggcagtccccatcgagaggccttgagtggctgggaaggacatactacgggtccaagtggtataatgattatgcagtatctgtgaaaagtcgaataaccatcaacccagacacatccaagaaccagttctccctgcagctgaactctgtgactcccgaggacacggctgtgtattactgtgcaagaggtcgcttaggggatgcttttgatatctgggccaagggacaatggtcaccgtctcttca (SEQ ID NO: 33)		
Full VL	DIQMTQSPSSLSASVGDRVTITCRASQSISSYLNWYQQKPGKAPKLLIYAASSLQSGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQSYSTPLTFGGGTKVDIKR (SEQ ID NO: 34)		
DNA	gacatccagatgacccagtctccatcctccctgtctgcatctgtaggagacagagtcaccatcacttgccgggcaagtcagagcattagcagctatttaaattggtatcagcagaaaccagggaaagcccctaagctcctgatctatgctgcatccagtttgcaaagtggggtcccatcaaggttcagtggcagtggatctgggacagatttcactctcaccatcagcagtctgcaacctgaagattttgcaacttactactgtcaacagagttacagtacccctctcactttcggcggagggaccaaagtggatatcaaacgt (SEQ ID NO: 35)		

Antigen	WT1 (Ext002 #5)
Peptide	RMFPNAPYL (SEQ ID NO: 1)
scFv	DIQMTQSPSSLSASVGDRVTITCRASQSISSYLNWYQQKPGKAPKLLIY AASSLQSGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQSYSTPLT FGGGTKVDIKR**SRGGGGSGGGGSGGGGSLEMA**QVQLQQSGPGLVK PSQTLSLTCAISGDSVSSNSAAWNWIRQSPSRGLEWLGRTYYGSKWY NDYAVSVKSRITINPDTSKNQFSLQLNSVTPEDTAVYYCARGRLGDAF DIWGQGTMVTVSS (SEQ ID NO: 36)
DNA	gacatccagatgacccagtctccatcctccctgtctgcatctgtaggagacagagtcaccatcacttg ccgggcaagtcagagcattagcagctatttaaattggtatcagcagaaaccagggaaagccccta agctcctgatctatgctgcatccagtttgcaaagtggggtcccatcaaggttcagtggcagtggatct gggacagatttcactctcaccatcagcagtctgcaacctgaagattttgcaacttactactgtcaaca gagttacgtaccctctcactttcggcggagggaccaaagtggatatcaaacgt**tctagaggtg gtggtggtagcggcggcggcggctctggtggtggtggatccctcgagatggcc**caggtac agctgcagcagtcaggtccaggactggtgaagccctcgcagaccctctcactcacctgtgccatct ccggggacagtgtctctagcaacagtgctgcttggaactggatcaggcagtccccatcgagaggc cttgagtggctgggaaggacatactacgggtccaagtggtataatgattatgcagtatctgtgaaaa gtcgaataaccatcaacccagacacatccaagaaccagttctccctgcagctgaactctgtgactc ccgaggacacggctgtgtattactgtgcaagaggtcgcttaggggatgcttttgatatctggggccaa gggacaatggtcaccgtctcttca (SEQ ID NO: 37)

[00113] In another embodiment, the antibody or antigen binding protein is an anti-WT1 scFv or antigen binding fragment thereof that has an antigen binding region that comprises the amino acid sequence of SEQ ID NO: 54 and specifically binds to a peptide with the amino acid sequence RMFPNAPYL (SEQ ID NO: 1) in conjunction with HLA-A0201. In other embodiments, the anti-WT-1 antibody is a scFv-Fc fusion protein or full length human IgG with VH and VL regions or CDRs selected from Table 3.

Table 3

Antigen	WT1 (Ext002 #13)		
Peptide	RMFPNAPYL (SEQ ID NO: 1)		
CDRs:	1	2	3
VH	GYSFTNFWIS (SEQ ID NO: 38)	RVDPGYSYSTYSPSF QG (SEQ ID NO: 39)	VQYSGYYDWFDP (SEQ ID NO: 40)
DNA	ggatacagcttcaccaact tctggatcagc (SEQ ID NO: 41)	agggttgatcctggctactctta tagcacctacagcccgtccttc caaggc (SEQ ID NO: 42)	gtacaatatagtggctactatg actggttcgacccc (SEQ ID NO: 43)
VL	SGSSSNIGSNTVN (SEQ ID NO: 44)	SNNQRPS (SEQ ID NO: 45)	AAWDDSLNGWV (SEQ ID NO: 46)

Antigen	WT1 (Ext002 #13)		
Peptide	RMFPNAPYL (SEQ ID NO: 1)		
DNA	tctggaagcagctccaac atcggaagtaatactgtaa ac (SEQ ID NO: 47)	agtaataatcagcggccctca (SEQ ID NO: 48)	gcagcatgggatgacagcct gaatggttgggtg (SEQ ID NO: 49)
Full VH	QMQLVQSGAEVKEPGESLRISCKGSGYSFTNFWISWVRQMPGKGLE WMGRVDPGYSYSTYSPSFQGHVTISADKSTSTAYLQWNSLKASDTA MYYCARVQYSGYYDWFDPWGQGTLVTVSS (SEQ ID NO: 50)		
DNA	cagatgcagctggtgcagtccggagcagaggtgaaagagcccggggagtctctgaggatctcct gtaagggttctggatacagcttcaccaacttctggatcagctgggtgcgccagatgcccgggaaa ggcctggagtggatggggagggttgatcctggctactcttatagcacctacagcccgtccttccaag gccacgtcaccatctcagctgacaagtctaccagcactgcctacctgcagtggaacagcctgaag gcctcggacaccgccatgtattactgtgcgagagtacaatatagtggctactatgactggttcgacc cctggggccagggaaccctggtcaccgtctcctca (SEQ ID NO: 51)		
Full VL	QAVVTQPPSASGTPGQRVTISCSGSSSNIGSNTVNWYQQVPGTAPK LLIYSNNQRPSGVPDRFSGSKSGTSASLAISGLQSEDEADYYCAAWD DSLNGWVFGGGTKLTVLG (SEQ ID NO: 52)		
DNA	caggctgtggtgactcagccaccctcagcgtctgggacccccgggcagagggtcaccatctcttgt tctggaagcagctccaacatcggaagtaatactgtaaactggtaccagcaggtcccaggaacgg cccccaaactcctcatctatagtaataatcagcggccctcaggggtccctgaccgattctctggctc caagtctggcacctcagcctccctggccatcagtgggctccagtctgaggatgaggctgattattac tgtgcagcatgggatgacagcctgaatggttgggtgttcggcggagggaccaagctgaccgtcct aggt (SEQ ID NO: 53)		
scFv	QAVVTQPPSASGTPGQRVTISCSGSSSNIGSNTVNWYQQVPGTAPK LLIYSNNQRPSGVPDRFSGSKSGTSASLAISGLQSEDEADYYCAAWD DSLNGWVFGGGTKLTVLG**SRGGGGSGGGGSGGGGSLEMA**QMQLV QSGAEVKEPGESLRISCKGSGYSFTNFWISWVRQMPGKGLEWMGR VDPGYSYSTYSPSFQGHVTISADKSTSTAYLQWNSLKASDTAMYYCA RVQYSGYYDWFDPWGQGTLVTVSS (SEQ ID NO: 54)		
DNA	caggctgtggtgactcagccaccctcagcgtctgggacccccgggcagagggtcaccatctcttgt tctggaagcagctccaacatcggaagtaatactgtaaactggtaccagcaggtcccaggaacgg cccccaaactcctcatctatagtaataatcagcggccctcaggggtccctgaccgattctctggctc caagtctggcacctcagcctccctggccatcagtgggctccagtctgaggatgaggctgattattac tgtgcagcatgggatgacagcctgaatggttgggtgttcggcggagggaccaagctgaccgtcct aggttctagaggtggtggtggtagcggcggcggcggctctggtggtggtggatccctcgagatgg cccagatgcagctggtgcagtccggagcagaggtgaaagagcccggggagtctctgaggatct cctgtaagggttctggatacagcttcaccaacttctggatcagctgggtgcgccagatgcccggga aaggcctggagtggatggggagggttgatcctggctactcttatagcacctacagcccgtccttcca aggccacgtcaccatctcagctgacaagtctaccagcactgcctacctgcagtggaacagcctga aggcctcggacaccgccatgtattactgtgcgagagtacaatatagtggctactatgactggttcga cccctggggccagggaaccctggtcaccgtctcctca (SEQ ID NO: 55)		

[00114] In another embodiment, the antibody or antigen binding protein is an anti-WT1 scFv or antigen binding fragment thereof that has an antigen binding region that comprises the amino acid sequence of SEQ ID NO: 72 and specifically binds to

a peptide with the amino acid sequence RMFPNAPYL (SEQ ID NO:1) in conjunction with HLA-A0201. In other embodiments, the anti-WT-1 antibody is a scFv-Fc fusion protein or full length human IgG with VH and VL regions or CDRs selected from Table 4.

Table 4

Antigen	WT1 (Ext002 #15)		
Peptide	RMFPNAPYL (SEQ ID NO: 1)		
CDRs:	1	2	3
VH	GYNFSNKWIG (SEQ ID NO: 56)	IIYPGYSDITYSPSFQG (SEQ ID NO: 57)	HTALAGFDY (SEQ ID NO: 58)
DNA	ggctacaactttagcaacaagtggatcggc (SEQ ID NO: 59)	atcatctatcccggttactcggacatcacctacagcccgtccttccaaggc (SEQ ID NO: 60)	cacacagctttggccggctttgactac (SEQ ID NO: 61)
VL	RASQNINKWLA (SEQ ID NO: 62)	KASSLES (SEQ ID NO: 63)	QQYNSYAT (SEQ ID NO: 64)
DNA	Cgggccagtcagaatatcaataagtggctggcc (SEQ ID NO: 65)	aaggcgtctagtttagaaagt (SEQ ID NO: 66)	caacaatataatagttatgcgacg (SEQ ID NO: 67)
Full VH	QVQLVQSGAEVKKPGESLKISCKGSGYNFSNKWIGWVRQLPGRGLEWIAIIYPGYSDITYSPSFQGRVTISADTSINTAYLHWHSLKASDTAMYYCVRHTALAGFDYWGLGTLVTVSS (SEQ ID NO: 68)		
DNA	caggtgcagctggtgcagtctggagcagaggtgaaaaagcccggagagtctctgaagatctcctgtaagggttctggctacaactttagcaacaagtggatcggctgggtgcgccaattgcccgggagaggcctggagtggatagcaatcatctatcccggttactcggacatcacctacagcccgtccttccaaggccgcgtcaccatctccgccgacacgtccattaacaccgcctacctgcactggcacagcctgaaggcctcggacaccgccatgtattattgtgtgcgacacacagctttggccggctttgactactggggcctggggcaccctggtcaccgtctcctca (SEQ ID NO: 69)		
Full VL	DIQMTQSPSTLSASVGDRVTITCRASQNINKWLAWYQQRPGKAPQLLIYKASSLESGVPSRFSGSGSGTEYTLTISSLQPDDFATYYCQQYNSYATFGQGTKVEIKR (SEQ ID NO: 70)		
DNA	gacatccagatgacccagtctccttccaccctgtctgcatctgtaggagacagagtcacaatcacttgccgggccagtcagaatatcaataagtggctggcctggtatcagcagagaccagggaaagcccctcagctcctgatctataaggcgtctagtttagaaagtggggtcccatctaggttcagcggcagtggatctgggacagaatacactctcaccatcagcagcctgcagcctgatgattttgcaacttattactgccaacaatataatagttatgcgacgttcggccaagggaccaaggtggaaatcaaacgt (SEQ ID NO: 71)		
scFv	DIQMTQSPSTLSASVGDRVTITCRASQNINKWLAWYQQRPGKAPQLLIYKASSLESGVPSRFSGSGSGTEYTLTISSLQPDDFATYYCQQYNSYATFGQGTKVEIKR**SRGGGGSGGGGSGGGGSLEMA**QVQLVQSGAEVKKPGESLKISCKGSGYNFSNKWIGWVRQLPGRGLEWIAIIYPGYSDITYSPSFQGRVTISADTSINTAYLHWHSLKASDTAMYYCVRHTALAGFDYWGLGTLVTVSS (SEQ ID NO: 72)		

Antigen	WT1 (Ext002 #15)
Peptide	RMFPNAPYL (SEQ ID NO: 1)
DNA	gacatccagatgacccagtctccttccaccctgtctgcatctgtaggagacagagtcacaatcacttg ccgggccagtcagaatatcaataagtggctggcctggtatcagcagagaccagggaaagcccct cagctcctgatctataaggcgtctagtttagaaagtggggtcccatctaggttcagcggcagtggatc tgggacagaatacactctcaccatcagcagcctgcagcctgatgattttgcaacttattactgccaac aatataatagttatgcgacgttcggccaagggaccaaggtggaaatcaaacgt**tctagaggtggt ggtggtagcggcggcggcggctctggtggtggtggatccctcgagatggcc**caggtgcag ctggtgcagtctggagcagaggtgaaaaagcccggagagtctctgaagatctcctgtaagggttct ggctacaactttagcaacaagtggatcggctgggtgcgccaattgcccgggagaggcctggagtg gatagcaatcatctatcccggttactcggacatcacctacagcccgtccttccaaggccgcgtcacc atctccgccgacacgtccattaacaccgcctacctgcactggcacagcctgaaggcctcggacac cgccatgtattattgtgtgcgacacacagctttggccggctttgactactggggcctgggcaccctggt caccgtctcctca (SEQ ID NO: 73)

[00115] In another embodiment, the antibody or antigen binding protein is an anti-WT1 scFv or antigen binding fragment thereof that has an antigen binding region that comprises the amino acid sequence of SEQ ID NO: 90 and specifically binds to a peptide with the amino acid sequence RMFPNAPYL (SEQ ID NO: 1) in conjunction with HLA-A0201. In other embodiments, the anti-WT-1 antibody is a scFv-Fc fusion protein or full length human IgG with VH and VL regions or CDRs selected from Table 5.

Table 5

Antigen	WT1 (Ext002 #18)		
Peptide	RMFPNAPYL (SEQ ID NO: 1)		
CDRs:	1	2	3
VH	GFTFDDYGMS (SEQ ID NO: 74)	GINWNGGSTGYADS VRG (SEQ ID NO: 75)	ERGYGYHDPHDY (SEQ ID NO: 76)
DNA	gggttcacctttgatgattat ggcatgagc (SEQ ID NO: 77)	ggtattaattggaatggtggt agcacaggttatgcagactc tgtgaggggc (SEQ ID NO: 78)	gagcgtggctacgggtacca tgatccccatgactac (SEQ ID NO: 79)
VL	GRNNIGSKSVH (SEQ ID NO: 80)	DDSDRPS (SEQ ID NO: 81)	QVWDSSSDHVV (SEQ ID NO: 82)
DNA	gggagaaacaacattgg aagtaaaagtgtgcac (SEQ ID NO: 83)	gatgatagcgaccggccctc a (SEQ ID NO: 84)	caggtgtgggatagtagtagt gatcatgtggta (SEQ ID NO: 85)
Full VH	EVQLVQSGGGVVRPGGSLRLSCAASGFTFDDYGMSWVRQAPGKG LEWVSGINWNGGSTGYADSVRGRFTISRDNAKNSLYLQMNSLRAE DTALYYCARERGYGYHDPHDYWGQGTLVTVSS (SEQ ID NO: 86)		

Antigen	WT1 (Ext002 #18)
Peptide	RMFPNAPYL (SEQ ID NO: 1)
DNA	gaagtgcagctggtgcagtctgggggaggtgtggtacggcctgggggtccctgagactctcct gtgcagcctctgggttcacctttgatgattatggcatgagctgggtccgccaagctccagggaag gggctggagtgggtctctggtattaattggaatggtggtagcacaggttatgcagactctgtgagg ggccgattcaccatctccagagacaacgccaagaactccctgtatctgcaaatgaacagtctg agagccgaggacacggccttgtattactgtgcgagagagcgtggctacgggtaccatgatccc catgactactggggccaaggcaccctggtgaccgtctcctca (SEQ ID NO: 87)
Full VL	QSVVTQPPSVSVAPGKTARITCGRNNIGSKSVHWYQQKPGQAPVL VVYDDSDRPSGIPERFSGSNSGNTATLTISRVEAGDEADYYCQVW DSSSDHVVFGGGTKLTVLG (SEQ ID NO: 88)
DNA	cagtctgtcgtgacgcagccgccctcggtgtcagtggccccaggaaagacggccaggattac ctgtgggagaaacaacattggaagtaaaagtgtgcactggtaccagcagaagccaggccag gcccctgtgctggtcgtctatgatgatagcgaccggccctcagggatccctgagcgattctctgg ctccaactctgggaacacggccaccctgaccatcagcagggtcgaagccggggatgaggcc gactattactgtcaggtgtgggatagtagtagtgatcatgtggtattcggcggagggaccaagct gaccgtcctaggt (SEQ ID NO: 89)
scFv	QSVVTQPPSVSVAPGKTARITCGRNNIGSKSVHWYQQKPGQAPVL VVYDDSDRPSGIPERFSGSNSGNTATLTISRVEAGDEADYYCQVW DSSSDHVVFGGGTKLTVLG**SRGGGGSGGGGSGGSLEMA**EVQLVQ SGGGVVRPGGSLRLSCAASGFTFDDYGMSWVRQAPGKGLEWVS GINWNGGSTGYADSVRGRFTISRDNAKNSLYLQMNSLRAEDTALYY CARERGYGYHDPHDYWGQGTLVTVSS (SEQ ID NO: 90)
DNA	cagtctgtcgtgacgcagccgccctcggtgtcagtggccccaggaaagacggccaggattac ctgtgggagaaacaacattggaagtaaaagtgtgcactggtaccagcagaagccaggccag gcccctgtgctggtcgtctatgatgatagcgaccggccctcagggatccctgagcgattctctgg ctccaactctgggaacacggccaccctgaccatcagcagggtcgaagccggggatgaggcc gactattactgtcaggtgtgggatagtagtagtgatcatgtggtattcggcggagggaccaagct gaccgtcctaggt**tctagaggtggtggtggtagcggcggcggcggctctggtggatccc tcgagatggcc**gaagtgcagctggtgcagtctgggggaggtgtggtacggcctgggggtcc ctgagactctcctgtgcagcctctgggttcacctttgatgattatggcatgagctgggtccgccaag ctccagggaaggggctggagtgggtctctggtattaattggaatggtggtagcacaggttatgca gactctgtgaggggccgattcaccatctccagagacaacgccaagaactccctgtatctgcaa atgaacagtctgagagccgaggacacggccttgtattactgtgcgagagagcgtggctacggg taccatgatccccatgactactggggccaaggcaccctggtgaccgtctcctca (SEQ ID NO: 91)

[00116] In another embodiment, the antibody or antigen binding protein is an anti-WT1 scFv or antigen binding fragment thereof that has an antigen binding region that comprises the amino acid sequence of SEQ ID NO: 108 and specifically binds to a peptide with the amino acid sequence RMFPNAPYL (SEQ ID NO: 1) in conjunction with HLA-A0201. In other embodiments, the anti-WT-1 antibody is a scFv-Fc fusion protein or full length human IgG with VH and VL regions or CDRs selected from Table 6.

Table 6

Antigen	WT1 (Ext002 #23)		
Peptide	RMFPNAPYL (SEQ ID NO. 1)		
CDRs:	1	2	3
VH	GFSVSGTYMG (SEQ ID NO. 92)	LLYSGGGTYHPASLQG (SEQ ID NO. 93)	GGAGGGHFDS (SEQ ID NO. 94)
DNA	gggttctccgtcagtggcactacatgggc(SEQ ID NO. 95)	cttctttatagtggtggcggcacataccacccagcgtccctgcagggc (SEQ ID NO. 96)	gaggggcaggaggtggccactttgactcc (SEQ ID NO. 97)
VL	TGSSSNIGAGYDVH (SEQ ID NO. 98)	GNSNRPS (SEQ ID NO. 99)	AAWDDSLNGYV (SEQ ID NO. 100)
DNA	actgggagcagctccaacatcggggcaggttatgatgtacac (SEQ ID NO. 101)	ggtaacagcaatcggccctca (SEQ ID NO. 102)	gcagcatgggatgacagcctgaatggttatgtc (SEQ ID NO. 103)
Full VH	EVQLVETGGGLLQPGGSLRLSCAASGFSVSGTYMGWVRQAPGKGLEWVALLYSGGGTYHPASLQGRFIVSRDSSKNMVYLQMNSLKAEDTAVYYCAKGGAGGGHFDSWGQGTLVTVSS (SEQ ID NO. 104)		
DNA	gaggtgcagctggtggagaccggaggaggcttgctccagccgggggggtccctcagactctcctgtgcagcctctgggttctccgtcagtggcacctacatgggctgggtccgccaggctccagggaagggactggagtgggtcgcacttctttatagtggtggcggcacataccacccagcgtccctgcagggccgattcatcgtctccagagacagctccaagaatatggtctatcttcaaatgaatagcctgaaagccgaggacacggccgtctattactgtgcgaaaggaggggcaggaggtggccactttgactcctggggccaaggcaccctggtgaccgtctcctca (SEQ ID NO. 105)		
Full VL	QSVLTQPPSVSGAPGQRVTISCTGSSSNIGAGYDVHWYQQLPGTAPKLLIYGNSNRPSGVPDRFSGSKSGTSASLAISGLQSEDEADYYCAAWDDSLNGYVFGTGTKLTVLG (SEQ ID NO. 106)		
DNA	cagtctgtgttgacgcagccgccctcagtgtctggggccccagggcagagggtcaccatctcctgcactgggagcagctccaacatcggggcaggttatgatgtacactggtaccagcagcttccaggaacagcccccaaactcctcatctatggtaacagcaatcggccctcaggggtccctgaccgattctctggctccaagtctggcacctcagcctccctggccatcagtgggctccagtctgaggatgaggctgattattactgtgcagcatgggatgacagcctgaatggttatgtcttcggaactgggaccaagctgaccgtcctaggt (SEQ ID NO. 107)		
scFv	QSVLTQPPSVSGAPGQRVTISCTGSSSNIGAGYDVHWYQQLPGTAPKLLIYGNSNRPSGVPDRFSGSKSGTSASLAISGLQSEDEADYYCAAWDDSLNGYVFG TGTKLTVLG**SRGGGGSGGGGSGGGGSLEMA**EVQLVETGGGLLQPGGSLRLSCAASGFSVSGTYMGWVRQAPGKGLEWVALLYSGGGTYHPASLQGRFIVSRDSSKNMVYLQMNSLKAEDTAVYYCAKGGAGGGHFDSWGQGTLVTVSS (SEQ ID NO. 108)		

Antigen	WT1 (Ext002 #23)
Peptide	RMFPNAPYL (SEQ ID NO. 1)
DNA	cagtctgtgttgacgcagccgccctcagtgtctggggccccagggcagagggtcaccatctcctgc actgggagcagctccaacatcggggcaggttatgatgtacactggtaccagcagcttccaggaac agcccccaaactcctcatctatggtaacagcaatcggccctcaggggtccctgaccgattctctggc tccaagtctggcacctcagcctccctggccatcagtgggctccagtctgaggatgaggctgattatta ctgtgcagcatgggatgacagcctgaatggttatgtcttcggaactgggaccaagctgaccgtccta ggt**tctagaggtggtggtggtagcggcggcggcggctctggtggtggtggatccctcgag atggcc**gaggtgcagctggtggagaccggaggaggcttgctccagccgggggggtccctcaga ctctcctgtgcagcctctgggttctccgtcagtggcacctacatgggctgggtccgccaggctccagg gaagggactggagtgggtcgcacttctttatagtggtggcggcacataccacccagcgtccctgca gggccgattcatcgtctccagagacagctccaagaatatggtctatcttcaaatgaatagcctgaaa gccgaggacacggccgtctattactgtgcgaaaggaggggcaggaggtggccactttgactcctg gggccaaggcaccctggtgaccgtctcctca (SEQ ID NO. 109)

[00117] Other embodiments of the disclosed antibodies and antigen binding proteins encompass those comprising light and heavy hypervariable regions and constant regions, for example as shown in Tables 7 (heavy chain), 8 (light chain) and 9 (constant regions).

Table 7

Group I	**CDR-H1**	**CDR-H2**	**CDR-H3**	**SEQ ID NO.**
EXT002-12(166)	SNAVAWN	RTYRGSTYY---ALSV	G-SNSAFDF	119-121
EXT002-5(184)	SNSAAWN	RTYYGSKWYNDYAVSV	GRLGDAFDI	122-124
EXT002-8(184)	SDGAAWN	RTYYRSKWYNDYAVSV	GDYYYGMDV	125-127
Consensus(191)	SNAAAWN	RTYYGSKWYNDYAVSV	G AFDI	128-130
Group II				
EXT002-14(163)	SYWIS	RIDPSDSYTNYSPSFQG	GD------YDFYLDP--	131-133

EXT002-25(163)	SYGIS	WISAYNGNTNYAQKLQG	DLYSSGWYESYYYGMDV	134-136
EXT002-3(186)	SYAIS	GIIPIFGTANYAQKFQG	RIP—P------YYGMDV	137-139
EXT002-30(163)	SYGIS	WISAHNGNTNYAQKLQG	DR-------VWFGDLSD	134, 140, 141
EXT002-33(163)	SYAIS	GIIPIFGTANYAQKEQG	NYDFWSG-----DAFDI	137, 142, 143
Consensus(188)	SYAIS	I P G TNYAQKFQG	FY GMDV	137, 144, 145
Group III				
EXT002-34(161)	DYGMS	GINWNGGSTGYADSV	ERGY-GYHDPHDY	146-148
EXT002-40(157)	NYTMN	SISLSGAYIYYADSL	EGYSSSVYDAFDL	149-151
EXT002-45(165)	SYGMH	GILSDGGKDYYVDSV	CSSN-YGNDAFDI	152-154
EXT002-48(165)	TYSMN	SISSGAYSIFYADSV	DQYYGDKWDAFDI	155-157
Consensus (170)	SYGMN	SISS GGSIYYADSV	E YY WDAFDI	158-160

Table 8

Group I	**CDR-L1**	**CDR-L2**	**CDR-L3**	**SEQ ID NOS.**
EXT002-1 (46)	CSGSSSNIGS-NTVN	SNNQRPSG	AAWDDSLNG--WVFG	161-163
EXT002-10 (46)	CSGSSSNIGS-NTVN	SNNQRPSG	EAWDDSLKG--PVFG	161,

				162, 164
EXT002-12 (22)	CTGSSSNIGAGYDVH	GNSNRPSG	QSYDSSLSADNYVFG	165-167
EXT002-13 (46)	CSGSSSNIGS-NTVN	SNNQRPSG	AAWDDSLNG--WVFG	161-163
EXT002-2 (46)	CSGSSSNIGR-NIVN	SNIERPSG	ASWDDSLNG--VLFG	168-170
EXT002-20 (46)	CSGSRSNIAS-NGVG	KNDQRPSG	SAWDDSLDGH-VVFG	171-173
EXT002-23 (46)	CTGSSSNIGAGYDVH	GNSNRPSG	AAWDDSLNG--YVFG	165, 166, 174
EXT002-25 (22)	CSGSSSNIGS-STVN	SNSQRPSG	AAWDDSLNG--VVFG	175-177
EXT002-3 (46)	CSGSSSNIGS-NYVY	RSNQRPSG	AAWDDSLNG--VVFG	178, 179, 177
EXT002-30 (22)	CSGSSSNIGR-NTVN	SNNQRPSG	AAWDDSLNG--YVFG	180, 162, 174
EXT002-33 (22)	CSGSSSNIGN-DYVS	DNNKRPSG	GTWDNSLSA--WVFG	181-183
EXT002-36 (22)	CSGSSSNIGS-NSVY	NNNQRPSG	ATWDDSLSG--WVFG	184-186
EXT002-40 (22)	CSGSSSNIGS-NYVY	RNNQRPSG	AAWDDSLSA--WVFG	178, 187, 188
EXT002-42 (46)	CSGSTSNIGS-YYVS	DNNNRPSG	GTWDSSLSA--WVFG	189-191
EXT002-45 (22)	CSGSSSNIGN-NYVS	DNNKRPSG	GTWDSSLSA--WVFG	192, 182, 191
EXT002-48 (22)	CSGSNSNIGT-NTVT	SNFERPSG	SAWDDSFNG--PVFG	193-195
EXT002-6 (46)	CSGSSSNIGS-NYVS	RNNQRPSG	AAWDDGLRG--YVFG	196, 187, 197
EXT002-9 (22)	CSGSSSNIGS-NTVN	SNNQRPSG	EAWDDSLKG--PVFG	161, 162, 164
Consensus (46)	CSGSSSNIGS N V	NNQRPSG	AAWDDSL G WVFG	161-163
Group II				
EXT002-24 (24)	RASQSISSYLN	AASSLQS	QQSYSTP--T	198-200
EXT002-31 (24)	RASQGISNYLA	AASTLQS	QKYNSAPGVT	201-203
EXT002-35 (24)	RASQSINGWLA	RASTLQS	QQSSSLP-FT	204-206

EXT002-5 (48)	RASQSISSYLN	AASSLQS	QQSYSTP-LT	198-200
EXT002-7 (48)	RASQGISYYLA	AASTLKS	QQLNSYP-LT	207-209
EXT002-B (48)	RASQSISSYLN	AASSLQS	QQSYSTP-WT	198-200
Consensus (48)	RASQSISSYLN	AASSLQS	QQSYSTP LT	198-200
Group III				
EXT002-16 (23)	GGNNIGSKSVH	DDSDRPS	QVWDSSSDHPV	210-212
EXT002-17 (47)	GGNNIGSKSVH	DDSDRPS	QVWDSSGDHPV	210, 211, 213
EXT002-19 (47)	GGNNIGSKSVH	YDSDRPS	QVWDSSSDHPV	210, 214, 212
EXT002-21 (19)	GGTNIGSRFVH	DDSDRPS	QVWDSSGDHPV	215, 211, 213
EXT002-22 (47)	GGNNVESKSVH	YDRDRPS	EVWDSGSDHPV	216-218
EXT002-32 (23)	GGKNIGSKSVH	YDSDRPS	QVWDSGSDHYV	219, 214, 220
EXT002-34 (23)	GGNNIGSKSVH	DDSDRPS	QVWISSGDRVI	210, 211, 221
EXT002-43 (23)	GGDNIGSQGVH	YDTDRPS	QVWGASSDHPV	222-224
Consensus (47)	GGNNIGSKSVH	YDSDRPS	QVWDSSSDHPV	210, 214, 212
Group IV				
EXT002-11 (47)	TGTSSDVGGYNYVS	DVSKRPS	GIYTYSDSW--V	225-227
EXT002-14 (23)	TGTSSDVGGYNYVS	DVGNRPS	SSYTSSSTR--V	225, 228, 229
EXT002-26 (23)	TGTRSDVGLYNYVA	DVIYRPG	SSYTNTGTV--L	230-232
EXT002-4 (47)	TGTSSDFGDYDYVS	DVSDRPS	QSYDSSLSGSGV	233-235
Consensus (47)	TGTSSDVGGYNYVS	DVS RPS	SSYTSS S V	225, 234, 229

Table 9

Constant Regions				
Human heavy chain constant region and IgG1 Fc domain sequence	ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGAL TSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNT KVDKRVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISR TPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYR VVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQ VYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKT TPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQK SLSLSPGK (SEQ ID NO. 236)			
Human light chain (kappa)	TVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNAL QSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQG LSSPVTKSFNRGEC (SEQ ID NO. 237)			
Human light chain (lambda)	QPKANPTVTLFPPSSEELQANKATLVCLISDFYPGAVTVAWKADGS PVKAGVETTKPSKQSNNKYAASSYLSLTPEQWKSHRSYSCQVTHEG STVEKTVAPTECS (SEQ ID NO. 238)			

[00118] The invention relates to recombinant antigen-binding proteins, antibodies and antigen binding fragments thereof that specifically recognize epitopes of a complex of a peptide/protein fragment derived from an intracellular protein, and an MHC class I molecule, for example, as the complex might be displayed at the cell surface during antigen presentation. The heavy and light chains of an antibody of the invention may be full-length (e.g., an antibody can include at least one, and preferably two, complete heavy chains, and at least one, and preferably two, complete light chains) or may include an antigen-binding portion (a Fab, $F(ab')_2$, Fv or a single chain Fv fragment ("scFv")). In other embodiments, the antibody heavy chain constant region is chosen from, e.g., IgG1, IgG2, IgG3, IgG4, IgM, IgA1, IgA2, IgD, and IgE, particularly chosen from, e.g., IgG1, IgG2, IgG3, and IgG4, more particularly, IgG1 (e.g., human IgG1). In another embodiment, the antibody light chain constant region is chosen from, e.g., kappa or lambda, particularly kappa.

[00119] The antibodies and antigen binding proteins of the present invention are intended to encompass bispecific antibodies, including bispecific T-cell engaging antibodies, that is, antibodies comprising two antibody variable domains on a single polypeptide chain that are able to bind two separate antigens. Where a first portion of a bispecific antibody binds an antigen on a tumor cell for example and a second portion of a bispecific antibody recognizes an antigen on the surface of a human immune effector cell, the antibody is capable of recruiting the activity of that effector cell by specifically binding to the effector antigen on the human immune effector cell. In some instances, bispecific antibodies, therefore, are able to form a link between effector cells, for example, T cells and tumor cells, thereby enhancing effector function.

[00120] In one embodiment, the constant region/framework region is altered, for example, by amino acid substitution, to modify the properties of the antibody (e.g., to increase or decrease one or more of: antigen binding affinity, Fc receptor binding, antibody carbohydrate, for example, glycosylation, fucosylation etc , the number of cysteine residues, effector cell function, effector cell function, complement function or introduction of a conjugation site). Furthermore, conjugation of the antibody to a

drug, toxin, radioisotope, cytokine, inflammatory peptide or cytotoxic agent is also contemplated.

[00121] In one embodiment, the antibody is an anti -WT1/A2 antibody and comprises the human IgG1 constant region and Fc domain shown in Table 9. In one embodiment, the anti-WT1/A2 antibody comprises a human kappa sequence, or a human lambda sequence having the sequence set forth in Table 9. The amino acid sequences for some complementarity determining regions (CDRs) of antibodies of the invention are shown in Tables 1-8.

[00122] The present invention is based on the identification of antigen-specific binding sequences from which a variety of antigen-binding proteins can be produced. In addition to antibodies specific for an antigen that represents a protein fragment (peptide)/HLA complex similar to that typically recognized by a T-cell receptor following antigen processing and presentation of the protein to the T-cell, identification of amino acid and nucleic sequences as disclosed herein for the preparation of antibodies can also be used to generate other antigen-binding molecules including chimeric antigen receptors (CARs), with specificity for the protein fragment (peptide)/HLA complex. These can be incorporated into cells to make them specifically ctyotoxic to the antigen expressing cell.

[00123] The present invention employs a novel approach to obtaining therapeutic antibodies to any protein, including those proteins that are inaccessible because they are not expressed on the cell surface. Nearly any intracytoplasmic or intranuclear protein (in addition to cell surface proteins) is a potential target for the approach described herein. This includes, but is not limited to, oncogenic proteins, transcription factors, enzymes, etc.

[00124] In order to target tumor antigens derived from intracellular or nuclear proteins, development of a therapeutic antibody an uncommon approach was required. This approach is to generate recombinant mAbs that recognize the peptide/MHC complex expressed on the cell surface, with the same specificity as a T-cell receptor (TCR). Such mAbs share functional homology with TCRs regarding target recognition, but confer higher affinity and capabilities of arming with potent cytotoxic agents that antibodies feature. Technically, TCR-like mAbs may be

generated by conventional hybridoma techniques known to those of skill in the art, to produce human, humanized or chimeric antibodies.

[00125] Furthermore, fully-human mAbs are preferred for therapeutic use in humans because murine antibodies cause an immunogenicity reaction, known as the HAMA (human anti-mouse antibodies) response (24, 25), when administered to humans, causing serious side effects, including anaphylaxis and hypersensitivity reactions. This immunogenicity reaction is triggered by the human immune system recognizing the murine antibodies as foreign because of slightly different amino acid sequences from natural human antibodies. Humanization methods known in the art (26, 27) can be employed to reduce the immunogenicity of murine-derived antibodies (28).

[00126] Recently, the use of phage display libraries has made it possible to select large numbers of Ab repertoires for unique and rare Abs against very defined epitopes (for more details on phage display see McCafferty et al., Phage antibodies: filamentous phage displaying antibody variable domains. *Nature*, 348: 552-554.) The rapid identification of human Fab or single chain Fv (ScFV) fragments highly specific for tumor antigen-derived peptide-MHC complex molecules has thus become possible (19-22). More recently, immuno-toxins, generated by fusing TCR-like Fab specific for melanoma Ag MART-1 26-35/A2 or gp100 280-288/A2 to a truncated form of Pseudomonas endotoxin, have been shown to inhibit human melanoma growth both *in vitro* and *in vivo* (23). In addition, by engineering full-length mAb using the Fab fragments, it is possible to directly generate a therapeutic human mAb, by-passing months of time-consuming work, normally needed for developing therapeutic mAbs. The present invention involves the development of a TCR-like, fully human mAb that recognizes, for example, the WT1 peptide/HLA-A2 complex (RMFPNAPYL, SEQ ID NO: 1) for cancer therapy.

[00127] Recombinant antibodies with TCR-like specificity represent a new and valuable tool for research and therapeutic applications in tumor immunology and immunotherapy. WT1 is a well-established and validated tumor antigen that has been investigated throughout the world as a marker, prognostic factor and therapeutic target. It was recently prioritized as the top priority tumor antigen by an NCI task force (29).

Identification of peptides with high predictive binding to HLA molecules

[00128] In one embodiment, the present invention relates to a method for the generation of antibodies that specifically bind to HLA-restricted peptides, which, when presented as part of a peptide/MHC complex are able to elicit a specific cytotoxic T-cell response. HLA class I molecules present endogenous derived peptides of about 8-12 amino acids in length to $CD8^+$ cytotoxic T lymphocytes. Peptides to be used in the method of the present invention are generally about 6-22 amino acids in length, and in some embodiments, between about 9 and 20 amino acids and comprise an amino acid sequence derived from a protein of interest, for example, human WT1protein (Genbank accession no. P19544) or an analog thereof.

[00129] Peptides suitable for use in generating antibodies in accordance with the method of the present invention can be determined based on the presence of HLA-A0201-binding motifs and the cleavage sites for proteasomes and immune-proteasomes using computer prediction models known to those of skill in the art. For predicting MHC class I binding sites, such models include, but are not limited to, ProPred1 (described in more detail in Singh and Raghava, *ProPred: prediction of HLA-DR binding sites*. *BIOINFORMATICS* 17(12):1236-1237 2001), and SYFPEITHI (see Schuler et al. *SYFPEITHI, Database for Searching and T-Cell Epitope Prediction.* in *Immunoinformatics Methods in Molecular Biology, vol 409(1): 75-93 2007)*

[00130] HLA-A*0201 is expressed in 39-46% of all caucasians and therefore, represents a suitable choice of MHC antigen for use in the present method. For preparation of one embodiment of a WT1 peptide antigen, amino acid sequences and predicted binding of putative $CD84^+$ epitopes to HLA-A0201 molecules were identified using the predictive algorithm of the SYFPEITHI database (see Schuler et al. *SYFPEITHI, Database for Searching and T-Cell Epitope Prediction.* in *Immunoinformatics Methods in Molecular Biology, vol 409(1): 75-93 2007).*

[00131] Once appropriate peptides have been identified, peptide synthesis may be done in accordance with protocols well known to those of skill in the art. Because of their relatively small size, the peptides of the invention may be directly synthesized in solution or on a solid support in accordance with conventional peptide synthesis

techniques. Various automatic synthesizers are commercially available and can be used in accordance with known protocols. The synthesis of peptides in solution phase has become a well-established procedure for large-scale production of synthetic peptides and as such is a suitable alternative method for preparing the peptides of the invention. (See for example, *Solid Phase Peptide Synthesis* by John Morrow Stewart and Martin et al. *Application of Almez-mediated Amidation Reactions to Solution Phase Peptide Synthesis*, Tetrahedron Letters Vol. 39, pages 1517-1520 1998.)

[00132] Each of the peptides used in the protocols described herein was purchased and synthesized by Genemed Synthesis, Inc. (San Antonio, TX) using fluorenylmethoxycarbonyl chemistry and solid-phase synthesis and purified by high-pressure liquid chromatography. The quality of the peptides was assessed by high-performance liquid chromatography analysis, and the expected molecular weight was observed using matrix-assisted laser desorption mass spectrometry. Peptides were sterile and 70% to 90% pure. The peptides were dissolved in DMSO and diluted in PBS (pH 7.4) or saline at 5 mg/mL and stored at −80°C.

[00133] Subsequent to peptide selection, binding activity of selected peptides is tested using the antigen-processing-deficient T2 cell line, which increases expression of HLA-A when stabilized by a peptide in the antigen-presenting groove. Briefly, T2 cells are pulsed with peptide for a time sufficient to induce HLA-A expression. HLA-A expression of T2 cells is then measured by immunostaining with a fluorescently labeled monoclonal antibody specific for HLA-A (for example, BB7.2) and flow cytometry. Fluorescence index (FI) is calculated as the mean fluorescence intensity (MFI) of HLA-A0201 on T2 cells as determined by fluorescence-activated cell-sorting analysis, using the formula FI= (MFI[T2 cells with peptide]/MFI [T2 cells without peptide] -1.

[00134] Fully human T-cell receptor (TCR)-like antibodies to Wilm's tumor oncognene protein (WT1) were produced using the method disclosed herein. TCR-like anti-WT1 antibodies generated by phage display technology are specific for a WT1 peptide/HLA complex similar to that which induces HLA-restricted cytotoxic CD8 T-cells.

[00135] The WT1 protein sequence was screened using the SYFPEITHI algorithm and WT1 peptides (for example, peptides designated 428, 328, and 122) were identified that had predicted high-affinity binding to multiple HLA molecules that are highly expressed in the Caucasian population. Peptide 428 spans WT1 amino acids 428-459, peptide 328 spans WT1 amino acids 328-349, and peptide 122 spans WT1 amino acids 122-140 (see Figure 1)

[00136] Heteroclitic peptides can also be designed by conservative amino acid substitutions of MHC-binding residues expected to enhance the affinity toward the MHC class 1 allele, as predicted by the prediction algorithm. WT1 peptide 122 comprises within it a known $CD8^+$ epitope (126-134). In one embodiment, therefore, a modified peptide of the peptide that spans the WT1 amino acid residues 126-134 and contains a modified amino acid at positions may be used. Peptides used for alanine mutagenesis of WT1A (otherwise designated RFM) were named based on the position where the substitution was made. Examples of WT1 peptides which may be used are shown in Table 10 along with irrelevant peptides, RHAMM-R3 and EW.

Table 10

WT1A (RMF)	RMFPNAPYL	SEQ ID NO.: 1
WT1A1-B	AMFPNAPYL	SEQ ID NO.: 110
WT1A-3	RMAPNAPYL	SEQ ID NO.: 111
WT1A-4	RMFANAPYL	SEQ ID NO.: 112
WT1A-5	RMFPAAPYL	SEQ ID NO.: 113
WT1A-7	RMFPNAAYL	SEQ ID NO.: 114
WT1A-8	RMFPNAPAL	SEQ ID NO.: 115
RHAMM-R3	ILSLELMKL	SEQ ID NO.: 116
EW	QLQNPSYDK	SEQ ID NO.: 117
RSDELVRHHNMHQRNMTKL		SEQ ID NO.: 118
PGCNKRYFKLSHLQMHSRKHTG		SEQ ID NO.: 119
SGQA**R**MFPNAPYLPSCLES		SEQ ID NO.: 120
SGQA**Y**MFPNAPYLPSCLES		SEQ ID NO.: 121

[00137] Once a suitable peptide has been identified, the target antigen to be used for phage display library screening, that is, a peptide/HLA complex (for example, WT1 peptide/HLA-A0201) is prepared by bringing the peptide and the histocompatibility antigen together in solution to form the complex.

Selecting a high affinity ScFV against a WT1 peptide

[00138] The next step is the selection of phage that bind to the target antigen of interest with high affinity, from phage in a human phage display library that either do not bind or that bind with lower affinity. This is accomplished by iterative binding of phage to the antigen, which is bound to a solid support, for example, beads or mammalian cells followed by removal of non-bound phage and by elution of specifically bound phage. In one embodiment, antigens are first biotinylated for immobilization to, for example, streptavidin-conjugated Dynabeads M-280. The phage library is incubated with the cells, beads or other solid support and non binding phage is removed by washing. Clones that bind are selected and tested.

[00139] Once selected, positive scFv clones are tested for their binding to HLA-A2/peptide complexes on live T2 cell surfaces by indirect flow cytometry. Briefly, phage clones are incubated with T2 cells that have been pulsed with Wt1-A peptide, or an irrelevant peptide (control). The cells are washed and then with a mouse anti-M13 coat protein mAb. Cells are washed again and labeled with a FITC-goat $(Fab)_2$ anti-mouse Ig prior to flow cytometry.

[00140] In other embodiments, the anti-WT1/A antibodies may comprise one or more framework region amino acid substitutions designed to improve protein stability, antibody binding, expression levels or to introduce a site for conjugation of therapeutic agents. These scFv are then used to produce recombinant human monoclonal Igs in accordance with methods known to those of skill in the art.

[00141] Methods for reducing the proliferation of leukemia cells is also included, comprising contacting leukemia cells with a WT1 antibody of the invention. In a related aspect, the antibodies of the invention can be used for the prevention or treatment of leukemia. Administration of therapeutic antibodies is known in the art.

Antibody Conjugates with Anti-Cancer Agents

[00142] Monoclonal antibodies represent the preferred vehicle for the targeted delivery of bioactive agents to cancer sites, including antibody-based delivery of cytotoxics, radionuclides or immunomodulatory cytokines. Conjugates of the antibodies of the invention with therapeutic agents, including without limitation, drugs (such as calecheamicin, aureastatin, doxorubicin), or toxins (such as ricin, diphtheria, gelonin) or radioisotopes emitting alpha or beta particles (such as, ^{90}Y, ^{131}I, ^{225}Ac, ^{213}Bi, ^{223}Ra and ^{227}Th), inflammatory peptides (such as IL2, TNF, IFN-γ) are encompassed by the invention.

[00143] *Pharmaceutical Compositions and Methods of Treatment*

[00144] WT1 antibodies of the present invention can be administered for therapeutic treatments to a patient suffering from a tumor or WT1-associated pathologic condition in an amount sufficient to prevent, inhibit, or reduce the progression of the tumor or pathologic condition. Progression includes, e.g, the growth, invasiveness, metastases and/or recurrence of the tumor or pathologic condition. Amounts effective for this use will depend upon the severity of the disease and the general state of the patient's own immune system. Dosing schedules will also vary with the disease state and status of the patient, and will typically range from a single bolus dosage or continuous infusion to multiple administrations per day (e.g., every 4-6 hours), or as indicated by the treating physician and the patient's condition.

[00145] The identification of medical conditions treatable by WT1antibodies of the present invention is well within the ability and knowledge of one skilled in the art. For example, human individuals who are either suffering from a clinically significant leukemic disease or who are at risk of developing clinically significant symptoms are suitable for administration of the present WT1 antibodies. A clinician skilled in the art can readily determine, for example, by the use of clinical tests, physical examination and medical/family history, if an individual is a candidate for such treatment.

[00146] Non-limiting examples of pathological conditions characterized by WT1 expression include chronic myelocytic leukemia, acute lymphoblastic leukemia (ALL), acute myeloid/myelogenous leukemia (AML) and myelodysplastic syndrome (MDS). Additionally, solid tumors, in general and in particular, tumors associated

with mesothelioma, ovarian cancer, gastrointestinal cancers, breast cancer, prostate cancer and glioblastoma are amenable to treatment using WT1 antibodies.

[00147] In another embodiment, therefore, the present invention provides a method of treating a medical condition by administering a WT1 antibody of the present invention in combination with one or more other agents. For example, an embodiment of the present invention provides a method of treating a medical condition by administering a WT1 antibody of the present invention with an antineoplastic or antiangiogenie agent. The WT1 antibody can be chemically or biosynthetically linked to one or more of the antineoplastic or antiangiogenic agents.

[00148] Any suitable method or route can be used to administer a WT1 antibody of the present invention, and optionally, to coadminister antineoplastic agents and/or antagonists of other receptors. Routes of administration include, for example, oral, intravenous, intraperitoneal, subcutaneous, or intramuscular administration. It should be emphasized, however, that the present invention is not limited to any particular method or route of administration.

[00149] It is noted that a WT1 antibody of the present invention can be administered as a conjugate, which binds specifically to the receptor and delivers a toxic, lethal payload following ligand-toxin internalization.

[00150] It is understood that WT1 antibodies of the invention will be administered in the form of a composition additionally comprising a pharmaceutically acceptable carrier. Suitable pharmaceutically acceptable carriers include, for example, one or more of water, saline, phosphate buffered saline, dextrose, glycerol, ethanol and the like, as well as combinations thereof. Pharmaceutically acceptable carriers may further comprise minor amounts of auxiliary substances such as wetting or emulsifying agents, preservatives or buffers, which enhance the shelf life or effectiveness of the binding proteins. The compositions of the injection may, as is well known in the art, be formulated so as to provide quick, sustained or delayed release of the active ingredient after administration to the mammal.

[00151] Other aspects of the invention include without limitation, the use of antibodies and nucleic acids that encode them for treatment of WT1 associated disease, for diagnostic and prognostic applications as well as use as research tools

for the detection of WT1 in cells and tissues. Pharmaceutical compositions comprising the disclosed antibodies and nucleic acids are encompassed by the invention. Vectors comprising the nucleic acids of the invention for antibody-based treatment by vectored immunotherapy are also contemplated by the present invention. Vectors include expression vectors which enable the expression and secretion of antibodies, as well as vectors which are directed to cell surface expression of the antigen binding proteins, such as chimeric antigen receptors.

[00152] Cells comprising the nucleic acids, for example cells that have been transfected with the vectors of the invention are also encompassed by the disclosure.

[00153] For use in diagnostic and research applications, kits are also provided that contain a WT1 antibody or nucleic acids of the invention, assay reagents, buffers, and the like.

[00154] The method of the present invention will now be described in more detail with respect to representative embodiments.

Materials

[00155] *Cell samples, cell lines and antibodies.* After informed consent on Memorial Sloan-Kettering Cancer Center Institutional Review Board approved protocols, peripheral blood mononuclear cells (PBMC) from HLA-typed healthy donors and patients were obtained by Ficoll density centrifugation. The sources for obtaining human mesothelioma cell lines are described previously (29). The cell lines include: H-Meso1A, JMN , VAMT, H2452 , H2373, H28, MSTO, Meso 11, Meso 34, Meso 37, Meso 47, and Meso 56. All cells were HLA typed by the Department of Cellular Immunology at Memorial Sloan-Kettering Cancer Center. Leukemia cell lines LAMA81, BV173, and 697, (WT1+, A0201+) were kindly provided by Dr. H. J. Stauss (University College London, London, United Kingdom). Melanoma cell line MeWo (WT1-, A201+), SKLY16 (B-cell lymphoma; WT1-, A0201+); K562, RwLeu4, and HL60, all WT1+ leukemias, and a TAP-deficient T2 cell line were obtained from the American Type Culture Collection. The cell lines were cultured in RPMI 1640 supplemented with 5% FCS, penicillin, streptomycin, 2 mmol/L glutamine, and 2-mercaptoethanol at 37 C/5% CO2.

[00156] Monoclonal Ab against human HLA-A2 (clone BB7.2) conjugated to FITC or APC, and its isotype control mouse IgG2b/FITC or APC, to human or mouse CD3, CD19, CD56, CD33, CD34 (BD Biosciences, San Diego), goat F(ab)2 anti-human IgG conjugated with PE or FITC and goat F(ab)2 anti-mouse Ig's conjugated to fluorescent (In Vitrogen, City) were purchased. Mouse mAb to HLA-class I (W6/32) was obtained from the MSKCC Monoclonal antibody Core Facility.

[00157] *Peptides*. All peptides were purchased and synthesized by Genemed Synthesis, Inc. (San Antonio, TX). Peptides were > 90% pure. (Table 1.) The peptides were dissolved in DMSO and diluted in saline at 5 mg/mL and frozen at-180C. Biotinylated single chain WT1peptide/HLA-A0201 and RHAMM-3/HLA-A0201 complexes were synthesized by refolding the peptides with recombinant HLA-A2 and beta2 microglobulen (β2M) at the Tetramer facility at MSKCC.

[00158] *Animals*. Eight to 10 week-old NOD.Cg-Prkdc scid IL2rgtm1Wjl/SzJ mice, known as NOD scid gamma (NSG), were purchased from the Jackson Laboratory (Bar Harbor, ME) or obtained from MSKCC animal breeding facility.

Methods

[00159] *Flow cytometry analysis*. For cell surface staining, cells were incubated with appropriate mAbs for 30 minutes on ice, washed, and incubated with secondary antibody reagents when necessary. Flow cytometry data were collected on a FACS Calibur (Becton Dickinson) and analyzed with FlowJo V8.7.1 and 9.4.8 software.

[00160] *Selection and characterization of scFv specific for WT1 peptide/HLA-A0201 complexes.* A human scFv antibody phage display library was used for the selection of mAb clones. In order to reduce the conformational change of MHC1 complex introduces by immobilizing onto plastic surfaces, a solution panning method was used in place of conventional plate panning. In brief, biotinylated antigens were first mixed with the human scFv phage library, then the antigen-scFv antibody complexes were ppulled down by streptavidin-conjugated Dynabeads M-280 through a magnetic rack. Bound clones were then eluted and were used to infect *E.Coli* XL1-Blue. The scFv phage clones expressed in the bacteria were purified (35, 36). Panning was performed for 3-4 cycles to enrich scFv phage clones binding to HLA-A0201/WT1 complex specifically. Positive clones were determined by standard ELISA method

against biotinylated single chain HLA-A0201/WT1 peptide complexes. Positive clones were further tested for their binding to HLA-A2/peptide complexes on live cell surfaces by flow cytometry, using a TAP-deficient, HLA-A0201+ cell line, T2. T2 cells were pulsed with peptides (50ug/ml) in the serum-free RPMI1640 medium, in the presence of 20 μg/ml β2 M ON. The cells were washed, and the staining was performed in following steps.

[00161] The cells were first stained with purified scFv phage clones, and followed by staining with a mouse anti-M13 mAb, and finally the goat F(ab)2 anti-mouse Ig's conjugate to FITC. Each step of the staining was done between 30 -60 minutes on ice and the cells were washed twice between each step of the staining.

[00162] *Engineering full length mAb using the selected ScFv fragments*. Full-length human IgG1 of the selected phage clones were produced in HEK293 and Chinese hamster ovary (CHO) cell lines, as described (37). In brief, antibody variable regions were subcloned into mammalian expression vectors, with matching human lambda or kappa light chain constant region and human IgG1 constant region sequences. Molecular weight of the purified full length IgG antibodies were measured under both reducing and non-reducing conditions by electrophoresis.

[00163] *Engineering chimeric antigen receptors and immune effector cells*. Nucleic acids that encode antibodies and antigen binding proteins identified herein can be used engineer recombinant immune effector cells. Methods and vectors to generate genetically modified T-cells, for example, are known in the art (See Brentjens et al., *Safety and persistence of adoptively transferred autologous CD19-targeted T cells in patients with relapsed or chemotherapy refractory B-cell leukemias* in Blood 118(18):4817-4828, November 2011).

[00164] *Characterization of the full-length human IgG1 for the WT1p/A2 complex.* Initially, specificities of the fully human IgG1 mAbs for the WT1peptide/A2 complex were determined by staining T2 cells pulsed with or without RMF or RHAMM-R3 control peptides, followed by secondary goat F(ab)2 anti-human IgG mAb conjugate to PE or FITC. The fluorescence intensity was measured by flow cytometry. The same method was used to determine the binding of the mAbs to fresh tumor cells and cell lines.

[00165] *Radioimmunoassays.* WT1 ab1 was labeled with 125-I (PerkinElmer) using the chloramine-T method (38). 100µg antibody was reacted with 1mCi 125-I and 20µg chloramine-T, quenched with 200µg Na metabisulfite, then separated from free 125-I using a 10DG column (company) equilibrated with 2% bovine serum albumin in PBS. Specific activities of products were in the range of 7-8 mCi/mg.

[00166] Hematopoietic cell lines, adherent cell lines (harvested with a non-enzymatic cell stripper (name)), PBMCs from normal donors and AML patients were obtained as described. Cells were washed once with PBS and re-suspended in 2% human serum in PBS at 10^7 cells/mL at 0^0. Cells (10^6 tube in duplicate) were incubated with125-I-labeled WT1 AB1 (1µg/mL) for 45 minutes on ice, then washed extensively with 1% bovine serum albumin in PBS at 0^0. To determine specific binding, a duplicate set of cells was assayed after pre-incubation in the presence of 50-fold excess unlabeled WT1 AB1 for 20 minutes on ice. Bound radioactivity was measured by a gamma counter, specific binding was determined, and the number of bound antibodies per cell was calculated from specific activity.

[00167] *Antibody-dependent cellular cytotoxicity (ADCC).* Target cells used for ADCC were T2 cells pulsed with or without WT1 or RHAAM-3 peptides, and tumor cell lines without peptide pulsing. WT1 ab1 or its isotype control human IgG1 at various concentrations were incubated with target cells and fresh PBMCs at different effector: target (E:T) ratio for 16 hrs. The supernatant were harvested and the cytotoxicity was measured by LDH release assay using Cytotox 96 non-radioreactive kit from Promega following their instruction. Cytotoxicity is also measured by standard 4 hours 51Cr-release assay.

[00168] *Transduction and selection of luciferase/GFP positive cells.* BV173 and JMN cells were engineered to express high level of GFP-luciferase fusion protein, using lentiviral vectors containing a plasmid encoding the luc/GFP (39). Using single cell cloning, only the cells showing high level GFP expression were selected by flow cytometry analysis and were maintained and used for the animal study.

[00169] *Therapeutic trials of the WT1 ab1 in a human leukemia xenograft NSG model.* Two million BV173 human leukemia cells were injected IV into NSG mice. On day 5, tumor engraftment was confirmed by firefly luciferase imaging in all mice

that were to be treated; mice were then randomly divided into different treatment groups. On day 6 and day 10, mAb WT1 ab1 or the isotype control mAb were injected IV. In animals that also received human effector cells with or without mAb, cells (CD34 and CD3-depleted healthy donor human PBMCs) were injected IV into mice (10^7 cells/mouse) 4 hr before the mAb injections. Tumor growth was assessed by luminescence imaging once to twice a week, and clinical activity was assessed daily.

[00170] *Selection and characterization of scFv specific for WT1 peptide/HLA-A0201 complexes.* Selection of an WT1-specific scFV is achieved using a 9-mer WT1-derived peptide comprising amino acids 126-134 (RMFPNAPYL, SEQ ID NO: 1) of WT1. This peptide has been shown to be processed and presented by HLA-A0201 to induce cytotoxic $CD8^+$ T cells that are capable of killing WT1-positive tumor cells.

[00171] Representative data from a patient with AML after 6 vaccinations with a WT1 RMF peptide are shown in Figure 2 as evidence that the WT1 peptides are immunogenic in humans. CD3 T cells were stimulated with WT1-A peptide (amino acids 126-134) and cytotoxicity was measured using a standard ^{51}Cr release assay against 697 ($A0201^+$ $WT1^+$) or SKLY-16 ($A0201^+$ $WT1^-$) cell lines. The SKLY-16 cells pulsed with WT1-A or irrelevant peptide EW were used as positive and negative controls for the specificity of the killing. Effector: Target (E:T) ratios are indicated on the X-axis. Data demonstrates that T cells killed $WT1^+$ tumor cells in a HLA- A0201-restricted manner.

[00172] Well established phage display libraries and screening methods known to those of skill in the art were used to select scFv fragments highly specific for an WT1-A peptide/HLA-A2 complex. In one embodiment, a human scFv antibody phage display library (7×10^{10} clones) was used for the selection of mAb clones. In order to reduce the conformational change of MHC1 complex introduced by immobilizing onto plastic surfaces, a solution panning method was used in place of conventional plate panning. In brief, biotinylated antigens were first mixed with the human scFv phage library, then the antigen-scFv phage antibody complexes were pulled down by streptavidin-conjugated Dynabeads M-280 through a magnetic rack.

[00173] Bound clones were then eluted and were used to infect *E.Coli* XL1-Blue. The scFv phage clones expressed in the bacteria were purified (35, 36). Panning was performed for 3-4 cycles to enrich scFv phage clones binding to HLA-A0201/WT1 complex specifically. Positive clones were determined by standard ELISA method against biotinylated single chain HLA-A0201/WT1 peptide complexes (Figure 3). Positive clones were further tested for their binding to HLA-A2/peptide complexes on live cell surfaces by flow cytometry, using a TAP-deficient, HLA-$A0201^+$ cell line, T2. T2 cells were pulsed with peptides (50μg/ml) in serum-free RPMI1640 medium, in the presence of 20 μg/ml β2 M overnight. The cells were washed, and staining was performed as follows.

[00174] The cells were first stained with purified scFv phage clones, followed by staining with a mouse anti-M13 mAb, and finally, a goat $F(ab)_2$ anti-mouse Ig conjugated to FITC. Each step of the staining was done for 30-60 minutes on ice. The cells were washed twice between each staining step. Results are shown in Figure 4. The phage clone of WT1 ab1 was shown to bind to T2 cells pulsed with only WT1-A peptide (RMFPNAPYL: abbreviated hereinafter as RMF), but not to T2 cells alone, T2 cells pulsed with control EW peptide, or heteroclitic peptide WT1-A.

[00175] Binding affinity of the full-length IgG1 of WT1 ab1 to the peptide/A0201 complex was tested by titration of WT1 ab1 at indicated concentrations. T2 cells were pulsed with 50 μg/ml or 10μg/ml, followed by secondary goat F(ab) anti-human IgG/PE. Results are shown in Figure 5.

[00176] Figure 6 shows the density of peptide/HLA-A0201 complex recognized by WT1 ab. T2 cells were pulsed overnight (ON) with RMF (upper panel) or RHAMM-R3 (lower panel) peptides at indicated concentrations, and binding of WT1 ab1, WT1 ab3 and WT1 ab5 at a concentration of 1 μg/ml was analyzed by flow cytometry.

Table 11: Summary of phage panning for WTI/A2

Phage libraries	*Rounds of panning*	*Number of single clone screened*	*Solution ELISA positive Rate*	*Number of Unique Clones*
scFv-spleen A	4	72	41/96	13
scFv-spleen B	4	47	3/47	2
scFv-spleen C	3	58	0/58	0
scFv-PBMC A	4	68	34/68	10
scFv-PBMC B	3	90	19/90	7
Fab-spleen A	4	12	2/12	0
Fab-spleen B	4	36	0/36	0
Fab-spleen C	4	24	2/24	1
Fab-spleen C	3	72	38/72	5
Fab-spleen D	4	72	4/72	1
Fab-spleen D	4	72	4/72	3

[00177] The positive scFv clones were tested for their binding to HLA-A2/peptide complexes on live cell surfaces by indirect flow cytometry on: (i) a TAP deficient HLA-A0201$^+$ T2 cells pulsed with WT1 peptide or irrelevant peptide; (ii) a WT1+ HLA-A0201$^+$ cell lines such as BV173, U266 and control WTI$^-$ HLA-A0201$^+$ cell line SKLY-16, or WT1$^+$ HLA-A0201$^-$ cell line, K562, without pulsing with the peptide. The latter

determine the recognition and binding affinity of the scFv to the naturally processed WT1p/A2 complex on tumor cells.

[00178] A total of 28 phage clones were screened for their ability to produce mAb specific for the WTI-A peptide/A2 complex. The recognition of the WT1p/A2 complex on live cells was measured by the binding of the phage scFv to T2 cells pulsed with the WTI-A peptide and the other HLA-A2-binding peptides (50 µg/ml). These include: T2 cells alone; T2 cells pulsed with WTI-A peptide; T2 cells pulsed with heteroclitic peptide WT1-A1; T2 cells pulsed with irrelevant EW peptide (HLA-A0201- binding 9-mer peptide, derived from Ewing sarcoma) or RHAMM-R3 (Figure 4).

Table 12

Clone #	Positive for binding toT2 pulsed with WT1A	Selected for Construction of full-length IgG1
1	+	
2	+	
3	+	+
4	+	
5	+	+
6	+	
7		
8	+	
9		
10		
11		
12		
13	+	+
14		
15	+	+

16		
17	+	
18	+	+
19	+	
20	+	
21		
22	+	
23	+	+
24-28		

Engineering full length mAb using the selected ScFv fragments.

[00179] Phage display technology allows for the rapid selection and production of antigen-specific scFv and Fab fragments, which are useful in and of themselves, or which can be further developed to provide complete antibodies, antigen binding proteins or antigen binding fragments thereof. Complete mAbs with Fc domains have a number of advantages over the scFv and Fab antibodies. First, only full length Abs exert immunological function such as CDC and ADCC mediated via Fc domain. Second, bivalent mAbs offer stronger antigen-binding affinity than monomeric Fab Abs. Third, plasma half- life and renal clearance will be different with the Fab and bivalent mAb. The particular features and advantages of each can be matched to the planned effector strategy. Fourth, bivalent mAb may be internalized at different rates than scFv and Fab, altering immune function or carrier function. Alpha emitters, for example, do not need to be internalized to kill the targets, but many drugs and toxins will benefit from internalization of the immune complex. In one embodiment, therefore, once scFv clones specific for WT1p/A2 were obtained from phage display libraries, a full length IgG mAb using the scFv fragments was produced.

[00180] To produce recombinant human monoclonal IgG in Chinese hamster ovary (CHO) cells, a full length IgG mAb was engineered based on a method known to

those of skill in the art (Tomomatsu et al., Production of human monoclonal antibodies against FceRIa by a method combining *in vitro* immunization with phage display. Biosci Biotechnol Biochem 73(7): 1465-1469 2009). Briefly, antibody variable regions were subcloned into mammalian expression vectors (Figure 7), with matching Lambda or Kappa light chain constant sequences and IgG1 subclass Fc (for example, see Table 9) (33,34). Purified full length IgG antibodies showed expected molecular weight under both reducing and non-reducing conditions (Figure 8). Kinetic binding analysis (35) confirmed specific binding of full length IgG to WT1/A2, with a KD in nanomolar range (Figures 9 and 10.)

EXAMPLE 1

Selection of ScFv specific for WT1p/A2 complex using a fully human phage display library.

[00181] Phage display against HLA-A0201/WT1 peptide complex was performed for 3-4 panning rounds to enrich the scFv phage clones binding to HLA-A0201/WT1 peptide complex specifically. Individual scFv phage clones positive for the WT1 peptide/A2 complex were determined by ELISA and the clones that possessed unique DNA coding sequences were subjected to further characterization. To test if the ScFv bound to the WT1p/A2 complex on live cells, the positive phage clones were tested for binding to a TAP deficient, HLA-A0201-positive cell line, T2. T2 cells can only present the exogenous peptides and therefore have been widely used for detection of specific epitopes presented by HLA-A2 molecules. A total 35 phage clones were screened on T2 cells and 15 clones showed specific binding to T2 cells pulsed with only WT1 RMF peptide, but not to T2 cells alone or pulsed with control RHAMM-3 peptide (Figure 4). The scFv phage clones were unable to bind to several tumor cell lines that are WT1- and HLA-A2 positive suggesting the affinity of the ScFv was weak, compared to full-length bivalent mAb.

EXAMPLE 2

Generation of full-length human IgG1.

[00182] Immunological function such as CDC and ADCC depend on the Fc domain of bivalent IgG. In addition, bivalent mAbs offer stronger antigen-binding avidity than

monomeric scFv Abs. Therefore, 6 ScFv phage clones among 15 positive phage clones were selected to produce the full-length human monoclonal IgG1 in HEK293 and Chinese hamster ovary (CHO) cells. In brief, variable regions of the mAbs were sub-cloned into mammalian expression vectors with matching human lambda or kappa light chain constant region and human IgG1 constant region sequences. Purified full length IgG antibodies showed expected molecular weight under both reducing and non-reducing conditions (Figure 8). Five clones were successfully engineered into human IgG1.

EXAMPLE 3

Specificity and binding avidity of the IgG1 mAb

Binding to human cell lines.

T2 cells, pulsed with or without RMF or RHAMM-3 peptides initially were used to determine the binding specificity of the mAb. Three out of five human IgG1, including WT1 ab1, showed specific binding to the T2 cells that were pulsed only with WT1 peptide, but not to T2 alone or T2 pulsed with control peptide RHAMM-R3. The binding avidity of the mAb were substantially enhanced (50 to 100 fold), compared to their parental scFv phage clones. Two mAbs among the five showed binding to T2 cells alone or pulsed with the control peptide RHAMM-R3, although the binding was greatly enhanced by pulsing the cells with RMF peptide. This suggested that these two mAb also had high avidity for epitopes on the HLA-A2 molecule alone and therefore were excluded from further investigation. This was not unexpected, as it has been a common problem for producing such mAb against peptide/MHC complexes, given the predominance of the MHC class I molecule epitopes within the complexes. It also suggests that the precise specificity of the mAb for the complexes might not be determined easily at the scFv stage, due to the lower affinity compared to the bivalent IgG1 mAb.

[00183] The binding affinity of the three remaining mAb specific for the WT1p/A2 complex first was investigated on T2 cells pulsed with or without RMF and control RHAMM-R3 peptides (50 ug/ml) by titration of the mAbs. Mab WT1 ab1 showed the strongest binding, down to a concentration of 0.01ug/ml. Isotype control human

IgG1 showed no binding at any concentrations tested (Figure 5). In addition to WT1 ab1, the two other mAb, WT1 ab3 and WT1 ab5, showed specific binding at a range of <1 ug/ml of the mAb concentrations used. The specific recognition of the mAb also depended on the antigenic density on the cell surface. T2 cells were pulsed with RMF or R3 peptides at 50, 25, 12.5, 6.25, 3.13 and 1.6 ug/ml; the test mAb were used at 1ug/ml for the T2 binding assay. WT1 ab1 could detect the RMF peptide/A2 complex on T2 cells in a concentration-dependent manner at concentrations as low as 1.6 ug/ml, with significantly higher fluorescence intensity than the other 2 mAb (Figure 6). These results further confirmed that the WT1 ab1 possessed the highest avidity for the RMFp/A0201 complex.

EXAMPLE 4

Epitope mapping.

[00184] To investigate with more precision the epitope for WT1 ab1 recognition, RMF peptides were substituted at positions 1, 3, 4, 5, 6, 7 and 8 with alanine and pulsed onto T2 cells and were tested for binding of WT1 ab1. Positions 2 and 9 of the RMF were left intact, as these are the anchor residues for peptide binding to the HLA-A0201 molecule. Except for position 1, alanine substitutions at other positions did not greatly affect the binding of the WT1 ab1, as compared to the native RMF peptide (Figure 19). However, substitution of position 1 by either alanine (WT1-A1-B) or tyrosine (WT1-A1), completely abrogated the binding of WT1 ab1. The loss of binding was not due to the reduction of peptide binding affinity to the HLA-A2 molecule, as both peptides showed the strongest binding in T2 stabilization assay using the mAb specific for the HLA-A2 molecule, clone BB7 (Figure 20). These results show that the arginine at position 1 of the RMF peptide is one of the most crucial for the WT1 ab1 recognition. The role of the residues at positions #2 and 9, could not be assessed.

[00185] The next important question was whether WT1 ab1 was able to recognize naturally processed WT1 epitope RMF presented by HLA-A0201 molecules on the cell surface. A panel of cell lines was selected based on the expression of WT1 mRNA and HLA genotyping (Table 12).

Table 12

Mesothelioma/solid tumor	HLA-A2 genotype	WT1 mRNA	WT1 AB binding	Ratio of BB7.2: Isotype
JMN	+	+	+	248
Meso 37	+	+	+	68
Meso 47	+ (02xx)	+	+	17
H2452	+	+	+	20
Meso34	+	+	+	37.3
Meso-56	+ (02xx)	+	+	23
H2373	+	+	-	1.6
MSTO	-	+	-	1.4
VAMT	-	3+	-	NT
Mewo	+	-	-	3
Leukemias and other hematopoietic cell lines				
BV173	+	++	+	196
BA25	+	?	+	117.5
ALL-3	+	+	+	60
U266	+	+	-	1.8
697	+	5+	-	4.1
LAMA	+	2+	-	6
SKLY-16	+	-	-	1.9
HL-60	-	3+	-	0.4
K562	-	2+	-	1.5
T2	+	NT	-	>20

[00186] WT1 mRNA expression level was estimated according to a previous study (Rena), by quantitative RT-PCR.

[00187] Among 7 human mesothelioma cell lines that are positive for both HLA-A0201 and WT1 mRNA, WT1 ab1 bound to 6 out of 7 cell lines, but not to the cells that were either HLA-A0201 negative (MSTO and VAMT) or WT1 mRNA negative, such as melanoma cell line, Mewo (Figure 21).

[00188] Similarly, among 9 leukemia cell lines tested, WT1 ab1 bound to 3 cell lines BV173 (Figure 22), BA25 and ALL-3, that are positive for both WT1 mRNA and HLA-A0201, but not to HLA-A2-negative cell lines HL60 and K562, that have been demonstrated to express a high level of WT1 transcripts in numerous studies.

[00189] As expected, intensity of binding of the WT1 AB1 also appeared to be directly associated with the expression level of HLA-A0201molecule, as shown in mesothelioma cells H2373, leukemia cell lines 697 and LAMA, and myeloma cell line U266. Although these cell lines were positive for both WT1 transcripts and HLA-A2, the expression level of the HLA-A2 was low (Table 12) and the mAb did not show binding. On the other hand, the results obtained with T2 cells argue against the possibility of WT1 ab1 binding to HLA- A0201 alone as T2 cells expressed a high level of HLA-A2 molecule. Notably, WT1 ab1 did not bind to T2 cells alone or pulsed with R3 and other HLA-A0201-binding peptides such as Ewing sarcoma-derived (EW) or the heteroclitic peptide for the RMF peptide, WT1-A1; these two peptides have been shown to have higher affinity for the HLA-A0201 molecule in T2 stabilization assay (28). These results provided strong evidences that WT1 ab1 recognition was specific for epitopes jointly composed of the RMF peptide and the A0201 molecule in a complex. The binding of the other two mAb, WT1 ab3 and WT1 ab5, to the BV173 and JMN cells was also weaker than WT1 ab1.

EXAMPLE 5

Quantitation of WT1 ab1Binding Sites on Cells.

[00190] A radioimmunoassay using 125I-labeled WT1 ab1 was used to confirm the specificity of the antibody for $WT1^+$ $HLA\text{-}A0201^+$ cell lines, to determine an affinity constant and to assess the number of antibody binding sites per cell on a panel of

cell lines. Scatchard analysis based on binding to JMN cells showed an avidity constant of about 0.2nM (Figure 23). This number was confirmed by interferometry using a Forte Bio device. 125-I-labeled WT1 ab1 was used to confirm the specificity of the antibody for $WT1^+$ $HLA\text{-}A0201^+$ cell lines, and to assess the number of antibody binding sites on a panel of cell lines (Figure 24). Because we cannot determine whether the bivalent mAb is binding to 1 or 2 complexes on the surface, total epitopes per cell could be as high as twice the number of mAb binding sites. Again, WT1 ab1 bound to JMN, ALL-3, BA25, BV173, which are positive for both HLA-A0201 and WT1 mRNA, but not HLA-A0201 negative (HL60) or WT1 mRNA negative (SKLY-16) cells. WT1 ab1 did not bind to 697 cells, which are both HLA-A0201 and WT1 positive, but contain low levels of HLA-A0201 (Table 12), confirming that a certain level of total MHC complex is needed to present sufficient WT1 peptide for WT1 ab1 binding. T2 pulsed with RMF bound the highest number of mAb (50,000 per cell), followed by JMN cells which bound ~$6x10^3$ WT1 ab1 molecules per cell, translating to between $6x10^3$ and $1.2x10^4$ RMFpeptide/A2 complexes per cell assuming monovalent or bivalent antibody binding, respectively. The three positive leukemia cell lines bound between 1 $x10^3$ and 2 $x10^3$ WT1 ab1 molecules, or 2 $x10^3$-4 $x10^3$ binding sites (Figure 24). These results were confirmed by quantitative flow cytometry.

EXAMPLE 6

WT1 ab1 Binding to leukemic patient samples.

[00191] We next investigated if WT1 ab1 is able to detect the RMF epitope on primary AML cells. Radioimmunoassay showed significant binding of the WT1 AB1 to AML blasts of patient 1, who is HLA-A2 positive and WT1 $mRNA^+$. WT1 ab1 bound to $CD33^+$ and $CD34^+$ double positive cells that account for more than 83% of the whole cell populations (Figure 25). WT1 ab1 did not bind to the cells of 3 other patients who are either HLA-A2 positive but mRNA negative or HLA-A2 negative. WT1 ab1 did not bind to PBMCs from either HLA-A2 positive or negative healthy donors. The results were confirmed by flow cytometry analysis. WT1 AB1 did not show significant binding to the blasts from the patients who were A0201 negative (Figure 26). The results were consistent with the results obtained with mRNA expression of the cells. These data confirm that the level of RMFp/HLA-A0201 on

the surface of leukemia cells is adequate to allow reactivity with the WT1 ab1 and the levels on WT1 negative healthy cells is not significant.

EXAMPLE 7

WT1 AB1 mediates ADCC against tumor cells

[00192] ADCC is considered to be one of the major effector mechanisms of therapeutic mAb in humans. In the presence of human PBMC, WT1 ab1 mediated dose-dependent PBMC ADCC against the T2 cells loaded with RMF peptide, but not T2 cells alone or T2 cells pulsed with control R3 peptide (Figure 27). Importantly, WT1 ab1 was able to mediate ADCC against naturally presented RMF epitope by HLA-A0201 molecule on tumor cells, such as the mesothelioma cell line, JMN (Figure 33), and the leukemia cell line BV173 (Figure 34), but not the HLA-A2 negative cells MSTO (Figure 28) or HL-60 (Figure 29). The killing was consistently observed at 1μg/ml or below of WT1 ab1 using PBMCs as effector cells from multiple healthy donors. Importantly, WT1 ab1 also killed primary A0201-positive AML blasts that were positive for the WT1 ab1 binding, but not the blasts that were HLA-A0201 negative (Figure 30). These results demonstrated that WT1 ab1 mediates specific ADCC against cells that naturally express RMF and HLA-A0201 complex at physiologic levels as well as on cell lines.

EXAMPLE 8

WT1 AB1 eliminates human leukemia cells in NSG mice

[00193] The efficacy of WT1 ab1 in vivo was tested in NOD SCID gamma (NSG) mice xenografted intravenously 6 days previously with BV173 bcr/abl positive acute lymphoblastic leukemia. At the time of treatment, mice had leukemia in their liver, spleen, and BM visible by luciferase imaging. NSG mice lack mature B-, T- and NK-cells, and we hypothesized that introducing human effector cells ($CD3^-$, $CD34^-$, PBMCs) along with WT1 ab1 treatment would recapitulate *in vivo* the ADCC-mediated anti-tumor effects observed in vitro. Injection of effectors along with two 100 μg doses of WT1 ab1 nearly ablated tumor growth compared to controls (Figure 31). This effect was durable over the course of the experiment (Figure 32). Interestingly, early on in the trials, effector cells alone or combined with control IgG

appeared to promote more rapid growth of leukemia relative to mice injected with leukemia alone, demonstrating that the anti-tumor effect was unrelated to the effectors by themselves. Several of the mice given effectors (with or without control mAb) died early in the experiment with massive infiltration of the BV173.

[00194] Surprisingly, WT1 ab1 treatment without human effectors also dramatically reduced tumor burden as well as the WT1 ab1 combined with effectors for approximately 30 days (Figure 32), though tumors eventually relapsed far more quickly in the WT1 ab1 alone group, when compared to WT1 ab1 combined with effectors group (Figure 32). We confirmed the effect of WT1 ab1 alone and titrated the dosage to evaluate potency. WT1 ab1 alone produced a marked reduction in tumor burden at early time points at all doses tested (25-100 µg times 2 doses). Tumors in all treatment groups relapsed slowly after antibody therapy was stopped; and by day 23 (13 days after the last antibody injection), significantly more tumor relapse could be observed in the 25 µg group compared to the 100 µg dose group, indicating a dose-response to WT1 ab1 therapy (Figure 33). Before treatment, mice displayed the largest tumor burden in the liver, which was quickly cleared by WT1 ab1. Upon relapse, tumor in the highest dose group appeared to develop mainly in bone marrow, while tumor returned more quickly to the liver in mice treated with the lowest dose.

EXAMPLE 9

Engineering antibodies to enhance their cytotoxic abilities.

[00195] Bispecific antibodies are constructed that recognize both WT1/A2 complex and CD3 on immune T cells as described (43,44) with a human IgG1 Fc. Bispecific antibodies are expected to recruit and target cytotoxic T cells to WT1/A2 positive cancer cells, while maintaining Fc effector functions and long half life *in vivo*. Three mechanisms are involved in the specific killing of cancer cells mediated by bispecific antibodies: i) killing by activated T cells; ii) ADCC activity; iii) CDC activity. Other formats of bispecific antibodies can be constructed, such tandem scFv molecules (taFv), diabodies (Db), or single chain diabodies (scDb), and fusion protein with human serum albumin (45, 46, 47, 48), but are devoid of Fc effector functions with distinct pharmacokinetic profiles.

[00196] WT1/A2 target specific-ADCC activity is enhanced by expressing antibodies recombinantly in glycol-engineered CHO cells as described in U.S. patents 8,025,879; 8,080,415; and 8,084,022. The modified oligosaccharide N-glycan on Asn297 alters effector functions as follows: 1) higher affinity binding to CD16/FcRIIIa for improved ADCC activity mediated by human Natural Killer cells; 2) reduced binding affinity to CD32b/FcRIIb, an inhibitory receptor expressed in multiple types of immune cells (except NK cells), for improved ADCC activity mediated by effector cells such as neutrophils and antigen presentation by macrophage and DC cells (50, 51, 52). Enhanced antibodies are expected to achieve better efficacy for cancer treatment *in vivo*.

[00197] Glycosylation (specifically fucosylation) variants of IgG Fc can be produced using host expression cells and methods described in U.S. patents 8,025,879; 8,080,415; and 8,084,022, the contents of which are incorporated by reference. Briefly, messenger RNA (mRNA) coding for heavy or light chain of the antibodies disclosed herein, is obtained by employing standard techniques of RNA isolation purification and optionally size based isolation. cDNAs corresponding to mRNAs coding for heavy or light chain are then produced and isolated using techniques known in the art, such as cDNA library construction, phage library construction and screening or RT-PCR using specific relevant primers. In some embodiments, the cDNA sequence may be one that is wholly or partially manufactured using known in vitro DNA manipulation techniques to produce a specific desired cDNA. The cDNA sequence can then be positioned in a vector which contains a promoter in reading frame with the gene and compatible with the low fucose-modified host cell.

[00198] Numerous plasmids that contain appropriate promoters, control sequences, ribosome binding sites, and transcription termination sites, and optionally convenient markers are known in the art, these include but are not limited to, vectors described in U.S. Pat. Nos. 4,663,283 and 4,456,748. In one embodiment, the cDNA coding for the light chain and that coding for the heavy chain may be inserted into separate expression plasmids. In an alternative embodiment, the cDNA coding for the light chain and that coding for the heavy chain may be inserted together in the same plasmid, so long as each is under suitable promoter and translation control. Results are shown in Figure 34.

References

1. Mundlos S, et al. Nuclear localization of the protein encoded by the Wilms' tumor gene WT1 in embryonic and adult tissues. *Development* 1993;119: 1329-41.

2. Keilholz U, et al. Wilms' tumor gene 1 (WT1) in human neoplasia. *Leukemia* 2005; 19: 1318-1323.

3. Inoue K, et al. WT1 as a new prognostic factor and a new marker for the detection of minimal residual disease in acute leukemia. Blood 1994; 84 (9): 3071-3079.

4. Ogawa H, et al. The usefulness of monitoring WT1 gene transcripts for the prediction and management of relapse following allogeneic stem cell transplantation in acute type leukemia. Blood 2003; 101 (5): 1698- 1704.

5. Yarnagarni T, et al. Growth Inhibition of Human Leukemic Cells by WT1(Wilms Tumor Gene) Antisense Oligodeoxynucleotides: Implications for the Involvement of WT1 in Leukemogenesis. *Blood* 1996; 87: 2878- 2884.

6. Bellantuono I, et al. Two distinct HLA-A0201-presented epitopes of th Wilms tumor antigen 1 can function as targets for leukemia-reactive CTL. *Blood* 2002; 100 (10): 3835-3837.

7. Gaiger A, et al. WT1- specific serum antibodies in patients with leukemia. *Clin. Cancer Res*. 2001; 7 (suppl 3): 761-765.

8. Oka Y, et al. WT1 peptide cancer vaccine for patients with hematopoietic malignancies and solid cancers. *The Scientific World Journal* 2007; 7: 649-665.

9. Kobayashi H, et al. Defining MHC class II T helper epitopes from WT1 antigen. *Cancer Immunol.Immunother*. 2006; 55 (7): 850-860.

10. Pinilla-Ibarz J, et al. Improved human T-cell responses against synthetic HLA-A0201 analog peptides derived from the WT1 oncoprotein. *Leukemia* 2006; 20 (11): 2025-2033.

11. May RJ, et al. Peptide epitopes from the Wilms tumor 1 oncoprotein stimulate CD4+ and CD8+ T cells that recognize and kill human malignant mesothelioma tumor cells. *Clin Cancer Res*. 2007; 13:4547-4555.

12. Keiholz U, et al. A clinical and immunologic phase 2 trial of Wils tumor gene product (WT1) peptide vaccination in patients with AML and MDS. Blood 2009; 113: 6541-6548.

13. Rezwani K, et al. Leukemia-associated antigen-specific T-cell responses following combined PR1 and WT1 peptide vaccination in patients with myeloid malignancies. Blood 2008; 111 (1): 236-242.

14. Maslak P, et al.. Vaccination with synthetic analog peptides derived from WT1 oncoprotein induces T cell responses in patients with complete remission from acute myeloid leukemia. *Blood* 2010; Accpt Minor rev.

15. Krug LM, et al. WT1 peptide vaccinations induce CD4 and CD8 T cell immune responses in patients with mesothelioma and non-small cell lung cancer. *Cancer Immunol Immunother* 2010; in revision.

16. Morris E, et al. Generation of tumor-specific T-cell therapies. *Blood Reviews* 2006; 20: 61-69.

17. Houghton AN et al. Monoclonal antibody therapies- a "constant" threat to cancer. *Nat Med* 2000; 6:373- 374.

18. Miederer M, et al. Realizing the potential of the Actinium-225 radionuclide generator in targeted alpha particle therapy applications. *Adv Drug Deliv Rev* 2008; 60 (12): 1371-1382.

19. Noy R, T-cell-receptor-like antibodies: novel reagents for clinical cancer immunology and immunotherapy. *Expert Rev Anticancer Ther* 2005: 5 (3): 523-536.

20. Chames P, et al. Direct selection of a human antibody fragment directed against the tumor T-cell epitope HLA-A1-MAGE-A1 from a nonimmunized phage-Fab library. Proc Nalt Acad Sci USA 2000; 97: 7969-7974.

21. Held G, et al. Dissecting cytotoxic T cell responses towards the NY-ESO-1 protein by peptide/MHC- specific antibody fragments. *Eur J Immunol.* 2004: 34:2919-2929.

22. Lev A, et al. Isolation and characterization of human recombinant antibodies endowed with the antigen- specific, major histocompatibility complex-restricted specificity of T cells directed toward the widely expressed tumor T cell-epitopes of the telomerase catalytic subunit. *Cancer Res* 2002; 62: 3184-3194.

23. Klechevsky E, et al. Antitumor activity of immunotoxins with T-cell receptor-like specificity against human melanoma xenografts. *Cancer Res* 2008; 68 (15): 6360-6367.

24. Azinovic I, et al. Survival benefit associated with human anti-mouse antibody (HAMA) in patients with B-cell malignancies. *Cancer Immunol Immunother* 2006; 55(12):1451-8.

25. Tjandra JJ, et al. Development of human anti-murine antibody (HAMA) response in patients. *Immunol Cell Biol* 1990; 68(6):367-76.

26. Riechmann L, et al. Reshaping human antibodies for therapy. *Nature* 1988; 332 (6162): 332:323.

27. Queen C, et al. A humanized antibody that binds to the interleukin 2 receptor. *Proc Natl Acad Sci USA* 1989; 86 (24): 10029–33.

28. Gerd R, et al. Serological Analysis of Human Anti-Human Antibody Responses in Colon Cancer Patients Treated with Repeated Doses of Humanized Monoclonal Antibody A33. *Cancer Res 2001*; 61, 6851– 6859.

29. Cheever MA, et al. The prioritization of cancer antigens: A national Cancer Institute pilot project for the acceleration of translational research. *Clin Cancer Res* 2009; 15 (17): 5323-5337.

30. Drakos E, et al. Differentiual expression of WT1 gene product in non-Hodgkin lymphomas. *Appl Immunohistochem Mol Morphol* 2005; 13 (2): 132-137.

31. Asemissen AM, et al. Identification of a highly immunogenic HLA-A*01-binding T cell epitope of WT1. *Clin Cancer Res* 2006; 12 (24):7476-7482.

32. Tomimatsu K, et al. Production of human monoclonal antibodies against FceRIa by a method combining in vitro immunization with phage display. *Biosci Biotechnol Biochem* 2009; 73 (7): 1465-1469.

33. Lidija P, et al. An integrated vector system for the eukaryotic expression of antibodies or their fragments after selection from phage display libraries. *Gene* 1997; 187(1): 9-18.

34. Lisa JH, et al. Crystallographic structure of an intact IgG1 monoclonal antibody. *Journal of Molecular Biology* 1998; 275 (5): 861-872.

35. Yasmina NA, et al. Probing the binding mechanism and affinity of tanezumab, a recombinant humanized anti-NGF monoclonal antibody, using a repertoire of biosensors. *Protein Science* 2008; 17(8): 1326–1335.

36. Roberts WK, et al. Vaccination with CD20 peptides induces a biologically active, specific immune response in mice. *Blood* 2002: 99 (10): 3748-3755.

37. Caron PC, Class K, Laird W, Co MS, Queen C, Scheinberg DA. Engineered humanized dimeric forms of IgG are more effective antibodies. *J Exp Med* 176:1191-1195. 1992.

38. McDevitt M, et al. Tumor targeting with antibody-functionalized, radiolabeled carbon nanotubes. *J. Nuclear Med* 2207; 48 (7))1180-1189.

39. Xue SA, et al. Development of a Wilms' tumor-specific T-cell receptor for clinical trials: engineered patient's T cells can eliminate autologous leukemia blasts in NOD/SCID mice. *Haematologica* 2010; 95 (1): 126-134.

40. McDevitt MR, et al. Tumor therapy with targeted atomic nanogenerators. *Science* 2001; 294 (5546): 1537-1540.

41. Borchardt PE, et al. Targeted Actinium-225 in vivo generators for therapy of ovarian cancer. *Cancer Res* 2003; 63: 5084-5090.

42. Singh Jaggi J, et al. Selective alpha-particle mediated depletion of tumor vasculature with vascular normalization. *Plos One* 2007; 2 (3): e267.

43. Yan W, et al. Enhancing antibody Fc heterodimer formation through electrostatic steering effects. *J. Biol. Chem*. 2010; 285: 19637-19646.

44. Rossi EA, et al. Stably tethered multi-functional structures of defined composition made by the dock and lock method for use in cancer targeting. *Proc Natl Aca Sci USA* 2006; 103:6841-6.

45. Ryutaro A, et al. Cytotoxic enhancement of a bispecific diabody by format conversion to tandem single-chain variable fragment (taFv). *J Biol Chem* 2011; 286: 1812-1818.

46. Anja L, et al. A recombinant bispecific single-chain antibody, CD19 × CD3, induces rapid and high lymphoma-directed cytotoxicity by unstimulated T lymphocytes. *Blood* 2000; 95(6): 2098-2103.

47. Weiner GJ, et al. The role of T cell activation in anti-CD3 x antitumor bispecific antibody therapy. *J. Immunology* 1994; 152(5): 2385-2392.

48. Dafne M, et al. Improved pharmacokinetics of recombinant bispecific antibody molecules by fusion to human serum albumin. *J Biol Chem* 2007; 282: 12650-12660.

49. Liu C, et al. Modified host cells and uses thereof, PCT/US2010/0081195.

50. Francisco J, et al. Neutrophils Contribute to the Biological Antitumor Activity of Rituximab in a Non-Hodgkin's Lymphoma Severe Combined Immunodeficiency Mouse Model. *Clin Cancer Res* 2003; 9: 5866.

51. Kavita M, et al. Selective blockade of inhibitory Fc receptor enables human dendritic cell maturation with IL-12p70 production and immunity to antibody-coated tumor cells. *Proc natl Aca Sci USA* 2005; 102(8): 2910–2915.

52. Raphael A, et al. Inhibitory Fc receptors modulate in vivo cytoxicity against tumor targets. *Nature Medicine* 2000; 6:443-446.

53. Milenic ED. Monoclonal antibody-based therapy strategies: providing options for the cancer patient. *Curr Pharm Des*. 2002; 8: 1794-1764.

54. Grillo-Lopez AJ. Anti-CD20 mAbs: modifying therapeutic strategies and outcomes in the treatment of lymphoma patients. *Expert Rev Anticancer Ther*. 2002: 2 (3): 323-329.

55. Jones KL & Buzdar AU. Evolving novel anti-Her2 strategies. *Lancet Oncol*. 2009: 10 (12): 1179-1187.

56. Reddy MM, Deshpande A & Sattler M. targeting JAK2 in the therapy of myeloproliferative neoplasms. Exper Opin Ther targets 2012: 3: 313-324.

57. Takeuchi K & Ito F. Receptor tyrosine kinases and targeted cancer therapeutics. Biol Pharm Bull. 2011; 34 (12) 1774-1780.

58. Roychowdhury S & Talpaz M. Managing resistance in chronic myeloid leukemia. Blood Rev. 2011; (6): 279- 290.

59. Konnig R. Interactions between MHC molecules and co-receptors of the TCR. *Curr Opin Immunol* 2002: 14 (1) 75-83.

60. Sergeeva A, Alatrash G, He H, Ruisaard K, Lu S, Wygant J, McIntyre BW, Ma Q, Li D, St John L, Clise-Dwyer K & Molldrem JJ. An anti-PR1/HLA-A2 T-cell receptor-like antibody mediated complement-dependent cytotoxicity against acute myeloid leukemia progenitor cells. *Blood* 2011; 117 (16): 4262-4272).

61. Takigawa N, Kiura K & Kishimoto T. Medical Treatment of Mesothelioma: Anything New? *Curr Oncol Rep* 2011; DOI 10.1007/s11912-011-0172-1.

62. Raja S, Murthy SC & Mason DP. Malignant Pleural Mesothelioma. *Curr Oncol Rep* 2011; DOI 10. 1007/s11912-0177-9.

63. Gerber JM, Qin L, Kowalski J, Smith D, Griffin CA, Vala MS, Collector MI, Perkins B, Zahurak M, Matsui W, Gocke CD, Sharkis S, Levitsky H & Jones RJ. Characterization of chronic myeloid leukemia stem cells. 2011; *Am J Hematol*. 86: 31-37.

64. Rezwani K, Yong AS, Savani BN, Mielke S, Keyvanfar K, Gostick E, Price DA, Douek DC & Barrett AJ. Graft-versus-leukemia effects associated with detectable Wilms tumor-1 specific T lymphocytes after allogeneic stem-cell transplantation for acute lymphoblastic leukemia. *Blood* 2007: 110 (6): 1924-1932.

65. Persic L, Roberts A, Wilton J et al. An integrated vector system for the eukaryotic expression of antibodies or their fragments after selection from phage display libraries. *Gene* 1997; 187(1): 9-18.

66. Cheng L, Xiang JY, Yan S et al. Modified host cells and uses thereof. *PCT/US*2010/0081195.

67. Lindmo T, Boven E, Cuttitta F, Fedorko J & Bunn PA Jr. Determination of the immunoreactive fraction of radiolabeled monoclonal antibodies by linear extrapolation to binding at infinite antigen excess. *J Immunol Methods*. 1984; 72 (1): 77-89.

68. Feng M, Zhang JL, Anver M, Hassan R & Ho M. *In vivo* imaging of human malignant mesothelioma growth orthotopically in the peritoneal cavity of nude mice. *J Cancer* 2011; 2: 123-131.

CLAIMS

What is claimed is:

1. An antigen-binding protein specific for a WT1 peptide/HLA complex, wherein the antigen binding protein is a full length antibody, a Fab fragment, a Fab' fragment or a $F(ab)_2$ fragment.

2. An antigen-binding protein specific for a WT1 peptide/HLA complex, wherein the antigen binding protein comprises:

A) (i) a heavy chain (HC) variable region comprising HC-CDR1, HC-CDR2 and HC-CDR3 respectively, comprising amino acid sequences SEQ ID NOS: 38, 39 and 40; and a light chain (LC) variable region comprising LC-CDR1, LC-CDR2 and LC-CDR3 respectively, comprising amino acid sequences selected from SEQ ID NOS: 44, 45 and 46;

(ii) a heavy chain (HC) variable region comprising HC-CDR1, HC-CDR2 and HC-CDR3 respectively, comprising amino acid sequences SEQ ID NOS: 2, 3, and 4; and a light chain (LC) variable region comprising LC-CDR1, LC-CDR2 and LC-CDR3 respectively, comprising amino acid sequences SEQ ID NOS: 8, 9 and 10;

(iii) a heavy chain (HC) variable region comprising HC-CDR1, HC-CDR2 and HC-CDR3 respectively, comprising amino acid sequences SEQ ID NOS: 20, 21and 22; and a light chain (LC) variable region comprising LC-CDR1, LC-CDR2 and LC-CDR3 respectively, comprising amino acid sequences SEQ ID NOS: 26, 27 and 28;

(iv) a heavy chain (HC) variable region comprising HC-CDR1, HC-CDR2 and HC-CDR3 respectively, comprising amino acid sequences SEQ ID NOS: 56, 57 and 58; and a light chain (LC) variable region comprising LC-CDR1, LC-CDR2 and LC-CDR3 respectively, comprising amino acid sequences SEQ ID NOS: 62, 63 and 64;

(v) a heavy chain (HC) variable region comprising HC-CDR1, HC-CDR2 and HC-CDR3 respectively, comprising amino acid sequences SEQ ID NOS: 74, 75 and 76; and a light chain (LC) variable region comprising LC-CDR1, LC-CDR2 and LC-CDR3 respectively, comprising amino acid sequences SEQ ID NOS: 80, 81 and 82; or

(vi) a heavy chain (HC) variable region comprising HC-CDR1, HC-CDR2 and HC-CDR3 respectively, comprising amino acid sequences SEQ ID NOS: 92, 93 and 94; and

a light chain (LC) variable region comprising LC-CDR1, LC-CDR2 and LC-CDR3 respectively, comprising amino acid sequences SEQ ID NOS: 98, 99 and 100; or

(B) a V_H and V_L comprising first and second amino acid sequences, respectively, selected from SEQ ID NOS: 50 and 52; 14 and 16; 32 and 34; 68 and 70; 86 and 88; and 104 and 106; or

(C) an amino acid sequence selected from SEQ ID NOS: 54, 18, 36, 72, 90, and108.

3. The antigen-binding protein of claim 2, wherein the antigen-binding protein is a full-length antibody or is an antigen-binding fragment of said antibody selected from the group consisting of Fab, Fab', $F(ab')_2$, Fv and single chain Fv (scFv) or is a chimeric antigen receptor.

4. The antigen-binding protein of any one of claims 1-3, wherein the antigen-binding protein comprises a human variable region framework region.

5. The antigen-binding protein of any one of claims 1-3, wherein the antigen-binding protein is fully human.

6. The antigen-binding protein of any preceding claim, wherein said WT1 peptide has the amino acid sequence RMFPNAPYL.

7. The antigen-binding protein of any preceding claim, wherein said HLA-A2 is HLA-A0201.

8. The antigen-binding protein of any one of claims 1 to 7 conjugated to a therapeutic agent.

9. The antigen-binding protein of claim 8, wherein said therapeutic agent is a drug, toxin, radioisotope, protein, or peptide.

10. An isolated nucleic acid that encodes an antigen-binding protein of any one of claims 1 to 7.

11. An isolated nucleic acid comprising:

(A) (i) first, second and third nucleotide sequences selected from the group consisting of SEQ ID NOS: 41, 42 and 43; 5, 6 and 7; 23, 24 and 25;58, 59 and 60; 77, 78 and 79; and 95, 96 and 97; and

(ii) fourth, fifth and sixth nucleotide sequences selected from the group consisting of SEQ ID NOS: 47, 48 and 49; 11, 12 and 13; 29, 30 and 31; 65, 66 and 67; 83, 84 and 85; and 101, 102 and 103; or

(B) first and second nucleotide sequences selected from the group consisting of SEQ ID NOS: 51 and 53; 15 and 17; 33 and 35; 69 and 71; 87 and 89 and 105 and 107; or

(C) a nucleotide sequence selected from the group consisting of SEQ ID NOS: 55, 19, 37, 73, 91 and 109.

12. A vector comprising a nucleic acid of claim 10 or claim 11.

13. A cell comprising a nucleic acid of claim 10 or claim 11.

14. The cell of claim 13, wherein said cell expresses on its surface an antigen-binding protein of any one of claims 1 to 3.

15. An *in vitro* method for detecting WT1 in a whole cell or tissue, said method comprising:

(A) contacting a cell or tissue with an antibody or antigen-binding fragment thereof that specifically binds to a WT1 peptide that is bound to HLA-A2, wherein said antibody comprises a detectable label;

(B) determining the amount of antibody bound to said cell or tissue by measuring the amount of detectable label associated with said cell or tissue, wherein the amount of bound antibody indicates the amount of WT1 in said cell or tissue.

16. The method of claim 15, wherein the antibody specifically binds to a WT1 peptide with the amino acid sequence RMFPNAPYL (SEQ ID NO: 1), bound to HLA-A2.

17. The method of claim 16, wherein the WT1 peptide is bound to HLA-A0201.

18. The method of claim 15, wherein the antibody is a full length antibody, a Fab fragment, a Fab' fragment or a $F(ab)_2$ fragment.

19. The method of claim 15, wherein the antibody is an isolated antibody or antigen-binding fragment thereof comprising one of:

(A) (i) a heavy chain (HC) variable region comprising HC-CDR1, HC-CDR2 and HC-CDR3 respectively, comprising amino acid sequences SEQ ID NOS: 38, 39 and 40; and a light chain (LC) variable region comprising LC-CDR1, LC-CDR2 and LC-CDR3 respectively, comprising amino acid sequences selected from SEQ ID NOS: 44, 45 and 46;

(ii) a heavy chain (HC) variable region comprising HC-CDR1, HC-CDR2 and HC-CDR3 respectively, comprising amino acid sequences SEQ ID NOS: 2, 3, and 4; and a light chain (LC) variable region comprising LC-CDR1, LC-CDR2 and LC-CDR3 respectively, comprising amino acid sequences SEQ ID NOS: 8, 9 and 10;

(iii) a heavy chain (HC) variable region comprising HC-CDR1, HC-CDR2 and HC-CDR3 respectively, comprising amino acid sequences SEQ ID NOS: 20, 21and 22; and a light chain (LC) variable region comprising LC-CDR1, LC-CDR2 and LC-CDR3 respectively, comprising amino acid sequences SEQ ID NOS: 26, 27 and 28;

(iv) a heavy chain (HC) variable region comprising HC-CDR1, HC-CDR2 and HC-CDR3 respectively, comprising amino acid sequences SEQ ID NOS: 56, 57 and 58; and a light chain (LC) variable region comprising LC-CDR1, LC-CDR2 and LC-CDR3 respectively, comprising amino acid sequences SEQ ID NOS: 62, 63 and 64;

(v) a heavy chain (HC) variable region comprising HC-CDR1, HC-CDR2 and HC-CDR3 respectively, comprising amino acid sequences SEQ ID NOS: 74, 75 and 76; and a light chain (LC) variable region comprising LC-CDR1, LC-CDR2 and LC-CDR3 respectively, comprising amino acid sequences SEQ ID NOS: 80, 81 and 82; or

(vi) a heavy chain (HC) variable region comprising HC-CDR1, HC-CDR2 and HC-CDR3 respectively, comprising amino acid sequences SEQ ID NOS: 92, 93 and 94; and a light chain (LC) variable region comprising LC-CDR1, LC-CDR2 and LC-CDR3 respectively, comprising amino acid sequences SEQ ID NOS: 98, 99 and 100; or

(B) a V_H and V_L comprising first and second amino acid sequences, respectively, selected from SEQ ID NOS: 50 and 52; 14 and 16; 32 and 34; 68 and 70; 86 and 88; and 104 and 106; or

(C) an amino acid sequence selected from SEQ ID NOS: 54, 18, 36, 72, 90, and108.

20. The method of claim 19, wherein the antigen binding protein is a full-length antibody or is an antigen-binding fragment of said antibody selected from the group consisting of Fab, Fab', $F(ab')_2$, Fv and single chain Fv (scFv) or is a chimeric antigen receptor.

21. A kit comprising an antigen-binding protein of any one of claims 1 to 9.

22. A pharmaceutical composition comprising an antigen-binding protein of any one of claims 1 to 9 and a pharmaceutically acceptable carrier.

23. An antigen-binding protein of any of claims 1 to 9 for use in a method for treatment of a subject having a WT1 positive disease, wherein the WT1-positive disease is a chronic leukemia or acute leukemia or $WT1^+$ cancer; orthe WT1-positive disease is selected from the group consisting of chronic myelocytic leukemia, multiple myeloma (MM), acute lymphoblastic leukemia (ALL), acute myeloid/myelogenous leukemia (AML), myelodysplastic syndrome (MDS), mesothelioma, ovarian cancer, gastrointestinal cancers, breast cancer, prostate cancer and glioblastoma.

24. The antigen-binding protein for use according to claim 22, wherein said antibody is a conjugate having a cytotoxic moiety linked thereto.

25. A nucleic acid of claim 10 or claim 11 for use in a method for treatment of a subject having a WT1-positive disease, wherein the WT1-positive disease is a chronic leukemia, acute leukemia or WT^+ cancer; or the WT1-positive disease is selected from the group consisting of chronic myelocytic leukemia, multiple myeloma, acute lymphoblastic leukemia (ALL), acute myeloid/myelogenous leukemia (AML), myelodysplastic syndrome (MDS), mesothelioma, ovarian cancer, gastrointestinal cancers, breast cancer, prostate cancer and glioblastoma.

26. A pharmaceutical composition comprising a nucleic acid of claim 10 or claim 11.

27. An *in vitro* method for killing a $WT1^+$ cell, said method comprising contacting the cell with an antigen-binding protein of any one of claims 1-9 .

28. The method of claim 27, wherein said antibody or antigen-binding fragment thereof has a cytotoxic moiety linked thereto.