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(71) Applicant: TEXAS TECH UNIVERSITY SYSTEM

[US/US]; Office of Research Commercialization, P.O. Box
42007, Lubbock, TX 79409-2007 (US).

(72) Inventors: LOWE, Devin, B.; 2009 River Oaks Circle,
Abilene, TX 79605 (US). WOOSTER, Amanda, L.; 2701
Nonesuch Road, Apt. 2003, Abilene, TX 79606 (US).

(74) Agent: CHALKER, Daniel, J. et al.; Chalker Flores, LLP,
14951 North Dallas Parkway, Suite 400, Dallas, TX 75254
(US).

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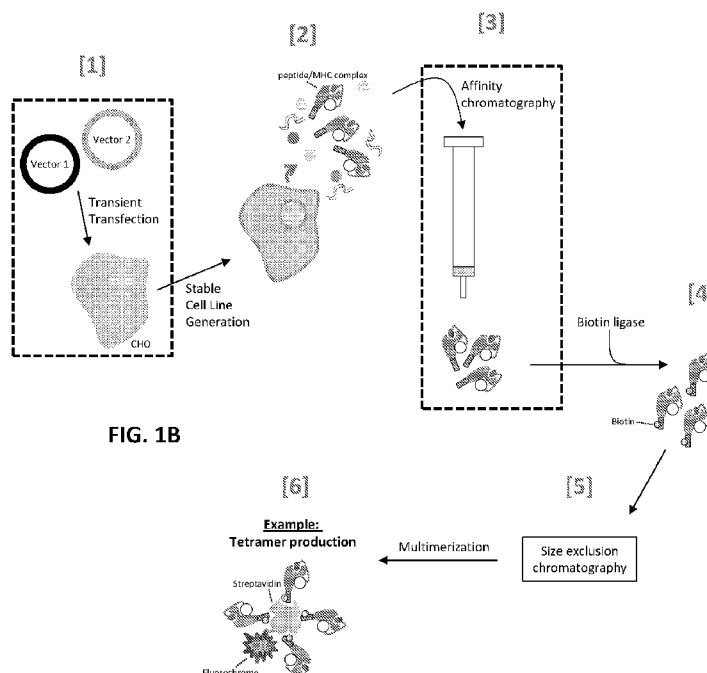


FIG. 1B

(57) Abstract: The present invention includes compositions and methods of making and using a fusion protein comprising a peptide, a first flexible linker, a β 2-microglobulin domain, a second flexible linker, a soluble major histocompatibility complex (MHC) heavy chain and a peptide tag.



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**METHOD TO EXPRESS, PURIFY, AND BIOTINYRATE EUKARYOTIC CELL-
DERIVED MAJOR HISTOCOMPATIBILITY COMPLEXES**

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority to U.S. Provisional Application Serial No. 62/746,198, filed
5 October 16, 2018, the entire contents of which are incorporated herein by reference.

TECHNICAL FIELD OF THE INVENTION

[0002] The present invention relates in general to the field of immunology, and more particularly to novel methods for expressing, purifying, and post-translationally modifying eukaryotic cell-derived major histocompatibility complexes.

10 **STATEMENT OF FEDERALLY FUNDED RESEARCH**

[0003] This invention was made with government support under R15 CA215874 and W81XWH-18-1-0293 awarded by the National Institutes of Health and the Department of Defense, respectively. The government has certain rights in the invention.

INCORPORATION-BY-REFERENCE OF MATERIALS FILED ON COMPACT DISC

15 [0004] The present application includes a Sequence Listing which has been submitted in ASCII format via EFS-Web and is hereby incorporated by reference in its entirety. Said ASCII copy, created on October 14, 2019, is named TECH2131WO_SeqList.txt and is 114, kilo bytes in size.

BACKGROUND OF THE INVENTION

20 [0005] Without limiting the scope of the invention, its background is described in connection with methods of making eukaryotic cell-derived major histocompatibility complexes (MHC).

[0006] The major histocompatibility complex (MHC) class I and II molecules play an integral role in T cell development and peripheral effector responses (Alcover et al., 2018). MHC class I is retained on the plasma membrane of nucleated cells and consists of a multi-unit heavy chain whose tertiary structure is stabilized by $\beta 2$ microglobulin through non-covalent forces (Wieczorek et al.,
25 2017). To provide specific binding to antigen specific CD8+ T cells, MHC class I usually retains a short 8-10 amino acid peptide within the MHC peptide binding groove that is derived from degraded intracellular proteins (hereafter referred to as peptide/MHC).

[0007] The present understanding of basic T cell properties and dynamics under a variety of normal and diseased settings has been greatly advanced by the ability to produce and purify peptide/MHC for use in assays to specifically engage the T cell receptor (TCR). Arguably the most widespread approach incorporates fluorochrome-conjugated peptide/MHC multimers (e.g., tetramers) for analyzing or isolating antigen-specific CD8⁺ T cells from biological samples (Khairmar et al., 2018; Soen et al., 2003). Peptide/MHC generation has continued similarly to the process outlined in the landmark work by Altman and colleagues (Altman et al., 1996). Briefly, β 2 microglobulin and MHC class I heavy chain (containing a BirA tail) are individually expressed in *E. coli* and later purified from inclusion bodies through a laborious lysis/solubilization process. A defined MHC class I peptide is then added alongside β 2 microglobulin and heavy chain in a precise folding reaction mixture that requires several days to complete prior to affinity chromatography (AC) purification of properly folded peptide/MHC and later biotinylation steps. Although this standard production process works to eventually yield excellent reagents for immunologic assays, there exist a number of major disadvantages. Namely, the standard method is [i] time consuming, [ii] requires substantial levels of raw ingredients (particularly purified MHC class I peptide), and [iii] cannot guarantee large-scale production of properly folded peptide/MHC molecules based on predicted peptide binders. For example, it is extremely difficult to stably produce MHC molecules bearing peptides with low-to-moderate affinity to the MHC peptide-binding groove.

[0008] Prior attempts to make peptide/MHC complexes include those of White and colleagues, which designed a soluble HIV-reactive MHC class I molecule (consisting of free heavy chain + linked peptide- β 2 microglobulin) for expression in baculovirus, which was capable of biotinylation/multimerization and identifying a particular T cell hybridoma by flow cytometry (White et al., 1999). An additional approach was by Greten and colleagues performed standard plasmid DNA transfection to produce a peptide- β 2 microglobulin-heavy chain linked protein in J588L cells that was only capable of dimerization due to mouse IgG fusion (Greten et al., 2002).

[0009] Thus a need remains for novel compositions, methods, vectors, cells, etc., that include novel constructs that allow for the production of peptide-MHC complexes in eukaryotic cells.

SUMMARY OF THE INVENTION

[0010] In one embodiment, the present invention includes a fusion protein comprising a peptide, a first flexible linker, a β 2-microglobulin domain, a second flexible linker, a soluble major

histocompatibility complex (MHC) heavy chain and a peptide tag. In one aspect, the peptide is an immunogenic peptide epitope. In another aspect, the MHC is a human, mouse, rat, hamster, horse, pig, cow, simian, avian, or chimeric MHC. In another aspect, the MHC is Class I MHC. In another aspect, the does not include a transmembrane sequence. In another aspect, the peptide tag is selected from wherein the peptide tag is selected from at least one of: a BirA tail, a myc, a FLAG, a glutathione-S-transferase, a His tag, a maltose binding protein, hemagglutinin (HA)-tag, a V5 tag, a T7 tag, a V9 tag, a NusA-tag, a thioredoxin-tag, or a fluorescent protein-tag, a Her2/neu-tag, a CD20-tag, or a GFP-tag. In another aspect, the first, the second, or both the first and second flexible linkers comprise glycine, serine or both glycine and serine and comprise 5 to 40 residues, but may be 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, or 39. In another aspect, the peptide is 8 to 16 residues long. In another aspect, the peptide is selected from at least one of: ABL1; ACPP; Ad5 E1A; AdV 5 Hexon; AdV Hexon; Ag85A; alfa fetoprotein; ASP-2; BA46; BALF4; BAP31; BCL-2; BCL-2A1; BCL-2L1; BCL-X; BCR-ABL; bcr-abl 210 kD fusion protein; Beta-gal; BGLAP; BMI1; BMLF1; BMRF1; BNP; BRAF 27; BRLF1; bZIP factor; BZLF1; C1orf59; CAMEL; Carbonic anhydrase; CB9L2; CD105; CD33; CD59; CEA; CEACAM; Chondromodulin; circadian clock protein PASD1; CP; CPS; cyclin-dependent kinase 4; CYP190; Cytochrome p450; DEP DC1; DLK1; Dutpase; E6; E7; EBNA 1; EBNA 3A; EBNA 3B; EBNA 6; EBV BRLF1; EBV EBNA3A; EBV LMP2; EDDR1; EGFR; EMNA 3A; EMV-1; Enhanced Green Fluorescent Protein; Env; EphA2; Erk1; ESAT-6; EZH2; FAP α ; FLT1; FOLR1; FOXM1; G250; GAD65; Gag; Gag-pol; Glypican 3; gp; gp100; gp33 (C9M); GPC3; H250; HA-1; HA-2; HA-8; HBB; HBsAg; HBV core; HBV polymerase; HBV surface antigen; HCMV IE1; HCMV pp65; HCV core; HCV E; HCV NS3; HCV NS4b; HCV NS5a; HCV NS5B; Heparanase; HER2; HER-2/neu; Histocompatibility antigen 60; HIV gag p24; HIV nef; HIV pol; HIV-1 env gp120; HIV-1 IIIB gp120; HIV-1 nef; HIV-1 p17; HIV-1 RT; HIV-1 US4 gp120; HJURP; HLA-A leader sequence peptide; HLA-A2; HLA-Cw3; chlamydia trachomatis MOMP ; HM1.24; HMMR; HMOX1; HO-1; hPSA; HPV 16 E6; HSP105; HSP90 alpha; hTERT; hTOM34p; hTRT; H-Y; IA-2; IAPP; IDO; IE-1; IE62; IGF; IGRP; IL13r; IL13Ra; Ilr1; Influenza A (PR8) NP ; Influenza A MP; Influenza A MP1; Influenza A MP2; Influenza A NP; Influenza A PB1; INF γ R; Insulin; Interferon gamma inducible protein (GILT) 30; IRS-2; ITGB8; K8.1; KIF20A; KLK; KLK3; L1; LANA; Large T antigen; Lengsin; Listeria monocytogenes Listeriolysin; LIVIN; LMP-1; LMP-2; LMP-2A; LMP-2A; LY6K; m139; m141; m145; M164; M2-

1; M38; M45; M57; MAGE-1; MAGE-10; MAGE-3; MAGE-4; MAGE-A1; MAGEA2; MAGEA3; MAGE-A5; MAGE-C1; MAGE-C2; MART-1; MC38 adpgk neoantigen; MC-38; MCL-1; MCMV IE1; MELK; Mena; Middle T antigen; Midkine; miHA_g SMCY; MOG precursor; MP; MPP11; MS4A1; MSLN; Mtb 16 kDa; Mtb 19 kDa; Mtb85A; mTERT; Mucin; muFAP α ; MuLV env ;
5 Murine Survivin; MYBPC-2; Mycobacterium bovis antigen 85-A; Mycobacterium tuberculosis ESAT-6; Mycobacterium tuberculosis TB10.4; Myelin basic protein; ND; NEF; NEF; Neu/Her-2/ErbB2 proto-oncoprotein; NG2; Non muscle Myosin-9; Non-muscle Myosin; NP; NP396; NP52; NPM1; NRP-1; NRP-2; NRP-V7 superagonist peptide 8.3 Tg NOD mouse; NS3; Nucleocapsid; Nuf2; NY-ESO-1; OVA; Ovalbumin (subdominant); P1A; P2X5a; p53; p56; P79; PAP-3; PASD1;
10 PAX-5; p-Cadherin; PDGFRbeta; PLAC1; PLAM csp; Plasmodium berghei ANKA acid phosphatase; Plasmodium berghei CSP; Plasmodium CSP; Plasmodium falciparum CSP; Plasmodium falciparum Liver stage antigen; PolA; Polymerase; Polyprotein; pp65; PPE; Pr1; PRAME; Prominin1; PSA; PSCA; PSM P2; PSMA; RGS5; RhoC; RNF43; RSV A strain F protein; RSV NP; RT; S protein; SAA; SART3; Sialidases ; SIV gag; SMCY; Spike GP; STEAP1; Surface
15 IgG (sA20-Ig) of A20; Survivin; Survivin-3a; SV40 T antigen; TACE; TARP; TARP 2M; Tax; TB10.3-4; Telomerase; TEM1; tgd057; TGF β ; TNPO3; topII; TPBG; TPR-protein; TRAG; TRP2; Trypanosoma cruzi ASP-2; Trypanosoma cruzi SP; TTK; Tyrosinase; Tyrosine-3-hydroxylase; Ubiquitin; UL105; UL138; UL44; USP9Y; V131; Vaccinia virus Copenhagen Protein G5 ; Vaccinia virus Host range protein 2 ; VACCL3_100; VEGFR1; VEGFR2; VEGFR2/KDR fragment
20 1; Vif; Vinculin; VP1; Vpu; VSV N; West Nile virus NY-99 polyprotein precursor; West Nile Virus polyprotein; WT1; Yellow Fever Virus 17D polyprotein; or ZnT-8. In another aspect, the MHC is selected from at least one of H-2 Db, H-2 Dd; H-2 Dk; H-2 Kb; H-2 Kd; H-2 Kk; H-2 Ld; HLA-A*0101; HLA-A*0201; HLA-A*0301; HLA-A*1101; HLA-A*2301; HLA-A*2402; HLA-A*2902; HLA-A*6801; HLA-B*0702; HLA-B*0801; HLA-B*1501; HLA-B*2705; HLA-B*3501;
25 HLA-B*4001; HLA-B*5101; or HLA-E*0101. In another aspect, the peptide is at least one of SEQ ID NO:1-599.

[0011] In another embodiment, the present invention includes a nucleic acid that expresses a fusion protein comprising a peptide, a first flexible linker, a β 2-microglobulin domain, a second flexible linker, a soluble major histocompatibility complex (MHC) heavy chain and a peptide tag. In one
30 aspect, the peptide is an immunogenic peptide epitope. In another aspect, the MHC is a human, mouse, rat, hamster, horse, pig, cow, simian, avian, or chimeric MHC. In another aspect, the MHC

is Class I MHC. In another aspect, the MHC does not include a transmembrane sequence. In another aspect, the peptide tag is selected from at least one of: a BirA tail, a myc, a FLAG, a glutathione-S-transferase, a His tag, a maltose binding protein, hemagglutinin (HA)-tag, a V5 tag, a T7 tag, a V9 tag, a NusA-tag, a thioredoxin-tag, or a fluorescent protein-tag, a Her2/neu-tag, a CD20-tag, or a GFP-tag. In another aspect, the first, the second, or both the first and second flexible linkers comprise glycine, serine or both glycine and serine and comprise 5 to 40 residues. In another aspect, the peptide is 8 to 16 residues long. In another aspect, the peptide is selected from at least one of: ABL1; ACPP; Ad5 E1A; AdV 5 Hexon; AdV Hexon; Ag85A; alfa fetoprotein; ASP-2; BA46; BALF4; BAP31; BCL-2; BCL-2A1; BCL-2L1; BCL-X; BCR-ABL; bcr-abl 210 kD fusion protein; Beta-gal; BGLAP; BMI1; BMLF1; BMRF1; BNP; BRAF 27; BRLF1; bZIP factor; BZLF1; C1orf59; CAMEL; Carbonic anhydrase; CB9L2; CD105; CD33; CD59; CEA; CEACAM; Chondromodulin; circadian clock protein PASD1; CP; CPS; cyclin-dependent kinase 4; CYP190; Cytochrome p450; DEP DC1; DLK1; Dufpase; E6; E7; EBNA 1; EBNA 3A; EBNA 3B; EBNA 6; EBV BRLF1; EBV EBNA3A; EBV LMP2; EDDR1; EGFR; EMNA 3A; EMV-1; Enhanced Green Fluorescent Protein; Env; EphA2; Erk1; ESAT-6; EZH2; FAP α ; FLT1; FOLR1; FOXM1; G250; GAD65; Gag; Gag-pol; Glypican 3; gp; gp100; gp33 (C9M); GPC3; H250; HA-1; HA-2; HA-8; HBB; HBsAg; HBV core; HBV polymerase; HBV surface antigen; HCMV IE1; HCMV pp65; HCV core; HCV E; HCV NS3; HCV NS4b; HCV NS5a; HCV NS5B; Heparanase; HER2; HER-2/neu; Histocompatibility antigen 60; HIV gag p24; HIV nef; HIV pol; HIV-1 env gp120; HIV-1 IIIB gp120; HIV-1 nef; HIV-1 p17; HIV-1 RT; HIV-1 US4 gp120; HJURP; HLA-A leader sequence peptide; HLA-A2; HLA-Cw3; chlamydia trachomatis MOMP; HM1.24; HMMR; HMOX1; HO-1; hPSA; HPV 16 E6; HSP105; HSP90 alpha; hTERT; hTOM34p; hTRT; H-Y; IA-2; IAPP; IDO; IE-1; IE62; IGF; IGRP; IL13r; IL13Ra; Ilr1; Influenza A (PR8) NP; Influenza A MP; Influenza A MP1; Influenza A MP2; Influenza A NP; Influenza A PB1; INF γ R; Insulin; Interferon gamma inducible protein (GILT) 30; IRS-2; ITGB8; K8.1; KIF20A; KLK; KLK3; L1; LANA; Large T antigen; Lengsin; Listeria monocytogenes Listeriolysin; LIVIN; LMP-1; LMP-2; LMP-2A; LMP-2A; LY6K; m139; m141; m145; M164; M2-1; M38; M45; M57; MAGE-1; MAGE-10; MAGE-3; MAGE-4; MAGE-A1; MAGEA2; MAGEA3; MAGE-A5; MAGE-C1; MAGE-C2; MART-1; MC38 adpgk neoantigen; MC-38; MCL-1; MCMV IE1; MELK; Mena; Middle T antigen; Midkine; miHAg SMCY; MOG precursor; MP; MPP11; MS4A1; MSLN; Mtb 16 kDa; Mtb 19 kDa; Mtb85A; mTERT; Mucin; muFAP α ; MuLV env; Murine Survivin; MYBPC-2;

Mycobacterium bovis antigen 85-A; Mycobacterium tuberculosis ESAT-6; Mycobacterium tuberculosis TB10.4; Myelin basic protein; ND; NEF; NEF; Neu/Her-2/ErbB2 proto-oncoprotein; NG2; Non muscle Myosin-9; Non-muscle Myosin; NP; NP396; NP52; NPM1; NRP-1; NRP-2; NRP-V7 superagonist peptide 8.3 Tg NOD mouse; NS3; Nucleocapsid; Nuf2; NY-ESO-1; OVA;

5 Ovalbumin (subdominant); P1A; P2X5a; p53; p56; P79; PAP-3; PASD1; PAX-5; p-Cadherin; PDGFRbeta; PLAC1; PLAM csp; Plasmodium berghei ANKA acid phosphatase; Plasmodium berghei CSP; Plasmodium CSP; Plasmodium falciparum CSP; Plasmodium falciparum Liver stage antigen; PolA; Polymerase; Polyprotein; pp65; PPE; Pr1; PRAME; Prominin1; PSA; PSCA; PSM P2; PSMA; RGS5; RhoC; RNF43; RSV A strain F protein; RSV NP; RT; S protein; SAA; SART3;

10 Sialidases; SIV gag; SMCY; Spike GP; STEAP1; Surface IgG (sA20-Ig) of A20; Survivin; Survivin-3a; SV40 T antigen; TACE; TARP; TARP 2M; Tax; TB10.3-4; Telomerase; TEM1; tgd057; TGFβ; TNPO3; topII; TPBG; TPR-protein; TRAG; TRP2; Trypanosoma cruzi ASP-2; Trypanosoma cruzi SP; TTK; Tyrosinase; Tyrosine-3-hydroxylase; Ubiquitin; UL105; UL138; UL44; USP9Y; V131; Vaccinia virus Copenhagen Protein G5; Vaccinia virus Host range protein

15 2; VACCL3_100; VEGFR1; VEGFR2; VEGFR2/KDR fragment 1; Vif; Vinculin; VP1; Vpu; VSV N; West Nile virus NY-99 polyprotein precursor; West Nile Virus polyprotein; WT1; Yellow Fever Virus 17D polyprotein; or ZnT-8. In another aspect, the MHC is selected from at least one of H-2 Db, H-2 Dd; H-2 Dk; H-2 Kb; H-2 Kd; H-2 Kk; H-2 Ld; HLA-A*0101; HLA-A*0201; HLA-A*0301; HLA-A*1101; HLA-A*2301; HLA-A*2402; HLA-A*2902; HLA-A*6801; HLA-

20 B*0702; HLA-B*0801; HLA-B*1501; HLA-B*2705; HLA-B*3501; HLA-B*4001; HLA-B*5101; or HLA-E*0101. In another aspect, the peptide is at least one of SEQ ID NO:1-599.

[0012] In another embodiment, the present invention includes a method of making a soluble eukaryotic-derived peptide/MHC complex comprising: expressing in a cell a fusion protein comprising a peptide, a first flexible linker, a β2-microglobulin domain, a second flexible linker, a

25 soluble major histocompatibility complex (MHC) heavy chain and a peptide tag. In another aspect, the method further comprises isolating the fusion protein from a supernatant. In another aspect, the method further comprises forming dimers, trimers, tetramers, or multimers of the fusion protein by mixing the fusion protein with one or more agents that bind to two or more fusion proteins. In

30 another aspect, the agent is selected from an antibody, a cross-linking agent, a ligase, an avidin, a streptavidin, a Protein A, or a J-chain. In another aspect, the peptide is an immunogenic peptide epitope. In another aspect, the MHC is a human, mouse, rat, hamster, horse, pig, cow, simian, avian

or chimeric MHC. In another aspect, the MHC is Class I MHC. In another aspect, the MHC does not include a transmembrane sequence. In another aspect, the peptide tag is selected from at least one of: a BirA tail, a myc, a FLAG, a glutathione-S-transferase, a His tag, a maltose binding protein, hemagglutinin (HA)-tag, a V5 tag, a T7 tag, a V9 tag, a NusA-tag, a thioredoxin-tag, or a fluorescent protein-tag, a Her2/neu-tag, a CD20-tag, or a GFP-tag. In another aspect, the first, the second, or both the first and second flexible linkers comprise glycine, serine or both glycine and serine and comprise 5 to 40 residues. In another aspect, the peptide is 8 to 16 residues long.

[0013] In another embodiment, the present invention includes a cell line expressing a fusion protein comprising a fusion protein comprising a peptide, a first flexible linker, a β 2-microglobulin domain, a second flexible linker, a soluble major histocompatibility complex (MHC) heavy chain and a peptide tag. In one aspect, the fusion protein is integrated into the genome by co-transfecting a fusion protein expressing vector with a transposase vector that expresses a transposase and wherein the fusion protein expressing vector, the transposase vector, or both further comprise a selectable marker. In one aspect, the peptide is an immunogenic peptide epitope. In another aspect, the MHC is a human, mouse, rat, hamster, horse, pig, cow, simian, avian, or chimeric MHC. In another aspect, the MHC is Class I MHC. In another aspect, the MHC does not include a transmembrane sequence. In another aspect, the peptide tag is selected from at least one of: a BirA tail, a myc, a FLAG, a glutathione-S-transferase, a His tag, a maltose binding protein, hemagglutinin (HA)-tag, a V5 tag, a T7 tag, a V9 tag, a NusA-tag, a thioredoxin-tag, or a fluorescent protein-tag, a Her2/neu-tag, a CD20-tag, or a GFP-tag. In another aspect, the first, the second, or both the first and second flexible linkers comprise glycine, serine or both glycine and serine and comprise 5 to 40 residues. In another aspect, the peptide is 8 to 16 residues long.

BRIEF DESCRIPTION OF THE DRAWINGS

[0014] For a more complete understanding of the features and advantages of the present invention, reference is now made to the detailed description of the invention along with the accompanying figures and in which:

[0015] FIG. 1A shows a representative DNA schematic of the synthetic peptide/MHC complex. The designed peptide/MHC molecule contains distinct peptide, beta-2-microglobulin, and MHC heavy chain regions that are linked via explicit glycine/serine amino acids. The MHC heavy chain lacks a transmembrane domain (designated sHeavy chain), ensuring that properly folded peptide/MHC

protein is secreted from cells into the culture medium. The peptide/MHC construct also contains a terminal BirA tail for enzymatic conjugation of biotin. FIG. 1B shows a standard workflow for expressing, purifying, and biotinylating eukaryotic-derived peptide/MHC molecules. [1] A suitable eukaryotic host cell line (such as CHO cells) is transiently transfected with two vectors that allow for transposon-directed integration of genes of interest. Vector 1 encodes the SB transposase (SB100X) while vector 2 is a transposon-compatible plasmid that encodes the synthetic peptide/MHC complex and puromycin resistance transgenes. [2] A stable cell line secreting peptide/MHC complexes is generated in as little as 2 weeks following antibiotic selection and expansion. [3] Spent culture media is processed through AC (against the sHeavy chain) to selectively purify peptide/MHC complexes. [4] A biotin ligase may then be employed to enzymatically conjugate biotin to the BirA tail of the synthetic peptide/MHC protein. [5] Following an additional polishing step such as size exclusion chromatography and [6] multimerization steps, the peptide/MHC reagent can be incorporated into immunologically-relevant assays that, for example, detect antigen-specific T cells. Abbreviation used: secretory MHC class I heavy chain (sHeavy chain), major histocompatibility complex (MHC)

[0016] FIGS. 2A and 2B show that stable CHO cells lines were established by a SB transposon system to secrete peptide/MHC molecules into culture media. (FIG. 2A) Evidence of extracellular peptide/MHC protein was first determined from cell-free supernatants by SDS-PAGE and coomassie blue staining. (FIG. 2B) Representative chromatogram of small scale AC purification of peptide/MHC-containing media. Cell-free supernatant was passed through an equilibrated agarose bead column containing the MHC class I-reactive antibody M1/42. After washing away unbound material, peptide/MHC protein was eluted, buffer-exchanged, and concentrated. Protein purity was then assessed using SDS-PAGE and coomassie blue staining. Arrow inset indicating peptide/MHC around the predicted molecular weight. Abbreviation used: protein ladder (L), affinity chromatography (AC), preparation (prep)

[0017] FIG. 3A shows Purified peptide/MHC (i.e., SIINFEKL/H-2Kb) identify was confirmed by western blot using monoclonal antibodies reactive to the β 2 microglobulin and BirA tail regions of the design molecule as depicted in FIG. 1A. FIG. 3B shows that peptide/MHC ligand binding was determined through immunoprecipitation and western blot following incubation of SIINFEKL/H-2Kb and a TCR-like antibody specific to this particular peptide/MHC class I complex. AC-purified

peptide/MHC was also incorporated as a positive control. Abbreviations used: protein ladder (L), SIINFEKL epitope + MHC class I (SIINFEKL/H-2Kb), isotype (Iso), positive (Pos), control (ctrl)

[0018] FIGS. 4A to 4C show that the peptide/MHC was biotinylated as detailed in the Materials and Methods and specific streptavidin binding initially confirmed through (FIG. 4A) western blot and (FIG. 4B) ELISA using wells coated with streptavidin. (FIG. 4C) Biotinylated peptide/MHC was also incubated with streptavidin-conjugated 5 μ m beads and washed extensively. Beads were then exposed to isotype, anti-SIINFEKL/H-2Kb, or anti-MHC monoclonal antibodies followed by washing steps and incubation with relevant secondary PE-conjugated antibodies. Specific ligand reactivity was subsequently determined by flow cytometry. Abbreviations used: protein ladder (L), SIINFEKL epitope + MHC class I (SIINFEKL/H-2Kb), biotinylated SIINFEKL/H-2Kb (b-SIINFEKL/H-2Kb), positive (Pos), isotype (Iso), control (ctrl), major histocompatibility complex (MHC), horseradish peroxidase (HRP), streptavidin (SA), primary antibody (1^o Ab)

[0019] FIG. 5 shows that the biotinylated peptide/MHC was incubated with PE-conjugated streptavidin to produce “small-scale” batches of SIINFEKL-reactive tetramers. Purified CD8⁺ T cells harvested from either wild-type or OT-1 mice were incubated with tetramers, washed, and stained with an anti-CD8 FITC antibody. Cells were again washed, fixed, and analyzed by flow cytometry for ligand binding. Abbreviations used: wild-type (WT), streptavidin (SA)

[0020] FIG. 6 shows the determination of the ability to construct and express a membrane-bound OVA-specific peptide/MHC molecule (i.e., SIINFEKL/H-2Kb). 4T1 cells were transiently transfected with two plasmids that allow transposon-directed genomic integration and cultured over a period of two weeks in puromycin-containing culture media (as outlined in FIG. 1B). The remaining “stably engineered” cells were then incubated with either an isotype control antibody or antibody that binds the OVA-specific epitope SIINFEKL within the constraints of MHC class I and assessed for reactivity by flow cytometry. Abbreviations used: SIINFEKL epitope + MHC class I (SIINFEKL/H-2Kb), major histocompatibility complex (MHC)

[0021] FIG. 7 is a DNA coding sequence of the synthetic SIINFEKL/H-2Kb molecule (SEQ ID NO: 600).

DETAILED DESCRIPTION OF THE INVENTION

[0022] While the making and using of various embodiments of the present invention are discussed in detail below, it should be appreciated that the present invention provides many applicable

inventive concepts that can be embodied in a wide variety of specific contexts. The specific embodiments discussed herein are merely illustrative of specific ways to make and use the invention and do not delimit the scope of the invention.

[0023] To facilitate the understanding of this invention, a number of terms are defined below.

5 Terms defined herein have meanings as commonly understood by a person of ordinary skill in the areas relevant to the present invention. Terms such as “a”, “an” and “the” are not intended to refer to only a singular entity, but include the general class of which a specific example may be used for illustration. The terminology herein is used to describe specific embodiments of the invention, but their usage does not limit the invention, except as outlined in the claims.

10 [0024] The present invention includes compositions, vectors, cells, and methods of making MHC class I-specific reagents such as fluorescently-labeled multimers (e.g., tetramers) that can be used to study CD8⁺ T cells under normal and diseased states. However, recombinant MHC class I components (comprising MHC class I heavy chain and β 2 microglobulin) are usually produced in bacteria following a lengthy purification protocol that requires additional non-covalent folding steps
15 with exogenous peptide for complete molecular assembly. The present inventors have developed an alternative and rapid approach to generating soluble and fully-folded MHC class I molecules in eukaryotic cell lines (such as CHO cells) using, e.g., a Sleeping Beauty transposon system. Importantly, this method generates stable cell lines that reliably secrete epitope-defined MHC class I molecules into the tissue media for convenient purification and eventual
20 biotinylation/multimerization. Additionally, MHC class I components are covalently linked, providing the opportunity to produce a diverse set of CD8⁺ T cell-specific reagents bearing peptides with various affinities to MHC class I.

[0025] As used herein, the term “gene” refers to a functional protein, polypeptide or peptide-encoding unit. As will be understood by those in the art, this functional term includes both genomic
25 sequences, cDNA sequences, or fragments or combinations thereof, as well as gene products, including those that may have been altered by the hand of man. Purified genes, nucleic acids, protein and the like are used to refer to these entities when identified and separated from at least one contaminating nucleic acid or protein with which it is ordinarily associated.

[0026] As used herein, the term “vector” is used in reference to nucleic acid molecules that transfer
30 DNA segment(s) from one cell to another. The vector may be further defined as one designed to

propagate specific sequences, or as an expression vector that includes a promoter operatively linked to the specific sequence, or one designed to cause such a promoter to be introduced. The vector may exist in a state independent of the host cell chromosome, or may be integrated into the host cell chromosome

5 [0027] As used herein, the term "host cell" refers to cells that have been engineered to contain nucleic acid segments or altered segments, whether archeal, prokaryotic, or eukaryotic. Thus, engineered, or recombinant cells, are distinguishable from naturally occurring cells that do not contain recombinantly introduced genes through the hand of man.

10 [0028] A nucleic acid is "operably linked" when it is placed into a functional relationship with another nucleic acid sequence. For example, DNA for a presequence or secretory leader is operably linked to DNA for a polypeptide if it is expressed as a preprotein that participates in the secretion of the polypeptide; a promoter or enhancer is operably linked to a coding sequence if it effects the transcription of the sequence; or a ribosome binding site is operably linked to a coding sequence if it is positioned so as to facilitate translation. Generally, "operably linked" refers to DNA sequences
15 being linked are contiguous and, in the case of a secretory leader, contiguous and in same reading frame. Enhancers do not have to be contiguous. Linking is accomplished by ligation at convenient restriction sites. If such sites do not exist, then synthetic oligonucleotide adaptors or linkers are used in accord with conventional practice.

20 [0029] As used herein, the terms "cell" and "cell culture" are used interchangeably and all such designations include progeny. Thus, the words "transformants" and "transformed cells" include the primary subject cell and cultures derived therefrom without regard for the number of transfers. It is also understood that all progeny may not be precisely identical in DNA content, due to deliberate or inadvertent mutations. Mutant progeny that have the same function or biological activity as screened for in the originally transformed cell are included. Different designations are will be clear
25 from the contextually clear.

[0030] As used herein, the term "plasmid" are designated by a lower case p preceded and/or followed by capital letters and/or numbers and refer to self-replicating circular DNA that include an origin of replication, and typically one or more selectable markers. The starting plasmids herein are commercially available, are publicly available on an unrestricted basis, or can be constructed from

such available plasmids in accord with published procedures. In addition, other equivalent plasmids are known in the art and will be apparent to the ordinary artisan.

[0031] As used herein, the term "selectable marker" refers to the use of a gene that encodes an enzymatic activity and which confers the ability to grow in medium lacking what would otherwise be an essential nutrient (e.g., the HIS3 gene in yeast cells); in addition, a selectable marker may confer resistance to an antibiotic or drug upon the cell in which the selectable marker is expressed. A review of the use of selectable markers in mammalian cell lines is provided in Sambrook, J., et al., MOLECULAR CLONING: A LABORATORY MANUAL, 2nd ed., Cold Spring Harbor Laboratory Press, New York (1989) pp.16.9-16.15.

[0032] As used herein the terms "protein", "polypeptide" or "peptide" refer to compounds comprising amino acids joined via peptide bonds and are used interchangeably.

[0033] As used herein, the terms "fusion protein" or "chimeric protein" refer to a hybrid protein, that includes portions of two or more different polypeptides, or fragments thereof, resulting from the expression of a polynucleotide that encodes at least a portion of each of the two polypeptides.

[0034] As used herein, the term "transformation," refers to a process by which exogenous DNA enters and changes a recipient cell. It may occur under natural or artificial conditions using various methods well known in the art. Transformation may rely on any known method for the insertion of foreign nucleic acid sequences into a prokaryotic or eukaryotic host cell. The method is selected based on the host cell being transformed and may include, but is not limited to, viral infection, electroporation, lipofection, and particle bombardment. Such "transformed" cells include stably transformed cells in which the inserted DNA is capable of replication either as an autonomously replicating plasmid or as part of the host chromosome.

[0035] As used herein, the term "transfection" refers to the introduction of foreign DNA into eukaryotic cells. Transfection may be accomplished by a variety of methods known to the art including, e.g., calcium phosphate-DNA co-precipitation, DEAE-dextran-mediated transfection, polybrene-mediated transfection, electroporation, microinjection, liposome fusion, lipofection, protoplast fusion, retroviral infection, and biolistics. Thus, the term "stable transfection" or "stably transfected" refers to the introduction and integration of foreign DNA into the genome of the transfected cell. The term "stable transfectant" refers to a cell that has stably integrated foreign DNA into the genomic DNA. The term also encompasses cells that transiently express the inserted

DNA or RNA for limited periods of time. Thus, the term "transient transfection" or "transiently transfected" refers to the introduction of foreign DNA into a cell where the foreign DNA fails to integrate into the genome of the transfected cell. The foreign DNA persists in the nucleus of the transfected cell for several days. During this time the foreign DNA is subject to the regulatory controls that govern the expression of endogenous genes in the chromosomes. As used herein, the term "transient transfectant" refers to cells that have taken up foreign DNA but have failed to integrate this DNA.

[0036] The present invention can be used to circumvent drawbacks in the prior art, in particular, stabilizing peptide binding to the MHC peptide binding groove. Previous efforts have revealed the ability to engineer and produce peptide/MHC molecules in bacteria by covalently joining the MHC class I peptide, $\beta 2$ microglobulin, and heavy chain with discrete amino acid linkers (designated single-chain trimers [SCTs]) (Yu et al., 2002). For most SCTs reported, these engineered proteins fold correctly and specifically engage CD8⁺ T cells as tetramers (Mitaksov et al., 2007), irrespective of the artificial linker design (Hansen et al., 2009). However, this particular SCT method still utilizes a bacterial expression system and requires substantial purification and refolding efforts.

[0037] The present inventors have developed an alternative method to potentially improve the production of peptide/MHC based on the SCT approach. The present invention has the ability to rapidly generate eukaryotic cell lines that stably express and secrete peptide/MHC into the tissue media for purification and biotinylation. This novel protocol provides a much faster/convenient route to generating properly folded peptide/MHC with minimal user intervention, especially for MHC class I targets with high demand (such as the model OVA epitope SIINFEKL). By using a SCT strategy, it was possible to generate MHC molecules presenting a range of class I peptides (i.e., low-to-high binding affinity), which can be reliably generated. Additionally, these eukaryotic-derived peptide/MHC molecules can be used to recapitulate binding dynamics with TCRs in downstream assays (Schmidt and Lill 2018).

[0038] Mice. Female 6-8-week-old C57BL/6J (stock #000664) and OT-1 (stock #003831) mice were purchased from The Jackson Laboratory (Bar Harbor, Maine, USA) and maintained in micro-isolator cages under sterile conditions. Animals were humanely euthanized and spleens/lymph nodes harvested and combined for Ficoll gradient centrifugation (GE HealthCare, Piscataway, NJ).

The lymphocyte interphase was then subjected to ACK lysis and eventual CD8⁺ T cell purification using MACS bead positive selection as instructed by the manufacturer (Miltenyi Biotec, Cambridge, MA). Purified CD8⁺ T cells were aliquoted in 90% FBS/10% DMSO and stored in liquid nitrogen until use. All mouse procedures were followed in accordance with TTUHSC IACUC-approved protocols.

[0039] Cell lines and culture. FreeStyle™ Chinese Hamster Ovary (CHO-S) (Thermo Fisher Scientific, Waltham, MA) and 4T1 (ATCC, Manassas, VA) cells were utilized for in vitro studies. CHO-S cells were passaged in FreeStyle™ CHO Expression Medium (Thermo Fisher Scientific) according to the manufacturer's recommendations. 4T1 cells are naturally deficient in H-2Kb expression and were grown in RPMI 1640 supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, and 10 mmol/l L-glutamine (all from Thermo Fisher Scientific). All cell lines were maintained in vented flasks at 37 °C with 5% CO₂.

[0040] Cloning strategy and construction of transposon expression vectors. Full-length mouse β2 microglobulin (NCBI Reference Sequence: NM_009735.3) and MHC class I heavy chain (H-2Kb) (NCBI Reference Sequence: NM_001001892.2), relevant sequences incorporated herein by reference, cDNA were synthesized from C57BL/6J splenocytes following TRIzol lysis (Thermo Fisher Scientific) and RT-PCR using oligo(dT) primers (SuperScript IV First-Strand Synthesis Kit; Thermo Fisher Scientific). Secretory MHC class I heavy chain was designed to not include the transmembrane domain. The leader signal, SIINFEKL epitope, and Gly/Ser amino acid linkers were ultimately added by overhang PCR as previously reported (Hansen et al., 2009) using the Phusion High-Fidelity DNA Polymerase (Thermo Scientific Fisher). PCR fragments were gel excised/purified and ligated (LigaFast™ Rapid DNA Ligation System; Promega, Madison, WI) into puc19 (NEB, Ipswich, MA) via SacI/HindIII restriction enzyme sites and fragments pieced together using the unique NheI/BamHI sites of the synthetic peptide/MHC sequence. The BirA AviTag™ amino acid sequence (GLNDIFEAQKIEWHE SEQ ID NO: 600) was subsequently added to the construct's terminus by overlap-extension PCR and cloned into a separate puc19 holding vector. Full-length peptide/MHC was then amplified and cloned into the pSBbi-pur transposon vector (kindly provided by Dr. Eric Kowarz [Addgene plasmid # 60523]) using the SfiI restriction enzyme sites (Kowarz et al., 2015). Plasmid transformations were carried out in chemically-competent NEB-5 alpha E. coli (NEB) using ampicillin selection. All vector constructs were confirmed by restriction enzyme digestion and DNA sequencing.

[0041] Sleeping Beauty (SB) transposon system. Parental cell lines were transiently transfected with transposon-related vectors using Lipofectamine reagent (Thermo Fisher Scientific) as directed by the manufacturer. A plasmid encoding the SB 100X transposase (pCMV[CAT]T7-SB100; designated Vector 1), a gift from Dr. Zsuzsanna Izsvak (Addgene plasmid # 34879), and a transposon plasmid containing the necessary inverted terminal repeats (pSBbi-pur; designated Vector 2) were used. Both vectors were provided concurrently to stably integrate peptide/MHC transgenes into cells. Briefly, 1×10^5 cells were plated in 24-well plates (Corning, Corning NY) and exposed to Vector 1 (12.5 ng)/Vector 2 (488 ng) in a total volume of 500 μ l media as similarly described (Kowarz et al., 2015). Culture media was replenished with 2 ml fresh media after 24 hrs and 48 hrs, whereupon cells were exposed to lethal doses of puromycin (CHO-S - 10 μ g/ml; 4T1 - 5 μ g/ml) (Invivogen, San Diego, CA). Fresh media containing puromycin was provided every 2-3 days as needed. Generally, actively dividing cells were ready for expansion by 2 weeks, with virtually all cell clones expressing peptide/MHC molecules.

[0042] SDS-PAGE and western blot. To remove extraneous tissue media components such as surfactants, CHO cell supernatants were passed onto PD10 columns (GE Healthcare) and concentrated/washed in PBS using a 30 MWCO Amicon centrifugal unit (MilliporeSigma, Burlington, MA). Protein concentration was determined using a BCA protein assay kit (Thermo Scientific Fisher) and sample aliquots stored at -80 °C until use. Protein purity was assessed with SDS-PAGE and coomassie blue staining while protein identity was confirmed through western blotting. Briefly, proteins were resolved on a 4%/12% polyacrylamide gel, transferred to a PVDF membrane (Amersham Hybond, 0.2 μ m; GE Healthcare), and blocked with 5% milk in PBST (0.1% Tween 20 in PBS) for 1 hr at RT. Membranes were then incubated with various primary antibodies (1 μ g/ml) in block solution with rocking at 4 °C overnight. Specific primary reagents included anti-mouse β_2 microglobulin (clone 893803; R&D Systems, Minneapolis, MN) or anti-BirA tail (clone Abc; Avidity, Aurora, CO) antibodies. Blots were then washed extensively with PBST and incubated in block with secondary HRP-conjugated goat antibodies specific to rat IgG (H+L) or mouse IgG (H+L) (Thermo Scientific Fisher) for 1 hr at RT. Washed blots were finally developed with a SignalFire ECL reagent (Cell Signaling, Danvers, MA) and exposed/imaged on a ChemiDoc™ Touch Imaging System (Bio-Rad, Hercules, CA). In separate experiments, blots containing biotinylated protein were probed with a Streptavidin-HRP reagent (Thermo Scientific Fisher) for 1 hr at RT to confirm streptavidin binding potential.

[0043] Immunoprecipitation. A Pierce Classic IP kit (Thermo Scientific Fisher) was used to investigate ligand binding of soluble peptide/MHC protein. Peptide/MHC protein was incubated overnight at 4 °C with 2 µg of the anti-SIINFEKL (clone eBio25-D1.16; Thermo Scientific Fisher) or mouse IgG isotype (clone MOPC-21; MP Biomedicals, Santa Ana, CA) antibodies. The anti-SIINFEKL antibody functions as a TCR-like antibody (Lowe et al., 2017), and binds SIINFEKL/H-2Kb much like SIINFEKL-reactive T cells such as OT-1 CD8⁺ T cells. Following purification of IgG containing complexes, samples were boiled and analyzed by western blot as described above.

[0044] Affinity chromatography (AC). The MHC class I-reactive M1/42 antibody (Bio X Cell, West Lebanon, NH) was covalently bound to an NHS-activated agarose bead column (HiTrap™ NHS-activated HP; GE Healthcare) manually as directed by the manufacturer. The column was attached to an ÄKTA™ start chromatography system (GE Healthcare) for automatic operation and equilibrated with 20 mM sodium phosphate, pH 7.0 (binding buffer). Cell-free supernatants were first desalted in PBS using PD-10 columns and diluted 1:2 in binding buffer prior to AC. Samples were then applied to the M1/42 column, unbound material washed away with binding buffer, and peptide/MHC molecules eluted using 0.1 M glycine, pH 2.7. Eluates were dispensed in tubes containing 1 M Tris, pH 9 and relevant fractions combined and concentrated/washed in PBS as explained above.

[0045] Biotinylation and tetramerization. Peptide/MHC was biotinylated using 2.5 µg BirA ligase (Avidity) overnight at 4 °C according to the manufacturer's guidelines. Reaction components were removed by successive washes in PBS using a 30 MWCO centrifugal filter (MilliporeSigma). Alternatively, larger reaction mixtures could be exclusively polished through size exclusion chromatography (Altman and Davis 2016) using a HiPrep™ 16/60 Sephacryl™ S-200 HR column (GE Healthcare) as indicated in FIG. 1B. In order to produce small-scale tetramer batches, biotinylated peptide/MHC was incubated with PE-conjugated streptavidin (BD Biosciences, San Jose, CA) at a 4:1 molar ratio in the dark for 30 min at RT. Tetramers were then stored at 4 °C shielded from light.

[0046] Enzyme-Linked Immunosorbent Assay (ELISA). To investigate the success of peptide/MHC biotinylation, a 96-well high protein binding plate (Corning) was first coated overnight at 4 °C with 4 µg/ml streptavidin (Promega) in 0.1 M sodium carbonate and then blocked (3% BSA/PBS) for 1 hr at RT. Biotinylated protein samples were diluted in block and added to

wells at various dilutions and incubated at RT for 1 hr. Wells were washed extensively with PBST and then exposed to 4 µg/ml of either a MHC class I-reactive (clone M1/42; Biolegend) or rat isotype control IgG (Biolegend) antibody in block. In separate wells, a positive control biotinylated irrelevant rat antibody (Biolegend) was incorporated to validate the extent of streptavidin binding.

5 The plate was again washed with PBST and relevant wells provided a goat anti-rat HRP (Thermo Scientific Fisher) or goat anti-mouse FcγR-specific HRP (Jackson ImmunoResearch, West Grove, PA) antibody in block for 1 hr at RT. Wells were washed with PBST and developed for 5 min after the addition of 200 µl 1-Step Ultra TMB (Thermo Scientific Fisher). Reactions were terminated with 100 µl TMB stop solution (KPL), and the absorbance immediately read at 450 nm using a
10 Cytation 5 Imaging Reader (Biotek, Winooski, VT).

[0047] Flow cytometry. To confirm gene expression, the 4T1 cell line (1×10^5 cells) was stained with relevant primary antibodies (anti-SIINFEKL/H-2Kb or mouse IgG isotype antibodies at 2 µg/ml) in FACS buffer (0.5% BSA/0.1% NaN₃ in PBS) for 20 min at 4 °C, washed, incubated with a PE-conjugated anti-mouse secondary antibody (Jackson ImmunoResearch), washed again, and
15 resuspended in FACS buffer for analysis (FIG. 6). To ensure proper orientation, biotinylated peptide/MHC was incubated with 5 µm PMMA beads conjugated to streptavidin (PolyAn, Berlin, Germany), washed, and incubated at 4 °C with correct pairs of primary and PE-conjugated secondary antibodies (as indicated in FIGS. 4A to 4C) prior to analysis in FACS buffer. Frozen CD8⁺ T cells were thawed, washed, and resuspended in FACS buffer so that each stain consisted of
20 2×10^5 viable CD8⁺ T cells. Cells were exposed to 50 nM dasatinib (Selleck Chemicals, Houston, TX) for 30 min at 37 °C and promptly incubated with peptide/MHC tetramers for 20 min at RT in the dark (Dolton et al., 2015). Cells were washed with FACS buffer and labeled with an anti-CD8-FITC antibody (clone 53-6.7; BioLegend, San Diego, CA) at 4 °C for 20 min shielded from light. After additional wash steps, cells were resuspended in fix solution (1% FBS, 2.5% formaldehyde in
25 PBS) and assessed by flow cytometry using a BD LSRFortessa. Single and double color stain analysis was carried out using FlowJo software. In the case of tetramer analysis, PE/FITC compensated events were first gated based on FSC/SSC profiles and subsequently evaluated by PE and FITC fluorescence.

[0048] Cloning, expression, and purification strategy for soluble eukaryotic-derived peptide/MHC.

30 The design of linked peptide/MHC class I molecules closely followed the previously reported generation of SCTs in bacteria (Hansen et al., 2009). Essentially, as presented in FIG. 1A, flexible

glycine/serine linkers join a particular peptide epitope, $\beta 2$ microglobulin, and MHC class I heavy chain. A BirA tail was also genetically encoded to the 3' end of the molecule for enzyme-directed biotinylation of secreted protein. Yet, a notable difference of this design involved omitting the transmembrane region of MHC class I (designated sHeavychain) to allow for functional and fully-
5 folded secreted protein. This process for eukaryotic expression centered on the use of a SB transposon system to stably integrate transgene content into relevant cell lines (FIG. 1B). Therefore, CHO cells would be transiently transfected with transposon-relevant plasmids (encoding separately a SB transposase and synthetic peptide/MHC molecule) and stable cells generated through antibiotic selection. After expanding relevant CHO cell clones, secreted peptide/MHC could be purified by
10 AC, biotinylated, and multimerized to produce, for example, tetramers capable of binding antigen-specific T cells.

[0049] Purification of secreted and fully-folded peptide/MHC protein. The suitability of the SB transposon system to stably integrate the peptide/MHC transgene was first determined. However, this particular peptide/MHC construct differed from the aforementioned soluble design in FIG. 1A
15 by encoding full-length MHC class I heavy chain (i.e., the murine MHC haplotype H-2Kb), thereby, ensuring cell surface expression for relative ease of detection. Briefly, 4T1 (H-2Kb null) cells were exposed to transposon vectors that expressed either a null or SIINFEKL/H-2Kb construct and puromycin resistant clones selected and expanded in culture. Cells were then stained with isotype or anti-SIINFEKL/H-2Kb antibodies and analyzed by flow cytometry using a PE-conjugated
20 secondary antibody. In comparison to 4T1-null cells, 4T1-SIINFEKL/H-2Kb cells expressed clear and robust levels of synthetic peptide/MHC (FIG. 7). These confirmatory results were expected, given the widespread use of the SB approach to induce expression of various protein classes in established cell lines and primary cells (Kebriaei et al., 2017).

[0050] CHO cells were next transfected with SB-related vectors as described in FIGS. 1A and 1B
25 to induce stable expression of soluble peptide/MHC. Parental CHO cells exhibited enhanced resistance to puromycin, requiring sustained culturing in high concentrations of puromycin at 10 $\mu\text{g/ml}$. However, the inventors were able to expand puromycin-resistant clones by 2 weeks post plasmid transfection. Cells were then grown to saturating conditions in culture for at least 4 days to generate suitable whole protein concentrations from a total volume of 20 ml media. Yet, the choice
30 of serum-free media contained a number of proprietary agents such as surfactants that could potentially interfere with protein purification and validation assays. The inventors, therefore, took

the precautionary step of desalting and concentrating cell-free supernatants in PBS prior to downstream analysis. The initial assessment of CHO-derived extracellular proteins by SDS-PAGE (under reducing conditions) and coomassie blue staining revealed distinct protein bands around a predicted 51 kDa molecular weight for synthetic peptide/MHC (FIG. 2A) that was not evident from CHO cells expressing a null construct (data not shown). This specific protein band was subsequently confirmed following AC as exhibited in FIG. 2B. That is, cell-free supernatants were passed onto an agarose bead column containing the MHC class I-reactive M1/42 antibody and bound material eluted and ultimately assessed again by SDS-PAGE. The inventors typically harvested at least 100 µg/ml AC-purified peptide/MHC from small-scale culturing efforts. However, the described protocol can be scaled-up (or down) depending on the desired peptide/MHC total yield. Although beyond the scope of this report, alternative culturing parameters and/or leader signals (Haryadi et al., 2015) may potentially enhance overall CHO secretion of peptide/MHC. The AC procedure appeared to provide substantial protein purity based on obtaining [i] one distinct chromatogram elution peak and [ii] a protein band comprising > 95% of detectable protein by coomassie blue staining.

[0051] Determination of peptide/MHC identity and upstream binding potential. Next, the inventors confirmed the identity of AC purified protein by western blot separate from MHC class I reactivity. Based on the linked design of polychain peptide/MHC molecules (FIG. 1A), the inventors incorporated monoclonal antibodies specific to mouse β2 microglobulin and the BirA tail peptide GLNDIFEAQKIEWHE (SEQ ID NO: 600). Developed blots clearly verified the integrity of synthetic peptide/MHC molecule expression, indicating no observable issues in CHO cells secreting these artificial proteins (FIG. 3A). An immunoprecipitation reaction was subsequently attempted in order to demonstrate ligand binding potential of linked SIINF EKL to the MHC class I binding groove. SIINF EKL/H-2Kb was incubated with 2 µg of anti-SIINF EKL/H-2Kb or isotype control antibodies overnight. As detailed in the Materials and Methods, IgG containing material was purified using protein A/G-complexed agarose and resolved by SDS-PAGE under reducing conditions. The presence of SIINF EKL/H-2Kb was further established through western blot using an anti-mouse β2 microglobulin antibody (FIG. 3B). Overall, these results help further validate the identity of soluble peptide/MHC from CHO cells and establish that these secreted proteins retain ligand binding specificity at the peptide/MHC class I interface.

[0052] Multimerization and functional assessment of eukaryotic-derived peptide/MHC. Biotinylated peptide/MHC arguably provides the greatest convenience to users for downstream assays. The inventors, therefore, designed a BirA tail to the terminal end of the peptide/MHC transgene by using a particular BirA tail peptide, GLNDIFEAQKIEWHE (SEQ ID NO: 600), which
5 offers a highly targeted site for enzymatic conjugation of biotin with minimal footprint (Beckett et al., 1999). AC purified peptide/MHC was subjected to BirA ligase activity in the presence of free biotin overnight. Excess biotin and other reaction components were removed by excessive washing in PBS using a 30 MWCO centrifugal unit, and the extent of biotinylation first assessed by western blot and ELISA. In the case of western blot analysis, SDS-PAGE-resolved biotinylated protein was
10 incubated with HRP-conjugated streptavidin to generate a specific peptide/MHC signal (FIG. 4A). Likewise, ELISA determination of biotinylated peptide/MHC clearly confirmed the functionality of the BirA tail region (FIG. 4B). Briefly, wells were coated overnight with streptavidin followed by various concentrations of peptide/MHC and biotinylated peptide/MHC. Detection was finally determined indirectly by MHC class I reactivity using the M1/42 antibody. Biotinylated
15 SIINFEKL/H-2Kb (as low as 0.25 µg/ml protein) was clearly evident in appropriate wells, with negligible reactivity occurring for conditions containing either unbiotinylated SIINFEKL/H-2Kb or biotinylated SIINFEKL/H-2Kb incubated with an isotype control. Next, the ability of immobilized/properly oriented biontinylated peptide/MHC to specifically engage ligands through the MHC class I peptide binding site was confirmed. Streptavidin was covalently attached to
20 PMMA 5 µM beads and incubated with saturating conditions of biotinylated SIINFEKL/H-2Kb. Beads were washed, incubated with appropriate primary reagents (i.e., anti-SIINFEKL/H-2Kb and anti-MHC class I agents), and the extent of MHC ligand binding determined by flow cytometry using secondary PE-conjugated antibodies. Ultimately, as initially validated by the immunoprecipitation shown in FIG. 3B, biotinylated peptide/MHC bound streptavidin and
25 specifically interacted with a TCR-like antibody that recapitulates CD8⁺ T cell interactions (FIG. 4C), indicating no observable functional issues with peptide/MHC post biotinylation and multimerization.

[0053] Next, the inventors assessed the ability of fluorescently-labeled multimers to specifically detect antigen-specific CD8⁺ T cells. Splenocytes and lymph nodes were harvested from wild-type
30 and OT-1 transgenic (i.e., SIINFEKL-reactive) mice and CD8⁺ T cells subsequently purified through magnetic bead selection. Biotinylated SIINFEKL/H-2Kb was then incubated with PE-

conjugated streptavidin following a 4:1 molar ratio, thereby, generating tetramers. In order to stabilize membrane dynamics of TCRs, CD8⁺ T cells were exposed to dasatinib (as previously described [Dolton et al., 2015]), followed by incubation with tetramers. After suitable wash steps, cells were specifically labeled with a FITC-conjugated anti-mouse CD8 antibody that displays minimal interference with TCR:MHC binding (Clement et al., 2011). Cells were fixed and double-positive events (CD8⁺/tetramer⁺) assessed by flow cytometry. As detailed in FIG. 5, CD8⁺ OT-1 cells clearly bound PE-labeled tetramers, with most cells displaying CD8 and tetramer positivity (in comparison to wild-type mice). Altogether, these data show the overarching strategy (as outlined in FIG. 1B) of stably producing eukaryotic-derived peptide/MHC that can be multimerized and used for immunologically-relevant assays such as CD8⁺ T cell identification.

[0054] Although MHC class I peptide candidates can be easily identified through *in silico* prediction methods (Andreatta and Nielsen 2016), free peptide occupancy of MHC class I molecules tends to be a rate limiting step in successfully generating stable peptide/MHC molecules from bacteria (Altman and Davis 2016). Considering the vital role MHC plays in human health (Cho and Sprent 2018), an inability to produce certain peptide/MHC reagents may adversely impact efforts on a number of fronts including diagnostics, therapies, and generalized scientific endeavors. For example, the burgeoning field of neoepitope identification for personalized cancer therapy could be stalled by those unique patient epitopes that exhibit high dissociation rates from MHC class I (Hu et al., 2018). One workaround to the issue of peptide occupancy has been in designing and expressing polychain SCTs that ensure full assembly of peptide, β 2 microglobulin, and MHC class I heavy chain through flexible linkers (Hansen et al., 2009). SCTs retained on the plasma membrane of eukaryotic cells maintain their native conformation and are highly resistant to exogenous peptide binding (Yu et al., 2002). Additionally, membrane-bound SCTs serve as effective targets for CD8⁺ T cell priming (Hung et al., 2007) and destruction (Yu et al., 2002). In the case of diagnostic determination of CD8⁺ T cell frequencies by tetramers, SCTs can be modified for expression/secretion in bacteria and biotinylation by way of an explicit amino acid sequence that directs BirA enzyme function (Mitaksov et al., 2007).

[0055] Previously, the present inventors developed a unique protocol to establish eukaryotic cell lines (such as CHO suspension cells) in as little as two weeks that stably secrete peptide/MHC molecules through transposon-directed delivery. Soluble peptide/MHC may then be biotinylated and utilized as multimers (via streptavidin), particularly for CD8⁺ T cell relevant assays. Currently,

CHO cells are an industry standard in producing FDA approved therapeutic recombinant proteins (Kuo et al., 2018). The inventors sought the advantages of the CHO cell line in order to [i] instigate post-translational modifications and [ii] be easily grown at high density under serum-free conditions in suspension cultures. However, other common cell line “protein workhorses” (e.g., HEK-293 cells) are amenable to the expression and characterization techniques outlined.

[0056] One advantage of the present invention is the rapid development of stable CHO cells secreting peptide/MHC using the SB transposon system. These studies incorporated the SB transposase SB100X, which has a high gene insertion efficiency at close-to-random chromosomal sites (Mátés et al., 2009). The SB approach provides the advantageous properties of viral transduction to insert transgenes without the disadvantages of either maintaining genomic material episomally (e.g., adeno-associated viruses) or near/in proto-oncogenes (e.g., retroviral vectors) (Kebriaei et al., 2017). Additionally, manufacturing high-quality viral particles can be a cumbersome task fraught with regulatory obstacles. The inventors generally experienced minimal difficulty in developing and expanding stable lines from parental cells after transiently transfecting plasmids that propagated the SB transposon system. The protocol is also amenable to freezing material at convenient stopping points. There was no apparent adverse effects to generating multimerized reagents when either cell-free CHO-derived supernatants or biotinylated peptide/MHC was frozen long-term at -80 °C. The method of the present invention is compatible with downstream tetramer production with the added benefits of convenient peptide/MHC expression that can incorporate a range of peptide affinities to the MHC peptide binding groove. Traditionally, to circumvent the low intrinsic affinity of the TCR with peptide/MHC, tetravalent multimers have been utilized to increase T cell avidity. However, these soluble peptide/MHC molecules can be utilized for higher order reagents to better discriminate low frequency T cells (or bind “difficult” TCRs) such as fluorochrome-conjugated dextramers since the production process involves incubating biotinylated peptide/MHC with a dextran backbone containing streptavidin (Dolton et al., 2014). Thus, the present invention can be used to reliably produce a range of soluble eukaryotic-derived peptide/MHC molecules for diagnostic, therapeutic, and investigative purposes.

[0057] Table 1. MHC, Peptide Sequences, and Target.

<u>ALLELE</u>	<u>PEPTIDE</u>	<u>TARGET</u>	<u>SEQ ID NO:</u>
H-2 Db	AAVKNWMTQTL	SIV gag	1

H-2 Db	AGPHNDMEI	p56	2
H-2 Db	AGVDNRECI	L1	3
H-2 Db	AIQGNVTSI	Mycobacterium tuberculosis ESAT-6	4
H-2 Db	AQLANDVVL	MC-38	5
H-2 Db	ASFRNLTHL	TPBG	6
H-2 Db	ASMTNMELM	MC38 adpgk neoantigen	7
H-2 Db	ASNENMDAM	NP	8
H-2 Db	ASNENMETM	NP	9
H-2 Db	ATFKNWPFL	Murine Survivin	10
H-2 Db	CMTWNQMNL	WT1	11
H-2 Db	CSANNSHHYI	gp	12
H-2 Db	FQPQNGQFI	NP396	13
H-2 Db	FSNSTNDILI	VEGFR2/KDR fragment 1	14
H-2 Db	GAVQNEVTL	HCV NS3	15
H-2 Db	HCIRNKSVI	PSA	16
H-2 Db	HCIRNKSVIL	hPSA	17
H-2 Db	HGIRNASFI	M45	18
H-2 Db	KAVYNFATC	gp	19
H-2 Db	KAVYNFATM	gp33 (C9M)	20
H-2 Db	KCSRNRQYL	miHAg SMCY	21
H-2 Db	KVPRNQDWL	gp100	22
H-2 Db	LGMSNRDFL	West Nile Virus polyprotein	23
H-2 Db	RAHYNIVTF	E7	24
H-2 Db	RMFPNAPYL	WT1	25
H-2 Db	RSPFSRVVHL	MOG precursor	26
H-2 Db	SCLENFRAYV	Po1A	27
H-2 Db	SGPSNTPPEI	Ad5 E1A	28
H-2 Db	SGVENPGGYCL	gp	29

H-2 Db	SHLVEALYL	IGF	30
H-2 Db	SQLLNAKYL	Plasmodium berghei ANKA acid phosphatase	31
H-2 Db	SSLENFRAYV	Po1A	32
H-2 Db	VILTNPISM	VEGFR2	33
H-2 Db	WMHHNMDLI	H-Y	34
H-2 Dd	AGPHNDMEI	p56	35
H-2 Dd	AGPPRYSRI	M164	36
H-2 Dd	IGPGRAFYA	HIV-1 US4 gp120	37
H-2 Dd	IPGRAFYA	Env	38
H-2 Dd	LGPISGHVL	pp65	39
H-2 Dd	RGPGRAFVTI	HIV-1 IIIB gp120	40
H-2 Dk	RRLGRTLII	Middle T antigen	41
H-2 Kb	AGLAYYSM	Polyprotein	42
H-2 Kb	ANYNFTLV	Trypanosoma cruzi SP	43
H-2 Kb	ATLTYRML	Yellow Fever Virus 17D polyprotein	44
H-2 Kb	DAPIYTNV	Beta-gal	45
H-2 Kb	EVYDFAFRDL	E6	46
H-2 Kb	FAPGNYPAL	NP	47
H-2 Kb	HILIIYSDV	MYBPC-2	48
H-2 Kb	HNTQYCNL	MAGE-A5	49
H-2 Kb	ICPMYARV	Beta-gal	50
H-2 Kb	ILSPFLPLL	HBV surface antigen	51
H-2 Kb	IMYNYPAM	TB10. 3-4	52
H-2 Kb	IMYNYPAML	Mycobacterium tuberculosis TB10. 4	53
H-2 Kb	ISHNFCNL	gp	54
H-2 Kb	KSPWFCTL	MuLV env	55

H-2 Kb	KVVRFDKL	Ovalbumin (subdominant)	56
H-2 Kb	LTFNYRNL	Histocompatibility antigen 60	57
H-2 Kb	MGLKFRQL	CP	58
H-2 Kb	RGYVYQGL	NP52	59
H-2 Kb	SCLEFWQRV	M57	60
H-2 Kb	SDYYFSWL	muFAP α	61
H-2 Kb	SIINFEKL	OVA	62
H-2 Kb	SIIVFNLL	MC-38	63
H-2 Kb	SIYRYYGL	OVA	64
H-2 Kb	SKYVFENV	MYBPC-2	65
H-2 Kb	SSIEFARL	gp	66
H-2 Kb	STYTFVRT	M38	67
H-2 Kb	SVLAFRRL	tgd057	68
H-2 Kb	SVYDFFVWL	TRP2	69
H-2 Kb	TSINFKI	P79	70
H-2 Kb	TSYKFESV	INF γ R	71
H-2 Kb	TVSEFLKL	Murine Survivin	72
H-2 Kb	TVYGFCLL	m139	73
H-2 Kb	VGRNFTNL	mTERT	74
H-2 Kb	VIDAFSRL	m141	75
H-2 Kb	VIYIFTVRL	VACCL3_100	76
H-2 Kb	VNHRFTLV	Trypanosoma cruzi ASP-2	77
H-2 Kb	VVYDFLKL	SV40 T antigen	78
H-2 Kb	VWLSVIWM	HBsAg	79
H-2 Kd	AMQMLKDTI	HIV gag p24	80
H-2 Kd	AMQMLKETI	HIV-1 gag p24	81
H-2 Kd	AYIDFEMKI	SART3	82
H-2 Kd	CYYASRTKL	m145	83
H-2 Kd	DYWGQGTSL	Surface IgG (sA20-Ig) of A20	84

H-2 Kd	EYILSLEEL	GPC3	85
H-2 Kd	GYETVITQL	PPE	86
H-2 Kd	GYKDGNEYI	Listeria monocytogenes Listeriolysin	87
H-2 Kd	HYLSTQSAL	Enhanced Green Fluorescent Protein (eGFP)	88
H-2 Kd	IYNVGQVSI	Sialidases	89
H-2 Kd	IYSTVASSL	ND	90
H-2 Kd	KYKNAVTEL	RSV A strain F protein	91
H-2 Kd	KYNKANVFL	NRP-V7 superagonist peptide 8.3 Tg NOD mouse	92
H-2 Kd	QYIHSANVL	Erk1	93
H-2 Kd	RYLKNGKETL	HLA-Cw3	94
H-2 Kd	SYIGSINNI	M2-1	95
H-2 Kd	SYIPSAEKI	Plasmodium berghei CSP	96
H-2 Kd	SYMLQALCI	TNP03	97
H-2 Kd	SYVPSAEQI	Plasmodium CSP	98
H-2 Kd	TYLPTNASL	HER2	99
H-2 Kd	TYQRTRALV	Influenza A (PR8) NP	100
H-2 Kd	TYVPANASL	Neu/Her-2/Erbb2 proto- oncoprotein	101
H-2 Kd	TYWPVVSDI	UL105	102
H-2 Kd	VYAGAMSGL	Mtb85A	103
H-2 Kd	YYIPHQSSL	Plasmodium falciparum Liver stage antigen	104
H-2 Kk	DYENDIEKKI	CPS	105
H-2 Kk	FETFEAKI	Tyrosine-3-hydroxylase	106
H-2 Kk	TENSGKDI	SMCY	107
H-2 Kk	TEWETGQI	ASP-2	108

H-2 Kk	VESTAGSL	ESAT-6	109
H-2 Kk	YENDIEKKI	PLAM csp	110
H-2 Ld	HPQKVTKFM	KLK3	111
H-2 Ld	IPQSLDSWWTSL	HBV surface antigen	112
H-2 Ld	LPYLGWLVF	P1A	113
H-2 Ld	MPVGGQSSF	Ag85A	114
H-2 Ld	MPYLIDFGL	VSV N	115
H-2 Ld	RPQASGVYM	NP	116
H-2 Ld	SPGAAGYDL	Dutpase	117
H-2 Ld	SPSYVYHQF	EMV-1	118
H-2 Ld	TPHPARIGL	Beta-gal	119
H-2 Ld	YPHFMPTNL	MCMV IE1	120
HLA-A*0101	ATDALMTGF	HCV NS3	121
HLA-A*0101	ATDALMTGY	Polyprotein	122
HLA-A*0101	CTELKLSDY	NP	123
HLA-A*0101	EADPTGHSY	MAGE-1	124
HLA-A*0101	EVDPIGHL	MAGE-3	125
HLA-A*0101	FTELTLGEF	Survivin	126
HLA-A*0101	IVDCLTEMY	USP9Y	127
HLA-A*0101	KSDICTDEY	Tyrosinase	128
HLA-A*0101	SADNNSEY	VP1	129
HLA-A*0101	SSDYVIPIGT	Tyrosinase	130
HLA-A*0101	TDLGQNLLY	AdV 5 Hexon	131
HLA-A*0101	TLDTLTAFY	MSLN	132
HLA-A*0101	TSEKRPFMCAY	WT1	133
HLA-A*0101	VSDGGPNLY	Influenza A PB1	134
HLA-A*0101	VTEHDTLLY	UL44	135
HLA-A*0101	YSEHPTFTSQY	HCMV pp65	136
HLA-A*0201	AILALLPAL	PSCA	137

HLA-A*0201	AIQDLCLAV	NPM1	138
HLA-A*0201	AIQDLCVAV	NPM1	139
HLA-A*0201	AITEVECFL	VP1	140
HLA-A*0201	ALCNTDSPL	iLR1	141
HLA-A*0201	ALDVYNGLL	ACPP	142
HLA-A*0201	ALFDIESKV	PSM P2	143
HLA-A*0201	ALIAPVHAV	Neg. Control	144
HLA-A*0201	ALISAFSGS	K8. 1	145
HLA-A*0201	ALKDVEERV	MAGE-C2	146
HLA-A*0201	ALLEIASCL	IDO	147
HLA-A*0201	ALLTSRLRFI	Telomerase	148
HLA-A*0201	ALMEQQHYV	ITGB8	149
HLA-A*0201	ALNVYNGLL	ACPP	150
HLA-A*0201	ALPFGFILV	IL13Ra	151
HLA-A*0201	ALPHIIDDEV	ND	152
HLA-A*0201	ALQPGTALL	PSCA	153
HLA-A*0201	ALSPVPPVV	BCL-2	154
HLA-A*0201	ALTPVVVTL	cyclin-dependent kinase 4	155
HLA-A*0201	ALVCYGPGI	FAP α	156
HLA-A*0201	ALVEMGHHA	Vpu	157
HLA-A*0201	ALWALPHAA	IE62	158
HLA-A*0201	ALWGPDAAA	Insulin	159
HLA-A*0201	ALWPWLLMAT	RNF43	160
HLA-A*0201	ALYDVVTKL	Polyprotein	161
HLA-A*0201	ALYLMELTM	CB9L2	162
HLA-A*0201	ALYVDSLFFL	PRAME	163
HLA-A*0201	AMASTEGNV	ESAT-6	164
HLA-A*0201	AMLVLLAEI	LANA	165
HLA-A*0201	AQCQETIRV	Midkine	166

HLA-A*0201	ATGEALWAL	IE62	167
HLA-A*0201	ATWAENIQV	West Nile virus NY-99 polyprotein precursor	168
HLA-A*0201	AVLDGLLSL	bZIP factor	169
HLA-A*0201	CINGVCWTV	NS3	170
HLA-A*0201	CLGGLTMTV	LMP-2A	171
HLA-A*0201	CLPSPSTPV	BMI1	172
HLA-A*0201	CLWCVPQLR	ABL1	173
HLA-A*0201	CVNGVCWTV	Polyprotein	174
HLA-A*0201	DIWDGIPHV	NRP-2	175
HLA-A*0201	DLMGYIPAV	HCV core	176
HLA-A*0201	DLMGYIPLV	HCV core	177
HLA-A*0201	EIWTSHYKV	FOLR1	178
HLA-A*0201	ELAGIGILTV	MART-1	179
HLA-A*0201	ELSDSLGPV	PASD1	180
HLA-A*0201	ELTLGEFLKL	Survivin	181
HLA-A*0201	ELVDGLLSL	bZIP factor	182
HLA-A*0201	FIDSYICQV	H-Y	183
HLA-A*0201	FILGIIITV	VI31	184
HLA-A*0201	FIYDFCIFGV	Lengsin	185
HLA-A*0201	FLAEDALNTV	EDDR1	186
HLA-A*0201	FLAMLKNTV	MAGE-C1	187
HLA-A*0201	FLDKGTYTL	BALF4	188
HLA-A*0201	FLDPRPLTV	CYP190	189
HLA-A*0201	FLFLRNFSL	TARP	190
HLA-A*0201	FLGKIWPS	Gag	191
HLA-A*0201	FLGYLILGV	PAP-3	192
HLA-A*0201	FLLSLFSLWL	ZnT-8	193
HLA-A*0201	FLLSLGIHL	HBV polymerase	194

HLA-A*0201	FLLTRILTI	S protein	195
HLA-A*0201	FLNKCETWV	DLK1	196
HLA-A*0201	FLPSDFFPSI	HBV core	197
HLA-A*0201	FLPSDFFPSV	CP	198
HLA-A*0201	FLPSPLFFFL	TARP 2M	199
HLA-A*0201	FLTPKKLQCV	PSA	200
HLA-A*0201	FLWGPRALV	MAGE-3	201
HLA-A*0201	FLYALALLL	LMP-2A	202
HLA-A*0201	FLYDDNQRV	topII	203
HLA-A*0201	FMNKFIYEI	alfa fetoprotein	204
HLA-A*0201	FVGEFFTDV	Glypican 3	205
HLA-A*0201	GILGFVFTL	MP	206
HLA-A*0201	GILTVSVAV	Mtb 16 kDa	207
HLA-A*0201	GLADQLIHL	Vif	208
HLA-A*0201	GLAPPQHLIRV	p53	209
HLA-A*0201	GLCTLVAML	BMLF1	210
HLA-A*0201	GLFKCGIAV	FAP α	211
HLA-A*0201	GLIQLVEGV	TRAG	212
HLA-A*0201	GLLGASVLGL	Telomerase	213
HLA-A*0201	GLLRFVTAV	NRP-1	214
HLA-A*0201	GLLSLEEL	bZIP factor	215
HLA-A*0201	GLMEEMSAL	Mena	216
HLA-A*0201	GLPVEYLQV	Mycobacterium bovis antigen 85-A	217
HLA-A*0201	GLQDCTMLV	HCV NS5B	218
HLA-A*0201	GLQHWVPEL	BA46	219
HLA-A*0201	GLSPTVWLSV	S protein	220
HLA-A*0201	GLSRYVARL	Polymerase	221
HLA-A*0201	GLYDGMEHL	MAGE-10	222
HLA-A*0201	GMLGMVSGL	NRP-1	223

HLA-A*0201	GVDPNIRTGV	Polyprotein	224
HLA-A*0201	GVLVGVALI	CEA	225
HLA-A*0201	GVRGRVEEI	BCR-ABL	226
HLA-A*0201	GVYDGREHTV	MAGE-4	227
HLA-A*0201	HIAGSLAVV	ZnT-8	228
HLA-A*0201	HLSTAFARV	G250	229
HLA-A*0201	HLVEALYLV	Insulin	230
HLA-A*0201	ILAKFLHWL	Telomerase	231
HLA-A*0201	ILDDNLYKV	Vaccinia virus Copenhagen Protein G5	232
HLA-A*0201	ILGFVFTLTV	MP	233
HLA-A*0201	ILGVLTSLV	DLK1	234
HLA-A*0201	ILHDGAYSL	HER2	235
HLA-A*0201	ILHNGAYSL	HER2	236
HLA-A*0201	ILKDFSILL	ZnT-8	237
HLA-A*0201	ILKEPVHGV	RT	238
HLA-A*0201	ILLWEIFTL	PDGFRbeta	239
HLA-A*0201	ILLWQPIPV	PAP-3	240
HLA-A*0201	ILMWEAVTL	VPI	241
HLA-A*0201	ILSLELMKL	HMMR	242
HLA-A*0201	IMDQVPFSV	gp100	243
HLA-A*0201	ITDQVPFSV	gp100	244
HLA-A*0201	KIFGSLAFL	HER2	245
HLA-A*0201	KLCPVQLWV	p53	246
HLA-A*0201	KLDVGNAEV	BAP31	247
HLA-A*0201	KLFGTSGQKT	EGFR	248
HLA-A*0201	KLGEFYNQMM	BNP	249
HLA-A*0201	KLHLYSHPI	Polymerase	250
HLA-A*0201	KLIANNTRV	Mycobacterium bovis antigen 85-A	251

HLA-A*0201	KLMSNSTDL	HSP105	252
HLA-A*0201	KLPQLCTEL	HPV 16 E6	253
HLA-A*0201	KLQCVDLHV	PSA	254
HLA-A*0201	KLQDASAEV	HM1. 24	255
HLA-A*0201	KLQVFLIVL	IAPP	256
HLA-A*0201	KLSGLGINAV	HCV NS3	257
HLA-A*0201	KLTPLCVTL	HIV-1 env gp120 848-856	258
HLA-A*0201	KLVALGINAV	Polyprotein	259
HLA-A*0201	KMLKEMGEV	RSV NP	260
HLA-A*0201	KTWGQYWQV	gp100	261
HLA-A*0201	KVAEELVHFL	MAGE-A3	262
HLA-A*0201	KVAELVHFL	MAGE-A3	263
HLA-A*0201	KVDDTFYYV	Vaccinia virus Host range protein 2	264
HLA-A*0201	KVLEYVIKV	MAGE-A1	265
HLA-A*0201	KVVEFLAML	MAGE-C1	266
HLA-A*0201	LAALPHSCL	RGS5	267
HLA-A*0201	LIAHNQVRQV	HER2	268
HLA-A*0201	LIDQYLYYL	VP1	269
HLA-A*0201	LILPLL FYL	NG2	270
HLA-A*0201	LLAARAIVAI	I1r1	271
HLA-A*0201	LLDFVRFMGV	EBNA 3B	272
HLA-A*0201	LLDVPTAAV	Interferon gamma inducible protein (GILT) 30	273
HLA-A*0201	LLFGLALIEV	MAGE-C2	274
HLA-A*0201	LLFGYPVYV	Tax	275
HLA-A*0201	LLFNILGGWV	HCV NS4b	276
HLA-A*0201	LLGRNSFEV	p53	277
HLA-A*0201	LLHETDSAV	PSMA	278

HLA-A*0201	LLLASIAAGL	LY6K	279
HLA-A*0201	LLLGPLGPL	Heparanase	280
HLA-A*0201	LLLIWFRPV	Large T antigen	281
HLA-A*0201	LLLLTVLTV	Mucin	282
HLA-A*0201	LLLNCLWSV	Spike GP	283
HLA-A*0201	LLLTVLTVV	Mucin	284
HLA-A*0201	LLMGTLGIVC	E7	285
HLA-A*0201	LLMWEAVTV	VP1	286
HLA-A*0201	LLNGWRWRL	ND	287
HLA-A*0201	LLQERGVAYI	PSMA	288
HLA-A*0201	LLTAALWYV	CD105	289
HLA-A*0201	LLVPTCVFLV	TEM1	290
HLA-A*0201	LLWNGPMAV	Polyprotein	291
HLA-A*0201	LMLGEFLKL	Survivin	292
HLA-A*0201	LMWYELSKI	gp	293
HLA-A*0201	LNIDLLWSV	IGRP	294
HLA-A*0201	LTFGWCFKL	HIV-1 Nef	295
HLA-A*0201	LTLGEFLKL	Survivin-3a	296
HLA-A*0201	LVWMACHSA	Nucleocapsid	297
HLA-A*0201	MLAVFLPIV	STEAP1	298
HLA-A*0201	MLDLQPETT	E7	299
HLA-A*0201	MLMAQEALAFI	CAMEL	300
HLA-A*0201	MLNIPSINV	pp65	301
HLA-A*0201	MMNDQLMFL	PSMA	302
HLA-A*0201	MVWESGCTV	IA-2	303
HLA-A*0201	NLFETPVEA	BA46	304
HLA-A*0201	NLVPMVATV	pp65	305
HLA-A*0201	NVWATHACV	Env	306
HLA-A*0201	PLFDFSWSL	BCL-2	307

HLA-A*0201	QLCPICRAPV	LIVIN	308
HLA-A*0201	QLFEELQEL	HO-1	309
HLA-A*0201	QLFNHTMFI	Non-muscle Myosin	310
HLA-A*0201	QLGEQCWTV	PSCA	311
HLA-A*0201	QLLDGFMITL	PASD1	312
HLA-A*0201	QLLIKAVNL	MPP11	313
HLA-A*0201	QMARLAWEA	LANA	314
HLA-A*0201	QQAHLWCV	ABL1	315
HLA-A*0201	RILGAVAKV	Vinculin	316
HLA-A*0201	RLAEYQAYI	SART3	317
HLA-A*0201	RLDDGDNFQL	West Nile Virus NY-99 polyprotein precursor	318
HLA-A*0201	RLLGNVLVCV	HBB	319
HLA-A*0201	RLLQETELV	HER2	320
HLA-A*0201	RLLVVYPWT	HBB	321
HLA-A*0201	RLMNDMTAV	HSP105	322
HLA-A*0201	RLNMFTPYI	chlamydia trachomatis MOMP	323
HLA-A*0201	RLSSCPVA	TGF β	324
HLA-A*0201	RLTPGVHEL	DLK1	325
HLA-A*0201	RLTSRVKAL	Telomerase	326
HLA-A*0201	RLVDDFLLV	Telomerase	327
HLA-A*0201	RLWQELSDI	circadian clock protein PASD1	328
HLA-A*0201	RMFPNAPYL	WT1	329
HLA-A*0201	RMPEAAPPV	p53	330
HLA-A*0201	RTLDKVLEV	HA-8	331
HLA-A*0201	RTLNAWVKV	Gag	332
HLA-A*0201	RVASPTSGV	IRS-2	333
HLA-A*0201	SIDWFMVTV	PLAC1	334
HLA-A*0201	SILLRDAGLV	TRAG	335

HLA-A*0201	SITEVECFL	VPI	336
HLA-A*0201	SLFEPPPPG	PSMA	337
HLA-A*0201	SLFLGILSV	MS4A1	338
HLA-A*0201	SLFNTVATL	Gag	339
HLA-A*0201	SLFNTVATLY	Gag	340
HLA-A*0201	SLGEQQYSV	WT1	341
HLA-A*0201	SLLFLLFSL	MSLN	342
HLA-A*0201	SLLMWITQC	NY-ESO-1	343
HLA-A*0201	SLLMWITQV	NY-ESO-1	344
HLA-A*0201	SLLNATAIAV	Env	345
HLA-A*0201	SLLQHLIGL	PRAME	346
HLA-A*0201	SLNQTVHSL	ND	347
HLA-A*0201	SLPPPGTRV	p53	348
HLA-A*0201	SLSEKTVLL	CD59	349
HLA-A*0201	SLSRFSWGA	Myelin basic protein	350
HLA-A*0201	SLVDVMPWL	Cytochrome p450	351
HLA-A*0201	SLVKHHMYI	Vif	352
HLA-A*0201	SLYNTVATL	Gag	353
HLA-A*0201	SLYNTVATLY	Gag	354
HLA-A*0201	SLYSFPEPEA	PRAME	355
HLA-A*0201	SMYRVFEVGV	H250	356
HLA-A*0201	SQADALKYV	EZH2	357
HLA-A*0201	STLCQVEPV	MPP11	358
HLA-A*0201	STPPPGTRV	p53	359
HLA-A*0201	TLADFDPRV	EphA2	360
HLA-A*0201	TLDYKPLSV	BMRF1	361
HLA-A*0201	TLFWLLTL	VEGFR1	362
HLA-A*0201	TLFWLLTL	FLT1	363
HLA-A*0201	TLNAVVKVV	Gag	364

HLA-A*0201	TLPGYPPHV	PAX-5	365
HLA-A*0201	TLPPAWQPFL	Survivin	366
HLA-A*0201	TLQDIVYKL	BMI1	367
HLA-A*0201	TLSNLSFPV	NG2	368
HLA-A*0201	TMNGSKSPV	Mena	369
HLA-A*0201	VIFDFLHCI	Large T antigen	370
HLA-A*0201	VIMPCSWWV	Chondromodulin	371
HLA-A*0201	VISNDVCAQV	KLK	372
HLA-A*0201	VIVMLTPLV	IA-2	373
HLA-A*0201	VIYHYVDDL	Po1	374
HLA-A*0201	VLAELVKQI	HCMV IE1	375
HLA-A*0201	VLGGFFLL	PSMA	376
HLA-A*0201	VLDFAPPGA	WT1	377
HLA-A*0201	VLDGLDVLL	PRAME	378
HLA-A*0201	VLEETSVML	IE-1	379
HLA-A*0201	VLFGLGFAI	IGRP	380
HLA-A*0201	VLHDDLLEA	HA-1	381
HLA-A*0201	VLLGAVCGV	NRP-1	382
HLA-A*0201	VLMIKALEL	Non muscle Myosin-9	383
HLA-A*0201	VLPLTVAEV	MSLN	384
HLA-A*0201	VLQELNVTV	Pr1	385
HLA-A*0201	VLQMKEEDV	iLR1	386
HLA-A*0201	VLSDFKTWL	HCV NS5a	387
HLA-A*0201	VLTDGNPPEV	Mtb 19 kDa	388
HLA-A*0201	VLRYGSFSV	gp100	389
HLA-A*0201	VMNILLQYVV	GAD65	390
HLA-A*0201	VVTGVLVYL	ZnT-8	391
HLA-A*0201	WLSLKTLLSL	BCL-2	392
HLA-A*0201	WLSLLVPFV	S protein	393

HLA-A*0201	YAYDGKDYIA	HLA-A2	394
HLA-A*0201	YIGEVLSV	HA-2	395
HLA-A*0201	YLEPGPVT	gp100	396
HLA-A*0201	YLEPGPVT	gp100	397
HLA-A*0201	YLFFYRKSV	mTERT	398
HLA-A*0201	YLGSYGFR	p53	399
HLA-A*0201	YLIELIDRV	TACE	400
HLA-A*0201	YLISGDSPV	CD33	401
HLA-A*0201	YLLEMLWRL	LMP-1	402
HLA-A*0201	YLLPRRGPR	HCV core	403
HLA-A*0201	YLNDHLEPWI	BCL-X	404
HLA-A*0201	YLNKIQNSL	Plasmodium falciparum CSP	405
HLA-A*0201	YLNRLHTWI	BCL-2	406
HLA-A*0201	YLVTVQPTCV	EGFR	407
HLA-A*0201	YLQLVFGIEV	MAGE-A2	408
HLA-A*0201	YLQQNWWTL	LMP-1	409
HLA-A*0201	YLQQNWWTL	LMP-1	410
HLA-A*0201	YLQVDLRFL	NRP-2	411
HLA-A*0201	YLQVNSLQTV	Telomerase	412
HLA-A*0201	YLQWIEFSI	Prominin1	413
HLA-A*0201	YLSCADLNL	CEA	414
HLA-A*0201	YLSGANLNL	CEACAM	415
HLA-A*0201	YLVGNVCIL	PASD1	416
HLA-A*0201	YLVSIPLHL	ND	417
HLA-A*0201	YLYQWLGAPV	BGLAP	418
HLA-A*0201	YMCSFLFNL	EZH2	419
HLA-A*0201	YMDGTMSQV	Tyrosinase	420
HLA-A*0201	YMLDLQPETT	E7	421
HLA-A*0201	YMNGTMSQV	Tyrosinase	422

HLA-A*0201	YTCPLCRAPV	SAA	423
HLA-A*0201	YTMDGEYRL	West Nile virus NY-99 polyprotein precursor	424
HLA-A*0201	YVLDHLIVV	BRLF1	425
HLA-A*0301	AIFQSSMTK	HIV pol	426
HLA-A*0301	ALLAVGATK	gp100	427
HLA-A*0301	ATGFKQSSK	bcr-abl 210 kD fusion protein	428
HLA-A*0301	ILRGSAHK	Influenza A (PR8) NP	429
HLA-A*0301	KLCLRFLSK	E6	430
HLA-A*0301	KLGGALQAK	IE-1	431
HLA-A*0301	KQSSKALQR	bcr-abl 210 kD fusion protein	432
HLA-A*0301	QVLKKIAQK	HMOX1	433
HLA-A*0301	QVPLRPMTYK	NEF	434
HLA-A*0301	RIAAWMATY	BCL-2L1	435
HLA-A*0301	RISTFKNWPK	Survivin-3a	436
HLA-A*0301	RLGLQVRKNK	RhoC	437
HLA-A*0301	RLLFFAPTR	MCL-1	438
HLA-A*0301	RLRAEAQVK	EMNA 3A	439
HLA-A*0301	RLRPGGKKK	Gag	440
HLA-A*0301	RVCEKMALY	HCV NS5B	441
HLA-A*0301	RVRAYTYSK	BRLF1	442
HLA-A*0301	SVLNYERARR	hTERT	443
HLA-A*0301	VTLTHPITK	Polyprotein	444
HLA-A*0301	YMPFIPLYR	Tyrosinase	445
HLA-A*1101	ACQGVGGPGHK	HIV gag p24	446
HLA-A*1101	ATIGTAMYK	EBV BRLF1	447
HLA-A*1101	AVDLSHFLK	NEF	448
HLA-A*1101	AVFDRKSDAK	EBNA 3B	449
HLA-A*1101	IVTDFSVIK	EBNA 3B	450

HLA-A*1101	KSMREEYRK	Influenza A MP2	451
HLA-A*1101	NTLEQTVKK	E6	452
HLA-A*1101	RMVLASTTAK	Influenza A MP1	453
HLA-A*1101	SIIPSGPLK	Influenza A MP	454
HLA-A*1101	SSCSSCPLSK	EBV LMP-2	455
HLA-A*1101	YVNVNMGK	HBV core antigen	456
HLA-A*2301	QYDPVAALF	pp65	457
HLA-A*2402	AFLPWHRLF	Tyrosinase	458
HLA-A*2402	AYACNTSTL	Survivin	459
HLA-A*2402	AYAQKIFKI	IE-1	460
HLA-A*2402	AYQGVQKQV	ESAT-6	461
HLA-A*2402	AYSQQTRGL	HCV NS3	462
HLA-A*2402	CYASGWGSI	PSA	463
HLA-A*2402	CYTWNQMNL	WT1	464
HLA-A*2402	DYCNVLNKEF	BRLF1	465
HLA-A*2402	DYLNEWGSRF	p-Cadherin	466
HLA-A*2402	DYLQYVLQI	BCL-2A1	467
HLA-A*2402	EYCPGGNLF	MELK	468
HLA-A*2402	EYILSLEEL	Glypican 3	469
HLA-A*2402	EYLQLVFGI	MAGEA2	470
HLA-A*2402	EYLVSGVW	HBV core	471
HLA-A*2402	EYRALQLHL	Carbonic anhydrase	472
HLA-A*2402	EYYELFVNI	DEP DC1	473
HLA-A*2402	GYCTQIGIF	C1orf59	474
HLA-A*2402	IMPKAGLLI	MAGE-A3	475
HLA-A*2402	IYTWIEDHF	FOXO1	476
HLA-A*2402	KLRGEVKQNL	hTOM34p	477
HLA-A*2402	KWLISPVKI	HJURP	478
HLA-A*2402	KYTSFPWLL	HBV polymerase	479

HLA-A*2402	KYYLRVRPLL	KIF20A	480
HLA-A*2402	LYQWLGAPV	BGLAP	481
HLA-A*2402	NYQPVWLCL	RNF43	482
HLA-A*2402	PYLFWLAAI	EBV LMP2	483
HLA-A*2402	QYDPVAALF	pp65	484
HLA-A*2402	RYCNLEGPPI	LY6K	485
HLA-A*2402	RYLKDQQLL	Env	486
HLA-A*2402	RYLRDQQLL	Env	487
HLA-A*2402	RYNAQCQETI	Midkine	488
HLA-A*2402	RYPLTFGW	HIV nef	489
HLA-A*2402	RYPLTFGWCF	NEF	490
HLA-A*2402	SFHSLHLLF	Tax	491
HLA-A*2402	SYRNEIAYL	TTK	492
HLA-A*2402	TFPDLESEF	MAGEA3	493
HLA-A*2402	TYACFVSNL	CEA	494
HLA-A*2402	TYFSLNNKF	AdV 5 Hexon	495
HLA-A*2402	TYGPVFMCL	LMP-2	496
HLA-A*2402	TYGPVFMSL	EBV LMP2	497
HLA-A*2402	TYLPTNASL	HER-2/neu	498
HLA-A*2402	VYALPLKML	pp65	499
HLA-A*2402	VYDFAFRDL	HPV16 E6	500
HLA-A*2402	VYFFLPDHL	gp100	501
HLA-A*2402	VYGFVRACL	hTRT	502
HLA-A*2402	VYGIRLEHF	Nuf2	503
HLA-A*2402	VYLRVRPLL	KIF20A	504
HLA-A*2402	VYYNWQYLL	IL13r	505
HLA-A*2902	IACP IVMRY	BRLF1	506
HLA-A*2902	KEYIDQEEL	HSP90 alpha	507
HLA-A*2902	KESTLHLVL	Ubiquitin	508

HLA-A*2902	LYNTVATLY	Gag	509
HLA-A*2902	SFDPIPIHY	Env	510
HLA-A*2902	SFNCRGEFFY	Env	511
HLA-A*6801	IVTDFSVIK	EBNA 3B	512
HLA-A*6801	TVSGNILTIR	NY-ESO-1	513
HLA-B*0702	APKKPKEPV	VP1	514
HLA-B*0702	APRGVRMAV	CAMEL	515
HLA-B*0702	APTKRKGEC	VP1	516
HLA-B*0702	DPRRRSRNL	HCV core	517
HLA-B*0702	GPGHKARVL	Gag-pol	518
HLA-B*0702	GPLCKADSL	VP1	519
HLA-B*0702	GPRLGVRAT	HCV core	520
HLA-B*0702	IPRRIRQGL	HIV-1 env gp120	521
HLA-B*0702	KPTLKEYVL	E7	522
HLA-B*0702	KPYSGTAYNAL	AdV Hexon	523
HLA-B*0702	KPYSGTAYNSL	AdV Hexon	524
HLA-B*0702	LPVSCPEDL	bZIP factor	525
HLA-B*0702	LPVSPRLQL	CEACAM	526
HLA-B*0702	LPWHRLFLL	Tyrosinase	527
HLA-B*0702	NPTAQSQVM	VP1	528
HLA-B*0702	QPEWFRNVL	Influenza A PB1	529
HLA-B*0702	QPRAPIRPI	EBNA 6	530
HLA-B*0702	RPHERNGFTVL	pp65	531
HLA-B*0702	RPPIFIRRL	EBNA 3A	532
HLA-B*0702	RPQGGSRPEFVKL	BMRF1	533
HLA-B*0702	RVRFFFPSL	MAGE-A1	534
HLA-B*0702	SPERKMLPC	VP1	535
HLA-B*0702	SPFFLLLLL	Mucin	536
HLA-B*0702	SPIVPSFDM	Influenza A NP	537

HLA-B*0702	SPSVDKARAEI	SMCY	538
HLA-B*0702	TPGPGVRYPL	NEF	539
HLA-B*0702	TPNQRQNVC	P2X5a	540
HLA-B*0702	TPRVTGGGAM	pp65	541
HLA-B*0702	VPQYGYLTL	VP1	542
HLA-B*0801	AAKGRGAAL	Neg. Control	543
HLA-B*0801	APLLRWVL	HMOX1	544
HLA-B*0801	DIYKRWII	Gag	545
HLA-B*0801	EIYKRWII	HIV p24 gag	546
HLA-B*0801	ELKRKMIYM	IE-1	547
HLA-B*0801	ELNRKMIYM	IE-1	548
HLA-B*0801	ELRRKMMYM	IE-1	549
HLA-B*0801	FLKEKGGL	HIV-1 nef	550
HLA-B*0801	FLRGRAYGL	EBNA 3A	551
HLA-B*0801	GEIYKRWII	HIV-1 gag p24	552
HLA-B*0801	GFKQSSKAL	bcr-abl 210 kD fusion protein	553
HLA-B*0801	HSKKKCDEL	Polyprotein	554
HLA-B*0801	LPHNHTDL	TPR-protein	555
HLA-B*0801	QAKWRLQTL	EBV EBNA3A	556
HLA-B*0801	QIKVRVDMV	IE-1	557
HLA-B*0801	RAKFKQLL	BZLF1	558
HLA-B*0801	YLKDQQLL	Env	559
HLA-B*1501	RLRPGGKKKY	HIV-1 p17	560
HLA-B*2705	ARMILMTHF	Polyprotein	561
HLA-B*2705	GRAFVTIGK	Env	562
HLA-B*2705	GRFGLATEK	BRAF 27	563
HLA-B*2705	GRFGLATVK	BRAF 27	564
HLA-B*2705	KRWIILGLNK	Gag	565
HLA-B*2705	KRWIILGLNKI	Gag	566

HLA-B*2705	KRWIILGLNKINR	Gag	567
HLA-B*2705	KRWIILGLNKIVR	Gag	568
HLA-B*2705	KRWIILGLNKIVR	Gag	569
HLA-B*2705	KRWIIMGL	Gag	570
HLA-B*2705	KRWIIMGLNK	HIV-1 Gag p24	571
HLA-B*2705	RMFPNAPYL	WT1	572
HLA-B*2705	RRWIQLGLQK	Gag	573
HLA-B*2705	SRYWAIRTR	Influenza A NP	574
HLA-B*3501	CPNSSIVY	HCV E	575
HLA-B*3501	EAAGIGILTY	MART-1	576
HLA-B*3501	EPDLAQCFY	Survivin-3a	577
HLA-B*3501	EPLPQGQLTAY	BZLF1	578
HLA-B*3501	EPLSQSQITAY	BZLF1	579
HLA-B*3501	HPNIEEVAL	HCV NS3	580
HLA-B*3501	HPVAEADYFEY	EBNA 1	581
HLA-B*3501	HPVGDADYFEY	EBNA 1	582
HLA-B*3501	HPVGEADYFEY	EBNA 1	583
HLA-B*3501	HPVGQADYFEY	EBNA 1	584
HLA-B*3501	IPSINVHHY	pp65	585
HLA-B*3501	IPYLDGTFY	AdV Hexon	586
HLA-B*3501	LPLNVGLPIIGVM	UL138	587
HLA-B*3501	LPSDFFPSV	CP	588
HLA-B*3501	MPFATPMEA	NY-ESO-1	589
HLA-B*3501	NPDIVIQY	HIV-1 RT	590
HLA-B*3501	VPLDEDFRKY	RT	591
HLA-B*3501	YPLHEQHGM	EBNA 3A	592
HLA-B*4001	IEDPPFNSL	EBV LMP2	593
HLA-B*4001	KEKGGLEGL	HIV-1 Nef	594
HLA-B*4001	REISVPAEIL	HCV NS5a	595

HLA-B*5101	IPFYGKAI	Polyprotein	596
HLA-B*5101	LPSDFFPSV	CP	597
HLA-B*5101	MPFATPMEA	NY-ES0-1	598
HLA-E*0101	VMAPRTLVL	HLA-A leader sequence peptide	599

[0058] It is contemplated that any embodiment discussed in this specification can be implemented with respect to any method, kit, reagent, or composition of the invention, and vice versa. Furthermore, compositions of the invention can be used to achieve methods of the invention.

5 [0059] It will be understood that particular embodiments described herein are shown by way of illustration and not as limitations of the invention. The principal features of this invention can be employed in various embodiments without departing from the scope of the invention. Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, numerous equivalents to the specific procedures described herein. Such equivalents are considered
10 to be within the scope of this invention and are covered by the claims.

[0060] All publications and patent applications mentioned in the specification are indicative of the level of skill of those skilled in the art to which this invention pertains. All publications and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

15 [0061] The use of the word “a” or “an” when used in conjunction with the term “comprising” in the claims and/or the specification may mean “one,” but it is also consistent with the meaning of “one or more,” “at least one,” and “one or more than one.” The use of the term “or” in the claims is used to mean “and/or” unless explicitly indicated to refer to alternatives only or the alternatives are mutually exclusive, although the disclosure supports a definition that refers to only alternatives and
20 “and/or.” Throughout this application, the term “about” is used to indicate that a value includes the inherent variation of error for the device, the method being employed to determine the value, or the variation that exists among the study subjects.

[0062] As used in this specification and claim(s), the words “comprising” (and any form of comprising, such as “comprise” and “comprises”), “having” (and any form of having, such as
25 “have” and “has”), “including” (and any form of including, such as “includes” and “include”) or “containing” (and any form of containing, such as “contains” and “contain”) are inclusive or open-

ended and do not exclude additional, unrecited elements or method steps. In embodiments of any of the compositions and methods provided herein, “comprising” may be replaced with “consisting essentially of” or “consisting of”. As used herein, the phrase “consisting essentially of” requires the specified integer(s) or steps as well as those that do not materially affect the character or function of the claimed invention. As used herein, the term “consisting” is used to indicate the presence of the recited integer (e.g., a feature, an element, a characteristic, a property, a method/process step or a limitation) or group of integers (e.g., feature(s), element(s), characteristic(s), property(ies), method/process steps or limitation(s)) only.

[0063] The term “or combinations thereof” as used herein refers to all permutations and combinations of the listed items preceding the term. For example, “A, B, C, or combinations thereof” is intended to include at least one of: A, B, C, AB, AC, BC, or ABC, and if order is important in a particular context, also BA, CA, CB, CBA, BCA, ACB, BAC, or CAB. Continuing with this example, expressly included are combinations that contain repeats of one or more item or term, such as BB, AAA, AB, BBC, AAABCCCC, CBBAAA, CABABB, and so forth. The skilled artisan will understand that typically there is no limit on the number of items or terms in any combination, unless otherwise apparent from the context.

[0064] As used herein, words of approximation such as, without limitation, “about”, “substantial” or “substantially” refers to a condition that when so modified is understood to not necessarily be absolute or perfect but would be considered close enough to those of ordinary skill in the art to warrant designating the condition as being present. The extent to which the description may vary will depend on how great a change can be instituted and still have one of ordinary skill in the art recognize the modified feature as still having the required characteristics and capabilities of the unmodified feature. In general, but subject to the preceding discussion, a numerical value herein that is modified by a word of approximation such as “about” may vary from the stated value by at least $\pm 1, 2, 3, 4, 5, 6, 7, 10, 12$ or 15% .

[0065] All of the compositions and/or methods disclosed and claimed herein can be made and executed without undue experimentation in light of the present disclosure. While the compositions and methods of this invention have been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations may be applied to the compositions and/or methods and in the steps or in the sequence of steps of the method described herein without

departing from the concept, spirit and scope of the invention. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the invention as defined by the appended claims.

5 [0066] To aid the Patent Office, and any readers of any patent issued on this application in interpreting the claims appended hereto, applicants wish to note that they do not intend any of the appended claims to invoke paragraph 6 of 35 U.S.C. § 112, U.S.C. § 112 paragraph (f), or equivalent, as it exists on the date of filing hereof unless the words “means for” or “step for” are explicitly used in the particular claim.

10 [0067] For each of the claims, each dependent claim can depend both from the independent claim and from each of the prior dependent claims for each and every claim so long as the prior claim provides a proper antecedent basis for a claim term or element.

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What is claimed is:

1. A fusion protein comprising a peptide, a first flexible linker, a β 2-microglobulin domain, a second flexible linker, a soluble major histocompatibility complex (MHC) heavy chain and a peptide tag.
- 5 2. The fusion protein of claim 1, wherein the peptide is an immunogenic peptide epitope.
3. The fusion protein of claim 1, wherein the MHC is a human, mouse, rat, hamster, horse, pig, cow, simian, avian, or chimeric MHC.
4. The fusion protein of claim 1, wherein the MHC is Class I MHC.
5. The fusion protein of claim 1, wherein the MHC does not include a transmembrane
18 sequence.
6. The fusion protein of claim 1, wherein the peptide tag is selected from wherein the peptide tag is selected from at least one of: a BirA tail, a myc, a FLAG, a glutathione-S-transferase, a His tag, a maltose binding protein, hemagglutinin (HA)-tag, a V5 tag, a T7 tag, a V9 tag, a NusA-tag, a thioredoxin-tag, or a fluorescent protein-tag, a Her2/neu-tag, a CD20-tag, or a GFP-tag.
- 15 7. The fusion protein of claim 1, wherein the first, the second, or both the first and second flexible linkers comprise glycine, serine or both glycine and serine and comprise 5 to 40 residues.
8. The fusion protein of claim 1, wherein the peptide is 8 to 16 residues long.
9. The fusion protein of claim 1, wherein the peptide is selected from at least one of: ABL1; ACPP; Ad5 E1A; AdV 5 Hexon; AdV Hexon; Ag85A; alfa fetoprotein; ASP-2; BA46; BALF4;
20 BAP31; BCL-2; BCL-2A1; BCL-2L1; BCL-X; BCR-ABL; bcr-abl 210 kD fusion protein; Beta-gal; BGLAP; BMI1; BMLF1; BMRF1; BNP; BRAF 27; BRLF1; bZIP factor; BZLF1; C1orf59; CAMEL; Carbonic anhydrase; CB9L2; CD105; CD33; CD59; CEA; CEACAM; Chondromodulin; circadian clock protein PASD1; CP; CPS; cyclin-dependent kinase 4; CYP190; Cytochrome p450; DEP DC1; DLK1; Dufpase; E6; E7; EBNA 1; EBNA 3A; EBNA 3B; EBNA 6; EBV BRLF1; EBV
25 EBNA3A; EBV LMP2; EDDR1; EGFR; EMNA 3A; EMV-1; Enhanced Green Fluorescent Protein; Env; EphA2; Erk1; ESAT-6; EZH2; FAP α ; FLT1; FOLR1; FOXM1; G250; GAD65; Gag; Gag-pol; Glypican 3; gp; gp100; gp33 (C9M); GPC3; H250; HA-1; HA-2; HA-8; HBB; HBsAg; HBV core; HBV polymerase; HBV surface antigen; HCMV IE1; HCMV pp65; HCV core; HCV E; HCV NS3;

HCV NS4b; HCV NS5a; HCV NS5B; Heparanase; HER2; HER-2/neu; Histocompatibility antigen 60; HIV gag p24; HIV nef; HIV pol; HIV-1 env gp120; HIV-1 IIIB gp120; HIV-1 nef; HIV-1 p17; HIV-1 RT; HIV-1 US4 gp120; HJURP; HLA-A leader sequence peptide; HLA-A2; HLA-Cw3; chlamydia trachomatis MOMP ; HM1.24; HMMR; HMOX1; HO-1; hPSA; HPV 16 E6; HSP105;

5 HSP90 alpha; hTERT; hTOM34p; hTRT; H-Y; IA-2; IAPP; IDO; IE-1; IE62; IGF; IGRP; IL13r; IL13Ra; Ilr1; Influenza A (PR8) NP ; Influenza A MP; Influenza A MP1; Influenza A MP2; Influenza A NP; Influenza A PB1; INF γ R; Insulin; Interferon gamma inducible protein (GILT) 30; IRS-2; ITGB8; K8.1; KIF20A; KLK; KLK3; L1; LANA; Large T antigen; Lengsin; Listeria monocytogenes Listeriolysin; LIVIN; LMP-1; LMP-2; LMP-2A; LMP-2A; LY6K; m139; m141;

10 m145; M164; M2-1; M38; M45; M57; MAGE-1; MAGE-10; MAGE-3; MAGE-4; MAGE-A1; MAGEA2; MAGEA3; MAGE-A5; MAGE-C1; MAGE-C2; MART-1; MC38 adpgk neoantigen; MC-38; MCL-1; MCMV IE1; MELK; Mena; Middle T antigen; Midkine; miHAg SMCY; MOG precursor; MP; MPP11; MS4A1; MSLN; Mtb 16 kDa; Mtb 19 kDa; Mtb85A; mTERT; Mucin; muFAP α ; MuLV env ; Murine Survivin; MYBPC-2; Mycobacterium bovis antigen 85-A;

15 Mycobacterium tuberculosis ESAT-6; Mycobacterium tuberculosis TB10.4; Myelin basic protein; ND; NEF; NEF; Neu/Her-2/ErbB2 proto-oncoprotein; NG2; Non muscle Myosin-9; Non-muscle Myosin; NP; NP396; NP52; NPM1; NRP-1; NRP-2; NRP-V7 superagonist peptide 8.3 Tg NOD mouse; NS3; Nucleocapsid; Nuf2; NY-ESO-1; OVA; Ovalbumin (subdominant); P1A; P2X5a; p53; p56; P79; PAP-3; PASD1; PAX-5; p-Cadherin; PDGFRbeta; PLAC1; PLAM csp; Plasmodium

20 berghei ANKA acid phosphatase; Plasmodium berghei CSP; Plasmodium CSP; Plasmodium falciparum CSP; Plasmodium falciparum Liver stage antigen; PolA; Polymerase; Polyprotein; pp65; PPE; Pr1; PRAME; Prominin1; PSA; PSCA; PSM P2; PSMA; RGS5; RhoC; RNF43; RSV A strain F protein; RSV NP; RT; S protein; SAA; SART3; Sialidases ; SIV gag; SMCY; Spike GP; Surface IgG (sA20-Ig) of A20; Survivin; Survivin-3a; SV40 T antigen; TACE; TARP; TARP 2M;

25 Tax; TB10.3-4; Telomerase; TEM1; tgd057; TGF β ; TNPO3; topII; TPBG; TPR-protein; TRAG; TRP2; Trypanosoma cruzi ASP-2; Trypanosoma cruzi SP; TTK; Tyrosinase; Tyrosine-3-hydroxylase; Ubiquitin; UL105; UL138; UL44; USP9Y; V131; Vaccinia virus Copenhagen Protein G5 ; Vaccinia virus Host range protein 2 ; VACCL3_100; VEGFR1; VEGFR2; VEGFR2/KDR fragment 1; Vif; Vinculin; VP1; Vpu; VSV N; West Nile virus NY-99 polyprotein precursor; West

30 Nile Virus polyprotein; WT1; Yellow Fever Virus 17D polyprotein; or ZnT-8.

10. The fusion protein of claim 1, wherein the MHC is selected from at least one of H-2 Db, H-2 Dd; H-2 Dk; H-2 Kb; H-2 Kd; H-2 Kk; H-2 Ld; HLA-A*0101; HLA-A*0201; HLA-A*0301; HLA-A*1101; HLA-A*2301; HLA-A*2402; HLA-A*2902; HLA-A*6801; HLA-B*0702; HLA-B*0801; HLA-B*1501; HLA-B*2705; HLA-B*3501; HLA-B*4001; HLA-B*5101; or HLA-E*0101.
- 5 11. The fusion protein of claim 1, wherein the peptide is at least one of SEQ ID NO:1-599.
12. A nucleic acid that expresses a fusion protein comprising a peptide, a first flexible linker, a β 2-microglobulin domain, a second flexible linker, a soluble major histocompatibility complex (MHC) heavy chain and a peptide tag.
13. The nucleic acid of claim 12, wherein the peptide is an immunogenic peptide epitope.
- 10 14. The nucleic acid of claim 12, wherein the MHC is a human, mouse, rat, hamster, horse, pig, cow, simian, avian, or chimeric MHC.
15. The nucleic acid of claim 12, wherein the MHC is Class I MHC.
16. The nucleic acid of claim 12, wherein the MHC does not include a transmembrane sequence.
17. The nucleic acid of claim 12, wherein the peptide tag is selected from at least one of: a BirA
15 tail, a myc, a FLAG, a glutathione-S-transferase, a His tag, a maltose binding protein, hemagglutinin (HA)-tag, a V5 tag, a T7 tag, a V9 tag, a NusA-tag, a thioredoxin-tag, or a fluorescent protein-tag, a Her2/neu-tag, a CD20-tag, or a GFP-tag.
18. The nucleic acid of claim 12, wherein the first, the second, or both the first and second flexible linkers comprise glycine, serine or both glycine and serine and comprise 5 to 40 residues.
- 20 19. The nucleic acid of claim 12, wherein the peptide is 8 to 16 residues long.
20. The nucleic acid of claim 12, wherein the peptide is selected from at least one of: ABL1; ACPP; Ad5 E1A; AdV 5 Hexon; AdV Hexon; Ag85A; alfa fetoprotein; ASP-2; BA46; BALF4; BAP31; BCL-2; BCL-2A1; BCL-2L1; BCL-X; BCR-ABL; bcr-abl 210 kD fusion protein; Beta-gal; BGLAP; BMI1; BMLF1; BMRF1; BNP; BRAF 27; BRLF1; bZIP factor; BZLF1; C1orf59;
25 CAMEL; Carbonic anhydrase; CB9L2; CD105; CD33; CD59; CEA; CEACAM; Chondromodulin; circadian clock protein PASD1; CP; CPS; cyclin-dependent kinase 4; CYP190; Cytochrome p450; DEP DC1; DLK1; Dutpase; E6; E7; EBNA 1; EBNA 3A; EBNA 3B; EBNA 6; EBV BRLF1; EBV EBNA3A; EBV LMP2; EDDR1; EGFR; EMNA 3A; EMV-1; Enhanced Green Fluorescent Protein;

Env; EphA2; Erk1; ESAT-6; EZH2; FAP α ; FLT1; FOLR1; FOXM1; G250; GAD65; Gag; Gag-pol; Glypican 3; gp; gp100; gp33 (C9M); GPC3; H250; HA-1; HA-2; HA-8; HBB; HBsAg; HBV core; HBV polymerase; HBV surface antigen; HCMV IE1; HCMV pp65; HCV core; HCV E; HCV NS3; HCV NS4b; HCV NS5a; HCV NS5B; Heparanase; HER2; HER-2/neu; Histocompatibility antigen

5 60; HIV gag p24; HIV nef; HIV pol; HIV-1 env gp120; HIV-1 IIIB gp120; HIV-1 nef; HIV-1 p17; HIV-1 RT; HIV-1 US4 gp120; HJURP; HLA-A leader sequence peptide; HLA-A2; HLA-Cw3; chlamydia trachomatis MOMP ; HM1.24; HMMR; HMOX1; HO-1; hPSA; HPV 16 E6; HSP105; HSP90 alpha; hTERT; hTOM34p; hTRT; H-Y; IA-2; IAPP; IDO; IE-1; IE62; IGF; IGRP; IL13r; IL13Ra; Ilr1; Influenza A (PR8) NP ; Influenza A MP; Influenza A MP1; Influenza A MP2;

10 Influenza A NP; Influenza A PB1; INF γ R; Insulin; Interferon gamma inducible protein (GILT) 30; IRS-2; ITGB8; K8.1; KIF20A; KLK; KLK3; L1; LANA; Large T antigen; Lengsin; Listeria monocytogenes Listeriolysin; LIVIN; LMP-1; LMP-2; LMP-2A; LMP-2A; LY6K; m139; m141; m145; M164; M2-1; M38; M45; M57; MAGE-1; MAGE-10; MAGE-3; MAGE-4; MAGE-A1; MAGEA2; MAGEA3; MAGE-A5; MAGE-C1; MAGE-C2; MART-1; MC38 adpgk neoantigen;

15 MC-38; MCL-1; MCMV IE1; MELK; Mena; Middle T antigen; Midkine; miHAg SMCY; MOG precursor; MP; MPP11; MS4A1; MSLN; Mtb 16 kDa; Mtb 19 kDa; Mtb85A; mTERT; Mucin; muFAP α ; MuLV env ; Murine Survivin; MYBPC-2; Mycobacterium bovis antigen 85-A; Mycobacterium tuberculosis ESAT-6; Mycobacterium tuberculosis TB10.4; Myelin basic protein; ND; NEF; NEF; Neu/Her-2/ErbB2 proto-oncoprotein; NG2; Non muscle Myosin-9; Non-muscle

20 Myosin; NP; NP396; NP52; NPM1; NRP-1; NRP-2; NRP-V7 superagonist peptide 8.3 Tg NOD mouse; NS3; Nucleocapsid; Nuf2; NY-ESO-1; OVA; Ovalbumin (subdominant); P1A; P2X5a; p53; p56; P79; PAP-3; PASD1; PAX-5; p-Cadherin; PDGFRbeta; PLAC1; PLAM csp; Plasmodium berghei ANKA acid phosphatase; Plasmodium berghei CSP; Plasmodium CSP; Plasmodium falciparum CSP; Plasmodium falciparum Liver stage antigen; PolA; Polymerase; Polyprotein; pp65;

25 PPE; Pr1; PRAME; Prominin1; PSA; PSCA; PSM P2; PSMA; RGS5; RhoC; RNF43; RSV A strain F protein; RSV NP; RT; S protein; SAA; SART3; Sialidases ; SIV gag; SMCY; Spike GP; Surface IgG (sA20-Ig) of A20; Survivin; Survivin-3a; SV40 T antigen; TACE; TARP; TARP 2M; Tax; TB10.3-4; Telomerase; TEM1; tgd057; TGF β ; TNPO3; topII; TPBG; TPR-protein; TRAG; TRP2; Trypanosoma cruzi ASP-2; Trypanosoma cruzi SP; TTK; Tyrosinase; Tyrosine-3-

30 hydroxylase; Ubiquitin; UL105; UL138; UL44; USP9Y; V131; Vaccinia virus Copenhagen Protein G5 ; Vaccinia virus Host range protein 2 ; VACCL3_100; VEGFR1; VEGFR2; VEGFR2/KDR

fragment 1; Vif; Vinculin; VP1; Vpu; VSV N; West Nile virus NY-99 polyprotein precursor; West Nile Virus polyprotein; WT1; Yellow Fever Virus 17D polyprotein; or ZnT-8.

21. The nucleic acid of claim 12, wherein the MHC is selected from at least one of H-2 Db, H-2 Dd; H-2 Dk; H-2 Kb; H-2 Kd; H-2 Kk; H-2 Ld; HLA-A*0101; HLA-A*0201; HLA-A*0301; HLA-A*1101; HLA-A*2301; HLA-A*2402; HLA-A*2902; HLA-A*6801; HLA-B*0702; HLA-B*0801; HLA-B*1501; HLA-B*2705; HLA-B*3501; HLA-B*4001; HLA-B*5101; or HLA-E*0101.

22. The nucleic acid of claim 12, wherein the peptide is SEQ ID NO:1-599.

23. A method of making a soluble eukaryotic-derived peptide/MHC complex comprising:

expressing in a cell a fusion protein comprising a peptide, a first flexible linker, a β 2-microglobulin domain, a second flexible linker, a soluble major histocompatibility complex (MHC) heavy chain and a peptide tag.

24. The method of claim 23, further comprising isolating the fusion protein from a supernatant.

25. The method of claim 23, further comprising forming dimers, trimers, tetramers, or multimers of the fusion protein by mixing the fusion protein with one or more agents that bind to two or more fusion proteins.

26. The method of claim 25, wherein the agent is selected from an antibody, a cross-linking agent, a ligase, an avidin, a streptavidin, a Protein A, or a J-chain.

27. The method of claim 23, wherein the peptide is an immunogenic peptide epitope.

28. The method of claim 23, wherein the MHC is a human, mouse, rat, hamster, horse, pig, cow, simian, avian or chimeric MHC.

29. The method of claim 23, wherein the MHC is Class I MHC.

30. The method of claim 23, wherein the MHC does not include a transmembrane sequence.

31. The method of claim 23, wherein the peptide tag is selected from at least one of: a BirA tail, a myc, a FLAG, a glutathione-S-transferase, a His tag, a maltose binding protein, hemagglutinin (HA)-tag, a V5 tag, a T7 tag, a V9 tag, a NusA-tag, a thioredoxin-tag, or a fluorescent protein-tag, a Her2/neu-tag, a CD20-tag, or a GFP-tag.

32. The method of claim 23, wherein the first, the second, or both the first and second flexible linkers comprise glycine, serine or both glycine and serine and comprise 5 to 40 residues.

33. The method of claim 23, wherein the peptide is 8 to 16 residues long.
34. A cell line expressing a fusion protein comprising a fusion protein comprising a peptide, a first flexible linker, a β 2-microglobulin domain, a second flexible linker, a soluble major histocompatibility complex (MHC) heavy chain and a peptide tag.
- 5 35. The cell line of claim 34, wherein the fusion protein is integrated into the genome by co-transfecting a fusion protein expressing vector with a transposase vector that expresses a transposase and wherein the fusion protein expressing vector, the transposase vector, or both further comprise a selectable marker.
36. The cell line of claim 34, wherein the peptide is an immunogenic peptide epitope.
- 10 37. The cell line of claim 34, wherein the MHC is a human, mouse, rat, hamster, horse, pig, cow, simian, avian, or chimeric MHC .
38. The cell line of claim 34, wherein the MHC is Class I MHC.
39. The cell line of claim 34, wherein the MHC does not include a transmembrane sequence.
40. The cell line of claim 34, wherein the peptide tag is selected from at least one of: a BirA tail, 15 a myc, a FLAG, a glutathione-S-transferase, a His tag, a maltose binding protein, hemagglutinin (HA)-tag, a V5 tag, a T7 tag, a V9 tag, a NusA-tag, a thioredoxin-tag, or a fluorescent protein-tag, a Her2/neu-tag, a CD20-tag, or a GFP-tag.
41. The cell line of claim 34, wherein the first, the second, or both the first and second flexible linkers comprise glycine, serine or both glycine and serine and comprise 5 to 40 residues.
- 20 42. The cell line of claim 34, wherein the peptide is 8 to 16 residues long.



FIG. 1A

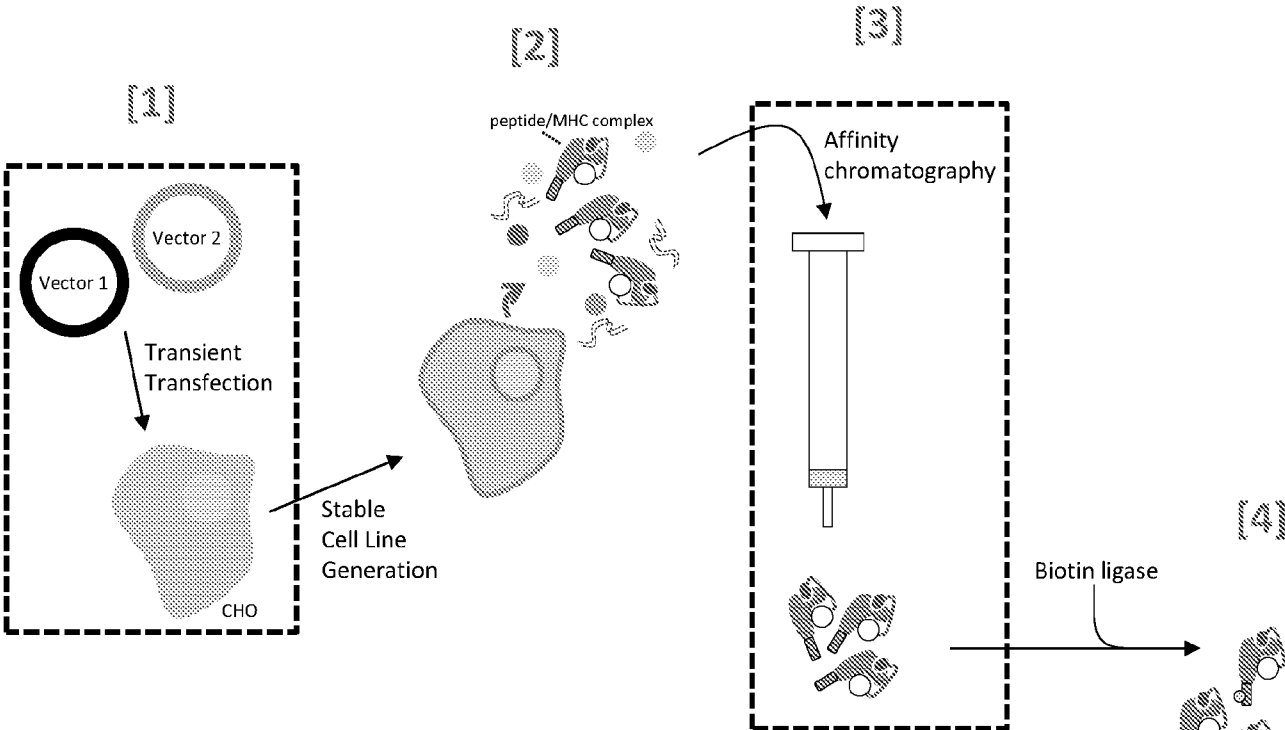
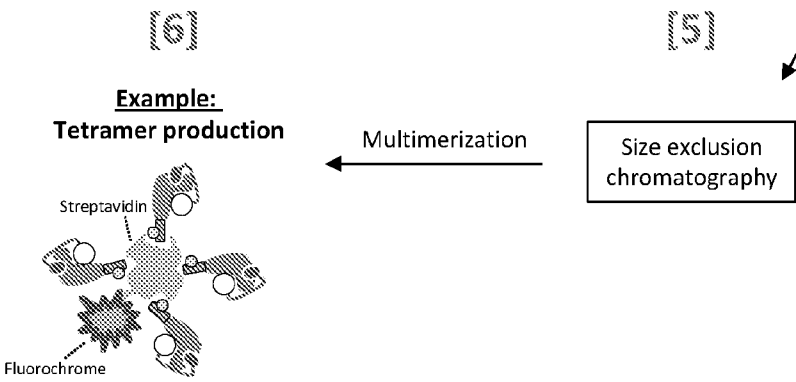


FIG. 1B



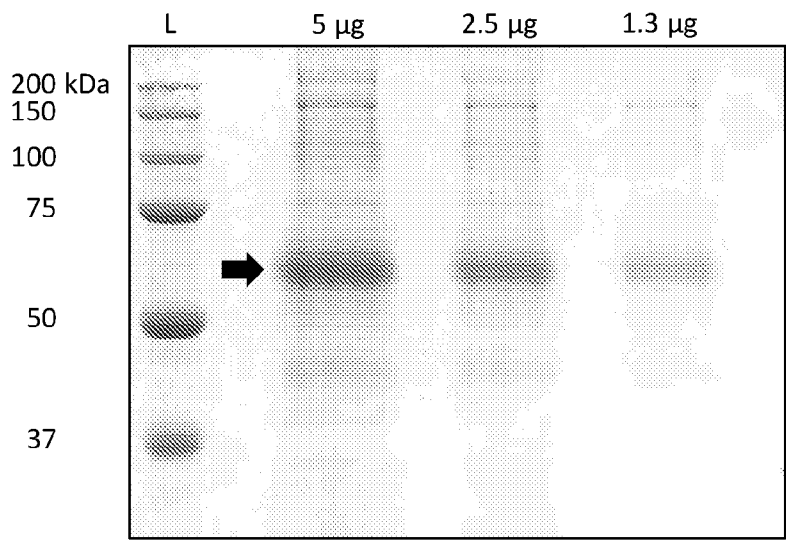


FIG. 2A

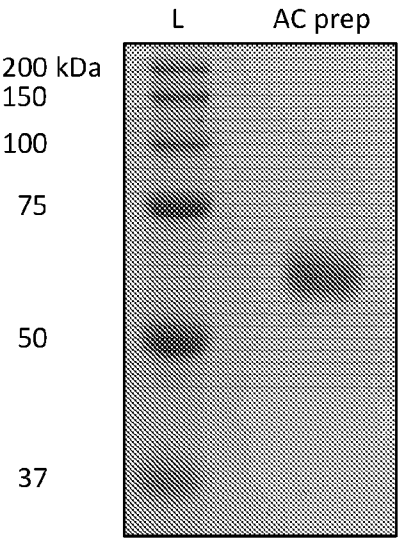
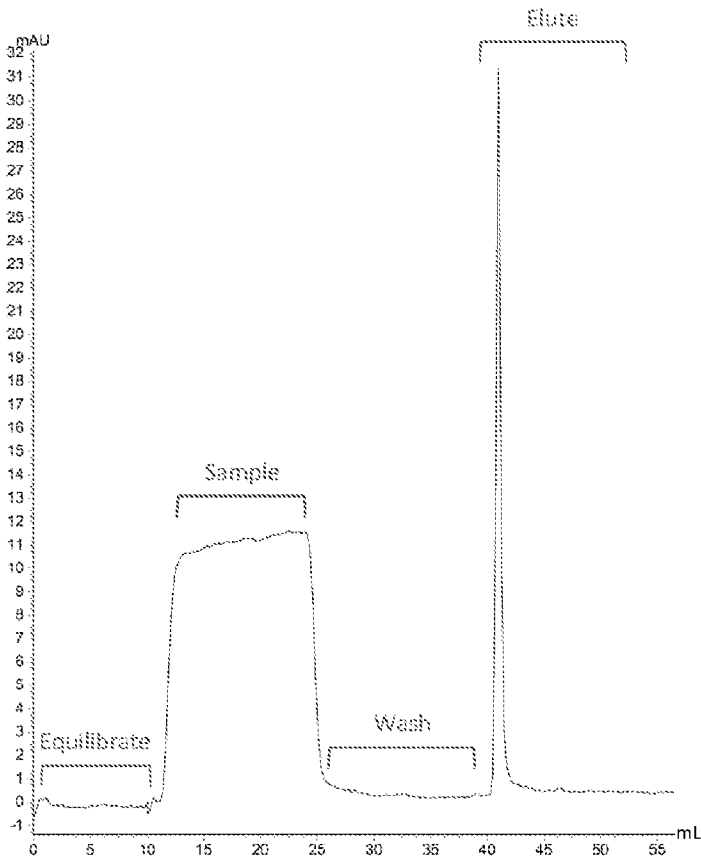


FIG. 2B

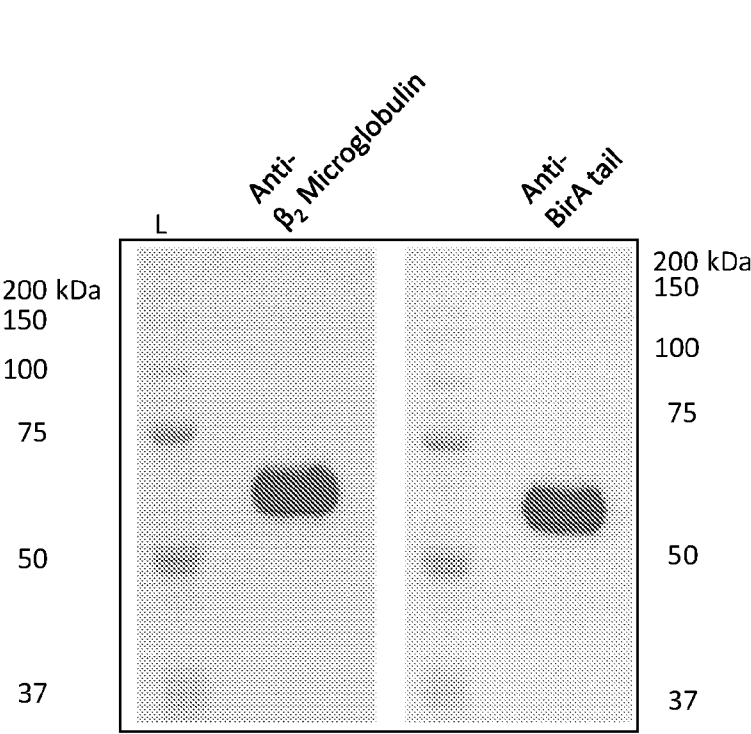


FIG. 3A

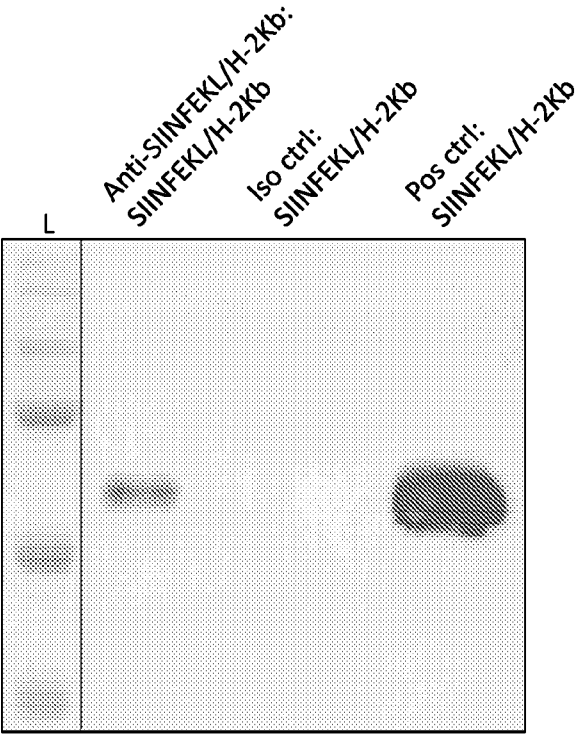


FIG. 3B

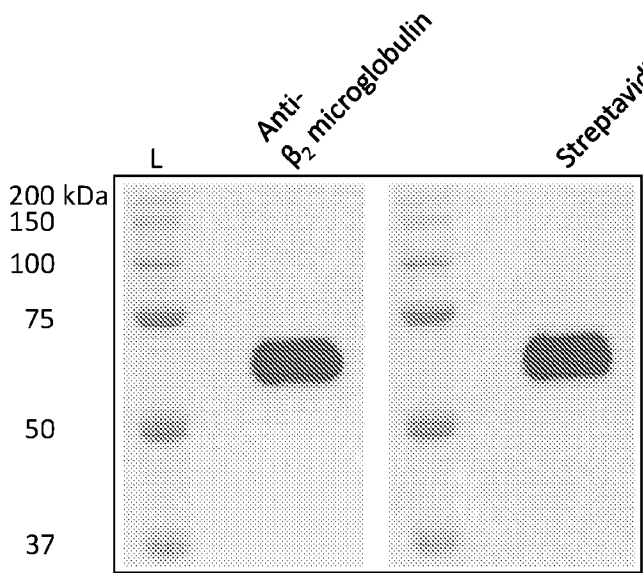


FIG. 4A

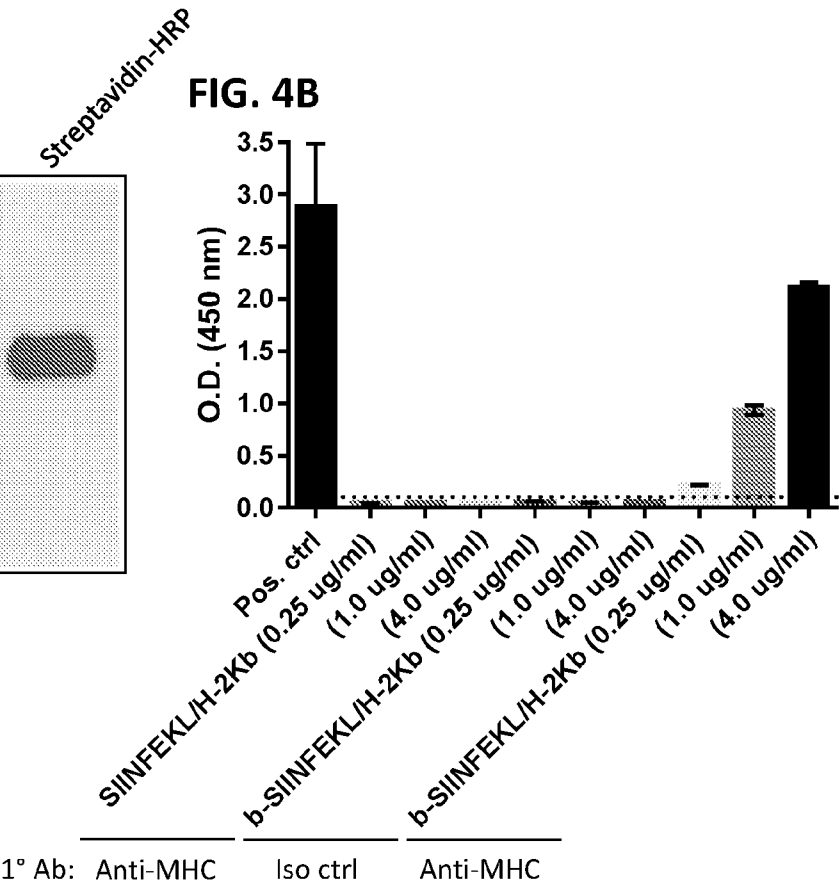
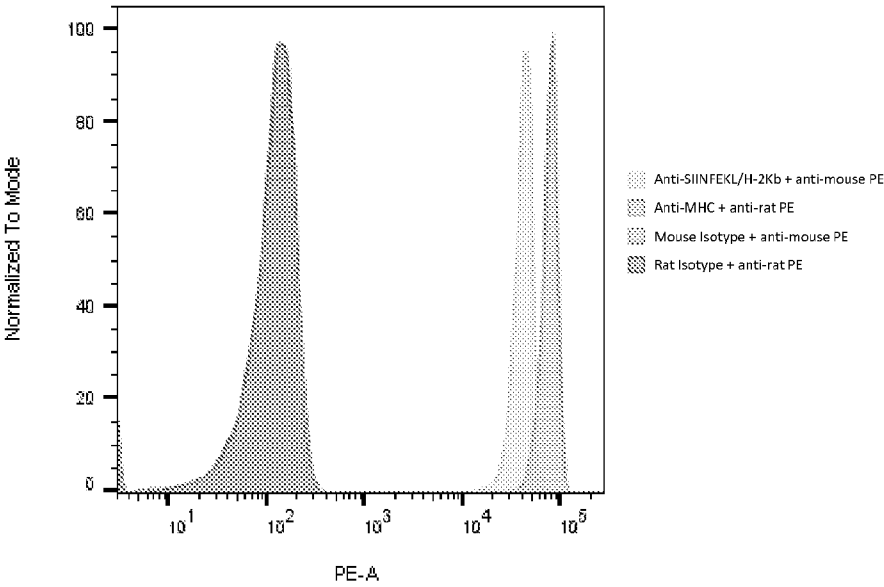
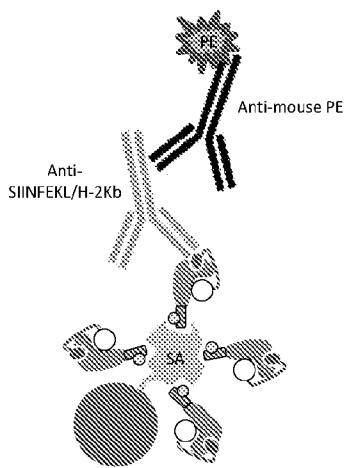


FIG. 4B

FIG. 4C



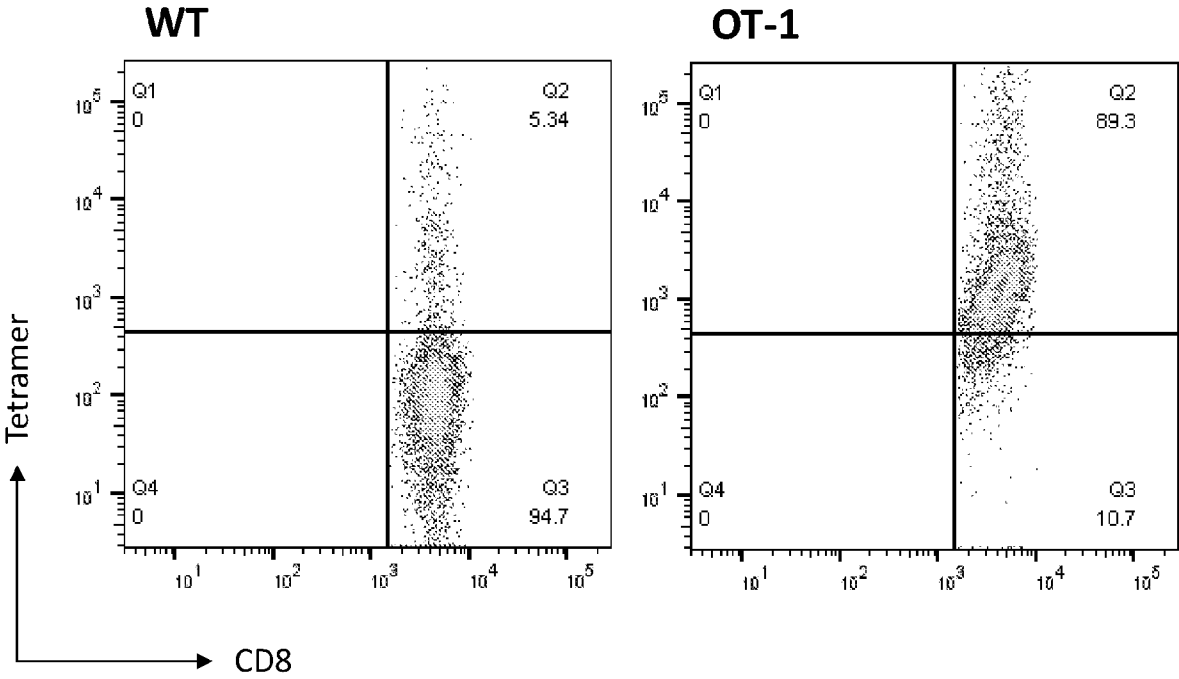
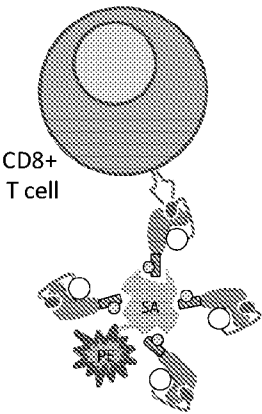


FIG. 5

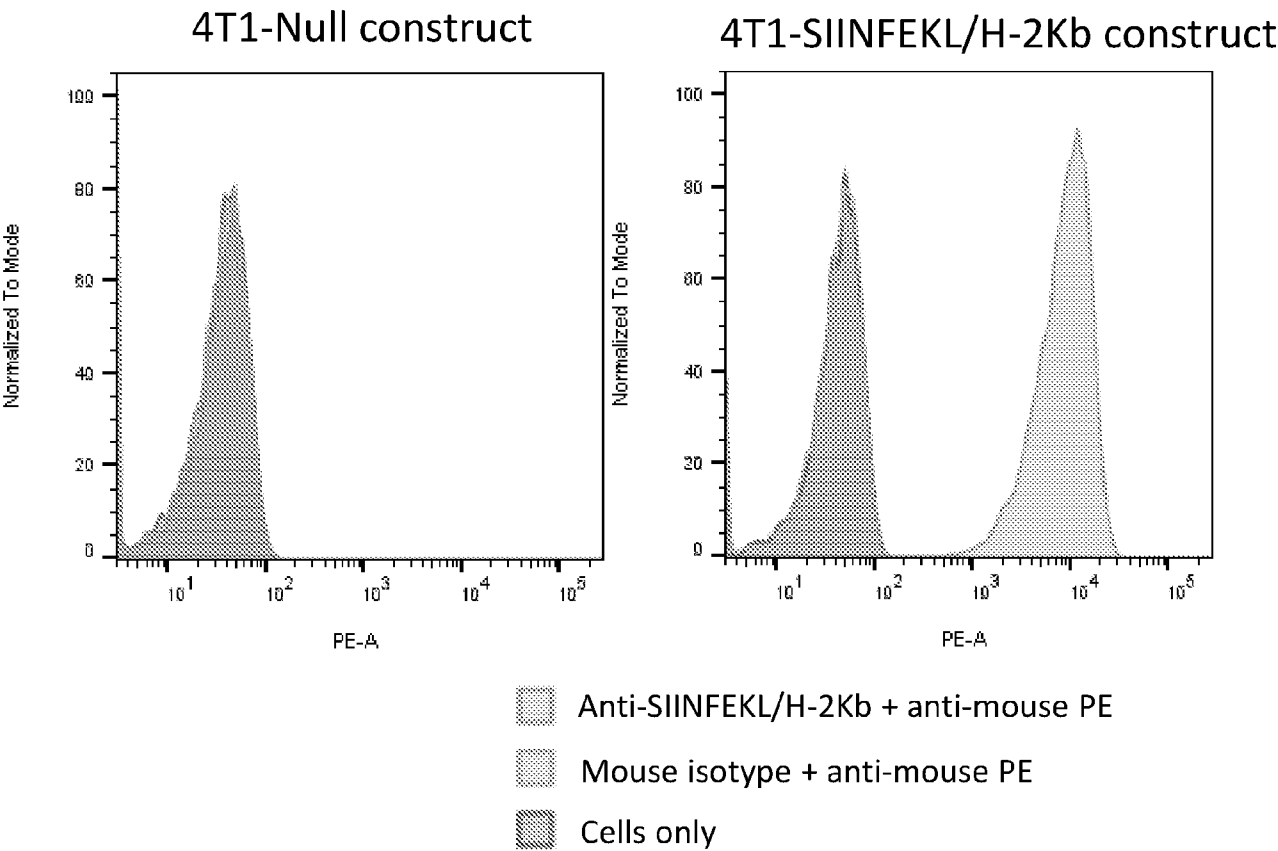


FIG. 6

FIG. 7

Leader signal SIINFEKL peptide
 ATGGCTCGCTCGGTGACCCTGGTCTTTCTGGTGCTTGCTCACTGACCGTTTGTATGCT AGTATCATTAATTCG
 Linker β_2 microglobulin
 AAAAACTTGGAGGAGGTGCTAGCGGTGGTGGAGGTAGCGGAGGTGGAGGAAGCATCCAGAAAACCCCTCAA
 ATTCAAGTATACTCACGCCACCCACCGAGAATGGGAAGCCGAACATACTGAACTGCTACGTAACACAGTTCCA
 CCCGCCTCACATTGAAATCAAATGCTGAAGAACGGGAAAAAAATTCCTAAAGTAGAGATGTCAGATATGTCCT
 TCAGCAAGGACTGGTCTTTCTATATCCTGGCTCACACTGAATTCACCCCCACTGAGACTGATACATACGCCTGCA
 Linker
 GAGTTAAGCATGCCAGTATGGCCGAGCCCAAGACCGTCTACTGGGATCGAGACATGGGCGGTGGTGGTTCCGG
 Secretory Heavy Chain (H-2Kb)
 TGGAGGCGGTTCCGGAGGTGGTGGATCCGGTGGTGGAGGTAGTGGCCCACTCGCTGAGGTATTTCTCACC
 GCCGTGTCCCGGCCCGCCTCGGGGAGCCCCGGTACATGGAAGTCGGCTACGTGGACGACACGGAGTTCGTGC
 GCTTCGACAGCGACGCGGAGAATCCGAGATATGAGCCGCGGGCGCGGTGGATGGAGCAGGAGGGGCCCGAG
 TATTGGGAGCGGGAGACACAGAAAGCCAAGGGCAATGAGCAGAGTTCCGAGTGGACCTGAGGACCCTGCTC
 GGCTACTACAACCAGAGCAAGGGCGGCTCTCACACTATTCAGGTGATCTCTGGCTGTGAAGTGGGGTCCGACG
 GGCGACTCCTCCGCGGGTACCAGCAGTACGCCTACGACGGCTGCGATTACATCGCCCTGAACGAAGACCTGAAA
 ACGTGGACGGCGGCGGACATGGCGGCGCTGATCACCAACACAAGTGGGAGCAGGCTGGTGAAGCAGAGAG
 ACTCAGGGCCTACCTGGAGGGCACGTGCGTGGAGTGGCTCCGCAGATACCTGAAGAACGGGAACGCGACGCT
 GCTGCGCACAGATTCCCCAAAGGCCCATGTGACCCATCACAGCAGACCTGAAGATAAAGTCACCCTGAGGTGCT
 GGGCCCTGGGCTTCTACCCTGCTGACATCACCTGACCTGGCAGTTGAATGGGGAGGAGCTGATCCAGGACAT
 GGAGCTTGTGGAGACCAGGCCTGCAGGGGATGGAACCTTCAGAAGTGGGCATCTGTGGTGGTGCCTCTTGGG
 AAGGAGCAGTATTACACATGCCATGTGTACCATCAGGGGCTGCCTGAGCCCCTACCCTGAGATGGGAGCCTCC
 BirA tail
 TCCATCCACTGCCGGCGGGCTGAACGATATTTTGAAGCCAGAAAATTGAATGGCATGAATGA

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US19/56281

A. CLASSIFICATION OF SUBJECT MATTER

IPC - A61K 38/17; C07K 14/74; C12N 15/62 (2020.01)

CPC - A61K 38/1774; C07K 14/70539; C12N 15/62

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

See Search History document

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

See Search History document

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

See Search History document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X -- Y	WO 2009/003492 A1 (DAKO DENMARK A/S) 08 January 2009; page 10, lines 7-10; page 11, lines 12-14; page 12, lines 27-35; page 13, lines 1-9; page 21, lines 3-6, 8-11; page 25, lines 4, 10-13; page 26, lines 28-29; page 27, lines 6-8; page 28, lines 1-11; page 52, lines 15-18; page 53, lines 14-15, 23-24; page 162, lines 27-29; page 163, lines 3-6; page 167, lines 1-4; page 212, lines 34; page 213, lines 1-3	1-8, 12-19, 23-34, 36-42 -- 9-10, 20-21, 35
Y	WO 2011/113819 A2 (IMMATICS BIOTECHNOLOGIES GMBH) 22 September 2011; abstract; page 4, third paragraph; page 5, first paragraph; page 9, fourth-fifth paragraphs; page 21, fourth paragraph; page 37, third-fourth paragraphs; page 38, first paragraph; table 2	9, 20
Y	WO 99/60120 A2 (AVIDEX LIMITED) 25 November 1999; abstract; page 59, lines 7-16	10, 21
Y	US 2015/0361451 A1 (SELEXIS S.A.) 17 December 2015; paragraphs [0079], [0090], [0130], [0317]	35
P, X	WOOSTER, Alet al. EXPRESSION AND CHARACTERIZATION OF SOLUBLE EPIOTOPE-DEFINED MAJOR HISTOCOMPATIBILITY COMPLEX (MHC) FROM STABLE EUKARYOTIC CELL LINES. January 2019; Journal of Immunological Methods. No. 464, pp. 22-30. entire document. DOI: 10.1016/j.jim.2018.10.006.	1-2, 4-6, 8, 12-13, 15-19, 23-27, 29-31, 33-34, 36, 38-40, 42

☐ Further documents are listed in the continuation of Box C.☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"D" document cited by the applicant in the international application

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

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

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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

"&" document member of the same patent family

Date of the actual completion of the international search

04 February 2020 (04.02.2020)

Date of mailing of the international search report

19 FEB 2020

Name and mailing address of the ISA/US

Mail Stop PCT, Attn: ISA/US, Commissioner for Patents

P.O. Box 1450, Alexandria, Virginia 22313-1450

Facsimile No. 571-273-8300

Authorized officer

Shane Thomas

Telephone No. PCT Helpdesk: 571-272-4300

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US19/56281

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

-Please See Supplemental Page-

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
Group I+, Claims 1-10, 12-21, 23-42 ; Human MHC H-2 Db (MHC); Bir A tail (peptide tag); and ABL1 (peptide)

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US19/56281

-***-Continued from Box No. III Observations where unity of invention is lacking-***-

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I+, Claims 1-42; Human MHC H-2 Db (MHC); Bir A tail (peptide tag); and ABL1 (peptide); are directed toward a fusion protein comprising a peptide, a first flexible linker, a β 2-microglobulin domain, a second flexible linker, a soluble major histocompatibility complex (MHC) heavy chain and a peptide tag.

The fusion protein will be searched to the extent that it encompasses a fusion protein encompassing human MHC H-2 Db (first exemplary MHC); Bir A tail (first exemplary peptide tag); and ABL1 (first exemplary peptide). Applicant is invited to elect additional MHC(s), peptide tag(s), and/or peptide(s), to be searched. Additional MHC(s), peptide tag(s), and/or peptide(s) will be searched upon the payment of additional fees. It is believed that claims 1-2, 3 (in-part), 4-5, 6 (in-part), 7-8, 9-10 (each in-part), 12-13, 14 (in-part), 15-16, 17 (in-part), 18-19, 20-21 (each in-part), 23-27, 28 (in-part), 29-30, 31 (in-part), 32-36, 37 (in-part), 38-39, 40 (in-part), and 41-42 encompass this first named invention and thus these claims will be searched without fee to the extent that they encompass a fusion protein encompassing a human MHC H-2 Db (MHC); Bir A tail (peptide tag); and ABL1 (peptide). Applicants must specify the claims that encompass any additionally elected MHC(s), peptide tag(s), and/or peptide(s). Applicants must further indicate, if applicable, the claims which encompass the first named invention, if different than what was indicated above for this group. Failure to clearly identify how any paid additional invention fees are to be applied to the "+" group(s) will result in only the first claimed invention to be searched/examined. An exemplary election would be fusion protein comprising myc (peptide tag).

No technical features are shared between the MHCs, peptide tags, and/or peptides, of Groups I+ and, accordingly, these groups lack unity a priori.

Additionally, even if Groups I+ were considered to share the technical features including: a fusion protein comprising a peptide, a first flexible linker, a β 2-microglobulin domain, a second flexible linker, a soluble major histocompatibility complex (MHC) heavy chain and a peptide tag; a nucleic acid that expresses a fusion protein comprising a peptide, a first flexible linker, a β 2-microglobulin domain, a second flexible linker, a soluble major histocompatibility complex (MHC) heavy chain and a peptide tag; a method of making a soluble eukaryotic-derived peptide/MHC complex comprising: expressing in a cell a fusion protein comprising a peptide, a first flexible linker, a β 2-microglobulin domain, a second flexible linker, a soluble major histocompatibility complex (MHC) heavy chain and a peptide tag; a cell line expressing a fusion protein comprising a fusion protein comprising a peptide, a first flexible linker, a β 2-microglobulin domain, a second flexible linker, a soluble major histocompatibility complex (MHC) heavy chain and a peptide tag; these shared technical features are previously disclosed by the publication entitled 'Peptide- β 2-microglobulin-MHC fusion molecules bind antigen-specific T cells and can be used for multivalent MHC-Ig complexes' by Greten, et al. (hereinafter 'Greten') in view of US 7,074,905 B2 to Rhode, et al. (hereinafter 'Rhode').

Greten discloses a fusion protein (abstract; figure 1) comprising a peptide (figure 1B), a first flexible linker (linker comprising both glycine and serine; figure 1B), a β 2-microglobulin domain (figure 1B), a second flexible linker (linker comprising both glycine and serine; figure 1B), a major histocompatibility complex (MHC) heavy chain (figure 1B); a nucleic acid that expresses a fusion protein (abstract; page 126, second column, second paragraph) comprising a peptide (figure 1B), a first flexible linker (linker comprising both glycine and serine; figure 1B), a β 2-microglobulin domain (figure 1B), a second flexible linker (linker comprising both glycine and serine; figure 1B), a major histocompatibility complex (MHC) heavy chain (figure 1B); a method of making a eukaryotic-derived peptide/MHC complex (abstract; page 126, second column, second paragraph) comprising: expressing in a cell a fusion protein (abstract; page 126, second column, second paragraph) comprising a peptide (figure 1B), a first flexible linker (linker comprising both glycine and serine; figure 1B), a β 2-microglobulin domain (figure 1B), a second flexible linker (linker comprising both glycine and serine; figure 1B), a major histocompatibility complex (MHC) heavy chain (figure 1B); a cell line expressing a fusion protein (J588L cells (a cell line) expressing a fusion protein; abstract; page 126, second column, second paragraph) comprising a fusion protein (figure 1B) comprising a peptide (figure 1B), a first flexible linker (linker comprising both glycine and serine; figure 1B), a β 2-microglobulin domain (figure 1B), a second flexible linker (linker comprising both glycine and serine; figure 1B), and a major histocompatibility complex (MHC) heavy chain (figure 1B). Greten further discloses soluble MHC molecules (page 133, second column, second paragraph).

Greten does not disclose a peptide tag.

Rhode discloses a peptide tag (soluble MHC complexes comprising a tag; abstract; column 6, lines 41-45; column 27, lines 13-26).

It would have been obvious to one of ordinary skill in the art at the time of the invention to modify the disclosure of Greten, to include a peptide tag, as taught by Rhode, in order to provide a superior method for identifying the protein of interest.

Since none of the special technical features of the Groups I+ inventions is found in more than one of the inventions, and since all of the shared technical features are previously disclosed by a combination of the Greten and Rhode references, unity of invention is lacking.