HLA-RESTRICTED, PEPTIDE-SPECIFIC ANTIGEN BINDING PROTEINS

Cross-Reference to Related Applications

[0001] This application is a national phase filing of PCT International Application PCT/US2012/024885, filed February 13, 2012, and published under PCT Article 21(2) in English as WO2012/109659 on August 16, 2012, which claims priority from U.S. Provisional Application No. 61/463,082, filed February 11, 2011, entitled GENERATION AND USE OF HLA-A2 RESTRICTED, PEPTIDE-SPECIFIC MONOCLONAL ANTIBODIES AND CHIMERIC ANTIGEN RECEPTORS. The contents of each of these applications are hereby incorporated by reference in their entirety into the present disclosure.

SEQUENCE LISTING

[0002] The instant application contains a Sequence Listing, created on February 2, 2012; the file, in PDF format, is designated Sequence_Listing.txt and is 58.6 kilobytes in size. The file is hereby incorporated by reference in its entirety into the instant application.

BACKGROUND OF THE INVENTION

Technical Field

[0003] The present invention relates generally to antigen-binding protein molecules involved in immune function. More particularly, the present invention relates to recombinant antibodies, chimeric antigen receptors and fragments thereof with specificity for an HLA-restricted peptide, where the peptide is derived from a cellular or viral protein of interest.

Background Information

[0004] Advances in adoptive T cell immunotherapy have led to several promising options for cancer patients in the past decade. T-cell based immunotherapy for cancer stemmed from studies which showed a correlation of increased numbers of tumor infiltrating lymphocytes (TILs) in surgical specimens and patient outcome. It is generally believed that this infiltration of TILs represents activation of an anti-tumor mechanism and that the infiltration was mediated through the expression of tumor associated antigens in the context of MHC. These findings eventually led researchers to try and take advantage of antigen-specific T cells for the treatment of cancer.

[0005] For induction of cytotoxic T- cell (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. Vaccinations with peptides derived from cellular and viral protein can induce HLA-A0201-restricted cytotoxic CD8 T cells, which are capable of killing tumor cells or virally-infected cells.

[0006] Antibodies are increasingly being used as therapeutic agents to fight cancer, autoimmune disease and infection. Therapeutic antibodies have been exploited based on their multiple mechanisms of action, which include the following: 1) naked antibodies killing tumor cells directly by ADCC or CDC (e.g. trastuzumab), 2) blocking or stimulating a cell membrane molecule to induce cell death (e.g. cetuximab), 3) neutralizing a secreted moiety (e.g. bevacizumab), 4) killing via an attached moiety such as a drug, toxin, radioisotope and 5) modulating the immune system via T cell effector functions.

[0007] In almost all cases, to generate a therapeutic benefit, antibodies have to possess critical properties including high affinity for their targeted antigen, minimal

acute and long-term side effects, and in specific applications, high affinity for human Fc receptors (4). In addition, the targeted antigen has to be expressed at high levels on tumors but not on normal tissues (specificity or selectivity), consistently expressed in the specific tumor among patients and within patients (low heterogeneity), and should either be essential for the survival of the cancer cell or unlikely to be down regulated.

[0008] To achieve these attributes, researchers can now reengineer existing antibodies to make them less immunogenic, modifying both protein and carbohydrate residues in the Fc regions to enhance ADCC and CDC, shrinking their sizes for potentially better tumor penetration, mutating the variable regions to improve affinity, increasing avidity by changing antibody valency, and constructing novel antibody-fusion proteins such as those for multi-step targeting (5) and for redirecting immune cells by way of a chimeric antigen receptor (CAR). Furthermore, researchers continue to define the structural attributes and the host characteristics responsible for success among currently approved antibodies (6).

[0009] With the objective of eliminating or neutralizing the pathogenic agent or disease target, including bacterial, viral or tumor targets, antigen-specific, antibody-based treatments are particularly attractive because of the antibody's exquisite specificity.

SUMMARY OF THE INVENTION

[0010] The present invention, therefore, is based on the identification of antigen-specific binding sequences from which a variety of antigen-binding proteins can be produced, for example, an antibody specific for an antigen that represents a complex of a protein fragment (peptide) and an HLA molecule similar to that typically recognized by a T-cell receptor following antigen processing and presentation of the protein to the T-cell. Phage display is used to select an initial antigen-binding

molecule that can be used to engineer the antigen-binding proteins of the invention, which include antibodies and chimeric antigen receptors (CARs).

[0011] In one aspect, therefore, the invention relates to an isolated antigen-binding protein or antigen-binding fragment thereof comprising one of:

(A) an antigen binding region having the amino acid sequence of one of SEQ ID NOS: 2, 5, 8, 10, 13, 14, 17, 20;

(B) an antigen binding region comprising a V_H and V_L, respectively, with amino acid sequences selected from SEQ ID NOs: 22 and 23; 24 and 25; 26 and 27; 28 and 29; 30 and 31; 32 and 33; 34 and 35; and 36 and 37; or

(C) (i) the following three light chain (LC) complementarity determining regions (CDRs):

(a) a LC CDR1 comprising the amino acid sequence of SEQ ID NO: 56; and

(b) a LC CDR2 and CDR3 comprising respectively, the amino acid sequence of SEQ ID NOs: 57 and 64, 58 and 65, 59 and 66, 60 and 67, 61 and 68, 61 and 69, 62 and 70 and 63 and 71; and

(ii) the following three heavy chain (HC) CDRs:

(a) a HC CDR1 comprising the amino acid sequence of SEQ ID NO: 38; and

(b) a LC CDR2 and CDR3 comprising respectively the amino acid sequence of one of SEQ ID NOs: 40 and 48, 41 and 49, 42 and 50, 43 and 51, 44 and 52, 45 and 53, 46 and 54 and 47 and 55.

[0012] In a related aspect, the invention relates to an isolated antigen-binding protein or antigen-binding fragment thereof, wherein the isolated antigen-binding protein is an antibody or a chimeric antigen receptor. The antibody is a full-length antibody**,** a substantially intact antibody**,** a Fab fragment, a $F(ab')_2$ fragment or a single chain variable fragment (scFv).

[0013] In the isolated antigen-binding protein, whether an antibody or CAR, the antigen-binding region specifically binds to an epitope of an HLA-peptide complex.

[0014] 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, a peptide with the amino acid sequence RMFPNAPYL (SEQ ID NO:1); a peptide with the amino acid sequence LLDFVRFMGV (SEQ ID NO:4); a peptide with the amino acid sequence RLTRFLSRV (SEQ ID NO: 7); a peptide with the amino acid sequence RIITSTILV (SEQ ID NO: 12); and a peptide with the amino acid sequence LLEEMFLTV (SEQ ID NO:19). In some embodiments, the peptide is recognized in associate with an HLA-A2 antigen.

[0015] In yet another aspect, the isolated antigen-binding protein of the invention is a scFv comprising an amino acid sequence selected from the group consisting of SEQ ID NOS: 2, 5, 8, 10, 13, 14, 17 and 20.

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

[0017] In another aspect, the invention relates to an immunoconjugate comprising a first component which is an antigen-binding protein, 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.

[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.

BRIEF DESCRIPTION OF THE DRAWINGS

[0019] **Figure 1** shows the binding of bacterial supernatant from individual EBNA3C scFv clones 315, 335, 327 and 345 (FIG 1A) and purified EBNA clone 315 scFv (FIG 1B) to various HLA-A2-peptide complexes demonstrating that clone 315 is highly specific for the HLA-A2-LLDFVRFMGV (SEQ ID NO: 4) complex.

[0020] **Figure 2** shows the binding of bacterial supernatant from individual WT-1 scFv clones 42, 43 and 45 (FIG 2A) and purified WT-1 clone 45 scFv (FIG 2B) to various HLA-A2-peptide complexes demonstrating that WT-1 clones 42, 43 and 45 are highly specific for the recombinant HLA-A2-RMFPNAPYL (SEQ ID NO: 1) complex.

[0021] **Figure 3** shows that HLA-A2 can be detected on TAP-deficient (TAP^-) T2 cells that were either pulsed or unpulsed with LLDFVRFMGV (SEQ ID NO: 4) or another (irrelevant) peptide (FIG 3A) but that EBNA clone 315 scFv recognizes T2 cells that have been pulsed with LLDFVRFMGV (SEQ ID NO: 4) but not unpulsed cells or cells pulsed with irrelevant peptide (FIG 3B) with a lower limit of detection at about 78nM (FIG 3C).

[0022] **Figure 4** shows that HLA-A2 can be detected on TAP-deficient (TAP^-) T2 cells that were either pulsed or unpulsed with RMFPNAPYL (SEQ ID NO: 1) or LLDFVRFMGV (SEQ ID NO: 4) (FIG 4A) but that WT-1 clone 45 scFv recognizes T2 cells that have been pulsed with RMFPNAPYL (SEQ ID NO: 1) but not unpulsed cells or cells pulsed with LLDFVRFMGV (SEQ ID NO: 4) (FIG 4B)

[0023] **Figure 5** shows that when DIMT (FIG 5A) and 6268A (FIG 5B) BLCLs are incubated with LLDFVRFMGV (SEQ ID NO: 4) (middle panel) or KLQCVDLHV (SEQ ID NO: 74) peptides (right panel) and stained with EBNA clone 315 scFv, only HLA-$A2^+$ DIMT peptide-pulsed with LLDFVRFMGV (SEQ ID NO: 4) could be stained, showing that EBNA clone 315 and LLDFVRFMGV (SEQ ID NO: 4) are HLA-A2

restricted; a time course (FIG 5C) shows that the HLA-A2-LLDFVRFMGV (SEQ ID NO: 4) complex is stable on the cell surface.

[0024] **Figure 6** shows the Tomlinson library vector used in PCR to add appropriate restriction enzyme sites to either side of the WT1 Clone 45 and EBNA Clone 315 scFv sequences (Figure 6A). Figure 6B shows the digested PCR products as they appeared on a 1% agarose gel following digestion with NheI and ApaI.

[0025] **Figure 7** shows the full IgG expression vector (Figure 7A) that was used to generate an expression vector for scFv-Fc fusion proteins (Figure 7B). *A*. The structure of the proprietary IgG expression vector (11381 bp). The vector expresses the heavy and light chains under two separate CMV promoters. The variable heavy chain (V_H) is fused to the first, second and third constant heavy chains ($CH_{1, 2, 3}$) and expressed under one promoter while the variable light chain (V_L) is fused to the constant light chain (C_L) and expressed under a different promoter. This vector was further modified to lack the first constant region of the heavy chain (CH_1), and this vector was used for the construction of scFv-Fc fusion proteins. *B*. After excision of the V_H from the IgG vector using NheI and ApaI, the pre-digested, purified scFv PCR products were ligated to the IgG vector to allow for the expression of the scFv fused to the $CH_{2, 3}$ domains (Fc).

[0026] **Figure 8** shows the results of binding studies using EBNA Clone 315 scFv-Fc in which purified EBNA Clone 315 scFv-Fc was shown to maintain its binding ability towards the recombinant complex when tested for binding on an ELISA plate coated with or without HLA-A2-LLDFVRFMGV (SEQ ID NO: 4) (Figure 8A) and when tested for binding on T2 cells pulsed with or without the LLDFVRFMGV (SEQ ID NO: 4) peptide (Figure 8B). When T2 cells were incubated with decreasing concentrations of the LLDFVRFMGV (SEQ ID NO: 4) peptide and subsequently stained with EBNA Clone 315 scFv-Fc, a lower limit of detection was demonstrated to be in the same range as the scFv (200 nM- 20 nM) (Figure 8C).

[0027] **Figure 9** shows the results of kinetics determination of EBNA Clone 315 to HLA-A2-LLDFVRFMGV (SEQ ID NO: 4) using surface plasmon resonance.

[0028] **Figure 10** shows the results of HLA-A2-LLDFVRFMGV (SEQ ID NO: 4) complex quantitation on T2 cells using fluorescently-conjugated EBNA Clone 315 scFv-Fc (Figure 10A). Fluorescently-conjugated EBNA Clone 315 scFv-Fc was tested for binding on T2 cells pulsed with (20, 10, or 5 μM) or without (0 μM) the LLDFVRFMGV (SEQ ID NO: 4) peptide in serum-free IMDM media at 37°C for 5 hours. The cells, in addition to beads containing known amounts of anti-human IgG_1 antibodies, were stained with the scFv-Fc and the cell's fluorescence intensity was correlated to that of the beads and their number of binding sites. Using these four peptide concentrations and corresponding number of complexes, a standard curve was created with an R^2 value of 0.9948. Figure 10B shows a close-up view of the lower end of the peptide and complex spectrum.

[0029] **Figure 11** shows the results of binding and specificity studies when purified WT1 Clone 45 scFv-Fc was tested for binding on an ELISA plate coated with or without HLA-A2-RMFPNAPYL (SEQ ID NO: 1) (Figure 11A). Figure 11B shows that when purified WT1 Clone 45 scFv-Fc was tested for binding on T2 cells pulsed with the RMFPNAPYL (SEQ ID NO: 1) or RLTRFLSRV (SEQ ID NO: 7) peptide (40 μM), the scFv-Fc (unfilled lines) was only able to recognize RMFPNAPYL (SEQ ID NO: 7) -pulsed T2 cells.

[0030] **Figure12** shows that when DIMT (top) and 6268A (bottom) BLCLs were incubated with RMFPNAPYL (SEQ ID NO: 1) (right panel) and the peptide-pulsed BLCLs were stained with WT1 Clone 315 scFv-Fc (unfilled lines) or a control scFv (filled lines), only the HLA-A2-positive RMFPNAPYL (SEQ ID NO: 1) peptide-pulsed DIMT BLCLs could be stained.

[0031] **Figure 13** shows EBNA Clone 315 scFv-Fc mediated ADCC (measured using ^{51}Cr release) of LLDFVRFMGV (SEQ ID NO: 4) peptide-pulsed cells.

[0032] **Figure 14** shows the MSCV-based vector (top left panel) containing an IRES-GFP sequence along with ampicilin-resistance used for transduction and expression of anti-EBNA CAR in NK92MI cells. The EBNA Clone 315 scFv sequence was cloned into the CAR gene (EBNA CAR) and further cloned into an MSCV-based vector (top left panel) which contained an IRES-GFP sequence along with ampicilin-resistance. The resulting CAR (top right panel) is composed of the scFv and hinge region on the extracellular surface, a transmembrane domain, along with 4-1BB and the CD3ζ chain present within the cell. After retroviral packaging using 293T GP2 cells and transduction into NK92MI cells, approximately 24% of the NK92MI cells contained the construct based on GFP expression (bottom left panel; unfilled lines) when compared to mock transduced (empty retrovirus) NK92MI cells (bottom left panel; filled lines). Of the GFP-positive cells, the top 20% were flow cytometrically sorted and expanded to yield a population of stably transduced cells which were greater than 90% GFP positive (bottom right panel). Retroviral transduction was done on three separate occasions, with 24% being the highest efficiency.

[0033] **Figure 15** shows EcoRI and XhoI digestion validation of the WT1 Clone 45 CAR vector. FIG 15A: Along with sequence validation, plasmids isolated from 8 different bacterial colonies, after ligation, transformation and EcoRI and XhoI digestion, were run on a 1% agarose gel. Based on the lambda HindIII and 100 bp markers, it was determined that the bands were the correct size (~1500 bp and ~6000 bp). FIG 15B: The structure of the resulting WT1 Clone 45 CAR vector has the same components as the original St. Jude CAR vector with the only difference being the scFv sequence.

[0034] **Figure 16** shows that Clone 315 CAR-expressing NK92MI cells can specifically detect the HLA-A2-EBNA3C complex on peptide-pulsed T2 cells via CD107a expression. T2 cells were pulsed with or without LLDFVRFMGV (SEQ ID

NO: 4) or YMFPNAPYL (SEQ ID NO: 76) peptides at 20 μM. CAR-equipped NK92MI cells were then cultured in media containing an anti-CD107a-PE conjugated antibody at 37ºC for 5 hours with or without peptide pulsed or unpulsed cells. FIG 16A: CAR-equipped NK92MI cells were gated based on GFP fluorescence and analyzed for CD107a expression. NK92MI cells which were cultured without any T2 cells or those which were cocultured with unpulsed and YMFPNAPYL (SEQ ID NO: 76) -pulsed T2 cells were unreactive while NK92MI cells which were cocultured with LLDFVRFMGV (SEQ ID NO: 4) -pulsed T2 cells led to a 27% increase in CD107a expression above background levels. FIG16B: T2 cells were pulsed with decreasing concentrations of LLDFVRFMGV (SEQ ID NO: 4) and subsequently cocultured with CAR-equipped NK92MI cells. NK92MI cells presented noticeable amounts of CD107a on their cell surface even when T2 cells were pulsed with only 10 nM of peptide.

[0035] **Figure 17** shows the results of flow cytometry in which HLA-$A2^+$ (DIMT) and HLA-$A2^-$ (6268A) BLCLs were pulsed with LLDFVRFMGV (SEQ ID NO: 4) and CAR-equipped NK92MI cells were then cultured in media containing an anti-CD107a-PE conjugated antibody with or without peptide pulsed or unpulsed cells. CAR-equipped NK92MI cells were gated based on GFP fluorescence and analyzed for CD107a expression. NK92MI cells which were cultured without any BLCL or those which were cocultured with LLDFVRFMGV (SEQ ID NO: 4) -pulsed 6268A BLCL were unreactive while NK92MI cells which were cocultured with unpulsed DIMT BLCL or LLDFVRFMGV(SEQ ID NO: 4) -pulsed DIMT BLCL led to a 0.5% and 25% increase in CD107a expression above background levels (pulsed 6268A) showing that EBNA Clone 315 CAR-expressing NK92MI cells can specifically detect the HLA-A2-EBNA3C complex on peptide-pulsed BLCLs via CD107a expression.

[0036] **Figure 18** shows the results of a ^{51}Cr release assay in whichT2 cells were pulsed with or without decreasing concentrations of LLDFVRFMGV (SEQ ID NO: 4). CAR-equipped NK92MI cells were cocultured with ^{51}Cr-labeled T2 cells at a 3:1 E:T

ratio demonstrating that EBNA Clone 315 CAR-expressing NK92MI cells can specifically detect the HLA-A2-EBNA3C complex on peptide-pulsed T2 cells. Even with 2 nM of peptide, peptide-specific cytotoxicity could be observed when compared to unpulsed T2 cells.

[0037] **Figure19** shows EBNA Clone 315 CAR-expressing NK92MI cells can specifically detect the HLA-A2-EBNA3C complex on peptide-pulsed BLCLs via ^{51}Cr release. BLCLs were pulsed with LLDFVRFMGV (SEQ ID NO: 4). CAR-equipped NK92MI cells were then cultured with ^{51}Cr labeled target cells. FIG 19A: CAR equipped NK92MI cells were able to specifically differentiate between peptide pulsed DIMT and 6268A BLCL, with a clear difference in cytotoxicity between the two different targets. FIG 19B: CAR-mediated killing of peptide-pulsed DIMT BLCL could be blocked using the EBNA Clone 315 scFv-Fc fusion protein, but not by an irrelevant, isotype-matched scFv-Fc, at a 20:1 E:T ratio.

[0038] **Figure 20** shows the results of a ^{51}Cr release assay in which CAR-equipped NK92MI cells were cultured with ^{51}Cr labeled, unpulsed BLCLs. FIG 20A: CAR-equipped NK92MI cells were more reactive towards the HLA-$A2^+$ DIMT and JG19 BLCL versus the HLA-$A2^-$ 6268A and GKO BLCL when cocultured in the absence of any exogenous peptide. FIG20B: CAR-mediated killing of unpulsed DIMT BLCL could be blocked using the EBNA Clone 315 scFv-Fc fusion protein but not by an irrelevant, isotype-matched scFv-Fc, at a 10:1 E:T ratio demonstrating that EBNA Clone 315 CAR-expressing NK92MI cells can specifically detect the HLA-A2-EBNA3C complex on HLA-$A2^+$ BLCLs.

[0039] **Figures 21** shows the results of a ^{51}Cr release assay of EBNA in which CD16(V)-expressing NK92MI cells were cultured with ^{51}Cr labeled, LLDFVRFMGV-(SEQ ID NO: 4) pulsed DIMT BLCL and either EBNA Clone 315 or an irrelevant scFv-Fc. At an E:T ratio of 15:1, EBNA Clone 315 scFv-Fc was able to kill 30-35% of target cells.

[0040] **Figure 22** shows the results of a ^{51}Cr release assay of EBNA in which Clone 315 CAR-expressing NK92MI cells were cultured with LLDFVRFMGV (SEQ ID NO: 4) -pulsed DIMT BLCL as above and either EBNA Clone 315 or an irrelevant scFv-Fc. At the same E:T ratio as in Figure 21, the CAR-equipped cells were able to kill 80-90% of target cells, with specific inhibition using EBNA Clone scFv-Fc demonstrating that CAR-mediated killing is more potent than scFv-Fc-mediated ADCC on peptide-pulsed DIMT BLCL.

[0041] **Figure 23** shows the MSCV-based vector (top left panel) containing an IRES-GFP sequence along with ampicilin-resistance used for transduction and expression of anti-WT1 CAR in NK92MI cells. A. The WT1 Clone 45 scFv sequence was cloned into the CAR gene (anti-WT1 CAR) and further cloned into the MSCV-based vector. The resulting CAR (top right panel) is composed of the scFv and hinge region on the extracellular surface, a transmembrane domain, along with 4-1BB and the CD3ζ chain present within the cell. B After retroviral packaging using 293T GP2 cells and transduction into NK92MI cells, approximately 27.5% of the NK92MI cells contained the construct based on GFP expression (bottom left panel; unfilled lines) when compared to mock transduced (empty retrovirus) NK92MI cells (bottom left panel; filled lines). Of the GFP-positive cells, the top 20% were flow cytometrically sorted and expanded to yield a population of stably transduced cells which were greater than 98% GFP positive (bottom right panel).

[0042] **Figure 24** shows the results of a ^{51}Cr release assay in which CAR-equipped NK92MI cells were able to specifically differentiate between peptide pulsed DIMT (■) and 6268A BLCL (●), with a clear difference in cytotoxicity between the two different targets demonstrating that NK92MI cells expressing WT1 Clone 45 CAR can specifically detect the HLA-A2-RMFPNAPYL (SEQ ID NO: 1) complex on peptide-pulsed BLCLs.

[0043] **Figure 25** shows that CAR-mediated killing of peptide-pulsed DIMT BLCL could be blocked using a commercial anti-HLA-A2 antibody (5 µg/ml), but not by an irrelevant, isotype-matched antibody (5 µg/ml), at a 9:1 E:T ratio.

[0044] **Figure 26** shows that NK92MI cells expressing WT1 Clone 45 CAR can specifically detect the HLA-A2-RMFPNAPYL (SEQ ID NO: 1) complex on DIMT BLCL via ^{51}Cr release. FIG 26A: CAR equipped NK92MI cells were able to specifically differentiate between DIMT and 6268A BLCL, with a clear difference in cytotoxicity between the two different targets. FIG26B CAR-mediated killing of DIMT BLCL could be blocked using the WT1 Clone 45 scFv-Fc fusion protein (20 µg/ml), but not by an irrelavent, isotype-matched scFv-Fc, at a 2:1 E:T ratio.

[0045] **Figure 27** shows that NK92MI cells expressing WT1 Clone 45 CAR can specifically detect the HLA-A2-RMFPNAPYL (SEQ ID NO: 1) complex on peptide-pulsed BLCLs via ^{51}Cr release. A CAR-equipped NK92MI cells were able to specifically differentiate between peptide pulsed DIMT and 6268A BLCL, with a clear difference in cytotoxicity between the two different targets.

[0046] **Figure 28** shows that NK92MI cells expressing WT1 Clone 45 CAR can specifically detect the HLA-A2-RMFPNAPYL (SEQ ID NO: 4) complex on 697 and OVCAR-3 cells via ^{51}Cr release.

DETAILED DESCRIPTION OF THE INVENTION

[0047] All patents, publications, applications and other references cited herein are hereby incorporated in their entirety into the present application.

[0048] 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.

[0049] The following abbreviations are used throughout the application:

[0050] **ADCC:** Antibody-dependent cellular cytotoxicity

[0051] **ALL:** Acute lymphocytic leukemia

[0052] **AML**: Acute myeloid leukemia

[0053] **APC:** Antigen presenting cell

[0054] **β2M:** Beta-2-microglobulin

[0055] **BiTE:** Bi-specific T cell engaging antibody

[0056] **BLCL:** EBV-transformed B-cell lymphoblastic cell line

[0057] **CAR:** Chimeric antigen receptor

[0058] **CDC:** Complement dependent cytotoxicity

[0059] **CMC:** Complement mediated cytotoxicity

[0060] **CDR:** Complementarity determining region (see also HVR below)

[0061] **C_L:** Constant domain of the light chain

[0062] **CH_1:** 1st constant domain of the heavy chain

[0063] **$CH_{1, 2, 3}$:** 1st, 2nd and 3rd constant domains of the heavy chain

[0064] **$CH_{2, 3}$:** 2nd and 3rd constant domains of the heavy chain

[0065] **CHO:** Chinese hamster ovary

[0066] **CTL:** Cytotoxic T cell

[0067] **EBNA3C:** Epstein-Barr nucleur antigen 3C

[0068] **EBV:** Epstein-Barr virus

[0069] **ECMV:** Encephalomyocarditis virus

[0070] **ER:** Endoplasmic reticulum

[0071] **E:T Ratio:** Effector:Target ratio

[0072] **Fab:** Antibody binding fragment

[0073] **FACS:** Flow assisted cytometric cell sorting

[0074] **FBS:** Fetal bovine serum

[0075] **GFP:** Green fluorescence protein

[0076] **HC:** Heavy chain

[0077] **HEL:** Hen egg lysozyme

[0078] **HLA:** Human leukocyte antigen

[0079] **HVR-H**: Hypervariable region-heavy chain (see also CDR)

[0080] **HVR-L**: Hypervariable region-light chain

[0081] **Ig:** Immunoglobulin

[0082] **IPTG:** isopropyl-1-thio-β-D-galactopyranoside

[0083] **IRES:** Internal ribosome entry site

[0084] **K_D:** Dissociation constant

[0085] **k_{off}:** Dissociation rate

[0086] **k_{on}:** Association rate

[0087] **MHC:** Major histocompatibility complex

[0088] **OPD:** O-phenylenediamine

[0089] **PEG:** Polyethylene glycol

[0090] **scFv:** Single-chain variable fragment

[0091] **SPR:** Surface plasmon resonance

[0092] **TB:** Terrific Broth

[0093] **TCE:** T cell epitope

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

[0095] **TIL:** Tumor infiltrating lymphocyte

[0096] **V_H:** Variable heavy chain

[0097] **V_L:** Variable light chain

[0098] **WT1:** Wilms tumor protein 1

[0099] 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.

[00100] 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, antigen receptors and fusion proteins.

[00101] "Antibody" and "antibodies" as those terms are known in the art refer to antigen binding proteins that arise in the context of the immune system. The term "antibody" as referred to herein includes whole, full length antibodies and any fragment thereof in which the "antigen-binding portion" or "antigen-binding region" is retained or single chains 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 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 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.

[00102] The term "antigen-binding portion" or "antigen-binding region" of an antibody (or simply "antigen portion"), as used herein, refers to that region or portion of the antibody that confers antigen specificity; fragments of antigen-binding proteins, for example, antibodies therefore, includes one or more fragments of an antibody that retain the ability to specifically bind to an antigen (e.g., an HLA-peptide 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).

[00103] 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 (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). Such single chain antibodies are also intended to be encompassed within the term "antigen-binding portion" of an antibody. 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.

[00104] 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.

[00105] Traditionally, the MHC-peptide complex could only be recognized by a T-cell receptor (TCR), limiting our ability to detect an epitope of interest to use of T cell-based readout assays. In the present disclosure, antigen binding proteins, including antibodies and chimeric antigen receptors, 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-EBNA and anti-WT1 antigen-binding proteins that recognize only the HLA-A2-LLDFVRFMGV (SEQ ID NO: 4) and HLA-A2-RMFPNAPYL (SEQ ID NO: 1) complexes, respectively. 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.

[00106] 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 and BLCLs were incubated in the presence of peptide, the cells could selectively recognize them using flow cytometry. In the case of one peptide, LLDFVRFMGV (SEQ ID NO: 4), the complex which the peptide formed with HLA could be detected on the surface of a BLCL even 24 hours after pulsing, further demonstrating the utility of these antibodies.

[00107] In some embodiments, the antigen binding proteins of the invention include antibodies that have the scFv sequence fused to the 2nd and 3rd constant domains of the heavy chain ($CH_{2, 3}$), forming the bottom third of the Fc region of a human immunoglobulin to yield a bivalent protein and fragments thereof, 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 using surface plasmon resonance (SPR), for targeted delivery of a therapeutic agent, to test for Fc-mediated cytotoxicity using CD16-expressing immune effector cells and many other applications.

[00108] The purified scFv-Fc fusion proteins were tested for binding to their targeted T-cell epitopes (TCEs) by way of ELISA and peptide-pulsed APCs. Once they were validated to maintain their specificity, one molecule, EBNA Clone 315 was used for affinity determination. That this molecule was able to bind bound to its targeted TCE through a 1:1 interaction with 10-100 fold greater affinity compared to a typical TCR-MHC-peptide complex interaction was demonstrated.

[00109] Correlation of peptide pulsing of APCs with antigen density was demonstrated. Fluorescently-conjugated scFv-Fc, combined with quantitation beads, allowed the approximation of the number of complexes that are formed when cells are incubated with different concentrations of peptide. Using this information, it was possible to approximate the sensitivity of an scFv and scFv-Fc fusion protein to be around 100 complexes, using flow cytometry.

[00110] Lastly, whether the Fc portion of the fusion protein maintained its effector function was tested. Using a scFv embodiment of the invention, CD16(V)-transduced NK92MI cells, and peptide-pulsed target cells, it was demonstrated that the antibody maintained its Fc-mediated effector functions by way of ADCC.

[00111] The results presented here highlight the specificity, sensitivity and utility of the antigen binding proteins of the invention in targeting MHC-peptide complexes.

[00112] In one embodiment, therefore, the present invention relates to recombinant antigen-binding molecules and portions thereof that recognize a complex of a peptide/protein fragment derived from an intracellular or viral protein, and an MHC

class I molecule, for example, as the complex might be appear at the cell surface for recognition by a T-cell.

[00113] The molecules of the invention are based on the identification and selection of a single chain variable fragment (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).

[00114] 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.

[00115] 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 well known to those of skill in the art.

[00116] In some embodiments, the constant region of the antibody is altered, e.g., mutated, to modify the properties of the antibody (e.g., to increase or decrease one or more of: Fc receptor binding, antibody carbohydrate, for example glycosylation or fucosylation, the number of cysteine residues, effector cell function, or complement function).

[00117] In one embodiment, the antigen binding protein is an anti-WT1/HLA-A2 antibody or fragment thereof having an antigen binding region that comprises the amino acid sequence of SEQ ID NO: 2 and specifically binds to a peptide with the amino acid sequence RMFPNAPYL (SEQ ID NO: 1) in conjunction with HLA-A2. 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 1.

Table 1

Antigen	WT1		
Peptide	RMFPNAPYL (SEQ ID NO: 1)		
CDRs:	1	2	3
VH	SYAMS (SEQ ID NO. 38)	QIDPWGQETLYADSVKG (SEQ ID NO. 40)	LTGRFDY (SEQ ID NO. 48)
VL	RASQSISSYLN (SEQ ID NO: 56)	SASQLQS (SEQ ID NO: 57)	QQGPGTPNT (SEQ ID NO. 64)
Full VH	EVQLLESGGGLVQPGGSLRLSCAASGFTFSSYAMSWVRQAPGKGLEWVSQIDPWGQETLYADS VKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAKLTGRFDYWGQGTLVTVS (SEQ ID NO: 22)		
Full VL	STDIQMTQSPSSLSASVGDRVTITCRASQSISSYLNWYQQKPGKAPKLLIYSASQLQSGVPSRFSGS GSGTDFTLTISSLQPEDFATYYCQQGPGTPNTFGQGTKVEIKRA (SEQ ID NO: 23)		
scFv clone 45	EVQLLESGGGLVQPGGSLRLSCAASGFTFSSYAMSWVRQAPGKGLEWVSQIDPWGQETLYADS VKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAKLTGRFDYWGQGTLVTVSSGGGGSGGGGS GGGGSTDIQMTQSPSSLSASVGDRVTITCRASQSISSYLNWYQQKPGKAPKLLIYSASQLQSGVPS RFSGSGSGTDFTLTISSLQPEDFATYYCQQGPGTPNTFGQGTKVEIKRA (SEQ ID NO: 2)		
DNA (5’ -3’)	GAGGTGCAGCTGTTGGAGTCTGGGGGAGGCTTGGTACAGCCTGGGGGGTCCCTGAGACTCT CCTGTGCAGCCTCTGGATTCACCTTTAGCAGCTATGCCATGAGCTGGGTCCGCCAGGCTCCAG GGAAGGGGCTGGAGTGGGTCTCACAGATTGATCCTTGGGGTCAGGAGACATTGTACGCAGA		

	CTCCGTGAAGGGCCGGTTCACCATCTCCAGAGACAATTCCAAGAACACGCTGTATCTGCAAAT GAACAGCCTGAGAGCCGAGGACACGGCCGTATATTACTGTGCGAAACTTACTGGTCGGTTTG ACTACTGGGGCCAGGGAACCCTGGTCACCGTCTCAAGCGGTGGAGGCGGTTCAGGCGGAGG TGGCAGCGGCGGTGGCGGGTCGACGGACATCCAGATGACCCAGTCTCCATCCTCCCTGTCTG CATCTGTAGGAGACAGAGTCACCATCACTTGCCGGGCAAGTCAGAGCATTAGCAGCTATTTA AATTGGTATCAGCAGAAACCAGGGAAAGCCCCTAAGCTCCTGATCTATTCGGCATCCCAGTTG CAAAGTGGGGTCCCATCAAGGTTCAGTGGCAGTGGATCTGGGACAGATTTCACTCTCACCAT CAGCAGTCTGCAACCTGAAGATTTTGCAACTTACTACTGTCAACAGGGTCCGGGGACTCCTAA TACGTTCGGCCAAGGGACCAAGGTGGAAATCAAACGGGCC (SEQ ID NO: 3)

[00118] In another embodiment, the antigen binding protein is an anti-EBNA3C antibody or fragment thereof that has an antigen binding region that comprises the amino acid sequence of SEQ ID NO: 5 and specifically binds to a peptide with the amino acid sequence LLDFVRFMGV (SEQ ID NO: 4) in conjunction with HLA-A2. In other embodiments, the anti-EBNA3C 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	EBNA3C		
Peptide	LLDFVRFMGV (SEQ ID NO: 4)		
CDRs	1	2	3
VH	GYAMS (SEQ ID NO: 39)	EIAPPGLNTRYADSVKG (SEQ ID NO: 41)	SDTAFDY (SEQ ID NO: 49)
VL	RASQSISSYLN (SEQ ID NO: 56)	LASNLQS (SEQ ID NO: 58)	QQAEYMPLT (SEQ ID NO: 65)
Full VH	EVQLLESGGGLVQPGGSLRLSCAASGFTFSGYAMSWVRQAPGKGLEWVSEIAPPGLNTRYADSV KGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAKSDTAFDYWGQGTLVTVS (SEQ ID NO: 24)		
Full VL	STDIQMTQSPSSLSASVGDRVTITCRASQSISSYLNWYQQKPGKAPKLLIYLASNLQSGVPSRFSGS GSGTDFTLTISSLQPEDFATYYCQQAEYMPLTFGQGTKVEIKRA (SEQ ID NO: 25)		
scFv clone 315	EVQLLESGGGLVQPGGSLRLSCAASGFTFSGYAMSWVRQAPGKGLEWVSEIAPPGLNTRYADSV KGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAKSDTAFDYWGQGTLVTVSSGGGGSGGGGSG GGGSTDIQMTQSPSSLSASVGDRVTITCRASQSISSYLNWYQQKPGKAPKLLIYLASNLQSGVPSR FSGSGSGTDFTLTISSLQPEDFATYYCQQAEYMPLTFGQGTKVEIKRA (SEQ ID NO: 5)		

DNA (5’ -3’)	GAGGTGCAGCTGTTGGAGTCTGGGGGAGGCTTGGTACAGCCTGGGGGGTCCCTGAGACTCT CCTGTGCAGCCTCTGGATTCACCTTTAGCGGCTATGCCATGAGCTGGGTCCGCCAGGCTCCAG GGAAGGGGCTGGAGTGGGTCTCAGAGATTGCGCCGCCTGGTTTGAATACACGTTACGCAGA CTCCGTGAAGGGCCGGTTCACTATCTCCAGAGACAATTCCAAGAACACGCTGTATCTGCAAAT GAACAGCCTGAGAGCCGAGGACACGGCCGTATATTACTGTGCGAAATCGGATACTGCTTTTG ACTACTGGGGCCAGGGAACCCTGGTCACCGTCTCGAGCGGTGGAGGCGGTTCAGGCGGAGG TGGCAGCGGCGGTGGCGGGTCGACGGACATCCAGATGACCCAGTCTCCATCCTCCCTGTCTG CATCTGTAGGAGACAGAGTCACCATCACTTGCCGGGCAAGTCAGAGCATTAGCAGCTATTTA AATTGGTATCAGCAGAAACCAGGGAAAGCCCCTAAGCTCCTGATCTATCTGGCATCCAATTTG CAAAGTGGGGTCCCATCAAGGTTCAGTGGCAGTGGATCTGGGACAGATTTCACTCTCACCAT CAGCAGTCTGCAACCTGAAGATTTTGCAACTTACTACTGTCAACAGGCGGAGTATATGCCTCT GACGTTCGGCCAAGGGACCAAGGTGGAAATCAAACGGGCC (SEQ ID NO: 6)

[00119] In yet another embodiment, the antigen binding protein is an anti-CCND1 antibody or fragment thereof that comprises the amino acid sequence of one of SEQ ID NOs: 8 or 10 and specifically binds to a peptide with the amino acids sequence RLTRFLSRV (SEQ ID NO: 7) in conjunction with HLA-A2. In other embodiments, the anti-CCND1 antibody is a scFv-Fc fusion or full length human IgG with VH and VL regions or CDRs selected from Tables 3 and 4.

Table 3

Antigen	CCND1		
Peptide	RLTRFLSRV (SEQ ID NO. 7)		
CDRs	1	2	3
VH	SYAMS (SEQ ID NO: 38)	TISDSDATDYADSVKG (SEQ ID NO: 42)	TTDYFDY (SEQ ID NO: 50)
VL	RASQSISSYLN (SEQ ID NO: 56)	YASYLQS (SEQ ID NO: 59)	QQSSSSPDT (SEQ ID NO: 66)
Full VH	EVQLLESGGGLVQPGGSLRLSCATSGFTFSSYAMSWVRQAPGKGLEWVSTISDSDATDYADSVK GRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAKTTDYFDYWGQGTLVTVS (SEQ ID NO: 26)		
Full VL	STDIQMTQSPSSLSASVGDRVTITCRASQSISSYLNWYQQKPGKAPKLLIYYASYLQSGVPSRFSGS GSGTDFTLTISSLQPEDFATYYCQQSSSSPDTFGQGTKVEIKRAA (SEQ ID NO: 27)		
scFv clone 5, 17	EVQLLESGGGLVQPGGSLRLSCATSGFTFSSYAMSWVRQAPGKGLEWVSTISDSDATDYADSVK GRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAKTTDYFDYWGQGTLVTVSSGGGGSGGGGSGG GGSTDIQMTQSPSSLSASVGDRVTITCRASQSISSYLNWYQQKPGKAPKLLIYYASYLQSGVPSRFS GSGSGTDFTLTISSLQPEDFATYYCQQSSSSPDTFGQGTKVEIKRAA (SEQ ID NO: 8)		
DNA (5’ -3’)	GAGGTGCAGCTGTTGGAGTCTGGGGGAGGCTTGGTACAGCCTGGGGGGTCCCTGAGACTCT CCTGTGCAACCTCTGGATTCACCTTTAGCAGCTATGCCATGAGCTGGGTCCGCCAGGCTCCAG GGAAGGGGCTGGAGTGGGTCTCAACTATTTCTGATAGTGATGCTACAGATTACGCAGACTCC GTGAAGGGCAGGTTCACCATCTCCAGAGACAATTCCAAGAACACGCTGTATCTGCAAATGAA CAGCCTGAGAGCCGAGGACACGGCCGTATATTACTGTGCGAAAACTACTGATTATTTTGACTA CTGGGGCCAGGGAACCCTGGTCACCGTCTCGAGCGGTGGAGGCGGTTCAGGCGGAGGTGG CAGCGGCGGTGGCGGGTCGACGGACATCCAGATGACCCAGTCTCCATCCTCCCTGTCTGCAT CTGTAGGAGACAGAGTCACCATCACTTGCCGGGCAAGTCAGAGCATTAGCAGCTATTTAAAT TGGTATCAGCAGAAACCAGGGAAAGCCCCTAAGCTCCTGATCTATTATGCATCCTATTTGCAA AGTGGGGTCCCATCAAGGTTCAGTGGCAGTGGATCTGGGACAGATTTCACTCTCACCATCAG CAGTCTGCAACCTGAAGATTTTGCAACTTACTACTGTCAACAGTCTTCTAGTTCTCCTGATACG TTCGGCCAAGGGACCAAGGTGGAAATCAAACGGGCGGCC (SEQ ID NO: 9)		

Table 4

Antigen	CCND1		
Peptide	RLTRFLSRV (SEQ ID NO: 7)		
CDRs:	1	2	3
VH	SYAMS (SEQ ID NO: 38)	DISDDGDATYYADSVKG (SEQ ID NO: 43)	SSTTFDY (SEQ ID NO: 51)
VL	RASQSISSYLN (SEQ ID NO: 56)	AASALQS (SEQ ID NO: 60)	QQGTDSPAT (SEQ ID NO: 67)
Full VH	EVQLLESGGGLVQPGGSLRLSCAASGFTFSSYAMSWVRQAPGKGLEWVSDISDDGDATYYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAKSSTTFDYWGQGTLVTVS (SEQ ID NO: 28)		
Full VL	STDIQMTQSPSSLSASVGDRVTITCRASQSISSYLNWYQQKPGKAPKLLIYAASALQSGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQGTDSPATFGQGTKVEIKRAA (SEQ ID NO: 29)		
scFv clone 43	EVQLLESGGGLVQPGGSLRLSCAASGFTFSSYAMSWVRQAPGKGLEWVSDISDDGDATYYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAKSSTTFDYWGQGTLVTVSSGGGGSGGGGSGGGGSTDIQMTQSPSSLSASVGDRVTITCRASQSISSYLNWYQQKPGKAPKLLIYAASALQSGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQGTDSPATFGQGTKVEIKRAA (SEQ ID NO: 10)		
DNA (5’ -3’)	GAGGTGCAGCTGTTGGAGTCTGGGGGAGGCTTGGTACAGCCTGGGGGGTCCCTGAGACTCTCCTGTGCAGCCTCTGGATTCACCTTTAGCAGCTATGCCATGAGCTGGGTCCGCCAGGCTCCAGGGAAGGGGCTGGAGTGGGTCTCAGATATTTCTGATGATGGTGATGCTACATATTACGCAGACTCCGTGAAGGGCAGGTTCACCATCTCCAGAGACAATTCCAAGAACACGCTGTATCTGCAAATGAACAGCCTGAGAGCCGAGGACACGGCCGTATATTACTGTGCGAAATCTTCTACTACTTTTGACTACTGGGGCCAGGGAACCCTGGTCACCGTCTCGAGCGGTGGAGGCGGTTCAGGCGGAGGTGGCAGCGGCGGTGGCGGGTCGACGGACATCCAGATGACCCAGTCTCCATCCTCCCTGTCTGCATCTGTAGGAGACAGAGTCACCATCACTTGCCGGGCAAGTCAGAGCATTAGCAGCTATTTAAATTGGTATCAGCAGAAACCAGGGAAAGCCCCTAAGCTCCTGATCTATGCTGCATCCGCCTTGCAAAGTGGGGTCCCATCAAGGTTCAGTGGCAGTGGATCTGGGACAGATTTCACTCTCACCATCAGCAGTCTGCAACCTGAAGATTTTGCAACTTACTACTGTCAACAGGGTACTGATAGTCCTGCTACGTTCGGCCAAGGGACCAAGGTGGAAATCAAACGGGCGGCC (SEQ ID NO: 11)		

[00120] In yet another embodiment, the antigen binding protein is an anti-HUD antibody or fragment thereof that comprises the amino acid sequence of one of SEQ ID NOs: 13, 14 and 17 and has an antigen-binding region that specifically binds to a

peptide with the amino acid sequence RIITSTILV (SEQ ID NO: 12) in conjunction with HLA-A2. In other embodiments, the anti-HUD antibody is a scFv-Fc fusion protein or full length human IgG with VH and VL regions or CDRs selected from Tables 5 - 7.

Table 5

Antigen	HUD		
Peptide	RIITSTILV (SEQ ID NO: 12)		
CDRs:	1	2	3
VH	SYAMS (SEQ ID NO: 38)	DIASTGYYTDYADSVKG (SEQ ID NO: 44)	NNASFDY (SEQ ID NO: 52)
VL	RASQSISSYLN (SEQ ID NO: 56)	DASTLQS (SEQ ID NO: 61)	QQTDSYPTT (SEQ ID NO: 68)
Full VH	EVQLLESGGGLVQPGGSLRLSCAASGFTFSSYAMSWVRQAPGKGLEWVSDIASTGYYTDYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAKNNASFDYWGQGTLVTVS (SEQ ID NO: 30)		
Full VL	STDIQMTQSPSSLSASVGDRVTITCRASQSISSYLNWYQQKPGKAPKLLIYDASTLQSGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQTDSYPTTFGQGTKVEIKR (SEQ ID NO: 31)		
scFv clone H128	EVQLLESGGGLVQPGGSLRLSCAASGFTFSSYAMSWVRQAPGKGLEWVSDIASTGYYTDYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAKNNASFDYWGQGTLVTVSSGGGGSGGGGSGGGGSTDIQMTQSPSSLSASVGDRVTITCRASQSISSYLNWYQQKPGKAPKLLIYDASTLQSGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQTDSYPTTFGQGTKVEIKR (SEQ ID NO: 13)		
DNA (5’ -3’)	GAGGTGCAGCTGTTGGAGTCTGGGGGAGGCTTGGTACAGCCTGGGGGGTCCCTGAGACTCTCCTGTGCAGCCTCTGGATTCACCTTTAGCAGCTATGCCATGAGCTGGGTCCGCCAGGCTCCAGGGAAGGGGCTGGAGTGGGTCTCGGATATTGCTTCTACTGGTTATTATACAGATTACGCAGACTCCGTGAAGGGCCGGTTCACCATCTCCAGAGACAATTCCAAGAACACGCTGTATCTGCAAATGAACAGCCTGAGAGCCGAGGACACGGCCGTATATTACTGTGCGAAAAATAATGCTAGTTTTGACTACTGGGGCCAGGGAACCCTGGTCACCGTCTCGAGCGGTGGAGGCGGTTCAGGCGGAGGTGGCAGCGGCGGTGGCGGGTCGACGGACATCCAGATGACCCAGTCTCCATCCTCCCTGTCTGCATCTGTAGGAGACAGAGTCACCATCACTTGCCGGGCAAGTCAGAGCATTAGCAGCTATTTAAATTGGTATCAGCAGAAACCAGGGAAAGCCCCTAAGCTCCTGATCTATGATGCATCCACTTTGCAAAGTGGGGTCCCATCAAGGTTCAGTGGCAGTGGATCTGGGACAGATTTCACTCTCACCATCAGCAGTCTGCAACCTGAAGATTTTGCAACTTACTACTGTCAACAGACTGATTCTTATCCTACTACGTTCGGCCAAGGGACCAAGGTGGAAATCAAACGG (SEQ ID NO: 15)		

Table 6

Antigen	HUD		
Peptide	RIITSTILV (SEQ ID NO: 12)		
CDRs:	1	2	3
VH	SYAMS (SEQ ID NO: 38)	SISSSGYYTDYADSVKG (SEQ ID NO: 45)	SASSFDY (SEQ ID NO: 53)
VL	RASQSISSYLN (SEQ ID NO: 56)	DASTLQS (SEQ ID NO: 61)	QQDDAYPTT (SEQ ID NO: 69)
Full VH	EVQLLESGGGLVQPGGSLRLSCAASGFTFSSYAMSWVRQAPGKGLEWVSSISSSGSYTDYADSVK GRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAKSASSFDYWGQGTLVTVS (SEQ ID NO: 32)		
Full VL	STDIQMTQSPSSLSASVGDRVTITCRASQSISSYLNWYQQKPGKAPKLLIYDASTLQSGVPSRFSGS GSGTDFTLTISSLQPEDFATYYCQQDDAYPTTFGQGTKVEIKR (SEQ ID NO: 33)		
scFv clone H78	EVQLLESGGGLVQPGGSLRLSCAASGFTFSSYAMSWVRQAPGKGLEWVSSISSSGSYTDYADSVK GRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAKSASSFDYWGQGTLVTVSSGGGGSGGGGSGG GGSTDIQMTQSPSSLSASVGDRVTITCRASQSISSYLNWYQQKPGKAPKLLIYDASTLQSGVPSRFS GSGSGTDFTLTISSLQPEDFATYYCQQDDAYPTTFGQGTKVEIKR (SEQ ID NO: 14)		
DNA (5’ -3’)	GAGGTGCAGCTGTTGGAGTCTGGGGGAGGCTTGGTACAGCCTGGGGGGTCCCTGAGACTCT CCTGTGCAGCCTCTGGATTCACCTTTAGCAGCTATGCCATGAGCTGGGTCCGCCAGGCTCCAG GGAAGGGGCTGGAGTGGGTCTCATCTATTAGTAGTTCTGGTAGTTATACAGATTACGCAGAC TCCGTGAAGGGCCGGTTCACCATCTCCAGAGACAATTCCAAGAACACGCTGTATCTGCAAAT GAACAGCCTGAGAGCCGAGGACACGGCCGTATATTACTGTGCGAAATCTGCTTCTTCTTTTGA CTACTGGGGCCAGGGAACCCTGGTCACCGTCTCGAGCGGTGGAGGCGGTTCAGGCGGAGGT GGCAGCGGCGGTGGCGGGTCGACGGACATCCAGATGACCCAGTCTCCATCCTCCCTGTCTGC ATCTGTAGGAGACAGAGTCACCATCACTTGCCGGGCAAGTCAGAGCATTAGCAGCTATTTAA ATTGGTATCAGCAGAAACCAGGGAAAGCCCCTAAGCTCCTGATCTATGATGCATCCACTTTGC AAAGTGGGGTCCCATCAAGGTTCAGTGGCAGTGGATCTGGGACAGATTTCACTCTCACCATC AGCAGTCTGCAACCTGAAGATTTTGCAACTTACTACTGTCAACAGGATGATGCTTATCCTACT ACGTTCGGCCAAGGGACCAAGGTGGAAATCAAACGG (SEQ ID NO: 16)		

Table 7

Antigen	HUD		
Peptide	RIITSTILV (SEQ ID NO: 12)		
CDRs:	1	2	3
VH	SYAMS (SEQ ID NO: 38)	SISSDGSYTDYADSVKG (SEQ ID NO: 46)	STDAFDY (SEQ ID NO: 54)
VL	RASQSISSYLN (SEQ ID NO: 56)	AASYLQS (SEQ ID NO: 62)	QQDNNYPTT (SEQ ID NO: 70)
Full VH	EVQLLESGGGLVQPGGSLRLSCAASGFTFSSYAMSWVRQAPGKGLEWVSSISSDGSYTDYADSVK GRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAKSTDAFDYWGQGTLVTVS (SEQ ID NO: 34)		
Full VL	STDIQMTQSPSSLSASVGDRVTITCRASQSISSYLNWYQQKPGKAPKLLIYAASYLQSGVPSRFSGS GSGTDFSLTISSLQPEDFATYYCQQDNNYPTTFGQGTKVEIKR (SEQ ID NO: 35)		
scFv clone H110	EVQLLESGGGLVQPGGSLRLSCAASGFTFSSYAMSWVRQAPGKGLEWVSSISSDGSYTDYADSVK GRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAKSTDAFDYWGQGTLVTVSSGGGGSGGGGSGG GGSTDIQMTQSPSSLSASVGDRVTITCRASQSISSYLNWYQQKPGKAPKLLIYAASYLQSGVPSRFS GSGSGTDFSLTISSLQPEDFATYYCQQDNNYPTTFGQGTKVEIKR (SEQ ID NO: 17)		
DNA (5’ -3’)	GAGGTGCAGCTGTTGGAGTCTGGGGGAGGCTTGGTACAGCCTGGGGGGTCCCTGAGACTCT CCTGTGCAGCCTCTGGATTCACCTTTAGCAGCTATGCCATGAGCTGGGTCCGCCAGGCTCCAG GGAAGGGGCTGGAGTGGGTCTCAAGTATTTCTTCTGATGGTAGTTATACAGATTACGCAGAC TCCGTGAAGGGCCGGTTCACCATCTCCAGAGACAATTCCAAGAACACGCTGTATCTGCAAAT GAACAGCCTGAGAGCCGAGGACACGGCCGTATATTACTGTGCGAAATCTACTGATGCTTTTG ACTACTGGGGCCAGGGAACCCTGGTCACCGTCTCGAGCGGTGGAGGCGGTTCAGGCGGAGG TGGCAGCGGCGGTGGCGGGTCGACGGACATCCAGATGACCCAGTCTCCATCCTCCCTGTCTG CATCTGTAGGAGACAGAGTCACCATCACTTGCCGGGCAAGTCAGAGCATTAGCAGCTATTTA AATTGGTATCAGCAGAAACCAGGGAAAGCCCCTAAGCTCCTGATCTATGCTGCATCCTATTTG CAAAGTGGGGTCCCATCAAGGTTCAGTGGCAGTGGATCTGGGACAGATTTCTCTCTCACCATC AGCAGTCTGCAACCTGAAGATTTTGCAACTTACTACTGTCAACAGGATAATAATTATCCTACTA CGTTCGGCCAAGGGACCAAGGTGGAAATCAAACGG (SEQ ID NO: 18)		

[00121] In yet another embodiment, the antigen binding protein is an anti-cdr2 antibody or fragment thereof that comprises the amino acid sequence of SEQ ID NO: 20 and specifically binds to a peptide with amino acids LLEEMFLTV (SEQ ID NO: 19) in conjunction with HLA-A2. In other embodiments, the anti-cdr2 antibody is a scFv-

Fc fusion protein or full length human IgG with VH and VL regions or CDRs selected from Table 8.

Table 8

Antigen	cdr2		
Peptide	LLEEMFLTV (SEQ ID NO: 19)		
CDRs:	1	2	3
VH	SYAMS (SEQ ID NO: 38)	TINYSGSGTTYADSVKG (SEQ ID NO: 47)	NAAYFDY (SEQ ID NO: 55)
VL	RASQSISSYLN (SEQ ID NO: 56)	GASGLQS (SEQ ID NO: 63)	QQSANAPTT (SEQ ID NO: 71)
Full VH	EVQLLESGGGLVQPGGSLRLSCAASGFTFSSYAMSWVRQAPGKGLEWVSTINYSGSGTTYADSV KGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAKNAAYFDYWGQGTLVTVS (SEQ ID NO: 36)		
Full VL	STDIQMTQSPSSLSASVGDRVTITCRASQSISSYLNWYQQKPGKAPKLLIYGASGLQSGVPSRFSGS GSGTDFTLTISSLQPEDFATYYCQQSANAPTTFGQGTKVEIKR (SEQ ID NO: 37)		
scFv clone L9	EVQLLESGGGLVQPGGSLRLSCAASGFTFSSYAMSWVRQAPGKGLEWVSTINYSGSGTTYADSV KGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAKNAAYFDYWGQGTLVTVSSGGGGSGGGGSG GGGSTDIQMTQSPSSLSASVGDRVTITCRASQSISSYLNWYQQKPGKAPKLLIYGASGLQSGVPSR FSGSGSGTDFTLTISSLQPEDFATYYCQQSANAPTTFGQGTKVEIKR (SEQ ID NO: 20)		
DNA (5’ -3’)	GAGGTGCAGCTGTTGGAGTCTGGGGGAGGCTTGGTACAGCCTGGGGGGTCCCTGAGACTCT CCTGTGCAGCCTCTGGATTCACCTTTAGCAGCTATGCCATGAGCTGGGTCCGCCAGGCTCCAG GGAAGGGGCTGGAGTGGGTCTCAACTATTAATTATTCTGGTTCTGGTACAACTTACGCAGACT CCGTGAAGGGCAGGTTCACCATCTCCAGAGACAATTCCAAGAACACGCTGTATCTGCAAATG AACAGCCTGAGAGCCGAGGACACGGCCGTATATTACTGTGCGAAAAATGCTGCTTATTTTGA CTACTGGGGCCAGGGAACCCTGGTCACCGTCTCGAGCGGTGGAGGCGGTTCAGGCGGAGGT GGCAGCGGCGGTGGCGGGTCGACGGACATCCAGATGACCCAGTCTCCATCCTCCCTGTCTGC ATCTGTAGGAGACAGAGTCACCATCACTTGCCGGGCAAGTCAGAGCATTAGCAGCTATTTAA ATTGGTATCAGCAGAAACCAGGGAAAGCCCCTAAGCTCCTGATCTATGGTGCATCCGGTTTG CAAAGTGGGGTCCCATCAAGGTTCAGTGGCAGTGGATCTGGGACAGATTTCACTCTCACCAT CAGCAGTCTGCAACCTGAAGATTTTGCAACTTACTACTGTCAACAGTCTGCTAATGCTCCTACT ACGTTCGGCCAAGGGACCAAGGTGGAAATCAAACGG (SEQ ID NO: 21)		

[00122] Embodiments of the antigen-binding proteins of the disclosure in accordance with Tables 1-8 include, but are not limited to the following:

[00123] an anti-WT-1 antibody which binds to an HLA-restricted peptide RMFPNAPYL (SEQ ID NO: 1) comprising: (i) an HVR-L1 sequence of RASQSISSYLN (SEQ ID NO: 56) (ii) an HVR-L2 sequence of SASQLQS (SEQ ID NO: 57) (iii) an HVR-L3 sequence of QQGPGTPNT (SEQ ID NO: 64) (iv) an HVR-H1 sequence of SYAMS (SEQ ID NO: 38) (v) an HVR-H2 sequence of QIDPWGQETLYADSVKG (SEQ ID NO: 40), and (vi) an HVR-H3 sequence of LTGRFDY (SEQ ID NO: 48);

[00124] an anti-EBNA3C antibody which binds to HLA-A2 restricted peptide LLDFVRFMGV (SEQ ID NO: 4) comprising: (i) an HVR-L1 sequence of RASQSISSYLN (SEQ ID NO: 56) (ii) an HVR-L2 sequence of LASNLQS (SEQ ID NO: 58) (iii) an HVR-L3 sequence of QQAEYMPLT (SEQ ID NO: 65) (iv) an HVR-H1 sequence of GYAMS (SEQ ID NO: 39) (v) an HVR-H2 sequence of EIAPPGLNTRYADSVKG (SEQ ID NO: 41), and (vi) an HVR-H3 sequence of SDTAFDY (SEQ ID NO: 49);

[00125] an anti-CCND1 antibody which binds to HLA-A2 restricted peptide RLTRFLSRV (SEQ ID NO: 7) comprising: (i) an HVR-L1 sequence of RASQSISSYLN (SEQ ID NO: 56) (ii) an HVR-L2 sequence of YASYLQS (SEQ ID NO: 59) (iii) an HVR-L3 sequence of QQSSSSPDT (SEQ ID NO: 66) (iv) an HVR-H1 sequence of SYAMS (SEQ ID NO: 38) (v) an HVR-H2 sequence of TISDSDATDYADSVKG (SEQ ID NO: 42), and (vi) an HVR-H3 sequence of TTDYFDY (SEQ ID NO: 50);

[00126] an anti-CCND1 antibody which binds to HLA-A2 restricted peptide RLTRFLSRV (SEQ ID NO: 7) comprising: (i) an HVR-L1 sequence of RASQSISSYLN (SEQ ID NO: 56) (ii) an HVR-L2 sequence of AASALQS (SEQ ID NO: 60) (iii) an HVR-L3 sequence of QQGTDSPAT (SEQ ID NO: 67) (iv) an HVR-H1 sequence of SYAMS (SEQ ID NO: 38) (v) an HVR-H2 sequence of DISDDGDATYYADSVKG (SEQ ID NO: 43), and (vi) an HVR-H3 sequence of SSTTFDY (SEQ ID NO: 51);

[00127] an anti-HUD antibody which binds to HLA-A2 restricted peptide RIITSTILV (SEQ ID NO: 12) comprising: (i) an HVR-L1 sequence of RASQSISSYLN (SEQ ID NO: 56) (ii) an HVR-L2 sequence of DASTLQS (SEQ ID NO: 61) (iii) an HVR-L3 sequence of QQTDSYPTT (SEQ ID NO: 68) (iv) an HVR-H1 sequence of SYAMS (SEQ ID NO: 38) (v) an HVR-H2 sequence of DIASTGYYTDYADSVKG (SEQ ID NO: 44), and (vi) an HVR-H3 sequence of NNASFDY (SEQ ID NO: 52);

[00128] an anti-HUD antibody which binds to HLA-A2 restricted peptide RIITSTILV (SEQ ID NO: 12) comprising: (i) an HVR-L1 sequence of RASQSISSYLN (SEQ ID NO: 56) (ii) an HVR-L2 sequence of DASTLQS (SEQ ID NO: 61) (iii) an HVR-L3 sequence of QQDDAYPTT (SEQ ID NO: 69) (iv) an HVR-H1 sequence of SYAMS (SEQ ID NO: 38) (v) an HVR-H2 sequence of SISSSGYYTDYADSVKG (SEQ ID NO: 45), and (vi) an HVR-H3 sequence of SASSFDY (SEQ ID NO: 53);

[00129] an anti-HUD antibody which binds to HLA-A2 restricted peptide RIITSTILV (SEQ ID NO: 12) comprising: (i) an HVR-L1 sequence of RASQSISSYLN (SEQ ID NO: 56) (ii) an HVR-L2 sequence of AASYLQS (SEQ ID NO: 62) (iii) an HVR-L3 sequence of QQDNNYPTT (SEQ ID NO: 70) (iv) an HVR-H1 sequence of SYAMS (SEQ ID NO: 38) (v) an HVR-H2 sequence of SISSDGSYTDYADSVKG (SEQ ID NO: 46), and (vi) an HVR-H3 sequence of STDAFDY (SEQ ID NO: 54); and

[00130] an anti-cdr2 antibody which binds to HLA-A2 restricted peptide LLEEMFLTV (SEQ ID NO: 19) comprising: (i) an HVR-L1 sequence of RASQSISSYLN (SEQ ID NO: 56) (ii) an HVR-L2 sequence of GASGLQS (SEQ ID NO: 63) (iii) an HVR-L3 sequence of QQSANAPTT (SEQ ID NO: 71) (iv) an HVR-H1 sequence of SYAMS (SEQ ID NO: 38) (v) an HVR-H2 sequence of TINYSGSGTTYADSVKG (SEQ ID NO: 47), and (vi) an HVR-H3 sequence of NAAYFDY (SEQ ID NO: 55).

[00131] EXAMPLES - General Procedures

Example 1: Production of biotinylated MHC-peptide complexes

[00132] Soluble MHC class I/peptide complexes were generated by overexpression of the HLA-A2 heavy chain (HC) and β2 microglobulin (β_2M) as recombinant proteins in *E. coli* and subsequent *in vitro* refolding and assembly in the presence of high concentrations of specific peptide (35, 36). To obtain soluble MHC/peptide complexes the HC sequence was mutagenized to remove the cytosolic and transmembrane regions. In order to specifically biotinylate refolded, monomeric MHC/peptide complexes, the HC was expressed as a fusion protein containing a specific biotinylation site at the C-terminus (37, 38). These short sequences are sufficient for enzymatic *in vitro* biotinylation of a single lysine residue within this sequence using the biotin protein ligase BirA (39). This procedure was carried out by the MSKCC Tetramer Core Facility.

Example 2: *Selection of phage on biotinylated MHC-peptide complexes*

Ex. 2.1: *Selection of phage on HLA-A2/EBNA3C (EBNA) complex*

[00133] The Tomlinson I + J human scFv phage display libraries (40), containing approximately 2.85 x 10^8 independent scFv clones, were used for selection according to previously published methods (22) with modifications. 7.5 x 10^{12} Phage, from the combination of both libraries, were first preincubated with streptavidin paramagnetic DYNABEADS(30 µl; Dynal, Oslo, Norway) and 150 µg unbiotinylated HLA-A2-YVDPVITSI (SEQ ID NO: 75) (irrelevant complex) in 1 ml of PBS to remove any phage which expressed an antibody that binds to streptavidin or the general framework of HLA-A2.

[00134] The DYNABEADS were subsequently captured using a magnet and the supernatant (phage and irrelevant complex mixture) transferred to a separate tube containing 7.5 µg of biotinylated HLA-A2-LLDFVRFMGV (SEQ ID NO: 4) (Epstein-Barr virus EBNA3C-derived) and 7.5 µg of biotinylated HLA-A2-NLVPMVATV (SEQ ID NO: 73) (Cytomedullovirus pp65-derived) and incubated at RT for 1 hour. The final mixture (1 ml) was then added to 200 µl of DYNABEADS (preincubated with 2% Milk and washed with PBS) and the contents were mixed for 15 min. at RT with

continuous rotation. The beads were then washed 10 times with PBS/0.1% TWEEN and 3 times with PBS and the bound phage were eluted from the DYNABEADS using 1 mg/ml trypsin in PBS (0.5 ml) for 15 min. at RT.

[00135] The phage were then used to infect TG1 *E. coli* (growing in log phase) at 37°C in 20 ml of LB for 1 hour. 10^{12} KM13 helper phage was subsequently added to the mixture, further incubated for an additional 30 minutes, and the cells pelleted using centrifugation (3000 rpm for 10 min.). The resulting cell pellet was resuspended in 200 ml LB + Ampicillin (100 µg/ml) + Kanamycin (50 µg/ml) and incubated overnight at 30°C.

[00136] The following morning, the overnight cultures were centrifuged at 3000 rpm for 15 min. and the supernatant (180 ml) was mixed with polyethylene glycol (PEG) on ice for 1 hour so as to precipitate the amplified phage from the previous round of selection. The PEG/phage mixture was then centrifuged at 3000 rpm for 20 min., and some of the resulting phage pellet used for subsequent rounds of panning while the rest was frozen down in 15% glycerol at -80°C. Subsequent rounds of panning were done using the same protocol as above with an increase in DYNABEADS washing steps and a decrease in the amount of biotinylated complexes used for selection.

[00137] After the final round of antibody selection (3rd or 4th round), the eluted phage were used to infect both TG1 and HB2151 *E. coli*; TG1 cells were cultured overnight as mentioned above while the HB2151 cells were plated on TYE + Ampicillin (100 µg/ml) agar plates. The next morning, individual colonies from the agar plate were picked and used to inoculate individual wells of a 48-well plate containing 400 µl LB + Ampicillin (100 µg/ml)/well. After incubation for 3-6 hours at 37°C, 200 µl of 50% glycerol solution was added to each well and the plates stored at -80°C as monoclonal stock cultures.

[00138] *Ex. 2.2: Selection of phage on HLA-A2-RMFPNAPYL (SEQ ID NO: 1) (WT-1) complex*

[00139] Selection was done similarly to the method above with slight modifications. 3.7 x 10^{12} Phage from the combination of both libraries, were first preincubated with streptavidin paramagnetic DYNABEADS (50 µl; Dynal, Oslo, Norway) and 20 µg unbiotinylated HLA-A2-NLVPMVATV (SEQ ID NO: 73) (irrelevant complex) in 1 ml of PBS to deplete the streptavidin and HLA-A2 binders. The DYNABEADS were subsequently captured using a magnet and the supernatant (phage and irrelevant complex mixture) transferred to a separate tube containing 5 µg of biotinylated HLA-A2-RMFPNAPYL (SEQ ID NO: 1) (WT1-derived) and incubated at RT for 1 hour. The final mixture (1 ml) was then added to 100 µl of DYNABEADS (preincubated with 2% Milk and washed with PBS) and the contents were mixed for 30 min. at RT with continuous rotation. The beads were then washed 10 times with PBS/0.1% TWEEN and 3 times with PBS and the bound phage were eluted from the DYNABEADS using 1 mg/ml trypsin in PBS (0.5 ml) for 20 min. at RT. All subsequent steps were performed as above.

Example 3: *Expression and purification of soluble scFv from HB2151*

[00140] Using the monoclonal glycerol stocks containing individual HB2151 clones, separate 48-well plates containing 400 µl LB + Ampicillin (100 µg/ml)/well were inoculated in a replica-plate type format using sterile pipette tips. The 48-well culture plates were subsequently incubated at 37^{o}C until the majority of the wells reached an OD600 of 0.4. 200 µl LB + Ampicillin (100 µg/ml) + isopropyl-1-thio-β-D-galactopyranoside (IPTG; 1mM final concentration) was subsequently added to each well to induce scFv production and the plates were further incubated overnight at 28^{o}C. The next morning, the plates were centrifuged at 3000 rpm for 15 min. and the supernatant used for scFv screening.

[00141] For large scale expression and purification, monoclonal glycerol stocks were used to inoculate 3 ml of Terrific Broth (TB) and incubated at 37°C until an OD600 of 0.8 was reached. Each 3 ml culture was subsequently divided amongst four flasks, each containing 250 ml TB + Ampicillin (100 µg/ml). The cultures were then incubated at 37°C until an OD600 of 0.4-0.5 was reach, after which IPTG was added to a final concentration of 0.5 mM and the cultures incubated overnight at 30°C. The next morning, the overnight cultures were centrifuged at 4000 rpm for 25 min. The supernatant was discarded and the pellets dissolved in 50 ml PBS + 10 mM imidazole. The cell suspensions were passed through a cell homogenizer (5000 pounds per square inch) and the resulting cell lysates were centrifuged at 12,000 rpm for 15 min. The supernatants were then passed over a 0.22 µm filter pre-layered with diatomaceous earth and the resulting filtrates loaded over VIVAPURE maxiprepMC Nickel affinity columns (Sartorius Stedim Biotech, Aubagne, France) using centrifugation (100 rpm for 5 min.). The columns were then washed 4 times using 10ml PBS + 30 mM imidazole (500 rpm for 3 min.) and the scFvs eluted using 20 ml PBS + 300 mM imidazole (500 rpm for 3 min.). The eluted scFvs were concentrated using 10,000 molecular weight cut-off membrane VIVASPIN centrifuge tubes at 3000 rpm for 30 min. (Sartorius Stedim Biotech) and dialyzed back into regular PBS. The final scFv products were subsequently stored at -80°C.

Example 4: Construction of scFv-Fc fusion protein and expression in DG44 CHO cells

[00142] Using a proprietary antibody expression vector (referred to herein as IgG Vector), similar to that of pFUSE—hIgG1-Fc1 (InvivoGen; San Diego, California), the construct was first modified to contain the CH_2, and CH_3 domains of a human IgG_1 (scFv-Fc Vector). Subsequently, the EBNA Clone 315 and WT1 Clone 45 scFv sequences were PCR amplified to contain the required NheI and ApaI restriction sites which would be compatible with the scFv-Fc vector. The resulting scFv PCR products and antibody expression plasmid were digested using the above enzymes

(NheI at 37°C for 2 hours and ApaI at 25°C for 2 hours) and then ligated together. The ligation products were then transformed into *E.coli*, plated on TYE + Amplicilin (100 µg/ml), colonies were picked and their plasmids sequenced at the MSKCC Sequencing Core Facility. Once the sequences were validated to have the correct scFv sequences upstream of the human IgG_1 CH_2 and CH_3 domains, the DNA (5-6 µg was electroporated (Amaxa NUCLEOFACTOR; Lonza, Switzerland) into 5 x 10^6 DG44 Chinese Hamster Ovary (CHO) Cells (Invitrogen) using Program U-030 and 100 µl Solution V. The cells were then cultured in OPTICHO media (Invitrogen) containing G418 (500 µg/ml; added 7 days post-electroporation) at a cell density of 1-5 x 10^6 DG44 per ml of media. The cells were then expanded to approximately 700 ml of culture media, which was centrifuged to remove the cells and supernatant used for antibody purification.

Example 5: Expression and purification of soluble scFv-Fc fusion protein

[00143] DG44 supernatant containing the soluble scFv-Fc fusion protein was purified using the KAPPASELECT affinity chromatograph medium (GE Healthcare). First, 1.5 ml of KAPPASELECT resin was loaded onto a column and activated with 20 ml of PBS. The supernatant was loaded onto the column using a peristaltic pump at a flow rate of approximately 1 ml/min. The column was subsequently washed using 40 ml of PBS until the flow-thru registered an OD280 of less than 0.05. The scFv-Fc fusion protein was then eluted from the resin using 10 ml citrate buffer (pH 2.0) and directly into 10ml of 1 M Tris for neutralization. The eluted scFv-Fc was subsequently concentrated using a 50,000 MWCO VIVASPIN centrifuge tube (Sartorius Stedim) and tested for its ability to bind to recombinant antigen using ELISA and the BIACORE T100 (GE Healthcare) as well as natively presented peptide on the surface of T2 cells using flow cytometry.

Example 6: Monoclonal ELISA with bacterial phage clones and purified scFv and scFv-Fc

[00144] Vinyl flat bottom microtiter plates (Thermo Fisher) were used for ELISA assays. Plates were initially coated overnight at 4^{o}C with BSA-biotin (10 µg/ml; 50 µl/well). The next morning, the contents were discarded and the plates incubated at RT with streptavidin (10 µg/ml; 50 µl/well) for 1 hour. The contents were discarded and the plates incubated with recombinant biotinylated HLA-A2-peptide complexes (5 µg/ml; 50 µl/well) at RT for 1 hour. The plates were then incubated with 2% Milk (150 µl/well) at RT for 1 hour. After blocking, the plates were washed 2 times with PBS and then incubated with bacterial supernatant from their respective HB2151 culture plate wells, purified scFv, or purified scFv-Fc at RT for 1 hour. The contents were discarded, the plates washed 5 times with PBS, and then incubated at RT for 1 hour with either a mouse-anti-myc tag antibody (Clone 9E10; Sigma Aldrich. 0.5 µg/ml; 100 µl/well in 0.5% Milk) to detect the scFv or a goat-anti-human-HRP (Jackson Immunoresearch Laboratories. 0.5 µg/ml; 100 µl/well in 0.5% Milk) to detect the scFv-Fc. The contents were discarded, the plates washed 5 times with PBS, and those receiving the scFv were further incubated with a goat-anti-mouse-HRP (Jackson Immunoresearch Laboratories. 0.5 µg/ml; 100 µl/well in 0.5% Milk) at RT for 1 hour while the plates receiving the scFv-Fc were developed using o-phenylenediamine (OPD) buffer (150 µl/well), which was made by combining 20 mg of OPD tablets in 40 ml of citrate phosphate buffer with 40 µl 30% hydrogen peroxide. The color reaction was stopped by adding 30 µl of 5N sulfuric acid to each well and the plates read using the Dynex MRX ELISA plate reader at 490 nm. Lastly, the contents of the scFv plates were discarded, the plates washed 5 times with PBS, and developed according to the method above.

Example 7: Cell lines and peptides

[00145] Tap-deficient HLA-A2^{+} T2 cells, 6268A, GKO (both HLA-A2^{-}), DIMT and JG19 (both HLA-A2^{+}) B-cell lymphoblastic cell lines (BLCLs) were used for antigen presentation studies. Cells were normally cultured in RPMI 1640 + 10% Fetal Bovine Serum (FBS). For antigen presentation, T2 cells were harvested and transferred to

serum-free IMDM + 10 µg/ml β2-microglobulin (β2M). The T2 cells would then be incubated with 20 µM or less of either LLDFVRFMGV SEQ ID NO: 4) -peptide (derived from EBNA3C) or any number of irrelavent peptides at 37°C for 5 hours. Studies with BLCLs were done in the same manner as with T2 cells with the occlusion of β_2M in the media. Pulse-Chase experiments with DIMT BLCLs were done by first pulsing the BLCLs in serum-free IMDM with 20 µM LLDFVRFMGV SEQ ID NO: 4) for 5 hours at 37°C. The cells were then washed with fresh RPMI 1640 + 10% FBS, transferred back into this culture medium and cultured further at 37°C for 5 and 24 hours, followed by flow cytometric analysis at each time point using EBNA Clone 315 scFv.

Example 8: Binding Kinetics Analysis

[00146] Kinetic measurements were performed by surface plasmon resonance using the BIACORE T100 (GE Biosciences). Briefly, the first two flow cells of a CM5 chip (GE Biosciences) were activated using the standard amine coupling reagents in HBS-EP running buffer (0.01 M HEPES, 0.15 M NaCl, 3 mM EDTA, 0.005% TWEEN 20) with flow cell 2 immobilized with the purified EBNA Clone 315 scFv-Fc fusion protein using 10 mM Acetate (pH 5). Subsequently, the target HLA-A2-peptide monomer (222 nM-13.875 nM) was injected over both the 1st (reference) and 2nd flow cells at 20 µl/min. for 120 sec., followed by the addition of running buffer for an extra 180 sec. Kinetics values were determined using the BIACORE T100 Evaluation Software 2.0 and 1:1 binding model (local Rmax).

Example 9: Flow Cytometry

[00147] Peptide-pulsed T2 cells and BLCLs were transferred to plastic polystyrene round-bottom tubes (Becton Dickinson Labware) and washed with PBS. The cells were subsequently incubated with 5 µg of either targeted or non-specific purified scFv or scFv-Fc on ice for 40 min. The cells were washed with PBS and then incubated with 1 µg of biotinylated mouse-anti-myc antibody (Clone 9E10; Sigma

Aldrich) or biotinylated mouse-anti-human IgG Fc-specific (Jackson Immunoresearch Laboratories) on ice for 30 min. The cells were washed with PBS and then incubated with streptavidin-PE (BD Biosciences). Lastly, the cells were washed once more with PBS and analyzed on the BD FACS Calibur.

[00148] For CD107a cytotoxicity assays, transduced-NK92MI cells and target T2 cells were cocultured in a 1:1 effector:target (E:T) ratio (2.5-5.0 x 10^5 cells each) in 200 µl complete Alpha Essential medium (12.5% horse serum and 12.5% FBS) (Invitrogen) + 10-15 µl anti-CD107a-PE at 37^{o}C for 5 Hours. The cell mixture was then washed with PBS and analyzed on the BD FACS Caliber.

[00149] For FACS sorting experiments, retrovirally transduced NK92MI cells were sorted based on GFP intensity using the BD Aria Flow Cytometer under the guidence of the MSKCC Flow Cytometry facility.

Example 10: Quantitation of HLA-A2-LLDFVRFMGV SEQ ID NO: 4) complexes on peptide-pulsed T2 cells

[00150] For MHC-peptide complex quantitation, the EBNA Clone 315 scFv-Fc was first directly conjugated to ALEXA FLUOR 647 using the APEX ALEXA FLUOR 647 Antibody Labeling Kit (Invitrogen). The kit yields about 10-20 µg of labeled antibody.

[00151] For quantitation, the QUANTUM SIMPLY CELLULAR anti-Human IgG kit was used (Bangs Laboratories) along with the technical assistance of Hong-fen Guo in our laboratory. Briefly, the kit is comprised of five microsphere populations; one blank and four labeled with increasing amounts of anti-human IgG. The beads and the peptide pulsed T2 cells (37^{o}C for 5 hours) were then labeled with the same fluorescently conjugated EBNA Clone 315 scFv-Fc on ice for 30 minutes. The cells were then washed with PBS and analyzed on the BD FACS Calibur along with the labeled beads. The Excel-based QuickCal analysis template that's provided with each kit aids in correlating fluorescence intensity with antigen density on the T2 cells. Each of the 4 data points are the average of duplicates.

Example 11: Construction of the WT-1 clone 45 chimeric antigen receptor

[00152] The original chimeric antigen receptor was obtained from Dr. Dario Campana from St. Jude Children's Hospital and previously described (41). For future compatibility purposes, a scFv-CD3ζ-4-1BB DNA construct (similar to that seen in the original chimeric immune receptor, with EcoRI and XhoI flanking the 5' and 3' ends) was purchased (pUC57 vector from Genescript; Piscataway, NJ) and contained an irrelevant scFv flanked by SfiI and NotI. The plasmid containing the EBNA Clone 315 scFv sequence (pIT2 vector from the Tomlinson library) was purified (Qiagen miniprep DNA isolation kit) from overnight culture of the bacterial stock in LB + Amplicilin (100 µg/ml). The scFv sequence was excised from the pIT2 vector using SfiI (50^{o}C for 2 hours) and NotI (37^{o}C for 2 hours) inserted into the purchased and predigested (SfiI and NotI) pUC57 vector. After ligation, the product was transformed into NEB 5-alpha competent *E. coli* (New England Biolabs), the cells plated on TYE + Amplicilin (100 µg/ml), colonies were picked and cultured in LB + Amplicilin (100 µg/ml), their plasmids purified and the product sizes were verified by gel electrophoresis. Plasmids which were found to have the correct ligation products were subsequently excised from the pUC57 vector using EcoRI (37^{o}C for 2 hours) and XhoI (37^{o}C for 2 hours) and used for insertion into the vector provided to us by the Campana laboratory. The ligation products were then transformed into *E.coli* as above, plated on TYE + Amplicilin (100 µg/ml), colonies were picked and their plasmids sequenced using the reverse primer 788A (5'-CCCTTGAACCTCCTCGTTCGACC-3') (SEQ ID NO: 72) at the MSKCC Sequencing Core Facility. Once the sequences were validated, the DNA was packaged into retrovirus and used to infect NK92MI cells.

Example 12: *Construction of the EBNA Clone 315 chimeric antigen receptor*

[00153] Due to compatibility issues, the pUC57 scFv-CD3ζ-4-1BB DNA construct purchased from Genescript and mentioned above was used to replace the WT1

Clone 45 scFv with the EBNA Clone 315 scFv. First, the plasmid containing the EBNA Clone 315 scFv sequence (pIT2 vector from the Tomlinson library) was purified (Qiagen miniprep DNA isolation kit) from overnight culture of the bacterial stock in LB + Amplicilin (100 µg/ml). The scFv sequence was excised from the pIT2 vector using SfiI (50^{o}C for 2 hours) and NotI (37^{o}C for 2 hours) and ligated to the predigested (SfiI and NotI) pUC57 vector. After ligation, the product was transformed into *E. coli*, colonies were picked, cultured overnight, their plasmids purified and the product sizes verified by gel electrophoresis. Plasmids which were found to have the correct ligation products were subsequently excised from the pUC57 vector using EcoRI (37^{o}C for 2 hours) and XhoI (37^{o}C for 1 minute). Due to the presence of a XhoI site inside of the EBNA Clone 315 scFv sequence, the DNA was partially digested with XhoI and then completely digested using EcoRI. This allowed for the isolation of the correct DNA fragment which kept the integrity of the scFv sequence while removing the entire CAR sequence from the pUC57 vector. After insertion into the vector provided to us by the Campana laboratory, the ligation products were then transformed into *E.coli* as above, plated on TYE + Amplicilin (100 µg/ml), colonies were picked and their plasmids sequenced using the reverse primer 788A (5'-CCCTTGAACCTCCTCGTTCGACC-3') (SEQ ID NO: 72) at the MSKCC Sequencing Core Facility. Once the sequences were validated, the DNA was packaged into retrovirus and used to infect NK92MI cells.

Example 13: Retroviral production, DNA packaging, and infection of NK92MI cells

[00154] To produce CAR-containing retrovirus, the following procedure was employed which used a 293T-based retroviral production cell line (GP2). Briefly, 7 µg of CAR DNA was combined with 3.5 µg of PCLAmpho helper construct and 3.5 µg pVSVg in 1 ml of serum-free DMEM. This mixture was then combined with 1 ml serum-free DMEM containing 36 µl of LIPOFECTAMINE 2000 (Invitrogen) and incubated at RT for 20 min. Afterwards, the DNA-LIPOFECTAMINE complex (2 ml) was mixed with GP2 cells (3-5 x 10^{6}) in 10 ml of DMEM + 10% FBS and cultured at

37°C for 72 hours. Subsequently, the supernatant (12 ml) was depleted of GP2 cells during recovery and incubated with 3 ml LENTI-X Concentrator solution (Clontech) at 4°C for 12-16 hours. Afterwards, the solution was centrifuged at 3000 rpm for 15 min., the supernatant discarded, and the pellet dissolved in 1 ml complete Alpha Essential medium containing 5 x 10^5 NK92MI cells. The cells were then incubated for 72 hours and checked by flow cytometry for CAR expression via GFP (the CAR gene is expressed under a CMV promoter which is followed by IRES-GFP).

Example14: Construction of scFv-Fc fusion protein and expression in DG44 CHO cells

[00155] Using a proprietary antibody expression vector similar to that of pFUSE—hIgG1-Fc1 (Invivogen; San Diego, California), the Clone 315 scFv sequence was first PCR amplified to contain the required NheI and ApaI restriction sites. The resulting PCR product and expression plasmid were digested using the above enzymes (NheI at 37°C for 2 hours and ApaI at 25°C for 2 hours) and ligated together. The ligation products were then transformed into *E.coli*, plated on TYE + Amplicilin (100 µg/ml), colonies were picked and their plasmids sequenced at the MSKCC Sequencing Core Facility. Once the sequences were validated to have the Clone 315 scFv sequence upstream of the human IgG_1 CH2 and CH3 domains, the DNA was electroporated (Amaxa NUCLEAOFACTOR; Lonza, Switzerland) into 5 x 10^6 DG44 Chinese Hamster Ovary (CHO) Cells (Invitrogen) using Program U-030 and 100 µl Solution V. The cells were then cultured in OPTICHO media (Invitrogen) containing G418 (500 µg/ml; added 7 days post-electroporation) at a cell density of 1-5 x 10^6 DG44 per ml of media.

Example 15: Retroviral production, DNA packaging, and infection of NK92MI cells

[00156] To produce CAR-containing retrovirus, the following procedure was employed which used a 293T-based retroviral production cell line (GP2). Briefly, 7 µg of CAR DNA was combined with 3.5 µg of PCLAmpho helper construct and 3.5 µg

pVSVg in 1 ml of serum-free DMEM. This mixture was then combined with 1 ml serum-free DMEM containing 36 µl of LIPOFECTAMINE 2000 (Invitrogen) and incubated at RT for 20 min. Afterwards, the DNA-LIPOFECTAMINE complex (2 ml) was mixed with GP2 cells (3-5 x 10^6) in 10 ml of DMEM + 10% FBS and cultured at 37°C for 72 hours. Subsequently, the supernatant (12 ml) was depleted of GP2 cells during recovery and incubated with 3 ml LENTI-X Concentrator solution (Clontech) at 4°C for 12-16 hours. Afterwards, the solution was centrifuged at 3000 rpm for 15 min., the supernatant discarded, and the pellet dissolved in 1 ml complete Alpha Essential medium containing 5 x 10^5 NK92MI cells. The cells were then incubated for 72 hours and checked by flow cytometry for CAR expression via GFP (the CAR gene is expressed under a CMV promoter which is followed by IRES-GFP).

Example 16: ^{51}Cr Release Cytotoxicity Assay

[00157] The capacity of CAR equipped NK92MI cells to lyse BLCLs was evaluated using a ^{51}Chromium release assay. Briefly, peptide pulsed or unpulsed ^{51}Cr-labeled BLCLs were plated in round-bottom 96-well plates (5 X 10^3 cells/well) in RPMI 1640 with 10% FBS. Subsequently, CAR equipped NK92MI cells were added to the BLCL containing wells at different effector (E)/target (T) ratios and incubated for 4 hours at 37°C, after which the cultures were depleted of cells and ^{51}Cr-release was measured in the supernatants. All E:T ratios were done in triplicate, with the average plotted on the graphs. % ^{51}Cr Release was determined using the following formula: ((Sample Release – Spontaneous Release) / (Total Release – Spontaneous Release)) X 100.

Example 17: Affinity selection of phage on virally-derived recombinant HLA-A2-peptide complexes

[00158] Biotinylated and non-biotinylated recombinant HLA-A2-peptide complexes presenting various different peptides previously shown to bind to HLA-A2 were obtained from the MSKCC Tetramer Core Facility. For selection purposes, the Tomlinson I and J phage display libraries were combined and first preincubated with

non-biotinylated, irrelevant HLA-A2-YVDPVITSI (SEQ ID NO: 75) complex along with streptavidin paramagnetic beads so that any phage which expresses an antibody that may bind to the general framework of HLA-A2, or the streptavidin beads themselves, are eventually discarded during the washing steps. Subsequently, the contents (phage and irrelevant complex) were incubated with biotinylated HLA-A2-LLDFVRFMGV (SEQ ID NO: 4) (EBNA3C) and biotinylated HLA-A2-NLVPMVATV (SEQ ID NO: 73) (pp65) simultaneously in equimolar ratios and captured using streptavidin paramagnetic beads. Once the beads were bound to the biotinylated complexes, the beads were washed with PBS containing TWEEN 20 and the bound phage were eluted from the beads using trypsin. After two additional rounds of selection, the recovered phage were used to infect HB2151 *E. coli* and plated on ampicillin-containing agar. The next morning, individual colonies were picked, cultured overnight in 48-well culture plates, and their supernatants tested for the presence of scFv on 96-well ELISA plates pre-coated with recombinant HLA-A2-peptide complexes.

[00159] The first three rounds of selection resulted in a 55-fold increase in phage recovery, based on output/input ratio, and scFvs which only bound to the HLA-A2-EBNA3C complex. Phage display selection results on recombinant HLA-A2-LLDFVRFMGV (SEQ ID NO: 4) and HLA-A2-NLVPMVATV (SEQ ID NO: 73) complexes are shown in Table 9.

Table 9

	Round 1*	Round 2*	Round 3*	Round 4**
Input	7.5×10^{12}	4.9×10^{12}	2.4×10^{12}	1.8×10^{12}
Output	4.7×10^{6}	6×10^{6}	8.4×10^{7}	1.25×10^{8}
Output/Input	6.3×10^{-7}	1.22×10^{-6}	3.5×10^{-5}	6.9×10^{-5}
Fold Enrichment (From Rd 1)	——	2	55.5	109.5
HLA-A2-EBNA3C Peptide-Specific Clones***	——	——	40/48 (83%)	37/48 (77%)
HLA-A2-pp65 Peptide-Specific Clones***	——	——	0/48 (0%)	0/48 (0%)

* Rd 1-3: Panning against Biotinylated-HLA-A2-pp65 Peptide + Biotinylated-HLA-A2-EBNA Peptide

** Rd 4: Panning against Biotinylated HLA-A2-EBNA3C Peptide Only

*** Relative signal at least 2-fold greater than background.

[00160] These results were somewhat surprising since both of the peptides on HLA-A2 were derived from viral-related proteins, which are not seen in the human protein repertoire. To confirm these findings, an additional round of selection was undertaken on just the HLA-A2-EBNA3C complex alone which resulted in a further amplification of recovered phage (109-fold) and a similar percentage of clones which bound to the HLA-A2-EBNA3C complex (83% positive after Round 3 and 77% after Round 4).

[00161] Bacterial supernatant from individual clones after 3 rounds of phage selection were tested for binding to recombinant, biotinylated-HLA-A2-peptide complexes on vinyl microtiter plates. While several clones resulted in cross-reactivity to more than just the targeted HLA-A2-LLDFVRFMGV (SEQ ID NO: 4) complex

(Clones 335 and 345), Clones 315 and 327 were found to have the desired specificity.

[00162] Purified EBNA Clone 315 scFv was retested against a similar panel of recombinant, biotinylated HLA-A2-peptide complexes. Purified EBNA Clone 315 scFv maintained its specificity over a panel of HLA-A2-peptide complex in addition to its inability to bind to the native peptide by itself. The anti-HLA-A2 antibody BB7.2 was included to demonstrate that all HLA-A2-peptide complexes are adherent and presented properly on the plate.

[00163] During the screening processes, therefore, several different scFv were found to bind to the targeted HLA-A2-EBNA3C complex, however only a few scFv sequences resulted in absolute specificity and did not bind to HLA-A2-peptide complexes of different origins (Figure 1A). Of those clones which were tested, EBNA Clones 315 and 327 had the same peptide sequence and were further characterized. After scFv purification, a subsequent validation ELISA demonstrates that EBNA Clone 315 maintained its specificity towards the targeted HLA-A2-EBNA complex, in addition to failing to bind to the LLDFVRFMGV (SEQ ID NO: 4) peptide by itself (Figure 1B). These initial binding assays demonstrate the TCR-like binding ability of this antibody.

Example 18: Affinity selection of phage on WT1-derived recombinant HLA-A2-peptide complex

[00164] Antibody selection using phage against biotinylated HLA-A2-RMFPNAPYL (SEQ ID NO: 1) (WT1-derived) was done in a similar manner to that which was described above. Briefly, the Tomlinson I and J phage display libraries were first combined and preincubated with non-biotinylated, irrelevant HLA-A2-NLVPMVATV (SEQ ID NO: 73) complex and streptavidin paramagnetic beads. Subsequently, the contents (phage and irrelevant complex) were incubated with biotinylated HLA-A2-RMFPNAPYL (SEQ ID NO: 1) and captured using fresh streptavidin paramagnetic

beads. Once bound to the biotinylated complex, the beads were washed with PBS containing TWEEN 20 and the bound phage were eluted from the beads using trypsin. After two additional rounds of selection, the recovered phage were used to infect HB2151 *E. coli* and plated on ampicillin-containing agar. The next morning, individual colonies were picked, cultured overnight in 48-well culture plates, and their supernatants tested for the presence of scFv on 96-well ELISA plates pre-coated with recombinant HLA-A2-peptide complexes.

[00165] The first three rounds of selection resulted in a 90-fold enrichment in phage when comparing the output/input ratios. Phage display selection results on recombinant HLA-A2-RMFPNAPYL (SEQ ID NO: 1) complex are shown in Table 10.

Table 10

	Round 1*	Round 2**	Round 3**
Input	3.7×10^{12}	5.6×10^{11}	1.55×10^{11}
Output	4.0×10^{6}	3.2×10^{6}	1.52×10^{7}
Output/Input	1.08×10^{-6}	5.7×10^{-6}	9.8×10^{-5}
Fold Enrichment (From Rd 1)	—	5.3	90.7
HLA-A2-RMFPNAPYL-Specific Clones***	—	—	3/48

* Rd 1: Panning against 5 µg Complex.

** Rd 2-3: Panning against 2.5 µg Complex.

*** Relative signal at least 3-fold greater than background (Irrelevant HLA-A2-Complex).

[00166] Bacterial supernatant from three individual clones after three rounds of phage selection were tested for binding to recombinant, biotinylated-HLA-A2-peptide complexes on vinyl microtiter plates. All three clones which were tested had the necessary specificity to only recognize the HLA-A2-RMFPNAPYL (SEQ ID NO: 1) complex. It was discovered that all three clones had the same DNA sequence. Purified WT1 Clone 45 scFv was retested against a similar panel of recombinant, biotinylated HLA-A2-peptide complexes. Purified WT1 Clone 45 scFv maintained its specificity over a panel of HLA-A2-peptide complex in addition to its inability to bind to the native peptide outside of the context of MHC. The anti-HLA-A2 antibody BB7.2 was included to demonstrate that all HLA-A2-peptide complexes are adherent and presented properly on the plate.

[00167] After screening 48 clones for binding to the specific HLA-A2-RMFPNAPYL complex, therefore, three clones were found to bind specifically to their targeted complex but failed to bind to complexes which displayed an irrelevant peptide (Figure 2A). Of the clones which were tested, all of them were found to have the same peptide sequence and WT1 Clone 45 was chosen for further characterization. After scFv purification, a subsequent validation ELISA demonstrates that WT1 Clone 45 maintained its specificity towards the targeted HLA-A2-WT1 complex, in addition to failing to bind to the RMFPNAPYL (SEQ ID NO: 1) peptide by itself (Figure 2B). These initial binding assays demonstrate the TCR-like binding ability of this antibody.

Example 19: Binding and specificity studies with purified EBNA Clone 315 and WT1 Clone 45 scFvs on peptide-pulsed T2 cells

[00168] To demonstrate that the isolated EBNA Clone 315 and WT1 Clone 45 scFvs are able to recognize and bind to their native complexes on the surface of peptide-pulsed antigen presenting cells (APCs), the TAP-deficient T2 cell line was used. T2 cells were first incubated for 5 hours at 37^{o}C with either LLDFVRFMGV (SEQ ID NO: 4) (EBNA3C-derived), RMFPNAPYL (SEQ ID NO: 1) (WT1-derived) or irrelevant peptide KLQCVDLHV (SEQ ID NO: 74) in serum-free medium containing β_2M. The

cells were subsequently washed and stained with the purified WT1 Clone 45, EBNA Clone 315 or an irrelevant scFv. In addition, peptide pulsed and unpulsed T2 cells were also incubated with an anti-HLA-A2-FITC (BB7.2) antibody. This BB7.2 staining control was included due to previous studies which demonstrate that if a peptide is able to bind HLA-A2 on the T2 cell surface, the HLA-A2 molecule is stabilized, and the stabilization can be visualized by an increase in fluorescence intensity (81). As shown in Figure 3A, T2 cells which have been pulsed with either the LLDFVRFMGV (SEQ ID NO: 4) or KLQCVDLHV (SEQ ID NO: 74) peptides resulted in a fluorescence shift, consistent with their binding to the HLA-A2 pocket. However, EBNA Clone 315 was only able to stain T2 cells pulsed with its specific target peptide LLDFVRFMGV (SEQ ID NO: 4) and not an irrelevant peptide (Figure 3B). Similar results were obtained when T2 cells were pulsed with either the RMFPNAPYL (SEQ ID NO: 1) or LLDFVRFMGV (SEQ ID NO: 4) peptides and stained with WT1 Clone 45 scFv. While both peptides were able to stabilize the HLA-A2 molecule (Fig. 4A), WT1 Clone 45 scFv was only able to detect the T2 cells pulsed with the RMFPNAPYL (SEQ ID NO: 1) peptide (Fig. 4B). This further validates their utility in detecting the native complex on the surface of cells.

[00169] Next, the detection sensitivity of the EBNA Clone 315 scFv using flow cytometry was evaluated in order to correlate sensitivity with antigen density using flow cytometric quantitative beads. Briefly, TAP-deficient T2 cells were pulsed with (solid, unfilled lines) or without (dashed, unfilled lines) LLDFVRFMGV (SEQ ID NO: 4) (Figure 3A, top, left panel) or KLQCVDLHV (SEQ ID NO: 74) peptides (Figure 3A top, right panel) at 20 μM in serum-free IMDM media at 37^{o}C for 5 hours. The cells were then stained with a mouse-anti-human HLA-A2-FITC conjugated antibody (unfilled lines) or a control mouse IgG_1-FITC conjugated antibody (filled lines) and analyzed on the FACS machine. Peptide-pulsed T2 cells from *A* were stained with EBNA Clone 315 scFv (unfilled lines) or a control scFv (filled lines) (Figure 3B). Only T2 cells which had been pulsed with the LLDFVRFMGV (SEQ ID NO: 4) peptide (left panel), but not ones which had been pulsed with KLQCVDLHV (SEQ ID NO: 74)

(right panel), could be stained by the EBNA Clone 315 scFv (Figure 3B). T2 cells were incubated with decreasing concentrations of the LLDFVRFMGV (SEQ ID NO: 4) peptide and subsequently stained with EBNA Clone 315 scFv as above (Figure 3C). Based on geometric mean fluorescence (control scFv background subtracted), the lower limit of detection corresponds with 78 nM of peptide used for pulsing.

[00170] By titrating down the amount of peptide used for incubation with the T2 cells, it was determined that concentrations as low as 78 nM were still able to produce a fluorescence signal above background when stained with EBNA Clone 315 scFv (Figure 3C). With decreasing concentrations of peptide used for loading, there was a corresponding reduction in overall HLA-A2 intensity (data not shown) as one would expect.

[00171] Similarly, Figure 4 shows that WT1 Clone 45 can recognize HLA-A2-RMFPNAPYL (SEQ ID NO: 1) on peptide-pulsed T2 cells. TAP-deficient T2 cells were pulsed with (solid, unfilled lines) or without (dashed, unfilled lines) RMFPNAPYL (SEQ ID NO: 1) (left panel) or LLDFVRFMGV (SEQ ID NO: 4) peptides (right panel) at 40 μM in serum-free IMDM media at 37^{o}C for 5 hours. The cells were then stained with a mouse-anti-human HLA-A2-FITC conjugated antibody (unfilled lines) or a control mouse IgG1-FITC conjugated antibody (filled lines) and analyzed on the FACS machine (Figure 4A). Peptide-pulsed T2 cells from *A* were stained with WT1 Clone 45 (unfilled lines) or a control scFv (filled lines). Only T2 cells which had been pulsed with the RMFPNAPYL (SEQ ID NO: 1) peptide (left panel), but not ones which had been pulsed with LLDFVRFMGV (SEQ ID NO: 4) (right panel), could be stained by the WT1 Clone 45 (Figure 4B).

Example 20: Demonstrating HLA restriction of the LLDFVRFMGV (SEQ ID NO: 4) peptide and EBNA Clone 315 using peptide-pulsed BLCLs

[00172] The expression of these peptides on BLCLs, especially since BLCLs are used routinely as APCs (82), was examined. Two BLCL lines were used, one HLA-$A2^+$ (DIMT) and one HLA-$A2^-$ (6268A) (Fig. 5A). The BLCLs were incubated in serum-free IMDM media for 5 hours at 37°C with either the specific LLDFVRFMGV (SEQ ID NO: 4) or irrelevant KLQCVDLHV(SEQ ID NO: 74) peptides. When incubated with the specific peptide, only the HLA-$A2^+$ DIMT BLCL could be stained by EBNA Clone 315 (Fig. 5B). Similarly to results seen with T2 cells, DIMT cells loaded with the irrelevant peptide, or 6268A loaded with the specific/irrelevant peptide, could not be stained with EBNA Clone 315. It is interesting to note that without peptide pulsing we were unsuccessful at staining DIMT. While our staining approach has been optimized to detect low levels of antigen through signal amplification involving secondary and tertiary reagents to detect the scFv, the amount of peptide that the cell naturally presents seems to be below our level of detection.

[00173] Subsequently, in an attempt to study the duration of peptide presentation on HLA-A2, a pulse-chase experiment was set up to monitor the levels of the HLA-A2-EBNA3C complex on DIMT cells over time. Initially, DIMT cells were incubated in serum-free IMDM media for 5 hours at 37°C with the LLDFVRFMGV (SEQ ID NO: 4) peptide. Afterwards, the cells were washed twice with RPMI + 10% FBS and further cultured in this media for an additional 5 hours and 24 hours. At each of these three time points, cells were harvested and stained with either the purified EBNA Clone 315 scFv or an irrelevant scFv. The results show that after pulsing the HLA-A2-EBNA3C complex could easily be detected on the cell surface (Fig. 5C). Interestingly, even after the cells were transferred to fresh media and cultured for an additional 5 and 24 hours, the MHC-peptide complex could still be detected, signifying that peptide-pulsed BLCLs are able to hold onto and present antigen for at least a day after the peptide had been removed from the media. This data further supports the use of autologous BLCLs in the generation of antigen specific T cells and the utility of TCE-specific antibodies like EBNA Clone 315 in precise visualization of TCE expression on APCs or target cells.

Example 21: Construction of EBNA Clone 315 and WT1 Clone 45 scFv-Fc fusion proteins

[00174] Initially, the scFv sequences were made compatible for cloning into a scFv-Fc expression vector by using PCR to add the desired restriction enzyme sites (NheI and ApaI) to either side of the EBNA Clone 315 and WT1 Clone 45 scFv sequences. The PCR reaction was done on the Tomlinson library vector which contained the WT1 Clone 45 and EBNA Clone 315 scFv sequences (Fig. 7A). After subsequent digestion using NheI and ApaI, the digested PCR products were removed from a 1% agarose gel and purified (Fig. 6B).

[00175] With regards to cloning and expression of the scFv-Fc fusion proteins, a proprietary vector obtained from Eureka therapeutics(IgG Vector) was used. The first constant heavy chain (CH_1) was removed from this vector, something which is typically done when generating Fc fusion proteins (83). Once generated, the vector was digested with NheI and ApaI and then ligated to the predigested PCR products from Figure 7B. The ligated products yielded a vector which expressed the EBNA Clone 315 or WT1 Clone 45 scFv genes in tandem to the $CH_{2,3}$ domains of a human IgG1 under a single CMV promoter (scFv-Fc Vector; Figure 7B). After further validation using DNA sequencing, the two fusion constructs were linearized using HindIII and ran on a 1% agarose gel. Digestion with HindIII also allowed us to block the expression of the light chain that is still present in the vector, which for all intensive purposes was undesired. As expected, both digested plasmids ran at the anticipated size (~11,000 bp) based on their location relative to the lambda HindIII marker. Each linearized plasmid was subsequently introduced into DG44 cells and cultured in OPTICHO media as described in Example 10 above.

Example 22: *Binding kinetics and sensitivity of EBNA Clone 315 scFv-Fc on recombinant HLA-A2-peptide complex and peptide-pulsed T2 cells*

[00176] To further understand the affinity of the interaction between EBNA Clone 315 and the HLA-A2-EBNA3C complex, surface plasmon resonance was used to determine the binding kinetics between these two proteins. First, the EBNA Clone 315 scFv-Fc was purified and its binding ability was tested using ELISA (Fig. 8A) along with flow cytometry via peptide-pulsed T2 cells at varying concentrations (Fig. 8B and C). These initial studies demonstrate that the antibody maintains its binding characteristics when expressed as a fusion protein In addition, it is important to note that the flow cytometric sensitivity of the scFv and scFv-Fc were very comparable (200nM – 20nM), further highlighting the utility of the scFv as a monomeric binding fragment.

[00177] Next, using the BIACORE T100 (GE Healthcare), a CM5 chip (flow cells 1 and 2) was initially activated for amine coupling based on manufacturer recommendation. The purified EBNA Clone 315 scFv-Fc was subsequently immobilized onto the second flow cell and the purified HLA-A2-EBNA3C complex passed over both flow cells as part of the soluble phase. After background subtraction (signal from flow cell 2 minus that of flow cell 1), the association rate (k_{on}) and dissociation rate (k_{off}) were determined (2.361×10^5 $M^{-1}s^{-1}$and 6.891×10^{-2} s^{-1}, respectively), resulting in an overall K_D (k_{off}/k_{on}) of 291 nM using a 1:1 binding model (Fig. 9); these kinetic rates were very similar to previously isolated Fabs against different MHC-peptide complexes (22, 31). Relative to published TCR:MHC Class I-peptide K_D measurements, which typically range in the neighborhood of 2-50 μM (84), our scFv:MHC Class I-peptide interaction seems to be at best 150-fold stronger, with the most significant improvement attributed to a slower k_{off}. Previous studies which support an affinity-based T cell activation model argue that a greater overall affinity or slower dissociation rate leads to higher interferon-gamma release and target cell lysis (85, 86).

[00178] Lastly, in an attempt to quantify the amount of HLA-A2-LLDFVRFMGV (SEQ ID NO: 4) complex on the surface of peptide-pulsed T2 cells, we decided to use flow

cytometric quantitation beads. This data will also be useful in determining the detection threshold of EBNA Clone 315 scFv and scFv-Fc. First, purified EBNA Clone 315 scFv-Fc was conjugated to a fluorescent label, ALEXA FLUOR 647 using a commercially available kit. Subsequently, T2 cells were pulsed with 20, 10, 5, or 0 μM of LLDFVRFMGV (SEQ ID NO: 4) peptide at 37ºC for 5 hours. After pulsing, the peptide-pulsed T2 cells, along with beads containing known quantities of anti-human IgG_1 antibodies, were incubated with the fluorescently-labeled EBNA Clone 315 scFv-Fc. Once the cells and beads were analyzed on the FACS machine, the fluorescence intensities were correlated to each other, resulting in an estimation of the number of complexes on the surface of the T2 cells relative to the quantity of peptide used for pulsing. These four values (337,091 sites with 20 μM, 149,688 sites with 10 μM, 76,040 sites with 5 μM, and no sites with 0 μM) were plotted on a graph and a trendline was used to create a standard curve ($R^2 = 0.9948$) (Fig. 10A). Furthermore, when looking at the lower end of the spectrum, we have determined that an amount less than 40 nM of peptide will correspond to less than 100 complexes on the surface of the cell (Fig. 10B), placing the detection level of the EBNA Clone 315 scFv-Fc fusion within that range.

Example 23: Binding and specificity studies of WT1 Clone 45 scFv-Fc on recombinant HLA-A2-peptide complex and peptide-pulsed cells

[00179] In order to do further studies regarding the presentation of the RMFPNAPYL (SEQ ID NO: 1) peptide on the surface of APCs, the WT1 Clone 45 scFv-Fc fusion protein was first purified and validated for binding to its targeted recombinant HLA-A2-peptide complex (Fig. 11A). As was shown with the scFv, the fusion protein maintained its binding ability on the ELISA plate. Next, we decided to check and see if the scFv-Fc maintained its binding ability and specificity on peptide-pulsed T2 cells. T2 cells were pulsed with the RMFPNAPYL (SEQ ID NO: 1) peptide or an irrelevant peptide in serum-free media with β_2M at 37ºC for 5 hours. Using flow cytometry, the scFv-Fc was able to detect T2 cells which had been pulsed with the RMFPNAPYL

(SEQ ID NO: 1) peptide, but failed to recognize the cells pulsed with an irrelevant peptide (Fig. 11B). These two assays further validated that the fusion protein acts in the same way as the original scFv.

[00180] Subsequently, we decided to test whether the binding of the RMFPNAPYL (SEQ ID NO: 1) peptide and scFv-Fc fusion protein were restricted to HLA-A2. HLA-$A2^+$ and HLA-$A2^-$ BLCLs (DIMT and 6268A, respectively) were pulsed with the RMFPNAPYL (SEQ ID NO: 1) peptide in serum-free media at 37°C for 5 hours. Similarly to EBNA Clone 315, the WT1 Clone 45 scFv-Fc fusion protein was only able to recognize the peptide pulsed DIMT and not the HLA-$A2^-$ 6268A BLCL (Fig. 12). These results demonstrate that the RMFPNAPYL (SEQ ID NO: 1) peptide is restricted to HLA-A2 and WT1 Clone 45 is only able to recognize it in the context of this complex.

Example 24: Antibody-dependent cellular cytotoxicity (ADCC) of EBNA Clone 315 scFv-Fc on peptide-pulsed cells

[00181] In addition to using the scFv-Fc for antigen presentation studies, we tested whether the truncated human IgG_1 Fc region is capable of inducing antibody-dependent cellular cytotoxicity (ADCC). In order to avoid variability amongst human donor lymphocytes, and in an effort to increase the chances of observing cytotoxicity, Hong-fen Guo in our laboratory generated a CD16(V)-transduced NK92MI cell line. This NK92 cell variant is transduced with both IL-2 and the human CD16 activating Fc receptor (FcγRIIIA) containing a high affinity polymorphism (valine instead of phenylalanine at position 158 on CD16) responsible for an enhancement in ADCC and clinical response to antibody-based immunotherapy (87, 88).

[00182] We used this cell line in combination with the EBNA Clone 315 scFv-Fc or an irrelevant, isotype-matched scFv-Fc to test whether the fusion protein can induce NK92MI-mediated ADCC against LLDFVRFMGV(SEQ ID NO: 4) -pulsed LUY (HLA-$A2^+$) BLCL. At an E:T ratio of 42:1, EBNA Clone 315 scFv-Fc led to greater killing

over background (with or without an irrelevant scFv-Fc) at the two highest concentrations tested (27-32% versus 13-15%) (Fig. 13). A similar magnitude of killing (over background) was also observed with other peptide-pulsed, HLA-$A2^+$ target BLCLs (DIMT and JG19). These results show that these truncated scFv-Fc fusion proteins maintain their Fc-mediated effector functions, despite being about 33% smaller than a full immunoglobulin.

Example 25: Construction and retroviral transduction of an HLA-A2-RMFPNAPYL(SEQ ID NO: 1) -specific chimeric antigen receptor into NK92MI cells

[00183] In order to generate a CAR specific for the HLA-A2-RMFPNAPYL (SEQ ID NO: 1) complex, the WT1 Clone 45 scFv would typically be fused to intracellular signaling domains of immune-modulatory proteins found in immune effector cells. A CAR expression vector (St. Jude CAR) in which a CD19-specific scFv is fused to the CD8α hinge/transmembrane region, 4-1BB and CD3ζ chain was obtained and modified so that the anti-CD19 scFv was replaced with a WT1 Clone 45 scFv. However, due to restriction enzyme incompatibility issues between the St. Jude CAR vector and the Tomlinson library vector used for PCR, the entire CAR gene, containing the WT1 Clone 45 scFv, was commercially synthesized by Genescript. The resulting WT1 pUC57 vector contained the desired WT1 Clone 45 CAR sequence flanked by EcoRI and XhoI.

[00184] An additional feature to the St. Jude CAR vector is an IRES-GFP sequence downstream of the CAR sequence. This allows for direct correlation of CAR expression with GFP without having to fuse both proteins together. In order to take advantage of this feature, we digested the WT1 pUC57 vector and St. Jude CAR vector using EcoRI and XhoI. Afterwards, the digested and undigested plasmids were run on a 1% agarose gel along with the lambda HindIII and 100 bp markers. The highlighted bands corresponded to the anticipated sizes of the St. Jude plasmid lacking the CAR sequence (~6500 bp) and the WT1 Clone 45 CAR sequence lacking the pUC57 plasmid (~1500 bp). These bands were excised from the gel, and after

DNA purification, the two were ligated together. After the ligation products were transformed into *E.coli*, 8 colonies were selected at random and their plasmids isolated. The isolated plasmids were then validated by sequencing to determine whether they contain the WT1 Clone 45 scFv sequence in the context of the CAR. In addition, the plasmids were also digested with EcoRI and XhoI and run on a 1% agarose gel along with lambda HindIII and 100 bp markers to validate their sizes. After demonstrating that both bands from each plasmid yielded the expected sizes, it was determined that the cloning was successful.

[00185] Once the WT1 Clone 45 CAR was generated (Fig. 23), the DNA was packaged into retrovirus using the 293T-based GP2 cell line. Once the retrovirus was generated in the culture media, it was recovered and concentrated. The concentrated virus was then used to infect 500,000 to 1,000,000 NK92MI cells in NK92MI growth media. After 3-4 days of culture, the NK92MI cells infected with the retrovirus were compared to mock-infected cells (infected with empty retrovirus) with regards to GFP expression using flow cytometry. While the infection efficiency was approximately 27.5%, flow assisted cytometric cell sorting (FACS) allowed us to enrich the GFP-positive population to more than 98% positive (Fig. 23).

Example 26: Construction and retroviral transduction of an HLA-A2-LLDFVRFMGV (SEQ ID NO: 4) -specific chimeric antigen receptor into NK92MI cells

[00186] Since the WT1 Clone 45 CAR required us to purchase the WT1 pUC57 vector, additional restriction sites were added to this construct for greater ease when swapping different scFvs. As a result, the EBNA Clone 315 scFv sequence could directly be cloned out of the Tomlinson vector from which it was derived.

[00187] The first cloning step involved the removal of the WT1 Clone 45 scFv from the WT1 pUC57 vector using SfiI and NotI. The same digestion was done to the Tomlinson vector containing the EBNA Clone 315 scFv sequence. Once digested, both plasmids were run on a 1% agarose gel along with lambda HindIII and 100 bp

markers. The highlighted bands corresponded to the WT1 pUC57 vector without a scFv, and the EBNA Clone 315 scFv excised from the Tomlinson vector. These bands were excised from the gel, the DNA was purified, and ligated together to yield the EBNA pUC57 vector. The ligation products were subsequently transformed into *E.coli*, 10 colonies were selected at random, their plasmids were purified, and each DNA digested with EcoRI alone or EcoRI and XhoI. As anticipated, due to an inherent XhoI site within every scFv sequence derived from the Tomlison vector (with the exception of WT1 Clone 45 scFv in the context of pUC57 since the site was removed when purchased as a CAR from Genescript), the double digestion yielded three separate bands.

[00188] For the second cloning step, in which the EBNA Clone 315 CAR sequence was excised from the pUC57 vector and added to the St. Jude CAR vector, a partial digestion of the EBNA pUC57 vector using XhoI was necessary. The EBNA pUC57 plasmids isolated from the 10 colonies above were combined and digested with XhoI at room temperature for 1 minute. The reaction was quickly stopped by adding it to 4 separate wells of a 1% agarose gel and running the DNA along with uncut plasmid, lambda HindIII and 100 bp markers. The highlighted bands were determined to be the expected size of the linearized EBNA pUC57 plasmid (~4300 bp); this linearized plasmid is a result of a random cut at either of the two XhoI sites. Subsequently, to obtain the complete CAR sequence (~1500 bp), the linearized plasmid was isolated from the gel and digested completely with EcoRI. The resulting double and single digests were run on a 1% agarose gel along with the lambda HindIII and 100 bp markers. The highlighted band corresponded to the anticipated size of the EBNA Clone 315 CAR gene, and as a result was excised from the gel, DNA purified, and ligated to the predigested (EcoRI and XhoI) St. Jude CAR vector. After the ligation products were transformed into *E.coli*, 10 colonies were selected at random and their plasmids isolated. The isolated plasmids were then validated by sequencing to determine whether they contain the EBNA Clone 315 scFv sequence in the context of the CAR. In addition, the plasmids were also digested with EcoRI

and run on a 1% agarose gel along with the lambda HindIII marker to validate their sizes. After demonstrating that the bands yielded the expected sizes, it was determined that the cloning was successful.

[00189] After the EBNA Clone 315 CAR was generated (Fig. 14), the DNA was packaged into retrovirus and used to infect NK92MI cells in the same way as with the WT1 Clone 45 CAR. After 3-4 days of culture, the GFP expression level of the infected NK92MI cells were compared to mock-infected cells. The initial infection efficiency was approximately 24%, and after flow assisted cytometric cell sorting (FACS), the GFP-positive population was enriched to more than 90% positive (Fig. 14).

Example 27: EBNA Clone 315 CAR-equipped NK92MI cells can detect cells bearing the specific HLA-A2-LLDFVRFMGV (SEQ ID NO: 4) complex via CD107a expression

[00190] Once the NK92MI cells were enriched for EBNA Clone 315 CAR expression, they were tested for their ability to recognize the targeted HLA-A2-EBNA3C complex. As an initial readout of NK92MI activation by target cells, we assayed for cell surface expression of CD107a, a marker of NK cell and T cell degranulation (90, 91). T2 cells were loaded with 20 μM of the targeted peptide (LLDFVRFMGV (SEQ ID NO: 4)), an irrelevant peptide (YMFPNAPYL (SEQ ID NO: 76)) or no peptide. Using a 1:1 E:T ratio, the T2 cells were cocultured with EBNA Clone 315 CAR-expressing NK92MI cells in the presence of an anti-CD107a-PE conjugated antibody at 37^{o}C for 5 hours. As shown in Fig. 16A, NK92MI cells equipped with the EBNA Clone 315 CAR did not react to unpulsed T2 cells or T2 cells pulsed with the irrelevant peptide, showing CD107a levels comparable to those of NK92MI cells cultured in the absence of targets. On the other hand, when the CAR-equipped NK92MI cells were cocultured with T2 cells that had been pulsed with the LLDFVRFMGV (SEQ ID NO: 4) peptide, 27% of GFP^{+} cells expressed CD107a above background levels. These results show that after scFv engineering, the CAR is able to maintain its specificity towards the targeted HLA-A2-peptide complex.

[00191] Next, in order to get a quantitative measurement of how sensitive this CAR is at activating NK92MI cells, we titrated down the LLDFVRFMGV (SEQ ID NO: 4) peptide concentration used to pulse T2 cells and measured their ability to activate the CAR-equipped NK92MI cells. As can be seen in Fig. 16B, the lower limit of response by the CAR-equipped NK92MI was at a peptide concentration of 10 nM, with a clear dose response curve beginning at the 600 nM concentration. Based on our earlier quantitation studies, this peptide concentration corresponds to approximately 25 complexes on the cell surface. Compared to the levels necessary for epitope detection using the EBNA Clone 315 scFv or scFv-Fc (200-20 nM), the CAR seems to be a more sensitive approach at detecting low levels of MHC-peptide complex on the surface of APCs using flow cytometric analysis.

[00192] While T2 cells can present any peptide of interest, BLCLs naturally present their own peptides on their MHC Class I molecules. Similarly to T2, these endogenous peptides can be replaced by simple incubation with a substitute peptide of high enough affinity. Using a 1:1 E:T ratio, HLA-$A2^+$ (DIMT) and HLA-$A2^-$ (6268A) BLCLs were pulsed with serum-free IMDM medium or medium containing the LLDFVRFMGV (SEQ ID NO: 4) , cocultured with EBNA Clone 315 CAR-expressing NK92MI cells as discussed above, and assayed for CD107a expression using flow cytometry. Peptide pulsed DIMT (HLA-$A2^+$) induced 25% of GFP^+ NK92MI cells to express CD107a (Fig. 17), in contrast to 0.54% for peptide pulsed 6268A (HLA-$A2^-$) and 1.09% for unpulsed DIMT. This data further demonstrates both peptide specificity and HLA-A2 exclusivity of the EBNA Clone 315 CAR.

Example 28: EBNA Clone 315 CAR-equipped NK92MI cells can destroy cells bearing the specific HLA-A2-LLDFVRFMGV (SEQ ID NO: 4) complex via ^{51}Cr release

[00193] While CD107a expression on NK cells and T cells reflect their activation, target cell lysis can also be measured using a conventional ^{51}Cr cytotoxicity assay. First, to get an idea of how sensitive the ^{51}Cr cytotoxicity assay is with regards to killing HLA-A2-EBNA3C expressing targets, T2 cells were pulsed with decreasing

concentrations of the LLDFVRFMGV (SEQ ID NO: 4) peptide at 37ºC for 3 hours and subsequently labeled with ^{51}Cr as described in the Materials and Methods. The labeled T2 cells were then cocultured with EBNA Clone 315 CAR-expressing NK92MI cells at 37ºC for 2 hours at a 3:1 E:T ratio. Similar to the results seen in the CD107a assay (Fig. 16B), EBNA Clone 315 CAR expressing NK92MI cells were able kill T2 cells in a peptide-dependent manner, with 13.2% of 2 nM peptide-pulsed T2 cells being killed compared to 10.1% with unpulsed T2 cells (Fig. 18). Relative to that which can be detected using flow cytometric antibody staining, the level of sensitivity is in the order of 10-100 fold greater in favor of the CAR using two separate assays (CD107a and ^{51}Cr).

[00194] Next, DIMT and 6268A BLCLs pulsed with the LLDFVRFMGV (SEQ ID NO: 4) peptide (20 μM) in serum-free IMDM (Fig 19A and B) were used as targets in the ^{51}Cr release assay. Similar to the results from the CD107a assay (Fig. 17), only the HLA-$A2^+$ DIMT BLCL were lysed by the CAR-equipped NK92MI cells (Fig. 19A), which could be blocked using the purified EBNA Clone 315 scFv-Fc (Fig. 19B). In addition, the ability to block cytotoxicity was not restricted to the scFv-Fc protein since a commercial anti-HLA-A2 (BB7.2) antibody also possessed blocking ability (data not shown). This blocking data recapitulates results seen with other MHC-restricted, peptide-specific antibodies on antigen-specific cytolytic T cells.

[00195] Although the lytic potential of the CAR-equipped NK92MI cells was clearly evident when targets were artificially pulsed with the relevant peptide, cytotoxicity against naturally processed HLA-A2-peptide complexes is of clinical relevance. Here, CAR-equipped NK92MI cells were tested against a panel of HLA-$A2^+$ (DIMT and JG19) and HLA-$A2^-$ (6268A and GKO) unpulsed BLCLs. While the level of cytoxicity was low, 23.0% for DIMT and 8.9% for JG19 (30:1 E:T ratio), this was highly significant when compared to 3.6% for GKO and 1.8% for 6268A (Fig. 20A). In addition, when the cytotoxicity assay was performed in the presence of EBNA Clone 315 scFv-Fc, the killing capacity could be reduced by approximately 46% when

compared to that with an irrelevant scFv-Fc or in the absence of antibody (Fig 20B). These findings demonstrate the utility and specificity of TCR-like CARs in reprogramming effector immune cells to engage antigen whose expression is below the detection limit using conventional flow cytometry.

[00196] Lastly, since both the EBNA Clone 315 CAR and scFv-Fc fusion protein have the same variable sequences used for detecting the HLA-A2-LLDFVRFMGV (SEQ ID NO: 4) complex, we decided to directly compare CAR-mediated cytotoxicity with ADCC since both approaches are currently being used independently for the treatment of cancer patients. First, the DIMT BLCL was pulsed with the LLDFVRFMGV (SEQ ID NO: 4) peptide at 20 μM in serum-free IMDM media at 37°C for 2 hours. The pulsed BLCL was then labeled with ^{51}Cr and cocultured with either EBNA Clone 315 CAR or CD16(V)-expressing NK92MI cells along with EBNA Clone 315 scFv-Fc or an irrelevant scFv-Fc at an E:T ratio of 15:1 for 3 hours at 37°C. At a EBNA Clone 315 scFv-Fc concentration of 0.5 μg/ml, CD16(V) NK92MI cells were able to kill about 30-35% of cells, compared to 10-15% with an irrelevant scFv-Fc or no antibody at all (Fig. 21). When the ADCC experiment was carried out using higher scFv-Fc concentrations, the cytotoxicity percentage did not change (data not shown). On the other hand, at the same E:T ratio, EBNA Clone 315 CAR-equipped NK92MI cells were able to kill 80-90% of the same peptide-pulsed target cells; and the EBNA Clone 315 scFv-Fc was included as a blocking control (Fig. 22). These results demonstrate that the CAR-mediated killing involving NK92MI cells is a far more potent means of target cell lysis compared to ADCC in our setting.

Example 29: WT1 Clone 45 CAR-equipped NK92MI cells can destroy cells bearing the specific HLA-A2-RMFPNAPYL (SEQ ID NO: 1) complex via ^{51}Cr release

[00197] Along with the EBNA Clone 315 CAR, we decided to test the cytolytic ability of the WT1 Clone 45 CAR in the context of NK92MI cells. First, DIMT and 6268A BLCLs were pulsed with the RMFPNAPYL (SEQ ID NO: 1) peptide (40 μg/ml) in serum-free IMDM at 37°C for 3-5 hours. Subsequently, the target cells were labeled

with ^{51}Cr and cocultured with the CAR-equipped NK92MI cells at 37^{o}C for 4 hours. Of the two peptide-pulsed BLCLs, only the HLA-A2^{+} DIMT could be lysed (~70% versus ~5% with 6268A) at a 10:1 E:T ratio (Fig. 24). In addition, CAR-mediated cytotoxicity could be blocked using a commercial anti-HLA-A2 antibody by approximately 45% (Fig. 25), further demonstrating specificity.

[00198] Next, we decided to tested the cytolytic capacity of WT1 Clone 45 CAR-equipped NK92MI cells against cell lines which might natively express the HLA-A2-RMFPNAPYL (SEQ ID NO: 1) complex. Due to previously published data (92), and conversations with Dr. Richard O'Reilly's laboratory here at MSKCC, researchers have demonstrated that WT1 can be constitutively activated in all BLCLs derived from EBV immortalization. More specifically, O'Reilly's group was able to show WT1 transcript in the DIMT BLCL (data not shown). As a result, we first decided to test WT1 Clone 45 CAR-mediated killing against unpulsed HLA-A2^{+} DIMT and HLA-A2^{-} 6268A BLCL. Similarly to what was seen with the EBNA Clone 315 CAR, WT1 Clone 45 CAR-equipped NK92MI cells were able to kill unpulsed DIMT at a lower capacity than peptide-pulsed DIMT. While the level of cytotoxicity was lower, ~35% for DIMT at a 20:1 E:T ratio, it was far greater when compared to 6268A (~5%) (Fig. 26A). In addition, when the cytotoxicity assay was performed in the presence of the WT1 Clone 45 scFv-Fc, the killing capacity could be reduced by approximately 43% relative to an irrelevant scFv-Fc or in the absence of antibody (Fig 26B). These findings correspond well with what was seen using the EBNA Clone 315 CAR and further demonstrate the utility and specificity of TCR-like CARs in reprogramming effector immune cells to engage antigen.

[00199] Lastly, CAR-mediated cytotoxicity against two cell lines which are HLA-A2-positive and previously shown to express WT1 was tested. OVCAR-3 is a cell line established from malignant ascites of a patient with progressive adenocarcinoma of the ovary (93) and later shown to contain WT1 mRNA (94). In addition, 697 is a human pre-B cell leukemia established from bone marrow cells obtained from a child

with relapsed acute lymphocytic leukemia (ALL) (95). Since then, several groups have shown that this cell line also expresses high levels of both WT1 transcript and protein (96, 97). WT1 Clone 45 CAR-expressing NK92MI cells were cocultured with ^{51}Cr labeled OVCAR-3 and 697 cells at 37^{o}C for 4 hours. CAR-equipped NK92MI cells were able to lyse approximately 20-30% of 697 and OVCAR-3 cells at a 20:1 E:T ratio, which decreased with the number of effector cells used in the assay. This data demonstrates that these two cell types are sensitive to WT1 Clone 45 CAR-equipped NK92MI cells and provides further evidence for their utility in the treatment of HLA-A2^{+}/WT1^{+} malignancies.

EQUIVALENTS

[00200] The foregoing written specification is considered to be sufficient to enable one skilled in the art to practice the invention. The foregoing description and Examples detail certain embodiments of the invention and describes the best mode contemplated by the inventors. It will be appreciated, however, that no matter how detailed the foregoing may appear, the invention may be practiced in many ways and the invention should be construed in accordance with the appended claims and any equivalents thereof.

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CLAIMS:

1. An antigen-binding protein comprising one of:

(A) an antigen binding region having the amino acid sequence of SEQ ID NO: 2;

(B) an antigen binding region comprising a V_H and a V_L respectively, with the amino acid sequences SEQ ID NOs: 22 and 23; or

(C))(i)

(a) a light chain(LC) complementarity determining region 1 (CDR1) comprising the amino acid sequence of SEQ ID NO: 56;

(b) a LC CDR2 comprising the amino acid sequence of SEQ ID NO: 57; and

(c) a LC CDR3 comprising the amino acid sequence of SEQ ID NO: 64; and

(ii) (a) a heavy chain (HC) complementarity determining region 1 (CDR1) comprising the amino acid sequence of SEQ ID NO: 38;

(b) a HC CDR2 comprising the amino acid sequence of SEQ ID NO: 40; and (c) a HC CDR3 comprising the amino acid sequence of SEQ ID NO: 48.

2. The antigen-binding protein of claim 1, wherein the isolated antigen-binding protein is an antibody.

3. The antigen-binding protein of claim 1, wherein the antigen-binding protein is a chimeric antigen receptor (CAR).

4. The antigen-binding protein of claim 2, wherein the antibody is a full-length antibody**,** an intact antibody**,** a Fab fragment, a $F(ab')_2$ fragment or a single chain variable fragment (scFv).

5. The antigen-binding protein of claim 6, wherein the antigen-binding protein specifically binds to an epitope on a human leukocyte antigen (HLA)/peptide complex.

6. The antigen-binding protein of claim 1, wherein the peptide of the HLA /peptide complex has the amino acid sequence RMFPNAPYL (SEQ ID NO:1).

7. The antigen-binding protein of claim 5, wherein the HLA of the HLA/peptide complex is HLA-A2.

8. A fusion protein comprising an antigen-binding protein of claim 1.

9. An immunoconjugate comprising an antigen-binding protein of claim 1 or antigen-binding fragment thereof.

10. The immunoconjugate of claim 9, comprising a second component having a second amino acid sequence.

11. The immunoconjugate according to claim 9, further comprising a cytotoxin.

12. The immunoconjugate of claim 10, wherein the second component is a binding protein or antibody having a binding specificity for a target that is different from the HLA-peptide complex.

13. A bispecific antibody comprising an antigen-binding protein of claim 1.

14. A pharmaceutical composition comprising an antigen-binding protein of claim 1.

15. An isolated single-chain variable fragment (scFv) comprising the amino acid sequence set forth in SEQ ID NO: 2.

16. An scFv comprising a V_H and a V_L linked by an amino acid spacer, wherein the V_H and V_L respectively comprise the amino acid sequence set forth in SEQ ID NO: 22 and 23.