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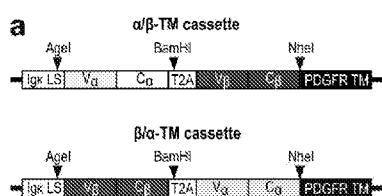
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(54) Title: HIGH AFFINITY ENGINEERED T-CELL RECEPTORS TARGETING CMV INFECTED CELLS

(57) Abstract: Provided herein are engineered T-cell receptors (TCRs) having nanomolar affinity for the immuno-dominant pp65 peptide residing between residues 495-503 (NLV) in complex with HLA-A2*02:01. The TCRs may be membrane-bound TCRs, soluble TCRs, chimeric TCRs, or chimeric antigen receptors. Also provided are methods of using the engineered TCRs to treat diseases, monitor disease progression, monitor vaccine efficacy, and detecting NLV/A2 presentation on the surface of cells.



FIGS. 1A

DESCRIPTION

HIGH AFFINITY ENGINEERED T-CELL RECEPTORS TARGETING CMV INFECTED CELLS

5 [0001] This application claims the benefit of United States Provisional Patent Application No. 62/804,642, filed February 12, 2019, the entirety of which is incorporated herein by reference.

 [0002] This invention was made with government support under Grant No. F32 GM111018 awarded by the National Institutes of Health and Grant No. DGE1610403 awarded
10 by the National Science Foundation. The government has certain rights in the invention.

BACKGROUND OF THE INVENTION

1. Field of the Invention

 [0003] The present invention relates generally to the field of medicine. More particularly, it concerns high affinity engineered T-cell receptors for detecting and targeting
15 CMV infected cells.

2. Description of Related Art

 [0004] Antibodies represent a large and growing class of successful therapeutics, by virtue of their abilities to tightly bind antigens that are secreted or expressed on a target cell surface. The structurally analogous T-cell receptor (TCR) provides access to a much wider
20 array of intracellular and extracellular antigens that are presented on a cell surface as proteolyzed peptides in complex with major histocompatibility complex (MHC). Like antibodies, TCR binding sites are formed by six complementarity determining regions (CDRs), three on the alpha variable domain and three on the beta variable domain with the CDR3 loops dominating TCR-peptide interactions (Rossjohn *et al.*, 2015). When a peptide-MHC complex
25 is recognized by the TCR expressed on a T cell, TCR activation, cytokine release, and cell killing can follow.

 [0005] There is considerable interest in using TCRs as therapeutics and reagents to monitor the presence of disease-related peptides. Engineered TCRs can be used in adoptive T-cell therapies to re-direct patient T cells to recognize a chosen target (Harris and Kranz, 2016),
30 while soluble TCRs can be used as antibody-like reagents to bind specific peptide MHC

complexes presented on a cell surface (Oates *et al.*, 2015). Proof-of-concept for these approaches has been demonstrated with TCRs targeting the immunodominant Gag epitope SL9 from HIV. When transduced into patient T cells, high affinity TCRs were able to control viral replication (Varela-Rohena *et al.*, 2008). When expressed as a soluble TCR linked to a CD3-specific single-chain antibody, the chimeric protein was able to redirect polyclonal CD8 T cells to kill CD4 T cells harboring reactivated HIV (Yang *et al.*, 2016).

[0006] Viral infection by cytomegalovirus (CMV) is also controlled by cytotoxic T cells in healthy individuals but causes disease in the very young, very old, and immunocompromised, with no vaccine candidate nearing licensure (Plotkin and Boppana, 2018). CMV-specific cytotoxic T cells primarily target peptides from the pp65 tegument protein, with the immuno-dominant peptide residing between residues 495-503 (sequence NLVPMVATV (SEQ ID NO: 41), hereafter called NLV) (Wills *et al.*, 1996; Saulquin *et al.*, 2000) in complex with HLA-A2*02:01 (hereafter called A2), the most common allele in North America. Adoptive transfer of NLV-specific T cells is sufficient to control infection in allogeneic hematopoietic stem cell transplantation patients with CMV infection (Feuchtinger *et al.*, 2010). Notably, NLV is presented on the infected cell surface early after infection, prior to *de novo* protein synthesis and in the presence of therapeutics blocking viral replication (Riddell *et al.*, 1991).

[0007] Identification of a validated, CMV-specific peptide-MHC complex suggests opportunities to monitor NLV-presenting cells, if an appropriate peptide-specific TCR is available. While hundreds of TCRs can recognize an immunodominant peptide, the NLV/A2 response is dominated by “public” clones whose CDR3 α and/ or CDR3 β sequences are shared among unrelated individuals (Trautmann *et al.*, 2005; Wang *et al.*, 2012). One of these, RA14, emerged as the dominant clone after rounds of immunosuppression and viral reactivation in a rheumatoid arthritis patient with asymptomatic CMV infection (Trautmann *et al.*, 2005). The RA14 CDR3 α contains the two most common public features observed in NLV-reactive TCRs: CDR3 α sequence x_nGNQF (where x_n indicates a variable number of residues), observed in 14% of all sequences across multiple donors and CDR3 β sequence Sx_nTGx_nYGY, observed in 13% of all sequences (Wang *et al.*, 2012). The RA14 TCR has been crystallized in complex with its ligand NLV/A2, revealing a typical TCR-pMHC binding interface characterized by high structural complementarity for the entire peptide (Trautmann *et al.*, 2009).

[0008] While RA14 appears to be an excellent candidate to monitor NLV/A2 presence and may be suitable for adoptive therapy applications, there are several limitations to using TCRs as soluble, antibody-like reagents. First, TCR-ligand binding affinities are much weaker than antibodies: RA14 has been reported to have a 6-30 μ M equilibrium affinity for NLV/A2 (Gras *et al.*, 2009; Gakamsky *et al.*, 2007), whereas antibody-ligand affinities are typically >1000-fold stronger (1-10 nM K_d). Second, despite considerable effort, soluble expression of TCRs continues to present challenges, with no generally successful strategies identified.

[0009] To address these shortcomings, TCRs have been engineered for increased stability, expression level, and affinity. This has been achieved using a single-chain format and yeast display (Shusta *et al.*, 1999; Holler *et al.*, 2000; Shusta *et al.*, 2000), but engineering of each unique TCR appears required to enable incorporation of properly folded protein into these formats (Aggen *et al.*, 2011). Phage display of the TCR extracellular regions with a stabilizing di-sulfide bond in between the constant domains (Boulter *et al.*, 2003) has also been used to identify several very high affinity human TCR variants (Varela-Rohena *et al.*, 2008; Li *et al.*, 2005; Liddy *et al.*, 2012). While successful, phage display lacks the eukaryotic protein folding machinery, which is likely required to allow expression of a greater range of TCRs and is not compatible with efficient FACS-based selection strategies. To produce soluble protein, single-chain or extracellular two-chain TCRs are most commonly refolded from bacterial inclusion bodies with varying levels of success (Aggen *et al.*, 2011). Some TCR sequences are amenable to soluble expression in bacteria with chaperone co-expression (Wulfig and Pluckthun, 1994; Maynard *et al.*, 2005; Gunnarsen *et al.*, 2013), in yeast (Shusta *et al.*, 1999), or in mammalian cells (Mosquera *et al.*, 2005; Walseng *et al.*, 2015; Foss *et al.*, 2016) but these represent only a fraction of TCRs of interest. Taken together, considerable effort is required to convert a TCR into a recombinant protein that can be used in biochemical assays or therapeutic methods.

SUMMARY OF THE INVENTION

[0010] In a first embodiment, there is provided an engineered T-cell receptor (TCR) comprising an alpha chain CDR3 having the amino acid sequence of any one of SEQ ID NOs: 1-7 and/or a beta chain CDR3 having the amino acid sequence of any one of SEQ ID NOs: 8-17. In some aspects, the engineered TCR is HLA-A2 restricted. In further aspects, the engineered TCR is HLA-A2*02:01 restricted. In other aspects, the engineered TCR specifically recognizes a peptide comprising an amino acid sequence of NLVPMVATV (SEQ ID NO: 41) in complex with HLA-A2*02:01. In certain aspects, the engineered TCR

comprises an alpha chain CDR3 having the amino acid sequence of SEQ ID NO: 2 and a beta chain CDR3 having the amino acid sequence of SEQ ID NO: 15. In several aspects, the engineered TCR comprises an alpha chain variable region having at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity to the amino acid sequence of any one of SEQ ID NOs: 18-24 and/or a beta chain variable region having at least 90% identity to the amino acid sequence of any one of SEQ ID NOs: 25-34.

[0011] In still further aspects, the engineered TCR comprises an alpha chain of any one of SEQ ID NOs: 18-24 and/or a beta chain of any one of SEQ ID NOs: 25-34. In specific aspects, the engineered TCR comprises an alpha chain of SEQ ID NO: 19 and a beta chain of SEQ ID NO: 32. In another aspect, the engineered TCR is further defined as a soluble TCR, wherein the soluble TCR does not comprise a transmembrane domain. In some aspects, the soluble TCR is linked to a CD3-specific antibody. In certain aspects, an extracellular domain of the TCR is fused to an antibody Fc domain. In several aspects, the alpha chain of the TCR extracellular domain is fused to the antibody Fc domain. In some particular aspects, the Fc domain is a human or mouse Fc domain. In another aspect, the engineered TCR further comprises an antibody hinge region.

[0012] In yet still further aspects, the engineered CAR is further defined as a chimeric antigen receptor. In another aspect, the engineered TCR further comprises a detectable label. In some aspects, the engineered TCR is covalently bound to a therapeutic agent. In some specific aspects, the therapeutic agent is an immunotoxin or a chemotherapeutic agent.

[0013] In an embodiment of the present disclosure, there is provided an engineered T-cell receptor (TCR) comprising an alpha chain CDR3 having the amino acid sequence of any one of SEQ ID NOs: 1-7 or 60-61 and/or a beta chain CDR3 having the amino acid sequence of any one of SEQ ID NOs: 8-17 or 62-63.

[0014] In some aspects, the engineered TCR is HLA-A2 restricted, such as HLA-A2*02:01 restricted. In particular aspects, the engineered TCR specifically recognizes a peptide comprising an amino acid sequence of NLVPMVATV (SEQ ID NO: 41) in complex with HLA-A2*02:01.

[0015] In certain aspects, the engineered TCR comprises an alpha chain CDR3 having the amino acid sequence of SEQ ID NO: 2 and a beta chain CDR3 having the amino acid sequence of SEQ ID NO: 15. In some aspects, the engineered TCR comprises an alpha chain

CDR3 having the amino acid sequence of SEQ ID NO: 60 and a beta chain CDR3 having the amino acid sequence of SEQ ID NO: 62. In particular aspects, the engineered TCR comprises an alpha chain CDR3 having the amino acid sequence of SEQ ID NO: 61 and a beta chain CDR3 having the amino acid sequence of SEQ ID NO: 63.

5 **[0016]** In some aspects, the engineered TCR comprises an alpha chain CDR1 of Table 1A-2 or Table 1C. In specific aspects, the engineered TCR comprises an alpha chain CDR1; CDR2 and CDR3 of Table 1C. In further aspects, the engineered TCR comprises a beta chain CDR1; CDR2 and CDR3 of Table 1D. For example, the engineered TCR may comprise an alpha chain CDR1; CDR2 and CDR3 of Table 1C; and a beta chain CDR1; CDR2 and CDR3
10 of Table 1D.

[0017] In certain aspects, the engineered TCR comprises an alpha chain variable region having at least 90% (e.g., 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99%) identity to the amino acid sequence of any one of those in Table 1C and/or a beta chain variable region having at least 90% (e.g., 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99%) identity to the amino
15 acid sequence of any one of those in Table 1D. In particular aspects, the engineered TCR comprises an alpha chain of any one of those in Table 1C and/or a beta chain of any one of those in Table 1D. In one specific aspect, the engineered TCR comprises an alpha chain of SEQ ID NO: 19 and a beta chain of SEQ ID NO: 32.

[0018] In some aspects, the engineered TCR is further defined as a soluble TCR,
20 wherein the soluble TCR does not comprise a transmembrane domain. In further aspects, the soluble TCR is linked to a CD3-specific antibody. In some aspects, an extracellular domain of the TCR is fused to an antibody Fc domain. For example, the alpha chain of the TCR extracellular domain may be fused to the antibody Fc domain. The Fc domain may be a human or mouse Fc domain. In additional aspects, the TCR further comprises an antibody hinge
25 region.

[0019] In certain aspects, the Fc domain exhibits reduced binding a viral Fc receptor (vFcR) relative to a wt Fc. In some aspects, the vFcR comprises CMV gp34 and/or gp68. In particular aspects, the vFcR is able to bind to human FcRIIIa and/or FcRn. In specific aspects, the Fc domain comprises one or more substitutions relative to wt Fc domain selected from a
30 substitution at S337; K334; G341; E345; T350; K338; H268; E294 and Q311. In some aspects, the Fc domain comprises substitutions selected from: (a) S337; (b) S337 and G341; (c) S337;

E345; and T350; (d) K334; and K338; (e) S337 and T350; (f) H268; and K334; and (g) H268; E294; Q311; and K334. In certain aspects, the Fc domain comprises one or more substitutions relative to wt Fc domain selected from S337F; K334E; G341E; E345K; T350I; K338I; H268L; E294K and Q311L, wherein the engineered Fc domain exhibits reduced binding a viral Fc receptor (vFcR) relative to a wt Fc. In some aspects, the Fc domain comprises substitutions selected from: (a) S337F; (b) S337F and G341E; (c) S337F; E345K; and T350I; (d) K334E; and K338I; (e) S337F and T350I; (f) H268L; and K334E; and (g) H268L; E294K; Q311L; and K334E. In specific aspects, the Fc domain comprises wherein the antibody Fc domain comprises one or more substitution selected from M252Y; S254T and T256E. In some aspects, the engineered antibody Fc domain comprises the following substitutions M252Y; S254T and T256E.

[0020] In some aspects, the engineered TCR is further defined as a chimeric antigen receptor.

[0021] In additional aspects, the TCR further comprises a detectable label. In some aspects, the engineered TCR is covalently bound to a therapeutic agent, such as an immunotoxin or a chemotherapeutic agent.

[0022] A further embodiment provides a polynucleotide encoding the engineered TCR of the present embodiment and aspects thereof (e.g., an engineered T-cell receptor (TCR) comprising an alpha chain CDR3 having the amino acid sequence of any one of SEQ ID NOs: 1-7 or 60-61 and/or a beta chain CDR3 having the amino acid sequence of any one of SEQ ID NOs: 8-17 or 62-63).

[0023] Also provided herein is an expression vector encoding the engineered TCR of the present embodiment and aspects thereof (e.g., an engineered T-cell receptor (TCR) comprising an alpha chain CDR3 having the amino acid sequence of any one of SEQ ID NOs: 1-7 or 60-61 and/or a beta chain CDR3 having the amino acid sequence of any one of SEQ ID NOs: 8-17 or 62-63). In some aspects, the sequence encoding the TCR is under the control of a promoter. The expression vector may be a viral vector, such as a retroviral vector. In other aspects, the expression vector is an episomal vector. In additional aspects, the vector further encodes a linker domain. The linker domain may be positioned between the alpha chain and beta chain. In some aspects, the linker domain comprises one or more cleavage sites, such as a

Furin cleavage site (e.g., RRKR) and/or a P2A cleavage site. In certain aspects, the one or more cleavage sites are separated by a spacer, such as SGSG or GSG.

[0024] Another embodiment provides a cell engineered to express the TCR of the present embodiment and aspects thereof (e.g., an engineered T-cell receptor (TCR) comprising an alpha chain CDR3 having the amino acid sequence of any one of SEQ ID NOs: 1-7 or 60-61 and/or a beta chain CDR3 having the amino acid sequence of any one of SEQ ID NOs: 8-17 or 62-63). In some aspects, the cell is a T cell, NK cell, invariant NK cell, NKT cell, mesenchymal stem cell (MSC), or induced pluripotent stem (iPS) cell. In particular aspects, the cell is an immune cell. The cell may be isolated from an umbilical cord. In particular aspects, the T cell is a CD8⁺ T cell, CD4⁺ T cell, or $\gamma\delta$ T cell. In some aspects, the T cell is a regulatory T cell (Treg).

[0025] A further embodiment provide a method for engineering the cell of the present embodiments or aspects thereof comprising contacting said immune cell with the TCR of the present embodiment and aspects thereof (e.g., an engineered T-cell receptor (TCR) comprising an alpha chain CDR3 having the amino acid sequence of any one of SEQ ID NOs: 1-7 or 60-61 and/or a beta chain CDR3 having the amino acid sequence of any one of SEQ ID NOs: 8-17 or 62-63) or the expression vector of the present embodiments or aspects thereof. In some aspects, the immune cell is a T cell, peripheral blood lymphocyte, NK cell, invariant NK cell, or NKT cell. In additional aspects, contacting is further defined as transfecting or transducing. In some aspects, transfecting comprises electroporating RNA encoding the TCR of the present embodiments into the immune cell. In further aspects, the method further comprises generating viral supernatant from the expression vector of the present embodiments prior to transducing the immune cell. In some aspects, the immune cell is a stimulated lymphocyte, such as a human lymphocyte. In particular aspects, stimulating comprises contacting the immune cell with or incubating the immune cell in OKT3 and/or IL-2. In additional aspects, the method further comprises sorting the immune cells to isolate TCR engineered T cells. In some aspects, the method further comprising performing T cell cloning by serial dilution. In certain aspects, the method further comprises expansion of the T cell clone by the rapid expansion protocol.

[0026] In another embodiment there is provided a polynucleotide encoding the engineered TCR of any of the embodiments and aspects described above.

[0027] Still a further embodiment of the invention provides a cell engineered to express the TCR of any of the embodiments and aspects described above. In some aspects, the cell is a T cell, NK cell, invariant NK cell, NKT cell, mesenchymal stem cell (MSC), or induced pluripotent stem (iPS) cell. In certain aspects, the cell is an immune cell. In another aspect, the cell is isolated from an umbilical cord. In several aspects, the T cell is a CD8⁺ T cell, CD4⁺ T cell, or $\gamma\delta$ T cell. In other aspects, the T cell is a regulatory T cell (Treg).

[0028] In still yet a further embodiment, there is provided a method for engineering the cell of any of the embodiments and aspects described above, comprising contacting said immune cell with the TCR of any of the embodiments and aspects described above or the expression vector of any of the embodiments and aspects described above. In certain aspects, the immune cell is a T cell, peripheral blood lymphocyte, NK cell, invariant NK cell, or NKT cell. In another aspect, contacting is further defined as transfecting or transducing. In some aspects, transfecting comprises electroporating RNA encoding the TCR of any of the embodiments and aspects described above into the immune cell. In additional aspects, the method further comprises generating viral supernatant from the expression vector of the

[0029] In further aspects, the immune cell is a stimulated lymphocyte. In some aspects, the stimulated lymphocyte is a human lymphocyte. In several aspects, stimulating comprises contacting the immune cell with or incubating the immune cell in OKT3 and/or IL-2. In additional aspects, the method further comprises sorting the immune cells to isolate TCR engineered T cells. In still further aspects, the method also comprises performing T cell cloning by serial dilution. In another aspects, the method further comprises expansion of the T cell clone by the rapid expansion protocol.

[0030] Yet a further embodiment provides use of a therapeutically effective amount of NLV/A2 TCR-specific cells according to any of the embodiments and aspects described above for the treatment of a disease or disorder in a patient. In certain aspects, the NLV/A2 TCR-specific cells are T cells.

[0031] Still a further embodiment of the invention provides use of a therapeutically effective amount of a soluble TCR according to any of the embodiments and aspects described above for the treatment of a disease or disorder in a patient.

[0032] In yet still a further embodiment of the invention there is provided a method of treating a disease or disorder in a subject in need thereof, monitoring disease progression in a subject, or monitoring vaccine efficacy in a subject, the method comprising administering an effective amount of the TCR-engineered cells of any of the embodiments and aspects described above or a soluble TCR according to any of the embodiments and aspects described above to the subject. In certain aspects, the subject has been identified as having an HLA-A2*02:01 allele. In some aspects, the TCR-engineered cell is a T cell or peripheral blood lymphocyte. In additional aspects, the T cell is a CD8⁺ T cell, CD4⁺ T cell, or Treg. In specific aspects, the cell is autologous. In other aspects, the cell is allogeneic. In certain aspects, the disease is a cancer. In a particular aspect, the cancer is a glioblastoma. In some aspects, an NLV peptide has been delivered to cells of the cancer. In several aspects, the subject is a human. In additional aspects, the cells are administered to the subject intravenously, intraperitoneally, or intratumorally. In certain aspects, the method further comprises administering a second anticancer therapy. In some aspects, the second anticancer therapy is a chemotherapy, immunotherapy, surgery, radiotherapy, or biotherapy. In certain aspects, the immunotherapy is an immune checkpoint inhibitor. In another aspect, the immune checkpoint inhibitor inhibits an immune checkpoint protein or ligand thereof selected from the group consisting of CTLA-4, PD-1, PD-L1, PD-L2, LAG-3, BTLA, B7H3, B7H4, TIM3, KIR, or adenosine A2a receptor (A2aR). In several aspects, the immune checkpoint inhibitor inhibits PD-1 or CTLA-4. In some aspects, the patient has been determined to have a cancer with a reactivated CMV infection.

[0033] Still a further embodiment provides a method of detecting NLV/A2 complexes on cells, the method comprising contacting the cells with a soluble TCR of any one of claims 11-16 and 18 and detecting binding of the soluble TCR to the cells. In certain aspects, the soluble TCR comprises a mouse Fc domain. In some aspects, the cells are peptide-pulsed antigen presenting cells. In other aspects, the antigen presenting cells have been pulsed with a peptide comprising an amino acid sequence of NLVPMVATV (SEQ ID NO: 41).

[0034] Yet still a further embodiment provides an immunoglobulin fusion complex comprising: (i) an α TCR variable domain fused to a first antibody Fc domain, wherein α TCR variable domain is bound to a β TCR variable domain by a disulfide bond; or (ii) a β TCR variable domain fused to a first antibody Fc domain, wherein β TCR variable domain is bound to an α TCR variable domain by a disulfide bond, wherein the α TCR variable domain and the

β TCR variable domain comprise amino acid substitutions that block N-linked glycosylation. In some aspects, the first antibody Fc domain is bound to a second antibody Fc domain by a disulfide bond. In certain aspects, the second antibody Fc domain is fused to an antibody heavy chain variable domain that is bound to an antibody light chain by a disulfide bond. In several

 5 aspects, the complex further comprises two immunoglobulin fusion complexes that are bound by disulfide bonds between the Fc domains. In some aspects, the second Fc domain is fused to a targeting moiety. In certain aspects, the targeting moiety is an antibody VH/VL domain. In another aspect, the targeting moiety binds to immune effector cells. In some aspects, the targeting moiety binds to CD3. In further aspects, the α TCR variable domain is bound to a

 10 β TCR variable domain by two disulfide bonds. In certain aspects, the α TCR variable domain and the β TCR variable domain comprise N to Q amino acid substitutions that block N-linked glycosylation. In other aspects, the α TCR variable domain and the β TCR variable domain specifically bind to a peptide in complex with HLA. In some specific aspects, the HLA is HLA-A2. In certain aspects, the peptide in complex with HLA is a cancer protein peptide, an

 15 autoimmune peptide or an infectious disease peptide. In another aspect, the peptide is a CMV peptide. In some particular aspects, the CDR3 domains of the TCR variable domains are those recited in the first embodiment described above.

[0035] In yet a further embodiment, there is provided a method of producing a TCR complex comprising (a) expressing one or more nucleic acid molecules in a mammalian cell

 20 encoding: (i) an α TCR variable domain fused to a first antibody Fc domain, wherein α TCR variable domain is bound to a β TCR variable domain by a disulfide bond; or (ii) a β TCR variable domain fused to a first antibody Fc domain, wherein β TCR variable domain is bound to an α TCR variable domain by a disulfide bond, wherein the α TCR variable domain and the β TCR variable domain comprise amino acid substitutions that block N-linked glycosylation;

 25 and (b) collecting or purifying the TCR complex. In some aspects, the mammalian cell is CHO cell. Also provided herein is a TCR complex produced by the present methods.

[0036] Further provided herein is an engineered antibody Fc domain comprising one or more substitutions relative to wt Fc domain selected from a substitution at S337; K334; G341; E345; T350; K338; H268; E294 and Q311, wherein the engineered Fc domain exhibits

 30 reduced binding a viral Fc receptor (vFcR) relative to a wt Fc. In some aspects, the Fc domain comprises substitutions selected from: (a) S337; (b) S337 and G341; (c) S337; E345; and T350; (d) K334; and K338; (e) S337 and T350; (f) H268; and K334; and (g) H268; E294; Q311; and

K334. In certain aspects, the Fc domain comprises one or more substitutions relative to wt Fc domain selected from S337F; K334E; G341E; E345K; T350I; K338I; H268L; E294K and Q311L, wherein the engineered Fc domain exhibits reduced binding a viral Fc receptor (vFcR) relative to a wt Fc. In certain aspects, the Fc domain comprises substitutions selected from: (a) S337F; (b) S337F and G341E; (c) S337F; E345K; and T350I; (d) K334E; and K338I; (e) S337F and T350I; (f) H268L; and K334E; and (g) H268L; E294K; Q311L; and K334E. In some aspects, the vFcR comprises CMV gp34 and/or gp68. For example, the vFcR may be CMV gp34. In particular aspects, the engineered Fc domain is able to bind to human FcRIIIa and/or FcRn. In some aspects, the engineered Fc domain comprises a CH1, CH2 and CH3 domain at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, or 95% identical to a human IgG1 CH1, CH2 and CH3. In some aspects, the engineered Fc domain is an IgG1 Fc domain or an IgG2 Fc domain. In some aspects, the engineered Fc domain is at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, or 95% identical to any one of SEQ ID NOs: 53-60. In additional aspects, the method Fc domain further comprises one or more substitutions selected from M252; S254 and T256. In some aspects, the Fc domain further comprises a substitution at M252; S254 and T256. In certain aspects, the Fc domain further comprises one or more substitution selected from M252Y; S254T and T256E. In some aspects, the Fc domain further comprises the following substitutions M252Y; S254T and T256E. In some aspects, the Fc domain further comprises an antibody variable domain or a TCR variable domain. The engineered Fc domain may be fused or conjugated to antibody variable domains or TCR variable domains that bind to a target of interest, such as a viral protein or a viral peptide bound to HLA. In some aspects, the target of interest is a CMV protein or a CMV peptide bound to HLA. In particular aspects, the engineered Fc domain is fused or conjugated to TCR variable domains that bind to NLVPMVATV (SEQ ID NO: 41) in complex with HLA-A2*02:01. Also provided herein is nucleic acid molecule encoding an engineered antibody Fc domain of the present embodiments and aspects thereof. Further provided herein is host cell expressing a nucleic acid molecule of the present embodiments. For example, the cell is a CHO cell.

[0037] Another embodiment provides a method of targeting a CMV infected cell comprising contacting the cell with molecule that binds to a CMV polypeptide or a CMV peptide bound to HLA, wherein the molecule is fused or conjugated to an engineered antibody Fc domain comprising one or more substitutions relative to wt Fc domain selected from a substitution at S337; K334; G341; E345; T350; K338; H268; E294; Q311; M252; S254; and T256, wherein the engineered Fc domain exhibits reduced binding a viral Fc receptor (vFcR)

relative to a wt Fc. In some aspects, the engineered antibody Fc domain comprises one or more substitution selected from M252Y; S254T and T256E. In certain aspects, the engineered antibody Fc domain comprises the following substitutions M252Y; S254T and T256E. In some aspects, the engineered antibody Fc domain is a domain in accordance with the present
5 embodiments or aspects thereof.

[0038] As used herein, “essentially free,” in terms of a specified component, is used herein to mean that none of the specified component has been purposefully formulated into a composition and/or is present only as a contaminant or in trace amounts. The total amount of the specified component resulting from any unintended contamination of a composition is
10 preferably below 0.01%. Most preferred is a composition in which no amount of the specified component can be detected with standard analytical methods.

[0039] As used herein in the specification and claims, “a” or “an” may mean one or more. As used herein in the specification and claims, when used in conjunction with the word “comprising”, the words “a” or “an” may mean one or more than one. As used herein, in the
15 specification and claim, “another” or “a further” may mean at least a second or more.

[0040] As used herein in the specification and claims, 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.

[0041] Other objects, features and advantages of the present invention will become
20 apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples, while indicating certain embodiments of the invention, are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

BRIEF DESCRIPTION OF THE DRAWINGS

[0042] The following drawings form part of the present specification and are included to further demonstrate certain aspects of the present invention. The invention may be better understood by reference to one or more of these drawings in combination with the detailed description of specific embodiments presented herein.

[0043] **FIGS. 1A-1C:** The NLV-specific human TCR RA14 displays on the surface of CHO-K1 cells. (FIG. 1A) The RA14 variable and constant regions were cloned in-frame with the mouse/human Ig heavy leader sequence (LS), a T2A peptide for cleavage, and the PDGFR transmembrane region (TM) with either the alpha (α/β -TM) or the beta chain (β/α -TM) in the first position. The cassettes were then cloned into a pcDNA3 mammalian expression vector. (FIG. 1B) Display of functional RA14 TCR was detected with a dual-stain approach, with an anti-V β 6-5 antibody-PE conjugate detecting expression of the TCR beta chain, and a peptide/A2 tetramer conjugated to APC used to assess ligand binding. (FIG. 1C) Plasmids encoding the TCR in both chain orientations and with the wild-type (WT) or engineered disulfide (ds) constant regions were transfected, stained two days later, and assayed for APC and PE signal via flow cytometry. Rainbow dots depict staining using a tetramer with the specific NLV peptide, while grey dots depict staining with a tetramer containing a control peptide from HCV. Control transfections without plasmid and with a plasmid lacking the alpha chain are also shown.

[0044] **FIGS. 2A-2B:** Structural interactions between RA14 CDR3 loops and NLV peptide/ A2. The crystal structure of the RA14 TCR complexed with NLV/A2 (PDB 3GSN) was used to guide library design. The A2 surface is shown in grey space-fill, the NLV peptide surface in pink space-fill and the RA14 structure in purple ribbon. The residues comprising the CDR3 α (SEQ ID NO: 39) (FIG. 2A) and CDR3 β (SEQ ID NO: 40) loops (FIG. 2B) are listed (IMGT definition), with those targeted for mutagenesis highlighted in red in the text and in the structure. Boxed residues form direct pMHC contacts in the wild-type crystal as analyzed by (Gras *et al.*, 2009).

[0045] **FIGS. 3A-3D:** RA14 variants with improved tetramer binding can be isolated by CHO display. The TCR display cassette optimized in FIG. 1 was mutagenized into two libraries following the strategy in FIG. 2, and cloned into a pPy vector to allow episomal maintenance (see U.S. Patent No. 7,919,270). The CDR3 α (FIG. 3A) and CDR3 β (FIG. 3B) libraries were separately transfected into CHO-T cells, stained and sorted over three rounds to enrich for improved tetramer binding. Untransfected cells and cells displaying Fab hu1B7 (Nguyen *et al.*, 2015) are shown as controls. The gate drawn is representative of the sorting gate used in round three, with the percentage of cells falling into the gate noted in red to facilitate comparisons. Individual clones from the round three sorts of the CDR3 α (FIG. 3C) and CDR3 β (FIG. 3D) libraries were re-transfected, stained, and checked for improved specific

tetramer binding compared to the wild-type sequence. Specific tetramer binding refers to the ratio of the AF647 signal (tetramer binding) to the PE signal (anti-TCR β display) calculated on a per-cell basis. The median fluorescence intensity for this new variable was normalized to the wild-type. Variants selected for further characterization are indicated by an asterisk (*). In this initial screening experiment there were no replicate transfections, therefore no error bars are indicated.

[0046] FIGS. 4A-4B: Combining selected CDR3 variants further improves NLV/A2 tetramer staining and activation. (FIG. 4A) The wild-type and improved alpha-chain variants ($\alpha 1$ and $\alpha 2$) were cloned in combination with the wild-type and improved beta-chain variants ($\beta V1$, $\beta V4$, $\beta V7$, $\beta V8$) in the display format and expressed in CHO-T cells. After two days, cells were stained and analyzed by flow cytometry, with specific tetramer binding quantified as in Figure 3. The standard deviation is shown for combinations that were tested multiple times. (FIG. 4B) Activation of human Jurkat T cells expressing RA14 and selected variants was measured by CD69 upregulation. Transfected cells were co-cultured with peptide-pulsed human T2 antigen-presenting cells. After 24 hours, TCR-positive cells (NLV-tetramer-binding and V β -positive) were further monitored for CD69 upregulation using anti-CD69- FITC antibody. Data shows the average and standard deviation of three independent experiments. ANOVA was used to compare tetramer MFI for each clone combination (****p<0.001).

[0047] FIGS. 5A-5C: Engineered RA14 TCR2ds-huFc variants show increased affinity for NLV/A2. (FIG. 5A) TCR2ds-huFc formats of each variant were purified by protein A and size-exclusion chromatography to isolate only the intact protein. Protein purity was evaluated by non-reduced and reduced 4-20% gradient SDS-PAGE gel (3 μ g protein per lane). (FIG. 5B) The tetramer binding activities of purified RA14 variants were compared by ELISA. Plates were coated with NLV/A2 tetramer, followed by TCR2ds-huFc and goat-anti-human Fc-HRP. Data shown are the average and range of duplicate series for a representative experiment; this was repeated several times with similar results. (FIG. 5C) The binding kinetics were measured by SPR. Each TCR2ds-huFc variant was immobilized on a CM5 chip at 2000-5000 RUs, after which monomeric NLV/A2 was injected at six concentrations between 3.9-500 nM. An in-line blank flow cell was used to assess background binding. Peptide specificity was evaluated with injections of monomeric HCV/ A2 at the maximum concentration used for NLV/A2 for each variant. All injections were performed in duplicate; shown are the data and fits with the kinetics reported in Table2.

[0048] FIGS. 6A-6D: Equilibrium binding affinity of RA14 and variants for NLV/ A2. The wild-type RA14 TCR2ds-huFc fusion and variants were immobilized on a CM5 chip at 2000-5000 RUs before injecting monomeric NLV/ A2 at concentrations ranging from 7.9 to 500 nM at a flowrate of 30 ul/min. The observed equilibrium response values were plotted against concentration using BIAevaluation 3.0 software. The equilibrium affinity Kd was calculated using least-squares fit to the data, assuming a 1:1 stoichiometry; numerical values listed in Table 2. No binding was observed with the control HCV/ A2 monomer at the highest concentrations used for each variant (shown in FIG. 7).

[0049] FIGS. 7A-7D: Expression and stability of the wild-type RA14 TCR as a soluble Fc fusion protein. (FIG. 7A) Several iterations of the TCR-Fc fusion protein were designed. In all scaffolds, the TCR α chain was fused to a human IgG1 hinge and Fc. Open circles represent native glycosylation sites. Additional modifications include a second disulfide bond (ds, green) in the TCR2ds-huFc format and the removal of predicted N-linked glycosylation sites (Δ gly, blue crosses) in the TCR2ds Δ gly-huFc format. (FIG. 7B) The purity of each protein A purified design was evaluated via reducing and non-reducing 4-20% gradient SDS-PAGE, with 3 μ g loaded per lane. (FIG. 7C) Protein homogeneity was analyzed by size exclusion chromatography. The arrow indicates the major peak collected for experiments using a glycosylated scaffold. Triangles indicate elution volumes for molecular weight standards: thyroglobulin with a 669 kDa size eluted at 8.65 ml; ferritin 440 kDa at 10.45 ml; aldolase 158 kDa at 12.77 ml; conalbumin 75 kDa at 14.32 ml; ovalbumin 44 kDa at 15.27 ml; carbonic anhydrase 29 kDa at 16.68 ml. Representative data are shown for each design. (FIG. 7D) The melting temperature of each design was analyzed by differential scanning fluorimetry. An average value for two separate normalized curves shown for each scaffold, from a representative experiment. For FIG. 7C and FIG. 7D, dotted black lines correspond to the TCR-Fc scaffold, the dashed green lines correspond to the TCR2ds-huFc scaffold, and the solid blue lines are used for the TCR2ds Δ gly-huFc scaffold.

[0050] FIGS. 8A-8B: ELISA shows no impact of TCR2ds-huFc de-glycosylation on pMHC binding. The tetramer binding activities of RA14 variants genetically deglycosylated (TCR2ds Δ gly-huFc) versus unmodified (TCR2ds-huFc) were compared by ELISA. Plates were coated with NLV/A2 tetramer at 1 μ g/mL, followed by serially diluted TCR2ds-huFc or TCR2ds Δ gly-huFc and 1:1000 goat-anti-human Fc-HRP. Data shown are the average and range of duplicate series; the experiment was repeated several times with similar results. No

coat and an irrelevant peptide (HCV/A2 tetramer) controls were performed in parallel and showed no binding.

[0051] FIGS. 9A-9C: High affinity TCR-mFc proteins bind peptide-pulsed antigen presenting cells. (FIG. 9A) The wild-type (WT) RA14 and improved variants were expressed as TCR2ds-mFc proteins with mouse IgG2a hinge and Fc domains to reduce binding to human Fc receptors expressed on T2 cells. SDS-PAGE was used to confirm protein purity (3 µg per lane). (FIG. 9B) Human T2 antigen presenting cells were pulsed overnight with 100 µM NLV or HCV peptide and stained with 1 µM TCR2ds-mFc and 1:500 anti-mouse Fc-AF647 before flow cytometric analysis. This experiment was performed twice with similar results. (FIG. 9C) Human T2 antigen presenting cells were pulsed overnight with NLV at 0, 0.5, 1, 5, 10, 25, 50, 100, 250, and 500 µM NLV or HCV peptide at 500 µM and stained with 1 µM αV2.βV8 in the TCR2dsΔgly-mFc format and 1:500 anti-mouse Fc-AF647 before flow cytometric analysis. This experiment was performed twice with similar results. Binding was significant over signals observed for HCV pulsed T2 cells to NLV peptide concentrations as low as 0.5 µM.

[0052] FIGS. 10A-10C: Genetic de-glycosylation of TCR2ds-huFc fusion proteins yields monodisperse protein. The purity of TCR2dsΔgly-huFc (solid lines) and TCR2ds-huFc (dashed lines) proteins for affinity matured RA14 variants was analyzed by size exclusion chromatography. Protein A purified protein was injected (100 µg in 100 µl) onto a Superdex S200 column on an Äkta FPLC. Triangles indicate elution volumes for the molecular weight standards: thyroglobulin with a 669 kDa size eluted at 8.65 ml; ferritin 440 kDa at 10.45 ml; aldolase 158 kDa at 12.77 ml; conalbumin 75 kDa at 14.32 ml; ovalbumin 44 kDa at 15.27 ml; carbonic anhydrase 29 kDa at 16.68 ml. Representative data are shown for each TCR variant in each format.

[0053] FIG. 11: Schematic representation of improved RA14 TCR variant, 341 (SEQ ID NO: 52) having the transmembrane linker on the alpha chain and eliminating the predicted glycosylation sites with N→Q amino acid substitutions.

[0054] FIG. 12: FACS comparison of α2β8 and 341 against the corresponding wild-type RA14.

[0055] FIG. 13: Graphical representation of enhancement in binding/expression (AF647/PE) shown in FIG. 12.

[0056] FIG. 14A-C: Expression and purification of the human Ob.1A12 TCR as a TCR-human Fc fusion protein from CHO cells. Human Ob 1A12 was isolated from a patient with multiple sclerosis. The protein was expressed as a TCR-human Fc fusion in CHO cells. In these constructs, the α chain was fused to human Fc. Both constant chains but not the variable domains were glycosylated. **A**, SDS-PAGE protein gel. Light chain (β): 30 kDa Heavy Chain (α Fc): 51kDa. **B**, Size exclusion chromatography using an S200 column on an Akta FPLC shows homogenous product. Molecular weight markers suggest size is similar to that expected for a glycosylated protein (~160 kDa plus sugars). **C**, ELISA demonstrates specific binding of the purified Ob.1A12 TCR-Fc protein to its corresponding peptide-MHC complex. Plates were coated with 85-99MBP/ DRB1*1501 protein or MHC with the control peptide CLIP, CLIP/ DRB1*1501 (both obtained from the NIH tetramer facility), purified TCR added and detected with goat anti-human Fc-HRP.

[0057] FIG. 15: Sequence changes in the CDRs conferring improved pp65/ A2 binding affinity to the RA14 TCR. Positions indicated by arrows are of greatest interest; no changes were identified outside these regions.

[0058] FIG. 16A-C: Selected variants show excellent expression characteristics and improved pp65/ A2 affinity. Selected TCR variants were expressed in CHO cells as TCR-mouse Fc fusion proteins and purified by protein A chromatography. **A**, SDS-PAGE gel shows protein purity and mono-dispersity, as well as the expected molecular weights under non-reducing (~150 kDa) and reducing conditions (~50 and 25 kDa bands). **B**, Protein monodispersity was evaluated using analytical size exclusion chromatography with an S200 Superdex column on an Akta FPLC (30 μ g each protein was injected, except 100 μ g was used for the α 2 β 8 TCR-mFc). **C**, An ELISA in which dilutions of the purified TCR-Fc proteins were used for coating, followed by monomeric pp65/A2 and streptavidin-HRP for detection shows that selected variants exhibit affinity increases, as measured by the TCR-Fc concentration resulting in 50% of the maximum ELISA signal. Controls include uncoated wells and an irrelevant peptide/A2 complex.

[0059] FIG. 17: Binding kinetics for selected TCR-Fc variants with the pp65/A2 ligand. SEC-purified TCR-Fc was immobilized on CM5 chips at ~500 RU. The monomeric pp65/ A2 was injected at concentrations ranging from 0.1 – 500 nM. The control pMHC complex, the GIL peptide from HCV complexed with the A2 MHC, was injected at the highest

concentration, 500 nM. Binding kinetics were determined using a 1:1 Langmuir fit. Experiments performed on a BIAcore X100 SPR instrument.

[0060] FIG. 18: Increased TCR affinity results in increased selectivity of binding to pp65 peptide-pulsed antigen presenting cells. Selected TCR variants were expressed in CHO cells as TCR-mouse Fc fusion proteins and purified. These were then used to stain human T2 antigen presenting cells which were pulsed with 100 μ M pp65 or control HCV peptide and detected with anti-mouse Fc (F(ab)2-AF647 in the presence of Fc block. The observed population mean fluorescence shift correlates with the TCR affinity.

[0061] FIG. 19: Engineered TCR sequences activate T cells in the presence of pp65-peptide-pulsed antigen presenting cells. Selected RA14 TCR sequences were expressed with the five described N-to-Q substitutions that eliminate N-linked glycosylation sites on the surface of human Jurkat cells as well as native TCR transmembrane and intracellular sequences. These cells were co-cultured w/ human T2 antigen presenting cells pulsed with NLV (pp65) or KLV (control flu) peptides and the resulting T cell activation monitored by CD69 upregulation & flow cytometry.

[0062] FIG. 20A-D: The vFcγRs gp34 and tgp68 were expressed in CHO cells and purified. Receptors were cloned from the AD169/GFP bacterial artificial chromosomal DNA, appended with strep and FLAG tags, and expressed transiently in CHO cells. **A**, After purification on a streptactin column, 4 μ g of reduced (R) and non-reduced (NR) protein was analyzed by SDS-PAGE. Each protein appeared as a single band and at approximately the correct size (66 kDa for glycosylated tgp68 and 68 kDa for glycosylated gp34). **B** and **C**, Protein monodispersity was assessed with size exclusion chromatography (SEC) using a Superdex200 column on an Akta FPLC and **D**, observed molecular weights for these glycosylated proteins estimated using the elution volumes of proteins with known sizes on the same S200 column.

[0063] FIG. 21A-C: Competition ELISAs indicate that gp34 and FcγRIIIA have overlapping epitopes and gp68 and FcRn have overlapping epitopes. **A**) Human IgG1 (purple shape was coated on high-binding plates. Receptor alone or mixed with competitor was added to plates and detected with anti-FLAG-HRP. **B**) Human IgG1 was coated on ELISA plates, and then a dilution series of FLAG-tagged FcRn was added and detected with anti-FLAG-HRP (negative control). Competition was performed by mixing each competitor (gp34,

5 tgp68, or excess IgG1) at 0.5 µg/ml (6.7 nM) with FcRn prior to addition to the plate. C) Human IgG1 was coated on ELISA plates, and then a dilution series of FLAG-tagged FcγRIIIA was added and detected with anti-FLAG-HRP (negative control). Competition was performed by mixing each competitor (gp34, tgp68, or excess IgG1) at 0.2 µg/ml (0.3 nM) with FcγRIIIA prior to addition to the plate.

[0064] FIG. 22A-B: Receptor binding data for the gp68 Fc escape variant YTE.

10 The YTE antibody changes (M252Y, S254T, T256E (Dall'Acqua *et al.* 2006) eliminate binding to the gp68 vFc receptor while actually increasing binding to the hist FcRn. Binding demonstrated using flow cytometry with yeast expressing the wild-type or YTE human IgG1 Fc as an Aga-2 fusion protein. Cells were then stained with an anti-human Fc-AF647 to monitor Fc display level and one of the four Fc receptors plus fluorescent detection (FcRn, FcγRIIIa, gp34 or gp68). Note, gp34 and gp68 bind Fc with nanomolar affinities while the host receptors bind with micromolar affinities; FcRn binding was monitored at low pH to increase binding affinity. YTE is known to slightly reduce binding to FcγRIIIa.

15 **[0065] FIG. 23A-B: Antibody Fc sequences that lack of binding to gp34 but retain binding to FcRn. A, sequence alignment from Kabat residues 220 to 353 of human IgG1. B, list of selected amino acid changes using Kabat numbering scheme.**

[0066] FIG. 24: Receptor binding data for selected gp34 Fc escape variants.

20 Variants selected by yeast display were analyzed for binding to gp34, human FcRn and human FcγRIIIa by flow cytometry. Yeast expressing the wild-type or selected human IgG1 Fc variants as an Aga-2 fusion protein were stained with each Fc receptor individually (FcRn, FcγRIIIa, gp34) followed by fluorescent detection. FcRn binding was monitored at low pH to increase binding affinity.

25 **[0067] FIG. 25A-C: Expression of the OT-II TCR-Fc in CHO cells followed by protein A chromatography purification. A, SDS-PAGE gel, B, size exclusion chromatography and C, ELISA with purified TCR-Fc protein.**

30 **[0068] FIG. 26A-C: DO11.10 and variants bind KJ1-26 as scTCR-Fc fusion proteins. Proteins were produced in CHO cells as scTCR-Fc fusion proteins using the Vβ-linker-Vα format and purified using protein A chromatography. A, Protein purity and size was assessed by SDS-PAGE under reducing and non-reducing conditions. B, Analytical**

SEC was used to assess the polydispersity of the purified protein preparations following seven days storage at 4°C using an S200 column. Shown are traces for DO11-Fc, 176-Fc, 817-Fc and the control 172.10-Fc. **C**, Binding to the KJ1-26 clonotypic antibody was assessed by ELISA. Purified scTCR-Fc proteins were titrated on KJ1-26 coated and blocked. Shown are DO11-Fc (circles), 176-Fc (squares), 817-Fc (triangles) and the 172.10-Fc control (inverted triangles). Shown are the mean and std dev for duplicates; data are representative of three replicate experiments.

[0069] FIG. 27A-C: DO11 and variants detect OVA/I-Ad on A20 antigen presenting cells as scTCR-Fc fusion proteins. **A**, Murine A20 cells were pulsed overnight with 100 µM OVA323-339 peptide or an equivalent volume of DMSO before staining with 0.5 µM of each TCR-Fc fusion variant and detection with anti-human Fc (AF-647) in the presence of Fc block. Shown are histograms resulting from staining with only the secondary antibody; the 172.10-Fc control, DO11-Fc, 176-Fc and 817-Fc. For all conditions, background staining to A20 cells without peptide pulsing are shown (hollow traces). **B**, The scTCR-Fc dissociation rates from peptide-pulsed cells were measured using flow cytometry. Cells were stained as above, with 10 µg/ml (KJ1-26 added at time zero to block scTCR rebinding. Temporal data were fit to a single exponential decay to calculate cellular off-rates. **C**, Tabulated off-rate data and half-life data ($t_{1/2} = \ln 2 / k_{off}$). Shown are the mean and std dev of replicates. Data are representative of 2-3 independent experiments. No comparisons were statistically significantly different by ANOVA.

DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS

[0070] While antibodies recognize secreted and surface-bound proteins, T-cell receptors (TCRs) recognize otherwise inaccessible disease-associated intracellular proteins when presented as a processed, nonameric peptide bound to a major histocompatibility complex (pMHC). TCRs have been primarily explored for cancer applications but could target infectious diseases, such as cytomegalovirus (CMV). However, TCRs are much more difficult to express and engineer than antibodies, and advanced methods are needed to enable their widespread use. Engineered TCRs can be used in adoptive T-cell therapies to re-direct patient T cells to recognize a chosen target, while soluble TCRs can be used as antibody-like reagents to bind specific peptide MHC complexes presented on a cell surface.

[0071] Provided herein are antibody-like reagents that specifically recognize CMV-infected cells by virtue of the immunodominant pp65 peptide in complex. These reagents have an increased affinity relative to a native TCR (from ~30 micromolar to 50 nanomolar) and a novel TCR-Fc format providing for very high expression levels. These reagents have multiple potential applications. For example, therapeutic uses of the engineered TCR include targeting brain cancers (*e.g.*, glioblastomas) where latent CMV is reactivated. Another use includes delivering the NLV peptide to cancer cells in a patient and then administering a therapeutic engineered TCR to the patient to detect the efficiency of NLV peptide delivery/display as well as re-target CMV-specific T cells. A therapeutic engineered TCR may be a bispecific molecule that binds to both NLV/A2 and to CD3, thereby re-targeting T cells to destroy virus-infected cells. In addition, the engineered TCRs may be used to monitor NLV/A2 expression on host cells, either *in vitro* or *in vivo*. This use includes monitoring NLV presentation after vaccination with novel CMV vaccines as well as detecting active infection in organ transplant recipients.

I. The Present Embodiments

[0072] Soluble TCRs have considerable potential as therapeutics and reagents to monitor disease progression or vaccine efficacy. In particular, T cells recognizing the immunodominant NLV peptide from CMV are sufficient to maintain clinical latency, suggesting NLV is a useful marker to monitor disease or vaccination status. Here, variants of the human CMV-specific TCR RA14 with nanomolar affinity for the cognate NLV/A2 complex were identified by selection on the CHO cell surface. These variants retained peptide selectivity and activity when expressed on the surface of human Jurkat T cells. Moreover, high level production of homogeneous protein was observed when the TCR domains were fused to an antibody Fc domain to create an antibody-like targeting molecule. This construct specifically detected NLV/A2 complexes on the surface of human antigen presenting cells at low display levels and provides proof-of-concept for a new TCR engineering strategy.

[0073] Since TCR engineering continues to be challenging with phage and yeast display platforms, it was reasoned that TCR expression on the near-native eukaryotic membrane might offer a more straightforward approach. High levels of active TCR were able to be displayed using a PDGFR transmembrane domain, modified only by inclusion of a previously described non-native disulfide bond between the TCR constant domains (Boulter *et al.*, 2003). Specific pMHC binding activity was measured as the ratio of NLV/A2 tetramer binding to TCR display level, with the wildtype RA14 showing a clear population of cells with

high specific activity (FIG. 1C). In contrast, phage or yeast display of scTCRs or paired TCR extracellular domains are restricted to well-behaved germline segments (*e.g.*, mouse TRBV13 and human TRAV12) (Li *et al.*, 2004; Maynard *et al.*, 2005) or require considerable engineering to achieve display of active protein, including identification of specific mutations and co-expression of chaperones (Maynard *et al.*, 2005; Gunnarsen *et al.*, 2013). Even so, variants with partially suppressed stop codons have been isolated, suggesting the proteins retain toxicity (Li *et al.*, 2005).

[0074] Eukaryotic display has been explored previously for TCR engineering. Importantly, these prior reports used completely native TCRs, with TCR extracellular, transmembrane, and intracellular signaling domains expressed on T cells (Kessels *et al.*, 2000; Chervin *et al.*, 2008; Malecek *et al.*, 2013). In contrast, use of the PDGFR transmembrane domain fused to just one TCR chain eliminated TCR dependence on CD3 co-expression, provided selection pressure for proper TCR assembly, and allowed the use of CHO cells. An episomal plasmid allowed the more cumbersome retroviral transfection system used in the earlier studies to be avoided while maintaining TCR expression for ~2 months, and while inclusion of a 2A peptide ensured equimolar expression of the alpha and beta chains. Since most therapeutic proteins are produced in CHO cells, this system allows for selection of TCRs with characteristics that are expected to be more predictive of the soluble protein when expressed in CHO cells. Importantly, this includes glycosylation sites that may affect ligand binding (Nguyen *et al.*, 2018) or TCR assembly (FIGS. 7A-D).

[0075] Eukaryotic display platforms have constrained library sizes due to their lower transfection efficiencies as compared to bacteria and yeast. Despite this, the three previously reported eukaryotic TCR libraries all produced interesting clones. Kessels *et al.* (2000) randomized seven codons in CDR3 β of the flu-specific F5 TCR to generate a library of $\sim 3 \times 10^4$ unique clones. After four rounds of flow cytometric sorting with labelled tetramer, they isolated a variant with physiologic affinity and newly acquired binding to an altered peptide ligand containing two residue changes. Similarly, Chervin *et al.* (2008) altered five codons in the mouse 2C TCR CDR3 α to yield a library of $\sim 10^3$ - 10^4 clones. After two rounds of flow cytometry, they isolated variant m100 with an estimated 1,900 nM affinity, representing a 4- to-15-fold improvement over the wild-type TCR. By contrast, an even higher affinity 2C variant, m33 with a ~ 32 nM affinity, was isolated from a similarly designed, but larger (5×10^5), yeast display library (Holler *et al.*, 2003).

[0076] The libraries provided here, while still of modest size (4×10^5 for CDR3 α and 1×10^6 for CDR3 β), were larger than those previously reported for eukaryotic TCR display systems. Taking into account a ~50% transfection efficiency, cell death, and dilution with blank plasmid, a single T-150 flask with adherent CHO cells can reasonably yield five-fold coverage of a 5×10^5 member library. With shaker flasks and suspension cells to facilitate scale up, a library of 10^7 should be achievable. While this remains smaller than many bacterial or yeast-display libraries, the stereotypical TCR-pMHC binding interaction in which the two CDR3 loops dominate peptide binding (Rossjohn *et al.*, 2015), can be exploited to generate targeted mutagenesis libraries rich in higher affinity variants.

[0077] From these libraries, variants with increased on-rate and decreased off-rate were isolated (Table 2). Importantly, the changes in specific tetramer binding observed during library screening anticipated the affinity improvements measured by SPR (FIGS. 3C-D). When the two selected chains were combined to create variant $\alpha V2.\beta V8$, the benefits were roughly additive, resulting in an overall ~60-fold improved NLV/A2 affinity as compared with the wild-type RA14. Specificity for the NLV peptide was retained, as binding to a control HCV/A2 complex was not detected even at 500 nM (Table 2). Analysis of previously engineered TCRs indicates that affinity improvements are typically due to large decreases in off-rate and small increases in on-rate, as was observed for RA14. Structurally, this has been mediated by increases in overall shape complementarity and formation of new contacts between the TCR and peptide residues, which retain the native TCR/pMHC binding angle (Sami *et al.*, 2007; Cole *et al.*, 2013). For example, four residue changes in CDR3 β of the A6 TCR formed 26 new peptide contacts that were sufficient to increase affinity for the tax/A2 complex from 3.2 μ M to 4 nM (Cole *et al.*, 2013).

[0078] As expected based on the significantly lower affinities present in endogenous TCR repertoires, the sequences identified here have not been reported in human sequencing studies of NLV/A2-binding T cells (Trautmann *et al.*, 2005; Wang *et al.*, 2012). In CDR3 α , two residues from the x_n GNQF motif were altered: the conserved glycine was not always observed in position α :109, while the enhanced on-rate $\alpha V2$ variant replaced the canonical α :Q115 with a histidine. Regardless, these modest sequence and affinity changes support the idea that this common public CDR3 α sequence is relatively optimized for NLV/A2 binding. Furthermore, the structure of the related TCR C7 (CDR3 α sequence: ITGNQF) in complex with NLV/A2 demonstrates the ability of this CDR3 α to preserve a similar peptide-binding

interaction while making small adjustments to accommodate a different CDR3 β (Yang *et al.*, 2015). Changes to the CDR3 β motif S_{Xn}TG_{Xn}YGY were more dramatic despite revealing V β :G111 to be absolutely conserved in all sequences recovered in this work; notably, residue V β :G111 was anticipated as crucial for binding from the initial analysis of the RA14-NLV/A2 crystal structure (Gras *et al.*, 2009). Among other CDR3 β residues, the motif residue V β :G115 was not always retained, but was preferentially replaced with hydrophobic residues, with five of ten clones using leucine. The other randomized CDR3 β positions all showed evidence of structural plasticity, with the slow off-rate β V8 variant preferring hydrophobic residues in all three modified positions (Table 1).

[0079] High-level expression of soluble TCR proteins continues to present challenges due to their low expression levels and weak heterodimerization properties. Common expression strategies include generation of single-chain TCRs, which typically requires identification of specific residue changes that support folding and expression in this format (Shusta *et al.*, 2000; Aggen *et al.*, 2011; Wulfig & Pluckthun, 1994); refolding of intact extracellular domains (van Bozel *et al.*, 2009), often with a modified disulfide bond supporting constant domain heterodimerization introduced at residues C α :T84C and C β :S79C (Boulter *et al.*, 2003); and TCR-antibody chimeras expressed in eukaryotes. For this latter approach, a variety of designs have been evaluated, including single-chain TCRs fused to a constant beta domain and then antibody heavy chain domains 1-3 (Mosquera *et al.*, 2005), complete TCR extracellular domains appended with constant kappa domains (Gregoire *et al.*, 1991) or an intact antibody fused to the TCR C-terminus (Lunde *et al.*, 2010; Lebowitz *et al.*, 1999) and simply replacing the antibody variable regions with TCR variable regions (Lunde *et al.*, 2010).

[0080] The present approach was to use the simplest antibody-like design that supported expression of active TCR material. This strategy was guided by the desire to employ established antibody purification processes and minimize the risks of proteolysis and immunogenicity in the resulting protein. After evaluation of several designs, substitution of the antibody Fab domains with the TCR extracellular domains emerged as the best approach. Specifically, the extracellular TCR α chain was fused to the antibody Fc domain with the β chain expressed *in trans*. Subsequent replacement of the human Fc for the corresponding mouse Fc domains allowed for detection of NLV/A2 complexes on peptide-pulsed antigen presenting cells (FIG. 9B).

[0081] Additional modifications were introduced to support proper assembly of the TCR-Fc chimera. In addition to the previously described engineered disulfide bond between the TCR constant domains (Boulter *et al.*, 2003), the human IgG1 upper and core hinge region introduced a second disulfide bond joining the TCR constant domains and two disulfide bonds stabilizing the Fc homodimer, while the free cysteine at position 85.1 was replaced with an alanine. Finally, two predicted N-linked glycosylation sites in the variable domains and three in the human constant TCR domains were eliminated by N-to-Q substitutions. Together, this allowed for production of 0.75-1 mg purified TCR2dsAgly-huFc protein from 50 mL media, matching typical antibody yields in the lab scale transient CHO cell expression system. This strategy has yielded similar production levels for three additional TCRs.

[0082] Prior attempts to replace the antibody Fab with the TCR extracellular domains were less successful (Lunde *et al.*, 2010; Ozawa *et al.*, 2012); but these in general did not include the antibody upper hinge region to provide an additional di-sulfide bond stabilizing the alpha and beta chains and did not remove glycosylation sites then thought to increase protein solubility. Although endogenous TCRs are highly glycosylated on the T-cell surface, analysis of TCR affinity and structure are typically performed on bacterially expressed TCR protein, which is aglycosylated. The expression system developed here allows for direct comparison of identical TCR proteins with and without glycosylation at specific sites. Removal of predicted N-linked glycosylation sites dramatically increased protein homogeneity as measured by size-exclusion chromatography and SDS-PAGE (FIG. 5A), with no negative impact on yield or thermal stability. Further investigation could provide insights into the impact of glycosylation on TCR function, as removal of glycosylation sites has been shown to increase the functional avidity of TCRs expressed on T cells (Kuball *et al.*, 2009).

[0083] The ability of TCRs to detect pMHC on the target cell surface is inextricably linked to not just the TCR-pMHC affinity but also the peptide display level and pMHC clustering on the cell surface. Peptide display level in turn reflects several factors, including antigen expression level, proteolysis sensitivity, and peptide-MHC affinity. For TCRs expressed with their native signaling machinery on T cells, an affinity of 1-5 μ M seems sufficient to confer maximal signaling responses (Irving *et al.*, 2012) while retaining the ability to respond to as few as 1-10 pMHC complexes per cell (Kageyama *et al.*, 1995; Sykulev *et al.*, 1996). For soluble TCRs, which lack the elaborate array of coreceptors that support cellular TCR-pMHC recognition, high affinity is crucial to allow detection of low levels of target

pMHC antigens. In this work, the ability of RA14 TCR variants to detect NLV/A2 correlated strongly with affinity for human T2 cells pulsed with 100 μ M NLV peptide (FIG. 9B). A subsequent peptide dosing experiment demonstrated that the α V2. β V8 variant could detect NLV/A2 complexes after pulsing T2 cells with as little as 0.5 μ M peptide (FIG. 9C).

5 [0084] The sensitivity exhibited by α V2. β V8 is relevant for detection in clinical settings. The NLV peptide was previously reported to present $\sim$ 100 molecules/cell when primary human fibroblasts were infected with an AD169 strain that retains the ability to suppress MHC display (Makler *et al.*, 2010). A bivalent TCR-like antibody with a 300 nM affinity was previously shown to detect the NLV/A2 complex after infection of primary human
10 fibroblasts with CMV, but has not been further developed (Makler *et al.*, 2010). The higher 50 nM affinity of the present engineered TCR suggests that the α V2. β V8 clone could be used to track pMHC display and demonstrates the feasibility of the present TCR engineering approach. Moreover, the flow cytometer sensitivity can be increased with an enzymatic amplification step (Anikeeva *et al.*, 2009) or a single molecule fluorescence assay (Bossi *et al.*,
15 2013).

 [0085] There is considerable interest in using TCRs to monitor the presence of disease-related peptides and a need to detect the NLV/A2 complex to support CMV diagnostics and therapeutics development. High-affinity, soluble TCRs directed at cancer antigens are currently under evaluation in the clinic as part of a bispecific molecule (Bossi *et al.*, 2014). As compared
20 to cancer antigens, infectious disease-associated antigens are more likely to be unique to diseased tissue, thereby reducing the risks of toxicity.

II. Engineered NLV/A2-specific TCRs

 [0086] In some embodiments, provided herein are engineered antigen receptors (*e.g.*, TCRs or CARs) having antigenic specificity for NLV/A2. The engineered antigen receptors
25 may comprise an alpha chain CDR3 having at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity to any one of SEQ ID NOs: 1-7 (or a sequence from Table 1C) and/or a beta chain CDR3 having at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity to any one of SEQ ID NOs: 8-17 (or a sequence from Table 1D). The engineered antigen receptors may comprise an alpha chain with
30 at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identity or similarity to any one of SEQ ID NOs: 18-24 (or a sequence from Table 1C) and/or a beta chain with at

least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identity or similarity to any one of SEQ ID NOs: 25-34 (or a sequence from Table 1D). Also provided herein are polynucleotides encoding the alpha chain and/or beta chain of the NLV/A2-specific antigen receptors provided herein. As used herein numbering of amino acids relative to TCR sequences uses the imgt numbering system (see, the world wide web at imgt.org/IMGTrepertoire/Proteins/proteinDisplays.php?species=human&latin=Homo%20sapiens&group=TRAV, incorporated herein by reference).

[0087] Exemplary antigen receptors, including CARs and recombinant TCRs, as well as methods for engineering and introducing the receptors into cells, include those described, for example, in International Patent Application Publication Nos. WO2000/14257, WO2013/126726, WO2012/129514, WO2014/031687, WO2013/166321, WO2013/071154, WO2013/123061; U.S. Patent Application Publication Nos. US2002/0131960, US2013/0287748, US2013/0149337; U.S. Patent Nos. 6,451,995, 7,446,190, 8,252,592, 8,339,645, 8,398,282, 7,446,179, 6,410,319, 7,070,995, 7,265,209, 7,354,762, 7,446,191, 8,324,353, and 8,479,118; and European Patent Application No. EP2537416. In some aspects, the genetically engineered antigen receptors include a CAR as described in U.S. Patent No. 7,446,190, and those described in International Patent Application Publication No. WO2014/055668.

[0088] In some embodiments, the engineered antigen receptors include recombinant T cell receptors (TCRs). A "T cell receptor" or "TCR" refers to a molecule that contains a variable α and β chains (also known as TCR α and TCR β , respectively) and that is capable of specifically binding to an antigen peptide bound to an MHC receptor. The TCRs may comprise an alpha chain CDR3 having at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity to any one of SEQ ID NOs: 1-7 (or a sequence from Table 1C) and/or a beta chain CDR3 having at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity to any one of SEQ ID NOs: 8-17 (or a sequence from Table 1D). The TCRs may comprise an alpha chain with at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identity or similarity to any one of SEQ ID NOs: 18-24 (or a sequence from Table 1C) and/or a beta chain with at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identity or similarity to any one of SEQ ID NOs: 25-34 (or a sequence from Table 1D).

[0089] In some embodiments, a TCR also can contain a constant domain, a transmembrane domain, and/or a short cytoplasmic tail. For example, in some aspects, each chain of the TCR can possess one N-terminal immunoglobulin variable domain, one immunoglobulin constant domain, a transmembrane region, and a short cytoplasmic tail at the C-terminal end. In some embodiments, a TCR is associated with invariant proteins of the CD3 complex involved in mediating signal transduction. Unless otherwise stated, the term "TCR" should be understood to encompass functional TCR fragments. The term also encompasses intact or full-length TCRs.

[0090] For purposes herein, reference to a TCR includes any TCR or functional fragment, such as an antigen-binding portion of a TCR that binds to a specific antigenic peptide bound in an MHC molecule, *i.e.*, MHC-peptide complex. An "antigen-binding portion" or "antigen-binding fragment" of a TCR, which can be used interchangeably, refers to a molecule that lacks a portion of the structural domains of a TCR, but that binds the antigen (*e.g.*, MHC-peptide complex) to which the full TCR binds. In some cases, an antigen-binding portion contains the variable domains of a TCR, such as variable α chain and variable β chain of a TCR, sufficient to form a binding site for binding to a specific MHC-peptide complex, such as generally where each chain contains three complementarity determining regions.

[0091] In some embodiments, the variable domains of the TCR chains associate to form loops, or complementarity determining regions (CDRs) analogous to immunoglobulins, which confer antigen recognition and determine peptide specificity by forming the binding site of the TCR molecule. Typically, like immunoglobulins, the CDRs are separated by framework regions (FRs). In some embodiments, CDR3 is the main CDR responsible for recognizing processed antigen, although CDR1 of the alpha chain has also been shown to interact with the N-terminal part of the antigenic peptide, whereas CDR1 of the beta chain interacts with the C-terminal part of the peptide. CDR2 is thought to recognize the MHC molecule. In some embodiments, the variable region of the β -chain can contain a further hypervariability (HV4) region.

[0092] In some embodiments, the TCR chains contain a constant domain. For example, like immunoglobulins, the extracellular portion of TCR chains (*e.g.*, α -chain, β -chain) can contain two immunoglobulin domains, a variable domain (*e.g.*, V α or V β ; typically amino acids 1 to 116 based on Kabat numbering (Kabat *et al.*, "Sequences of Proteins of Immunological Interest, US Dept. Health and Human Services, Public Health Service National Institutes of

Health, 1991, 5th ed.) at the N-terminus, and one constant domain (*e.g.*, α -chain constant domain or C α , typically amino acids 117 to 259 based on Kabat, β -chain constant domain or C β , typically amino acids 117 to 295 based on Kabat) adjacent to the cell membrane. For example, in some cases, the extracellular portion of the TCR formed by the two chains contains
5 two membrane-proximal constant domains, and two membrane-distal variable domains containing CDRs. The constant domains of the TCR contain short connecting sequences in which a cysteine residue forms a disulfide bond, making a link between the two chains. In some embodiments, a TCR may have an additional cysteine residue in each of the α and β chains such that the TCR contains two disulfide bonds in the constant domains.

10 **[0093]** In some embodiments, the TCR chains can contain a transmembrane domain. In some embodiments, the transmembrane domain is positively charged. In some cases, the TCR chains contains a cytoplasmic tail. In some cases, the structure allows the TCR to associate with other molecules like CD3. For example, a TCR containing constant domains with a transmembrane region can anchor the protein in the cell membrane and associate with
15 invariant subunits of the CD3 signaling apparatus or complex.

20 **[0094]** In some embodiments, the TCR may be a heterodimer of two chains α and β or it may be a single-chain TCR construct. In some embodiments, the TCR is a heterodimer containing two separate chains (α and β chains) that are linked, such as by a disulfide bond or disulfide bonds. In some embodiments, a TCR for a target antigen (*e.g.*, NLV/A2) is identified and introduced into the cells. In some embodiments, a nucleic acid encoding the TCR can be obtained from a variety of sources. In some embodiments, the TCR is obtained from a biological source, such as from cells such as from a T-cell hybridomas. In some embodiments, the T cells can be a cultured T-cell hybridoma or clone.

Table 1A. CDR3 sequences for α chain variants.

Variant	SEQ ID NO:	CDR3 α
α 1	1	CARNSGNP
α 2	2	CARNYGNH
α 3	3	CARNYINT
α 4	4	CARSFGNP
α 5	5	CARSVGNS
α 6	6	CARLLANL
α 7	7	CARGSWNQ
2S-16	60	CARNYGNQ
2S-20	61	CARNYGNP
WT	39	CARNTGNQ

Table 1A-2. CDR1 sequences for α chain variants.

Variant	SEQ ID NO:	CDR1 α
a2b8	64	SNFYA
R2G	65	LDFWA
2S-16	66	SNFWA
2S-20	66	SNFWA
WT	64	SNFYA

5 **Table 1B.** CDR3 sequences for β chain variants.

Variant	SEQ ID NO:	CDR3 β
β 1	8	AAAPVTGGVYLYT
β 2	9	ASSRVTGGVYLYT
β 3	10	ASSPITGSTYIYT
β 4	11	ASSPITGAPYLYT
β 5	12	ASSPVTGSSYGYT
β 6	13	ASSWITGSIYTYT
β 7	14	ASSPVTGSSYWYT
β 8	15	ASSLVTGGVYLYT
β 9	16	ASSLVTGIPYLYT
β 10	17	ASSRVTGSVYGYT
2S-16	62	ASSLVTGSVYLYT
2S-20	63	ASSWVTGGVYLGT
WT	40	ASSPVTGGIYGYT

Table 1C. Protein sequences for α chain variable regions. Underlined text indicates the CDR region; gray text indicates N-to-Q changes introduced for deglycosylation; gray text in the CDRs indicates a change in the CDR sequence selected during library sorting and associated with increased affinity.

10

Variant	SEQ ID NO:	Variable domain alpha sequence
RA14 WT	18	ILNVEQSPQSLHVQEGDSTQFTCSFPSSNFEYALHWYRWETAKSPEALFVMTLNGDEKKK GRISATLNTKEGYSYLYIKGSQPEDSATYLCARNTGNQFYFGTGTSLTVIP
$\alpha 1$	19	ILNVEQSPQSLHVQEGDSTQFTCSFPSSNFEYALHWYRWETAKSPEALFVMTLNGDEKKK GRISATLNTKEGYSYLYIKGSQPEDSATYLCARNSGNFFYFGTGTSLTVIP
$\alpha 2$	20	ILNVEQSPQSLHVQEGDSTQFTCSFPSSNFEYALHWYRWETAKSPEALFVMTLNGDEKKK GRISATLNTKEGYSYLYIKGSQPEDSATYLCARNYGNFFYFGTGTSLTVIP
$\alpha 3$	21	ILNVEQSPQSLHVQEGDSTQFTCSFPSSNFEYALHWYRWETAKSPEALFVMTLNGDEKKK GRISATLNTKEGYSYLYIKGSQPEDSATYLCARNYINTFYFGTGTSLTVIP
$\alpha 4$	22	ILNVEQSPQSLHVQEGDSTQFTCSFPSSNFEYALHWYRWETAKSPEALFVMTLNGDEKKK GRISATLNTKEGYSYLYIKGSQPEDSATYLCARSGNFFYFGTGTSLTVIP
$\alpha 5$	23	ILNVEQSPQSLHVQEGDSTQFTCSFPSSNFEYALHWYRWETAKSPEALFVMTLNGDEKKK GRISATLNTKEGYSYLYIKGSQPEDSATYLCARSGVGNFFYFGTGTSLTVIP
$\alpha 6$	24	ILNVEQSPQSLHVQEGDSTQFTCSFPSSNFEYALHWYRWETAKSPEALFVMTLNGDEKKK GRISATLNTKEGYSYLYIKGSQPEDSATYLCARLIANLFYFGTGTSLTVIP
$\alpha 7$	25	ILNVEQSPQSLHVQEGDSTQFTCSFPSSNFEYALHWYRWETAKSPEALFVMTLNGDEKKK GRISATLNTKEGYSYLYIKGSQPEDSATYLCARSGSNQFYFGTGTSLTVIP
341	26	ILNVEQSPQSLHVQEGDSTQFTCSFPSSNFEYALHWYRWETAKSPEALFVMTLNGDEKKK GRISATLNTKEGYSYLYIKGSQPEDSATYLCARSGSNQFYFGTGTSLTVIP
R2G	64	ILNVEQSPQSLHVQEGDSTQFTCSFPSSNFEYALHWYRWETAKSPEALFVMTLNGDEKKK GRISATLNTKEGYSYLYIKGSQPEDSATYLCARNYGNFFYFGTGTSLTVIP
2S16	65	ILNVEQSPQSLHVQEGDSTQFTCSFPSSNFEYALHWYRWETAKSPEALFVMTLNGDEKKK GRISATLNTKEGYSYLYIKGSQPEDSATYLCARNYGNQFYFGTGTSLTVIP
2S20	66	ILNVEQSPQSLHVQEGDSTQFTCSFPSSNFEYALHWYRWETAKSPEALFVMTLNGDEKKK GRISATLNTKEGYSYLYIKGSQPEDSATYLCARNYGNFFYFGTGTSLTVIP

Table 1D. Protein sequences for β chain variable regions. Underlined text indicates the CDR region; gray_text indicates N-to-Q changes introduced for deglycosylation; gray text in the

CDRs indicates a change in the CDR sequence selected during library sorting and associated with increased affinity.

Var iant	SEQ ID NO:	Variable domain beta sequence
RA 14 WT	27	AGVTQTPKFQVLKTGQSMTLQCAQDMNHEYMSWYRQDPGMGLRLIHYSVGAGITDQGEVP NGYNVSRSTTEDFPLRLLSAAPSQTSVYFCASSPVTGGIYGYTFGSGTRLTVE
β1	28	AGVTQTPKFQVLKTGQSMTLQCAQDMNHEYMSWYRQDPGMGLRLIHYSVGAGITDQGEVP NGYQVSRSTTEDFPLRLLSAAPSQTSVYFCASSPVTGGVYLYTFGSGTRLTVE
β2	29	AGVTQTPKFQVLKTGQSMTLQCAQDMNHEYMSWYRQDPGMGLRLIHYSVGAGITDQGEVP NGYQVSRSTTEDFPLRLLSAAPSQTSVYFCASSPVTGGVYLYTFGSGTRLTVE
β3	30	AGVTQTPKFQVLKTGQSMTLQCAQDMNHEYMSWYRQDPGMGLRLIHYSVGAGITDQGEVP NGYQVSRSTTEDFPLRLLSAAPSQTSVYFCASSPVTGSIYLYTFGSGTRLTVE
β4	31	AGVTQTPKFQVLKTGQSMTLQCAQDMNHEYMSWYRQDPGMGLRLIHYSVGAGITDQGEVP NGYQVSRSTTEDFPLRLLSAAPSQTSVYFCASSPVTGAPYLYTFGSGTRLTVE
β5	32	AGVTQTPKFQVLKTGQSMTLQCAQDMNHEYMSWYRQDPGMGLRLIHYSVGAGITDQGEVP NGYQVSRSTTEDFPLRLLSAAPSQTSVYFCASSPVTGSSYGYTFGSGTRLTVE
β6	33	AGVTQTPKFQVLKTGQSMTLQCAQDMNHEYMSWYRQDPGMGLRLIHYSVGAGITDQGEVP NGYQVSRSTTEDFPLRLLSAAPSQTSVYFCASSPVTGSIYLYTFGSGTRLTVE
β7	34	AGVTQTPKFQVLKTGQSMTLQCAQDMNHEYMSWYRQDPGMGLRLIHYSVGAGITDQGEVP NGYQVSRSTTEDFPLRLLSAAPSQTSVYFCASSPVTGSSYGYTFGSGTRLTVE
β8	35	AGVTQTPKFQVLKTGQSMTLQCAQDMNHEYMSWYRQDPGMGLRLIHYSVGAGITDQGEVP NGYQVSRSTTEDFPLRLLSAAPSQTSVYFCASSPVTGGVYLYTFGSGTRLTVE
β9	36	AGVTQTPKFQVLKTGQSMTLQCAQDMNHEYMSWYRQDPGMGLRLIHYSVGAGITDQGEVP NGYQVSRSTTEDFPLRLLSAAPSQTSVYFCASSPVTGIFLYTFGSGTRLTVE
β10	37	AGVTQTPKFQVLKTGQSMTLQCAQDMNHEYMSWYRQDPGMGLRLIHYSVGAGITDQGEVP NGYQVSRSTTEDFPLRLLSAAPSQTSVYFCASSPVTGSIYLYTFGSGTRLTVE
341	38	AGVTQTPKFQVLKTGQSMTLQCAQDMNHEYMSWYRQDPGMGLRLIHYSVGAGITDQGEVP NGYQVSRSTTEDFPLRLLSAAPSQTSVYFCASSPVTGGVYLYTFGSGTRLTVE
2S1 6	67	AGVTQTPKFQVLKTGQSMTLQCAQDMNHEYMSWYRQDPGMGLRLIHYSVGAGITDQGEVP NGYQVSRSTTEDFPLRLLSAAPSQTSVYFCASSPVTGSIYLYTFGSGTRLTVE
2S2 0	68	AGVTQTPKFQVLKTGQSMTLQCAQDMNHEYMSWYRQDPGMGLRLIHYSVGAGITDQGEVP NGYQVSRSTTEDFPLRLLSAAPSQTSVYFCASSPVTGGVYGYTFGSGTRLTVE

A. Soluble TCRs and BiTEs

5 [0095] In addition, the present disclosure provides soluble TCRs. Furthermore, soluble bispecific T cell-engaging molecules (BiTEs) can be generated by linking the engineered NLV/A2-specific TCR to CD3- or CD16-specific Fab fragments or CD3- or CD16-specific single-chain antibodies. These bispecific molecules can bind the cell surface of a CMV-
10 infected cell via their TCR domain binding to the NLV/A2 complex, and the CD3-specific domain would crosslink the TCR. This would result in cellular activation and elimination of the target cell. Thus, these soluble bispecific TCR constructs can be used for treating patients (e.g., transplant patients with active CMV disease or brain cancer patients) directly.

[0096] In the context of this application, “solubility” is defined as the ability of the TCR to be purified as a mono-disperse heterodimer in phosphate buffered saline (PBS) (KCl

2.7 mM, KH_2PO_4 1.5 mM, NaCl 137 mM, and Na_2PO_4 8 mM, pH 7.1-7.5. Life Technologies, Gibco BRL) at a concentration of 1 mg/ml and for more than 90% of said TCR to remain as a mono disperse heterodimer after incubation at 25° C for 1 hour.

[0097] The soluble TCR can be fused to an antibody Fc region to create an antibody-TCR chimera. For this, the antibody Fc is fused to the TCR extracellular domains. For example, the TCR α chain may be fused to the Fc and the TCR β chain may be expressed *in trans*. The chimera may include the antibody hinge region. The antibody Fc may be derived from a mouse or human antibody.

[0098] The soluble TCR can be used as a probe for diagnostic evaluation of peptide/MHC in cells, either in vitro or in vivo, or to direct therapeutic molecules to sites of interest in vivo. The soluble TCR molecules also could be labeled with tracers, such as a fluorescent probe or radioactive probe, and then used for diagnostic evaluation of the presentation of peptide/MHC in cells. Furthermore, the soluble TCR molecules could be linked with therapeutic molecules, such as toxin, and then directed to the sites for the treatment of infected cells.

[0099] In some embodiments, the present disclosure provides soluble TCRs, such as an engineered NLV/A2-specific TCR provided herein. Soluble TCRs may be used for investigating specific TCR-pMHC interactions or as a diagnostic tool to detect infection or response to vaccination. Soluble TCRs may have applications in staining, for example to stain cells for the presence of a particular peptide antigen presented in the context of the MHC. Similarly, soluble TCRs can be used to deliver a therapeutic agent, for example a cytotoxic compound or an immunostimulating compound, to cells presenting a particular antigen. In some aspects, the TCR is linked to another molecule that delivers a cell in proximity to the tumor. In further aspects, the TCR delivers a toxin, a cytokine, costimulatory ligand, or inhibitor ligand and directs the molecule, cell or compound to the target cells expressing the peptide-MHC.

[00100] In some aspects, the present disclosure provides a soluble TCR that comprises (i) all or part of a TCR α chain (*e.g.*, SEQ ID NOs: 18-24), except the transmembrane domain thereof, and (ii) all or part of a TCR β chain (*e.g.*, SEQ ID NOs: 25-34), except the transmembrane domain thereof, wherein (i) and (ii) each comprise a functional variable domain

and at least a part of the constant domain of the TCR chain, and are linked by a disulfide bond between constant domain residues that is not present in native TCR.

[00101] In some aspects, the soluble TCR comprises a TCR α or γ chain extracellular domain dimerized to a TCR β or δ chain extracellular domain respectively, by means of a pair of C-terminal dimerization peptides, such as leucine zippers (International Patent Publication No. WO 99/60120; U.S. Patent No. 7,666,604).

[00102] A soluble TCR of the present disclosure may be provided in substantially pure form, or as a purified or isolated preparation. For example, it may be provided in a form which is substantially free of other proteins.

[00103] The TCR of the present disclosure may alternatively or additionally be associated with (*e.g.*, covalently or otherwise linked to) a therapeutic agent which may be, for example, a toxic moiety for use in cell killing, or an immunostimulating agent such as an interleukin or a cytokine. The present disclosure also provides a method for delivering a therapeutic agent to a target cell, which method comprises contacting potential target cells with a TCR under conditions to allow attachment of the TCR to the target cell, said TCR being specific for the TCR ligand and having the therapeutic agent associated therewith.

[00104] In particular, the soluble TCR can be used to deliver therapeutic agents to the location of cells presenting a particular antigen. This would be useful in many situations and, in particular, against certain types of tumors. A therapeutic agent could be delivered such that it would exercise its effect locally but not only on the cell it binds to. Thus, one particular strategy envisages anti-tumor molecules linked to T cell receptors specific for NLV/A2 in order to kill glioblastoma cells.

[00105] Many therapeutic agents could be employed for this use, for instance radioactive compounds, enzymes (perforin for example) or chemotherapeutic agents (cisplatin for example). To ensure that toxic effects are exercised in the desired location, the toxin could be inside a liposome linked to streptavidin so that the compound is released slowly. This will prevent damaging effects during the transport in the body and ensure that the toxin has maximum effect after binding of the TCR to the relevant antigen presenting cells.

B. Chimeric Antigen Receptors

[00106] In various aspects, the antigen binding region of the TCR may be included in a chimeric antigen receptor (CAR) as the extracellular domain comprising an antigen binding region. The CAR may be transfected into cells (*e.g.*, autologous or allogeneic cells) that may be used in an adoptive cell transfer therapy. In some embodiments, the CAR is humanized to reduce immunogenicity (hCAR).

[00107] The CARs generally include an extracellular antigen (or ligand) binding domain linked to one or more intracellular signaling components, in some aspects via linkers and/or transmembrane domain(s). Such molecules typically mimic or approximate a signal through a natural antigen receptor, a signal through such a receptor in combination with a costimulatory receptor, and/or a signal through a costimulatory receptor alone. In some embodiments, the CAR includes an antigen-binding portion or portions of a TCR.

[00108] The arrangement of the antigen-binding domain of a CAR may be multimeric. The hinge portion of the CAR may in some embodiments be shortened or excluded (*i.e.*, generating a CAR that only includes an antigen binding domain, a transmembrane region, and an intracellular signaling domain). In some embodiments, the hinge region may have the first cysteine maintained, or mutated by a proline or a serine substitution, or be truncated up to the first cysteine. In some embodiments, the hinge portion of may comprise or consist of an 8-14 amino acid peptide (*e.g.*, a 12 AA peptide), a portion of CD8 α , or the IgG4 Fc. In some embodiments, the antigen binding domain may be suspended from cell surface using a domain that promotes oligomerization, such as CD8 alpha. In some embodiments, the antigen binding domain may be suspended from cell surface using a domain that is recognized by monoclonal antibody (mAb) clone 2D3 (mAb clone 2D3 described, *e.g.*, in Singh *et al.*, 2008).

[00109] The endodomain or intracellular signaling domain of a CAR can generally cause or promote the activation of at least one of the normal effector functions of an immune cell comprising the CAR. For example, the endodomain may promote an effector function of a T cell such as, *e.g.*, cytolytic activity or helper activity including the secretion of cytokines. The effector function in a naive, memory, or memory-type T cell may include antigen-dependent proliferation. The terms “intracellular signaling domain” or “endodomain” refers to the portion of a CAR that can transduce the effector function signal and/or direct the cell to perform a specialized function. While usually the entire intracellular signaling domain may be included in a CAR, in some cases a truncated portion of an endodomain may be

included. Generally, endodomains include truncated endodomains, wherein the truncated endodomain retains the ability to transduce an effector function signal in a cell.

[00110] In some embodiments, an endodomain comprises the zeta chain of the T cell receptor or any of its homologs (*e.g.*, eta, delta, gamma, or epsilon), MB1 chain, B29, Fc RIII, Fc RI, and combinations of signaling molecules, such as CD3 ζ and CD28, CD27, 4-1BB, DAP-10, OX40, and combinations thereof, as well as other similar molecules and fragments. Intracellular signaling portions of other members of the families of activating proteins can be used, such as Fc γ RIII and Fc ϵ RI. In some embodiments, an endodomain may comprise the human CD3 ζ intracellular domain.

[00111] The antigen-specific extracellular domain and the intracellular signaling-domain are preferably linked by a transmembrane domain. Transmembrane domains that may be included in a CAR include, *e.g.*, the human IgG4 Fc hinge and Fc regions, the human CD4 transmembrane domain, the human CD28 transmembrane domain, the transmembrane human CD3 ζ domain, or a cysteine mutated human CD3 ζ domain, or a transmembrane domain from a human transmembrane signaling protein such as, *e.g.*, the CD16 and CD8 and erythropoietin receptor.

[00112] In some embodiments, the endodomain comprises a sequence encoding a costimulatory receptor such as, *e.g.*, a modified CD28 intracellular signaling domain, or a CD28, CD27, OX-40 (CD134), DAP10, or 4-1BB (CD137) costimulatory receptor. In some embodiments, both a primary signal initiated by CD3 ζ , an additional signal provided by a human costimulatory receptor may be included in a CAR to more effectively activate a transformed T cells, which may help improve *in vivo* persistence and the therapeutic success of the adoptive immunotherapy. As noted in Table 2, the endodomain or intracellular receptor signaling domain may comprise the zeta chain of CD3 alone or in combination with an Fc γ RIII costimulatory signaling domains such as, *e.g.*, CD28, CD27, DAP10, CD137, OX40, CD2, 4-1BB. In some embodiments, the endodomain comprises part or all of one or more of TCR zeta chain, CD28, CD27, OX40/CD134, 4-1BB/CD137, Fc ϵ RI γ , ICOS/CD278, IL-2R β /CD122, IL-2R α /CD132, DAP10, DAP12, and CD40. In some embodiments, 1, 2, 3, 4 or more cytoplasmic domains may be included in an endodomain. For example, in some CARs it has been observed that at least two or three signaling domains fused together can result in an additive or synergistic effect.

[00113] In some aspects, an isolated nucleic acid segment and expression cassette including DNA sequences that encode a CAR may be generated. A variety of vectors may be used. In some preferred embodiments, the vector may allow for delivery of the DNA encoding a CAR to immune such as T cells. CAR expression may be under the control of regulated eukaryotic promoter such as, *e.g.*, the MNDU3 promoter, CMV promoter, EF1alpha promoter, or Ubiquitin promoter. Also, the vector may contain a selectable marker, if for no other reason, to facilitate their manipulation *in vitro*. In some embodiments, the CAR can be expressed from mRNA *in vitro* transcribed from a DNA template.

[00114] Chimeric antigen receptor molecules are recombinant and are distinguished by their ability to both bind antigen and transduce activation signals *via* immunoreceptor activation motifs (ITAM's) present in their cytoplasmic tails. Receptor constructs utilizing an antigen-binding moiety (for example, generated from single chain antibodies (scFv)) afford the additional advantage of being "universal" in that they can bind native antigen on the target cell surface in an HLA-independent fashion. For example, a scFv constructs may be fused to sequences coding for the intracellular portion of the CD3 complex's zeta chain (ζ), the Fc receptor gamma chain, and sky tyrosine kinase.

[00115] In some embodiments, a TCR is included in a CAR as the antigen binding domain (*e.g.*, as a scFv region) and the CAR further comprises a hinge region, a transmembrane region, and an endodomain.

Table 2. Regions that may be included in an anti-NLV/A2 targeting CAR

Hinge/Scaffold
12 AA (peptide)
t-20 AA (peptide)
CD8 α
IgG4 Fc
2D3
IgG4 Fc Δ EQ (IgG4Fc N40Q)
IgG4 Fc Δ Q (IgG4Fc L18E N40Q)
t-12AA + t-20AA
mKate
phiLov
dsRed
Venus
eGFP
CH3 HA
mTFP-1

CD8 α + t-20AA
Double t-20 AA
t-20AA + CD8 α
CD8 α + Leucine Zipper Basep1
CD8 α + Leucine Zipper Acid1

Transmembrane domain
CD28
CD137 (4-1BB)
CD8 α
CD3 ζ

Endo-domain (signaling domain)
CD28 + CD3 ζ
CD28 + CD27 + CD3 ζ
CD28 + OX40 + CD3 ζ
CD28 + 4-1BB + CD3 ζ
CD28 + CD27 + OX40 + CD3 ζ
CD28 + 4-1BB + CD27 + CD3 ζ
CD28 + 4-1BB + OX40 + CD3 ζ
4-1BB + CD3 ζ
4-1BB + OX40 + CD3 ζ
4-1BB + CD27 + CD3 ζ
CD27 + CD3 ζ
CD27 + OX 40 + CD3 ζ
CD28 Δ + CD3 ζ
CD28 Δ + CD27 + CD3 ζ
CD28 Δ + OX40 + CD3 ζ
CD28 Δ + 4-1BB + CD3 ζ
CD28 Δ + 4-1BB + OX40 + CD3 ζ
CD28 Δ + CD27 + OX40 + CD3 ζ
CD28 Δ + 4-1BB + CD27 + CD3 ζ
4-1BB + ICOS + CD3 ζ
CD28 + ICOS + CD3 ζ
ICOS + CD3 ζ
CD3 ζ
CD28 only

ζ -zeta; Δ -mutant; Note = 4-1BB is also referred to as CD137; “+” refers to the fusion of the different regions.

III. Engineered NLV/A2-specific Cells

[00116] Antigen-specific cells can be generated by using the NLV/A2-specific

- 5 TCRs provided herein. For example, the TCR or CAR sequence may be inserted into a vector (e.g., retroviral or lentiviral vector) that is introduced into host cells, such as T cells (e.g., CD4⁺

T cells, CD8⁺ T cells, $\gamma\delta$ T cells, or Tregs), NK cells, invariant NK cells, NKT cells, mesenchymal stem cells (MSCs), or induced pluripotent stem (iPS) cells to generate antigen-specific cells that can be used for adoptive cell therapy. For example, the host cells may be autologous or allogeneic cells (*e.g.*, isolated from an umbilical cord).

5 **[00117]** Further provided herein are cells, such as T cells, NK cells, invariant NK cells, or NKT cells, engineered to express the NLV/A2-specific TCR or CAR provided herein. These effector immune cells may express a TCR together with CD3 molecules or other signaling domains linked to the TCR, which would initiate the signal transduction in these cells.

10 **[00118]** The engineered immune cells may be constructed using any of the many well-established gene transfer methods known to those skilled in the art. In certain embodiments, the engineered cells are constructed using viral vector-based gene transfer methods to introduce nucleic acids encoding an NLV/A2-specific TCR. The viral vector-based gene transfer method may comprise a lentiviral vector, a retroviral vector, an adenoviral or an
15 adeno-associated viral vector. In certain embodiments, the engineered cells are constructed using non-viral vector-based gene transfer methods to introduce nucleic acids encoding a NLV/A2-specific TCR or CAR. The vector for the TCR may comprises the alpha chain polypeptide and the beta chain polypeptide, which may be linked by a linker domain or IRES
20 cleavage site and/or a P2A cleavage site, which may be separated by a spacer, such as SGSG or GSG. In certain embodiments, the non-viral vector-based gene transfer method comprises a gene-editing method selected from the group consisting of a zinc-finger nuclease (ZFN), a transcription activator-like effector nuclease (TALENs), and a clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) nuclease. In certain
25 embodiments, the non-viral vector-based gene editing method comprises a transfection or transformation method selected from the group consisting of lipofection, nucleofection, virosomes, liposomes, polycation or lipid:nucleic acid conjugates, naked DNA, artificial virions, and agent-enhanced uptake of DNA.

IV. Methods of Use

30 **[00119]** As used herein, the terms “treat,” “treatment,” “treating,” and the like refer to the process of ameliorating, lessening, or otherwise mitigating the symptoms of a

disease or condition in a subject by, for example, administering a therapeutic agent to the subject, or by performing a surgical, clinical, or other medical procedure on the subject.

[00120] As used herein, the terms “subject” or “patient” are used interchangeably herein to refer to an individual, *e.g.*, a human or a non-human organism, such as a primate, a mammal, or a vertebrate.

[00121] As used herein, the terms “therapeutically effective” or “therapeutically beneficial” and the like refer to a therapeutic agent, or a surgical, clinical, or other medical procedure that ameliorates, mitigates or otherwise relieves one or more symptoms of a disease, disorder, or condition, thereby enhancing the well-being of a subject having a disease, disorder, or condition by, for example, reducing the frequency or severity of the signs or symptoms of a disease, disorder, or condition. Thus, a therapeutically effective or therapeutically beneficial cancer treatment may, for example, reduce the size of a tumor, reduce the growth rate of a tumor, reduce the likelihood of tumor dissemination or metastasis.

[00122] Provided herein are methods for treating a disease or disorder in a subject comprising administering to the subject a therapeutically effective amount of a soluble TCR of the present embodiments to a population of NLV/A2-specific cells, such as T cells, NK cells, invariant NK cells, NKT cells, MSCs, or iPS cells, produced by any of the methods provided herein. Also provided are methods of using a soluble TCR of the present embodiments to detect the presence of NLV/A2 in the surface of a cell, either *in vitro* or *in vivo*.

[00123] Provided herein are methods for treating or delaying progression of cancer in an individual comprising administering to the individual an effective amount of an NLV/A2-specific T cell therapy, wherein the patient has a cancer where CMV has been reactivated. Adoptive T cell therapies with genetically engineered TCR-transduced T cells (conjugate TCR to other bioreactive proteins (*e.g.*, anti-CD3)) are also provided herein.

[00124] Tumors for which the present treatment methods are useful include any malignant cell type expressing NLV due to reactivation of CMV, such as those found in a solid tumor or a hematological tumor. Exemplary solid tumors can include, but are not limited to, a tumor of an organ selected from the group consisting of pancreas, colon, cecum, stomach, brain, head, neck, ovary, kidney, larynx, sarcoma, lung, bladder, melanoma, prostate, and breast. Exemplary hematological tumors include tumors of the bone marrow, T or B cell malignancies, leukemias, lymphomas, blastomas, myelomas, and the like. Further examples of cancers that may be treated using the methods provided herein include, but are not limited

to, brain cancer (glioblastoma), lung cancer (including small-cell lung cancer, non-small cell lung cancer, adenocarcinoma of the lung, and squamous carcinoma of the lung), cancer of the peritoneum, gastric or stomach cancer (including gastrointestinal cancer and gastrointestinal stromal cancer), pancreatic cancer, cervical cancer, ovarian cancer, liver cancer, bladder cancer, breast cancer, colon cancer, colorectal cancer, endometrial or uterine carcinoma, salivary gland carcinoma, kidney or renal cancer, prostate cancer, vulval cancer, thyroid cancer, various types of head and neck cancer, and melanoma.

[00125] In certain embodiments, the method further comprises a step of performing lymphodepletion prior to administration of the therapeutically effective amount of the population of NLV/A2-specific TCR cells. In certain embodiments, the lymphodepletion comprises non-myeloablative lymphodepleting chemotherapy. In certain embodiments, the non-myeloablative lymphodepleting chemotherapy comprises administration of cyclophosphamide and fludarabine.

[00126] In certain embodiments, the method further comprises a step of administering a T-cell growth factor that promotes the growth and activation of autologous T cells to the subject, either concomitantly with the autologous T cells or subsequently to the autologous T cells. In certain embodiments, the T cell growth factor comprises any suitable growth factor that promotes the growth and activation of the autologous T-cells. In certain embodiments, the T cell growth factor is selected from the group consisting of interleukin (IL)-2, IL-7, IL-15, and IL-12, and combinations thereof (*e.g.*, IL-2 and IL-7, IL-2 and IL-15, IL-7 and IL-15, IL-2, IL-7 and IL-15, IL-12 and IL-7, IL-12 and IL-15, or IL-12 and IL-2).

[00127] In certain embodiments, the therapeutically effective amount of the population of NLV/A2-specific TCR cells produced by any of the methods provided herein is administered to the subject intravenously, intratumorally, or intraperitoneally. The appropriate dosage of the cell therapy may be determined based on the type of cancer to be treated, the severity and course of the disease, the clinical condition of the individual, the individual's clinical history and response to the treatment, and the discretion of the attending physician.

A. Combination Therapies

[00128] In certain embodiments, the compositions and methods of the present embodiments involve an antigen-specific cell population or soluble TCR in combination with at least one additional therapy. The additional therapy may be radiation therapy, surgery (*e.g.*, lumpectomy and a mastectomy), chemotherapy, gene therapy, DNA therapy, viral therapy,

RNA therapy, immunotherapy, bone marrow transplantation, nanotherapy, monoclonal antibody therapy, or a combination of the foregoing. The additional therapy may be in the form of adjuvant or neoadjuvant therapy.

[00129] In some embodiments, the additional therapy is the administration of small molecule enzymatic inhibitor or anti-metastatic agent. In some embodiments, the additional therapy is the administration of side-effect limiting agents (*e.g.*, agents intended to lessen the occurrence and/or severity of side effects of treatment, such as anti-nausea agents, *etc.*). In some embodiments, the additional therapy is radiation therapy. In some embodiments, the additional therapy is surgery. In some embodiments, the additional therapy is a combination of radiation therapy and surgery. In some embodiments, the additional therapy is gamma irradiation. In some embodiments, the additional therapy is a chemotherapy such as, *e.g.*, dacarbazine, or temozolomide. The additional therapy may be one or more of the chemotherapeutic agents known in the art.

[00130] A T cell therapy or soluble TCR may be administered before, during, after, or in various combinations relative to an additional cancer therapy, such as immune checkpoint therapy. The administrations may be in intervals ranging from concurrently to minutes to days to weeks. In embodiments where the T cell therapy or soluble TCR is provided to a patient separately from an additional therapeutic agent, one would generally ensure that a significant period of time did not expire between the time of each delivery, such that the two compounds would still be able to exert an advantageously combined effect on the patient. In such instances, it is contemplated that one may provide a patient with the antibody therapy and the anti-cancer therapy within about 12 to 24 or 72 h of each other and, more particularly, within about 6-12 h of each other. In some situations it may be desirable to extend the time period for treatment significantly where several days (2, 3, 4, 5, 6, or 7) to several weeks (1, 2, 3, 4, 5, 6, 7, or 8) lapse between respective administrations.

[00131] Various combinations may be employed. For the example below an antigen-specific T cell therapy, peptide, or TCR is “A” and an anti-cancer therapy is “B”:

A/B/A B/A/B B/B/A A/A/B A/B/B B/A/A A/B/B/B B/A/B/B
 B/B/B/A B/B/A/B A/A/B/B A/B/A/B A/B/B/A B/B/A/A
 B/A/B/A B/A/A/B A/A/A/B B/A/A/A A/B/A/A A/A/B/A

[00132] Administration of any compound or therapy of the present embodiments to a patient will follow general protocols for the administration of such compounds, taking into account the toxicity, if any, of the agents. Therefore, in some embodiments there is a step of monitoring toxicity that is attributable to combination therapy.

5

1. Chemotherapy

[00133] A wide variety of chemotherapeutic agents may be used in accordance with the present embodiments. The term “chemotherapy” refers to the use of drugs to treat cancer. A “chemotherapeutic agent” is used to connote a compound or composition that is administered in the treatment of cancer. These agents or drugs are categorized by their mode
10 of activity within a cell, for example, whether and at what stage they affect the cell cycle. Alternatively, an agent may be characterized based on its ability to directly cross-link DNA, to intercalate into DNA, or to induce chromosomal and mitotic aberrations by affecting nucleic acid synthesis.

[00134] Examples of chemotherapeutic agents include alkylating agents, such as
15 thiotepa and cyclophosphamide; alkyl sulfonates, such as busulfan, improsulfan, and piposulfan; aziridines, such as benzodopa, carboquone, meturedopa, and uredopa; ethylenimines and methylamelamines, including altretamine, triethylenemelamine, trietylenephosphoramidate, triethylenethiophosphoramidate, and trimethylolomelamine; acetogenins (especially bullatacin and bullatacinone); a camptothecin (including the synthetic
20 analogue topotecan); bryostatin; callystatin; CC-1065 (including its adozelesin, carzelesin and bizelesin synthetic analogues); cryptophycins (particularly cryptophycin 1 and cryptophycin 8); dolastatin; duocarmycin (including the synthetic analogues, KW-2189 and CB1-TM1); eleutherobin; pancratistatin; a sarcodictyin; spongistatin; nitrogen mustards, such as chlorambucil, chlornaphazine, cholophosphamide, estramustine, ifosfamide,
25 mechlorethamine, mechlorethamine oxide hydrochloride, melphalan, novembichin, phenesterine, prednimustine, trofosfamide, and uracil mustard; nitrosureas, such as carmustine, chlorozotocin, fotemustine, lomustine, nimustine, and ranimustine; antibiotics, such as the enediyne antibiotics (*e.g.*, calicheamicin, especially calicheamicin gammall and calicheamicin omegaII); dynemicin, including dynemicin A; bisphosphonates, such as clodronate; an
30 esperamicin; as well as neocarzinostatin chromophore and related chromoprotein enediyne antibiotic chromophores, aclacinomysins, actinomycin, authrarnycin, azaserine, bleomycins, cactinomycin, carabacin, carminomycin, carzinophilin, chromomycinis, dactinomycin,

daunorubicin, detorubicin, 6-diazo-5-oxo-L-norleucine, doxorubicin (including morpholino-doxorubicin, cyanomorpholino-doxorubicin, 2-pyrrolino-doxorubicin and deoxydoxorubicin), epirubicin, esorubicin, idarubicin, marcellomycin, mitomycins, such as mitomycin C, mycophenolic acid, nogalarnycin, olivomycins, peplomycin, potfiromycin, puromycin, 5 quelamycin, rodorubicin, streptonigrin, streptozocin, tubercidin, ubenimex, zinostatin, and zorubicin; anti-metabolites, such as methotrexate and 5-fluorouracil (5-FU); folic acid analogues, such as denopterin, pteropterin, and trimetrexate; purine analogs, such as fludarabine, 6-mercaptopurine, thiamiprine, and thioguanine; pyrimidine analogs, such as ancitabine, azacitidine, 6-azauridine, carmofur, cytarabine, dideoxyuridine, doxifluridine, 10 enocitabine, and floxuridine; androgens, such as calusterone, dromostanolone propionate, epitio stanol, mepitio stanane, and testolactone; anti-adrenals, such as mitotane and trilostane; folic acid replenisher, such as frolic acid; aceglatone; aldophosphamide glycoside; aminolevulinic acid; eniluracil; amsacrine; bestabucil; bisantrene; edatraxate; defofamine; demecolcine; diaziquone; elformithine; elliptinium acetate; an epothilone; etoglucid; gallium nitrate; 15 hydroxyurea; lentinan; lonidainine; maytansinoids, such as maytansine and ansamitocins; mitoguazone; mitoxantrone; mopidanmol; nitraerine; pentostatin; phenamet; pirarubicin; losoxantrone; podophyllinic acid; 2-ethylhydrazide; procarbazine; PSKpolysaccharide complex; razoxane; rhizoxin; sizofiran; spirogermanium; tenuazonic acid; triaziquone; 2,2',2''-trichlorotriethylamine; trichothecenes (especially T-2 toxin, verracurin A, roridin A and 20 anguidine); urethan; vindesine; dacarbazine; mannomustine; mitobronitol; mitolactol; pipobroman; gacytosine; arabinoside ("Ara-C"); cyclophosphamide; taxoids, *e.g.*, paclitaxel and docetaxel gemcitabine; 6-thioguanine; mercaptopurine; platinum coordination complexes, such as cisplatin, oxaliplatin, and carboplatin; vinblastine; platinum; etoposide (VP-16); ifosfamide; mitoxantrone; vincristine; vinorelbine; novantrone; teniposide; edatrexate; 25 daunomycin; aminopterin; xeloda; ibandronate; irinotecan (*e.g.*, CPT-11); topoisomerase inhibitor RFS 2000; difluoromethylornithine (DMFO); retinoids, such as retinoic acid; capecitabine; carboplatin, procarbazine, plicomycin, gemcitabine, navelbine, farnesyl-protein transferase inhibitors, transplatinum, and pharmaceutically acceptable salts, acids, or derivatives of any of the above.

2. Radiotherapy

[00135] Other factors that cause DNA damage and have been used extensively include what are commonly known as γ -rays, X-rays, and/or the directed delivery of radioisotopes to tumor cells. Other forms of DNA damaging factors are also contemplated,

such as microwaves, proton beam irradiation (U.S. Patents 5,760,395 and 4,870,287), and UV-irradiation. It is most likely that all of these factors affect a broad range of damage on DNA, on the precursors of DNA, on the replication and repair of DNA, and on the assembly and maintenance of chromosomes. Dosage ranges for X-rays range from daily doses of 50 to 200 roentgens for prolonged periods of time (3 to 4 wk), to single doses of 2000 to 6000 roentgens. Dosage ranges for radioisotopes vary widely, and depend on the half-life of the isotope, the strength and type of radiation emitted, and the uptake by the neoplastic cells.

3. Immunotherapy

[00136] The skilled artisan will understand that additional immunotherapies may be used in combination or in conjunction with methods of the embodiments. In the context of cancer treatment, immunotherapeutics, generally, rely on the use of immune effector cells and molecules to target and destroy cancer cells. Rituximab (RITUXAN®) is such an example. The immune effector may be, for example, an antibody specific for some marker on the surface of a tumor cell. The antibody alone may serve as an effector of therapy or it may recruit other cells to actually affect cell killing. The antibody also may be conjugated to a drug or toxin (chemotherapeutic, radionuclide, ricin A chain, cholera toxin, pertussis toxin, etc.) and serve as a targeting agent. Alternatively, the effector may be a lymphocyte carrying a surface molecule that interacts, either directly or indirectly, with a tumor cell target. Various effector cells include cytotoxic T cells and NK cells.

[00137] Antibody-drug conjugates have emerged as a breakthrough approach to the development of cancer therapeutics. Cancer is one of the leading causes of deaths in the world. Antibody-drug conjugates (ADCs) comprise monoclonal antibodies (MAbs) that are covalently linked to cell-killing drugs. This approach combines the high specificity of MAbs against their antigen targets with highly potent cytotoxic drugs, resulting in “armed” MAbs that deliver the payload (drug) to tumor cells with enriched levels of the antigen. Targeted delivery of the drug also minimizes its exposure in normal tissues, resulting in decreased toxicity and improved therapeutic index. The approval of two ADC drugs, ADCETRIS® (brentuximab vedotin) in 2011 and KADCYLA® (trastuzumab emtansine or T-DM1) in 2013 by FDA validated the approach. There are currently more than 30 ADC drug candidates in various stages of clinical trials for cancer treatment (Leal *et al.*, 2014). As antibody engineering and linker-payload optimization are becoming more and more mature, the discovery and development of new ADCs are increasingly dependent on the identification and validation of

new targets that are suitable to this approach and the generation of targeting MAbs. Two criteria for ADC targets are upregulated/high levels of expression in tumor cells and robust internalization.

[00138] In one aspect of immunotherapy, the tumor cell must bear some marker that is amenable to targeting, *i.e.*, is not present on the majority of other cells. Many tumor markers exist and any of these may be suitable for targeting in the context of the present embodiments. Common tumor markers include CD20, carcinoembryonic antigen, tyrosinase (p97), gp68, TAG-72, HMFG, Sialyl Lewis Antigen, MucA, MucB, PLAP, laminin receptor, erb B, and p155. An alternative aspect of immunotherapy is to combine anticancer effects with immune stimulatory effects. Immune stimulating molecules also exist including: cytokines, such as IL-2, IL-4, IL-12, GM-CSF, gamma-IFN, chemokines, such as MIP-1, MCP-1, IL-8, and growth factors, such as FLT3 ligand.

[00139] Examples of immunotherapies currently under investigation or in use are immune adjuvants, *e.g.*, *Mycobacterium bovis*, *Plasmodium falciparum*, dinitrochlorobenzene, and aromatic compounds (U.S. Patents 5,801,005 and 5,739,169; Hui and Hashimoto, 1998; Christodoulides *et al.*, 1998); cytokine therapy, *e.g.*, interferons α , β , and γ , IL-1, GM-CSF, and TNF (Bukowski *et al.*, 1998; Davidson *et al.*, 1998; Hellstrand *et al.*, 1998); gene therapy, *e.g.*, TNF, IL-1, IL-2, and p53 (Qin *et al.*, 1998; Austin-Ward and Villaseca, 1998; U.S. Patents 5,830,880 and 5,846,945); and monoclonal antibodies, *e.g.*, anti-CD20, anti-ganglioside GM2, and anti-p185 (Hollander, 2012; Hanibuchi *et al.*, 1998; U.S. Patent 5,824,311). It is contemplated that one or more anti-cancer therapies may be employed with the antibody therapies described herein.

[00140] In some embodiments, the immunotherapy may be an immune checkpoint inhibitor. Immune checkpoints either turn up a signal (*e.g.*, co-stimulatory molecules) or turn down a signal. Inhibitory immune checkpoints that may be targeted by immune checkpoint blockade include adenosine A2A receptor (A2AR), B7-H3 (also known as CD276), B and T lymphocyte attenuator (BTLA), cytotoxic T-lymphocyte-associated protein 4 (CTLA-4, also known as CD152), indoleamine 2,3-dioxygenase (IDO), killer-cell immunoglobulin (KIR), lymphocyte activation gene-3 (LAG3), programmed death 1 (PD-1), T-cell immunoglobulin domain and mucin domain 3 (TIM-3) and V-domain Ig suppressor of T cell activation (VISTA). In particular, the immune checkpoint inhibitors target the PD-1 axis and/or CTLA-4.

[00141] The immune checkpoint inhibitors may be drugs such as small molecules, recombinant forms of ligand or receptors, or, in particular, are antibodies, such as human antibodies (*e.g.*, International Patent Publication WO2015016718; Pardoll, *Nat Rev Cancer*, 12(4): 252-64, 2012; both incorporated herein by reference). Known inhibitors of the immune checkpoint proteins or analogs thereof may be used, in particular chimerized, humanized or human forms of antibodies may be used. As the skilled person will know, alternative and/or equivalent names may be in use for certain antibodies mentioned in the present disclosure. Such alternative and/or equivalent names are interchangeable in the context of the present disclosure. For example, it is known that lambrolizumab is also known under the alternative and equivalent names MK-3475 and pembrolizumab.

[00142] In some embodiments, the PD-1 binding antagonist is a molecule that inhibits the binding of PD-1 to its ligand binding partners. In a specific aspect, the PD-1 ligand binding partners are PDL1 and/or PDL2. In another embodiment, a PDL1 binding antagonist is a molecule that inhibits the binding of PDL1 to its binding partners. In a specific aspect, PDL1 binding partners are PD-1 and/or B7-1. In another embodiment, the PDL2 binding antagonist is a molecule that inhibits the binding of PDL2 to its binding partners. In a specific aspect, a PDL2 binding partner is PD-1. The antagonist may be an antibody, an antigen binding fragment thereof, an immunoadhesin, a fusion protein, or oligopeptide. Exemplary antibodies are described in U.S. Patent Nos. US8735553, US8354509, and US8008449, all incorporated herein by reference. Other PD-1 axis antagonists for use in the methods provided herein are known in the art such as described in U.S. Patent Application No. US20140294898, US2014022021, and US20110008369, all incorporated herein by reference.

[00143] In some embodiments, the PD-1 binding antagonist is an anti-PD-1 antibody (*e.g.*, a human antibody, a humanized antibody, or a chimeric antibody). In some embodiments, the anti-PD-1 antibody is selected from the group consisting of nivolumab, pembrolizumab, and CT-011. In some embodiments, the PD-1 binding antagonist is an immunoadhesin (*e.g.*, an immunoadhesin comprising an extracellular or PD-1 binding portion of PDL1 or PDL2 fused to a constant region (*e.g.*, an Fc region of an immunoglobulin sequence). In some embodiments, the PD-1 binding antagonist is AMP- 224. Nivolumab, also known as MDX-1106-04, MDX-1106, ONO-4538, BMS-936558, and OPDIVO®, is an anti-PD-1 antibody described in WO2006/121168. Pembrolizumab, also known as MK-3475, Merck 3475, lambrolizumab, KEYTRUDA®, and SCH-900475, is an anti-PD-1 antibody

described in WO2009/114335. CT-011, also known as hBAT or hBAT-1, is an anti-PD-1 antibody described in WO2009/101611. AMP-224, also known as B7-DCIg, is a PDL2-Fc fusion soluble receptor described in WO2010/027827 and WO2011/066342.

[00144] Another immune checkpoint that can be targeted in the methods
5 provided herein is the cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), also known as CD152. The complete cDNA sequence of human CTLA-4 has the Genbank accession number L15006. CTLA-4 is found on the surface of T cells and acts as an “off” switch when bound to CD80 or CD86 on the surface of antigen-presenting cells. CTLA4 is a member of the immunoglobulin superfamily that is expressed on the surface of Helper T cells and transmits
10 an inhibitory signal to T cells. CTLA4 is similar to the T-cell co-stimulatory protein, CD28, and both molecules bind to CD80 and CD86, also called B7-1 and B7-2 respectively, on antigen-presenting cells. CTLA4 transmits an inhibitory signal to T cells, whereas CD28 transmits a stimulatory signal. Intracellular CTLA4 is also found in regulatory T cells and may be important to their function. T cell activation through the T cell receptor and CD28 leads to
15 increased expression of CTLA-4, an inhibitory receptor for B7 molecules.

[00145] In some embodiments, the immune checkpoint inhibitor is an anti-CTLA-4 antibody (*e.g.*, a human antibody, a humanized antibody, or a chimeric antibody), an antigen binding fragment thereof, an immunoadhesin, a fusion protein, or oligopeptide.

[00146] Anti-human-CTLA-4 antibodies (or VH and/or VL domains derived
20 therefrom) suitable for use in the present methods can be generated using methods well known in the art. Alternatively, art recognized anti-CTLA-4 antibodies can be used. For example, the anti-CTLA-4 antibodies disclosed in: US 8,119,129, WO 01/14424, WO 98/42752; WO 00/37504 (CP675,206, also known as tremelimumab; formerly ticilimumab), U.S. Patent No. 6,207,156; Hurwitz *et al.* (1998) *Proc Natl Acad Sci USA* 95(17): 10067-10071; Camacho *et al.* (2004) *J Clin Oncology* 22(145): Abstract No. 2505 (antibody CP-675206); and Mokyr *et al.* (1998) *Cancer Res* 58:5301-5304 can be used in the methods disclosed herein. The
25 teachings of each of the aforementioned publications are hereby incorporated by reference. Antibodies that compete with any of these art-recognized antibodies for binding to CTLA-4 also can be used. For example, a humanized CTLA-4 antibody is described in International
30 Patent Application No. WO2001014424, WO2000037504, and U.S. Patent No. 8,017,114; all incorporated herein by reference.

[00147] An exemplary anti-CTLA-4 antibody is ipilimumab (also known as 10D1, MDX-010, MDX-101, and Yervoy®) or antigen binding fragments and variants thereof (see, *e.g.*, WO 01/14424). In other embodiments, the antibody comprises the heavy and light chain CDRs or VRs of ipilimumab. Accordingly, in one embodiment, the antibody comprises the CDR1, CDR2, and CDR3 domains of the VH region of ipilimumab, and the CDR1, CDR2 and CDR3 domains of the VL region of ipilimumab. In another embodiment, the antibody competes for binding with and/or binds to the same epitope on CTLA-4 as the above-mentioned antibodies. In another embodiment, the antibody has at least about 90% variable region amino acid sequence identity with the above-mentioned antibodies (*e.g.*, at least about 90%, 95%, or 99% variable region identity with ipilimumab).

[00148] Other molecules for modulating CTLA-4 include CTLA-4 ligands and receptors such as described in U.S. Patent Nos. US5844905, US5885796 and International Patent Application Nos. WO1995001994 and WO1998042752; all incorporated herein by reference, and immunoadhesins such as described in U.S. Patent No. US8329867, incorporated herein by reference.

4. Surgery

[00149] Approximately 60% of persons with cancer will undergo surgery of some type, which includes preventative, diagnostic or staging, curative, and palliative surgery. Curative surgery includes resection in which all or part of cancerous tissue is physically removed, excised, and/or destroyed and may be used in conjunction with other therapies, such as the treatment of the present embodiments, chemotherapy, radiotherapy, hormonal therapy, gene therapy, immunotherapy, and/or alternative therapies. Tumor resection refers to physical removal of at least part of a tumor. In addition to tumor resection, treatment by surgery includes laser surgery, cryosurgery, electrosurgery, and microscopically-controlled surgery (Mohs' surgery).

[00150] Upon excision of part or all of cancerous cells, tissue, or tumor, a cavity may be formed in the body. Treatment may be accomplished by perfusion, direct injection, or local application of the area with an additional anti-cancer therapy. Such treatment may be repeated, for example, every 1, 2, 3, 4, 5, 6, or 7 days, or every 1, 2, 3, 4, and 5 weeks or every 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, or 12 months. These treatments may be of varying dosages as well.

5. Other Agents

[00151] It is contemplated that other agents may be used in combination with certain aspects of the present embodiments to improve the therapeutic efficacy of treatment. These additional agents include agents that affect the upregulation of cell surface receptors and GAP junctions, cytostatic and differentiation agents, inhibitors of cell adhesion, agents that increase the sensitivity of the hyperproliferative cells to apoptotic inducers, or other biological agents. Increases in intercellular signaling by elevating the number of GAP junctions would increase the anti-hyperproliferative effects on the neighboring hyperproliferative cell population. In other embodiments, cytostatic or differentiation agents can be used in combination with certain aspects of the present embodiments to improve the anti-hyperproliferative efficacy of the treatments. Inhibitors of cell adhesion are contemplated to improve the efficacy of the present embodiments. Examples of cell adhesion inhibitors are focal adhesion kinase (FAKs) inhibitors and Lovastatin. It is further contemplated that other agents that increase the sensitivity of a hyperproliferative cell to apoptosis, such as the antibody c225, could be used in combination with certain aspects of the present embodiments to improve the treatment efficacy.

B. Pharmaceutical Compositions

[00152] In select embodiments, it is contemplated that a cell expressing a TCR as disclosed herein, a protein containing the variable regions of a TCR, or a DNA encoding the variable regions of a TCR of the present invention may be comprised in a composition and administered to a subject to induce a therapeutic response in the subject. A therapeutic composition for pharmaceutical use in a subject may comprise a TCR composition disclosed herein, such as a soluble TCR (optionally attached to an imaging agent), and a pharmaceutically acceptable carrier.

[00153] The phrases “pharmaceutical,” “pharmaceutically acceptable,” or “pharmacologically acceptable” refers to molecular entities and compositions that do not produce an adverse, allergic or other untoward reaction when administered to an animal, such as, for example, a human, as appropriate. As used herein, “pharmaceutically acceptable carrier” includes any and all solvents, dispersion media, coatings, surfactants, antioxidants, preservatives (*e.g.*, antibacterial agents, antifungal agents), isotonic agents, absorption delaying agents, salts, preservatives, drugs, drug stabilizers, gels, binders, excipients, disintegration agents, lubricants, sweetening agents, flavoring agents, dyes, such like materials and

combinations thereof, as would be known to one of ordinary skill in the art (see, for example, Remington: The Science and Practice of Pharmacy, 21st edition, Pharmaceutical Press, 2011, incorporated herein by reference). Except insofar as any conventional carrier is incompatible with the active ingredient, its use in the vaccine compositions of the present invention is contemplated.

[00154] A person having ordinary skill in the medical arts will appreciate that the actual dosage amount of a therapeutic composition administered to an animal or human patient can be determined by physical and physiological factors such as body weight, severity of condition, the type of disease being treated, previous or concurrent therapeutic interventions, idiopathy of the patient and on the route of administration. The practitioner responsible for administration will, in any event, determine the concentration of active ingredient(s) in a composition and appropriate dose(s) for the individual subject.

[00155] A therapeutic composition disclosed herein can be administered intravenously, intradermally, intraarterially, intraperitoneally, intralesionally, intracranially, intraarticularly, intraprostatically, intrapleurally, intratracheally, intranasally, intravitreally, intravaginally, intrarectally, topically, intratumorally, intramuscularly, intraperitoneally, subcutaneously, subconjunctivally, intravesicularly, mucosally, intrapericardially, intraumbilically, intraocularly, orally, topically, locally, and by inhalation, injection, infusion, continuous infusion, lavage, and localized perfusion. A therapeutic composition may also be administered to a subject *via* a catheter, in cremes, in lipid compositions, by ballistic particulate delivery, or by other method or any combination of the forgoing as would be known to one of ordinary skill in the art.

[00156] While any suitable carrier known to those of ordinary skill in the art may be employed in the pharmaceutical compositions of this invention, the type of carrier will vary depending on the mode of administration. For parenteral administration, such as subcutaneous injection, the carrier preferably comprises water, saline, alcohol, a fat, a wax or a buffer. For oral administration, any of the above carriers or a solid carrier, such as mannitol, lactose, starch, magnesium stearate, sodium saccharine, talcum, cellulose, glucose, sucrose, and magnesium carbonate, may be employed. Biodegradable microspheres (*e.g.*, polylactic galactide) may also be employed as carriers for the pharmaceutical compositions of this invention. Suitable biodegradable microspheres are disclosed, for example, in U.S. Patents 4,897,268 and 5,075,109.

[00157] In other embodiments, a composition comprises an immobilized or encapsulated TCR or soluble TCR disclosed herein and a support substrate. In these embodiments, a support substrate can include, but is not limited to, a lipid microsphere, a lipid nanoparticle, an ethosome, a liposome, a niosome, a phospholipid, a sphingosome, a surfactant, a transferosome, an emulsion, or a combination thereof. The formation and use of liposomes and other lipid nano- and microcarrier formulations is generally known to those of ordinary skill in the art, and the use of liposomes, microparticles, nanocapsules and the like have gained widespread use in delivery of therapeutics (*e.g.*, U.S. Patent 5,741,516, specifically incorporated herein in its entirety by reference). Numerous methods of liposome and liposome-like preparations as potential drug carriers, including encapsulation of peptides, have been reviewed (U.S. Patents 5,567,434; 5,552,157; 5,565,213; 5,738,868 and 5,795,587, each of which is specifically incorporated in its entirety by reference).

[00158] A soluble TCR may be formulated into a composition in a neutral or salt form. Pharmaceutically acceptable salts, include the acid addition salts (formed with the free amino groups of the protein) and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids such as acetic, oxalic, tartaric, mandelic, and the like. Salts formed with the free carboxyl groups can also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, histidine, procaine and the like.

[00159] In any case, the composition may comprise various antioxidants to retard oxidation of one or more component. Additionally, the prevention of the action of microorganisms can be brought about by preservatives such as various antibacterial and antifungal agents, including but not limited to parabens (*e.g.*, methylparabens, propylparabens), chlorobutanol, phenol, sorbic acid, thimerosal or combinations thereof.

[00160] Sterile injectable solutions are prepared by incorporating the active peptides in the required amount in the appropriate solvent with various of the other ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the various sterilized active ingredients into a sterile vehicle that contains the basic dispersion medium and/or the other ingredients. In the case of sterile powders for the preparation of sterile injectable solutions, suspensions or emulsion, the preferred methods of preparation are vacuum-drying or freeze-drying techniques which yield a powder

of the active ingredient plus any additional desired ingredient from a previously sterile-filtered liquid medium thereof. The liquid medium should be suitably buffered if necessary and the liquid diluent first rendered isotonic prior to injection with sufficient saline or glucose. The preparation of highly concentrated compositions for direct injection is also contemplated, where the use of DMSO as solvent is envisioned to result in extremely rapid penetration, delivering high concentrations of the active agents to a small area.

[00161] The composition must be stable under the conditions of manufacture and storage, and preserved against the contaminating action of microorganisms, such as bacteria and fungi. It will be appreciated that endotoxin contamination should be kept minimally at a safe level, for example, less than 0.5 ng/mg protein.

[00162] In particular embodiments, prolonged absorption of an injectable composition can be brought about by the use in the compositions of agents delaying absorption, such as, for example, aluminum monostearate, gelatin or combinations thereof.

V. Examples

[00163] The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventor to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

Materials & Methods

[00164] *Display of recombinant RA14 variants on the CHO cell surface.* The amino acid sequences of the of the extracellular alpha and beta chains of the human TCR RA14 were obtained from the protein data bank (PDB 3GSN; Gras *et al.*, 2009), with the constant regions including the native-PESSC and -C on the alpha and beta chain C-termini, respectively. Minor changes were introduced into this sequence: V α :1I and V β :M2A were added to match the germline TRAV24 and TRBV6-5 sequences (IMGT), and α A78V (PDB residue 152) substitution was made to match the germline TRAC gene (UniProt). These

sequences were then reverted to DNA with CHO cell optimized codons and synthesized as a gblock (IDT). These were assembled into a cassette with a murine IgH leader sequence (Smith *et al.*, 2009) followed by one TCR chain, a T2A cleavage site with furin cut site (bold) and GSG linker (italics; sequence: **RRKR***GS***GEGRG**SLLT**CGDVEEN**PGP (SEQ ID NO: 43)), then the second TCR chain fused to a PDGFR transmembrane region (FIG. 1A) (Chng *et al.*, 2015). The TCR chains were cloned in both orientations, alpha-T2A-beta and beta-T2A-alpha. The constant regions were further modified to create a disulfide variant (ds) by introducing the amino acid substitutions V α :T84C, V β :S79C, and V β :C85.1A to move the terminal disulfide to a more central position and remove a free cysteine (Boulter *et al.*, 2003). The cassette was cloned into a pcDNA3 (Invitrogen) backbone for transient expression and moved into a pPyEBV backbone (Acyte Biotech) for semi-stable replication used during library sorting (Nguyen *et al.*, 2018).

[00165] *CHO cell transfection for TCR display analysis.* CHO-T cells (Acyte Biotech) were grown in CHO-S-SFM II media (Gibco) supplemented with 2x GlutaMax and penicillin/streptomycin. For transfection, cells were spun and resuspended at a concentration of 1.5×10^6 cells/mL, with 2 mL plated per well in a 6-well plate. For each well, 250 μ L of OptiMEM (ThermoFisher) was mixed with 10 μ L of Lipofectamine 2000 (ThermoFisher) and added to another tube with 250 μ L of OptiMEM and 4 μ g of DNA. The solution was mixed and allowed to equilibrate for 30 minutes at room temperature, before adding the solution to the appropriate well. The next day, cells were fed an additional 1 mL of media.

[00166] *TCR library design and cloning.* Two separate libraries were generated, one targeting the CDR3 α and the other targeting CDR3 β with saturation mutagenesis. The targeted region was defined as the single continuous stretch of residues in each CDR3 having direct contact with the pMHC (Gras *et al.*, 2009), as well as an additional residue on either side to confer additional loop flexibility (V α :107-115, V β :108-115; FIGS. 2A-B). To limit library size and retain binding affinity, three residues forming hydrogen bonds with the peptide (V α :114, V β :110, V β :114) were not randomized.

[00167] Libraries were generated using overlap PCR with degenerate codons (CDR3 α : NNS-NNS-NNS-AAC-NNS (SEQ ID NO: 44) and CDR3 β : NBS-VBC-ACC-VBC-VBC-VBC-TAC-NBS (SEQ ID NO: 45)) and Q5 hot-start master mix (NEB). The PCR insert and pPyEBV backbone were both digested with restriction enzymes flanking each targeted region (for CDR3 α , *AgeI* and *NheI*; for CDR3 β , *BamHI* and *NheI*, NEB), gel

extracted, and desalted. For each library ~1 µg of vector was ligated with insert at a 3:1 (CDR3α) or 6:1 (CDR3β) ratio overnight using T4 ligase (NEB). The following day, ligations were desalted and transformed into fresh NEB10β electrocompetent cells. After one hour of recovery, dilutions were plated and incubated, and colony counts used to estimate the library size. The library was grown to an OD₆₀₀ of 2 in liquid culture, which was then used to make frozen stocks and inoculate a new flask for overnight growth. Library DNA was prepared using a Maxiprep kit (Qiagen).

[00168] *Tetramer preparation.* Biotinylated HLA-A*0201 monomer loaded with the pp65₄₉₅₋₅₀₃ NLV peptide from human CMV or a control HCV peptide (Biolegend and the NIH tetramer facility) at 100 µg/mL was combined in a 4:1 molar ratio with streptavidin-conjugated to APC, AF647, or AF488 (ThermoFisher). Streptavidin was added slowly over 1 hour on ice to favor the formation of tetramer. Biotin (30 µM) was then added to block any unfilled biotin binding sites, and the tetramers incubated overnight at 4 °C.

[00169] *Flow cytometry and analysis.* Transfected CHO-K1 or CHO-T cells (~1x10⁶) were centrifuged (250g for 10 min) and resuspended in 100 µL PBS plus 2% FBS (Sigma) with 2 µg/mL NLV or HCV tetramer and a 1:50 dilution of anti-Vβ6-5-PE (Beckman Coulter). Cells were stained on ice for 1 hour, then rinsed and resuspended in 0.5 mL PBS plus 2% FBS. Samples were run on a Fortessa cytometer (BD Biosciences). A forward- and side-scatter gate was used to select live cells, with TCR-positive cells defined as having a PE signal greater than or equal to 10³. To compare binding activity of TCR variants, NLV tetramer binding was normalized by TCR display level by dividing the AF647 signal by the PE signal on a per-cell basis for the TCR-positive population. All analyses were performed with FlowJo software.

[00170] *Library transfection and sorting.* Confluent CHO-T cells (1 or 2 T-150 flasks) were transfected as above, with quantities scaled accordingly. Library DNA was diluted 1:4 with an inert yeast plasmid as carrier DNA (pCTCON) (Colby *et al.*, 2004) so that each CHO cell received at most one TCR expressing pPy plasmid. Flasks were also transfected with the wild-type pPy_RA14 as a positive control and pPy_hu4D5 expressing the anti-HER2 Fab as a negative control. Two days later, cells were scanned for TCR surface display using the anti-Vb6-5 antibody to evaluate transfection efficiency, and media replaced with half-strength selective media (CHO-S-SFM II plus 2x GlutaMax plus 150 µg/mL Hygromycin). Four or five days after transfection, cells were expanded and transferred into

full-strength selective media with 300 µg/mL hygromycin. Cells were maintained in selective media until reaching confluence in a T-150 (~2 weeks).

[00171] For sorting, $\sim 1 \times 10^7$ live cells were centrifuged (250g, 5 min) and resuspended in OptiMEM plus 1% BSA and stained with anti-TCR Vβ6-5 and NLV tetramer as described above. Cells were sorted using a FACS Aria, with the sort gate drawn to collect most fluorescent ~1-2% of the population, biased towards cells showing higher tetramer binding at the same TCR display level, to collect $\sim 1 \times 10^5$ cells. The sorted cells were expanded for ~1 week, and the process repeated two times. To recover plasmid from sorted CHO cells, genomic DNA was prepared from the pooled population one week after each sort. The region of interest (CDR3α or 3β, depending on the library) was PCR-amplified using flanking primers and 500 ng template DNA. PCR product was ligated into a TOPO vector (Invitrogen/Thermo Fisher) or digested and ligated into empty pPy backbone. After transformation, 10-40 individual colonies were sequenced from each round of sorting.

[00172] *Expression of soluble TCR-Fc fusion proteins.* To express soluble TCR-Fc fusion proteins, DNA encoding the RA14 extracellular alpha and beta chains was cloned into pcDNA3.0 plasmids downstream of a human IgG heavy chain leader sequence. Both the native human TCR constant domains and versions with the additional inter-chain disulfide introduced were used. The Cα domain was followed by the upper hinge sequence of human IgG1 (VEPKSC (SEQ ID NO: 46)) and then the Fc domains. The native IgG1 light chain cysteine was inserted at the C-terminus of Cβ to pair with the cysteine in the upper hinge and further stabilize the TCR constant domains. Additional modifications included the removal of five N-linked glycosylation sites predicted by NetNGlyc 1.0 Server (DTU Bioinformatics; available on the world wide web at cbs.dtu.dk/services/NetNGlyc/) by introducing α:N20Q, α:N177Q, α:N188Q, β:N65Q, and β:N184Q substitutions (FIG. 5A).

[00173] In one version, the construct consisted of alpha-T2A-beta-huIgG1 hinge, CH2 and CH3 domains, in another these two chains were encoded on separate pcDNA3.0-based plasmids, with either alpha or beta chain fused to the Fc. Both human IgG1 and mouse IgG2a hinge-Fc sequences were used (Uniprot #P01857 and #P01863, respectively). Constructs containing the mouse IgG2a Fc retained the human IgG1 VEPKSC before the mouse hinge-Fc sequence. Cloning was performed using Q5 hot-start polymerase

(NEB) and either traditional digestion/ligation or Gibson assembly methods and confirmed by Sanger sequencing.

[00174] For large-scale expression, two T-150 flasks of adherent CHO-K1 cells (ATCC #CCL-61) were grown to confluency and transfected using the same cell/media/reagent ratio described above for each variant. Cells were grown in high-glucose DMEM (Sigma) with 10% low-IgG FBS (ThermoFisher) and no antibiotics at 37 °C with 5% CO₂. Media was replaced the day after transfection and cells were transferred to a 32 °C, 5% CO₂ incubator for one week. Media was harvested and loaded onto a protein A column using an FPLC (ÅKTAPure, GE healthcare) using 100 mM phosphate, 150 mM NaCl, pH 7.2 and eluted with 100 mM glycine, pH 2.5. The eluate was immediately neutralized with 1M Tris, pH 8, and buffer-exchanged into PBS, pH 7.4, using a centrifugal filter (Amicon or Centricon).

[00175] *Protein biophysical characterization.* Purified TCR-Fc proteins (3 µg each) were prepared in reducing or non-reducing 6x SDS loading buffer and incubated for five minutes at 80 °C or 42 °C, respectively. Samples were run on a 4-20% gradient gel (BioRad) and stained with GelCode Blue (ThermoFisher Scientific). Size exclusion chromatography was performed with 100 µg purified protein in 100 µL using a Superdex S200 column and Åkta FPLC with PBS as the running buffer and Gel Filtration Calibration Kit High and Low molecular weight standards (GE Healthcare). For affinity variants, the peak eluting at ~10.5 mL containing properly assembled, bivalent TCR-Fc was collected and concentrated. To monitor thermal stability, protein was prepared at 200 µg/mL and diluted with protein thermal shift dye (ThermoFisher Scientific) following the recommended protocol. Samples were heated at 1 °C/min on a RT-PCR machine measuring fluorescence.

[00176] *Protein-protein binding assays.* For ELISA analyses, high-protein binding plates (Costar) were coated with 1 µg/mL of NLV/A2, HCV/A2 tetramer or nothing in PBS overnight at 4 °C, before blocking with 5% milk in PBS with 0.05% Tween-20 (PBS-T) for one hour at room temperature. The plate was washed three times with PBS-T, purified TCR-Fc titrated in 1:5 dilution steps from 10 µg/mL and incubated for one hour. After washing again, 1:1000 dilution of goat-anti-human Fc-HRP conjugate (Southern Biotech) was added to the plate for one hour. After a final wash, the plate was developed with TMB (Fisher Scientific), quenched with 1N HCl, and absorbance measured at 450 nm on a

Molecular Devices Spectramax. All plated volumes were 50 μ L. Data were analyzed with Graphpad Prism 5.

[00177] Dynamic and equilibrium binding kinetics were obtained using a BIAcore 3000 instrument. The purified TCR-Fc was immobilized on a CM5 sensor chip (GE Healthcare) via EDC/NHS coupling using a sodium acetate buffer at pH 4.0 for a total of 2000-5000 response units, with a blank flow cell used as the reference channel. Monomeric NLV/A2 was injected at concentrations ranging from 4 to 1000 nM at 30 μ L/min for two minutes and allowed to dissociate for six minutes, which resulted in a return to baseline without regeneration. The negative control HCV/A2 monomer was injected at the highest concentration for all variants. All data was measured at 25 °C. On rate, off rate, and equilibrium affinity analyses were performed using BIAEvaluation 3.0 software and fit using the 1:1 Langmuir binding model. All injections were performed twice and final kinetic values reported are the average and standard deviation for the entire dataset.

[00178] *Staining peptide-pulsed antigen-presenting cells.* Human TAP-deficient T2 lymphoblasts (174 x CEM.T2; ATCC #CRL-1992) expressing empty HLA-A2 were cultured in IMDM media supplemented with 4 mM glutamine at 37 °C and 5% CO₂. The pp65₄₉₅₋₅₀₃ peptide NLVPMVATV (SEQ ID NO: 41) and control HCV₁₄₀₆₋₁₄₁₅ peptide KLVALGINAV (SEQ ID NO: 42) were produced by solid phase synthesis (Peptide 2.0) and dissolved in DMSO for a final peptide stock of 50 mM. T2 cells in a six-well plate (2 mL/well) containing 10⁶ cells/mL were adjusted to 100 μ M or the indicated peptide concentration and incubated at 37 °C. After 24 hours, 5x10⁵ cells per sample were stained on ice for one hour using 2 μ M of purified TCR-mFc in PBS with 1% FBS (PBS-F) with 50 μ g/mL human Fc block (BD Biosciences) in a 50 μ L volume. Cells were washed twice with PBS-F and bound TCR-mFc detected with a 1:500 dilution of goat-anti-mouse Fc-AF647 (Jackson Immunobiology) for another hour. After a final wash, cells were resuspended and assayed for AF647 signal using a flow cytometer (Fortessa, BD Biosciences).

[00179] *Activation of human Jurkat T cells expressing RA14 variants.* A pcDNA3.1-derived plasmid with a CMV promoter was modified to support transient expression of a signaling-competent TCR comprised of the RA14 variable regions and mouse constant/transmembrane regions. First, a Kozak sequence with optimal ribosome binding site (sequence: CC ACC ATG G), multiple cloning site, and stop codon followed by a HindIII site and terminal SV40 PolyA tail signal were added to pcDN3.1. Next, separate

plasmids containing the TCR alpha and beta chains were cloned. The pRA14a alpha chain plasmid includes a murine alpha chain TCR signal sequence from IMGT TRAV5D-4 (amino acid sequence: MKTYAPTLFMFLWLQLDGMSQ (SEQ ID NO: 47)) in-frame with the human TCR alpha variable region, both flanked by restriction sites *EcoRI* and *AflIII* and the murine TCR alpha constant region (TRAC*01) and alpha transmembrane domain between restriction sites *AflIII* and *HindIII*. The pRA14b beta chain plasmid was constructed similarly, but with the murine beta chain signal sequence from IMGT TRBV13-2 (MGSRLFFVLSSLLCSKHM (SEQ ID NO: 48)) and human variable beta domain flanked by *EcoRI* and *AflIII* sites and beta constant region (TRBC*02) and beta transmembrane domain between restriction sites *AflIII* and *HindIII*. Signal sequences were encoded by oligonucleotides, constant regions amplified from mouse DO11.10 hybridoma mRNA by RT-PCR. Altered RA14 variable regions were introduced by PCR amplification followed by digestion/ligation into the *EcoRI* and *AflIII* sites.

[00180] Human Jurkat T-cells, clone E6-1 (ATCC #TIB-152) were grown in RPMI 1640 media with 10% FBS and 100 units/mL penicillin-streptomycin (Sigma) and transfected as previously described (Goyarts *et al.*, 1998). Briefly, 10^6 cells per transfection were centrifuged at 250g for five minutes, resuspended in 5 mL OptiMEM and incubated at room temperature for eight minutes. Cells were centrifuged as before and resuspended in 400 μ L OptiMEM. Cells were then mixed with 7.5 μ g of each alpha and beta plasmid in a 4 mm electroporation cuvette (Fisher) and incubated for eight minutes before pulsing exponentially with 250V, 950 μ F, and $\infty \Omega$ on a Biorad GenePulser. After an eight-minute recovery period, cells were rescued with 7 mL of RPMI 1640 (supplemented with 10% FBS and antibiotics) in a T-25 flask at 37°C and 5% CO₂. After 18-24 hours, recombinant TCR expression was monitored by flow cytometry, with the RA14 display level monitored by the anti-TRBV6-5 antibody-PE and binding activity monitored by NLV/A2 tetramer-AF647, as described above.

[00181] The ability of transfected RA14 variants to activate Jurkat cells was monitored by CD69 upregulation after incubation with peptide-pulsed T2 cells. T2 cells (10^5) were pulsed with NLV and HCV peptides at 0.1 μ M for four hours. T2 cells were washed once in RPMI to remove excess peptide. Transfected Jurkat cells (10^5) were co-cultured with pulsed T2 cells at a TCR-positive effector: target ratio of 1:1. After 24 hours, cells were collected and incubated with 50 μ g/mL human Fc block (BD Biosciences) for 10

minutes before adding anti-TRBV6-5-PE, NLV/A2 tetramer-AF647, and anti-CD69-FITC (Biolegend; 1 μ L of each antibody and 5 nM of tetramer per 50 μ L staining volume) for one hour on ice. Cells were scanned on a Fortessa cytometer (BD Biosciences) and gated for display of RA14 variants (PE signal $\geq$ 500) to exclude T2 cells. All analyses were performed with FlowJo.

Example 1 – Display of pp65 NLV-specific TCR RA14 on the CHO cell surface

[00182] To first determine the level of recombinant TCR displayed on the CHO cell surface, the truncated extracellular alpha and beta chains of the human RA14 TCR were cloned into a pcDNA3-based plasmid with a CMV promoter, human IgH leader sequence, one TCR chain, and T2A peptide sequence followed by the second TCR chain fused in-frame to a platelet-derived growth factor receptor (PDGFR)-derived transmembrane region (FIG. 1A). As only the second chain is fused to the transmembrane region and chain order can impact yields (Maynard *et al.*, 2005), the chains were cloned in both the α/β -TM and β/α -TM orientations. Similarly, since moving the terminal inter-chain di-sulfide bond to the C α :T84C and C β :S79C position (IMGT numbering used throughout) and removing the free cysteine at position C β 85.1 has been reported to improve expression of soluble and phage/yeast displayed TCRs (Boulter *et al.*, 2003; Ho *et al.*, 1998), these modifications were also tested in each chain orientation.

[00183] After cloning and sequence confirmation, midi-prepped plasmid DNA was transiently transfected into CHO-T cells and TCR surface display assessed by flow cytometry two days later. The presence of TCR on the cell surface was monitored by an antibody binding the human variable beta chain (V β 6-5-PE), while NLV/A2 tetramers conjugated to APC were used to assess ligand binding activity. A tetramer presenting an unrelated peptide from hepatitis C virus (HCV1406-1415 sequence: KLVALGINAV (SEQ ID NO: 42); hereafter called HCV) complexed with A2 was used to evaluate peptide specificity (FIG. 1B).

[00184] Flow cytometry showed varying expression patterns for each vector design, with the PE+/APC+ population indicative of cells binding tetramer and displaying TCR (FIG. 1C). Cells transfected with empty pcDNA3 vector showed minimal binding to either reagent, while a β -TM only construct bound the V β antibody, indicating that unpaired TCR chains will not be detected by tetramer binding. By contrast, cells transfected with constructs containing both TCR chains presented a diagonal double-positive population, indicating a

correlation between tetramer staining and surface display over a range of expression levels. Staining with the control HCV tetramer also showed no binding, indicating that the displayed TCRs retained peptide specificity. All samples included a population of unstained, non-expressing cells, which is expected for eukaryotes with unsynchronized growth. While inclusion of the modified di-sulfide bond greatly increased the specific tetramer binding activity (tetramer binding: TCR display ratio), chain order had less impact. A small but consistent improvement was detected in specific tetramer binding activity for the α/β -TM configuration. Accordingly, this design with the modified di-sulfide was selected for further use.

Example 2 – Design of CDR3 α and CDR3 β libraries

[00185] Analysis of the RA14-NLV/A2 crystal structure revealed that RA14 engages nearly all solvent-exposed peptide residues and forms hot spots with peptide residues P4:Pro, P5:Met and P8:Thr (Gras *et al.*, 2009). In the alpha chain, CDR3 α :N114 forms a key hydrogen bond with P5:Met which is also present in structure of the related TCR C7 with NLV/A2 (Yang *et al.*, 2015), which has a nearly identical CDR3 α . In the beta chain, CDR3 β :T110 forms multiple hydrogen bonds with P8:Pro. To identify high affinity RA14 variants, separate CDR3 α and CDR3 β libraries were designed, which allowed a larger sequence space for each CDR to be probed. Three anchor residues (V α :N114, V β :T110 and V β :Y114) were retained while four or six residues flanking these contacts were randomized to optimize the TCR-pMHC interface (FIG. 2).

[00186] To create each library, primers incorporating degenerate codons were designed to maximize amino acid diversity while keeping the theoretical library sizes (1×10^6 for CDR3 α , 4×10^6 for CDR3 β) near $\sim 10^6$, a limitation determined by mammalian cell culture volume constraints. Mutagenized cassettes were generated using overlap PCR with these primers, followed by overlap extension PCR to produce full-length inserts. These were digested and ligated into the pPyEBV vector, which allows semi-stable, selectable transfection in CHO-T cells (Nguyen *et al.*, 2018). After transformation into *E. coli*, actual library sizes were estimated as 4×10^5 for the CDR3 α library and 1×10^6 for the CDR3 β library, with diversity confirmed by DNA sequencing.

Example 3 – Selection of RA14 variants with improved tetramer binding

[00187] Pooled library plasmids were diluted with a 1:4 molar ratio of carrier DNA, to ensure each cell will acquire at most one library clone, and transfected into CHO-T cells (Nguyen *et al.*, 2018). After two weeks of growth under antibiotic selection to eliminate
5 cells lacking the pPy plasmid, cells were stained with AlexaFluor-647 (AF647)-labelled NLV/A2-tetramers to detect ligand binding and anti-V β 6-5-PE to detect surface TCR display. Each library was sorted by FACS to collect the 1-2% of cells with the highest ratio of tetramer binding to TCR display. After sorting, each library was allowed one week of recovery before being sorted again, for a total of three rounds. Comparison of the libraries at each step
10 demonstrated enrichment of clones with high levels of tetramer binding (FIGS. 3A-B). The CDR3 α and CDR3 β libraries each showed a 5-10-fold increase in the number of cells falling within the gated area per round.

[00188] TCR sequences were recovered from pooled cells after round three sorting by PCR amplification from total cellular DNA, followed by re-cloning into the pPyEBV
15 plasmid for sequencing. Analysis of 20-35 colonies revealed seven unique CDR3 α and 10 unique CDR3 β sequences (Tables 3A-B). The wild-type residues were largely retained at positions V β :109 and V β :111 within the CDR3 β library, while positions V β :112 and V β :113 were highly variable. RA14 covers an unusually high percent of the exposed peptide upon binding, and in particular that the CDR3 β contacts are mostly backbone-mediated, which may
20 explain the high variation possible in these residues (Gras *et al.*, 2009). Finally, larger residues were frequently found in the flanking sites, which may have been preferentially enriched if they were able to introduce additional peptide contacts.

Table 3A. Amino acid sequences of RA14 CDR3 α variants isolated from round 3 sorting.

Variant	SEQ ID NO:	CDR3 α										
IMGT #		104	105	106	107	108	109	114	115			
WT	39	C	A	R	N	T	G	N	Q			
Library	49	C	A	R	*	*	*	N	*			
CDR3 α 1	1	C	A	R	N	S	G	N	P			
CDR3 α 2	2	C	A	R	N	Y	G	N	H			
CDR3 α 3	3	C	A	R	N	Y	I	N	T			
CDR3 α 4	4	C	A	R	S	F	G	N	P			
CDR3 α 5	5	C	A	R	S	V	G	N	S			
CDR3 α 6	6	C	A	R	L	L	A	N	L			
CDR3 α 7	7	C	A	R	G	S	W	N	Q			

Table 3B. Amino acid sequences of RA14 CDR3 β variants isolated from round 3 sorting.

Variant	SEQ ID NO:	CDR3 β														
IMGT #		105	106	107	108	109	110	111	112	113	114	115	116	117		
WT	40	A	S	S	P	V	T	G	G	I	Y	G	Y	T		
Library	50	A	S	S	*	*	T	*	*	*	Y	*	Y	T		
CDR3 β 1	8	A	S	S	P	V	T	G	G	V	Y	L	Y	T		
CDR3 β 2	9	A	S	S	R	V	T	G	G	V	Y	L	Y	T		
CDR3 β 3	10	A	S	S	P	I	T	G	S	T	Y	I	Y	T		
CDR3 β 4	11	A	S	S	P	I	T	G	A	P	Y	L	Y	T		
CDR3 β 5	12	A	S	S	P	V	T	G	S	S	Y	G	Y	T		
CDR3 β 6	13	A	S	S	W	I	T	G	S	I	Y	T	Y	T		
CDR3 β 7	14	A	S	S	P	V	T	G	S	S	Y	W	Y	T		
CDR3 β 8	15	A	S	S	L	V	T	G	G	V	Y	L	Y	T		
CDR3 β 9	16	A	S	S	L	V	T	G	I	P	Y	L	Y	T		
CDR3 β 10	17	A	S	S	R	V	T	G	S	V	Y	G	Y	T		

Example 4 – Characterization of RA14 variants with improved tetramer binding

[00189] All identified CDR variants were transfected into fresh CHO-T cells in combination with the complementary wild-type chain and analyzed for TCR display level and tetramer binding as single clones. To compensate for TCR expression level differences, the specific tetramer binding activity (tetramer-AF647 signal/ anti-TCR-PE signal) was compared on a per cell basis for each clone. The median of this distribution was then normalized to the median value obtained for wild-type RA14 to report a fold-increase as compared to RA14 (FIGS. 3C-D). A similar process was performed after staining with the control HCV tetramers, revealing that peptide restriction was retained for all selected variants. Only two CDR3 α variants (α V1, α V2) showed binding similar to or improved over wild-type, with the best variant (α V2) having a 2.3-fold increase in normalized binding. In contrast, all 10 of the CDR3 β variants showed significant improvements over wild-type, with 1.8- to 3.5-fold improved specific tetramer binding. The variants with the greatest specific tetramer binding activity (α V1, α V2, β V1, β V4, β V7, and β V8) were selected for further analysis.

[00190] Next, it was determined whether the best alpha and beta variants could be combined to further improve tetramer binding, as reported in other studies (Li *et al.*, 2005). The wild-type and selected alpha (α V1, α V2) and beta (β V1, β V4, β V7, β V8) variants were combined pairwise, transfected and analyzed as before. The combinations generally showed further improved specific tetramer binding (FIG. 4A), with some variation in TCR display levels among clones. As surface display levels can be useful predictors of soluble expression yields (Shusta *et al.*, 1999), the α V2 and β V8 combination was selected as the lead candidate. Variant α V2 contains the V α :T108Y and V α :Q115H substitutions, while β V8 contains V β :P108L and the frequently-observed V β :I113V and V β :G115L mutations (Tables 3A-B).

[00191] To better understand the impact of the selected CDR changes on TCR function, TCR activation was evaluated after transfection into human Jurkat T cells. The native RA14 and selected α V2 and β V8 variable regions were combined with murine constant regions followed by human TCR transmembrane regions on a pcDNA-based plasmid. Mouse constant regions were used to prevent mispairing with the endogenous human TCR expressed in Jurkat cells (Cohen *et al.*, 2006). After electroporation, Jurkat cells were co-cultured with human T2 antigen-presenting cells pre-incubated with 0.1 μ M NLV or control HCV peptide and activation was measured by CD69-upregulation using flow cytometry 24 hours later (FIG. 4B). All TCRs tested showed activation in the presence of NLV but not HCV-pulsed T2 cells.

Moreover, the engineered TCRs showed significantly improved activation (2.2-2.7-fold) as compared to the wild-type RA14. Although the selection strategy was based upon TCR display levels and tetramer binding, these data suggest that features required for TCR activation were also retained (Sibener *et al.*, 2018), which is not always the case for affinity matured TCRs.

5 **Example 5 – Production of RA14 TCR as a soluble Fc-fusion protein**

[00192] In order to fully characterize our engineered TCRs and to support future applications of high affinity NLV-binding TCRs, it was necessary to produce these as soluble proteins. TCRs are notoriously difficult to produce, with no generally successful strategies yet identified (Maynard *et al.*, 2005 and Gunnarsen *et al.*, 2013). Since TCRs are naturally
10 produced by mammalian cells and since fusion of a poorly expressed protein to an antibody Fc domain generally increases expression level, increases avidity through bivalency, and provides a convenient detection handle for immunoassays, a TCR-Fc format was selected for production in CHO cells. Antibody expression plasmids were modified (Nguyen *et al.*, 2015) to express the wild-type RA14 variable and constant domains with one chain fused to the human IgG1
15 upper and core hinge region, followed by human Fc domains.

[00193] Several constructs were evaluated by small scale CHO expression and ELISA to compare expression level, including a one plasmid system with the two chains separated by a T2A peptide and versions with either the beta or alpha chain fused to the Fc. For these, constant TCR domains with the modified inter-chain disulfide bond were used
20 (named TCRds-huFc; Boulter *et al.*, 2003) in addition to a second disulfide-bond joining the base of the TCR constant domains contributed by the antibody upper hinge sequence (named TCR2ds-huFc). The highest expression levels, approaching that observed with antibodies, were obtained with a two-plasmid system in which the α -chain was fused to the Fc.

[00194] To compare RA14 and the engineered variants in the TCR2ds-huFc
25 format, the α V2 and β V8 domains were cloned into the two-plasmid expression system and transfected pairwise with plasmids encoding the wild-type RA14 chains to produce four variants: wild-type RA14 (WT), α V2. β WT, α WT. β V8, and α V2. β V8. After protein A purification, size exclusion chromatography (SEC) was performed to isolate the dominant peak (eluting at ~10.5 mL) containing protein with the highest tetramer binding activity and
30 appearing to correspond to intact, bivalent protein. SDS-PAGE analysis showed similar, high levels of purity for all variants (FIG. 5A). Protein stability was compared by thermal

unfolding, using differential scanning fluorimetry and using the inflection point of the first unfolding event to compare TCR domain stabilities. The β V8 changes are mildly destabilizing, inducing a >4 °C decrease in melting temperature as compared to RA14. In contrast, the α V2 changes increase thermal stability >2 °C, and partially compensate for the presence of the less stable β V8 in the combined α V2. β V8 variant (Table 4).

Table 4. Affinity and stability of RA14 TCR-Fc variants.

Variant	T _m , glycosylated (°C)	T _m , deglycosylated (°C)	k _a × 10 ⁵ , (M ⁻¹ s ⁻¹)	k _d × 10 ⁻² , (s ⁻¹)	χ ²	K _d = k _d /k _a (nM)	Equilibrium K _d (nM)
αWT.βWT	67.9 ± 0.4	68.8 ± 0.3	0.95 ± 0.1	29 ± 0.2	1.7 ± 0.6	3092 ± 400	1480 ± 60
α2.βWT	70.2 ± 0.5	71.3 ± 0.1	3.1 ± 0.8	15 ± 1	14 ± 2	514 ± 100	570 ± 13
αWT.β8	63.7 ± 0.7	64.1 ± 0.7	1.3 ± 0.02	3.2 ± 0.1	6.1 ± 0.6	243 ± 7	224 ± 7
α2.β8	65.2 ± 0.1	67.0 ± 0.5	3.5 ± 0.02	1.8 ± 0.1	1.4 ± 0.1	53 ± 1	62 ± 1
R2G	64.3 ± 0.7	ND	3.89 ± 0.01	0.36 ± 1 × 10 ⁻²	0.7 ± 0.04	9.2 ± 0.01	ND
2S-16	64.4 ± 0.6	ND	4.96 ± 0.17	0.029 ± 4 × 10 ⁻³	0.1 ± 0.01	0.59 ± 0.07	ND

Example 6 – Engineered variants show improved pMHC affinity as soluble TCR2ds-huFc fusion

[00195] To provide an initial assessment of NLV/A2 binding, the ability of purified TCR2ds-huFc variants to bind immobilized pMHC were compared in an ELISA. The NLV or HCV tetramer was coated onto an ELISA plate, TCR2ds-huFc protein titrated and detected by anti-human-Fc-HRP (FIG. 5B). No binding was detected for any variant to the control surface, while RA14 provided a clear dose-response curve on NLV tetramer coated wells, with a detection limit near 0.1 µg/mL. Both variants including the β V8 chain showed a distinct increase in binding, with the detection limit ~30-fold lower than observed for the other variants. In contrast, the α V2.βWT showed improved detection but a much shallower slope than observed for RA14.

[00196] Surface plasmon resonance was used to rigorously quantify binding of the four TCR-Fc variants to NLV/A2. SEC-purified TCR2ds-huFc was coupled to the sensor surface, with varying concentrations of NLV/A2 or HCV/A2 monomer passed over the surface. The WT RA14 exhibited the slow on-rates ($9.5 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$) and fast off-rates (0.29 sec^{-1}) typical of TCRs, resulting in a calculated affinity of $3.1 \text{ } \mu\text{M}$ for the bivalent TCR2ds-huFc format. This compares well to the $6.3 \text{ } \mu\text{M}$ K_d previously reported for monovalent RA14 using an immobilized pMHC orientation, but is tighter than the $27.7 \text{ } \mu\text{M}$ reported using immobilized TCR (Gras *et al.*, 2009; Gakamsky *et al.*, 2007). By contrast, the α V2 CDR changes affected only the on-rate, with a three-fold increase, while the β V8 CDR changes affected only the off-rate, with a 10-fold decrease (FIG. 5C). When combined, these changes showed synergy, conferring a 50 nM affinity for α V2. β V8, a 60-fold improved affinity over RA14 (Table 4). Equilibrium binding analyses yielded similar data (Table 4, FIGS. 6A-D). No variant exhibited detectable binding to the control HCV/HLA-A2 monomer at the highest concentrations used (FIG. 5C).

Example 7 – Genetic deglycosylation yields homogeneous TCR-Fc protein

[00197] The success of affinity maturation prompted efforts to further improve soluble TCR-Fc expression. Specifically, reducing SDS-PAGE showed broad bands with approximately the expected size, while SEC showed two or three distinct peaks, indicating the presence of incompletely-assembled TCR-Fc species (FIGS. 7B-C). This prompted the evaluation of two hypotheses: first, that TCR-Fc assembly was improved by stronger α - β chain interactions from the additional disulfide bond (Boulter *et al.*, 2003), and second, that assembly was sterically inhibited by TCR glycosylation, since RA14 includes several predicted N-linked glycosylation sites near chain interfaces.

[00198] To evaluate these possibilities, several constructs were compared: one with only the engineered di-sulfide bond between the constant domains (TCRds-huFc), one with an additional di-sulfide bond (TCR2ds-huFc) and one in which five predicted N-linked glycosylation sites were disrupted by N-to-Q amino acid substitutions (TCR2ds Δ gly-huFc; FIG. 7A). SDS-PAGE analysis shows that genetic de-glycosylation resulted in sharper protein bands (FIG. 7B). Introduction of the additional disulfide bond eliminated the minor SEC peak eluting at $\sim 13.5 \text{ mL}$, while de-glycosylation of the doubly-disulfide bonded format eliminated the second minor peak at $\sim 12.5 \text{ mL}$, resulting in a single, monodisperse product on SEC (FIG. 7C & 10A-C).

[00199] To evaluate the potential impact of these changes on protein stability, the thermal stability of each version was monitored, as above (FIG. 7D). The melting point of the RA14 TCRds-huFc increased slightly with the addition of the second disulfide bond and slightly again after deglycosylation to 68.8 °C (Table 4). This melting temperature is in the range of values typically reported for antibody Fab domains (67-79 °C; Nguyen *et al.*, 2015). It was subsequently confirmed that the SEC-purified glycosylated and genetically deglycosylated versions of each TCR2ds-Fc variant bind NLV tetramer similarly in an ELISA (FIGS. 8A-B).

Example 8 – Engineered TCR proteins stain pMHC-displaying cells

[00200] To demonstrate the potential utility of high affinity TCR-Fc fusion proteins to detect CMV-positive cells, they were used to stain peptide-pulsed antigen presenting cells. To prevent background staining due to binding between the human Fc on the TCR2ds-huFc proteins and human Fcγ receptors on the T2 cells, the human IgG1 hinge and Fc domains were replaced with their murine IgG2a counterparts to create TCR2ds-mFc constructs, which achieved similar yields and purity as the TCR2ds-huFc format (FIG. 9A). Human T2 antigen presenting cells were incubated overnight with 100 μM purified NLV or HCV peptide, stained with 1 μM purified TCR2ds-mFc followed by anti-mouse Fc-Alexa647 and analyzed by flow cytometry. Cells incubated with the HCV control peptide showed no shift in fluorescence as compared to cells incubated only with the anti-mouse Fc-AF647. A clear correlation in fluorescence shift with TCR affinity was observed, which increased from no detectable staining for RA14 to a distinct population for αV2.βV8 (FIG. 9B). Finally, to determine the peptide detection sensitivity, the peptide dose was serially diluted before incubation with T2 cells and staining with 1 μM α2β8 TCR2dsΔgly-mFc. Signals did not saturate at high peptide concentrations, as is expected for these TAP-deficient cells which upregulate MHC levels in the presence of peptide and were detectable down to 0.5 μM peptide (FIG. 9C).

Example 9 – Further improved RA14 TCR variant, 341

[00201] Using nearly the same method described above, a variant with further increased affinity due to changes in CDR1 was identified. For this library, the alpha chain was appended with the PDGFR transmembrane anchor, which allowed us to select for enhanced alpha-beta chain pairing with an anti-beta chain antibody (FIG. 11). Additionally,

the five predicted glycosylation sites were eliminated with amino acid substitutions at positions V α :N20Q, C α :N90Q, α :N109Q, V β :N77Q and C β :N85.6Q, since these changes improved assembly of purified TCR-Fc protein and are expected to also enhance TCR assembly when expressed on the eukaryotic cell surface. To generate this library, four residues in CDR 1 β and 1 α of the deglycosylated α 2. β 8 were randomized using degenerate oligonucleotides (red residues in FIG. 11). Finally, instead of the KLV control peptide complexed with HLA A2, a similar peptide from influenza was used to monitor promiscuous peptide binding, here called GIL (full sequence: GILGFVFTL (SEQ ID NO: 51)), also complexed with HLA A2. Three rounds of FACS selection, performed as above, resulted in clone 341 with a single residue change at position V α N29D and 7.8-fold enhanced binding over wild-type when displayed on CHO cells (Table 5 and FIG. 12). In the same experiment, α 2. β 8 was 3-fold better than wild-type and clone 341 ~2.5-fold better than α 2. β 8 (Table 5). Quantification of the flow data corresponding to FIG. 12 is shown below in Table 5 as well as in FIG. 13.

Table 5. Quantification of flow data from FIG. 12 to document improved binding to the NLV/A2 tetramer of TCR variants expressed on the CHO cell surface.

Ratio of NLV/A2 binding: TCR beta chain display (A647:PE)			
	Stain with 10 nM NLV/A2	Stain with 5 nM NLV/A2	Stain with 10 nM GIL/A2 control
WT	0.24	0.15	0.032
α 2. β 8	0.72	0.4	0.049
341	1.86	0.97	0.043
Fold better than WT			
α 2. β 8	3.00	2.67	
341	7.75	6.47	
Fold better than α2.β8			
α 2. β 8	1.00	1.00	
341	2.58	2.43	

[00202] None of the clones (wild-type RA14, α 2. β 8, nor 341) binds to the control peptide when complexed with HLA-A2. Controlling for display level, there is an absolute improvement in binding.

Example 10: Further development of sub-nanomolar TCR-Fc variants

[00203] Additional affinity maturation of pp65/HLA-A2-specific TCRs was performed. Further mutations in CDR3 α , CDR3 β , and CDR1 α that have resulted in 300-fold

improvements in affinity, down to ~10 nM Kd. These variants were used to create a final “shuffled” library comprising selected mutations or wild-type residue to remove detrimental or neutral mutations and identify beneficial or synergistic mutations. This library was generated using degenerate oligonucleotides and subjected to three additional rounds of FACS sorting after expression on the CHO cell surface and staining with fluorescent pMHC-tetramer-AF647. This resulted in identification of variants 2S-16, which has an affinity of 0.6 nM, as measured by SPR (see FIGs. 15-17). This represents a 1000-fold improved affinity as compared to the wild-type RA14 and improves the complex half-life from 2.3 seconds to 39 minutes. The affinity improved variants, especially the highest affinity 2S-16 variant, show an improved ability to detect pp65-pulsed human cells and exhibit decreased signal: noise (FIG. 18). When expressed on the surface of T cells with endogenous TCR transmembrane regions and exposed to human T2 cells pulsed with either the pp65 or a control peptide, the TCRs retain the ability to activate the T cell (FIG. 19).

Example 11: Antibody Fc variants that escape capture by CMV viral Fc receptors (vFcR)

[00204] HCMV has evolved an impressive array of immune evasion strategies[7] that allow the virus to infect and establish latency at some point in the majority of humans. It is clear that HCMV infection stimulates a robust adaptive immune response,[8] but spread of clinical strains of HCMV is not inhibited by antibodies from seropositive individuals,[9] about 1% of seropositive mothers still transmit HCMV to their fetuses,[10] the best attempts at immunization are only ~50% effective,[11, 12] and prophylactic IVIG administration of HCMV-specific antibodies to pregnant women have not provided particularly impressive fetal protection.[13] For example, the neutralizing anti-HCMV monoclonal antibody MSL-109 prevented hCMV infection *in vitro*, but not in the clinic. This antibody binds the glycoprotein gH on the surface of infected cells, but HCMV quickly became resistant to the antibody.[14]

[00205] The ability of antibodies to eliminate hCMV-infected cells and prevent cell-to-cell spread [9] appears to be hampered by expression of viral Fc gamma receptors (vFcγRs). HCMV induces the expression of vFcγRs in infected cells, which causes capture and degradation of antibodies targeting HCMV.[15] In theory, antibody-cellular cytotoxicity (ADCC) and antibody-dependent cellular phagocytosis (ADCP) should be important components of an immune response to viral infection, but data supporting this role is limited.

Data suggest that previously tested antibody therapeutics such as the anti-gH MSL-109 have been negatively impacted by the immune evasion mediated by vFcγRs.[14] When vFcγRs are expressed on the infected cell's surface, antibodies that bind exposed antigens, such as a pMHC complex or gH, are then bound by vFcγRs and internalized via bipolar bridging[15, 16] for degradation or repackaging for further evasion.[14] In HCMV strains lacking the vFcγRs gp34 and gp68, HCMV antibodies were much more potently able to bind human FcγRs and induce ADCC *in vitro*. [17] This immune response blunts the ability of antibodies to initiate ADCC and stimulate local immune responses.

[00206] Human Fc receptors in the FcγRI, FcγRII, and FcγRIII families all have overlapping binding sites on the human IgG1 Fc hinge, while FcRn binds between CH2 and CH3. To determine whether the binding sites of the human and viral Fc receptors can be uncoupled, gp34 and gp68 proteins were first cloned and purified. The gp34 protein (residues 24-182) and a truncated version of gp68 (tgp68; residues 68-289) were expressed, purified, and characterized (FIG. 20). The genes encoding the vFcγRs were amplified from the AD169/GFP[18] BAC (with an added strep tag for purification and detection and expressed in CHO cells. After purification, both proteins are homogeneous and monodisperse. Gp34 expresses as a homodimer of ~30 kDa glycosylated monomers, while gp68 expresses as a monomer of ~68 kDa.

[00207] The human and viral Fc receptors were used in competition ELISAs to determine that gp34 competitively inhibits FcγRIIIa binding while tgp68 competitively inhibits FcRn binding (FIG. 21). These data show that gp34 likely shares a partially overlapping epitope with FcγRIIIA, while gp68 likely binds near the FcRn binding site, and the two vFcγRs do not share an epitope. Prior mutagenesis studies support the conclusions that gp68 binds in the CH2-CH3 hinge.[19]

[00208] Additional ELISAs evaluated binding of the Fc receptors to multiple established Fc variants. The vFcγRs were minimally impacted by mutations known to impact FcRn and FcγRIIIA binding, with the exception of YTE,[20] which improves binding to FcRn, but abolishes binding to tgp68 (FIG. 22). This indicates that the vFcγRs do not bind identical epitopes to either FcRn or FcγRIIIA.

[00209] Using yeast Fc display, additional antibody Fc variants were identified that have lost the ability to bind two vFcγRs but retain native binding to the host Fcγ receptors, including FcγRIIIA which is required for ADCC and FcRn which mediates antibody recycling and is crucial for the long antibody half-life in serum. The human IgG1 Fc region was mutagenized and screened by yeast display to identify variants with reduced binding to HCMV vFcγRs but retain binding to host FcRn and FcγRIIIa. Libraries were created by error prone PCR on the CH2 domain with a 0.25% error rate to yield a final library of 1.6×10^6 variants. The hinge region was excluded from mutagenesis as it includes the FcγR3A binding site. The library was initially sorted by flow cytometry to select variants that maintain binding to FcγRIIIA. After regrowth, they were then sorted for variants which lost gp34 binding by staining with 20 nM FcγRIIIa-PE in the presence of an excess (1.5 μM) of unlabeled, purified gp34 as competitor. Selected sequences are shown in FIG. 23 and binding to viral and host Fc receptors in FIG. 24.

Example 12: Expression of additional TCR constructs in CHO cells

[00210] To demonstrate the generality of this CHO cell system for expression and engineering of TCRs, other than the RA14 TCR, additional TCR constructs were studied.

[00211] The Ob.1A12 human TCR recognizing the myelin basic protein peptide formed by residues 85-99 when presented by the class II DRB1*1501 MHC molecule was derived from a patient with relapsing-remitting multiple sclerosis (Wucherpfennig *et al.*, 1994). Notably, as an autoimmune-associated TCR, this exhibits a very low affinity (estimated at $K_d > 100 \mu M$) for its pMHC ligand. This TCR was expressed in CHO cells as a TCR-Fc fusion protein with the alpha chain fused to the human Fc domain and purified using protein A chromatography. Protein gel shows the protein purity, while size exclusion chromatography shows that the protein is monodisperse (FIG. 14).

[00212] Two classic mouse TCRs were also used as model systems. The OT-II and the DO11.10 mouse TCRs both bind the same ovalbumin peptide (residues 323-339) when bound by the class II MHC I-Ab (OT-II) or I-Ad (DO11.10). These T cell hybridomas, originally elicited by vaccination of mice with hen egg white ovalbumin (OVA) (White *et al.*, 1983), are well understood model system used to study CD4⁺ T cell responses (Scott *et al.*, 1998). Class II MHCs present exogenous peptides and are thus important for infection resistance and autoimmunity. Data presented in FIGs. 26-28 demonstrate that these TCR-Fc

fusion constructs produced in CHO cells could likewise be successfully, expressed and purified and displayed the selective binding to target peptide.

* * *

[00213] All of the methods disclosed and claimed herein can be made and executed
5 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 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. More specifically, it will be apparent that certain
10 agents which are both chemically and physiologically related may be substituted for the agents
described herein while the same or similar results would be achieved. 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.

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WHAT IS CLAIMED IS:

1. An engineered T-cell receptor (TCR) comprising an alpha chain CDR3 having the amino acid sequence of any one of SEQ ID NOs: 1-7 and/or a beta chain CDR3 having the amino acid sequence of any one of SEQ ID NOs: 8-17.
2. The engineered TCR of claim 1, wherein the engineered TCR is HLA-A2 restricted.
3. The engineered TCR of claim 1, wherein the engineered TCR is HLA-A2*02:01 restricted.
4. The engineered TCR of claim 1, wherein the engineered TCR specifically recognizes a peptide comprising an amino acid sequence of NLVPMVATV (SEQ ID NO: 41) in complex with HLA-A2*02:01.
5. The engineered TCR of claim 1, wherein the engineered TCR comprises an alpha chain CDR3 having the amino acid sequence of SEQ ID NO: 2 and a beta chain CDR3 having the amino acid sequence of SEQ ID NO: 15.
6. The engineered TCR of claim 1, wherein the engineered TCR comprises an alpha chain variable region having at least 90% identity to the amino acid sequence of any one of SEQ ID NOs: 18-24 and/or a beta chain variable region having at least 90% identity to the amino acid sequence of any one of SEQ ID NOs: 25-34.
7. The engineered TCR of claim 6, wherein the engineered TCR comprises an alpha chain variable region having at least 95% identity to the amino acid sequence of any one of SEQ ID NOs: 18-24 and/or a beta chain variable region having at least 95% identity to the amino acid sequence of any one of SEQ ID NOs: 25-34.
8. The engineered TCR of claim 6, wherein the engineered TCR comprises an alpha chain having at least 99% identity to the amino acid sequence of any one of SEQ ID NOs: 18-24 and/or a beta chain having at least 99% identity to the amino acid sequence of any one of SEQ ID NOs: 25-34.
9. The engineered TCR of claim 1, wherein the engineered TCR comprises an alpha chain of any one of SEQ ID NOs: 18-24 and/or a beta chain of any one of SEQ ID NOs: 25-34.

10. The engineered TCR of claim 1, wherein the engineered TCR comprises an alpha chain of SEQ ID NO: 19 and a beta chain of SEQ ID NO: 32.
11. The engineered TCR of any one of claims 1-10, wherein the engineered TCR is further defined as a soluble TCR, wherein the soluble TCR does not comprise a transmembrane domain.
12. The engineered TCR of claim 11, wherein the soluble TCR is linked to a CD3-specific antibody.
13. The engineered TCR of claim 11, wherein an extracellular domain of the TCR is fused to an antibody Fc domain.
14. The engineered TCR of claim 13, wherein the alpha chain of the TCR extracellular domain is fused to the antibody Fc domain.
15. The engineered TCR of claim 13 or 14, wherein the Fc domain is a human or mouse Fc domain.
16. The engineered TCR of any one of claims 13-15, further comprising an antibody hinge region.
17. The engineered TCR of any one of claims 1-10, wherein the engineered CAR is further defined as a chimeric antigen receptor.
18. The engineered TCR of any one of claims 1-16, further comprising a detectable label.
19. The engineered TCR of any one of claims 1-16, wherein the engineered TCR is covalently bound to a therapeutic agent.
20. The engineered TCR of claim 19, wherein the therapeutic agent is an immunotoxin or a chemotherapeutic agent.
21. A polynucleotide encoding the engineered TCR of any one of claims 1-20.
22. An expression vector encoding the engineered TCR of any one of claims 1-20.
23. The expression vector of claim 22, wherein the sequence encoding the TCR is under the control of a promoter.

24. The expression vector of claim 22, wherein the expression vector is a viral vector.
25. The expression vector of claim 24, wherein the viral vector is a retroviral vector.
26. The expression vector of claim 22, wherein the expression vector is an episomal vector.
27. The expression vector of claim 22, wherein the vector further encodes a linker domain.
28. The expression vector of claim 27, wherein the linker domain is positioned between the alpha chain and beta chain.
29. The expression vector of claim 27, wherein the linker domain comprises one or more cleavage sites.
30. The expression vector of claim 29, wherein the one or more cleavage sites are a Furin cleavage site and/or a P2A cleavage site.
31. The expression vector of claim 30, wherein the Furin cleavage site is RRKR.
32. The expression vector of claim 28, wherein the one or more cleavage sites are separated by a spacer.
33. The expression vector of claim 32, wherein the spacer is SGSG or GSG.
34. A cell engineered to express the TCR of any one of claims 1-17.
35. The cell of claim 34, wherein the cell is a T cell, NK cell, invariant NK cell, NKT cell, mesenchymal stem cell (MSC), or induced pluripotent stem (iPS) cell.
36. The cell of claim 34, wherein the cell is an immune cell.
37. The cell of claim 34, wherein the cell is isolated from an umbilical cord
38. The cell of claim 35, wherein the T cell is a CD8⁺ T cell, CD4⁺ T cell, or $\gamma\delta$ T cell.
39. The cell of claim 35, wherein the T cell is a regulatory T cell (Treg).
40. A method for engineering the cell of any one of claims 34 comprising contacting said immune cell with the TCR of any one of claims 1-17 or the expression vector of any one of claims 22-33.

41. The method of claim 40, wherein the immune cell is a T cell, peripheral blood lymphocyte, NK cell, invariant NK cell, or NKT cell.
42. The method of claim 40, wherein contacting is further defined as transfecting or transducing.
43. The method of any one of claims 40-42, wherein transfecting comprises electroporating RNA encoding the TCR of any one of claims 1-17 into the immune cell.
44. The method of any one of claims 40-42, further comprising generating viral supernatant from the expression vector of claim 22 prior to transducing the immune cell.
45. The method of any one of claims 40-44, wherein the immune cell is a stimulated lymphocyte.
46. The method of claim 45, wherein the stimulated lymphocyte is a human lymphocyte.
47. The method of claim 45, wherein stimulating comprises contacting the immune cell with or incubating the immune cell in OKT3 and/or IL-2.
48. The method of any one of claims 40-47, further comprising sorting the immune cells to isolate TCR engineered T cells.
49. The method of claim 48, further comprising performing T cell cloning by serial dilution.
50. The method of claim 49, further comprising expansion of the T cell clone by the rapid expansion protocol.
51. Use of a therapeutically effective amount of NLV/A2 TCR-specific cells according to any one of claims 34-39 for the treatment of a disease or disorder in a patient.
52. The use of claim 51, wherein the NLV/A2 TCR-specific cells are T cells.
53. Use of a therapeutically effective amount of a soluble TCR according to any one of claims 11-16 for the treatment of a disease or disorder in a patient.
54. A method of treating a disease or disorder in a subject in need thereof, monitoring disease progression in a subject, or monitoring vaccine efficacy in a subject, the method

comprising administering an effective amount of the TCR-engineered cells of any one of claims 34-39 or a soluble TCR according to any one of claims 11-16 to the subject.

55. The method of claim 54, wherein the subject has been identified as having an HLA-A2*02:01 allele.

56. The method of claim 54, wherein the TCR-engineered cell is a T cell or peripheral blood lymphocyte.

57. The method of claim 56, wherein the T cell is a CD8⁺ T cell, CD4⁺ T cell, or Treg.

58. The method of claim 56, wherein the cell is autologous.

59. The method of claim 56, wherein the cell is allogeneic.

60. The method of claim 54, wherein the disease is a cancer.

61. The method of claim 60, wherein the cancer is a glioblastoma.

62. The method of claim 60, wherein an NLV peptide has been delivered to cells of the cancer.

63. The method of claim 54, wherein the subject is a human.

64. The method of claim 56, wherein the cells are administered to the subject intravenously, intraperitoneally, or intratumorally.

65. The method of any one of claims 54-64, further comprising administering a second anticancer therapy.

66. The method of claim 65, wherein the second anticancer therapy is a chemotherapy, immunotherapy, surgery, radiotherapy, or biotherapy.

67. The method of claim 66, wherein the immunotherapy is an immune checkpoint inhibitor.

68. The method of claim 67, wherein the immune checkpoint inhibitor inhibits an immune checkpoint protein or ligand thereof selected from the group consisting of CTLA-4, PD-1, PD-L1, PD-L2, LAG-3, BTLA, B7H3, B7H4, TIM3, KIR, or adenosine A2a receptor (A2aR).

69. The method of claim 67, wherein the immune checkpoint inhibitor inhibits PD-1 or CTLA-4.
70. The method of claim 60, wherein the patient has been determined to have a cancer with a reactivated CMV infection.
71. A method of detecting NLV/A2 complexes on cells, the method comprising contacting the cells with a soluble TCR of any one of claims 11-16 and 18 and detecting binding of the soluble TCR to the cells.
72. The method of claim 71, wherein the soluble TCR comprises a mouse Fc domain.
73. The method of claim 71, wherein the cells are peptide-pulsed antigen presenting cells.
74. The method of claim 73, wherein the antigen presenting cells have been pulsed with a peptide comprising an amino acid sequence of NLVPMVATV (SEQ ID NO: 41).
75. An engineered T-cell receptor (TCR) comprising an alpha chain CDR3 having the amino acid sequence of any one of SEQ ID NOs: 1-7 or 60-61 and/or a beta chain CDR3 having the amino acid sequence of any one of SEQ ID NOs: 8-17 or 62-63.
76. The engineered TCR of claim 75, wherein the engineered TCR is HLA-A2 restricted.
77. The engineered TCR of claim 75, wherein the engineered TCR is HLA-A2*02:01 restricted.
78. The engineered TCR of claim 75, wherein the engineered TCR specifically recognizes a peptide comprising an amino acid sequence of NLVPMVATV (SEQ ID NO: 41) in complex with HLA-A2*02:01.
79. The engineered TCR of claim 75, wherein the engineered TCR comprises an alpha chain CDR3 having the amino acid sequence of SEQ ID NO: 2 and a beta chain CDR3 having the amino acid sequence of SEQ ID NO: 15.
80. The engineered TCR of claim 75, wherein the engineered TCR comprises an alpha chain CDR3 having the amino acid sequence of SEQ ID NO: 60 and a beta chain CDR3 having the amino acid sequence of SEQ ID NO: 62.

81. The engineered TCR of claim 75, wherein the engineered TCR comprises an alpha chain CDR3 having the amino acid sequence of SEQ ID NO: 61 and a beta chain CDR3 having the amino acid sequence of SEQ ID NO: 63.
82. The engineered TCR of claim 75, wherein the engineered TCR comprises an alpha chain CDR1 of Table 1A-2 or Table 1C.
83. The engineered TCR of claim 75, wherein the engineered TCR comprises an alpha chain CDR1; CDR2 and CDR3 of Table 1C.
84. The engineered TCR of claim 75, wherein the engineered TCR comprises a beta chain CDR1; CDR2 and CDR3 of Table 1D.
85. The engineered TCR of claim 75, wherein the engineered TCR comprises an alpha chain CDR1; CDR2 and CDR3 of Table 1C; and a beta chain CDR1; CDR2 and CDR3 of Table 1D.
86. The engineered TCR of claim 75, wherein the engineered TCR comprises an alpha chain variable region having at least 90% identity to the amino acid sequence of any one of those in Table 1C and/or a beta chain variable region having at least 90% identity to the amino acid sequence of any one of those in Table 1D.
87. The engineered TCR of claim 86, wherein the engineered TCR comprises an alpha chain variable region having at least 95% identity to the amino acid sequence of any one of those in Table 1C and/or a beta chain variable region having at least 95% identity to the amino acid sequence of any one of those in Table 1D.
88. The engineered TCR of claim 86, wherein the engineered TCR comprises an alpha chain having at least 99% identity to the amino acid sequence of any one of those in Table 1C and/or a beta chain having at least 99% identity to the amino acid sequence of any one of those in Table 1D.
89. The engineered TCR of claim 75, wherein the engineered TCR comprises an alpha chain of any one of those in Table 1C and/or a beta chain of any one of those in Table 1D.

90. The engineered TCR of claim 75, wherein the engineered TCR comprises an alpha chain of SEQ ID NO: 19 and a beta chain of SEQ ID NO: 32.
91. The engineered TCR of any one of claims 75-90, wherein the engineered TCR is further defined as a soluble TCR, wherein the soluble TCR does not comprise a transmembrane domain.
92. The engineered TCR of claim 91, wherein the soluble TCR is linked to a CD3-specific antibody.
93. The engineered TCR of claim 91, wherein an extracellular domain of the TCR is fused to an antibody Fc domain.
94. The engineered TCR of claim 93, wherein the alpha chain of the TCR extracellular domain is fused to the antibody Fc domain.
95. The engineered TCR of claim 93 or 94, wherein the Fc domain is a human or mouse Fc domain.
96. The engineered TCR of any one of claims 93-95, further comprising an antibody hinge region.
97. The engineered TCR of claim 95, wherein the Fc domain exhibits reduced binding a viral Fc receptor (vFcR) relative to a wt Fc.
98. The engineered TCR of claim 97, wherein the vFcR comprises CMV gp34 and/or gp68.
99. The engineered TCR of claim 97, wherein the vFcR is able to bind to human FcRIIIa and/or FcRn.
100. The engineered TCR of claim 97, wherein the Fc domain comprises one or more substitutions relative to wt Fc domain selected from a substitution at S337; K334; G341; E345; T350; K338; H268; E294 and Q311.
101. The engineered TCR of claim 100, comprising substitutions selected from: (a) S337; (b) S337 and G341; (c) S337; E345; and T350; (d) K334; and K338; (e) S337 and T350; (f) H268; and K334; and (g) H268; E294; Q311; and K334.

102. The engineered TCR of claim 100, comprising one or more substitutions relative to wt Fc domain selected from S337F; K334E; G341E; E345K; T350I; K338I; H268L; E294K and Q311L, wherein the engineered Fc domain exhibits reduced binding a viral Fc receptor (vFcR) relative to a wt Fc.

103. The engineered TCR of claim 100, comprising substitutions selected from: (a) S337F; (b) S337F and G341E; (c) S337F; E345K; and T350I; (d) K334E; and K338I; (e) S337F and T350I; (f) H268L; and K334E; and (g) H268L; E294K; Q311L; and K334E.

104. The engineered TCR of claim 97, comprising wherein the antibody Fc domain comprises one or more substitution selected from M252Y; S254T and T256E.

105. The engineered TCR of claim 104, wherein the engineered antibody Fc domain comprises the following substitutions M252Y; S254T and T256E.

106. The engineered TCR of any one of claims 75-90, wherein the engineered TCR is further defined as a chimeric antigen receptor.

107. The engineered TCR of any one of claims 75-96, further comprising a detectable label.

108. The engineered TCR of any one of claims 75-96, wherein the engineered TCR is covalently bound to a therapeutic agent.

109. The engineered TCR of claim 108, wherein the therapeutic agent is an immunotoxin or a chemotherapeutic agent.

110. A polynucleotide encoding the engineered TCR of any one of claims 75-109.

111. An expression vector encoding the engineered TCR of any one of claims 75-109.

112. The expression vector of claim 111, wherein the sequence encoding the TCR is under the control of a promoter.

113. The expression vector of claim 111, wherein the expression vector is a viral vector.

114. The expression vector of claim 113, wherein the viral vector is a retroviral vector.

115. The expression vector of claim 111, wherein the expression vector is an episomal vector.

116. The expression vector of claim 111, wherein the vector further encodes a linker domain.
117. The expression vector of claim 116, wherein the linker domain is positioned between the alpha chain and beta chain.
118. The expression vector of claim 116, wherein the linker domain comprises one or more cleavage sites.
119. The expression vector of claim 118, wherein the one or more cleavage sites are a Furin cleavage site and/or a P2A cleavage site.
120. The expression vector of claim 119, wherein the Furin cleavage site is RRKR.
121. The expression vector of claim 117, wherein the one or more cleavage sites are separated by a spacer.
122. The expression vector of claim 121, wherein the spacer is SGSG or GSG.
123. A cell engineered to express the TCR of any one of claims 75-106.
124. The cell of claim 123, wherein the cell is a T cell, NK cell, invariant NK cell, NKT cell, mesenchymal stem cell (MSC), or induced pluripotent stem (iPS) cell.
125. The cell of claim 123, wherein the cell is an immune cell.
126. The cell of claim 123, wherein the cell is isolated from an umbilical cord
127. The cell of claim 124, wherein the T cell is a CD8⁺ T cell, CD4⁺ T cell, or $\gamma\delta$ T cell.
128. The cell of claim 124, wherein the T cell is a regulatory T cell (Treg).
129. A method for engineering the cell of any one of claims 123 comprising contacting said immune cell with the TCR of any one of claims 75-106 or the expression vector of any one of claims 111-122.
130. The method of claim 129, wherein the immune cell is a T cell, peripheral blood lymphocyte, NK cell, invariant NK cell, or NKT cell.
131. The method of claim 129, wherein contacting is further defined as transfecting or transducing.

132. The method of any one of claims 129-131, wherein transfecting comprises electroporating RNA encoding the TCR of any one of claims 75-106 into the immune cell.
133. The method of any one of claims 129-131, further comprising generating viral supernatant from the expression vector of claim 111 prior to transducing the immune cell.
134. The method of any one of claims 129-133, wherein the immune cell is a stimulated lymphocyte.
135. The method of claim 134, wherein the stimulated lymphocyte is a human lymphocyte.
136. The method of claim 134, wherein stimulating comprises contacting the immune cell with or incubating the immune cell in OKT3 and/or IL-2.
137. The method of any one of claims 129-136, further comprising sorting the immune cells to isolate TCR engineered T cells.
138. The method of claim 137, further comprising performing T cell cloning by serial dilution.
139. The method of claim 138, further comprising expansion of the T cell clone by the rapid expansion protocol.
140. Use of a therapeutically effective amount of NLV/A2 TCR-specific cells according to any one of claims 123-128 for the treatment of a disease or disorder in a patient.
141. The use of claim 140, wherein the NLV/A2 TCR-specific cells are T cells.
142. Use of a therapeutically effective amount of a soluble TCR according to any one of claims 91-96 for the treatment of a disease or disorder in a patient.
143. A method of treating a disease or disorder in a subject in need thereof, monitoring disease progression in a subject, or monitoring vaccine efficacy in a subject, the method comprising administering an effective amount of the TCR-engineered cells of any one of claims 123-128 or a soluble TCR according to any one of claims 91-96 to the subject.
144. The method of claim 143, wherein the subject has been identified as having an HLA-A2*02:01 allele.

145. The method of claim 143, wherein the TCR-engineered cell is a T cell or peripheral blood lymphocyte.
146. The method of claim 145, wherein the T cell is a CD8⁺ T cell, CD4⁺ T cell, or Treg.
147. The method of claim 145, wherein the cell is autologous.
148. The method of claim 145, wherein the cell is allogeneic.
149. The method of claim 143, wherein the disease is a cancer.
150. The method of claim 149, wherein the cancer is a glioblastoma.
151. The method of claim 149, wherein an NLV peptide has been delivered to cells of the cancer.
152. The method of claim 143, wherein the subject is a human.
153. The method of claim 145, wherein the cells are administered to the subject intravenously, intraperitoneally, or intratumorally.
154. The method of any one of claims 143-153, further comprising administering a second anticancer therapy.
155. The method of claim 154, wherein the second anticancer therapy is a chemotherapy, immunotherapy, surgery, radiotherapy, or biotherapy.
156. The method of claim 155, wherein the immunotherapy is an immune checkpoint inhibitor.
157. The method of claim 156, wherein the immune checkpoint inhibitor inhibits an immune checkpoint protein or ligand thereof selected from the group consisting of CTLA-4, PD-1, PD-L1, PD-L2, LAG-3, BTLA, B7H3, B7H4, TIM3, KIR, or adenosine A2a receptor (A2aR).
158. The method of claim 156, wherein the immune checkpoint inhibitor inhibits PD-1 or CTLA-4.
159. The method of claim 149, wherein the patient has been determined to have a cancer with a reactivated CMV infection.

160. A method of detecting NLV/A2 complexes on cells, the method comprising contacting the cells with a soluble TCR of any one of claims 91-96 and 107 and detecting binding of the soluble TCR to the cells.

161. The method of claim 160, wherein the soluble TCR comprises a mouse Fc domain.

162. The method of claim 160, wherein the cells are peptide-pulsed antigen presenting cells.

163. The method of claim 162, wherein the antigen presenting cells have been pulsed with a peptide comprising an amino acid sequence of NLVPMVATV (SEQ ID NO: 41).

164. An immunoglobulin fusion complex comprising:

(i) an α TCR variable domain fused to a first antibody Fc domain, wherein α TCR variable domain is bound to a β TCR variable domain by a disulfide bond; or

(ii) a β TCR variable domain fused to a first antibody Fc domain, wherein β TCR variable domain is bound to an α TCR variable domain by a disulfide bond,

wherein the α TCR variable domain and the β TCR variable domain comprise amino acid substitutions that block N-linked glycosylation.

165. The complex of claim 164, wherein the first antibody Fc domain is bound to a second antibody Fc domain by a disulfide bond.

166. The complex of claim 165, wherein the second antibody Fc domain is fused to an antibody heavy chain variable domain that is bound to an antibody light chain by a disulfide bond.

167. The complex of claim 164, comprising two immunoglobulin fusion complexes that are bound by disulfide bonds between the Fc domains.

168. The complex of claim 164, wherein the second Fc domain is fused to targeting moiety.

169. The complex of claim 168, wherein targeting moiety is an antibody VH/VL domain.

170. The complex of claim 168, wherein targeting moiety binds to immune effector cells.

171. The complex of claim 168, wherein targeting moiety binds to CD3.

172. The complex of claim 164, wherein α TCR variable domain is bound to a β TCR variable domain by two disulfide bonds.
173. The complex of claim 164, wherein the α TCR variable domain and the β TCR variable domain comprise N to Q amino acid substitutions that block N-linked glycosylation.
174. The complex of claim 164, wherein the α TCR variable domain and the β TCR variable domain comprise N to Q amino acid substitutions that block all N-linked glycosylation.
175. The complex of claim 164, wherein the α TCR variable domain and the β TCR variable domain specifically bind to a peptide in complex with HLA.
176. The complex of claim 175, wherein the HLA is HLA-A2.
177. The complex of claim 175, wherein the peptide in complex with HLA is a cancer protein peptide, an autoimmune peptide or an infectious disease peptide.
178. The complex of claim 175, wherein the peptide is a CMV peptide.
179. The complex of claim 178, wherein the CDR3 domains of the TCR variable domains are those recited in claim 75.
180. A method of producing a TCR complex comprising:
- (a) expressing one or more nucleic acid molecules in a mammalian cell encoding:
 - (i) an α TCR variable domain fused to a first antibody Fc domain, wherein α TCR variable domain is bound to a β TCR variable domain by a disulfide bond; or
 - (ii) a β TCR variable domain fused to a first antibody Fc domain, wherein β TCR variable domain is bound to an α TCR variable domain by a disulfide bond,wherein the α TCR variable domain and the β TCR variable domain comprise amino acid substitutions that block N-linked glycosylation; and
 - (b) collecting or purifying the TCR complex.
181. The method of claim 180, wherein the mammalian cell is CHO cell.
182. A TCR complex produced by a method of claim 180 or 181.

183. An engineered antibody Fc domain comprising one or more substitutions relative to wt Fc domain selected from a substitution at S337; K334; G341; E345; T350; K338; H268; E294 and Q311, wherein the engineered Fc domain exhibits reduced binding a viral Fc receptor (vFcR) relative to a wt Fc.

184. The engineered antibody Fc domain of claim 183, comprising substitutions selected from: (a) S337; (b) S337 and G341; (c) S337; E345; and T350; (d) K334; and K338; (e) S337 and T350; (f) H268; and K334; and (g) H268; E294; Q311; and K334.

185. The engineered antibody Fc domain of claim 183, comprising one or more substitutions relative to wt Fc domain selected from S337F; K334E; G341E; E345K; T350I; K338I; H268L; E294K and Q311L, wherein the engineered Fc domain exhibits reduced binding a viral Fc receptor (vFcR) relative to a wt Fc.

186. The engineered antibody Fc domain of claim 185, comprising substitutions selected from: (a) S337F; (b) S337F and G341E; (c) S337F; E345K; and T350I; (d) K334E; and K338I; (e) S337F and T350I; (f) H268L; and K334E; and (g) H268L; E294K; Q311L; and K334E.

187. The engineered antibody Fc domain of any one of claims 183-186, wherein the vFcR comprises CMV gp34 and/or gp68.

188. The engineered antibody Fc domain of claim 187, wherein the vFcR is CMV gp34.

189. The engineered antibody Fc domain of any one of claims 183-186, wherein the engineered Fc domain is able to bind to human FcRIIIa and/or FcRn.

190. The engineered antibody Fc domain of any one of claims 183-186, wherein the engineered Fc domain comprises a CH1, CH2 and CH3 domain at least 85%, 89% or 95% identical to a human IgG1 CH1, CH2 and CH3.

191. The engineered antibody Fc domain of any one of claims 183-186, wherein the engineered Fc domain is an IgG1 Fc domain.

192. The engineered antibody Fc domain of any one of claims 183-186, wherein the engineered Fc domain is an IgG2 Fc domain.

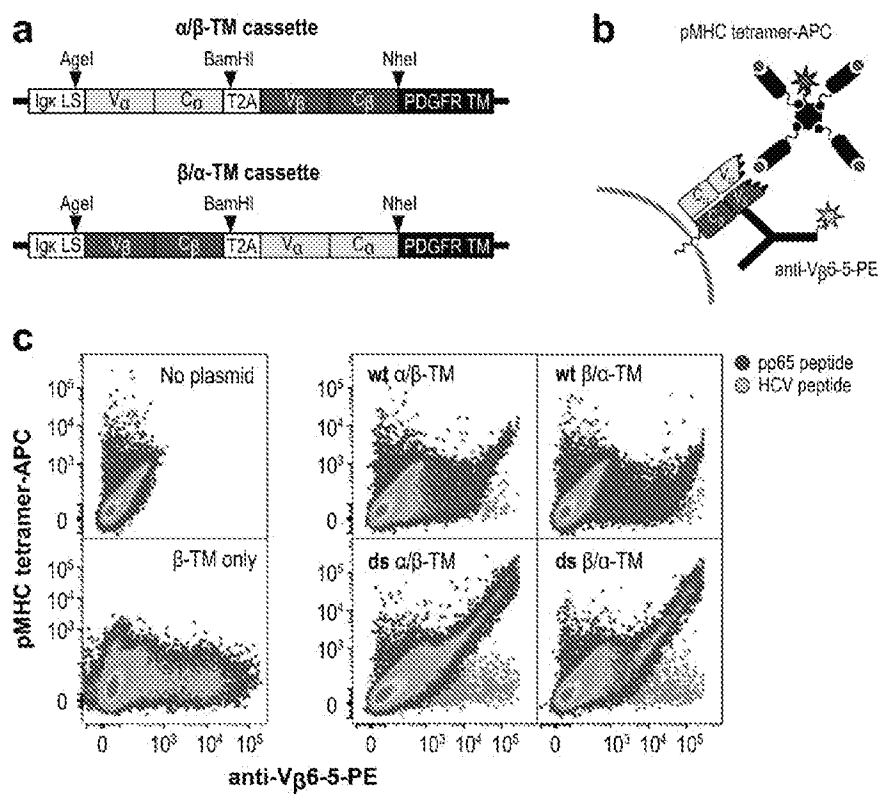
193. The engineered antibody Fc domain of any one of claims 183-186, wherein the engineered Fc domain is at least 85%, 90% or 95% identical to any one of SEQ ID NOs: 53-60.
194. The engineered antibody Fc domain of any one of claims 183-186, further comprising one or more substitution selected from M252; S254 and T256.
195. The engineered antibody Fc domain of any one of claims 183-186, further comprising a substitution at M252; S254 and T256.
196. The engineered antibody Fc domain of any one of claims 183-186, further comprising one or more substitution selected from M252Y; S254T and T256E.
197. The engineered antibody Fc domain of any one of claims 183-186, further comprising the following substitutions M252Y; S254T and T256E.
198. The engineered antibody Fc domain of any one of claims 183-197, further comprising an antibody variable domain or a TCR variable domain.
199. The engineered antibody Fc domain of any one of claims 183-197, wherein the engineered Fc domain is fused or conjugated to antibody variable domains or TCR variable domains that bind to a target of interest.
200. The engineered antibody Fc domain of claim 199, wherein target of interest is a viral protein or a viral peptide bound to HLA.
201. The engineered antibody Fc domain of claim 199, wherein target of interest is a CMV protein or a CMV peptide bound to HLA.
202. The engineered antibody Fc domain of claim 199, wherein the engineered Fc domain is fused or conjugated to TCR variable domains that bind to NLVPMVATV (SEQ ID NO: 41) in complex with HLA-A2*02:01.
203. A nucleic acid molecule encoding an engineered antibody Fc domain of any one of claims 183-197.
204. A host cell expressing a nucleic acid molecule of claim 203.
205. The host cell of claim 204, wherein the cell is a CHO cell.

206. A method of targeting a CMV infected cell comprising contacting the cell with molecule that binds to a CMV polypeptide or a CMV peptide bound to HLA, wherein the molecule is fused or conjugated to an engineered antibody Fc domain comprising one or more substitutions relative to wt Fc domain selected from a substitution at S337; K334; G341; E345; T350; K338; H268; E294; Q311; M252; S254; and T256, wherein the engineered Fc domain exhibits reduced binding a viral Fc receptor (vFcR) relative to a wt Fc.

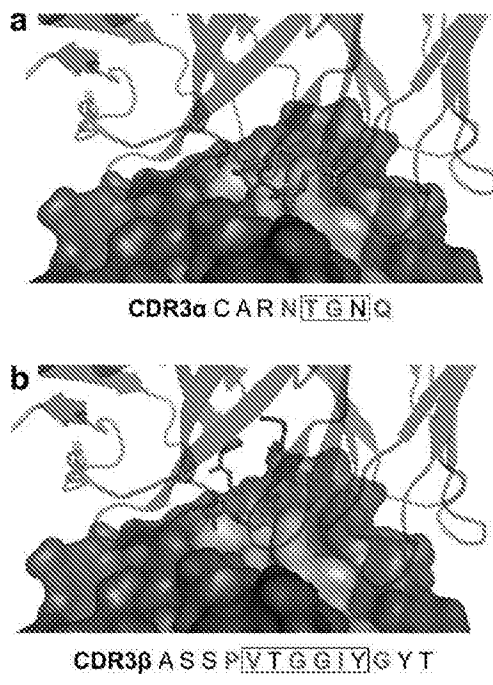
207. The method of claim 206, wherein the engineered antibody Fc domain comprises one or more substitution selected from M252Y; S254T and T256E.

208. The method of claim 207, wherein the engineered antibody Fc domain comprises the following substitutions M252Y; S254T and T256E.

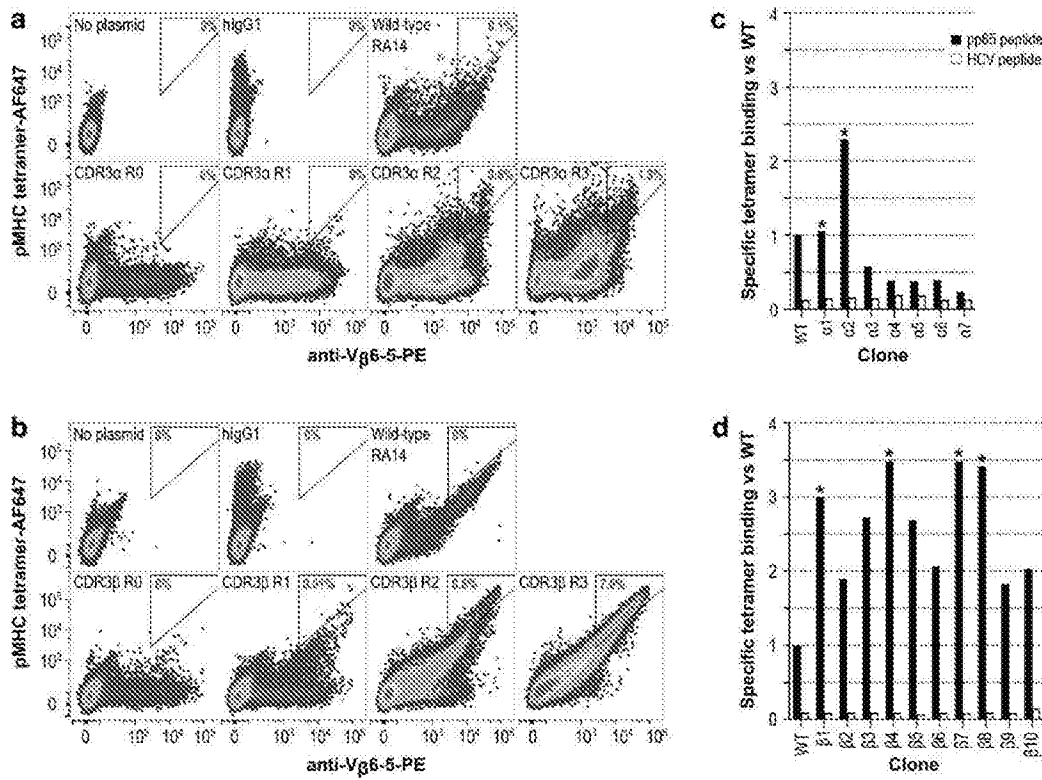
209. The method of any one of claims 206-208, wherein the engineered antibody Fc domain is a domain in accordance with anyone of claims 183-197.



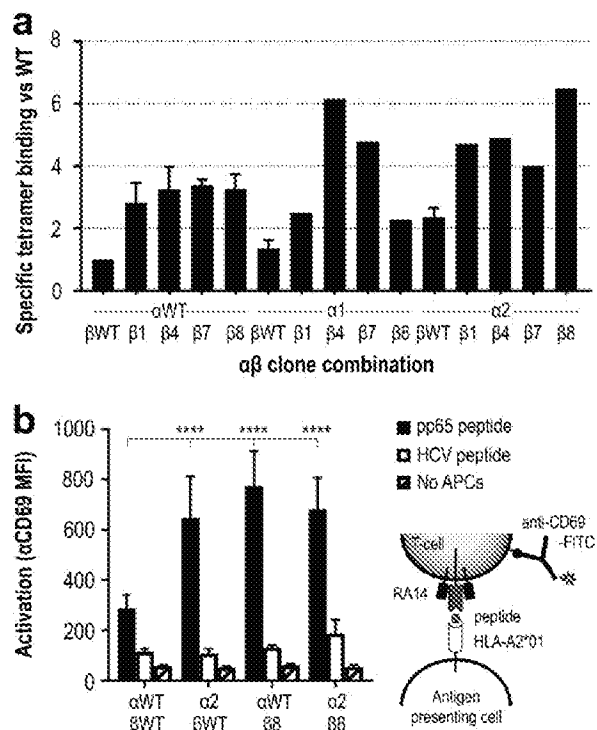
FIGS. 1A-1C



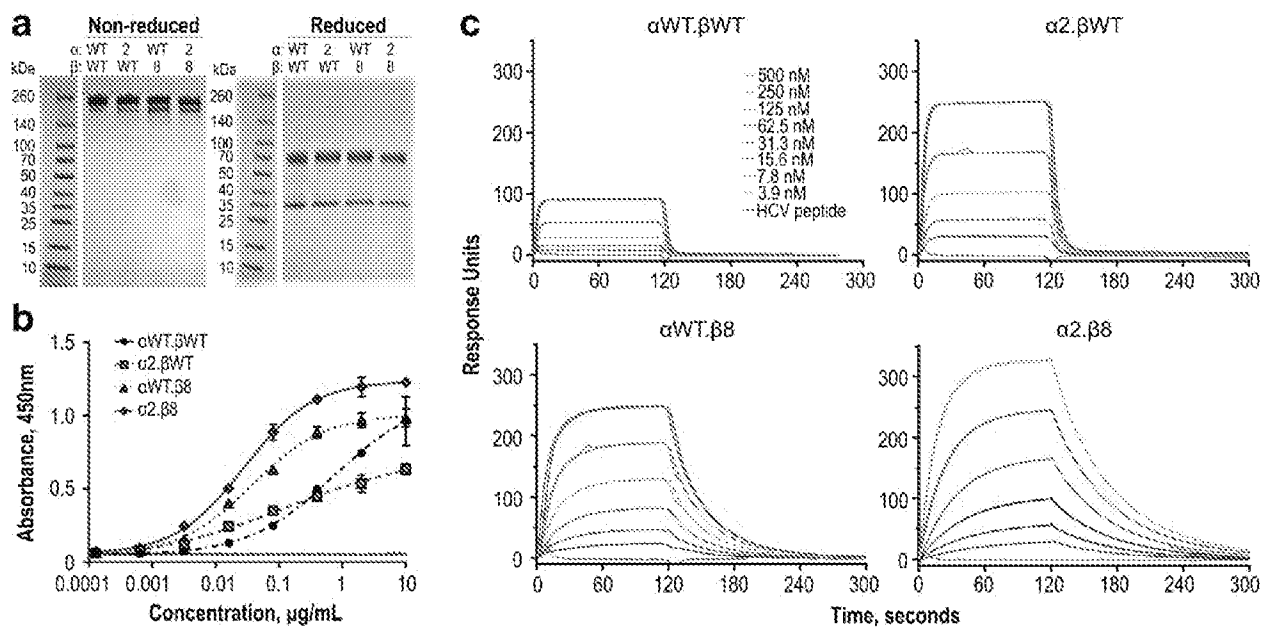
FIGS. 2A & 2B



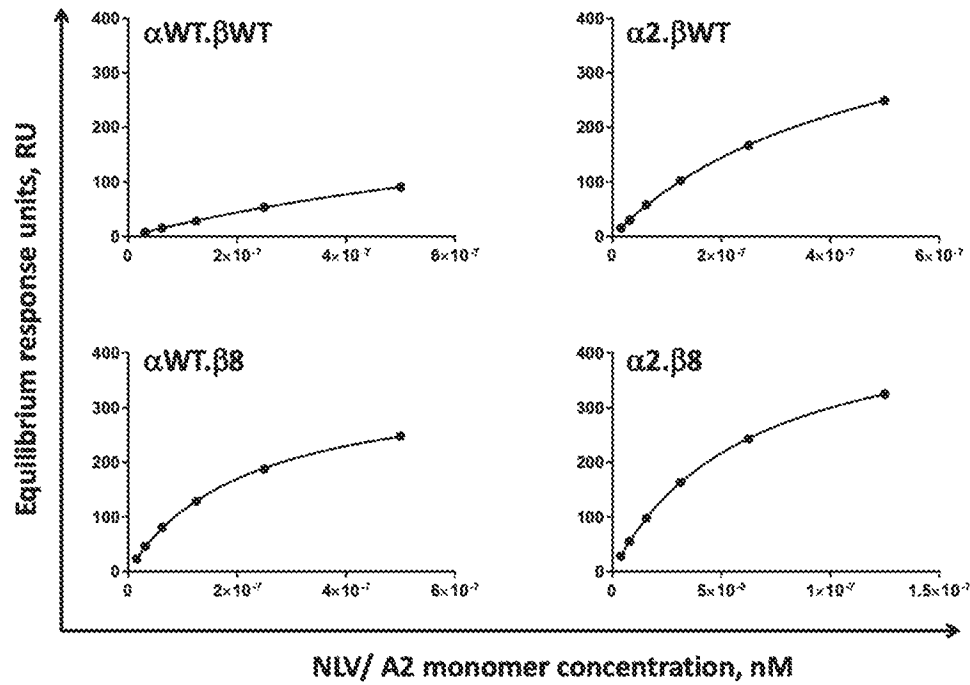
FIGS. 3A-3D



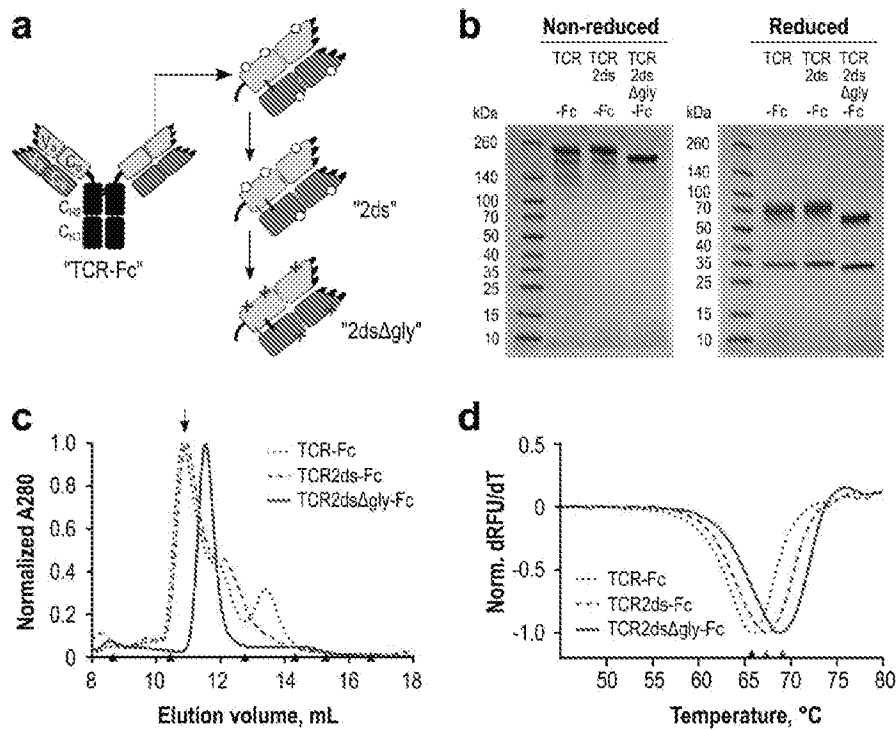
FIGS. 4A & 4B



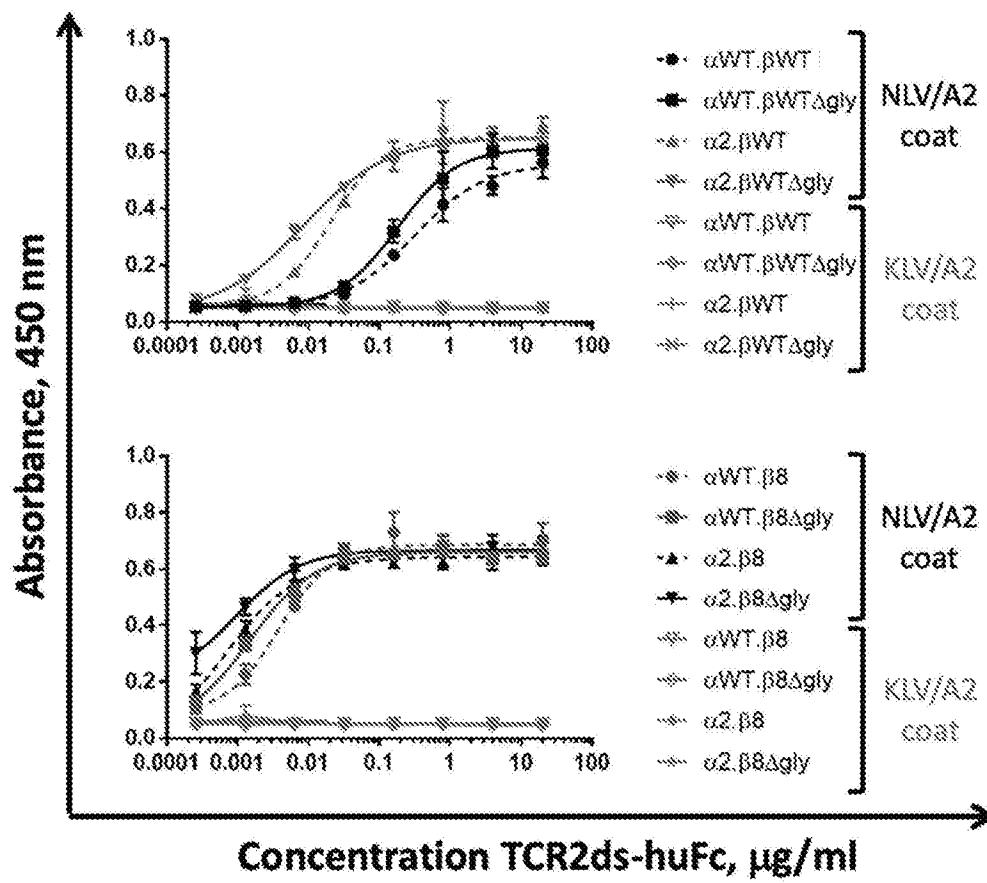
FIGS. 5A-5C



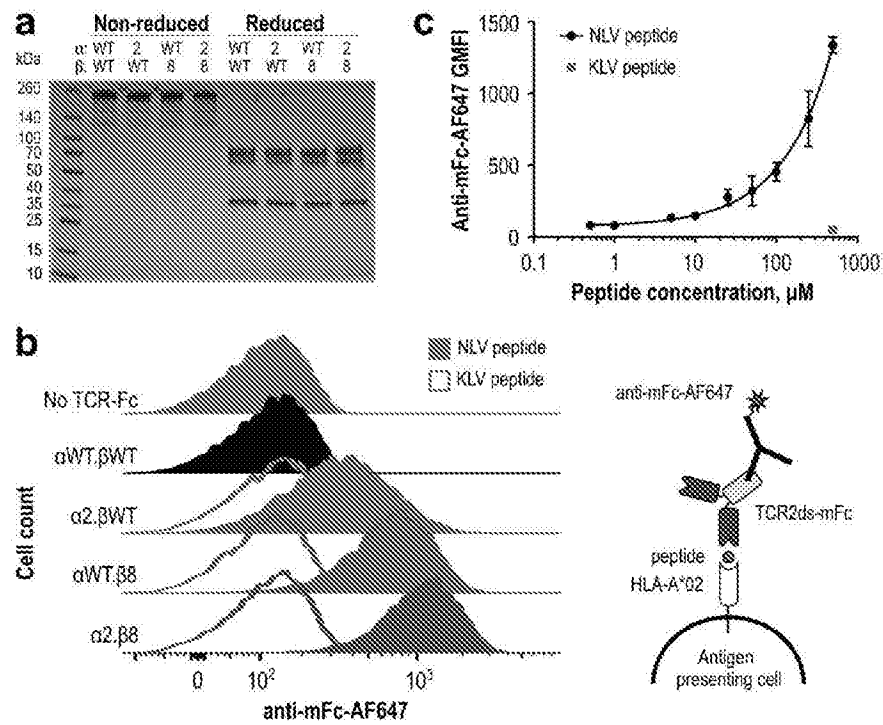
FIGS. 6A-6D



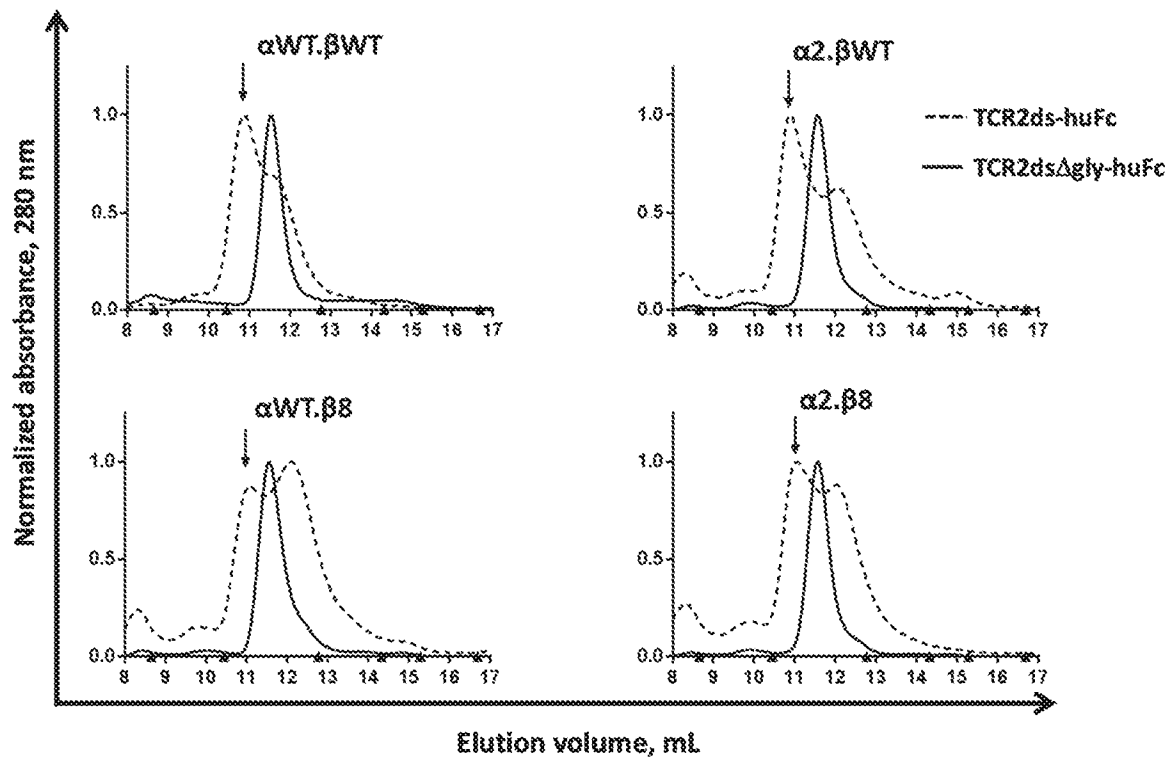
FIGS. 7A-7D



FIGS. 8A & 8B



FIGS. 9A-9C



FIGS. 10A-10C

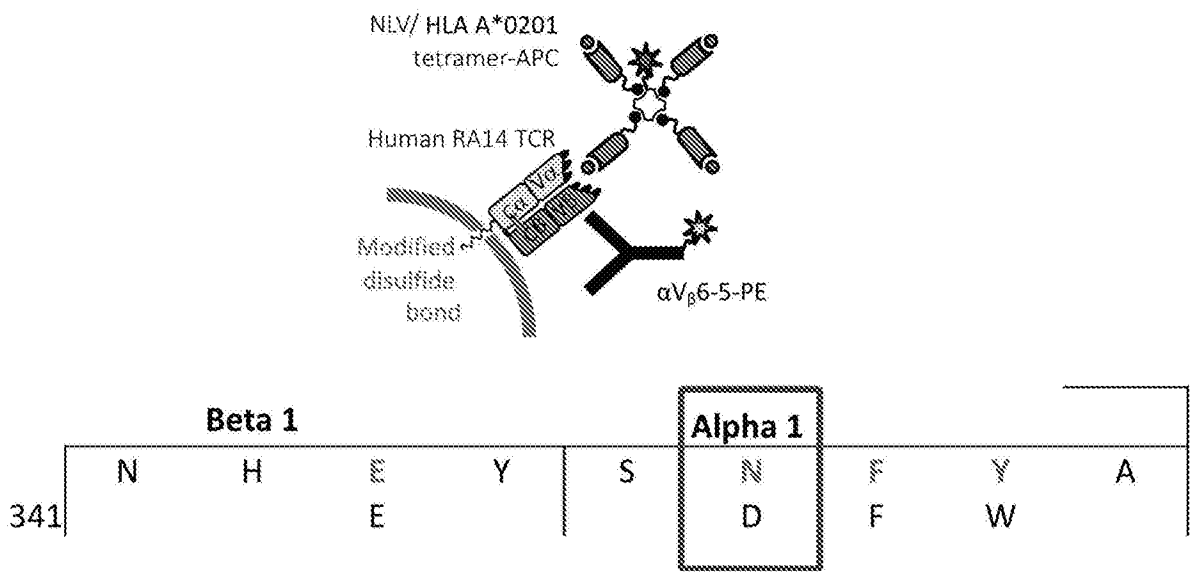


FIG. 11

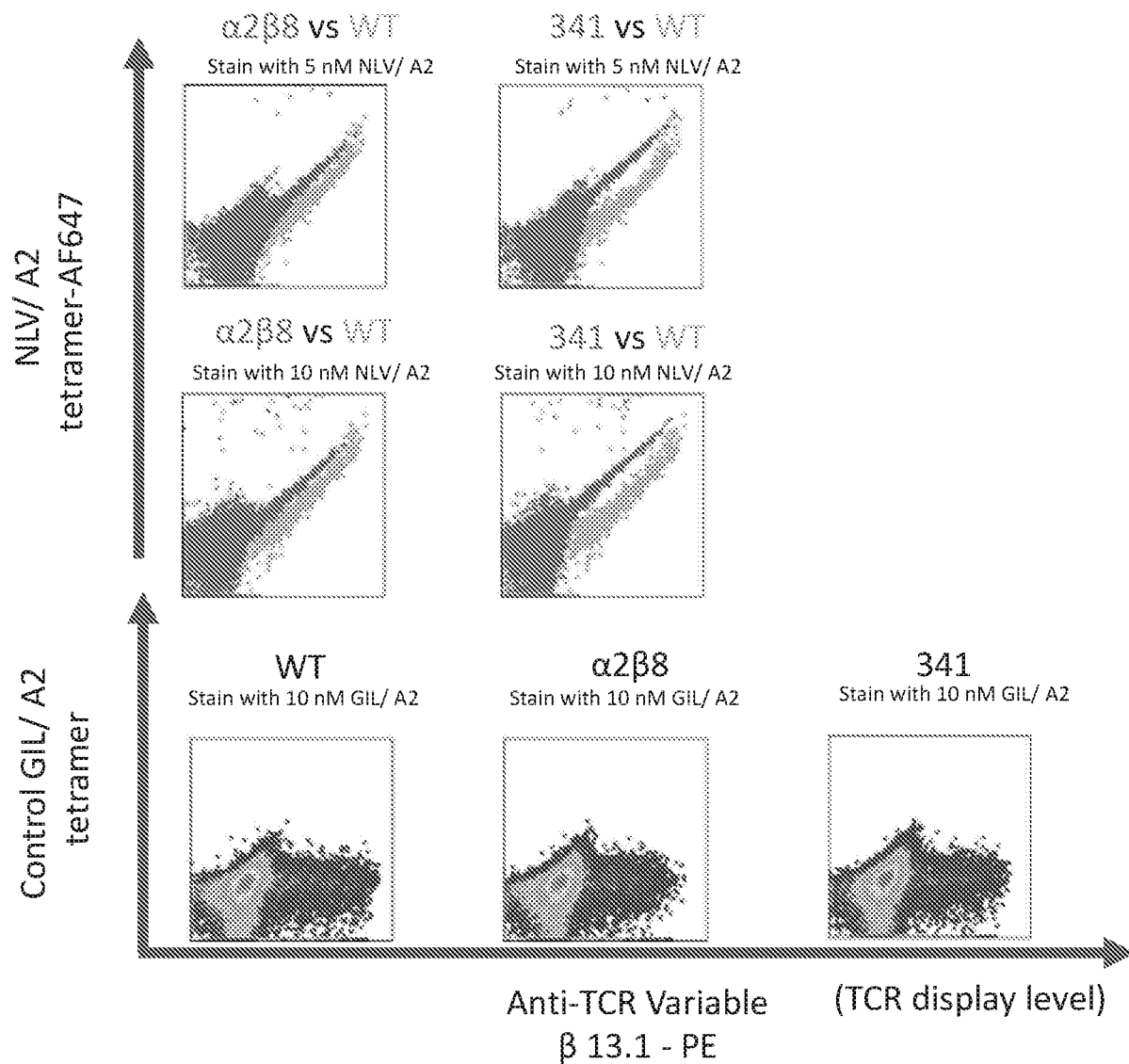


FIG. 12

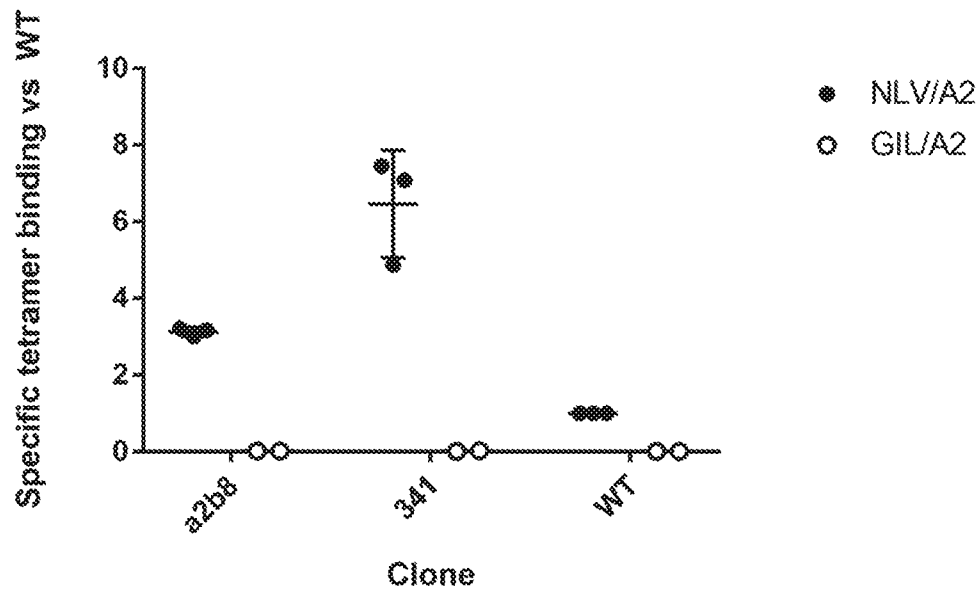


FIG. 13

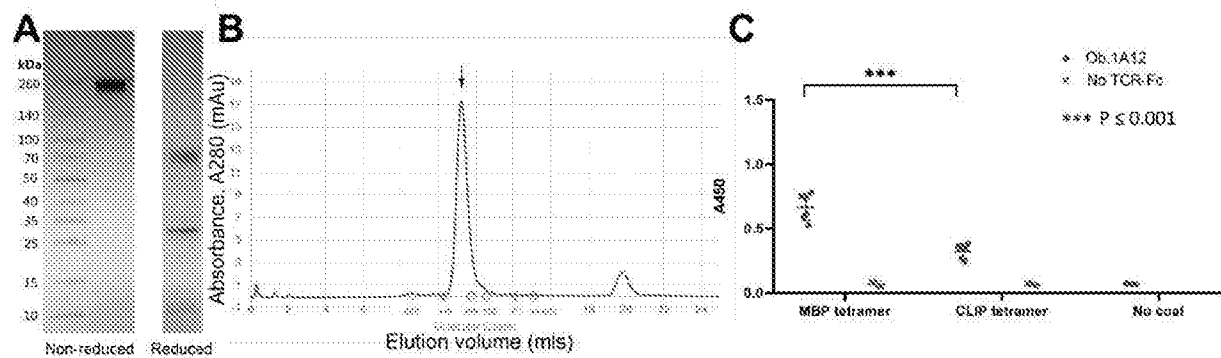


FIG. 14A-C

CDRα1										CDRα3					
IMGT #	28	29	36	37	38	104	105	106	107	108	109	114	115		
WT	S	N	F	Y	A	C	A	R	N	T	G	N	Q	(SEQ ID NO: 64; SEQ ID NO: 39)	
a2b8	*	*	*	*	*	*	*	*	*	Y	*	*	§		
R2G	L	D	*	W	*	*	*	*	*	Y	*	*	§		
2S-16	*	*	*	W	*	*	*	*	*	Y	*	*	*		
2S-20	*	*	*	W	*	*	*	*	*	Y	*	*	§		
				↑						↑			↑		

CDRβ3												
IMGT #	105	106	107	108	109	110	111	112	113	114	115	117
WT	A	S	S	P	V	T	G	G	I	Y	G	T
a2b8	*	*	*	L	*	*	*	*	V	*	L	*
R2G	*	*	*	L	*	*	*	*	V	*	L	*
2S-16	*	*	*	L	*	*	*	S	V	*	L	*
2S-20	*	*	*	W	*	*	*	*	V	*	*	*
				↑					↑		↑	

FIG. 15

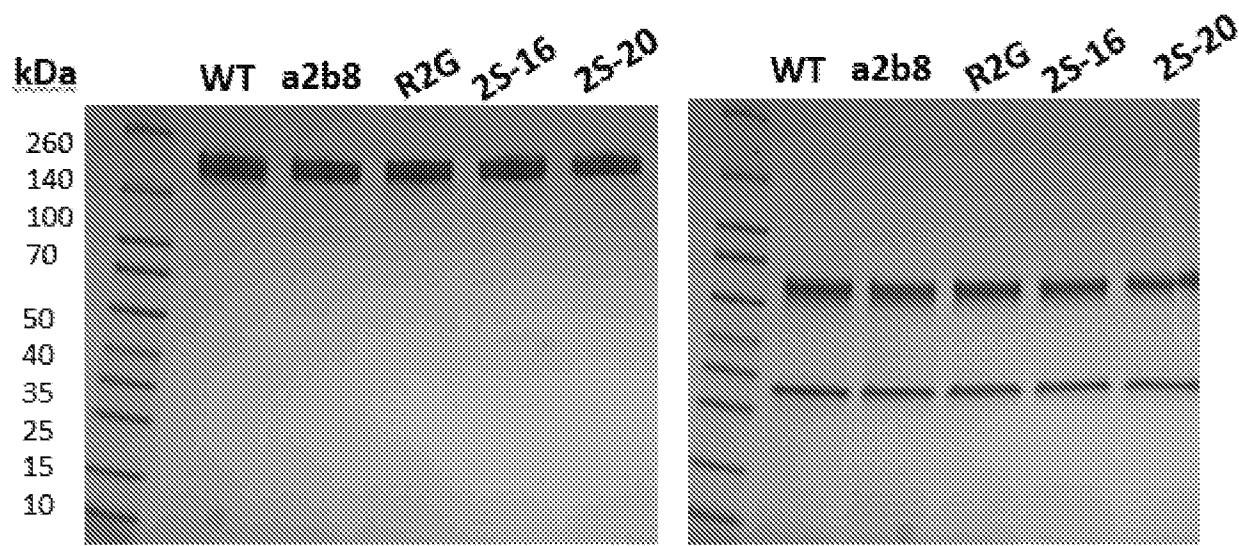


FIG. 16A

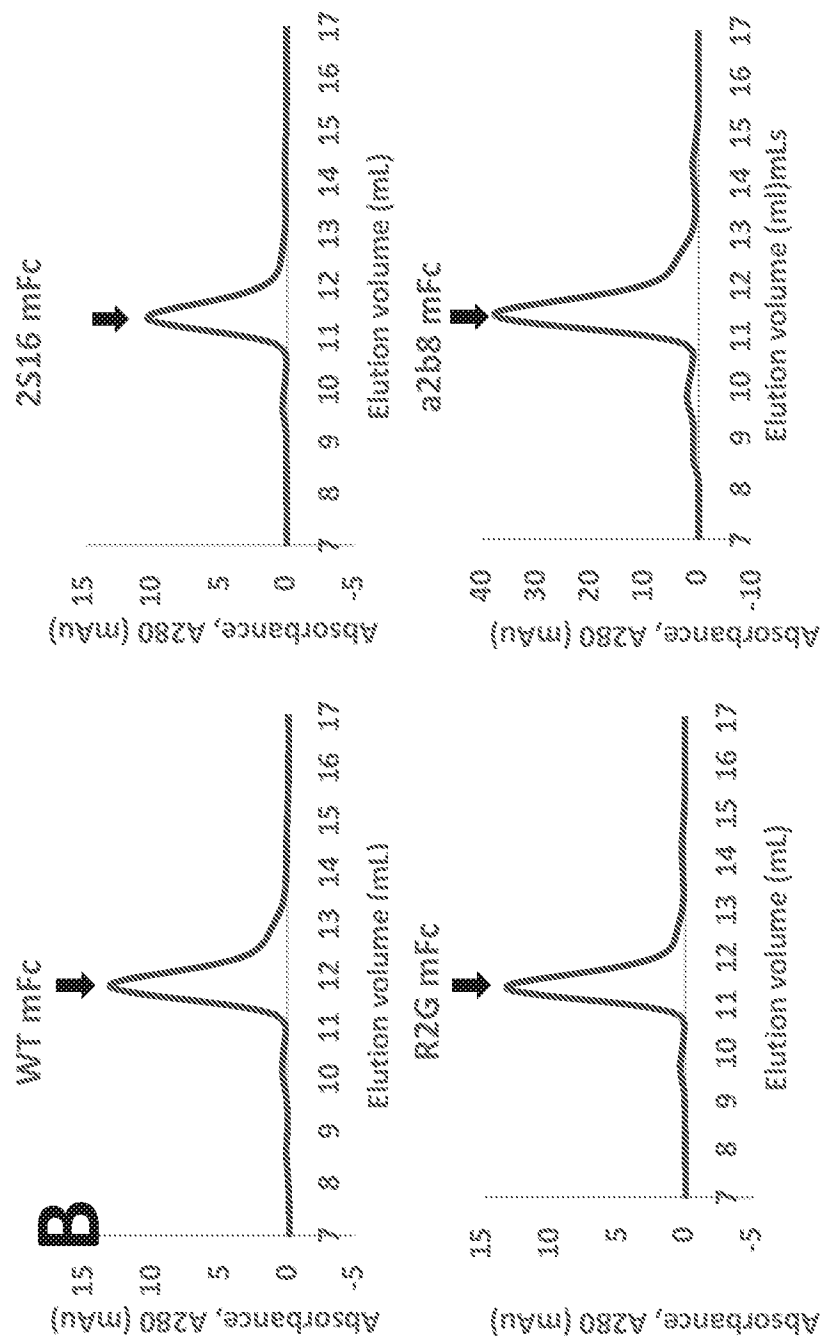


FIG. 16B

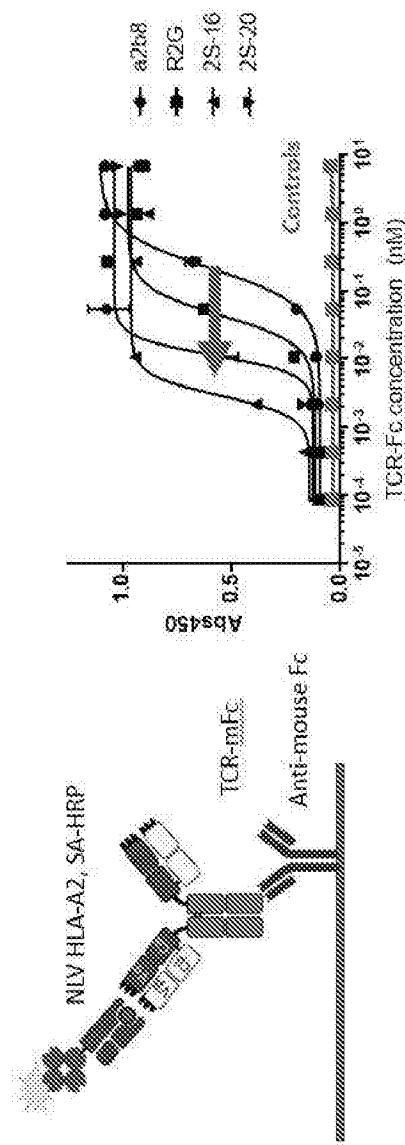


FIG. 16C

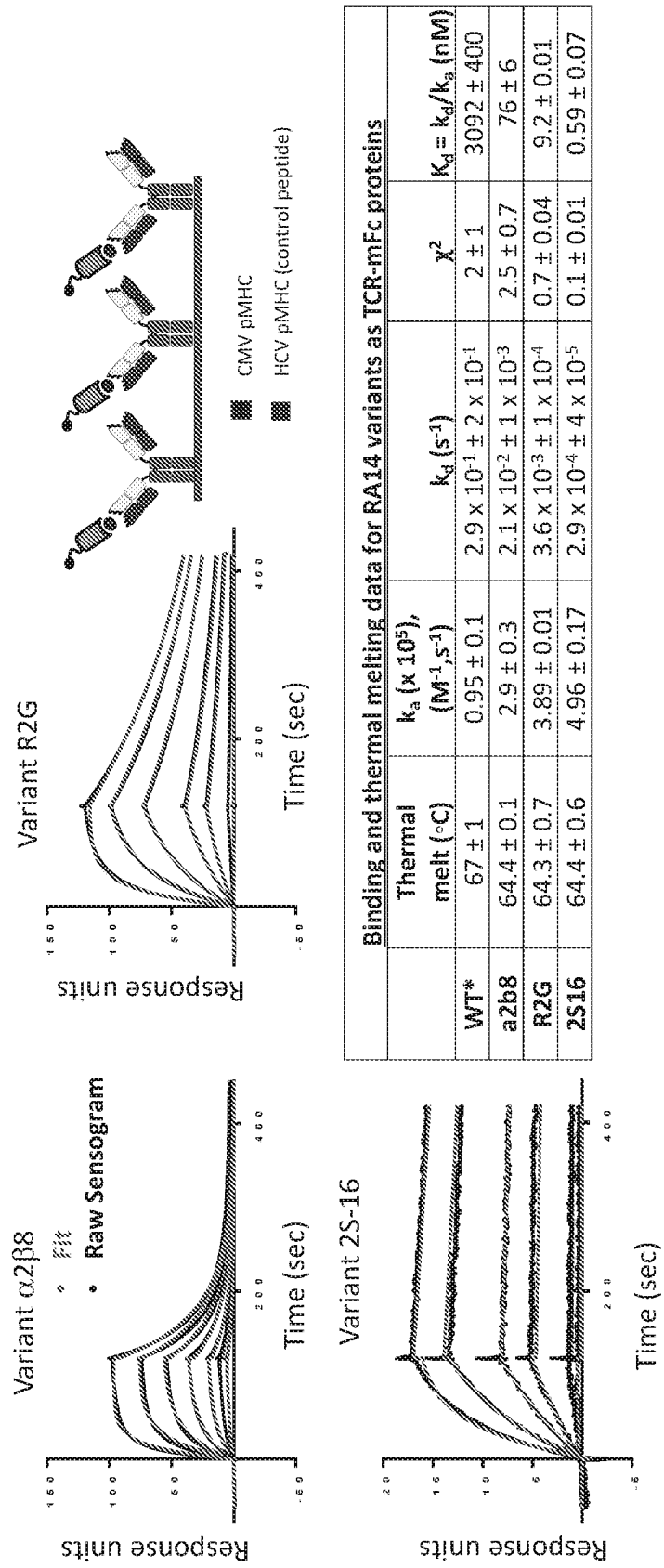


FIG. 17

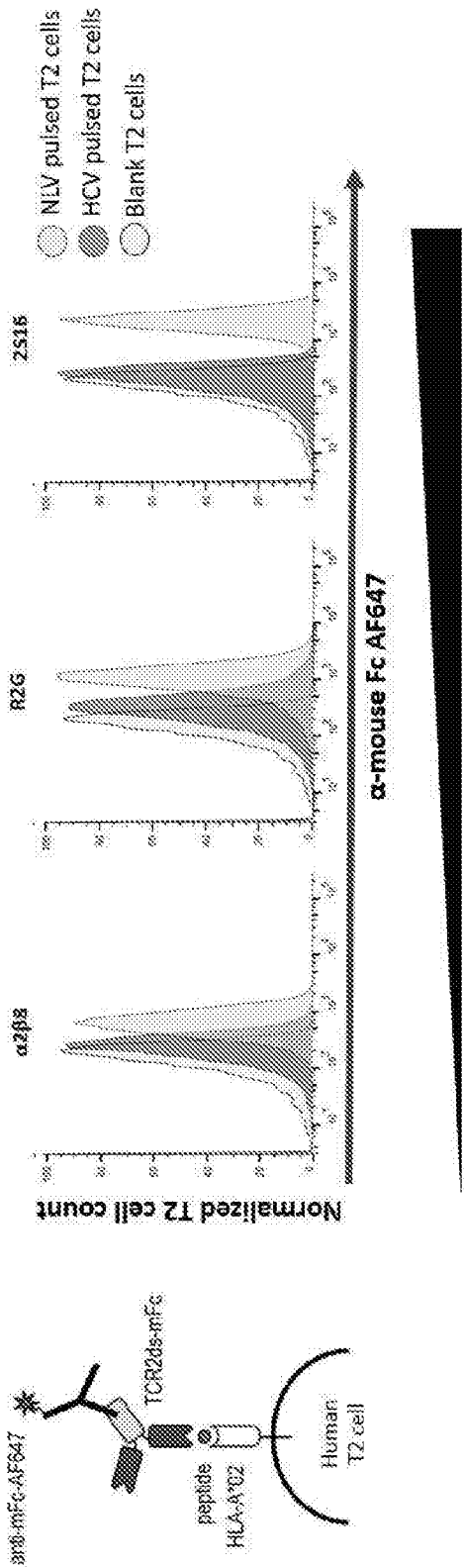


FIG. 18

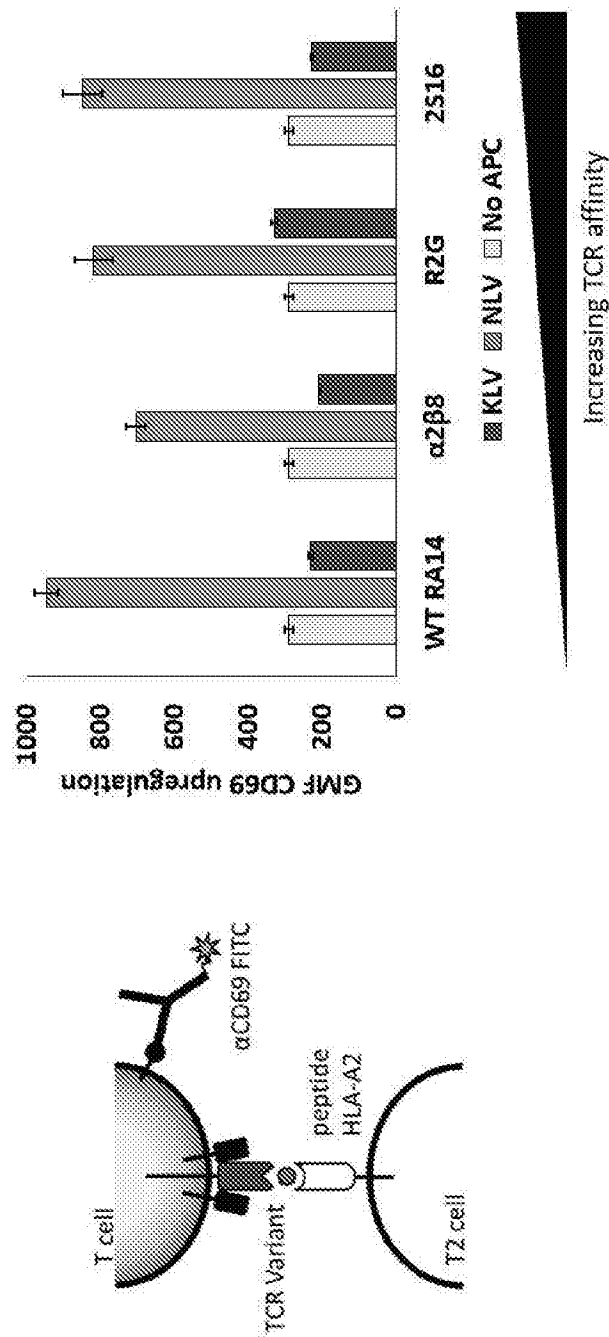


FIG. 19

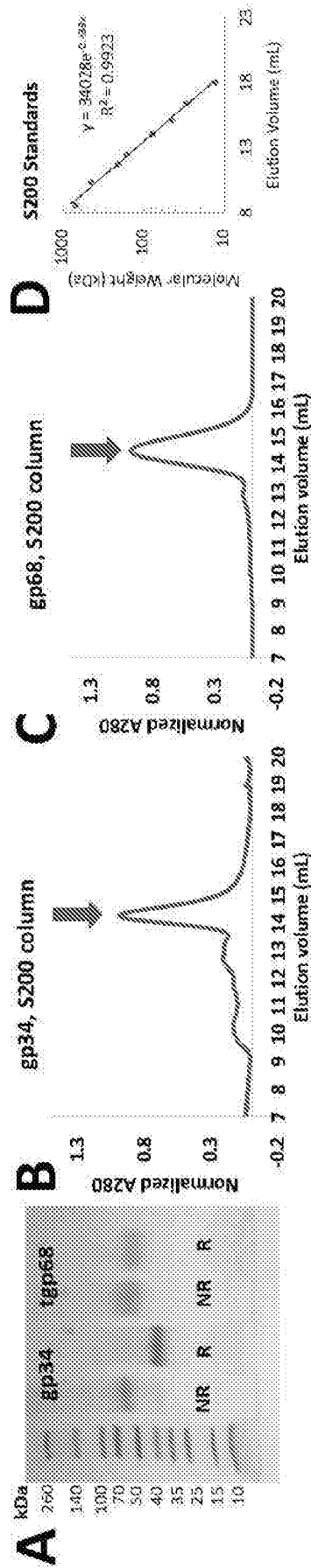


FIG. 20A-D

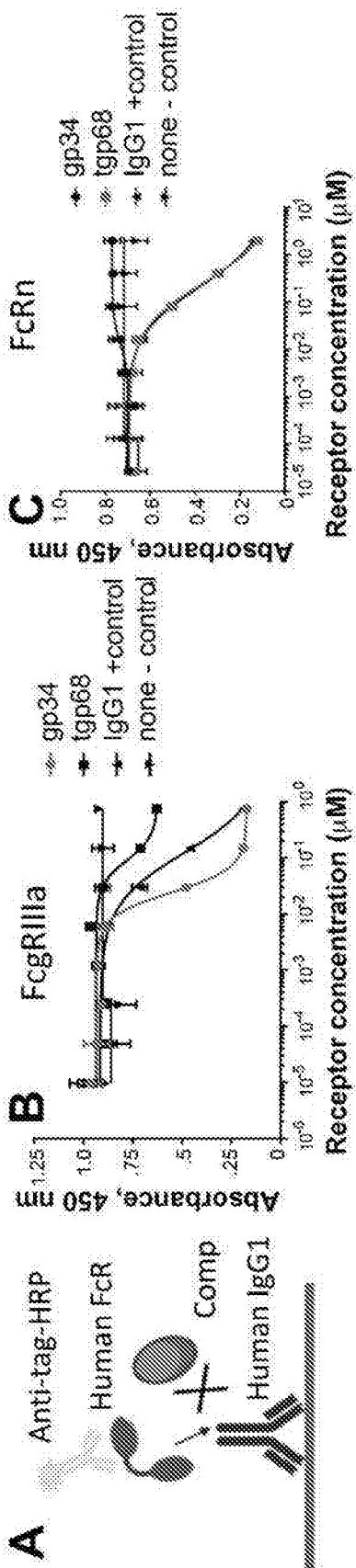


FIG. 21A-C

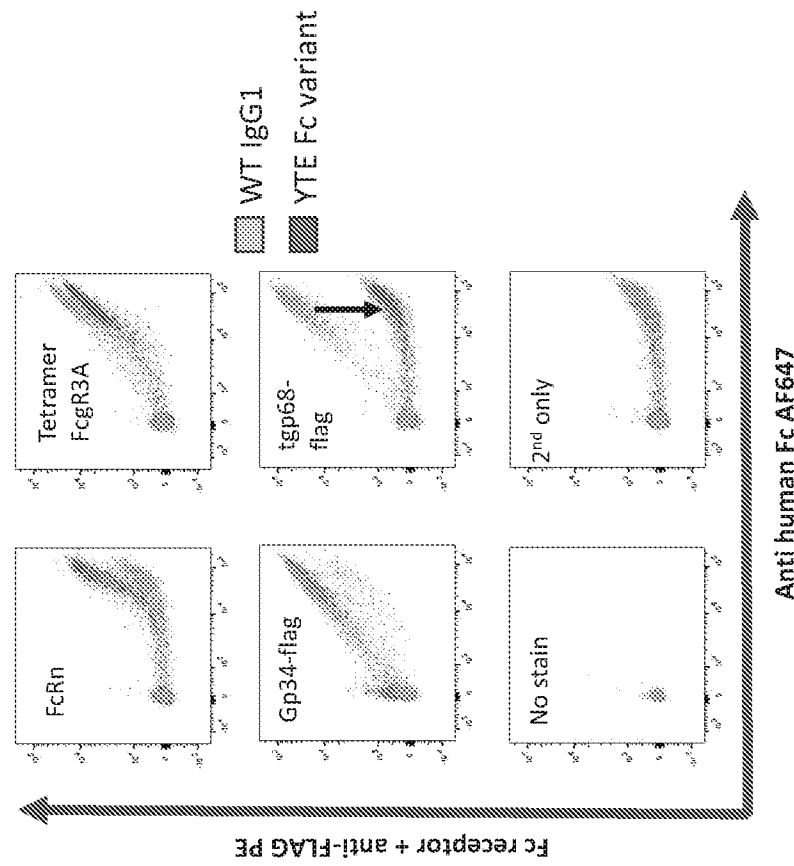
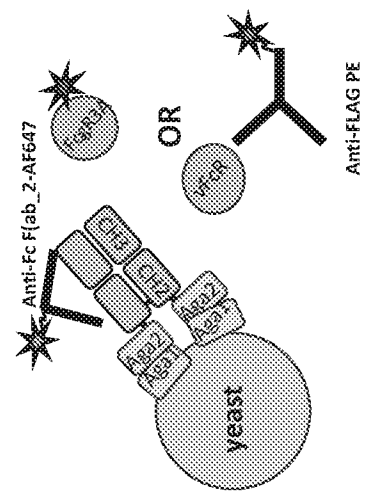


FIG. 22



A. WT Human IgG1: sequence alignment from residue 220 to 353 (no changes outside this region)

WTIgG1 CDKTHTCPCPAPPELLGGPSVFLFPPKPKDITIMISRTPEVTCVVVDVSHEDPEVKFNWYVDGV (SEQ ID NO: 53)
 38-4 CDKTHTCPCPAPPELLGGPSVFLFPPKPKDITIMISRTPEVTCVVVDVSHEDPEVKFNWYVDGV (SEQ ID NO: 54)
 38-7 CDKTHTCPCPAPPELLGGPSVFLFPPKPKDITIMISRTPEVTCVVVDVSHEDPEVKFNWYVDGV (SEQ ID NO: 55)
 38-12 CDKTHTCPCPAPPELLGGPSVFLFPPKPKDITIMISRTPEVTCVVVDVSHEDPEVKFNWYVDGV (SEQ ID NO: 56)
 38-13 CDKTHTCPCPAPPELLGGPSVFLFPPKPKDITIMISRTPEVTCVVVDVSHEDPEVKFNWYVDGV (SEQ ID NO: 57)
 38-14 CDKTHTCPCPAPPELLGGPSVFLFPPKPKDITIMISRTPEVTCVVVDVSHEDPEVKFNWYVDGV (SEQ ID NO: 58)
 4-3 CDKTHTCPCPAPPELLGGPSVFLFPPKPKDITIMISRTPEVTCVVVDVSHEDPEVKFNWYVDGV (SEQ ID NO: 59)
 4-7 CDKTHTCPCPAPPELLGGPSVFLFPPKPKDITIMISRTPEVTCVVVDVSHEDPEVKFNWYVDGV (SEQ ID NO: 60)
 YTE CDKTHTCPCPAPPELLGGPSVFLFPPKPKDITITREPEVTCVVVDVSHEDPEVKFNWYVDGV (SEQ ID NO: 70)

B.

gp34 escape variants:

3S-4: S337F
 3S-7: S337F, G341E
 3S-12: S337F, E345K, T350I
 3S-13: K334E, K338I
 3S-14: S337F, T350I
 4-3: H268L, K334E
 4-7: H268L, E294K, Q311L, K334E

gp68 escape variants

YTE: M252Y, S254T, T256E

FIG. 23A-B

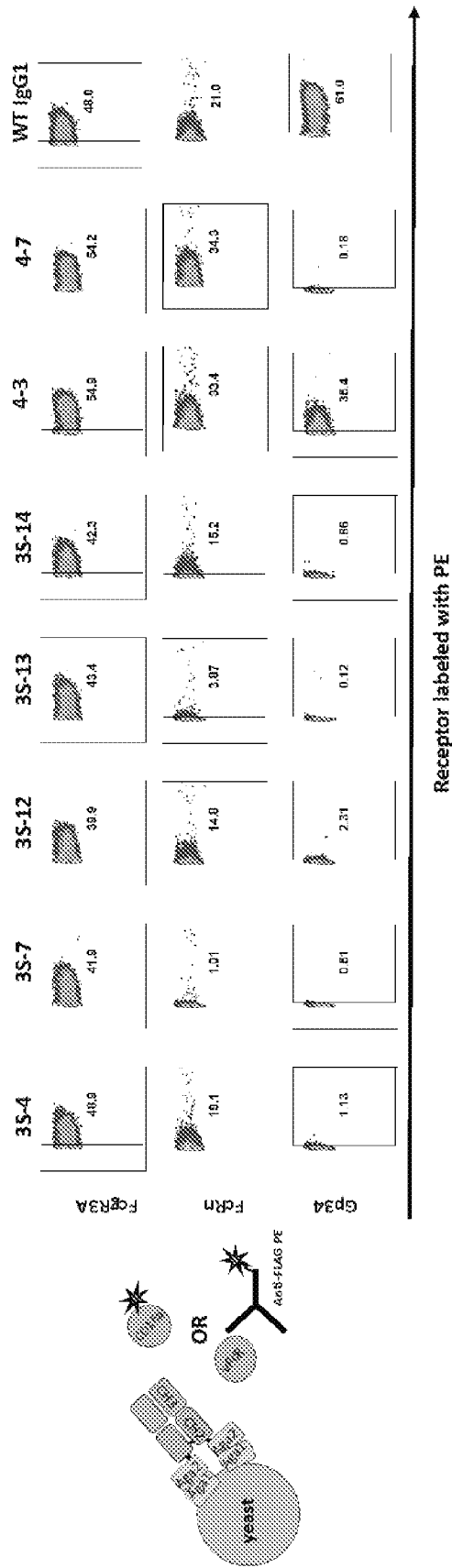
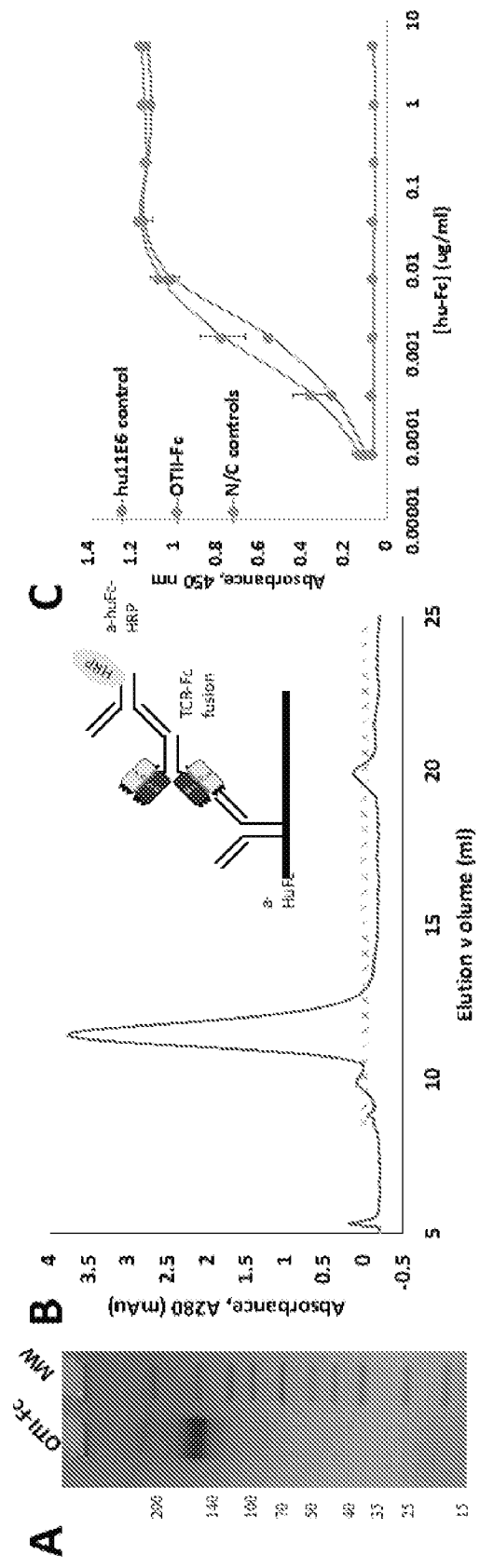


FIG. 24



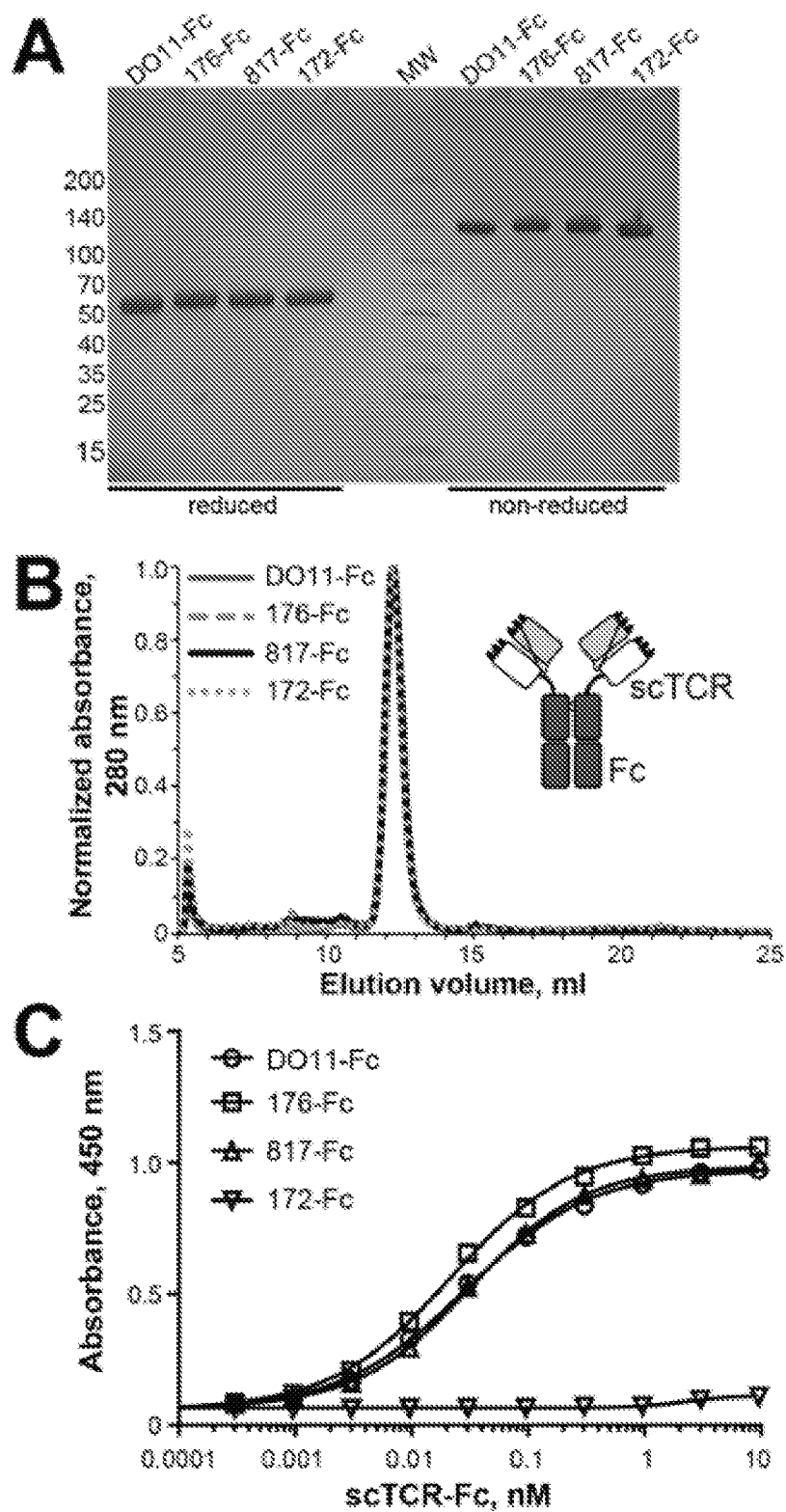


FIG. 26A-C

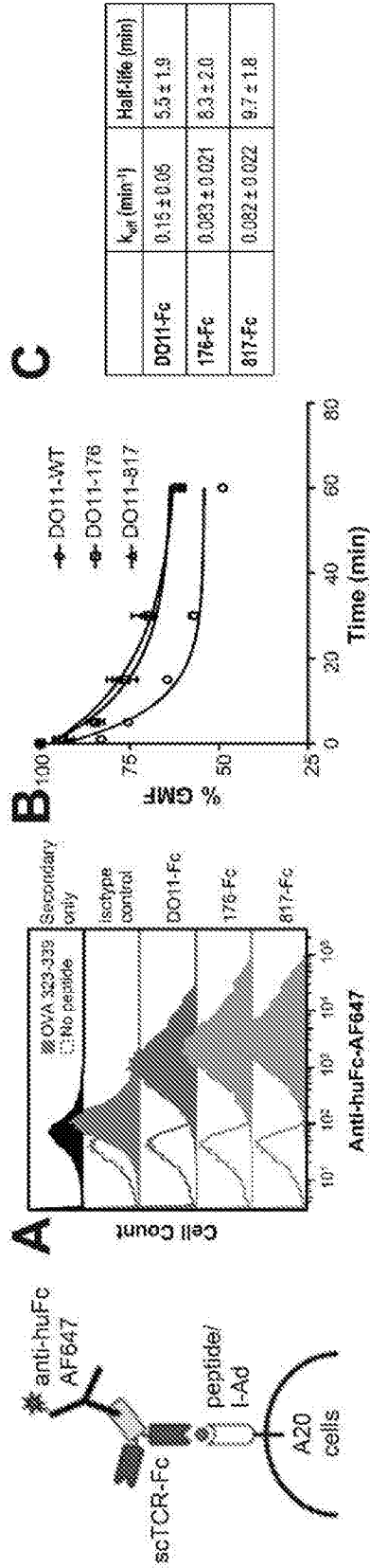


FIG. 27A-C

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2020/017940

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - A61K 35/12; A61K 35/14; A61K 38/00; A61K 38/16 (2020.01)

CPC - A61K 35/12; A61K 38/00; A61K 38/177; C07K 14/705; C07K 14/7051 (2020.05)

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

USPC - 424/93.21; 424/93.71; 530/387.1; 424.133.1 (keyword delimited)

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 2011/091078 A2 (XENCOR, INC. et al) 28 July 2011 (28.07.2011) entire document	183-185, 189-192 ----- 178, 186-188, 193-197, 206-208
Y	US 2018/0118852 A1 (REGENERON PHARMACEUTICALS, INC.) 03 May 2018 (03.05.2018) entire document	164-172, 175-178, 180-182
Y	WO 2006/125962 A2 (AVIDEX LTD et al) 30 November 2006 (30.11.2006) entire document	164-172, 175-178, 180-182
Y	KUBALL et al. "Increasing functional avidity of TCR-redirected T cells by removing defined N-glycosylation sites in the TCR constant domain," Journal of Experimental Medicine, 26 January 2009 (26.01.2009), Vol. 206, Iss. 2, Pgs. 463-475. entire document	164-172, 175-178, 180-182
Y	US 2002/0150891 A1 (HOOD et al) 17 October 2002 (17.10.2002) entire document	164-172, 175-178, 180-182
Y	US 2017/0166622 A1 (TCR2 THERAPEUTICS INC.) 15 June 2017 (15.06.2017) entire document	166
Y	WO 2015/017548 A2 (AMGEN INC.) 05 February 2015 (05.02.2015) entire document	166, 167
Y	US 2018/0208657 A1 (JULIUS-MAXIMILIANS-UNIVERSITAT WURZBURG) 26 July 2018 (26.07.2018) entire document	168-171



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

"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

13 May 2020

Date of mailing of the international search report

27 MAY 2020

Name and mailing address of the ISA/US

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

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

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Blaine R. Copenheaver

PCT Helpdesk: 571-272-4300

PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2020/017940

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 2016/0297886 A1 (RESEARCH DEVELOPMENT FOUNDATION) 13 October 2016 (13.10.2016) entire document	186
Y	SPRAGUE et al. "The Human Cytomegalovirus Fc Receptor gp68 Binds the Fc CH2-CH3 Interface of Immunoglobulin G," Journal of Virology, 11 March 2008 (11.03.2008), Vol. 82, No.7, Pgs. 3490–3499. entire document	187, 188, 206-208
Y	US 2002/0136724 A1 (MOHLER) 26 September 2002 (26.09.2002) entire document	193
Y	US 8,802,820 B2 (CHAMBERLAIN et al) 12 August 2014 (12.08.2014) entire document	194-197, 207, 208
P, X	WAGNER et al. "Human cytomegalovirus-specific T-cell receptor engineered for high affinity and soluble expression using mammalian cell display," Journal of Biological Chemistry, 22 February 2019 (22.02.2019), Vol. 294, Iss. 15, Pgs. 5790-5804. entire document	1-14, 17, 75-94, 106, 164-178, 180-197, 206-208

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2020/017940

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.: 15, 16, 18-74, 95-105, 107-163, 179, 198-205, 209
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:

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.:

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.