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(54) Title: ENGINEERED HOST CELLS AND METHODS OF USE THEREOF

(57) Abrégé/Abstract:
Provided herein are engineered host cells suitable for expression of functional T cell receptors (TCR) and methods of using these cells to identify TCRs that specifically bind to a desired peptide-major histocompatibility complex (MHC) complex.

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(54) **Title**: ENGINEERED HOST CELLS AND METHODS OF USE THEREOF

(57) **Abstract**: Provided herein are engineered host cells suitable for expression of functional T cell receptors (TCR) and methods of using these cells to identify TCRs that specifically bind to a desired peptide-major histocompatibility complex (MHC) complex.

ENGINEERED HOST CELLS AND METHODS OF USE THEREOF

RELATED APPLICATIONS

[001] This application claims the benefit of U.S. Provisional Application No: 62/217,677, filed September 11, 2015, which is incorporated by reference herein in its entirety.

FIELD OF THE INVENTION

[002] The present disclosure provides engineered host cells suitable for expression of functional T cell receptors (TCR) and methods of using these cells to identify TCRs that specifically bind to a desired peptide-major histocompatibility complex (MHC) complex.

BACKGROUND

[003] In mammals, the acquired immune system comprises two major components: the humoral immune response, involving antibodies, and the cellular immune response, involving T cells. T cells are the major mediators of adaptive cellular immunity and exert their action through the TCR-mediated recognition of a peptide epitope bound to a major histocompatibility complex (MHC) molecule. The immune system contains a large number of T cells that encompass a broad range of peptide-MHC specificities. However, negative and positive selection processes in the thymus result in a restriction on T cell diversity, limiting the spectrum of *in vivo* T cell reactivity. For example, negative selection leads to the removal of the high affinity T cell repertoire specific for self-antigens, and this includes T cells with desirable specificities, such as for self-antigens expressed on tumor tissues.

[004] Cancer immunotherapy, the enhancement of a patient's immune response to cancerous cells, holds great promise. Active immunotherapy is carried out by stimulation of the endogenous immune system of tumor-bearing patients. Passive, or adoptive, immunotherapy involves the transfer of immune-competent cells into the patient. One approach to adoptive therapy is the use of gene therapy techniques to introduce TCRs specific for known cancer-specific peptide-MHC complexes into the T cells of cancer patients. However, this approach is reliant upon obtaining TCRs that bind specifically to cancer-specific peptide-MHC complexes with suitable affinity.

[005] Accordingly, there is a need in the art for novel TCRs that specifically bind to a desired peptide-MHC complex (*e.g.*, a peptide-MHC complex comprising a cancer-specific peptide antigen), and for technologies to discover such TCRs.

BRIEF SUMMARY OF THE INVENTION

[006] Provided herein are engineered host cells suitable for expression of functional TCRs and methods of using these cells to identify TCRs that specifically bind to a desired peptide-MHC complex.

[007] In one aspect, the disclosure provides a cell (e.g., an isolated cell) derived from a lymphocyte comprising a first polynucleotide encoding a recombinant co-receptor, or a first chain thereof, wherein the cell expresses CD3 but does not express an endogenous TCR on the cell surface. In certain embodiments, the CD3 is endogenous CD3. In certain embodiments, the CD3 is recombinant CD3. In certain embodiments, the cell expresses the recombinant co-receptor, or a first chain thereof, on the cell surface, and/or the cell expresses the recombinant co-receptor, or a first chain thereof, on the cell surface in a regulatable manner, and/or the cell does not express an endogenous co-receptor on the cell surface, and/or the endogenous co-receptor is CD8 or CD4.

[008] In another aspect, the disclosure also provides a cell (e.g., an isolated cell) derived from a lymphocyte comprising a first polynucleotide encoding a recombinant co-receptor, or a first chain thereof, wherein the lymphocyte is an invariant NKT (iNKT) cell or a diverse NKT (dNKT) cell. In certain embodiments, the cell expresses the recombinant co-receptor, or a first chain thereof, on the cell surface. In certain embodiments, the cell does not express an endogenous co-receptor on the cell surface, and/or the endogenous co-receptor is CD8 or CD4.

[009] In another aspect, the disclosure provides a cell (e.g., an isolated cell) derived from a lymphocyte comprising a first polynucleotide encoding a recombinant co-receptor, or a first chain thereof, wherein the cell is capable of expressing the recombinant co-receptor, or a first chain thereof, on the cell surface in a regulatable manner. In certain embodiments, the cell expresses the recombinant co-receptor, or a first chain thereof, on the cell surface in a regulatable manner. In certain embodiments, the cell does not express an endogenous co-receptor on the cell surface. In certain embodiments, the endogenous co-receptor is CD8 or CD4.

[010] Each of the following embodiments apply to the cells of the invention described herein.

[011] In certain embodiments, the recombinant co-receptor, or a first chain thereof, comprises an extracellular region, a transmembrane region, and a cytoplasmic region, wherein the extracellular region is linked to the transmembrane region, and the transmembrane region is linked to the cytoplasmic region.

[012] In certain embodiments, the amino acid sequence of the cytoplasmic region of the recombinant co-receptor, or a first chain thereof, can be at least 90% identical, optionally 91, 92, 93, 94, 95, 96, 97, 98, 99, or 100% identical, to the amino acid sequence of a corresponding cytoplasmic region of a naturally occurring co-receptor, or a first chain thereof, from the same species as the lymphocyte.

[013] In certain embodiments, the amino acid sequence of the transmembrane region and cytoplasmic region together of the recombinant co-receptor, or a first chain thereof, can be at least 90% identical, optionally 91, 92, 93, 94, 95, 96, 97, 98, 99, or 100% identical, to the amino acid sequence of a corresponding transmembrane region and cytoplasmic region together of a naturally occurring co-receptor, or a first chain thereof, from the same species as the lymphocyte.

[014] In certain embodiments, the amino acid sequence of the extracellular region of the recombinant co-receptor, or a first chain thereof, is at least 90% identical, optionally 91, 92, 93, 94, 95, 96, 97, 98, 99, or 100% identical, to the amino acid sequence of a corresponding extracellular region of a naturally occurring co-receptor, or a first chain thereof, from a different species than the lymphocyte. In certain embodiments, the naturally occurring co-receptor, or a first chain thereof, from a different species than the lymphocyte is human.

[015] In certain embodiments, the cytoplasmic region of the recombinant co-receptor, or a first chain thereof, is at least 90% identical, optionally 91, 92, 93, 94, 95, 96, 97, 98, 99, or 100% identical, to the amino acid sequence of a corresponding cytoplasmic region of a naturally occurring co-receptor, or a first chain thereof, from the same species as the lymphocyte, and/or the amino acid sequence of the transmembrane region and cytoplasmic region together of the recombinant co-receptor, or a first chain thereof, can be at least 90% identical, optionally 91, 92, 93, 94, 95, 96, 97, 98, 99, or 100% identical, to the amino acid sequence of a corresponding transmembrane region and cytoplasmic region together of a naturally occurring co-receptor, or a first chain thereof, from the same species as the lymphocyte, and, the amino acid sequence of the extracellular region of the recombinant co-receptor, or a first chain thereof, is at least 90% identical, optionally 91, 92, 93, 94, 95, 96,

97, 98, 99, or 100% identical, to the amino acid sequence of a corresponding extracellular region of a naturally occurring co-receptor, or a first chain thereof, from a different species than the lymphocyte. In one embodiment, the naturally occurring co-receptor, or a first chain thereof, from a different species than the lymphocyte is human.

[016] In certain embodiments, the recombinant co-receptor is CD8 or CD4.

[017] In certain embodiments, the cell as disclosed herein further comprises a second polynucleotide encoding a second chain of the recombinant co-receptor, wherein the second chain comprises an extracellular region, a transmembrane region, and a cytoplasmic region, wherein the extracellular region of the second chain is linked to the transmembrane region of the second chain, and the transmembrane region of the second chain is linked to the cytoplasmic region of the second chain. In certain embodiments, the amino acid sequence of the cytoplasmic region of the second chain is at least 90% identical, optionally 91, 92, 93, 94, 95, 96, 97, 98, 99, or 100% identical, to the amino acid sequence of a corresponding cytoplasmic region of a naturally occurring co-receptor chain from the same species as the lymphocyte. In certain embodiments, the amino acid sequence of the transmembrane region and cytoplasmic region together of the second chain is at least 90% identical, optionally 91, 92, 93, 94, 95, 96, 97, 98, 99, or 100% identical, to the amino acid sequence of a corresponding transmembrane region and cytoplasmic region together of a naturally occurring co-receptor chain from the same species as the lymphocyte. In certain embodiments, the amino acid sequence of the extracellular region of the second chain is at least 90% identical, optionally 91, 92, 93, 94, 95, 96, 97, 98, 99, or 100% identical, to the amino acid sequence of a corresponding extracellular region of a naturally occurring co-receptor chain from a different species than the lymphocyte. The naturally occurring co-receptor chain can be from a different species than the lymphocyte is human.

[018] Therefore, in certain embodiments, the amino acid sequence of the cytoplasmic region of the second chain is at least 90% identical, optionally 91, 92, 93, 94, 95, 96, 97, 98, 99, or 100% identical, to the amino acid sequence of a corresponding cytoplasmic region of a naturally occurring co-receptor chain from the same species as the lymphocyte, and/or the amino acid sequence of the transmembrane region and cytoplasmic region together of the second chain is at least 90% identical, optionally 91, 92, 93, 94, 95, 96, 97, 98, 99, or 100% identical, to the amino acid sequence of a corresponding transmembrane region and cytoplasmic region together of a naturally occurring co-receptor chain from the same species as the lymphocyte, and the amino acid sequence of the extracellular region of the second

chain is at least 90% identical, optionally 91, 92, 93, 94, 95, 96, 97, 98, 99, or 100% identical, to the amino acid sequence of a corresponding extracellular region of a naturally occurring co-receptor chain from a different species than the lymphocyte. In one embodiment, the naturally occurring co-receptor chain from a different species than the lymphocyte is human.

[019] In certain embodiments, the recombinant co-receptor is CD8. In certain embodiments, the cytoplasmic region of the first chain of the recombinant co-receptor comprises the amino acid sequence of residues 183-212 of SEQ ID NO: 1. In certain embodiments, the transmembrane region and the cytoplasmic region of the first chain of the recombinant co-receptor together comprise the amino acid sequence of residues 162-212 of SEQ ID NO: 1. In certain embodiments, the extracellular region of the first chain of the recombinant co-receptor comprises the amino acid sequence of residues 1-161 of SEQ ID NO: 1. In certain embodiments, the cytoplasmic region of the second chain of the recombinant co-receptor comprises the amino acid sequence of residues 171-187 of SEQ ID NO: 2. In certain embodiments, the transmembrane region and the cytoplasmic region of the second chain of the recombinant co-receptor together comprise the amino acid sequence of residues 150-187 of SEQ ID NO: 2. In certain embodiments, the extracellular region of the second chain of the recombinant co-receptor comprises the amino acid sequence of residues 1-149 of SEQ ID NO: 2. Therefore, in certain embodiments, the cytoplasmic region of the first chain of the recombinant co-receptor comprises the amino acid sequence of residues 183-212 of SEQ ID NO: 1, and/or the transmembrane region and the cytoplasmic region of the first chain of the recombinant co-receptor together comprise the amino acid sequence of residues 162-212 of SEQ ID NO: 1, for example, wherein the extracellular region of the first chain of the recombinant co-receptor comprises the amino acid sequence of residues 1-161 of SEQ ID NO: 1. In one embodiment, the cytoplasmic region of the second chain of the recombinant co-receptor comprises the amino acid sequence of residues 171-187 of SEQ ID NO: 2, and/or the transmembrane region and the cytoplasmic region of the second chain of the recombinant co-receptor together comprise the amino acid sequence of residues 150-187 of SEQ ID NO: 2. In one embodiment, the extracellular region of the second chain of the recombinant co-receptor comprises the amino acid sequence of residues 1-149 of SEQ ID NO: 2.

[020] In certain embodiments, the recombinant co-receptor is a chimeric CD8 comprising: a) a chimeric CD8α chain comprising an extracellular region of human CD8α and transmembrane and cytoplasmic regions of mouse CD8α, and b) a chimeric CD8β chain comprising an extracellular region of human CD8β and transmembrane and cytoplasmic

regions of mouse CD8β. In one embodiment, the chimeric CD8α chain comprises the amino acid sequence of SEQ ID NO: 1, and the chimeric CD8β chain comprises the amino acid sequence of SEQ ID NO: 2, for example, wherein the amino acid sequence of the chimeric CD8α chain consists of the amino acid sequence of SEQ ID NO: 1, and the amino acid sequence of the chimeric CD8β chain consists of the amino acid sequence of SEQ ID NO: 2.

[021] In another aspect, the disclosure also provides an isolated cell derived from a mouse lymphocyte, the cell comprising: a) a first polynucleotide encoding a chimeric CD8α chain comprising an extracellular region of human CD8α and transmembrane and cytoplasmic regions of mouse CD8α, and b) a second polynucleotide encoding a chimeric CD8β chain comprising an extracellular region of human CD8β and transmembrane and cytoplasmic regions of mouse CD8β, wherein the cell expresses CD3 but does not express endogenous TCR on the cell surface. In certain embodiments, the chimeric CD8α chain comprises the amino acid sequence of SEQ ID NO: 1, and the chimeric CD8β chain comprises the amino acid sequence of SEQ ID NO: 2. In certain embodiments, the amino acid sequence of the chimeric CD8α chain consists of the amino acid sequence of SEQ ID NO: 1, and the amino acid sequence of the chimeric CD8β chain consists of the amino acid sequence of SEQ ID NO: 2.

[022] In certain embodiments, the surface expression of the recombinant co-receptor or chain thereof is regulatable.

[023] In certain embodiments, the first polynucleotide is flanked by a first pair of compatible site-specific recombination sequences.

[024] In certain embodiments, the second polynucleotide is flanked by a second pair of compatible site-specific recombination sequences.

[025] In certain embodiments, a portion of the first polynucleotide, the portion encoding the transmembrane region and/or the cytoplasmic region, is flanked by a first pair of compatible site-specific recombination sequences.

[026] In certain embodiments, a portion of the second polynucleotide, the portion encoding the transmembrane region and/or the cytoplasmic region, is flanked by a second pair of compatible site-specific recombination sequences.

[027] In certain embodiments, the first pair of compatible site-specific recombination sequences does not cross-react with the second pair of compatible site-specific recombination sequences.

[028] In certain embodiments, the surface expression of the recombinant co-receptor, or chain thereof, can be attenuated by contacting the first polynucleotide and/or the second polynucleotide with a cognate site-specific recombinase that recognizes the first pair of compatible site-specific recombination sequences of the first polynucleotide and/or the second pair of compatible site-specific recombination sequences of the second polynucleotide.

[029] In certain embodiments, the surface expression of the recombinant co-receptor, or chain thereof, can be abrogated by contacting the first polynucleotide and/or the second polynucleotide with a cognate site-specific recombinase that recognizes the first pair of compatible site-specific recombination sequences of the first polynucleotide and/or the second pair of compatible site-specific recombination sequences of the second polynucleotide.

[030] Therefore, in certain embodiments, the first pair of compatible site-specific recombination sequences does not cross-react with the second pair of compatible site-specific recombination sequences, for example, wherein the surface expression of the recombinant co-receptor, or chain thereof, can be attenuated by contacting the first polynucleotide and/or the second polynucleotide with a cognate site-specific recombinase that recognizes the first pair of compatible site-specific recombination sequences of the first polynucleotide and/or the second pair of compatible site-specific recombination sequences of the second polynucleotide, or wherein the surface expression of the recombinant co-receptor, or chain thereof, can be abrogated by contacting the first polynucleotide and/or the second polynucleotide with a cognate site-specific recombinase that recognizes the first pair of compatible site-specific recombination sequences of the first polynucleotide and/or the second pair of compatible site-specific recombination sequences of the second polynucleotide.

[031] In certain embodiments, the cell further comprises a third polynucleotide encoding a cognate site-specific recombinase that recognizes the first and/or the second pair of compatible site-specific recombination sequences. In certain embodiments, the transcription of the third polynucleotide is regulated by an inducible promoter.

[032] In certain embodiments, the first and second pairs of site-specific recombination sequences are compatible pairs of lox sites and the cognate site-specific recombinase is a Cre recombinase.

[033] In certain embodiments, the compatible pairs of lox sites are compatible pairs of loxP sites. Therefore, in one embodiment, the first and second pairs of site-specific recombination sequences are compatible pairs of lox sites and the cognate site-specific recombinase is a Cre recombinase, for example, wherein the compatible pairs of lox sites are compatible pairs of loxP sites.

[034] In certain embodiments, the recombinant co-receptor is CD8. In certain embodiments, the first polynucleotide comprises the polynucleotide sequence of residues 1-675 of SEQ ID NO: 3. In certain embodiments, the first polynucleotide comprises the polynucleotide sequence of SEQ ID NO: 3. In certain embodiments, the second polynucleotide comprises the polynucleotide sequence of residues 1-600 of SEQ ID NO: 4. In certain embodiments, the second polynucleotide comprises the polynucleotide sequence of SEQ ID NO: 4. In certain embodiments, the first chain of the recombinant co-receptor comprises the amino acid sequence of SEQ ID NO: 25. In certain embodiments, the first chain of the recombinant co-receptor comprises the amino acid sequence of SEQ ID NO: 5. In certain embodiments, the second chain of the recombinant co-receptor comprises the amino acid sequence of SEQ ID NO: 26. In certain embodiments, the second chain of the recombinant co-receptor comprises the amino acid sequence of SEQ ID NO: 6. Therefore, in one embodiment of the present invention, the recombinant co-receptor is CD8, for example, wherein the first polynucleotide comprises the polynucleotide sequence of residues 1-675 of SEQ ID NO: 3 or the first polynucleotide comprises the polynucleotide sequence of SEQ ID NO: 3, and/or wherein the second polynucleotide comprises the polynucleotide sequence of residues 1-600 of SEQ ID NO: 4 or the second polynucleotide comprises the polynucleotide sequence of SEQ ID NO: 4. In another embodiment, the recombinant co-receptor is CD8, and the first chain of the recombinant co-receptor comprises the amino acid sequence of SEQ ID NO: 25 or SEQ ID NO: 5, and/or the second chain of the recombinant co-receptor comprises the amino acid sequence of SEQ ID NO: 26 or SEQ ID NO: 6.

[035] In certain embodiments, the transcription of the first polynucleotide is regulated by an inducible promoter, and/or the transcription of the second polynucleotide is regulated by an inducible promoter.

[036] In certain embodiments, the cell further comprises one or more reporter systems. In certain embodiments, the one or more reporter systems provide an indication of one or more activities of the cell selected from the group consisting of nuclear factor of activated T-cell (NFAT) signaling, activator protein-1 (AP1) signaling, NFκB/Rel signaling, and calcium flux. In certain embodiments, the one or more reporter systems comprise a polynucleotide

comprising a promoter region operably linked to a polynucleotide sequence encoding a detectable marker. In certain embodiments, the promoter region comprises one or more transcriptional response elements selected from the group consisting of nuclear factor of activated T-cell (NFAT) response elements, activator protein-1 (AP1) response elements, and NFκB/Rel response elements. In certain embodiments, the detectable marker is selected from the group consisting of a fluorescent protein, a cell surface marker, and an antibiotic selection marker. In certain embodiments, the promoter region is an IL-2 promoter. In certain embodiments, the IL-2 promoter is a minimal IL-2 promoter. In certain embodiments, the IL-2 promoter comprises one or more NFAT binding sites. In certain embodiments, the IL-2 promoter comprises three NFAT binding sites. In certain embodiments, the detectable marker is enhanced green fluorescent protein (EGFP). In certain embodiments, the promoter region is an IL-2 promoter comprising three NFAT binding sites and the detectable marker is enhanced green fluorescent protein (EGFP).

[037] In certain embodiments, the cell is derived from a single cell, wherein when the cell is expanded to a plurality of cells of at least 10^5 and transduced or transfected with separate polynucleotide vectors encoding a TCR α chain and a TCR β chain to express a recombinant TCR comprising the TCR α chain and the TCR β chain under conditions that maximize transduction or transfection efficiency, at least 20% of the plurality of cells express the recombinant TCR on the cell surface. In certain embodiments, the cell has previously been subjected to one or more rounds of transduction or transfection.

[038] In certain embodiments, the lymphocyte is a T cell, B cell, or an NK cell. In certain embodiments, the lymphocyte is a T cell that is selected from the group consisting of a $CD8^+$ cytotoxic T cell, a $CD8^+$ regulatory T cell, a $CD4^+$ cytotoxic T cell, a $CD4^+$ helper T cell and a $CD4^+$ regulatory T cell. In certain embodiments, the lymphocyte is a mammalian lymphocyte. In certain embodiments, the lymphocyte is a murine lymphocyte. In certain embodiments, the lymphocyte is selected from the group consisting of a RAG1 or RAG2 knockout murine cell, an immature murine T cell, a mouse DO-11.10.7 58 α- β- (58-/-) cell, and a mouse Bw5147 cell. In certain embodiments, the lymphocyte is a mouse lymphocyte. Therefore, in another embodiment of a cell of the present invention, the lymphocyte is a T cell, B cell, or an NK cell, for example, the lymphocyte is a T cell that is selected from the group consisting of a $CD8^+$ cytotoxic T cell, a $CD8^+$ regulatory T cell, a $CD4^+$ cytotoxic T cell, a $CD4^+$ helper T cell and a $CD4^+$ regulatory T cell, and/or wherein the lymphocyte is a mammalian lymphocyte, for example, a murine lymphocyte, such as a lymphocyte selected

from the group consisting of a RAG1 or RAG2 knockout murine lymphocyte, an immature murine T cell, a mouse DO-11.10.7 58 α- β- (58-/-) cell, and a mouse Bw5147 cell. In one embodiment, the lymphocyte is a mouse lymphocyte.

[039] In certain embodiments, the cell further expresses a recombinant TCR on the cell surface, the recombinant TCR comprising a TCR α chain and a TCR β chain, wherein the TCR α chain and the TCR β chain each comprises a variable region and a non-variable region, each non-variable region comprising a constant region, a transmembrane region, and a cytoplasmic region, wherein for each chain, the variable region is linked to the constant region, the constant region is linked to the transmembrane region, and the transmembrane region is linked to the cytoplasmic region. In certain embodiments, for each of the TCR α chain and the TCR β chain, the amino acid sequence of the cytoplasmic region is at least 60% identical, optionally 70, 80, 90, or 100% identical, to the amino acid sequence of a corresponding cytoplasmic region of a naturally occurring TCR chain from the same species as the lymphocyte. In certain embodiments, for each of the TCR α chain and the TCR β chain, the amino acid sequence of the transmembrane region and the cytoplasmic region, together, is at least 90% identical, optionally 91, 92, 93, 94, 95, 96, 97, 98, 99, or 100% identical, to the amino acid sequence of a corresponding region of a naturally occurring TCR chain from the same species as the lymphocyte. In certain embodiments, the amino acid sequence of the transmembrane region and the cytoplasmic region of the TCR α chain, together, comprises the amino acid sequence of SEQ ID NO: 14. In certain embodiments, the amino acid sequence of the transmembrane region and the cytoplasmic region of the TCR β chain, together, comprises the amino acid sequence selected from the group consisting of SEQ ID NOs: 16, 18 and 20. In certain embodiments, for each of the TCR α chain and the TCR β chain, the amino acid sequence of the non-variable region is at least 90% identical, optionally 91, 92, 93, 94, 95, 96, 97, 98, 99, or 100% identical, to the amino acid sequence of a corresponding non-variable region of a naturally occurring TCR chain from the same species as the lymphocyte. In certain embodiments, the non-variable region of the TCR α chain comprises the amino acid sequence of SEQ ID NO: 13. In certain embodiments, the non-variable region of the TCR β chain comprises the amino acid sequence selected from the group consisting of SEQ ID NOs: 15, 17, and 19.

[040] Therefore, in certain embodiments, the cell further expresses a recombinant TCR on the cell surface, the recombinant TCR comprising a TCR α chain and a TCR β chain, wherein the TCR α chain and the TCR β chain each comprises a variable region and a non-variable region, each non-variable region comprising a constant region, a transmembrane region, and

a cytoplasmic region, wherein for each chain, the variable region is linked to the constant region, the constant region is linked to the transmembrane region, and the transmembrane region is linked to the cytoplasmic region. In one embodiment, for each of the TCR α chain and the TCR β chain, the amino acid sequence of the cytoplasmic region is at least 60% identical, optionally 70, 80, 90, or 100% identical, to the amino acid sequence of a corresponding cytoplasmic region of a naturally occurring TCR chain from the same species as the lymphocyte. In another embodiment, for each of the TCR α chain and the TCR β chain, the amino acid sequence of the transmembrane region and the cytoplasmic region, together, is at least 90% identical, optionally 91, 92, 93, 94, 95, 96, 97, 98, 99, or 100% identical, to the amino acid sequence of a corresponding region of a naturally occurring TCR chain from the same species as the lymphocyte, for example, wherein the amino acid sequence of the transmembrane region and the cytoplasmic region of the TCR α chain, together, comprises the amino acid sequence of SEQ ID NO: 14, and/or wherein the amino acid sequence of the transmembrane region and the cytoplasmic region of the TCR β chain, together, comprises the amino acid sequence selected from the group consisting of SEQ ID NOs: 16, 18 and 20. In another embodiment, for each of the TCR α chain and the TCR β chain, the amino acid sequence of the non-variable region is at least 90% identical, optionally 91, 92, 93, 94, 95, 96, 97, 98, 99, or 100% identical, to the amino acid sequence of a corresponding non-variable region of a naturally occurring TCR chain from the same species as the lymphocyte, for example, wherein the non-variable region of the TCR α chain comprises the amino acid sequence of SEQ ID NO: 13, and/or the non-variable region of the TCR β chain comprises the amino acid sequence selected from the group consisting of SEQ ID NOs: 15, 17, and 19.

[041] In certain embodiments, the amino acid sequence of each variable region is derived from a corresponding variable region of a TCR chain from a different species than the lymphocyte. In one embodiment, the TCR from a different species than the lymphocyte is human.

[042] In certain embodiments, the amino acid sequence of each variable region is derived from a corresponding variable region of a TCR chain from a different species than the lymphocyte, for example, wherein for each of the TCR α chain and the TCR β chain, the amino acid sequence of the variable region and the constant region, together, is derived from a corresponding region of a TCR chain from a different species than the lymphocyte. In one embodiment, the TCR from a different species than the lymphocyte is human.

[043] In certain embodiments, the recombinant TCR is a fusion molecule comprising a co-stimulatory signaling region. In certain embodiments, the co-stimulatory signaling region is

fused to the cytoplasmic region of the TCR α chain or the cytoplasmic region of the TCR β chain. In certain embodiments, the co-stimulatory signaling region comprises the signaling region of a co-stimulatory molecule selected from the group consisting of CD27, CD28, 4-1BB, OX40, CD30, CD40, ICOS, lymphocyte function-associated antigen-1 (LFA-1), CD2, CD7, LIGHT, NKG2C, NKG2D, B7-H3, and a ligand that specifically binds CD83. In certain embodiments, the co-stimulatory signaling region comprises the signaling region of CD28, the signaling region of 4-1BB, or a combination thereof. In certain embodiments, the fusion molecule further comprises a CD3ζ signaling region.

[044] Therefore, in certain embodiments, the recombinant TCR is a fusion molecule comprising a co-stimulatory signaling region, for example, wherein the co-stimulatory signaling region is fused to the cytoplasmic region of the TCR α chain or the cytoplasmic region of the TCR β chain, and/or wherein the co-stimulatory signaling region comprises the signaling region of a co-stimulatory molecule selected from the group consisting of CD27, CD28, 4-1BB, OX40, CD30, CD40, ICOS, lymphocyte function-associated antigen-1 (LFA-1), CD2, CD7, LIGHT, NKG2C, NKG2D, B7-H3, and a ligand that specifically binds CD83. In one embodiment, the co-stimulatory signaling region comprises the signaling region of CD28, the signaling region of 4-1BB, or a combination thereof, and/or the fusion molecule further comprises a CD3ζ signaling region.

[045] In certain embodiments, the expression of the recombinant TCR is coupled to the expression of (1) at least one antibiotic selection marker; (2) at least one screening marker; or (3) a combination thereof, for example, wherein the at least one screening marker is a fluorescent protein or a cell surface marker.

[046] In certain embodiments, the recombinant TCR is derived from a source selected from the group consisting of fetal thymus, cord blood, tumor infiltrating lymphocytes (TILs) isolated from a cancer patient, a vaccinated subject, a subject naturally exposed to an antigen of interest, a healthy subject, and a synthetic library, for example, wherein the vaccinated subject is a human who has received an HPV vaccine, an EBV vaccine, or a cancer vaccine, or the vaccinated subject is a transgenic mouse expressing human major histocompatibility complex (MHC) that has been immunized with an antigen of interest, and/or wherein the vaccinated subject is a transgenic mouse expressing HLA-A2 that has been immunized with an antigen of interest.

[047] In certain embodiments of the present invention described above, the cell expresses (1) a recombinant TCR α chain and a recombinant TCR β chain, (2) a recombinant TCR α chain and multiple different recombinant TCR β chains, (3) a recombinant TCR β chain and

multiple different recombinant TCR α chains, or (4) multiple different recombinant TCR α chains and multiple different recombinant TCR β chains.

[048] In another aspect, the disclosure also provides a cellular library comprising a plurality of any one of the cells disclosed herein. In certain embodiments, the cellular library has a TCR diversity of at least 10^7, 10^8, or 10^9. In another aspect, the present invention relates to a cellular library comprising a plurality of any one of the cells of the invention, wherein the cell further expresses a cellular TCR on the cell surface. The embodiments disclosed herein for a cell of the invention also apply to a cellular library of the invention.

[049] In another aspect, the disclosure also provides a method for the isolation and identification of a TCR with a desired characteristic comprising subjecting a plurality of any one of the cells or the cellular libraries disclosed herein to a selection process comprising selecting for the desired characteristic in the presence of a desired peptide-MHC complex, isolating a cell that expresses a TCR, and determining that the TCR of the isolated cell has the desired characteristic. The embodiments disclosed herein for a cell of the invention wherein the cell further expresses a cellular TCR on the cell surface and the embodiments disclosed herein for a cellular library of the invention also apply to the method of the invention.

[050] In another aspect, the disclosure also provides a method for the identification of at least one polynucleotide encoding a TCR with a desired characteristic, comprising: (a) providing a plurality of any one of the cells disclosed, (b) transducing or transfecting the cells with a plurality of expression constructs encoding a plurality of TCRs, (c) expressing the TCRs on the surface of the cells, (d) selecting cells expressing the TCRs for the desired characteristic in the presence of a desired peptide-MHC complex, (e) isolating the at least one polynucleotide encoding the TCR with the desired characteristic from the selected cells, and (f) identifying the at least one polynucleotide encoding the TCR with the desired characteristic isolated in step (e). The embodiments disclosed herein for a cell of the invention also apply to the method of the invention. Preferably, the method is an *in vitro* method.

[051] In another aspect, the disclosure also provides a method for the identification of at least one polynucleotide encoding a TCR with a desired characteristic, comprising: (a) providing a plurality of any one of the cells disclosed herein; (b) transducing or transfecting the cells with a plurality of expression constructs encoding a plurality of TCRs, (c) expressing the TCRs on the surface of the cells, (d) selecting cells expressing the TCRs for

the desired characteristic in the presence of a desired peptide-MHC complex, (e) modulating an activity of the recombinant co-receptor or chain thereof in the cells selected in step (d), (f) further selecting cells expressing the TCRs produced by step (e) for the desired characteristic in the presence of the desired peptide-MHC complex, (g) isolating the at least one polynucleotide encoding the TCR with the desired characteristic from the cells selected in step (f), and (h) identifying the at least one polynucleotide encoding the TCR with the desired characteristic isolated in step (g). The embodiments disclosed herein for a cell of the invention also apply to the method of the invention. Preferably, the method is an *in vitro* method.

[052] In certain embodiments, step (d) is performed in the presence of surface expression of the recombinant co-receptor or chain thereof, and step (e) comprises abrogation of cell surface expression of the recombinant co-receptor or chain thereof.

[053] In certain embodiments, the plurality of cells of step (a) comprise the any one of cells disclosed herein, and in step (e), the cell surface expression of the recombinant co-receptor or chain thereof is abrogated by contacting the site-specific recombination sequences in the first and/or second polynucleotide with a cognate site-specific recombinase. In certain embodiments, the cognate site-specific recombinase is expressed in the cells under the control of an inducible promoter. In certain embodiments, the cognate site-specific recombinase is introduced into the cells selected in step (d) prior to step (e). In certain embodiments, the cognate site-specific recombinase is introduced by transfecting or transducing the cells selected in step (d) with an expression construct encoding the site-specific recombinase. In certain embodiments, the cognate site-specific recombinase is introduced as a protein. In certain embodiments, the site-specific recombination sequences are lox sites and the cognate site-specific recombinase is a Cre recombinase. In certain embodiments, the site-specific recombination sequences are lox sites and the cognate site-specific recombinase is a Cre recombinase fusion protein comprising a protein transduction domain. In certain embodiments, the protein transduction domain is from HIV Tat. Therefore, in certain embodiments of the methods of the invention, step (d) is performed in the presence of surface expression of the recombinant co-receptor or chain thereof, and step (e) comprises abrogation of cell surface expression of the recombinant co-receptor or chain thereof, for example, wherein the plurality of cells of step (a) comprise the any one of cells disclosed herein, and wherein in step (e), the cell surface expression of the recombinant co-receptor or chain thereof is abrogated by contacting the site-specific recombination sequences in the first and/or second polynucleotide with a cognate site-specific recombinase, and/or wherein the

cognate site-specific recombinase is expressed in the cells under the control of an inducible promoter. In another embodiment, the cognate site-specific recombinase is introduced into the cells selected in step (d) prior to step (e), in particular, wherein the cognate site-specific recombinase is introduced by transfecting or transducing the cells selected in step (d) with an expression construct encoding the site-specific recombinase, or wherein the cognate site-specific recombinase is introduced as a protein. In one embodiment, the site-specific recombination sequences are lox sites and the cognate site-specific recombinase is a Cre recombinase, for example, wherein the site-specific recombination sequences are lox sites and the cognate site-specific recombinase is a Cre recombinase fusion protein comprising a protein transduction domain, in particular wherein the protein transduction domain is from HIV Tat.

[054] In another aspect, the disclosure also provides a method for the identification of at least one polynucleotide encoding a TCR with a desired characteristic, comprising: (a) providing a plurality of cells any one of the cells disclosed herein, (b) transducing or transfecting the cells with a plurality of expression constructs encoding a plurality of TCRs, (c) expressing the TCRs on the surface of the cells, (d) selecting cells expressing the TCRs for the desired characteristic in the presence of an MHC molecule complexed with a peptide of interest, wherein the MHC molecule is a variant of a wild type MHC molecule, wherein the variant MHC molecule binds to a co-receptor with reduced affinity compared to the affinity of the wild type MHC molecule for the co-receptor, (e) isolating the at least one polynucleotide encoding the TCR with the desired characteristic from the cells selected in step (d), and (f) identifying the at least one polynucleotide encoding the TCR with the desired characteristic isolated in step (e). The embodiments disclosed herein for a cell of the invention also apply to the method of the invention. Preferably, the method is an *in vitro* method.

[055] In certain embodiments of any of the methods described above, the co-receptor is CD8 and the variant MHC molecule is a variant MHC class I molecule comprising an α chain that is a variant of a wild type MHC class I α chain, for example, wherein the co-receptor is human CD8 and the variant MHC class I molecule is a variant HLA-A2 comprising an α chain that is a variant of wild type HLA-A2 α chain, and/or wherein the variant HLA-A2 α chain comprises an A245V mutation or a D227K/T228A mutation, numbered according to the mature protein sequence.

[056] In certain embodiments of any of the methods described above, the desired characteristic is selected from the group consisting of high selectivity for the desired peptide-MHC complex, high affinity for the desired peptide-MHC complex, and demonstration of a positive functional readout in the presence of the desired peptide-MHC complex. In certain embodiments, the functional readout is selected from the group consisting of expression of activation marker, triggering of signaling pathway, production of cytokine, calcium flux, proliferation, and activation of reporter system. In certain embodiments, the activation marker is CD69 or CD137. In certain embodiments, the signaling pathway is NFAT signaling, IL-2 signaling, activator protein-1 signaling, or NFκB/Rel signaling. In certain embodiments, the cytokine is IL-2, TNF-α, or IFN-γ. In certain embodiments, the proliferation is proliferation of T cells. In certain embodiments, the functional readout is the activation of one or more reporter systems in the cells. In certain embodiments, the reporter system is IL-2-EGFP reporter system. Therefore, certain embodiments of any of the methods of the invention described above, the desired characteristic is selected from the group consisting of high selectivity for the desired peptide-MHC complex, high affinity for the desired peptide-MHC complex, and demonstration of a positive functional readout in the presence of the desired peptide-MHC complex, for example, wherein the functional readout is selected from the group consisting of expression of activation marker, triggering of signaling pathway, production of cytokine, calcium flux, proliferation, and activation of reporter system, in particular a reporter system as described above for a cell of the invention, for example, wherein the activation marker is CD69 or CD137. In one embodiment, the signaling pathway is NFAT signaling, IL-2 signaling, activator protein-1 signaling, or NFκB/Rel signaling. In one embodiment, the cytokine is IL-2, TNF-α, or IFN-γ. In one embodiment, the proliferation is proliferation of T cells. In one embodiment, the functional readout is the activation of one or more reporter systems in the cells. In one embodiment, the reporter system is IL-2-EGFP reporter system.

[057] In certain embodiments of any of the methods described above, the plurality of expression constructs encode (i) a TCR α chain and a TCR β chain, (ii) a TCR α chain and multiple different TCR β chains, (iii) a TCR β chain and multiple different TCR α chains, or (iv) multiple different TCR α chains and multiple different TCR β chains.

[058] In certain embodiments of any of the methods described above, the desired peptide-MHC complex comprises an MHC class I or MHC class II molecule. In certain embodiments, the desired peptide-MHC complex is a cell free peptide-MHC complex. In certain

embodiments, the desired peptide-MHC complex comprises a dimer, a tetramer, or a multimer of MHC molecules. In certain embodiments, the desired peptide-MHC complex is displayed on the surface of a cell. In a exemplary embodiment of any of the methods of the invention described above, the desired peptide-MHC complex is displayed on the surface of a cell, for example, wherein the cell is a mammalian cell, a yeast cell, or an insect cell, or the cell is a T2 cell or an antigen-presenting cell. In certain embodiments, the cell is a mammalian cell, a yeast cell, or an insect cell. In certain embodiments, the cell is a T2 cell or an antigen-presenting cell. Therefore, in one embodiment of any of the methods of the invention described above, the desired peptide-MHC complex comprises an MHC class I or MHC class II molecule, and/or the desired peptide-MHC complex is a cell free peptide-MHC complex, in particular wherein the desired peptide-MHC complex comprises a dimer, a tetramer, or a multimer of MHC molecules.

[059] In certain embodiments of any of the methods described above, the peptide is a phosphopeptide or a phosphopeptide mimetic.

[060] In certain embodiments of any of the methods described above, in step (b), the plurality of expression constructs encoding a plurality of TCRs is transduced into the cells using retroviral vector particles comprising an envelope of PVC-211 murine leukemia virus, wherein the retroviral vector particle comprises a retroviral expression construct encoding the TCR α chain or the TCR β chain.

[061] In certain embodiments of any of the methods described above, each TCR comprises a TCR α chain and a TCR β chain, wherein the TCR α chain and the TCR β chain are encoded by separate expression constructs.

BRIEF DESCRIPTION OF THE FIGURES

[062] **Figure 1** is a bar graph showing relative IL-2 levels in culture supernatant measured by ELISA after overnight co-incubation of MTCD8 mCD8αβ- chiCD8αβ+ chiC58αβ+ cells with anti-CD3 and anti-CD28 antibodies. The y-axis shows optical density (OD) signal.

[063] **Figures 2A, 2B, 2C, 2D, and 2E:** Figure 2A is a set of flow cytometry plots showing the co-staining of PE-labeled HLA-A*0201 Mart-1 tetramer and APC-labeled anti-TCR β chain antibody on AK-D10 cells, AK-D10 cells expressing chimeric DMF4 (AK-D10 chiDMF4), and AK-D10 cells expressing chimeric DMF5 (AK-D10 chiDMF5). The percentage of tetramer+ TCR+ cells is indicated in each plot. The rightmost plot shows an overlay of the AK-D10 chiDMF4 cells and the AK-D10 chiDMF5 cells. Figures 2B and 2C

are bar graphs showing relative IL-2 production measured by ELISA following overnight incubation of the AK-D10 chiDMF4 cells (Figure 2B) or the AK-D10 chiDMF5 cells (Figure 2C) with DimerX loaded with a Mart-1 peptide ELAGIGILTV (SEQ ID NO: 30) (DimerX/Mart-1) or DimerX alone without any peptide (DimerX) at indicated concentrations. The y axis shows optical density (OD) signal. Figures 2D and 2E are bar graphs showing results from intracellular staining of IL-2 and TNFα, respectively, of AK-D10 cells expressing chimeric TCR following overnight incubation with non-loaded DimerX (grey bars) or DimerX loaded with a Mart-1 peptide ELAGIGILTV (SEQ ID NO: 30) (for AK-D10 chiDMF4 and AK-D10 chiDMF5) or a NY-ESO-1 peptide SLLMWITQV (SEQ ID NO: 31) (for AK-D10 chiC58) (black bars). The y-axis shows the percentage (%) of TCR+ IL-2+ (Figure 2D) or TCR+ TNFα+ (Figure 2E) cells.

[064] **Figure 3** is a set of flow cytometry plots showing the expression of chimeric CD8 on the surface of LOT-D08 chiCD8α^{flox}+ chiCD8β^{flox}+ cells and the subsequent elimination of chimeric CD8 from the cell surface following transduction with a Cre recombinase. The percentage of chiCD8α+ chiCD8β+ cells is indicated in each plot.

[065] **Figures 4A, 4B, and 4C** are results from an assay testing binding of HLA-A*02:01 Mart-1 tetramer to BB8 cells (MTCD8 mCD8αβ- chiCD8α^{flox}+ chiCD8β^{flox}+ IL-2-$(NFAT)_3$-EGFP+) expressing chimeric DMF4 (chiDMF4) or DMF5 (chiDMF5). Figures 4A and 4B are a set of flow cytometry plots showing BB8 cells expressing chimeric DMF4 either transduced to express a Cre recombinase (bottom) or not transduced (top). The pairs of parameters shown on the X and Y axes are indicated in the figures. Figure 4C is a bar graph showing binding of HLA-A*02:01 Mart-1 tetramer to BB8 cells expressing chimeric DMF4 or DMF5. The cells were either transduced to express a Cre recombinase (marked as "CD8-") or not transduced (marked as "CD8+"). The y-axis shows median fluorescence intensity (MFI) detected for tetramer binding as measured by flow cytometry.

[066] **Figure 5** is a bar graph showing relative IL-2 levels in culture supernatant produced by AK-D10 cells (marked as "chiC58-") or AK-D10 cells expressing both a reporter construct and chimeric TCR C58 (marked as "chiC58+") after incubation with either anti-CD3 antibody (left) or anti-CD3 and anti-CD28 antibodies (right), measured by ELISA. The y axis shows optical density (OD) signal.

[067] **Figures 6A, 6B, and 6C** are a set of flow cytometry plots showing EGFP expression. The cells tested are AK-D10 cells expressing an IL-2 reporter construct and chimeric TCR

DMF4 (AK-D10 IL-2-$(NFAT)_3$-EGFP+ chiDMF4+) incubated in the presence (Figure 6A) or absence (Figure 6B) of anti-CD3 antibody or AK-D10 cells expressing only the IL-2 reporter construct (AK-D10 IL-2-$(NFAT)_3$-EGFP+) incubated with anti-CD3 antibody (Figure 6C). The percentage of EGFP positive cells is indicated in each plot.

[068] **Figures 7A, 7B, and 7C** are a set of flow cytometry plots from an assay in which AK-D10 cells expressing an IL-2 reporter construct and chimeric TCR C259 (AK-D10 IL-2-$(NFAT)_3$-EGFP+ chiC259+) were incubated with T2 cells pulsed with a NY-ESO-1 peptide SLLMWITQV (SEQ ID NO: 31) (Figure 7A), incubated with non-pulsed T2 cells (Figure 7B), or incubated in the absence of T2 cells (Figure 7C) and measured for TCR and EGFP expression using flow cytometry. The percentage of TCR^{high} EGFP+ cells is indicated in the upper right panel of each plot. The percentage of TCR^{low} EGFP+ cells is indicated in the lower right panel of each plot.

[069] **Figure 8** is a bar graph showing results from an assay in which AK-D10 cells expressing a reporter construct IL-2-$(NFAT)_3$-EGFP and chimeric TCR C58 (marked as "chiC58") or C259 (marked as "chiC259") were co-cultured with T2 cells pulsed with a NY-ESO-1 peptide SLLMWITQV (SEQ ID NO: 31) or co-cultured with non-pulsed T2 cells and analyzed for the expression of TCR, EGFP, CD69, and CD137 using flow cytometry. The percentage (%) of TCR+ EGFP+ cells, TCR+ CD69+ cells, or TCR+ CD137+ cells is shown.

[070] **Figures 9A and 9B** are graphs showing results from an assay in which AK-D10 cells expressing a reporter construct IL-2-$(NFAT)_3$-EGFP and chimeric TCR DMF4 (marked as "chiDMF4", Figure 9A) or DMF5 (marked as "chiDMF5", Figure 9B) were co-cultured with T2 cells pulsed with various concentrations of a Mart-1 peptide ELAGIGILTV (SEQ ID NO: 30) and measured for TCR and EGFP expression using flow cytometry. The percentage (%) of TCR+ EGFP+ cells is plotted against concentrations of the Mart-1 peptide.

[071] **Figures 10A and 10B** are graphs showing results from an assay in which BB8 cells expressing chimeric TCR DMF4 or DMF5 were transduced to express a Cre recombinase (marked as "chiDMF4 Cre" in Figure 10A and "chiDMF5 Cre" in Figure 10B) or not transduced (marked as "chiDMF4" in Figure 10A and "chiDMF5" in Figure 10B). The cells were then co-cultured with T2 cells pulsed with 50 μg/ml, 5 μg/ml, or 0.5 μg/ml of a Mart-1 peptide ELAGIGILTV (SEQ ID NO: 30) and measured for TCR, CD8, and EGFP expression using flow cytometry. The percentage (%) of TCR+ EGFP+ cells is plotted against lengths of activation.

DETAILED DESCRIPTION

[072] Disclosed herein are methods for the generation and screening of highly diversified T cell receptor (TCR) cellular libraries, allowing for the rapid identification and isolation of antigen-reactive therapeutic TCRs for immune therapy applications.

[073] In a preferred embodiment of the present invention, a cell according to the present invention is not a human embryonic cell.

[074] All instances of "isolated cells" described herein are additionally contemplated as cells that may be, but need not be, isolated. All instances of "isolated polynucleotides" described herein are additionally contemplated as polynucleotides that may be, but need not be, isolated. All instances of "cells" described herein are additionally contemplated as cells that may be, but need not be, isolated. All instances of "polynucleotides" described herein are additionally contemplated as polynucleotides that may be, but need not be, isolated.

I. DEFINITIONS

[075] Unless otherwise defined herein, scientific and technical terms used herein have the meanings that are commonly understood by those of ordinary skill in the art. In the event of any latent ambiguity, definitions provided herein take precedence over any dictionary or extrinsic definition. Unless otherwise required by context, singular terms shall include pluralities and plural terms shall include the singular. The use of "or" means "and/or" unless stated otherwise. The use of the term "including", as well as other forms, such as "includes" and "included", is not limiting.

[076] It is noted here that as used in this specification and the appended claims, the singular forms "a", "an", and "the" also include plural reference, unless the context clearly dictates otherwise.

[077] The term "about" or "approximately" means within 10%, and more preferably within 5% (or 1% or less) of a given value or range.

[078] As used herein, the term "isolated cell" refers to a cell that has been removed from its in-vivo location.

[079] As used herein, the terms "T cell receptor" and "TCR" are used interchangeably and refer to full length heterodimeric αβ or γδ TCRs, antigen-binding fragments of TCRs, and molecules comprising TCR CDRs or variable regions. Examples of TCRs include, but are

not limited to, full-length TCRs, antigen-binding fragments of TCRs, soluble TCRs lacking transmembrane and cytoplasmic regions, single-chain TCRs containing variable regions of TCRs attached by a flexible linker, TCR chains linked by an engineered disulfide bond, monospecific TCRs, multi-specific TCRs (including bispecific TCRs), TCR fusions, human TCRs, humanized TCRs, chimeric TCRs, recombinantly produced TCRs, and synthetic TCRs. The term encompasses wild-type TCRs and genetically engineered TCRs (*e.g.*, a chimeric TCR comprising a chimeric TCR chain which includes a first portion from a TCR of a first species and a second portion from a TCR of a second species).

[080] As used herein, the term "TCR variable region" is understood to encompass amino acids of a given TCR which are not included within the non-variable region as encoded by the TRAC gene for TCR α chains and either the TRBC1 or TRBC2 genes for TCR β chains. In some embodiments, a TCR variable region encompasses all amino acids of a given TCR which are encoded by a TRAV gene or a TRAJ gene for a TCR α chain or a TRBV gene, a TRBD gene, or a TRBJ gene for a TCR β chain (see, *e.g.*, T cell receptor Factsbook, (2001) LeFranc and LeFranc, Academic Press, ISBN 0-12-441352-8, which is incorporated by reference herein in its entirety).

[081] As used herein, the term "constant region" with respect to a TCR refers to the extracellular portion of a TCR that is encoded by the TRAC gene for TCR α chains and either the TRBC1 or TRBC2 genes for TCR β chains. The term constant region does not include a TCR variable region encoded by a TRAV gene or a TRAJ gene for a TCR α chain or a TRBV gene, a TRBD gene, or a TRBJ gene for a TCR β chain (*see*, *e.g.*, T cell receptor Factsbook, (2001) LeFranc and LeFranc, Academic Press, ISBN 0-12-441352-8, which is incorporated by reference herein in its entirety).

[082] As used herein, the term a "recombinant" molecule refers to a molecule that is not naturally present in the cell in which it is expressed. In certain embodiments, the recombinant molecule, *e.g.*, co-receptor or chain thereof, is from a different species than the cell in which it is expressed.

[083] As used herein, the term an "endogenous" molecule refers to a molecule that is naturally present in the cell in which it is expressed.

[084] As used herein, the term "co-stimulatory signaling region" refers to the intracellular portion of a co-stimulatory molecule that is responsible for mediating intracellular signaling events.

[085] As used herein, the term "extracellular" with respect to a recombinant protein refers to the portion or portions of a recombinant transmembrane protein that are located outside of a cell.

[086] As used herein, the term "transmembrane" with respect to a recombinant protein refers to the portion or portions of a recombinant transmembrane protein that are embedded in the plasma membrane of a cell.

[087] As used herein, the term "cytoplasmic" with respect to a recombinant protein refers to the portion or portions of a recombinant protein that are located in the cytoplasm of a cell.

[088] As used herein, the term "chimeric" protein refers to a recombinant protein in which a first region of a first recombinant protein is fused in frame, optionally with a peptide linker, with a second region of a second recombinant protein. In one embodiment, the first and second recombinant proteins are from the same species and the first region is different from the second region. In another embodiment, the first and second recombinant proteins are from different species.

[089] As used herein, the term "CD3" refers to "cluster of differentiation 3", or functional portion thereof. An example of CD3 includes, but is not limited to, a complex comprising six polypeptides forming three dimers: CD3ε/CD3γ, CD3ε/CD3δ, and CD3ζ/CD3ζ.

[090] As used herein, the term "CD8" refers to "cluster of differentiation 8", a co-receptor for TCR, or functional portion thereof. Examples of CD8 include, but are not limited to, a CD8α/CD8β heterodimer, a CD8α/CD8α homodimer, and a CD8β/CD8β homodimer. The term encompasses wild-type CD8 and genetically engineered CD8 (*e.g.*, a chimeric CD8 comprising a chimeric CD8 chain which includes a first portion from a CD8 of a first species and a second portion from a CD8 of a second species).

[091] As used herein, the term "CD4" refers to "cluster of differentiation 4", a co-receptor for TCR, or functional portion thereof. The term encompasses wild-type CD4 and genetically engineered CD4 (*e.g.*, a chimeric CD4 comprising a first portion from a CD4 of a first species and a second portion from a CD4 of a second species).

[092] As used herein, the term "co-receptor" refers to a cell surface molecule that binds to a peptide-MHC complex together with a TCR and potentiates T cell activation. Examples of co-receptors include, without limitation, CD4 and CD8.

[093] As used herein, the term "internal ribosomal entry site (IRES) element" refers to a structure to which a ribosome can bind that does not need to be at the 5′ end of the mRNA. It can therefore direct a ribosome to initiate translation at a second initiation codon within an mRNA, allowing more than one polypeptide to be produced from a single mRNA. Exemplary IRES elements include, but are not limited to, IRES elements isolated from poliovirus, rhinovirus, encephalomyocarditis virus, foot-and-mouth disease virus, hepatitis A and hepatitis C virus (*see*, for example, Mokrejs *et al.* (2006) Nucleic Acids Res. 34 (Database issue): D125–30, which is incorporated by reference herein in its entirety).

[094] As used herein, the term "TCR engagement" refers to the binding of a TCR to its ligand. In certain embodiments, the ligand is a peptide-MHC complex.

[095] As used herein, the terms "major histocompatibility complex" and "MHC" are used interchangeably and refer to an MHC class I molecule and/or an MHC class II molecule.

[096] As used herein, the term "MHC class I" refers to a dimer of an MHC class I α chain and a β2-microglobulin chain and the term "MHC class II" refers to a dimer of an MHC class II α chain and an MHC class II β chain.

[097] As used herein, the term "soluble MHC" refers to an MHC molecule lacking transmembrane and cytoplasmic regions.

[098] As used herein, the term "peptide-MHC complex" refers to an MHC molecule (MHC class I or MHC class II) with a peptide bound in the art-recognized peptide binding pocket of the MHC.

[099] As used herein, the terms "DO-11.10.7 58 α- β- cell" and "58-/- cell" are used interchangeably and refer to a mouse T cell hybridoma cell line as described in Letourneur and Malissen Eur J Immunol. 1989; 19(12):2269-74, which is incorporated by reference herein in its entirety.

[0100] As used herein, the term "T2 cell" refers to a human lymphoblast cell line as described in Salter RD, *et al.* Genes regulating HLA class I antigen expression in T-B lymphoblast hybrids. Immunogenetics 21: 235-246, 1985, which is incorporated by reference herein in its entirety.

[0101] As used herein, the term "Bw5147 cell" refers to a mouse T lymphocyte cell line as described in Ralph P. Retention of lymphocyte characteristics by myelomas and theta^{+}-

lymphomas: sensitivity to cortisol and phytohemagglutinin. J. Immunol. 110: 1470-1475, 1973, which is incorporated by reference herein in its entirety.

[0102] As used herein the term “site-specific recombination” refers to a recombination event that is effected between two compatible sequence-specific recombination sites on a single nucleic acid molecule by a cognate site-specific recombinase. A pair of site-specific recombination sites are said to be “compatible” if the members of the pair can direct the recombination between the site-specific recombination sites by a cognate site-specific recombinase. In one embodiment, the recombination event results in the excision of the intervening nucleotide sequences between the site-specific recombination sites. Pairs of compatible site-specific recombination sites are said to be “non-cross-reactive” if any one pair of compatible site-specific recombination sites is unable to recombine with any other pair of compatible site-specific recombination sites.

[0103] As used herein the term "site-specific recombinase" refers to an enzyme that mediates site-specific recombination. Examples of site-specific recombinase include, without limitation, Cre recombinase and FLP recombinase. A “cognate” site-specific recombinase refers to a recombinase that can recognize and effectuate recombination between a pair of compatible sequence-specific recombination sites. For example, bacteriophage P1 Cre recombinase recognizes lox recombination sites whereas yeast FLP recombinase recognizes FRT recombination sites.

[0104] As used herein, the term “Cre recombinase” refers to a protein that is able to mediate site-specific recombinase activity of the Cre protein of bacteriophage P1 (Hamilton, D. L., et al., J. Mol. Biol. 178:481-486 (1984), which is incorporated by reference herein in its entirety). The Cre protein of bacteriophage P1 mediates site-specific recombination between specialized recombination sequences, known as “loxP” sequences. See, *e.g.*, Hoess et al., Proc. Natl. Acad. Sci. USA 79:3398-3402 (1982) and Sauer, B. L., U.S. Pat. No. 4,959,317, which are incorporated by reference herein in their entireties. The term encompasses any derivatives of a naturally occurring Cre recombinase that retain the ability to effectuate recombination between two compatible lox sites.

[0105] Recombination products are dependent on the location and relative orientation of the lox sites. When two lox sequences having an identical orientation exist within the same DNA molecule, a DNA sequence flanked by the two lox sequences is excised by the Cre recombinase to form a circular molecule (excision reaction). Conversely, when two lox

sequences exist in different DNA molecules, one of which is a circular DNA, the circular DNA is inserted into the other DNA molecule via the lox sequences (insertion reaction). In another embodiment, the Cre recombinase can be an optimized Cre recombinase. *See*, for example, International Patent Application Publication No. WO 2014158593, which is incorporated by reference herein in its entirety. In one embodiment, the Tat-Cre recombinase includes a nuclear localization signal.

[0106] As used herein, the term "FLP recombinase" refers to a protein having the site-specific recombinase activity of yeast FLP (*Saccharomyces cerevisiae*). The FLP protein encoded by yeast 2 μ DNA mediates site-specific recombination between a pair of compatible FRT recombination sites. See, for example, Babineau et al., J. Biol. Chem., 260, 12313-12319 (1985), which is incorporated by reference herein in its entirety. The term encompasses any derivatives of a naturally occurring FLP recombinase that retain the ability to effectuate recombination between two compatible FRT sites. Recombination products are dependent on the location and relative orientation of the FRT sites. When two FRT sequences having an identical orientation exist within the same DNA molecule, a DNA sequence flanked by the two FRT sequences is excised by the FLP recombinase to form a circular molecule (excision reaction). Conversely, when two FRT sequences exist in different DNA molecules, one of which is a circular DNA, the circular DNA is inserted into the other DNA molecule via the FRT sequences (insertion reaction). In one embodiment, the FLP recombinase can be an optimized FLP recombinase. *See*, for example, U.S. Patent Application Publication No. 2010/0050279, which is incorporated by reference herein in its entirety.

[0107] In one embodiment, the term "site-specific recombinase" refers to a Cre:FLP fusion protein that is able to recombine pair of compatible FRT or lox recombination sites. *See*, for example, Djukanovic *et al.* Plant Biotechnol J. 2008 Oct;6(8):770-81, which is incorporated by reference herein in its entirety.

[0108] As used herein, the term "recombinase fusion protein comprising a protein transduction domain" refers to a fusion protein comprising a site-specific DNA recombinase domain (such as Cre or FLP), a protein transduction domain (*e.g.*, derived from TAT, VP22, FGF or Antp) and a nuclear localization domain. *See*, for example, International Patent Application Publication No. WO 2003076561, which is incorporated by reference herein in its entirety. In one embodiment, the recombinase fusion protein is a "TAT-Cre" fusion protein comprising a Cre DNA recombinase domain fused to the basic membrane

translocation peptide of HIV Tat protein, or cell penetrating fragments thereof. Recombinant TAT-Cre protein is commercially available, *e.g.* from Excellgen (Cat. No.: EG-1001) or EMD-Millipore (Cat. No.: SCR508).

[0109] As used herein, the term "lox site" refers to any art-recognized *lox* recombination site, or variant thereof, which includes the 34 base pair loxP site in bacteriophage P1 as well as a number of variant lox sites including, but not limited to, Lox 511, Lox 5171, Lox 2272, M2, M3, M7, M11, Lox71 and Lox66 (Missirlis *et al.* BMC Genomics 7: 73. 1471-2164, which is incorporated by reference herein in its entirety). Examples of non-cross-reactive compatible pairs of mutant lox sites are disclosed in U.S. Patent Nos. 7,696,335; 7,060,499 and 7,696,335, which are incorporated herein by reference in their entireties.

[0110] As used herein, the term "FRT site" refers to any art-recognized yeast FRT recombination site, or variant thereof, which includes a 34 base pair FRT site, in which a spacer region of 8 base pairs is flanked by two inverted repeats of 13 base pairs. *See*, for example, Jayaram *et al.*, Proc. Natl. Acad. Sci. 82, 5875-5879 (1985); Umlauf S. W. *et al.*, EMBO Journal, 7, 1845-1852 (1988); Lee J. *et al.*, EMBO Journal, 18, 784-791, 1999, which are incorporated by reference herein in their entireties. Examples of non-cross-reactive compatible pairs of mutant FRT sites are disclosed, for example, in U.S. Patent Nos. 7,476,539 and 7,736,897, which are incorporated herein by reference in their entireties.

[0111] As used herein, the term "transduction" or "transduced" refers to introduction of a polynucleotide into a host cell genome using a retrovirus.

[0112] As used herein, the term "stably expressing" refers to expression of a gene from a polynucleotide introduced into a host cell genome.

[0113] As used herein, the term "promoter" refers to a regulatory region of DNA that is in operable linkage with a nucleotide sequence, *e.g.*, a protein coding sequence. The promoter contains specific DNA sequences and response elements that are recognized by proteins known as transcription factors. These factors bind to and recruit RNA polymerase II to the promoter thereby facilitating transcription of the downstream nucleotide sequence. The transcribed mRNA comprising a protein coding sequence is then translated to produce the expressed protein.

[0114] As used herein, the term "operable linkage" refers to the configuration between a promoter sequence and a protein coding region that allows for the transcription of the protein coding region in a host cell.

[0115] As used herein, the term “regulatable” means that the transcription of a protein coding region can be activated in a negative manner, or "down-regulated"; or in a positive manner, or "up-regulated". In one embodiment, the expression of a protein coding sequence can be regulated by an inducible promoter. In another embodiment, the surface expression of a protein coding sequence can be down regulated by site-specific recombination after excision of at least a portion of the protein coding sequence.

[0116] As used herein, the term “mutagenizing conditions” refers to conditions that result in the genetic alteration of a polynucleotide (*e.g.*, a polynucleotide encoding a TCR) in a cell. Non-limiting mutagenizing conditions include cells that have been engineered to express an activation-induced (cytidine) deaminase (AID) gene.

[0117] As used herein, the term “specifically binds to” refers to the ability of a TCR to bind to an antigen (*e.g.*, a peptide-MHC complex) as such binding is understood by one skilled in the art. For example, a TCR that specifically binds to an antigen can bind to other antigens, generally with lower affinity as determined by, *e.g.*, immunoassays, BIAcore®, or other assays known in the art (*see*, *e.g.*, Savage et al., Immunity. 1999, 10(4):485-92, which is incorporated by reference herein in its entirety). In a specific embodiment, a TCR that specifically binds to an antigen binds to the antigen with an association constant (K_a) that is at least 2-fold, 5-fold, 10-fold, 50-fold, 100-fold, 500-fold, 1,000-fold, 5,000-fold, or 10,000-fold greater than the K_a when the TCR binds to another antigen.

[0118] As used herein, the term “high affinity” refers to the ability of a TCR to bind to an antigen (*e.g.*, a peptide-MHC complex) with an association constant (K_a) of at least 10^4 M^{-1}, *e.g.*, at least 10^5 M^{-1}, at least 10^6 M^{-1}, at least 10^7 M^{-1}, at least 10^8 M^{-1}, at least 10^9 M^{-1}, at least 10^{10} M^{-1}, at least 10^{11} M^{-1}, or at least 10^{12} M^{-1}.

[0119] As used herein, the term “high selectivity” refers to a TCR that specifically binds to a desired antigen (*e.g.*, a desired peptide-MHC complex) with an association constant (Ka) that is at least 5-fold, 10-fold, 50-fold, 100-fold, 500-fold, 1,000-fold, 5,000-fold, or 10,000-fold greater than the Ka when the TCR binds to another antigen (*e.g.*, a peptide-MHC complex).

[0120] As used herein, the term “mimetic” refers to a molecule that biologically mimics the action or activity of some other molecule. An example of a mimetic molecule is a phosphomimetic molecule, which mimics the action or activity of a phosphorylated molecule; thus, a phosphopeptide mimetic is a peptide that mimics the action or activity of a phosphorylated peptide by, *e.g.*, replacing a phosphorylated amino acid residue in the

phosphorylated peptide with a phosphomimetic residue. Non-limiting examples of phosphomimetic groups or residues include O-boranophospho, borono, O-dithiophospho, phosphoramide, H-phosphonate, alkylphosphonate, phosphorothiolate, phosphodithiolate and phosphorofluoridate, any of which may be derivatized on Tyr, Thr, Ser, Arg, Lys, or His residues. In certain embodiments, an Asp or Glu residue is used as a phosphomimetic. The Asp or Glu residue can substitute for a phospho-Tyr, phospho-Thr, phospho-Ser, phospho-Arg, phospho-Lys and/or phospho-His residue in a peptide.

[0121] As used herein, a cell "does not express" endogenous TCR if the expression of the endogenous TCR in this cell is not detectable using art-recognized methods, *e.g.*, RT-PCR or flow cytometry analysis. As used herein, a cell "does not express" endogenous CD4 or CD8 if the expression level of the endogenous CD4 or CD8 in this cell is not detectable using art-recognized methods, *e.g.*, RT-PCR or flow cytometry analysis.

[0122] As used herein, the surface expression of a protein is "abrogated" if 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% of the cells that previously expressed the protein on the cell surface no longer express the protein on the cell surface and/or if the surface expression level of the protein on a given cell is reduced by at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99%.

[0123] As used herein, the term "TCR diversity" of a TCR cellular library refers to the number of TCRs expressed on the cell surface that include distinct combinations of TCR α chain amino acid sequence and TCR β chain amino acid sequence.

[0124] In the following, preferred embodiments of a cell, cellular library and/or method of the invention are provided.

II. GENERATION OF HOST CELL LINES SUITABLE FOR TCR DISPLAY

1) Selection of a host cell line for TCR gene library expression and cell surface display

[0125] The TCR display technology disclosed herein employs a host cell line that provides a suitable environment for the expression and proper assembly of functional T cell receptors on the host cell surface. In addition, *e.g.*, to high transduction efficiency and rapid doubling time, it is advantageous for the selected host cell line to faithfully reproduce protein trafficking to cellular compartments where post-translational modification (*e.g.*, glycosylation, palmitoylation, and/or phosphorylation) occurs and where the interaction with chaperone

proteins ensures proper folding of the nascent polypeptides. Accessory membrane proteins then facilitate membrane deposition where CD3 stabilizes the assembly of the expressed TCR on the cell surface. In addition, the peptide-MHC complex recognition and signaling activity of the assembled TCR protein is dependent on co-receptors, surface expressed CD3 as well as adhesion and accessory proteins, for example, CD2 and LFA-1.

[0126] In one embodiment, the host cell is a vertebrate cell, preferably from a mammal, including but not limited to, a mouse or human. In another embodiment, the host cell is derived from a lymphocyte, for example a cell of the T or B lymphocyte lineage. In one embodiment, the host cell can be derived from a T cell progenitor, a developing T cell or a mature T lymphocyte.

[0127] In one embodiment, the host cell of the T cell lineage is isolated from fetal liver, embryonic thymus, adult thymus or a thymoma. A T lymphocyte can also be derived from T cells resulting from the differentiation of a pluripotent stem cell, including but not limited to, embryonic stem cells, induced pluripotent stem (iPS) cells, adult stem cells or $CD34^+$ hematopoietic stem cells.

[0128] In another embodiment, the host cell can originate from a T lymphocyte of a genetically engineered animal, such as a knockout mouse in which the disruption of a regulatory gene leads to the accumulation of T cell progenitors that are blocked at a defined stage of T cell development (Bradley *et al.* Mamm. Genome 2012 Oct;23(9-10):580-6, which is incorporated by reference herein in its entirety). Examples of mutant mice with arrested T cell development include, but are not limited to, SCID mice as well as null mutations of GATA3, RAG1, RAG2, ku80, TCR β, CD3epsilon, Lck/Fyn double knockout, ZAP-70/Syk double knockout, LAT, SLP-76, GADS, VAV1/2/3 triple knock out, TCRα, CD3δ, RAG1/RAG2 double knockout or ZAP-70 (Miosge and Goodnow, *Immunology and Cell Biology* (2005) 83, 318–335, which is incorporated by reference herein in its entirety).

[0129] In one embodiment, a host cell can be, *e.g.*, a $CD8^+$ cytotoxic T cell, a $CD8^+$ regulatory T cell, a $CD4^+$ cytotoxic T cell, a $CD4^+$ helper T (including T_H1, T_H2, T_H3, T_H17, Th9, or T_{FH} subtypes), a $CD4^+$ regulatory T cell, or an invariant NKT (iNKT) cell. In other embodiments, a host cell can be a B cell or an NK cell.

[0130] The host cells listed above that are primary cells can be immortalized into cell lines by retroviral transfection with Telomerase Reverse Transcriptase (Barsov, E.V., Methods Mol Biol. 2009;511:143-58, which is incorporated by reference herein in its entirety) as well

as other techniques that are well known in the art (see, for example, published U.S. Patent Application Nos. 2005/0123521, 2010/0279401 and International PCT application PCT/US1989/001526, or Robek, M.D. and Ratner, L., Virology, 1999, 73: 4856-4865, each of which is incorporated by reference herein in its entirety). Immortalized host cell lines that retain the requisite properties summarized above are then selected based on doubling time, retroviral transduction efficiency and the amount of IL-2 secretion or reporter activity after TCR activation, as disclosed herein.

[0131] In one embodiment, the host cell can be derived from murine pre-B 1624-5 cell lines that possess the intracellular components needed to assemble and express T cell receptors (see, for example, U.S. Patent No. 8,748,353, which is incorporated herein by reference in its entirety).

[0132] In another embodiment, the host cell can be derived from the DO-11.10.7 mouse T cell hybridoma, 58 alpha-beta- (58-/-), which does not express functional T cell receptor alpha/beta chains on the cell surface, but does express CD3 on its cell surface (see, *e.g.*, Letourneur and Malissen Eur J Immunol. 1989;19(12):2269-74, which is incorporated herein by reference in its entirety).

[0133] In another embodiment, the host cell can be derived from the murine thymoma cell line Bw5147 that does not express functional T cell receptor alpha/beta chains.

2) Genetic disruption of endogenous gene expression in the host cell line

[0134] Depending on the particular experimental design of the TCR cellular library screen, the expression of one or more endogenous host cell genes involved in TCR activity and antigen-MHC recognition is abolished to avoid interference with a transduced T cell receptor library and/or co-receptors (*e.g.*, CD4 and/or CD8).

[0135] Gene silencing in the host cell can be achieved using a number of methods that are well known in the art.

[0136] For example, mutant host cell lines that no longer express a cell surface marker on the cell surface can be generated by somatic cell mutagenesis, using, for example, a chemical mutagen, gamma irradiation (*e.g.*, the 58 α-/β- cell line described in Letourneur and Malissen Eur J Immunol. 1989;19(12):2269-74, which is incorporated by reference herein in its entirety) or transposon-mediated insertional mutagenesis (described, for example, in U.S. Patent Nos. 8,592,211 and 7,767,454, which are incorporated by reference herein in their

entireties). The resulting mutant host cell lines can then be screened for defects in the expression of cell surface markers by FACS selection.

[0137] The expression of a targeted endogenous gene can also be abolished by conventional knockout via locus-specific homologous recombination (see, for example, U.S. Patent No. 5,530,178 (CD8 knockout mouse) or U.S. Patent No. 5,698,765 (CD4 knockout mouse), which are incorporated by reference herein in their entireties).

[0138] In addition, the expression of any selected endogenous target gene can be disrupted using a number of genome editing technologies, including, but not limited to, designer zinc fingers (described, for example, in U.S. Patent No. 8,106,255 (general methodology) and U.S. Patent No. 8,956,828 (targeted disruption of T cell receptor genes using engineered zinc finger protein nuclease, which are incorporated by reference herein in their entireties), transcription activator-like effector nucleases (TALENs; described, for example, in U.S. Patent No. 8,614,092, which is incorporated by reference herein in its entirety), homing meganucleases (described, for example, in U.S. Patent No. 7,842,489, which is incorporated by reference herein in its entirety) or CRISPR/cas genome editing systems (described, for example, in U.S. Patent No. 8,697,359, which is incorporated by reference herein in its entirety).

[0139] In one embodiment, endogenous host cell gene expression can also be abolished using site-directed recombination technology, including, but not limited to, a Cre recombinase (described for example in U.S. Patent No. 4,959,317, which is incorporated by reference herein in its entirety) or FLP recombinase (described, for example, in U.S. Patent No. 5,885,836, which is incorporated by reference herein in its entirety).

[0140] In another embodiment, host cell expression of a targeted gene can be silenced by RNA interference (RNAi; described, for example, in U.S. Patent No. 8,329,463, which is incorporated by reference herein in its entirety). For example, sequence-specific small-hairpin RNAs (shRNAs) have been shown to inhibit endogenous TCR genes and/or co-receptors (*e.g.*, TCR-α and TCR-β, see for example, U.S. Patent Application Publication No. 2012/0321667, which is incorporated by reference herein in its entirety). By blocking expression of one or more of these proteins, the T cell is no longer able to produce one or more of the key components of the TCR complex, thereby destabilizing the TCR complex and preventing cell surface expression of a functional TCR.

[0141] In one embodiment, any one of the above-referenced methodologies is implemented to generate a T lymphocyte derived host cell line that does not express endogenous TCR on its cell surface.

[0142] In an embodiment, a T lymphocyte derived host cell line does not express endogenous TCR (i.e., TCRα and TCRβ) or endogenous CD4 on its cell surface (referred to herein as $TCR\alpha^{-}/\beta^{-}$ $CD4^{-}$ host cell).

[0143] In another embodiment, a mouse T lymphocyte derived host cell line does not express mouse TCR (i.e., mTCRα and mTCRβ) or mouse CD4 on its cell surface (referred to herein as $mTCR\alpha^{-}/\beta^{-}$ $mCD4^{-}$ host cell).

[0144] In another embodiment, a T lymphocyte derived host cell line does not express endogenous TCR (i.e., TCRα and TCRβ) or endogenous CD8 (*e.g.*, CD8α and CD8β) on its cell surface (referred to herein as $TCR\alpha^{-}/\beta^{-}$ $CD8^{-}$ host cell).

[0145] In another embodiment, a mouse T lymphocyte derived host cell line does not express mouse TCR (i.e., mTCRα and mTCRβ) or mouse CD8 (*e.g.*, mCD8α and mCD8β) on its cell surface (referred to herein as $mTCR\alpha^{-}/\beta^{-}$ $mCD8^{-}$ host cell).

[0146] In another embodiment, a T lymphocyte derived host cell line does not express endogenous TCR (i.e., TCRα and TCRβ) or endogenous MHC I on its cell surface (referred to herein as $TCR\alpha^{-}/\beta^{-}$ MHC I^{-} host cell).

[0147] In another embodiment, a mouse T lymphocyte derived host cell line does not express mouse TCR (i.e., mTCRα and mTCRβ) or mouse MHC I on its cell surface (referred to herein as $mTCR\alpha^{-}/\beta^{-}$ mMHC I^{-} host cell).

[0148] In another embodiment, a T lymphocyte derived host cell line does not express endogenous TCR (i.e., TCRα and TCRβ) or endogenous MHC II on its cell surface (referred to herein as $TCR\alpha^{-}/\beta^{-}$ MHC II^{-} host cell).

[0149] In another embodiment, a mouse T lymphocyte derived host cell line does not express mouse TCR (i.e., mTCRα and mTCRβ) or mouse MHC II on its cell surface (referred to herein as $mTCR\alpha^{-}/\beta^{-}$ mMHC II^{-} host cell).

3) Generation of a host cell line expressing a recombinant T cell co-receptor

[0150] The transmembrane glycoproteins CD8 and CD4 are characteristic of distinct populations of T lymphocytes whose antigen responses are restricted by Major Histocompatibility Complex class I and II proteins (MHC, also referred to as HLA in man), respectively. CD4 is expressed on the cell surface as a monomer whereas CD8 is expressed as a dimer. CD4 and CD8 act as co-receptors of the T cell receptor by binding to their cognate MHC, thereby facilitating the engagement of the T cell receptor with the MHC bound antigen peptide. $CD4^+$ T cells respond to antigen in association with MHC class II molecules, and $CD8^+$ T cells respond to antigen in association with MHC class I molecules. Studies *in vitro* (Laugel *et al.*, J. Biol. Chem. 2007;282(33):23799-810, which is incorporated by reference herein in its entirety) indicate that the CD8 co-receptor substantially enhances binding efficiency at suboptimal TCR/peptide-MHC I affinities through effects on both association and dissociation rates. The trimolecular interactions among TCR, peptide-MHC I, and CD8 has also been shown to be cooperative (Jiang et al., Immunity. 2011;34(1):13-23). The extent of co-receptor dependence was therefore found to be inversely correlated to TCR/peptide-MHC I affinity.

[0151] To evaluate the affinity of a T cell receptor for a peptide-MHC complex during a TCR cellular library screen, a $TCR\alpha^-/\beta^-$ CD4 or CD8 negative $CD3^+$ host cell can be engineered to express a recombinant CD4 or CD8 co-receptor.

[0152] In an embodiment, a $TCR\alpha^-/\beta^-$ $CD4^-$ $CD3^+$ host cell can be engineered to express a recombinant CD4 (*e.g.*, Human CD4: UniProtKB - P01730; Mouse CD4: UniProtKB - P06332).

[0153] In another embodiment, a $TCR\alpha^-/\beta^-$ $CD4^-$ $CD3^+$ host cell can be engineered to express a recombinant chimeric CD4.

[0154] In another embodiment, a human $TCR\alpha^-/\beta^-$ $CD4^-$ $CD3^+$ host cell can be engineered to express the extracellular region of a recombinant mouse CD4 protein (*e.g.*, residues 27 – 394 of UniProtKB - P06332).

[0155] In another embodiment, a mouse $TCR\alpha^-/\beta^-$ $CD4^-$ $CD3^+$ host cell can be engineered to express the extracellular region of a recombinant human CD4 protein (*e.g.*, residues 26 – 396 of UniProtKB - P01730).

[0156] In another embodiment, a human TCRα^-/β^- $CD4^-$ $CD3^+$ host cell can be engineered to express a recombinant chimeric CD4 protein having the extracellular region of mouse CD4 polypeptide fused in frame with human CD4 transmembrane and cytoplasmic regions.

[0157] In another embodiment, a non-human TCRα^-/β^- $CD4^-$ $CD3^+$ host cell can be engineered to express a recombinant chimeric CD4 protein having the extracellular region of the human CD4 polypeptide fused in frame with a non-human CD4 transmembrane and cytoplasmic regions.

[0158] In another embodiment, a mouse TCRα^-/β^- $CD4^-$ $CD3^+$ host cell can be engineered to express a recombinant chimeric CD4 protein having the extracellular region of the human CD4 polypeptide fused in frame with mouse CD4 transmembrane and cytoplasmic regions.

[0159] The amino acid sequences encoding the human extracellular regions of the chimeric human/mouse CD4 can be at least 90% identical, optionally 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100% identical, to the corresponding amino acid sequences of a wild type human CD4.

[0160] The amino acid sequences encoding the mouse transmembrane region and the cytoplasmic region of the chimeric human/mouse CD4 can be at least 90% identical, optionally 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100% identical, to the corresponding amino acid sequences of a wild type mouse CD4.

[0161] In another embodiment, a TCRα^-/β^- $CD8^-$ $CD3^+$ host cell can be engineered to express recombinant CD8α and CD8β.

[0162] Exemplary mouse CD8α and CD8β amino acid sequences are listed below.

Mouse CD8α polypeptide isoform 1(UniProtKB: P01731-1):

KPQAPELRIFPKKMDAELGQKVDLVCEVLGSVSQGCSWLFQNSSSKLPQPTFVVYMA
SSHNKITWDEKLNSSKLFSAMRDTNNKYVLTLNKFSKENEGYYFCSVISNSVMYFSS
VVPVLQKVNSTTTKPVLRTPSPVHPTGTSQPQRPEDCRPRGSVKGTGLDFACDIYIW
APLAGICVALLLSLIITLI*CYHRSRKRVCKCPRPLVRQEGKPRPSEKIV* (SEQ ID NO.: 21) (bold: extracellular region; normal: transmembrane; italic: cytoplasmic region)

Mouse CD8β polypeptide (UniProtKB: P10300):

LIQTPSSLLVQTNHTAKMSCEVKSISKLTSIYWLRERQDPKDKYFEFLASWSSSKGV
LYGESVDKKRNIILESSDSRRPFLSIMNVKPEDSDFYFCATVGSPKMVFGTGTKLTV

VDVLPTTAPTKKTTLKMKKKKQCPFPHPETQKGLTCSLTTLSLLVVCILLLLAFLGV AVYF*YCVRRRARIHFMKQFHK* (SEQ ID NO.: 22) (bold: extracellular region; normal: transmembrane; italic: cytoplasmic region)

[0163] Exemplary human CD8α and CD8β amino acid sequences are listed below:

Human CD8α polypeptide isoform 1 (UniProtKB: P01732-1):

SQFRVSPLDRTWNLGETVELKCQVLLSNPTSGCSWLFQPRGAAASPTFLLYLSQNKP KAAEGLDTQRFSGKRLGDTFVLTLSDFRRENEGYYFCSALSNSIMYFSHFVPVFLPA KPTTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDFACDIYIWAPLAGT CGVLLLSLVIT*LYCNHRNRRRVCKCPRPVVKSGDKPSLSARYV* (SEQ ID NO.: 23) (bold: extracellular region; normal: transmembrane; italic: cytoplasmic region)

Human CD8β polypeptide isoform 1 (UniProtKB: P10966-1):

LQQTPAYIKVQTNKMVMLSCEAKISLSNMRIYWLRQRQAPSSDSHHEFLALWDSAKG TIHGEEVEQEKIAVFRDASRFILNLTSVKPEDSGIYFCMIVGSPELTFGKGTQLSVV DFLPTTAQPTKKSTLKKRVCRLPRPETQKGPLCSPITLGLLVAGVLVLLVSLGVAI*H LCCRRRRARLRFMKQFYK* (SEQ ID NO.: 24) (bold: extracellular region; normal: transmembrane; italic: cytoplasmic region)

[0164] In another embodiment, a TCRα$^-$/β$^-$ CD8$^-$ CD3$^+$ host cell can be engineered to express recombinant human/mouse chimeric CD8α and human/mouse chimeric CD8β (chiCD8αβ$^+$).

[0165] In another embodiment, a human TCRα$^-$/β$^-$ CD8$^-$ CD3$^+$ host cell can be engineered to express a recombinant chimeric CD8 protein having the extracellular region of mouse CD8α and mouse CD8β.

[0166] In another embodiment, a mouse TCRα$^-$/β$^-$ CD8$^-$ CD3$^+$ host cell can be engineered to express a recombinant chimeric CD8 protein having the extracellular region of human CD8α and human CD8β.

[0167] In other examples, recombinant chimeric human/mouse CD8α and human/mouse CD8β polypeptides can be engineered where a human CD8α extracellular region is fused in frame with a mouse CD8α transmembrane and cytoplasmic regions and a human

CD8β extracellular region is fused in frame with a mouse CD8β transmembrane and cytoplasmic regions.

[0168] The amino acid sequences encoding the human extracellular regions of the chimeric human/mouse CD8α and human/mouse CD8β polypeptides can be at least 90% identical, optionally 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100% identical, to the corresponding amino acid sequences of a wild type human CD8α and CD8β, respectively.

[0169] The amino acid sequences encoding the mouse transmembrane region and the cytoplasmic region of the chimeric human/mouse CD8α and human/mouse CD8β polypeptides can be at least 90% identical, optionally 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100% identical, to the corresponding amino acid sequences of a wild type mouse CD8α and CD8β, respectively.

[0170] Identity of related polypeptides can be readily calculated by known methods. Such methods include, but are not limited to, those described in Computational Molecular Biology, Lesk, A. M., ed., Oxford University Press, New York (1988); Biocomputing: Informatics and Genome Projects, Smith, D. W., ed., Academic Press, New York (1993); Computer Analysis of Sequence Data, Part 1, Griffin, A. M., and Griffin, H. G., eds. Humana Press, New Jersey (1994); Sequence Analysis in Molecular Biology, von Heinje, G., Academic Press (1987); Sequence Analysis Primer, Gribskov, M. and Devereux, J., eds., M. Stockton Press, New York (1991); and Carillo *et al.*, SIAM J. Applied Math, 48:1073 (1988), all the contents of which are incorporated by reference herein in their entireties. Methods of determining the relatedness or percent identity of two polypeptides are designed to give the largest match between the sequences tested. Preferred computer program methods to determine identity between two sequences include, but are not limited to, the GCG program package, including GAP (Devereux *et al.*, Nucl. Acid. Res., 12:387 (1984), which is incorporated by reference herein in its entirety; Genetics Computer Group, University of Wisconsin, Madison, Wis., BLASTP, BLASTN, and FASTA (Altschul *et al.*, J. Mol. Biol., 215:403-410 (1990), which is incorporated by reference herein in its entirety). The BLASTX program is publicly available from the National Center for Biotechnology Information (NCBI) and other sources (BLAST Manual, Altschul *et al.* NCB/NLM/NIH Bethesda, Md. 20894; Altschul *et al.*, supra (1990)). The well-known Smith Waterman algorithm may also be used to determine identity.

[0171] In one embodiment, the chimeric CD8α and CD8β comprise the amino acid sequences of SEQ ID NOs.: 1 and 2, respectively.

[0172] For expression in a TCRα^-/β^- $CD8^-$ $CD3^+$ host cell, the selected CD8α and CD8β coding regions can be cloned downstream of a suitable promoter in an expression cassette, *e.g.*, a retroviral or lentiviral expression cassette.

[0173] For expression in a TCRα^-/β^- $CD4^-$ $CD3^+$ host cell, the selected CD4 coding region can be cloned downstream of a suitable promoter in an expression cassette, *e.g.*, a retroviral or lentiviral expression cassette.

[0174] The promoter can be a constitutive or regulatable promoter depending on the design of the TCR cellular library screen.

[0175] Exemplary embodiments of constitutive promoters include, but are not limited to, viral promoters from polyoma, adenovirus, cytomegalovirus (CMV) and simian virus 40 (SV40). In an exemplary configuration, the protein coding sequences are flanked upstream (i.e., 5') by the human cytomegalovirus IE promoter and downstream (i.e., 3') by an SV40 poly(A) signal. The human cytomegalovirus IE promoter is described in Boshart *et al.* (1985) Cell 41:521 530, which is incorporated by reference herein in its entirety. Other ubiquitously expressing promoters which can be used include the HSV-TK promoter, β-actin promoters and the EF-1α promoter. In certain embodiments, the constitutive promoter can be T cell-specific, *e.g.*, a CD3δ T cell-specific promoter.

[0176] Suitable regulatable expression systems should have, *e.g.*, the following properties: a low level of basal expression in the non-induced state; inducers that do not promote pleiotropic effects; high levels of expression in the induced state; highly specific induction of expression of a candidate nucleic acid of interest; and modulation of the level of induced expression. Examples of regulatable expression systems having such properties include, but are not limited to: a Tet inducible system (see, *e.g.*, Gossen *et al.* (1992) Proc. Natl. Acad. Sci. USA 89:5547 5551; Gossen *et al.* (1995) Science 268:1766 1769, which are incorporated by reference herein in their entireties); a FK506/rapamycin inducible system (see, *e.g.*, Spencer *et al.* (1993) Science 262:1019 1024; Belshaw *et al.* (1996) Proc. Natl. Acad. Sci. USA 93:4604 4607, which are incorporated by reference herein in their entireties); a RU486/mifepristone inducible system (Wang *et al.* Proceedings of the National Academy of Sciences (1994) 91(17):8180-4, which is incorporated by reference herein in its entirety); a cumate inducible system (Mullick *et al.* BMC Biotechnol. 2006 Nov 3;6:43, which is incorporated by reference herein in its entirety) or an ecdysone inducible system (for review, see Rossi *et al.* (1989) Curr. Op. Biotech. 9:451 456, which is incorporated by reference

herein in its entirety). Many constitutive, tissue-specific and inducible promoters are now commercially available from vendors such as Origene, Promega, Invitrogen, System Biosciences and Invivogen.

[0177] In certain embodiments, CD8α and CD8β are cloned into separate expression vectors.

[0178] In other embodiments, the expression of CD8α and CD8β can be linked on the same retroviral expression construct. This can be accomplished by, for example, expressing CD8α and CD8β, or fragments thereof, from one promoter and separating the coding regions for CD8α and CD8β, or fragments thereof, by an IRES element.

[0179] Alternatively, co-expression of the two chains of CD8 can be achieved by cloning two separate expression cassettes into a single retroviral backbone, such that the expression of each individual binding protein chain is separately controlled.

[0180] In another embodiment, CD8α and CD8β chains can be expressed from the same vector by the use of bi-directional promoters that confer transcriptional activities in opposite directions. This option has the advantage that promoter interference does not occur, which may negatively affect expression levels of promoters in close proximity.

[0181] Depending on the screening strategy, a co-receptor expression cassette can be designed to include site-specific recombination sequences flanking the co-receptor's coding region or a portion of the coding region. Thus, upon integration of the transduced co-receptor expression cassette into the TCR negative, co-receptor negative host cell, the nucleotide sequence located between two site-specific recombination sites can be excised by expressing the corresponding site-specific recombinase in the host cell.

[0182] In another embodiment, the co-receptor expression cassette comprises an intervening DNA sequence flanked by site-specific recombination sequences that are inserted to disrupt translation of the co-receptor's coding region. Co-receptor gene translation can then be restored by site-specific recombinase-mediated excision of the intervening DNA sequence located between the site-specific recombination sequences.

[0183] Exemplary site-specific recombination systems that may be used in the present invention include, but are not limited to, the CRE-LOX or FLP/FRT systems (reviewed in Ann. Rev. Biochem. (2006) 75: 567-605 and Gaj *et al.* Biotechnol Bioeng. 2014 Jan; 111(1): 1–15., which are incorporated herein by reference in their entireties).

[0184] Expression of the appropriate site-specific recombinase can be driven by a constitutive, T cell-specific or inducible promoter, as described above.

[0185] In other embodiments, a site-specific recombinase can be delivered to a TCRα^-/β^- host cell as a fusion protein, *e.g.*, a Tat-Cre fusion protein (Joshi *et al.* Genesis (2002) 33:48-54; Peitz *et al.*, (2002) Proc. Natl. Acad. Sci. USA 99:4489-94, the contents of which are incorporated by reference herein in their entireties). A TAT-Cre has been shown to induce greater than 95% recombination efficiency in fibroblasts and murine embryonic stem cells *in vitro*.

[0186] In another embodiment, a Cre recombinase can be linked to the ligand-binding domain of an estrogen receptor (see, *e.g.*, Feil *et al.*, (1996) PNAS 93:10887-10890, which is incorporated herein by reference in its entirety). This allows for the nuclear translocation of Cre in response to treatment of cells with tamoxifen.

[0187] In another embodiment, a TCRα^-/β^- mCD8$^-$ CD3$^+$ host cell is transduced with a co-receptor expression cassette that contains lox recombination sites flanking the mouse transmembrane and cytoplasmic regions of the chimeric human/mouse CD8α coding region and human/mouse CD8β coding regions (chiCD8$\alpha\beta^+$/flox$^+$).

[0188] In another embodiment, a TCRα^-/β^- mCD4$^-$ CD3$^+$ host cell is transduced with a co-receptor expression cassette that contains lox recombination sites flanking the mouse transmembrane and cytoplasmic regions of the chimeric human/mouse CD4 coding region (chiCD4$^+$/flox$^+$).

4) *Generation of a derivative host cell line having one or more reporter constructs for the measurement of antigen-MHC specific TCR signaling*

[0189] The T cell receptor complex is a multi-subunit assembly comprising a variable TCR$\alpha\beta$ or TCR$\gamma\delta$ heterodimer and non-variable signal transduction subunits that contain immunoreceptor tyrosine based activation motifs (ITAMs) within their cytoplasmic regions (*e.g.*, CD3). TCR engagement by a peptide-MHC complex triggers the association of Lck, a Src type tyrosine kinase, with a CD4 or CD8 co-receptor and the phosphorylation of two Tyr within each ITAM motif of the intracellular region of CD3. ZAP70, a Syk type tyrosine kinase, then associates with the ITAM motifs activated by Lck. Thereafter, the signal is transmitted to serine/threonine protein phosphatases (for example, by calcium signaling via calmodulin - calcineurin), serine/threonine protein kinases (for example, PKC, MAPK super

family) and the like via various adaptor proteins (for example, LAT and SLP-76). These events ultimately lead to the activation of three key transcription factors: NFκB, NFAT and AP-1, each of which play a pivotal role in the induction of early cytokine production (*e.g.*, interleukins (such as IL-2, -4, -6, and -17), TNF-α, and IFN-γ), which is required for clonal expansion of the activated T lymphocyte.

[0190] By incorporating a peptide/MHC-TCR responsive reporter construct into a host cell, the interaction between a recombinantly expressed TCR and/or co-receptor with a peptide-MHC complex and the subsequently induced T cell signaling activity can be determined in real-time from a reporter readout.

[0191] Reporter proteins providing readout of signaling activity are well known in the art and include, but are not limited to, cell-surface markers and bioluminescent (luciferase) or fluorescent proteins (*e.g.*, green fluorescent protein (GFP), enhanced green fluorescent protein (EGFP), yellow fluorescent protein (YFP), blue fluorescent protein (BFP) and red fluorescent protein (RFP), recently reviewed by Senutovitch *et al.* Exp. Biol. Med. (2015); 240(6): 795–808, which is incorporated by reference herein in its entirety).

[0192] In certain embodiments, each of the signaling events induced by TCR activation can be individually monitored using promoters having appropriate transcription response elements (TREs), for example, NFκB/Rel, NFAT or AP-1. An array of signal transduction pathway GFP or luciferase reporters are now commercially available from, *e.g.*, Sambio Sciences/Qiagen, Addgene and Invitrogen. Individual TREs, or combinations thereof, can be expressed in host cells.

III. GENERATION OF A CELLULAR TCR DISPLAY LIBRARY

[0193] In order to provide a source of DNA containing TCR α variable region Vα and Jα genes, and TCR β variable region Vβ, Jβ and Dβ genes, poly A^+ RNA is prepared from, for example, human fetal thymus, cord blood, tumor infiltrating lymphocytes (from a patient), a vaccinated patient, such as one vaccinated for human papillomavirus (HPV), Epstein–Barr virus (EBV), or for a cancer, or a HLA-A2 transgenic mouse vaccinated with an antigen of interest.

[0194] cDNAs encoding TCR β chains can be generated from mRNA isolated, for example, from pre-T lymphocytes having completed V β and DJ β rearrangement. These T lymphocytes can be $CD44^{low}$ $CD25^+$ DN3 or DN4 $CD44^-$ $CD25^-$ T lymphocytes.

[0195] cDNAs encoding TCR α chains can be generated, for example, from mRNA isolated from immature double positive T lymphocytes having completed Vα and Jα rearrangement. These lymphocytes can be $CD4^+$ $CD8^+$ $CD3^{low}$ T lymphocytes.

[0196] cDNA is prepared and used as a template for polymerase chain reaction (PCR)-based amplification of the TCR variable regions. The isolated PCR products comprising the TCR α and TCR β variable region open reading frames (ORFs) are then cloned into expression systems that allow the gene product of the various variable region genes to be expressed in a functional form on the cell surface of a host cell that does not express endogenous TCR on the cell surface.

[0197] For the expression of TCRs encoded by separate expression constructs, it is preferable that expression of different polypeptide chains are linked to different selection markers, thereby allowing separate selection for the expression of corresponding expression constructs.

[0198] Selection markers conferring resistance to antibiotics useful for the selection of the transduced or transfected host cells include, *e.g.*, genes for puromycin, neomycin, hygromycin B, mycophenolic acid, histidinol, bleomycin, and phleomycin resistance. Cell surface proteins and fluorescent proteins are also suitable for use as selection markers.

[0199] In one embodiment, cDNA sequences encoding the variable regions of human TCR α or TCR β are amplified by PCR and cloned into a chimeric TCR α or TCR β expression vector backbone.

[0200] Specifically, a TCR α expression cassette contains a promoter, a human TCR signal sequence, a cloning site followed by a nucleotide sequence encoding mouse TCR α non-variable region. Nucleotide sequences encoding human TCR α variable regions are amplified out of the TCR α cDNA library by PCR and cloned into the cloning site of the TCR α expression cassette so as to be in frame with the mouse TCR α non-variable region. The resulting library is then transformed into *E. coli*. and plasmid DNA is introduced into host cells to generate a recombinant chimeric human/mouse TCR α library.

[0201] In a similar manner, a TCR β expression cassette contains a promoter, a human TCR signal sequence, a cloning site followed by a nucleotide sequence encoding mouse TCR β non-variable region. Nucleotide sequences encoding human TCR β variable regions are amplified out of the TCR β cDNA library by PCR and cloned into the cloning site of the TCR

β expression cassette so as to be in frame with the mouse TCR β non-variable region. The resulting library is then transformed into *E. coli*. and plasmid DNA is introduced into host cells to generate a recombinant chimeric human/mouse TCR β library.

[0202] In one embodiment, cDNA sequences encoding the extracellular regions of human TCR α or TCR β are amplified by PCR and cloned into a chimeric TCR α or TCR β expression vector backbone.

[0203] Specifically, a TCR α expression cassette contains a promoter, a human TCR signal sequence, a cloning site followed by a nucleotide sequence encoding mouse TCR α transmembrane and cytoplasmic regions. Nucleotide sequences encoding human TCR α extracellular regions are amplified out of the TCR α cDNA library by PCR and cloned into the cloning site of the TCR α expression cassette so as to be in frame with the mouse TCR α transmembrane and cytoplasmic regions. The resulting library is then transformed into *E. coli*. and plasmid DNA is introduced into host cells to generate a recombinant chimeric human/mouse TCR α library.

[0204] In a similar manner, a TCR β expression cassette contains a promoter, a human TCR signal sequence, a cloning site followed by a nucleotide sequence encoding mouse TCR β transmembrane and cytoplasmic regions. Nucleotide sequences encoding human TCR β extracellular regions are amplified out of the TCR β cDNA library by PCR and cloned into the cloning site of the TCR β expression cassette so as to be in frame with the mouse TCR β transmembrane and cytoplasmic regions. The resulting library is then transformed into *E. coli*. and plasmid DNA is introduced into host cells to generate a recombinant chimeric human/mouse TCR β library.

[0205] In certain embodiments, the signaling region of a co-stimulatory molecule is fused to the C terminal end of a TCR α and/or TCR β cytoplasmic region. The co-stimulatory molecule can be selected from the group consisting of CD27, CD28, 4-1BB, OX40, CD30, CD40, ICOS, lymphocyte function-associated antigen-1 (LFA-1), CD2, CD7, LIGHT, NKG2C, NKG2D, B7-H3, a ligand that specifically binds with CD83, TCR ζ chain or any combination thereof. T

[0206] In one embodiment, a TCRα$^-$/β$^-$ CD8$^-$ CD3$^+$ chiCD8αβ$^+$/flox$^+$ TRE-GFP host cell line is transduced or transfected with the TCR α gene library followed by selection for stable transformants. Expression of TCR α in the transformants can be confirmed by RT-PCR

analysis. The resulting clones are pooled and then transduced or transfected with the TCR β gene library followed by selection of the stable double transformants.

[0207] In other embodiments, a TCRα^-/β^- $CD8^-$ $CD3^+$ chiCD8$\alpha\beta^+$/$flox^+$ TRE-GFP host cell line is first transduced or transfected with the TCR β gene library followed by the TCR α gene library.

[0208] In another embodiment, a TCRα^-/β^- $CD4^-$ $CD3^+$ chi$CD4^+$/$flox^+$ TRE-GFP host cell line is transduced or transfected with the TCR α gene library followed by selection for stable transformants. Expression of TCR α in the transformants can be confirmed by RT-PCR. The resulting clones are pooled and then transduced or transfected with the TCR β gene library followed by selection of the stable double transformants.

[0209] In other embodiments, a TCRα^-/β^- $CD4^-$ $CD3^+$ chi$CD4^+$/$flox^+$ TRE-GFP host cell line is first transduced or transfected with the TCR β gene library followed by the TCR α gene library.

[0210] In certain embodiments, the TCR gene library is introduced into a host cell line by retroviral transduction. Retroviral transduction can be controlled to ensure that the majority of retrovirally transduced cells are genetically modified by only one recombinant retroviral construct, resulting in clonal expression of at least one TCR protein. In one embodiment, the retroviral transduction is controlled by exploiting multiplicity of infection (MOI). The MOI can be 10, 1, 0.5, 0.1 or less. In another embodiment, retroviral transduction is controlled to ensure that after transduction less than 5-6% of cells express the recombinant protein that is encoded by the retroviral expression vector.

[0211] The separate expression of TCR α and TCR β chains from different expression vectors offers the advantage that collections of expression vectors encoding a diverse collection of TCR α chains can randomly be combined with collections of expression vectors encoding a diverse collection of TCR β chains. This TCR α and TCR β chain shuffling can create a large degree of diversity of different binding specificities, even when the total number of TCR α and TCR β chain collections are limited (*e.g.*, 10^4 different TCR α chains, randomly combined with 10^4 different TCR β chains, theoretically result in 10^8 TCRs, each of which may have distinct specificities).

[0212] TCRα^-/β^- $CD8^-$ $CD3^+$ chiCD8$\alpha\beta^+$/$flox^+$ TRE-GFP or TCRα^-/β^- $CD4^-$ $CD3^+$ chi$CD4^+$/$flox^+$ TRE-GFP reporter host cells receiving recombinant complementary TCR α

and TCR β chains express functional TCRs on the cell surface which can then be enriched by fluorescence-activated cell sorting (FACS) or magnetic activated cell sorting (MACS) to generate a highly diverse cellular TCR display library.

IV. SCREENING OF A CELLULAR TCR DISPLAY LIBRARY

[0213] Disclosed herein are methods for the isolation and identification of a T cell receptor (TCR) with a desired characteristic comprising transducing or transfecting host cells that do not express endogenous TCR with a library of expression constructs encoding different recombinant TCRs to generate a recombinant TCR cellular library in which cells express different recombinant TCRs on their cell surface, selecting cells within the cellular library for a desired characteristic in the presence of a desired peptide-MHC complex and isolating and identifying the expression construct encoding the recombinant TCR with the desired characteristic.

[0214] Examples of a desired characteristic include, but are not limited to, high selectivity for the desired peptide-MHC complex, high affinity for the desired peptide-MHC complex, and demonstration of a positive functional readout in the presence of the desired peptide-MHC complex.

1) Screening cellular TCR display libraries in the presence or absence of co-receptor cell surface expression

[0215] Disclosed herein are methods for the isolation and identification of at least one nucleic acid encoding a T cell receptor (TCR) that specifically binds to a desired peptide-MHC complex in the presence and absence of the cell surface expression of a co-receptor, *e.g.*, CD4 or CD8.

[0216] In one embodiment, mouse TCRα^-/β^- co-receptor$^-$ CD3$^+$ host cells expressing a recombinant co-receptor are transduced or transfected with a gene library of recombinant TCR α and TCR β, as disclosed herein. Transformants having TCRα/β surface expression are then selected. Host cells expressing cell surface recombinant TCR proteins that bind to a desired peptide-MHC complex with the desired affinity or selectivity (herein referred to as co-receptor dependent binders) are enriched by FACS or MACS from the non-binding host cell population. Nucleotide sequences encoding TCRs that bind the desired peptide-MHC

complex in the presence of the co-receptor with the desired affinity or selectivity can then be isolated by PCR and sequenced.

[0217] In certain embodiments, the polynucleotide encoding the recombinant co-receptor or a portion of the recombinant co-receptor is flanked by site-specific recombination sites. To identify host cell clones expressing high affinity TCR in the co-receptor dependent binder population, the recombinant co-receptor expression cassette is deleted using a cognate site-specific recombinase as disclosed herein and cells no longer expressing the recombinant co-receptor on their cell surface are enriched. This co-receptor negative cell population is then rescreened for cell surface recombinant TCR proteins that bind to a desired peptide-MHC complex (herein referred to as co-receptor independent binders). Nucleotide sequences encoding TCRs that bind the desired peptide-MHC complex in the absence of the co-receptor can then be isolated by PCR and sequenced. In certain embodiments, a co-receptor independent binder is able to specifically bind to a cognate ligand (*e.g.*, a peptide-MHC complex) in the absence of the co-receptor and/or able to mediate signaling in the presence of a cognate ligand (*e.g.*, a peptide-MHC complex) in the absence of the co-receptor.

[0218] In one embodiment, an activity of a recombinant co-receptor can be modulated. In one embodiment, the cell surface expression of the recombinant co-receptor can be regulated using an inducible promoter. In another embodiment, the cell surface expression of the recombinant co-receptor can be regulated using the Cre/loxP system. The TCR library can then be screened for binding to the desired peptide-MHC complex as a function of decreasing expression of the recombinant co-receptor as described above.

[0219] Any T cell antigen that can be associated with the MHC class I or II protein and presented to T cells may be used to screen a cellular TCR display library. The peptide antigen can be chemically synthesized or derived from a natural source. In one embodiment, the peptide antigen is derived from a tumor cell.

[0220] The methods and compositions disclosed herein are broadly applicable to the expression, screening and identification of TCRs that specifically bind to an antigen of interest. However, any (functional) fragment of a TCR with any naturally occurring or artificially engineered modification may be used. With regard to full-length TCRs, any kind of artificially engineered or designed modifications of TCR binding regions can be exploited, for example, those generated by site-, or region-directed mutagenesis, fusion of naturally

occurring sequences from different TCRs, randomization of CDR sequences, DNA shuffling, and error-prone PCR.

a) CD8 dependent TCR activation

[0221] In one embodiment, the TCRα^-/β^- $CD8^-$ $CD3^+$ chiCD8$\alpha\beta^+$/$flox^+$ TRE-GFP host cell library expressing recombinant TCRs is assayed for binding to any T cell epitope of interest in the context of various MHC class I alleles in the form of specific 9- to 11-mer peptides complexed to MHC tetramers, dimers, or other multimeric complexes either directly or indirectly fluorescently labeled.

[0222] In one embodiment, the cells bearing an appropriate TCR can be captured and enriched by FACS and the α and β chains recovered and characterized.

[0223] In other embodiments, peptide-MHC class I engagement by the cell surface expression of an individual TCR can be determined by the expression of activation marker (*e.g.*, CD69 or CD137), triggering of signaling pathway (*e.g.*, NFAT signaling, IL-2 signaling, activator protein-1 signaling, or NFκB/Rel signaling), production of cytokine (*e.g.*, IL-2, TNF-α, or IFN-γ), calcium flux, cell proliferation (*e.g.*, proliferation of T cells), and activation of reporter system (*e.g.*, IL-2-EGFP reporter system).

[0224] As a co-receptor, CD8 can also bind to the α3 domain of the same MHC class I molecules as the TCR to facilitate TCR signaling. Mutagenesis data indicate that residues 223-229 of the human MHC class I α chain are essential for CD8-MHC class I interaction (Salter *et al.* Nature 345:41-46 1990).

[0225] The role of the CD8-α3 interaction in T cell engagement of a desired peptide-MHC class I complex can be investigated using the TCR cellular library screen disclosed herein.

[0226] In one embodiment, a mouse TCRα^-/β^- $CD8^+$ $CD3^+$ TRE-GFP host cell library expressing recombinant TCRs can be contacted with a desired peptide-human/mouse chimeric MHC class I protein having one or more amino acid mutations at residues 223-229 of the human MHC class I α3 domain. TCR activation is assessed by the reporter readout, i.e., GFP production. GFP^+ cells are then isolated by FACS and the variable region of the recombinant TCR is amplified by PCR and sequenced.

[0227] In another embodiment, the peptide-human/mouse chimeric MHC class I protein having one or more amino acid mutations at residues 223-229 of the human MHC class I α3 domain is expressed on a cell, *e.g.*, an antigen presenting cell.

b) CD8 independent TCR activation

[0228] In another embodiment, the TCRα^-/β^- $CD8^-$ $CD3^+$ chiCD8$\alpha\beta^+$/$flox^+$ TRE-GFP host cell library expressing recombinant TCRs is subjected to a two round screening protocol.

[0229] In the first round, the TCR cell library is screened for peptide-MHC I activation in the presence of the cell surface expression of the chimeric human/mouse CD8. Positive TCR cell clones that engage the target peptide-MHC I complexes in the presence of the chimeric CD8 co-receptor are then identified.

[0230] In the second round, delivery of a Cre recombinase to the identified positive TCR cell clones results in the deletion of the recombinant chimeric CD8 co-receptor and the subsequent removal of the CD8 co-receptor from the cell surface.

[0231] FACS sorted positive TCR cell clones that no longer express chimeric CD8 on their cell surface are again screened for peptide-MHC I activation. Positive TCR cell clones that engage the target peptide-MHC I complexes in the absence of CD8 co-receptor are then identified as high affinity binders.

[0232] In another embodiment, high affinity TCRs are isolated that bind to a desired peptide-MHC I complex having one or more amino acid mutations at residues 223-229 of the MHC I α3 domain.

[0233] In one embodiment, a mouse TCRα^-/β^- $CD8^-$ $CD3^+$ TRE-GFP host cell library expressing recombinant TCRs on the cell surface is contacted with a peptide-chiMHC I protein having one or more amino acid mutations at residues 223-229 of the MHC I α3 domain. TCR activation is assessed by the reporter readout, i.e., GFP production. GFP^+ cells are then isolated by FACS and the variable region of the recombinant TCRs is amplified by PCR and sequenced.

[0234] In another embodiment, the peptide-chiMHC I protein having one or more amino acid mutations at residues 223-229 of the MHC I α3 domain is expressed on a cell, *e.g.*, an antigen presenting cell.

c) CD4 dependent TCR activation

[0235] In one embodiment, the TCRα^-/β^- $CD4^-$ $CD3^+$ chiCD4$^+$/$flox^+$ TRE-GFP host cell library expressing recombinant TCRs is assayed for binding to any T cell epitope of interest

in the context of various MHC class II alleles, either directly or indirectly fluorescently labeled.

[0236] In one embodiment, the cells bearing an appropriate TCR can be captured and enriched by FACS and the α and β chains recovered and characterized.

[0237] In other embodiments, peptide-MHC II engagement by the cell surface expression of an individual TCR can be determined by the expression of activation marker (*e.g.*, CD69 or CD137), triggering of signaling pathway (*e.g.*, NFAT signaling, IL-2 signaling, activator protein-1 signaling, or NFκB/Rel signaling), production of cytokine (*e.g.*, IL-2, TNF-α, or IFN-γ), calcium flux, cell proliferation (*e.g.*, proliferation of T cells), and activation of reporter system (*e.g.*, IL-2-EGFP reporter system).

[0238] In another embodiment, $TCR\alpha^{-}/\beta^{-}$ $CD4^{+}$ $CD3^{+}$ TRE-GFP host cell library expressing recombinant TCRs can be screened for TCR activation after presentation with peptide-chiMHC II protein in which the human β-chain β2 domain comprises one or more mutations that attenuate or abrogate binding of the variant MHC II to CD4 (Rolf Li-Yun KönigHuang & Ronald N. Germain (1992) Nature 356, 796 – 798, which is incorporated herein in its entirety).

[0239] In another embodiment, $TCR\alpha^{-}/\beta^{-}$ $CD4^{+}$ $CD3^{+}$ TRE-GFP host cell library expressing recombinant TCRs can be screened for TCR activation after presentation with a peptide-chiMHCII expressed on the surface of a cell.

d) CD4 independent TCR activation

[0240] In another embodiment, the $TCR\alpha^{-}/\beta^{-}$ $CD4^{-}$ $CD3^{+}$ $chiCD4/flox^{+}$ TRE-GFP host cell library expressing recombinant TCRs is subjected to a two round screening protocol.

[0241] In the first round, the TCR cell library is screened for peptide-MHC II activation in the presence of the cell surface expression of the chimeric human/mouse CD4. Positive TCR cell clones that engage the target peptide-MHC II complexes in the presence of the chimeric CD4 co-receptor are then identified.

[0242] In the second round, delivery of a Cre recombinase to the identified positive TCR cell clones results in the deletion of the recombinant chimeric CD4 co-receptor and the subsequent removal of the CD4 co-receptor from the cell surface.

[0243] FACS sorted positive TCR cell clones that no longer express CD4 on their cell surface are again screened for peptide-MHC II activation. Positive TCR cell clones that

engage the target peptide-MHC II complexes in the absence of CD4 co-receptor are then identified.

e) In vitro evolution of a TCR variable region

[0244] Directed protein evolution is a very powerful tool to generate TCRs for a specific peptide-MHC complex. The process involves engineering or modifying a TCR so that mutants of the TCR exhibit different characteristics (*e.g.*, increased affinity and/or enhanced signaling capability) for the cognate peptide-MHC complex (the original antigen that the wild-type TCR cells was specific for). Thus, the wild-type TCR can be used as a template for producing mutagenized libraries in one or more of the CDRs, and mutants with different characteristics (*e.g.*, higher affinity and/or enhanced signaling capability) can be selected by binding to the cognate peptide-MHC complex.

[0245] In one embodiment, variable regions of TCR α/β can be mutated by somatic hypermutation in the presence of Activation-induced cytidine deaminase (AID), an enzyme that deaminates cytosine base to produce uracil, which is recognized as a thymine. Exemplary methods of using AID for in vitro mutagenesis are disclosed in, for example, U.S. Patent Nos. 8,685,897 and 8,603,950, which are incorporated by reference herein in their entireties). *In vivo* methods of AID mutagenesis are disclosed, for example, in U.S. Patent Application No. 2012/0309011, which is incorporated herein in its entirety.

[0246] Some examples of high affinity include an equilibrium binding constant for a target ligand of between about 10^{-4} M and 10^{-12} M and all individual values and ranges therein. This range encompasses affinities between those reported to be wild type affinities (10^{-4} to 10^{-6} M), and those which have been isolated by directed evolution (about 10^{-12} M).

[0247] Those of skill in the art, through standard mutagenesis techniques, in conjunction with the assays described herein, can obtain altered TCR sequences and test them for particular binding affinity and/or specificity. Useful mutagenesis techniques known in the art include, without limitation, de novo gene synthesis, oligonucleotide-directed mutagenesis, region-specific mutagenesis, linker-scanning mutagenesis, and site-directed mutagenesis by PCR (see, *e.g.*, Sambrook *et al.* (1989) and Ausubel *et al.* (1999)).

[0248] In obtaining variant TCR coding sequences, those of ordinary skill in the art will recognize that TCR-derived proteins may be modified by certain amino acid substitutions, additions, deletions, and post-translational modifications, without loss or reduction of biological activity. In particular, it is well known that conservative amino acid substitutions,

that is, substitution of one amino acid for another amino acid of similar size, charge, polarity and conformation, are unlikely to significantly alter protein function.

[0249] Additional mutations in any region or regions of the variable region may result in a stabilized protein. In one embodiment, one or more additional mutations are in one or more of CDR1, CDR2, HV4, CDR3, FR2, and FR3 of a TCR α chain or β chain. The regions used for mutagenesis can be determined by directed evolution, where crystal structures or molecular models are used to generate regions of the TCR which interact with the ligand of interest (antigen, for example). In other examples, the variable region can be reshaped, by adding or deleting amino acids to engineer a desired interaction between the TCR and the ligand.

2) Screening cellular TCR display libraries in the presence or absence of peptide-variant MHC complexes

[0250] Disclosed herein are methods for the isolation and identification of a T cell receptor (TCR) with a desired characteristic comprising transducing or transfecting host cells that do not express endogenous TCR with a library of expression constructs encoding different recombinant TCRs to generate a recombinant TCR cellular library in which cells express different recombinant TCRs on their cell surface, selecting cells expressing the TCRs for the desired characteristic in the presence of an MHC molecule complexed with a peptide of interest, where the MHC molecule comprises an MHC chain that is a variant of a wild type MHC chain, and where the variant MHC chain binds to a co-receptor with reduced affinity compared to the affinity of the wild type MHC chain for the co-receptor, and isolating and identifying the expression construct encoding the recombinant TCR with the desired characteristic.

[0251] Examples of a desired characteristic include, but are not limited to, high selectivity for the desired peptide-variant MHC complex, high affinity for the desired peptide-variant MHC complex, and demonstration of a positive functional readout in the presence of the desired peptide-variant MHC complex.

a) TCR activation by peptide-variant MHC I complexes

[0252] In one embodiment, a T cell receptor (TCR) with a desired characteristic is isolated and identified by a method comprising transducing or transfecting host cells that do not express endogenous TCR with a library of expression constructs encoding different

recombinant TCRs to generate a recombinant TCR cellular library in which cells express different recombinant TCRs on their cell surface, selecting cells within the cellular library for a desired characteristic in the presence of a desired peptide-variant MHC I complex, where the MHC I molecule comprises an MHC I α chain that is a variant of a wild type MHC I α chain, and where the variant MHC I α chain binds to the CD8 co-receptor with reduced affinity compared to the affinity of the wild type MHC I α chain for the CD8 co-receptor, and isolating and identifying the expression construct encoding the recombinant TCR with the desired characteristic.

[0253] Examples of a desired characteristic include, but are not limited to, a T cell receptor (TCR) having high selectivity or high affinity for the desired peptide-variant MHC complex.

[0254] In one embodiment, recombinant TCRs are selected in the presence of a desired peptide-variant MHC I complex by measuring a functional readout of TCR activation, for example, the activation of one or more reporter systems.

[0255] In one embodiment, the desired peptide-MHC I complex is a cell free peptide-MHC I complex. In one embodiment, the desired peptide-MHC I complex comprises a dimer, a tetramer, or a multimer. In one embodiment, the desired peptide-MHC I complex is expressed on the surface of a cell, *e.g.*, a mammalian cell, a yeast cell, or an insect cell. In one embodiment, the mammalian cell is a T2 cell.

[0256] In one embodiment, the variant MHC I α chain is a variant of HLA-A2 α chain. In one embodiment, the variant MHC I molecule comprises one or more mutations within the α 3 region of the MHC I α chain that attenuates or prevents binding to the CD8 co-receptor. For example, the variant MHC I molecule can have an A245V mutation or a D227K/T228A mutation, numbered according to the mature protein sequence.

[0257] Examples of α3 region mutations are described, for example, in Pittet *et al.* J Immunol. 2003 171(4):1844-9 ; Dutoit *et al.* J Immunol. 2003 170(10):5110-7 and U.S. Patent Publication No. 2004/0146520, which are incorporated by reference herein in their entireties.

[0258] In one embodiment, the desired peptide-variant MHC I complex is a cell free peptide-variant MHC I complex. In one embodiment, the desired peptide-variant MHC I complex comprises a dimer, a tetramer, or a multimer. In one embodiment, the desired peptide-variant MHC I complex is expressed on the surface of a cell, *e.g.*, a mammalian cell, a yeast cell, or an insect cell.

b) TCR activation by peptide-variant MHC II complexes

[0177] In one embodiment, a T cell receptor (TCR) with a desired characteristic is isolated and identified by a method comprising transducing or transfecting host cells that do not express endogenous TCR with a library of expression constructs encoding different recombinant TCRs to generate a recombinant TCR cellular library in which cells express different recombinant TCRs on their cell surface, selecting cells within the cellular library for a desired characteristic in the presence of a desired peptide-variant MHC II complex, where the MHC II molecule comprises an MHC II β chain that is a variant of a wild type MHC II β chain, and where the variant MHC II β chain binds to the CD4 co-receptor with reduced affinity compared to the affinity of the β chain of a wild type MHC II for the CD4 co-receptor, and isolating and identifying the expression construct encoding the recombinant TCR with the desired characteristic.

[0259] In one embodiment, a variant MHC class II β-chain comprises one or mutations in the β2 domain that attenuate or abrogate binding of the variant MHC II to CD4 (Rolf Li-Yun KönigHuang & Ronald N. Germain (1992) Nature 356, 796 – 798, which is incorporated herein in its entirety).

[0260] Examples of a desired characteristic include, but are not limited to, a T cell receptor (TCR) having high selectivity or high affinity for the desired peptide-variant MHC II complex.

[0261] In one embodiment, recombinant TCRs are selected in the presence of a desired peptide-variant MHC II complex by measuring a functional readout of TCR activation, for example, the activation of one or more reporter systems.

[0180] In one embodiment, the desired peptide-variant MHC II complex is a cell free peptide- MHC II complex. In one embodiment, the desired peptide-variant MHC II complex comprises a dimer, a tetramer, or a multimer. In one embodiment, the desired peptide-variant MHC II complex is expressed on the surface of a cell, *e.g.*, a mammalian cell, a yeast cell, or an insect cell. In one embodiment, the mammalian cell is an antigen-presenting cell (APC).

V. EXAMPLES

[0262] Examples have been set forth below for the purpose of illustration and to describe certain specific embodiments of the invention. However, the scope of the claims is not to be in any way limited by the examples set forth herein. Various changes and modifications to the

disclosed embodiments will be apparent to those skilled in the art and such changes and modifications including, without limitation, those relating to the packaging vectors, cell lines and/or methods of the invention may be made without departing from the spirit of the invention and the scope of the appended claims.

[0263] The practice of the invention employs, unless otherwise indicated, conventional molecular biological and immunological techniques within the skill of the art. Such techniques are well known to the skilled worker, and are explained fully in the literature. See, *e.g.*, Bailey, J. E. and Ollis, D. F., Biochemical Engineering Fundamentals, McGraw-Hill Book Company, NY, 1986; Current Protocols in Immunology, John Wiley & Sons, Inc., NY, N.Y. (1991-2015), including all supplements; Current Protocols in Molecular Biology, John Wiley & Sons, Inc., NY, N.Y. (1987-2015), including all supplements; Sambrook, *et al.*, Molecular Cloning: A Laboratory Manual, 2nd Edition, Cold Spring Harbor, N.Y. (1989); and Harlow and Lane, Antibodies, a Laboratory Manual, Cold Spring Harbor, N.Y. (1989), all the contents of which are incorporated by reference herein in their entireties.

Example 1: Generation of a murine T cell line suitable for TCR display

[0264] To develop a T cell line for TCR display, a murine thymoma derived TCR negative mouse CD8 positive cell line ("MTCD8") was used as a starting point. MTCD8 cells were cultured in SF-IMDM medium (Amimed) supplemented with 2% fetal calf serum (FCS, Amimed) and 0.1% β-mercaptoethanol in Erlenmeyer flasks (80 rpm in a Multitron Standard incubator, INFORS HT) or filter cap cell culture flasks (Cellstar) at 37°C under 10% CO_2.

[0265] The cell line MTCD8 was modified as described below to generate a recipient cell line for mammalian TCR display.

1) Generation of MTCD8-derived cell line expressing a human-mouse chimeric CD8

[0266] To facilitate the expression of human TCR in MTCD8 cells, CD8 α and β chains were expressed in MTCD8 cells as chimeric proteins comprising human CD8 extracellular region fused to mouse CD8 transmembrane and intracellular regions. The resulting chimeric CD8α (chiCD8α) and CD8β (chiCD8β) comprise the amino acid sequences of SEQ ID NOs.: 1 and 2, respectively.

[0267] Briefly, chiCD8α and chiCD8β genes were each packaged separately into replication-incompetent retroviral particles. HEK 293 cells (ATCC) were co-transfected with three

different vectors: a pVPack vector encoding gag-pol genes (pVPack vector system, Stratagene), a second vector encoding the PVC 211 env gene, and a retroviral vector encoding chimeric CD8α or CD8β. Transfection was conducted using the FuGENE 6 Transfection Reagent (Roche Applied Science) according to the manufacturer's instructions. The resulting retroviral supernatant was harvested two or three days later and used directly to transduce MTCD8 cells.

[0268] MTCD8 cells expressing chimeric CD8α and CD8β (MTCD8 chiCD8αβ+) were enriched using anti-human CD8α FITC (eBioscience, Cat. No.: 11-0086) and anti-human CD8β APC (BD Biosciences, Cat. No.: 641058) by fluorescence-activated cell sorting (FACS). FACS was performed using BD FACSAria I or II with FACSDiva software (Becton-Dickinson). Each round of FACS enrichment was performed using a calibrated amount of FITC- or APC-labeled antibody per 1.0×10^6 cells for 30 minutes at 4°C. Fc receptor blocker (BD Biosciences, Cat. No.: 553142) was used to eliminate background binding of the Fc region of the detection antibodies to MTCD8 cells. MTCD8 cells that did not express chimeric CD8 were used to detect non-specific binders. All the washes between labeling reactions were conducted by centrifuging cells for 5 minutes at 300 g at 4°C in PBS (PAA) with 2% FCS. After appropriate rounds of bulk enrichment, a population of MTCD8 chiCD8αβ+ cells became detectable by flow cytometry.

2) Depletion of mouse CD8 from MTCD8 cells or derivatives

[0269] MTCD8 cells or MTCD8 cells expressing chimeric CD8 as generated above were sorted by Fluorescence-activated cell sorting (FACS) to enrich cells that did not express mouse CD8α or CD8β. The following antibodies were used: anti-mouse CD8α APC (BD Biosciences, Cat. No.: 561093), anti-mouse CD8β PE (BD Biosciences, Cat. No.: 550798), anti-human CD8α PE (eBioscience, Cat. No.: 12-0086), and anti-human CD8β APC (BD Biosciences, Cat. No.: 641058). FACS was performed using FACSAria I or II with FACSDiva software (Becton-Dickinson). Each round of FACS enrichment was performed using a calibrated amount of PE- or APC-labeled antibody per 1.0×10^6 cells for 30 minutes at 4°C. Fc receptor blocker (BD Biosciences, Cat. No.: 553142) was used to eliminate background binding of the Fc region of the detection antibodies to MTCD8 cells or derivatives. All the washes between labeling reactions were conducted by centrifuging cells for 5 minutes at 300 g at 4°C in PBS (PAA) with 2% FCS.

[0270] After appropriate rounds of bulk enrichment, a population of MTCD8 cells that did not express mouse CD8 became detectable by flow cytometry (MTCD8 mCD8αβ- chiCD8αβ- and MTCD8 mCD8αβ- chiCD8αβ+) and single T cells were sorted in 96-well plates. These single T cells grew into T cell clones in about one week and cells in these clones did not express mouse CD8 on their surface anymore.

[0271] Subsequently, these single cell clones were analyzed by flow cytometry to confirm the lack of the surface expression of mouse CD8α and CD8β and wherever applicable, the positive surface expression of chimeric CD8α and CD8β. The flow cytometry analysis was performed in 96-well plates (Cellstar) on BD FACSCalibur. All the flow cytometer data were analyzed using the FlowJo software (Treestar).

3) Selection of clones based on transduction efficiency, APC background, and signaling capability

[0272] In this example, suitable clones of (i) MTCD8 mCD8αβ- chiCD8αβ+ and (ii) MTCD8 mCD8αβ- chiCD8αβ- were selected that satisfied the following characteristics: (i) high transduction efficiency, (ii) low APC background, and (iii) strong TCR-mediated signaling capability.

[0273] Briefly, MTCD8 mCD8αβ- chiCD8αβ+ cells and MTCD8 mCD8αβ- chiCD8αβ- cells were replica plated and transduced to express the α and β chains of chimeric TCR C58 (MTCD8 mCD8αβ- chiCD8αβ+ chiC58αβ+ and MTCD8 mCD8αβ- chiCD8αβ- chiC58αβ+). C58, which refers to c58c61 in U.S. Patent No. 8,367,804 (herein incorporated by reference in its entirety), is a TCR specific for a peptide derived from NY-ESO-1 in the context of HLA-A*0201. C58 was expressed as a chimeric TCR with human variable regions fused to murine non-variable regions to ensure proper anchoring and interaction with murine CD3 and proper triggering of murine signaling pathways. The α and β chains of chimeric C58 comprise the amino acid sequences of SEQ ID NOs: 7 and 8, respectively. The transduced cells were stained using a calibrated amount of anti-mouse TCR PE (BD Biosciences, Cat. No.: 553172) per 1.0×10^6 cells for 30 minutes at 4°C. Fc receptor blocker (BD Biosciences, Cat. No.: 553142) was used to eliminate background binding of the Fc region of the detection antibody to MTCD8 cell derivatives. To detect non-specific binders, TCR negative MTCD8 cells were used. All the washes were conducted by centrifuging cells for 5 minutes at 300 g at 4°C in PBS (PAA) with 2% FCS. The flow cytometry was performed using a FACSCalibur (Becton Dickinson) and data were analyzed using the FlowJo software

(Treestar). Most clones showed 0-30% positivity for TCR expression after transduction. The clones with high transduction efficiency (20-30% positivity for TCR expression) and low APC background staining were selected (data not shown).

[0274] The MTCD8 mCD8αβ- chiCD8αβ+ chiC58αβ+ and MTCD8 mCD8αβ- chiCD8αβ- chiC58αβ+ cells generated above were activated and tested for IL-2 expression to identify clones with strong TCR-mediated signaling capability. Briefly, the cells were activated with plate-bound anti-CD3 antibody (eBioscience, Cat. No.:16-0032) and soluble anti-CD28 antibody (eBioscience, Cat. No.: 16-0281). The supernatant was harvested and tested for IL-2 expression using a mouse IL-2 ELISA kit (eBioscience, Cat. No.: 88-7024). Figure 1 shows exemplary IL-2 production data of individual clones from one testing. Only clones that showed strong IL-2 production were selected.

[0275] The selected MTCD8 mCD8αβ- chiCD8αβ+ clone was named AK-D10 and the selected MTCD8 mCD8αβ- chiCD8αβ- clone was named LOT-D08.

4) Expression of chimeric DMF4 and DMF5 in AK-D10 cells

[0276] Next, chimeric DMF4 and DMF5, both of which are MART-1 reactive TCRs in the context of HLA-A*0201, were expressed in the AK-D10 cells (MTCD8 mCD8αβ- chiCD8αβ+) generated above. Both DMF4 and DMF5 TCRs are described in U.S. Patent No. 8,088,379 (herein incorporated by reference in its entirety). To ensure proper interaction with murine CD3 and proper triggering of murine signaling pathways, both DMF4 and DMF5 were expressed as chimeric TCRs in which human variable regions were fused to murine non-variable regions. The α and β chains of chimeric DMF4 comprise the amino acid sequences of SEQ ID NOs: 9 and 10, respectively. The α and β chains of chimeric DMF5 comprise the amino acid sequences of SEQ ID NOs: 11 and 12, respectively. Chimeric DMF4 or DMF5 TCR α and β chain genes were each packaged separately into replication-incompetent retroviral particles. HEK 293 cells (ATCC) were co-transfected with three different vectors: a pVPack vector encoding gag-pol genes (pVPack vector system, Stratagene), a second vector encoding the PVC 211 env gene, and a retroviral vector encoding chimeric TCR α or β chains. Transfection was conducted using the FuGENE 6 Transfection Reagent (Roche Applied Science) according to the manufacturer's instructions. The resulting retroviral supernatant was harvested three days later and used directly to transduce AK-D10 cells.

[0277] AK-D10 cells expressing chimeric DMF4 or DMF5 (AK-D10 chiTCRαβ+) were enriched using HLA-A*0201 Mart-1 (ELAGIGILTV) (SEQ ID NO: 30) tetramer PE (MBL, Cat. No.: T01008) and hamster anti-mouse TCR β chain antibody APC (BD Biosciences, Cat. No.: 553174) by fluorescence-activated cell sorting (FACS) using BD FACSAria I or II with FACSDiva software (Becton-Dickinson). Each round of FACS enrichment was performed using a calibrated amount of PE-labeled antigen or APC-labeled antibody per 1.0×10^6 cells for 30 minutes at 4°C. Fc receptor blocker (BD Biosciences, Cat. No.: 553142) was used to eliminate background binding of the Fc region of the detection antibody to AK-D10 cells. TCR negative AK-D10 cells were used to detect non-specific binders. All the washes between labeling reactions were conducted by centrifuging cells for 5 minutes at 300 g at 4°C in PBS (PAA) with 2% FCS.

[0278] After an appropriate number of bulk enrichment steps, a population of AK-D10 cells expressing chimeric DMF4 or DMF5 became detectable by flow cytometry. Figure 2A shows co-staining of PE-labeled HLA-A*0201 Mart-1 (ELAGIGILTV (SEQ ID NO: 30)) tetramer and APC-labeled anti-TCR β chain antibody. Consistent with the fact that DMF5 has higher affinity than DMF4, under similar TCR expression levels, the cells expressing chimeric DMF5 showed stronger binding to the peptide-MHC tetramer than the cells expressing chimeric DMF4 did.

5) Correlation of TCR/peptide-MHC interaction and TCR-mediated signaling strength

[0279] In this example, AK-D10 cells expressing chimeric DMF4 or DMF5 as generated above were activated using peptide-MHC complexes and then tested for IL-2 production. DimerX (BD Biosciences, Cat. No.: 551263) is a HLA-A2:Ig fusion protein consisting of three extracellular major histocompatibility complex (MHC) class I HLA-A2 domains that are fused to the VH regions of mouse IgG_1. DimerX can be loaded with any peptide restricted to HLA-A2. Peptide loading was conducted by incubating DimerX and peptide of interest overnight at 37°C.

[0280] AK-D10 cells expressing chimeric DMF4 or DMF5 on the surface were activated using DimerX loaded with Mart-1 peptide ELAGIGILTV (SEQ ID NO: 30) at different concentrations (2 μg/ml, 1 μg/ml, and 0.5 μg/ml). Briefly, 96-well plates were coated with DimerX/peptide complexes and incubated for 3 hours at 37°C, followed by a wash with PBS. Subsequently, 1×10^5 AK-D10 cells expressing chimeric DMF4 or DMF5 were resuspended in 150 μl of SF-IMDM and added to 96-well plates. After overnight incubation, the

supernatant was tested for IL-2 expression using a mouse IL-2 ELISA kit (eBioscience, Cat. No.: 88-7024). DimerX/peptide complexes induced dose-dependent IL-2 production in AK-D10 cells expressing chimeric DMF4 (Figure 2B) or chimeric DMF5 (Figure 2C). DimerX alone without any peptide was used as a negative control.

[0281] AK-D10 cells expressing chimeric DMF4, DMF5, or C58 were activated using DimerX/peptide complexes in an assay similar to the assay described above and tested for IL-2 and TNFα production using intracellular cytokine staining (ICS). Briefly, five hours before staining, the cells were treated with monensin (eBioscience, Cat. No.: 00-4505). The cells were then fixed and permeabilized with Cytofix-Cytoperm (BD Biosciences, Cat. No.: 554714) for intracellular staining according to the manufacturer's instructions. Flow cytometry staining was conducted using a calibrated amount of anti-mouse IL-2 PE (eBioscience, Cat. No.: 12-7021) or anti-mouse TNFα PE (eBioscience, Cat. No.: 12-7321) per 1.0×10^6 cells for 30 minutes at 4°C. Fc receptor blocker (BD Biosciences, Cat. No.: 553142) was used to eliminate background binding of the Fc regions of the detection antibodies to AK-D10 cells. AK-D10 cells expressing chimeric TCR without DimerX/peptide activation were used as negative controls. All the washes between labeling reactions were conducted by centrifuging cells for 5 minutes at 300 g at 4°C in PBS (PAA) with 2% FCS. Cells were then subjected to flow cytometry analysis. TCR positive cells were gated and the percentage of TCR+ IL-2+ and TCR+ TNFα+ cells was determined using the FlowJo software (Treestar). Consistent with IL-2 production detected by ELISA, the cells expressing chimeric TCR showed positive IL-2 (Figure 2D) and TNFα (Figure 2E) intracellular staining after activation using DimerX/peptide complexes.

Example 2: Generation of a cellular TCR display library

[0282] The screening system described in the present Example is based on retroviral-mediated expression of full-length TCRs in mammalian cells, *e.g.*, the MTCD8 cell or its derivatives. TCR α and β chain libraries are expressed from separate vectors by consecutive transduction. Retroviral transduction is an efficient method for the stable genetic modification of mammalian cells. The number of transduced α or β chains per cell can be regulated by modifying the multiplicity of infection (MOI) of viral titer, such that each cell produces on average, *e.g.*, only one α and/or β chain, one α chain and multiple β chains, one β chain and multiple α chains, or multiple α and β chains. Separate retroviral expression vectors for TCR α or β chains are generated which transcriptionally couple the expression of

a gene of interest (TCR α or β chains) via an internal ribosome entry site (IRES) to a reporter gene. Exemplary reporter genes include, but are not limited to, surface proteins such as human CD6 and CD7, and antibiotic resistance genes such as puromycin resistance genes.

1) Generation of a human TCR gene library of high diversity

[0283] $CD4^+$ or $CD8^+$ T cells separated by MACS (Miltenyi Biotec) or similar methods from leukapheresis materials from human donors were used to generate TCR α or β gene libraries. The source of the cells included healthy adult donors and cord blood. Additional sources can include fetal thymus, tumor infiltrating lymphocytes (TILs) isolated from cancer patients, and PBMCs from vaccinated patients.

[0284] Total mRNA was prepared from isolated T cells and cDNA was synthesized using SMARTer® RACE 5'/3' cDNA Synthesis Kit (Clontech Laboratories, Cat. No.: 634860). Library construction was performed separately per α chain or β chain family in order to achieve the desired high complexity of at least 1.0×10^9 colony-forming units. The variable regions of TCR α or β families were PCR amplified with family-specific primers, digested with restriction enzymes, and ligated into TCR α or β retroviral expression vector. Empty retroviral TCR α and β chain expression constructs contained the coding information for either α or β chain non-variable region and a restriction enzyme-flanked non-coding stuffer sequence upstream of the non-variable regions that can be replaced by cloned variable TCR α and β chain coding regions from human or synthetic origin. In cases where murine host cells were used to display human TCRs, the TCR expressing vectors were constructed to comprise human TCR variable regions fused to murine TCR non-variable regions to ensure proper anchoring and interaction with murine CD3 and proper triggering of murine signaling pathways. Single TCR α and β coding regions from selected TCRs were cloned to generate cells expressing a specific recombinant TCR on the cell surface. Furthermore, this method generated cells displaying diverse repertoires of TCR α and β chains isolated by RT-PCR from human T cell sources using TCR α and β chain specific primers, or generated by gene synthesis.

[0285] *E.coli* DH10B was transformed with the ligated TCR α or β chain library and plated on LB (Amp) agar plates. Bacteria colonies were collected and plasmid DNA was purified. DNA preparations of all TCR α or β families were pooled. The resultant TCR α or β chain library was quality controlled by performing next-generation sequencing (NGS). In NGS analysis, 1-3 μg of purified PCR product was used for sequencing reactions using MiSeq

from Illumina in multiplexed runs. Sequence data obtained were filtered for complete TRAV-, TRAJ-, TRBV-, TRBD-, and TRBJ-reads, and cluster analyses were performed using the CD-HIT set of applications (www.cd-hit.org). Relative representation of TCR α or β families as percentage of total genes sequenced was analyzed.

2) Generation of a human TCR cellular library of high diversity

[0286] The murine thymoma derived TCR negative cell line MTCD8 and its derivatives (*e.g.*, LOT-D08 and AK-D10 cells generated above) were selected as host cells for human TCR cellular libraries based in part on high permissiveness for transduction with ecotropic, MLV-enveloped retroviral particles. MTCD8 cells are deficient for endogenous mouse TCR expression and contain all necessary cellular components for expression, pairing, folding, signaling, and surface display of human TCRs. In addition, these cells grow in suspension and have a very short doubling time of approximately 10-12 hours, thus enabling fast expansion.

[0287] Illustrated in the following examples is the generation of a TCR cellular library in two phases: the TCR β chains were introduced in phase 1, and the TCR α chains were introduced in phase 2.

3) Retroviral particle production

[0288] TCR α and β chain gene libraries were each packaged separately into replication-incompetent retroviral particles. HEK 293 cells (ATCC) were co-transfected with three different vectors: a pVPack vector encoding gag-pol genes (pVPack vector system, Stratagene), a vector encoding the PVC 211 env gene, and a retroviral TCRα-IRES-human CD7 or TCRβ-IRES-human CD6 expression vector. Transfection was performed using FuGENE 6 Transfection Reagent (Roche Applied Science) according to the manufacturer's instructions. The resulting retroviral supernatant was harvested three days later and either used directly or kept frozen at -80°C. Retroviral particle titers of either TCR α or β chain retroviral particle stocks were determined experimentally by performing a test-transduction of MTCD8 cells and measuring human CD7 or human CD6 surface expression on the transduced population of MTCD8 cells. Based on the titration results, a dilution was calculated that yielded a desired average copy number of TCR α and β chains per transduced cell. Roughly, a 5% or less transduction rate gives >90% single copy integration of either the α or β chain. Subsequently, MTCD8 cells or derivatives were spin-transduced with retroviral

supernatant for 3 hours at 30°C. TCR α and β chain gene libraries were transduced separately and sequentially. Transduction was performed in 6-well plates (Cellstar) with 1.5 × 10^8 cells per plate and in Eppendorf centrifuges. Following transduction, cells were transferred to Erlenmeyer flasks, with a cell concentration of 3.0 × 10^6 cells per 1 ml, and allowed to expand for 24 hours.

4) Transducing TCR β chains (phase 1)

[0289] In this example, TCR β chains were selected from a cord blood source (up to 3 x 10^8 different β chains), to: a) be 100% homologous to germline and b) represent all known functional β gene families in a ratio corresponding to their normal distribution in human peripheral blood. The TCRβ-IRES-human CD6 retroviral expression vectors were packaged into replication-deficient retroviral particles, which were then used to transduce MTCD8 cells or derivatives. Successful β chain gene transfer and subsequent expression were evidenced by the presence of human CD6 on the cell surface. After transduction, approximately 6% of the cells expressed human CD6, indicating successful integration and expression of single β chain genes. TCR β chain-expressing cells were isolated by enriching CD6 positive cells using fluorescence-activated cell sorting (FACS). Following sorting, TCR β chain-expressing cells were recovered and expanded for two days. The expanded TCR β chain cellular library was frozen in ready-to-use aliquots. Selection markers other than CD6 can also be used.

5) Transducing TCR α chains (phase 2)

[0290] The TCR α chain gene library was introduced in phase 2. Similar to the TCR β chain constructs, TCR α genes were co-expressed with an internal ribosome entry site (IRES)-coupled human CD7 marker gene. The human CD7 marker allows for titration of the retroviral particle-containing supernatant generated on empty MTCD8 cells, such that less than 5% of the transduced cells express TCR α chains. Under these conditions, approximately 90% of the cells contain a single integrated TCR α gene copy.

[0291] Following transduction of the TCR α-chain library, cells were stained for TCR expression using an anti-mouse TCRβ-PE antibody (BD Biosciences, Cat. No.: 553172) and analyzed by flow cytometry. TCR-expressing cells were then isolated by magnetic-activated cell sorting (MACS) using anti-Biotin Microbeads (Miltenyi Biotec, Cat. No.: 130-090-485). Around 3.0-5.0 x 10^9 live cells were recovered after FACS or MACS from each TCR α chain

transduction. More than 85% of these cells expressed surface TCR. The cells were then recovered and expanded for two days.

6) Quality control of cellular library

[0292] Finally, at the end of the expansion phase, all the cells were pooled and frozen at -80°C in cryobags, each bag containing approximately 1.0×10^9 viable cells.

[0293] The percentage of cells that have integrated one TCR α or one TCR β gene copy per cell was analyzed by amplifying the integrated TCR α or β gene and its adjacent vector regions from genomic DNA from ten single cell clones per transduction, and directly sequencing the PCR product. If the PCR product yielded one single clearly readable TCR α or β gene sequence, it was concluded that there was only one gene copy present in the cell of origin. In the cases where an overlap of at least two different TCR α or β gene sequences appeared after the co-amplified common vector region, it was concluded that there are at least two TCR α or TCR β copies present in that cell. From this analysis, it was possible to determine the percentage of the cells in the TCR cellular library that contained only one TCR α and one TCR β gene copy integrated into the host cell.

7) Generation of a murine TCR gene library of high diversity

[0294] Alternatively, TCR α and β gene libraries can be generated based on TCRs isolated from animals (*e.g.*, HLA-A2 transgenic mice immunized with a target of interest). Murine TCR gene libraries can be constructed similarly as described above for human TCR gene libraries. Subsequently, similar protocols can be followed to generate a cellular library containing cells that do not express TCR endogenously displaying murine TCRs on the cell surface.

Example 3: Screening of TCR cellular library using peptide-MHC complexes

1) Screening protocol

[0295] Recombinant peptide-MHC complexes were purchased or synthesized and quality-checked for function prior to use. The TCR cellular library generated above was screened using labeled (*e.g.*, fluorescent-labeled) peptide-MHC complexes (*e.g.*, MHC tetramers, MHC dextramers, or DimerX loaded with peptides) by Fluorescence-activated cell sorting (FACS) using BD FACSAria I or II with FACSDiva software (Becton-Dickinson).

Fluorescent-labeled anti-TCR antibody was used to visualize TCR expression level. Fc receptor blocker (BD Biosciences, Cat. No.: 553142) was used to eliminate background binding. The TCR cellular library was first screened using a calibrated amount of fluorescent-labeled MHC tetramers or DimerX/peptide complexes per 1.0×10^6 cells for 30 minutes at 4°C. To deplete non-specific binders, the positive hits from the first screening were further incubated with a calibrated amount of MHC tetramers with a negative control peptide or DimerX without a peptide per 1.0×10^6 cells for 15 minutes at 4°C. The cells that bound to MHC tetramers with a negative control peptide or DimerX without a peptide were gated out. All the washes between labeling reactions were conducted by centrifuging cells for 10 minutes at 300 rpm at 4°C in PBS (PAA) with 2% FCS.

[0296] After an appropriate number of bulk enrichment steps, a population of TCR-expressing MTCD8 cells or derivatives that clearly and specifically recognized the antigen of interest became visible in flow cytometry analysis, and single T cells were sorted in 96-well plates. These single T cells grew into T cell clones in about one week.

[0297] Subsequently, these single cell clones were analyzed by flow cytometry to verify that they bound the antigen of interest and did not show non-specific background binding. In addition, the TCRs expressed on the surface of these single cell clones were analyzed for other properties, such as signaling and cross-reactivity towards other peptide-MHC complexes.

[0298] In addition to screening TCR-expressing cells based on their binding to peptide-MHC complexes of interest, TCR-expressing cells can also be screened based on functional readouts (*e.g.*, expression of activation marker, triggering of signaling pathway, production of cytokine, calcium flux, cell proliferation, and activation of reporter assay) in the presence of the peptide-MHC complexes of interest. Hits from the binding screening can be compared with hits from the functional screening to identify TCRs that bind specifically to peptide-MHC complexes of interest and initiate relevant signaling pathways.

[0299] It may be possible to identify TCRs directed against self-antigens in a TCR library derived from T cells from cord blood or fetal thymus, partially because of the presence of a large number of immature T cells. These cells express TCRs against a myriad of self-antigens and have yet to be removed in T cell tolerance checkpoints as part of immune system maturation. With a two-tiered screening approach based on binding to peptide-MHC

complexes of interest and then counter-screening against MHC molecules without peptides or with negative control peptides, non-poly-reactive TCRs are enriched.

[0300] In addition to the natural occurrence of self-reactive TCRs, a second mechanism may contribute to the likelihood of isolating self-reactive TCRs from the TCR library. As described above, when the TCR library is generated, the original cognate TCR α and β pairs as present in the T cells from human donors are separated, and each α or β chain is re-paired with a large number of non-original complementary β chains or α chains, respectively. This strategy leads to the expression of novel TCRs possibly showing altered properties such as different affinity or specificity for self-antigens.

[0301] The highly-diverse TCR library combined with stringent, antigen-specific screening methods allow for the isolation and identification of TCRs targeting human self-antigens or antigens that share high similarity with self-antigens.

2) TCR α and β chain gene recovery

[0302] TCR α and β genes were recovered from individual T cell clones by plate-based PCR amplification: T cells were lysed and PCR for TCR α and β chain variable regions was performed separately, directly on the cell lysate, without prior isolation of genomic DNA. The amplified PCR product encoding either the TCR α or β chain was then cloned as a pool into appropriate expression vectors in a single restriction/ligation step using Type IIS restriction enzymes. In parallel, the PCR product was sequenced: if the T cell clone contained a single copy of α and β chain, reliable sequence information could be obtained at this stage. In cases where the T cell clones contained two or more integrated TCR α and/or β chains, individual chains were cloned into standard vectors and sequenced separately. The resulting ligated plasmid pool was transformed into E. coli, and DNA was recovered by bulk miniprep from the transformed cell-pool (no prior plating). The TCRs identified in this step were further analyzed for binding affinity, specificity, signaling capabilities, and/or other characteristics.

3) Alternative cellular library compositions

[0303] The high efficiency of TCR gene transfer by retroviral transduction of MTCD8 derivatives as described herein allows generation of a variety of different types of cellular libraries customized for specific uses.

4) Multi-copy TCR α and/or β chain libraries for de novo screens

[0304] By varying the multiplicity of infection, the average number of TCR α and β chain genes that are integrated into the genome of recipient MTCD8 cells or derivatives can be controlled. This strategy was used in the following variations.

[0305] To generate a cellular library that encompassed a maximal level of TCR diversity, libraries were constructed to express multiple different TCR α chains and multiple different TCR β chains per cell. Not all of the TCR α and β chains in a given cell would productively pair and thus many would fail to be presented on the surface. Still, by offering multiple options per cell, it was ensured that a much larger percentage of transduced cells expressed at least one functional TCR on the cell surface. This multi-copy library design offers two benefits: first, the library generation process is much more efficient (fewer drop-outs) and second, the TCR diversity in the resulting library may be substantially higher compared to using single-copy libraries. A potential further advantage is that the avidity effect caused by expressing many identical TCRs on the T cell surface is much reduced, which can in theory lead to the identification of higher affinity TCRs.

[0306] Using multi-copy libraries requires a second step to identify which TCR α chains pair with which β chains from a given T cell clone. A process was established as an exemplary approach to recovering α and β pairs: single T cells that demonstrated target-specific binding were sorted, positive T cell clones were pooled together, all TCR α and β chains from these pooled target-specific T cells were recovered using the recovery procedure described above (usually about 100-200 T cell clones), and a second cellular library was constructed by combining the now much smaller set of recovered TCR α and β chains in a single-copy format. A TCR screen was then repeated with this much smaller and more focused cellular library, and TCRs of interest were recovered following steps described above. In combination, these two steps would allow for coverage of much larger TCR diversity in comparison to using a single copy library from the beginning.

5) Guided selection

[0307] Guided selection is a screening process that starts with an existing TCR with desired characteristics (for instance, a mouse TCR, a TCR isolated from a human patient, or a TCR isolated in a de novo screen) and sequentially replacing the individual chains. In an exemplary study, the original TCR β chain was replaced by screening the original TCR α

chain in combination with a TCR β chain library, and then the selected TCR β chains were screened together with a diverse TCR α chain library to find a complementary new human TCR α chain (or vice versa). The high efficiency of the retroviral transduction process in combination with the fact that TCR α and β chain genes are in separate expression libraries allow for rapid generation of new libraries with any TCR composition.

Example 4: CD8-dependent and -independent screening

[0308] CD8 forms a dimer, consisting of a pair of CD8 chains. The most common form of CD8 is composed of a CD8 α chain and a CD8 β chain. The extracellular IgV-like region of CD8 α chain interacts with the α3 portion of the MHC class I molecule. This interaction keeps the TCR of the cytotoxic T cell and the peptide-MHC complex of the target cell bound closely together during antigen-specific activation. The specificity of T cell activation depends on the interaction of peptide-MHC complexes and TCR. Other signals via co-receptors such as CD4 and CD8 or co-stimulatory interactions such as CD28 and CD80/CD86 appear to act as amplifiers that increase the magnitude and/or duration of the TCR signals, and do not act independently. Different TCRs may show different levels of dependence on CD8 for binding and signaling. Generally speaking, TCRs that are CD8-independent tend to have higher-affinity towards peptide-MHC complexes. By enhancing the affinity of an existing TCR, it may be possible to change the T cell activation mediated by this TCR from CD8-dependent to CD8-independent. In addition, CD8-independence is a desired feature for developing soluble TCRs as therapeutics. Therefore, it is of significant interest to be able to interrogate the level of CD8-dependence of a TCR or a library of TCRs by modulating or abrogating the expression of CD8 on the display library/cell clone during library screening and clone characterization. This allows for a direct assessment in the same cell or cell population of the relative influence of CD8 on the binding strength and specificity, and the resulting intracellular signalling events of the interaction between TCR and the MHC-peptide complex.

[0309] Such a screening system can be achieved by using the Cre/loxP system. A Cre recombinase is a tyrosine recombinase enzyme derived from the P1 Bacteriophage. This enzyme uses a topoisomerase I like mechanism to catalyze site specific recombination events between two DNA recognition sites (loxP sites). The 34 base pair (bp) loxP recognition site consists of two 13 bp palindromic sequences, which flank an 8 bp spacer region. DNA

between two loxP sites oriented in the same direction will be excised as a circular loop of DNA.

[0310] In this example, chimeric CD8α and CD8β comprising loxP sites flanking the transmembrane and cytoplasmic regions were expressed in LOT-D08 cells. Upon Cre-mediated recombination, the transmembrane and cytoplasmic regions of chimeric CD8α and CD8β are excised and consequently the host T cells do not express membrane-bound chimeric CD8.

1) Generation of LOT-D08 cells expressing chimeric CD8 flanked by loxP sites

[0311] Chimeric CD8α and CD8β, which comprise human CD8 extracellular region fused to mouse CD8 transmembrane and cytoplasmic regions, were further modified to include two loxP sites flanking the mouse transmembrane and cytoplasmic regions (chiCD8α^{flox} and chiCD8β^{flox}).

[0312] The chiCD8α^{flox} and chiCD8β^{flox} genes were each packaged separately into replication-incompetent retroviral particles. HEK 293 cells (ATCC) were co-transfected with three different vectors: a pVPack vector encoding gag-pol genes (pVPack vector system, Stratagene), a vector encoding the PVC 211 env gene, and a retroviral expression vector encoding chiCD8α^{flox} or chiCD8β^{flox}. Transfection was performed using FuGENE 6 Transfection Reagent (Roche Applied Science) according to the manufacturer's instructions. The resulting retroviral supernatant was harvested three days later and used directly to transduce LOT-D08 cells.

[0313] LOT-D08 cells expressing chiCD8α^{flox} and chiCD8β^{flox} (LOT-D08 chiCD8α^{flox}+ chiCD8β^{flox}+) were enriched using anti-human CD8α FITC (eBioscience, Cat. No.: 11-0086) and anti-human CD8β APC (BD Biosciences, Cat. No.: 641058) by fluorescence-activated cell sorting (FACS). FACS was performed using BD FACSAria I or II with FACSDiva software (Becton-Dickinson). Each round of FACS enrichment was performed using a calibrated amount of FITC- or APC-labeled antibody per 1.0×10^6 cells for 30 minutes at 4°C. Fc receptor blocker (BD Biosciences, Cat. No.: 553142) was used to eliminate background binding of the Fc regions of detection antibodies to LOT-D08 cells. For detection of non-specific binders, LOT-D08 cells were used. All the washes between labeling reactions were conducted by centrifuging cells for 5 minutes at 300 g at 4°C in PBS (PAA) with 2% FCS.

[0314] Subsequently, the LOT-D08 chiCD8α^{flox}+ chiCD8β^{flox}+ cells generated were analyzed by flow cytometry to verify the surface expression of chiCD8α^{flox} and chiCD8β^{flox}. The flow cytometry analysis was performed using a BD FACSAria I or II with FACSDiva software (Becton-Dickinson). All the flow cytometer data were analyzed using the FlowJo software (Treestar).

2) Expressing Cre in LOT-D08 chiCD8α^{flox}+ chiCD8β^{flox}+ cells

[0315] LOT-D08 chiCD8α^{flox}+ chiCD8β^{flox}+ cells generated above were transduced with an expression vector encoding a Cre recombinase. After expression of Cre, the cell clones were analyzed by flow cytometry to verify the lack of surface expression of chimeric CD8.

[0316] The Cre recombinase gene was packaged into replication-incompetent retroviral particles. HEK 293 cells (ATCC) were co-transfected with three different vectors: a pVPack vector encoding gag-pol genes (pVPack vector system, Stratagene), a vector encoding the PVC 211 env gene, and a retroviral vector encoding Cre-IRES-puromycin resistance gene. Transfection was performed using FuGENE 6 Transfection Reagent (Roche Applied Science) according to the manufacturer's instructions. The resulting retroviral supernatant was harvested three days later and either used directly or kept frozen at -80°C. Retroviral particle titers from Cre retroviral particle stocks were determined experimentally by performing a test-transduction of LOT-D08 cells and measuring puromycin resistance of the transduced population of LOT-D08 cells. The Cre recombinase was transduced in 1.5 ml Eppendorf tubes (Eppendorf) with 5×10^5 cells per tube. Following transduction, the cells were transferred to filter cap cell culture flasks (Cellstar), with a cell concentration of 1×10^5 cells per 1 ml, and allowed to expand for 24 hours at 37°C under 10% CO_2 before puromycin was added to start selection.

[0317] LOT-D08 chiCD8α^{flox}+ chiCD8β^{flox}+ cells expressing Cre (LOT-D08 chiCD8α^{flox}+ chiCD8β^{flox}+ Cre+) were analyzed by flow cytometry to confirm that these cells did not express chimeric CD8 on the cell surface using anti-human CD8α and anti-human CD8β antibodies. Fc receptor blocker (BD Biosciences, Cat. No.: 553142) was used to eliminate background binding of the Fc regions of detection antibodies to MTCD8 derivatives. All the washes between labeling reactions were conducted by centrifuging cells for 5 minutes at 300 g at 4°C in PBS (PAA) with 2% FCS. The flow cytometer data were analyzed using the FlowJo software (Treestar). As shown in Figure 3, transduction of the Cre recombinase effectively eliminated the expression of chimeric CD8 on the cell surface.

3) CD8 dependence of TCR binding to peptide-MHC complexes

[0318] Next, the impact of Cre recombinase-mediated CD8 deletion on the TCR/peptide-MHC interaction was examined. The display cells used in this example were LOT-D08 chiCD8α^{flox}+ chiCD8β^{flox}+ cells transduced with a reporter construct comprising an IL-2 promoter and three NFAT binding sites operably linked to a nucleotide sequence encoding EGFP. These cells were named BB8 cells. See Example 5 for more details on the reporter construct. BB8 cells expressing chimeric TCR DMF4 or DMF5 were transduced to express the Cre recombinase as described above. The cells with or without Cre expression were stained using 0.2 μg/ml of iTAg Tetramer/PE-HLA-A*02:01 Mart-1 (ELAGIGILTV) (SEQ ID NO: 30) (MBL, Cat. No.: T01008). TCR expression was examined using anti-mouse TCRβ antibody (BD Biosciences, Cat. No.: 553174). The presence or absence of surface CD8 expression was confirmed using anti-human CD8α-PE (eBioscience, Cat. No.: 12-0086). After staining, cells were washed and analyzed using FACSCalibur (BD Biosciences).

[0319] While Cre expression did not impact surface TCR levels (Figures 4A and 4B), the binding of chimeric DMF4 to Mart-1/HLA-A*0201 tetramer was reduced by 30% when CD8 expression was abolished by the Cre recombinase (Figure 4C). In contrast, the high-affinity chimeric TCR DMF5 exhibited similar binding to tetramer in the presence or absence of CD8 (Figure 4C). This study demonstrates that the display cells expressing loxP-flanked CD8 can be used to interrogate CD8-dependence of TCRs. The tetramer used in this study contains an A245V mutation in the MHC heavy chain α3 domain, which was shown to reduce overall binding strength of MHC to CD8 (*see*, *e.g.*, Bodinier *et al.*, Nat Med. 2000 Jun;6(6):707-10, which is herein incorporated by reference in its entirety). Using wild type tetramer with stronger binding to CD8 may be able to increase sensitivity and allow for detection of even smaller differences between TCR/tetramer binding in the presence or absence of CD8.

4) CD8-dependent and -independent screening using the Cre/loxP system

[0320] LOT-D08 chiCD8α^{flox}+ chiCD8β^{flox}+ cells with or without reporter constructs are used to generate a diverse human TCR cellular library as described above. More specifically, TCR α and β chain gene libraries generated previously are each packaged separately into replication-incompetent retroviral particles and transduced into LOT-D08 chiCD8α^{flox}+ chiCD8β^{flox}+ cells sequentially. The resulting cellular library is quality-checked and then screened using peptide-MHC complexes of interest as described above.

[0321] Further, in order to identify CD8-independent TCRs, the cells that bind to peptide-MHC complexes in the screening above are transduced with a Cre-IRES-puromycin resistance gene construct as described previously. Upon expression of the Cre recombinase, the transmembrane and cytoplasmic regions of chiCD8α and chiCD8β are deleted and chimeric CD8 is no longer expressed on the cell surface.

[0322] After puromycin selection, TCR-expressing LOT-D08 chiCD8α^{flox}+ chiCD8β^{flox}+ Cre+ cells are screened again using a calibrated amount of MHC-peptide complexes of interest followed by TCR α and β chain gene recovery using aforementioned methods.

[0323] With the ability to switch between CD8 positive and CD8 negative systems within a screening campaign, the display cell line LOT-D08 chiCD8α^{flox}+ chiCD8β^{flox}+ provides a versatile system that can be used readily to identify CD8-dependent low-to-intermediate affinity TCRs as well as CD8-independent high affinity TCRs.

5) CD8-dependent and -independent screening using mutated MHC molecules

[0324] Alternatively, when using cells constitutively expressing CD8, CD8-dependent and -independent screenings can be achieved by using wild type MHC class I molecules or MHC class I mutations that affect the CD8/MHC class I interaction. A number of such MHC class I mutants have been identified, including HLA-A2 A245V, which reduces, without completely abolishing, the binding of HLA-A2 to CD8 and HLA-A2 D227K/T228A, which abrogates CD8 binding without affecting the peptide-MHC/TCR interaction. A TCR cellular library can be screened directly against tetramers containing the HLA-A2 A245V mutant or the HLA-A2 D227K/T228A mutant loaded with peptides of interest. Alternatively, a two-step screening approach is employed. In this approach, a TCR cellular library expressing CD8 and TCRs on the cell surface is first screened using tetramers containing wild type HLA-A2 loaded with peptides of interest. The positive hits identified in this first step are then screened against tetramers containing the HLA-A2 A245V mutant or the HLA-A2 D227K/T228A mutant loaded with peptides of interest. CD8-independent high affinity TCRs can be isolated and identified.

Example 5: Screening TCR cellular library using reporter assays

[0325] TCR cellular library can also be screened based on functional outcomes of interactions with peptide-MHC complexes. Possible readouts include expression of activation marker (*e.g.*, CD69 or CD137), triggering of signaling pathway (*e.g.*, NFAT

signaling, IL-2 signaling, activator protein-1 signaling, or NFκB/Rel signaling), calcium fluxing, cell proliferation (*e.g.*, proliferation of T cells), as well as production of cytokine (*e.g.*, IL-2, TNF-α, or IFN-γ).

[0326] Alternatively, a derivative display cell line can be generated to express one or more reporter constructs. Commonly used reporter constructs encode a selectable marker (*e.g.*, fluorescent or luminescent proteins, surface-expressed proteins, or antibiotic resistance markers) under the control of a target promoter (*e.g.*, an IL-2 promoter comprising NFAT binding sites, an NFκB promoter comprising NFκB transcriptional response element, or an activator protein-1 promoter comprising TPA-induced transcriptional response element).

[0327] In this example, a derivative display cell line comprising an IL-2-EGFP reporter construct was generated. After activation of T cells and subsequent activation of the IL-2 signaling cascade, the IL-2 promoter is activated, leading to synthesis of EGFP. The readout, *e.g.*, EGFP expression in this case, can be used to qualitatively or quantitatively measure the strength of IL-2 signaling using, *e.g.*, flow cytometry analysis. The reporter construct was retrovirally transduced into the display cell line AK-D10 generated above.

[0328] Similar reporter constructs were also introduced into other display cells such as LOT-D08 cells and LOT-D08 cells expressing chiCD8α^{flox} and chiCD8β^{flox} generated above.

1) Generation of AK-D10 cells comprising an IL-2 reporter construct

[0329] A study was first conducted to examine the feasibility of using IL-2 production as a functional readout of T cell activation. Briefly, AK-D10 cells or AK-D10 cells expressing the chimeric TCR C58 were stimulated using anti-CD3 antibody or an anti-CD3/anti-CD28 antibody cocktail and then examined for activation by measuring IL-2 production. Anti-CD3 antibody (eBioscience, Cat. No.:16-0032) or anti-CD3 and anti-CD28 (eBioscience, Cat. No.: 16-0281) antibodies were coated on a 96-well plate at a concentration of 5 μg/ml and incubated for 3 hours at 37°C. Subsequently, 1 x 10^5 cells resuspended in 200 μl of SF-IMDM were added to each well and incubated for 24 hours at 37°C and 10% CO_2. IL-2 production in the culture supernatant of activated AK-D10 cells with or without C58 expression was examined using a mouse IL-2 ELISA kit (eBioscience, Cat. No.: 88-7024).

[0330] Under anti-CD3 or anti-CD3/anti-CD28 antibody stimulation, only AK-D10 cells expressing the chimeric TCR C58, but not AK-D10 cells that lacked TCR expression, secreted IL-2 (Figure 5).

[0331] After establishing that IL-2 production could serve as a useful functional readout of T cell activation, AK-D10 cells were transduced to express IL-2-EGFP reporter constructs, which provided a surrogate readout of IL-2 production. Three reporter constructs were generated, in which a minimal IL-2 promoter, an IL-2 promoter comprising three NFAT binding sites, or an IL-2 promoter comprising six NFAT binding sites was operably linked to a nucleotide sequence encoding EGFP (mIL-2-EGFP, IL-2-$(NFAT)_3$-EGFP, and IL-2-$(NFAT)_6$-EGFP, respectively). The reporter constructs comprised a hygromycin B resistance gene as a selection marker. The reporter construct genes were each packaged separately into replication-incompetent retroviral particles. HEK 293 cells (ATCC) were co-transfected with three different vectors: a pVPack vector encoding gag-pol genes (pVPack vector system, Stratagene), a vector encoding the PVC 211 env gene, and a retroviral vector encoding one of the three reporter constructs. Transfection was performed using FuGENE 6 Transfection Reagent (Roche Applied Science) according to the manufacturer's instructions. The resulting retroviral supernatant was harvested three days later and used directly to transduce AK-D10 cells generated above. The AK-D10 cells expressing the reporter constructs were selected using hygromycin B antibiotic at a working concentration of 800 μg/ml. After a selection time of 4 days, single T cells from a population of hygromycin-resistant AK-D10 cells were sorted in 96-well plates. These single T cells grew into T cell clones in about one week. Subsequently, these single cell clones were transduced with a TCR, activated with a cognate peptide-MHC complex or anti-CD3 antibody, and analyzed by flow cytometry to confirm that the reporter gene was turned on as evidenced by EGFP expression.

2) Activation of AK-D10 cells comprising an IL-2 reporter construct using anti-CD3 antibody

[0332] In this example, AK-D10 cells expressing an IL-2-$(NFAT)_3$-EGFP reporter construct and the chimeric TCR DMF4 (AK-D10 IL-2-$(NFAT)_3$-EGFP+ chiDMF4+) were activated in the presence or absence of plate-bound anti-CD3 antibody as described above and then EGFP expression was examined using flow cytometry. All flow cytometry data were analyzed using the FlowJo software (Treestar). AK-D10 cells expressing only the IL-2 reporter construct but not recombinant TCR were used as a negative control.

[0333] Activation of the IL-2 reporter construct, as evidenced by EGFP expression, was only observed in DMF4-expressing cells co-cultured with anti-CD3 antibody (Figure 6A). In the

absence of the anti-CD3 antibody (Figure 6B), or in the absence of TCR expression (Figure 6C), minimal reporter activation was detected.

3) Activation of AK-D10 cells or BB8 cells comprising an IL-2 reporter construct using T2 cells pulsed with peptides

[0334] Next, a series of studies were conducted to examine activation of AK-D10 cells or BB8 cells expressing an IL-2 reporter construct and a recombinant TCR by peptide-pulsed T2 cells.

[0335] In a first study, two NY-ESO-1 specific TCRs were tested: C58, which has been described above, and C259, which is a TCR specific for a peptide derived from NY-ESO-1 in the context of HLA-A*0201. *See*, *e.g.*, Rapoport *et al.*, Nat Med. 2015, 21(8): 914–921; Robbins *et al.*, J Clin Oncol. 2011, 29(7): 917–924; Robbins *et al.*, J Immunol. 2008, 180(9): 6116–6131; and U.S. Patent No. 8,008,438, each of which is herein incorporated by reference in its entirety. C259 was expressed as a chimeric TCR with human variable regions fused to murine non-variable regions to ensure proper anchoring and interaction with murine CD3 and proper triggering of murine signaling pathways. The α and β chains of chimeric C259 comprise the amino acid sequences of SEQ ID NOs: 35 and 36, respectively.

[0336] T2 cells (1.0×10^6 cells in 200 µl PBS) were pulsed with 50 µg/ml NY-ESO-1 antigen peptide SLLMWITQV (SEQ ID NO: 31) (IBA Lifesciences, Cat. No.: 6-7013-901) for 3 hours at 37°C. Next, 5.0×10^4 AK-D10 cells transduced to express an IL-2 reporter construct and chimeric TCR C58 (AK-D10 IL-2-$(NFAT)_3$-EGFP+ chiC58+) or chimeric TCR C259 (AK-D10 IL-2-$(NFAT)_3$-EGFP+ chiC259+) were co-incubated with 5.0×10^4 pulsed T2 cells in 200 µl SF-IMDM media for 16 hours at 37°C and 10% CO_2. As controls, AK-D10 derivatives were also incubated with non-pulsed T2 cells or incubated in the absence of T2 cells. After incubation, cells were stained for TCR expression using anti-mouse TCRβ antibody (BD Biosciences, Cat. No.: 553174). To assess expression of T cell activation markers CD69 and CD137, cells were stained using anti-mouse CD69-BV421 (Biolegend, Cat. No.: 104528) and anti-mouse CD137-APC (eBioscience, Cat. No.: 17-1371). Cells were then subjected to flow cytometry analysis. TCR positive cells were gated and the percentage of TCR+ EGFP+, TCR+ CD69+, and TCR+ CD137+ cells was determined using the FlowJo software (Treestar).

[0337] Co-incubation with T2 cells pulsed with cognate peptides enhanced activation of the IL-2 reporter construct as evidenced by EGFP expression (Figures 7A-7C and Figure 8).

Consistently, co-culture with pulsed T2 cells also upregulated expression of T cell activation markers CD69 and CD137 (Figure 8). Notably, surface TCR expression was down-regulated upon activation (Figures 7A-7C), highlighting that the IL-2 reporter assay developed here may be particularly useful in identifying TCRs with superior signaling capability. A binding based screening, *e.g.*, using MHC tetramers, may be less informative if enhanced tetramer binding is more than offset by down-regulated surface TCR expression.

[0338] In a second study, the sensitivity of the IL-2-EGFP reporter construct was examined using T2 cells pulsed with a dose titration of cognate peptides. Briefly, 22.0 x 10^6 T2 cells were pulsed with a Mart-1 antigen peptide ELAGIGILTV (SEQ ID NO: 30) (P&E, Cat. No.: EP04197) ranging from 0.125 μM to 256 μM for 3 hours at 37°C. For peptide pulsing, a negative control peptide ILLWQPIPV (SEQ ID NO: 34) (Genscript, custom order) was added to maintain a constant total peptide concentration of 512 μM. Subsequently, 5.0 x 10^4 AK-D10 cells transduced to express an IL-2 reporter construct and chimeric TCR DMF4 (AK-D10 IL-2-$(NFAT)_3$-EGFP+ chiDMF4+) or chimeric TCR DMF5 (AK-D10 IL-2-$(NFAT)_3$-EGFP+ chiDMF5+) were co-incubated with 5.0 x 10^4 pulsed T2 cells in 200 μl SF-IMDM media for 12 hours at 37°C and 10% CO_2. Cells were stained for TCR expression using anti-human TCRα/β-APC (BD Biosciences, Cat. No.: 563826) and analyzed by flow cytometry. TCR positive cells were gated and the percentage of TCR+ EGFP+ cells was determined using the FlowJo software (Treestar).

[0339] As shown in Figures 9A and 9B, T2 cells pulsed with cognate peptides activated the IL-2-EGFP reporter construct in a dose-dependent manner.

[0340] A third study looked at how activation of the IL-2-EGFP reporter construct was influenced by factors such as peptide concentrations used to pulse T2 cells, lengths of activation, and the presence or absence of CD8. T2 cells (5.0 x 10^5 cells in 200 μl PBS) were pulsed with 50 μg/ml, 5 μg/ml, or 0.5 μg/ml of a Mart-1 antigen peptide ELAGIGILTV (SEQ ID NO: 30) (P&E, Cat. No.: EP04197) for 3 hours at 37°C. BB8 cells (MTCD8 mCD8αβ-chiCD8α^{flox}+ chiCD8β^{flox}+ IL-2-$(NFAT)_3$-EGFP+) expressing chimeric TCR DMF4 or DMF5 were transduced to express a Cre recombinase or were not transduced. Next, 5.0 x 10^4 BB8 derivatives were co-incubated with 5.0 x 10^4 pulsed T2 cells in 200 μl SF-IMDM media at 37°C and 10% CO_2 over a time course of 0 – 25 hours. Cells were then stained for TCR and CD8 using anti-mouse TCRβ-APC (BD Biosciences, Cat. No.: 553174) and anti-human CD8-PE (BD Biosciences, Cat. No.: 555635) and analyzed by flow cytometry. TCR

positive cells were gated and the percentage of TCR+ EGFP+ cells was determined using the FlowJo software (Treestar).

[0341] As shown in Figures 10A and 10B, activation of the IL-2-EGFP reporter construct is a function of time within the measured duration. The extent of activation increased as T2 cells were pulsed with increasing concentrations of cognate peptides. CD8 staining confirmed that transduction of the Cre recombinase successfully depleted surface CD8 expression (data not shown), which decreased Il-2 signaling activation induced by peptide-pulsed T2 cells.

Example 6: Isolating TCRs from human donors

[0342] In this example, T cells from human subjects were screened for TCRs specific for a target of interest. More specifically, TCRs specific for HPV E6 or E7 derived peptides were isolated from human donors. T cells from humans who have been vaccinated against E6 or E7 can also be used to isolate TCRs specific for E6 or E7 derived peptides.

1) Isolating TCRs from in vitro stimulated human T cells

[0343] Human T cells were isolated from PBMCs using standard methods. In order to identify TCRs specific for HPV E6 or E7 derived peptides, isolated T cells were activated with (i) autologous dendritic cells loaded with a peptide pool PepMix™ HPV 16 (protein E6 or E7) from JPT peptides, (ii) T2 cells loaded with HLA-A2 restricted peptides derived from HPV E6 or E7, or (iii) peptide-MHC complexes (DimerX loaded with E6 or E7 derived peptides). In the latter two cases, the E6-derived peptide KLPQLCTEL (SEQ ID NO: 31) and the E7-derived peptide GTLGIVCPI (SEQ ID NO: 32) were used to load T2 cells and DimerX. Alternatively, isolated T cells can also be activated using antigen-presenting cells transfected with in vitro–transcribed (IVT) RNA encoding the antigens of interest.

[0344] Subsequently, the activated cells were harvested, stained with a fluorescent-conjugated anti-IFNγ antibody (Secretion Assay Kit, Miltenyi Biotec) as well as a T cell specific antibody (anti-CD8 or anti-CD4 antibody), and bulk sorted or single cell sorted for cells that were double positive for IFNγ/CD8 or IFNγ/CD4. Sorting was conducted using a BD FACSAria flow cytometer (BD Biosciences). Alternatively, the activated cells can also be stained using anti-IL-2 antibody together with anti-CD8 or anti-CD4 antibody, and then sorted for IL-2/CD8 or IL-2/CD4 double positive cells.

[0345] Bulk or single cell sorted activated T cells are expanded using, *e.g.*, recombinant IL-2, anti-CD3/anti-CD28 antibodies, or the cognate peptide-MHC complexes. TCR α and β chains are cloned directly from single cell sorted or bulk sorted antigen-specific CD8+ or CD4+ T lymphocytes. The cloned TCRs are directly tested in functional assays or used for library generation.

2) Isolating TCRs from T cells sorted using peptide-MHC complexes of interest

[0346] Human T cells were isolated from PBMCs using standard methods. Isolated T cells were stained using DimerX loaded with an E6-derived peptide KLPQLCTEL (SEQ ID NO: 31) or an E7-derived peptide GTLGIVCPI (SEQ ID NO: 32) and bulk or single cell sorted using a BD FACSAria flow cytometer (BD Biosciences). No activation of T cells was conducted.

[0347] Bulk or single cell sorted T cells are expanded using, *e.g.*, recombinant IL-2, anti-CD3/anti-CD28 antibodies, or the specific peptide-MHC complex used in sorting. TCR α and β chains are cloned directly from single cell sorted or bulk sorted antigen-specific CD8+ or CD4+ T lymphocytes. The cloned TCRs are directly tested in functional assays or used for library generation.

Table 1 Amino acid sequences of exemplary co-receptors, TCRs, MHCs and peptide antigens

SEQ ID NO:	Description (protein sequences unless otherwise indicated)	Sequence
1	Chimeric CD8α	SQFRVSPLDRTWNLGETVELKCQVLLSNPTSGCSWLFQPRGAAASP TFLLYLSQNKPKAAEGLDTQRFSGKRLGDTFVLTLSDFRRENEGYY FCSALSNSIMYFSHFVPVFLPAKPTTTPAPRPPTPAPTIASQPLSL RPEACRPAAGGAVHTRGLDFACDIWAPLAGICVALLLSLIITLICY HRSRKRVCKCPRPLVRQEGKPRPSEKIV
2	Chimeric CD8β	LQQTPAYIKVQTNKMVMLSCEAKISLSNMRIYWLRQRQAPSSDSHH EFLALWDSAKGTIHGEEVEQEKIAVFRDASRFILNLTSVKPEDSGI YFCMIVGSPELTFGKGTQLSVVDFLPTTAQPTKKSTLKKRVCRLPR PETQKGPLCSPLSLLVVCILLLLAFLGVAVYFYCVRRRARIHFMKQ FHK

SEQ ID NO:	Description (protein sequences unless otherwise indicated)	Sequence
3	Chimeric CD8α with loxp sites (nucleotide sequence)	AGCCAGTTCAGAGTGTCCCCCCTGGACAGAACCTGGAACCTGGGCG AGACAGTGGAACTGAAGTGCCAGGTGCTGCTGAGCAACCCCACCAG CGGCTGTAGCTGGCTGTTCCAGCCTAGAGGCGCCGCTGCCAGCCCT ACCTTTCTGCTGTACCTGAGCCAGAACAAGCCCAAGGCCGCCGAGG GCCTGGACACCCAGAGATTCAGCGGCAAGAGACTGGGCGACACCTT CGTGCTGACCCTGAGCGACTTCAGAAGAGAGAACGAGGGCTACTAC TTCTGCAGCGCCCTGAGCAACAGCATCATGTACTTCAGCCACTTCG TGCCCGTGTTTCTGCCCGCCAAGCCTACCACAACCCCTGCCCCTAG ACCTCCTACCCCAGCCCCTACAATCGCCAGCCAGCCTCTGTCTCTG AGGCCCGAGGCTTGTAGACCAGCTGCTGGCGGAGCCGTGCACACAA GAGGCCTGGACTTCGCCTGCGACATAACTTCGTATAGCATACATTA TACGAAGTTATCTATCTGGGCTCCACTGGCCGGCATTTGTGTGGCA CTGCTGCTGTCCCTGATCATCACCCTGATCTGCTACCACAGATCCC GGAAGCGCGTGTGCAAGTGCCCTAGACCACTCGTGCGGCAGGAAGG CAAGCCCAGACCCAGCGAGAAGATTGTCTGAATAACTTCGTATAGC ATACATTATACGAAGTTAT
4	Chimeric CD8β with loxp sites (nucleotide sequence)	CTGCAGCAGACCCCCGCCTACATCAAGGTGCAGACCAACAAGATGG TCATGCTGAGCTGCGAGGCCAAGATCAGCCTGAGCAACATGAGAAT CTACTGGCTGAGACAGAGACAGGCCCCCAGCAGCGACAGCCACCAC GAGTTTCTGGCCCTGTGGGACAGCGCCAAGGGCACCATCCATGGCG AGGAAGTGGAACAGGAAAAGATCGCCGTGTTCAGGGACGCCAGCAG ATTCATCCTGAACCTGACCAGCGTGAAGCCCGAGGACAGCGGAATC TACTTCTGCATGATCGTGGGCAGCCCCGAGCTGACCTTCGGCAAGG GAACACAGCTGAGCGTGGTGGACTTCCTGCCTACCACCGCCCAGCC CACCAAGAAGTCTACCCTGAAGAAAAGAGTGTGCAGACTGCCCAGA CCCGAGACACAGAAAGGCCCTCTGTGCAGCCCTATAACTTCGTATA GCATACATTATACGAAGTTATCTCTGAGCCTGCTGGTCGTGTGCAT CCTCCTGCTGCTGGCTTTTCTGGGCGTGGCCGTGTACTTCTACTGC GTGCGGAGAAGGGCCAGGATCCACTTTATGAAGCAGTTCCACAAAT GAATAACTTCGTATAGCATACATTATACGAAGTTAT
5	Chimeric CD8α with loxp sites (amino acid sequence)	SQFRVSPLDRTWNLGETVELKCQVLLSNPTSGCSWLFQPRGAAASP TFLLYLSQNKPKAAEGLDTQRFSGKRLGDTFVLTLSDFRRENEGYY FCSALSNSIMYFSHFVPVFLPAKPTTTPAPRPPTPAPTIASQPLSL RPEACRPAAGGAVHTRGLDFACDITSYSIHYTKLSIWAPLAGICVA LLLSLIITLICYHRSRKRVCKCPRPLVRQEGKPRPSEKIV
6	Chimeric CD8β with loxp sites (amino acid sequence)	LQQTPAYIKVQTNKMVMLSCEAKISLSNMRIYWLRQRQAPSSDSHH EFLALWDSAKGTIHGEEVEQEKIAVFRDASRFILNLTSVKPEDSGI YFCMIVGSPELTFGKGTQLSVVDFLPTTAQPTKKSTLKKRVCRLPR PETQKGPLCSPITSYSIHYTKLSLSLLVVCILLLLAFLGVAVYFYC VRRRARIHFMKQFHK
7	Chimeric C58 α chain	KQEVTQIPAALSVPEGENLVLNCSFTDSAIYNLQWFRQDPGKGLTS LLLITPWQREQTSGRLNASLDKSSGRSTLYIAASQPGDSATYLCAV RPLLDGTYIPTFGRGTSLIVHPYIQNPEPAVYQLKDPRSQDSTLCL FTDFDSQINVPKTMESGTFITDKTVLDMKAMDSKSNGAIAWSNQTS FTCQDIFKETNATYPSSDVPCDATLTEKSFETDMNLNFQNLSVMGL RILLLKVAGFNLLMTLRLWSS
8	Chimeric C58 β chain	NAGVTQTPKFQVLKTGQSMTLQCAQDMNHEYMSWYRQDPGMGLRLI HYSVAIQTTDQGEVPNGYNVSRSTIEDFPLRLLSAAPSQTSVYFCA SSYLGNTGELFFGEGSRLTVLEDLRNVTPPKVSLFEPSKAEIANKQ KATLVCLARGFFPDHVELSWWVNGKEVHSGVSTDPQAYKESNYSYC LSSRLRVSATFWHNPRNHFRCQVQFHGLSEEDKWPEGSPKPVTQNI SAEAWGRADCGITSASYHQGVLSATILYEILLGKATLYAVLVSGLV LMAMVKKKNS

SEQ ID NO:	Description (protein sequences unless otherwise indicated)	Sequence
9	Chimeric DMF4 α chain	GQQLNQSPQSMFIQEGEDVSMNCTSSSIFNTWLWYKQDPGEGPVLL IALYKAGELTSNGRLTAQFGITRKDSFLNISASIPSDVGIYFCAGG TGNQFYFGTGTSLTVIPYIQNPEPAVYQLKDPRSQDSTLCLFTDFD SQINVPKTMESGTFITDKTVLDMKAMDSKSNGAIAWSNQTSFTCQD IFKETNATYPSSDVPCDATLTEKSFETDMNLNFQNLSVMGLRILLL KVAGFNLLMTLRLWSS
10	Chimeric DMF4 β chain	DAGITQSPRHKVTETGTPVTLRCHQTENHRYMYWYRQDPGHGLRLI HYSYGVKDTDKGEVSDGYSVSRSKTEDFLLTLESATSSQTSVYFCA ISEVGVGQPQHFGDGTRLSILEDLRNVTPPKVSLFEPSKAEIANKQ KATLVCLARGFFPDHVELSWWVNGKEVHSGVSTDPQAYKESNYSYC LSSRLRVSATFWHNPRNHFRCQVQFHGLSEEDKWPEGSPKPVTQNI SAEAWGRADCGITSASYQQGVLSATILYEILLGKATLYAVLVSTLV VMAMVKRKNS
11	Chimeric DMF5 α chain	QKEVEQNSGPLSVPEGAIASLNCTYSDRGSQSFFWYRQYSGKSPEL IMFIYSNGDKEDGRFTAQLNKASQYVSLLIRDSQPSDSATYLCAVN FGGGKLIFGQGTELSVKPYIQNPEPAVYQLKDPRSQDSTLCLFTDF DSQINVPKTMESGTFITDKTVLDMKAMDSKSNGAIAWSNQTSFTCQ DIFKETNATYPSSDVPCDATLTEKSFETDMNLNFQNLSVMGLRILL LKVAGFNLLMTLRLWSS
12	Chimeric DMF5 β chain	IAGITQAPTSQILAAGRRMTLRCTQDMRHNAMYWYRQDLGLGLRLI HYSNTAGTTGKGEVPDGYSVSRANTDDFPLTLASAVPSQTSVYFCA SSLSFGTEAFFGQGTRLTVVEDLRNVTPPKVSLFEPSKAEIANKQK ATLVCLARGFFPDHVELSWWVNGKEVHSGVSTDPQAYKESNYSYCL SSRLRVSATFWHNPRNHFRCQVQFHGLSEEDKWPEGSPKPVTQNIS AEAWGRADCGITSASYQQGVLSATILYEILLGKATLYAVLVSTLVV MAMVKRKNS
13	Mouse TCR α chain non-variable region (A0A075B662)	XIQNPEPAVYQLKDPRSQDSTLCLFTDFDSQINVPKTMESGTFITD KTVLDMKAMDSKSNGAIAWSNQTSFTCQDIFKETNATYPSSDVPCD ATLTEKSFETDMNLNFQNLSVMGLRILLLKVAGFNLLMTLRLWSS X can be any amino acid
14	Mouse TCR α chain transmembrane and cytoplasmic regions	SVMGLRILLLKVAGFNLLMTLRLWSS
15	Mouse TCR β chain non-variable region (Trbc1) (A0A0A6YWV4)	XDLRNVTPPKVSLFEPSKAEIANKQKATLVCLARGFFPDHVELSWW VNGKEVHSGVSTDPQAYKESNYSYCLSSRLRVSATFWHNPRNHFRC QVQFHGLSEEDKWPEGSPKPVTQNISAEAWGRADCGITSASYQQGV LSATILYEILLGKATLYAVLVSTLVVMAMVKRKNS X can be any amino acid
16	Mouse TCR β chain (Trbc1) transmembrane and cytoplasmic regions	EILLGKATLYAVLVSTLVVMAMVKRKNS
17	Mouse TCR β chain non-variable region (Trbc1) (A0A075B5J3)	XDLRNVTPPKVSLFEPSKAEIANKQKATLVCLARGFFPDHVELSWW VNGKEVHSGVSTDPQAYKESNYSYCLSSRLRVSATFWHNPRNHFRC QVQFHGLSEEDKWPEGSPKPVTQNISAEAWGRADCGITSASYQQGV LSATILYEILLGKATLYAVLVSTLVVMAMVRNR X can be any amino acid
18	Mouse TCR β chain (Trbc1) transmembrane and cytoplasmic regions	EILLGKATLYAVLVSTLVVMAMVRNR

SEQ ID NO:	Description (protein sequences unless otherwise indicated)	Sequence
19	Mouse TCR β chain non-variable region (Trbc2) (A0A075B5J4)	XDLRNVTPPKVSLFEPSKAEIANKQKATLVCLARGFFPDHVELSWW VNGKEVHSGVSTDPQAYKESNYSYCLSSRLRVSATFWHNPRNHFRC QVQFHGLSEEDKWPEGSPKPVTQNISAEAWGRADCGITSASYHQGV LSATILYEILLGKATLYAVLVSGLVLMAMVKKKNS X can be any amino acid
20	Mouse TCR β chain (Trbc2) transmembrane and cytoplasmic regions	EILLGKATLYAVLVSGLVLMAMVKKKNS
21	Mouse CD8α isoform 1	KPQAPELRIFPKKMDAELGQKVDLVCEVLGSVSQGCSWLFQNSSSK LPQPTFVVYMASSHNKITWDEKLNSSKLFSAMRDTNNKYVLTLNKF SKENEGYYFCSVISNSVMYFSSVVPVLQKVNSTTTKPVLRTPSPVH PTGTSQPQRPEDCRPRGSVKGTGLDFACDIYIWAPLAGICVALLLS LIITLICYHRSRKRVCKCPRPLVRQEGKPRPSEKIV
22	Mouse CD8β	LIQTPSSLLVQTNHTAKMSCEVKSISKLTSIYWLRERQDPKDKYFE FLASWSSSKGVLYGESVDKKRNIILESSDSRRPFLSIMNVKPEDSD FYFCATVGSPKMVFGTGTKLTVVDVLPTTAPTKKTTLKMKKKKQCP FPHPETQKGLTCSLTTLSLLVVCILLLLAFLGVAVYFYCVRRRARI HFMKQFHK
23	Human CD8α isoform 1	SQFRVSPLDRTWNLGETVELKCQVLLSNPTSGCSWLFQPRGAAASP TFLLYLSQNKPKAAEGLDTQRFSGKRLGDTFVLTLSDFRRENEGYY FCSALSNSIMYFSHFVPVFLPAKPTTTPAPRPPTPAPTIASQPLSL RPEACRPAAGGAVHTRGLDFACDIYIWAPLAGTCGVLLLSLVITLY CNHRNRRRVCKCPRPVVKSGDKPSLSARYV
24	Human CD8β isoform 1	LQQTPAYIKVQTNKMVMLSCEAKISLSNMRIYWLRQRQAPSSDSHH EFLALWDSAKGTIHGEEVEQEKIAVFRDASRFILNLTSVKPEDSGI YFCMIVGSPELTFGKGTQLSVVDFLPTTAQPTKKSTLKKRVCRLPR PETQKGPLCSPITLGLLVAGVLVLLVSLGVAIHLCCRRRRARLRFM KQFYK
25	Chimeric CD8α with loxp sites (amino acid sequence)	SQFRVSPLDRTWNLGETVELKCQVLLSNPTSGCSWLFQPRGAAASP TFLLYLSQNKPKAAEGLDTQRFSGKRLGDTFVLTLSDFRRENEGYY FCSALSNSIMYFSHFVPVFLPAKPTTTPAPRPPTPAPTIASQPLSL RPEACRPAAGGAVHTRGLDFACDITSYSIHYTKLXIWAPLAGICVA LLLSLIITLICYHRSRKRVCKCPRPLVRQEGKPRPSEKIV X is S, F, L, Y, C, or W
26	Chimeric CD8β with loxp sites (amino acid sequence)	LQQTPAYIKVQTNKMVMLSCEAKISLSNMRIYWLRQRQAPSSDSHH EFLALWDSAKGTIHGEEVEQEKIAVFRDASRFILNLTSVKPEDSGI YFCMIVGSPELTFGKGTQLSVVDFLPTTAQPTKKSTLKKRVCRLPR PETQKGPLCSPITSYSIHYTKLXLSLLVVCILLLLAFLGVAVYFYC VRRRARIHFMKQFHK X is S, F, L, Y, C, or W
27	HLA-A2 α chain	GSHSMRYFFTSVSRPGRGEPRFIAVGYVDDTQFVRFDSDAASQRME PRAPWIEQEGPEYWDGETRKVKAHSQTHRVDLGTLRGYYNQSEAGS HTVQRMYGCDVGSDWRFLRGYHQYAYDGKDYIALKEDLRSWTAADM AAQTTKHKWEAAHVAEQLRAYLEGTCVEWLRRYLENGKETLQRTDA PKTHMTHHAVSDHEATLRCWALSFYPAEITLTWQRDGEDQTQDTEL VETRPAGDGTFQKWAAVVVPSGQEQRYTCHVQHEGLPKPLTLRWEP SSQPTIPIVGIIAGLVLFGAVITGAVVAAVMWRRKSSDRKGGSYSQ AASSDSAQGSDVSLTACKV

SEQ ID NO:	Description (protein sequences unless otherwise indicated)	Sequence
28	HLA-A2 α chain A245V	GSHSMRYFFTSVSRPGRGEPRFIAVGYVDDTQFVRFDSDAASQRME PRAPWIEQEGPEYWDGETRKVKAHSQTHRVDLGTLRGYYNQSEAGS HTVQRMYGCDVGSDWRFLRGYHQYAYDGKDYIALKEDLRSWTAADM AAQTTKHKWEAAHVAEQLRAYLEGTCVEWLRRYLENGKETLQRTDA PKTHMTHHAVSDHEATLRCWALSFYPAEITLTWQRDGEDQTQDTEL VETRPAGDGTFQKWVAVVVPSGQEQRYTCHVQHEGLPKPLTLRWEP SSQPTIPIVGIIAGLVLFGAVITGAVVAAVMWRRKSSDRKGGSYSQ AASSDSAQGSDVSLTACKV
29	HLA-A2 α chain D227K/T228A	GSHSMRYFFTSVSRPGRGEPRFIAVGYVDDTQFVRFDSDAASQRME PRAPWIEQEGPEYWDGETRKVKAHSQTHRVDLGTLRGYYNQSEAGS HTVQRMYGCDVGSDWRFLRGYHQYAYDGKDYIALKEDLRSWTAADM AAQTTKHKWEAAHVAEQLRAYLEGTCVEWLRRYLENGKETLQRTDA PKTHMTHHAVSDHEATLRCWALSFYPAEITLTWQRDGEDQTQKAEL VETRPAGDGTFQKWAAVVVPSGQEQRYTCHVQHEGLPKPLTLRWEP SSQPTIPIVGIIAGLVLFGAVITGAVVAAVMWRRKSSDRKGGSYSQ AASSDSAQGSDVSLTACKV
30	Mart-1 peptide	ELAGIGILTV
31	E6 peptide	KLPQLCTEL
32	E7 peptide	GTLGIVCPI
33	NY-ESO-1 peptide	SLLMWITQV
34	peptide	ILLWQPIPV
35	Chimeric C259 α chain	QEVTQIPAALSVPEGENLVLNCSFTDSAIYNLQWFRQDPGKGLTS LLLIPFWQREQTSGRLNASLDKSSGRSTLYIAASQPGDSATYLCA VRPLYGGSYIPTFGRGTSLIVHPYIQNPEPAVYQLKDPRSQDSTL CLFTDFDSQINVPKTMESGTFITDKTVLDMKAMDSKSNGAIAWSN QTSFTCQDIFKETNATYPSSDVPCDATLTEKSFETDMNLNFQNLS VMGLRILLLKVAGFNLLMTLRLWSS
36	Chimeric C259 β chain	AGVTQTPKFQVLKTGQSMTLQCAQDMNHEYMSWYRQDPGMGLRLI HYSVSVGMTDQGEVPNGYNVSRSTTEDFPLRLLSAAPSQTSVYFC ASSYVGNTGELFFGEGSRLTVLEDLRNVTPPKVSLFEPSKAEIAN KQKATLVCLARGFFPDHVELSWWVNGKEVHSGVSTDPQAYKESNY SYCLSSRLRVSATFWHNPRNHFRCQVQFHGLSEEDKWPEGSPKPV TQNISAEAWGRADCGITSASYQQGVLSATILYEILLGKATLYAVL VSTLVVMAMVKRKNS

[0348] The invention is not to be limited in scope by the specific embodiments described herein. Indeed, various modifications of the invention in addition to those described will become apparent to those skilled in the art from the foregoing description and accompanying figures. Such modifications are intended to fall within the scope of the appended claims.

[0349] Any patent, patent application, publication, or other disclosure material identified in the specification is hereby incorporated by reference in its entirety and for all purposes to the same extent as if each such individual reference (*e.g.*, patent, patent application, publication, or other disclosure material) was specifically and individually indicated to be incorporated by reference in its entirety for all purposes. Any material, or portion thereof, that is said to be incorporated by reference herein, but which conflicts with existing definitions, statements, or

other disclosure material set forth herein, is only incorporated by reference to the extent that no conflict arises between that incorporated by reference material and the present disclosure material.

[0350] Other embodiments are within the following claims.

CLAIMS

What is claimed is:

1. An isolated cell derived from a lymphocyte, the cell comprising a first polynucleotide encoding a recombinant co-receptor, or a first chain thereof, wherein the cell expresses CD3 but does not express endogenous T cell receptor (TCR) on the cell surface.

2. The cell of claim 1, wherein the CD3 is endogenous CD3.

3. The cell of claim 1, wherein the CD3 is recombinant CD3.

4. An isolated cell derived from a lymphocyte, the cell comprising a first polynucleotide encoding a recombinant co-receptor, or a first chain thereof, wherein the lymphocyte is an invariant NKT (iNKT) cell or a diverse NKT (dNKT) cell.

5. The cell of any one of claims 1-4, wherein the cell expresses the recombinant co-receptor, or a first chain thereof, on the cell surface.

6. An isolated cell derived from a lymphocyte, the cell comprising a first polynucleotide encoding a recombinant co-receptor, or a first chain thereof, wherein the cell is capable of expressing the recombinant co-receptor, or a first chain thereof, on the cell surface in a regulatable manner.

7. The cell of any one of claims 1-6, wherein the cell expresses the recombinant co-receptor, or a first chain thereof, on the cell surface in a regulatable manner.

8. The cell of any one of claims 1-7, wherein the cell does not express an endogenous co-receptor on the cell surface.

9. The cell of claim 8, wherein the endogenous co-receptor is CD8 or CD4.

10. The cell of any one of claims 1-9, wherein the recombinant co-receptor, or a first chain thereof, comprises an extracellular region, a transmembrane region, and a cytoplasmic

region, wherein the extracellular region is linked to the transmembrane region, and the transmembrane region is linked to the cytoplasmic region.

11. The cell of claim 10, wherein the amino acid sequence of the cytoplasmic region of the recombinant co-receptor, or a first chain thereof, is at least 90% identical, optionally 91, 92, 93, 94, 95, 96, 97, 98, 99, or 100% identical, to the amino acid sequence of a corresponding cytoplasmic region of a naturally occurring co-receptor, or a first chain thereof, from the same species as the lymphocyte.

12. The cell of claim 10, wherein the amino acid sequence of the transmembrane region and cytoplasmic region together of the recombinant co-receptor, or a first chain thereof, is at least 90% identical, optionally 91, 92, 93, 94, 95, 96, 97, 98, 99, or 100% identical, to the amino acid sequence of a corresponding transmembrane region and cytoplasmic region together of a naturally occurring co-receptor, or a first chain thereof, from the same species as the lymphocyte.

13. The cell of any one of claims 10-12, wherein the amino acid sequence of the extracellular region of the recombinant co-receptor, or a first chain thereof, is at least 90% identical, optionally 91, 92, 93, 94, 95, 96, 97, 98, 99, or 100% identical, to the amino acid sequence of a corresponding extracellular region of a naturally occurring co-receptor, or a first chain thereof, from a different species than the lymphocyte.

14. The cell of claim 13, wherein the naturally occurring co-receptor, or a first chain thereof, from a different species than the lymphocyte is human.

15. The cell of any one of claims 1-14, wherein the recombinant co-receptor is CD8 or CD4.

16. The cell of any one of claims 1-15, further comprising a second polynucleotide encoding a second chain of the recombinant co-receptor, wherein the second chain comprises an extracellular region, a transmembrane region, and a cytoplasmic region, and wherein the extracellular region of the second chain is linked to the transmembrane region of the second chain, and the transmembrane region of the second chain is linked to the cytoplasmic region of the second chain.

17. The cell of claim 16, wherein the amino acid sequence of the cytoplasmic region of the second chain is at least 90% identical, optionally 91, 92, 93, 94, 95, 96, 97, 98, 99, or 100% identical, to the amino acid sequence of a corresponding cytoplasmic region of a naturally occurring co-receptor chain from the same species as the lymphocyte.

18. The cell of claim 16, wherein the amino acid sequence of the transmembrane region and cytoplasmic region together of the second chain is at least 90% identical, optionally 91, 92, 93, 94, 95, 96, 97, 98, 99, or 100% identical, to the amino acid sequence of a corresponding transmembrane region and cytoplasmic region together of a naturally occurring co-receptor chain from the same species as the lymphocyte.

19. The cell of any one of claims 16-18, wherein the amino acid sequence of the extracellular region of the second chain is at least 90% identical, optionally 91, 92, 93, 94, 95, 96, 97, 98, 99, or 100% identical, to the amino acid sequence of a corresponding extracellular region of a naturally occurring co-receptor chain from a different species than the lymphocyte.

20. The cell of claim 19, wherein the naturally occurring co-receptor chain from a different species than the lymphocyte is human.

21. The cell of any one of claims 16-20, wherein the recombinant co-receptor is CD8.

22. The cell of claim 21, wherein the cytoplasmic region of the first chain of the recombinant co-receptor comprises the amino acid sequence of residues 183-212 of SEQ ID NO: 1.

23. The cell of claim 21, wherein the transmembrane region and the cytoplasmic region of the first chain of the recombinant co-receptor together comprise the amino acid sequence of residues 162-212 of SEQ ID NO: 1.

24. The cell of any one of claims 21-23, wherein the extracellular region of the first chain of the recombinant co-receptor comprises the amino acid sequence of residues 1-161 of SEQ ID NO: 1.

25. The cell of claim 21, wherein the cytoplasmic region of the second chain of the recombinant co-receptor comprises the amino acid sequence of residues 171-187 of SEQ ID NO: 2.

26. The cell of claim 21, wherein the transmembrane region and the cytoplasmic region of the second chain of the recombinant co-receptor together comprise the amino acid sequence of residues 150-187 of SEQ ID NO: 2.

27. The cell of any one of claims 21, 25, and 26, wherein the extracellular region of the second chain of the recombinant co-receptor comprises the amino acid sequence of residues 1-149 of SEQ ID NO: 2.

28. The cell of claim 21, wherein the recombinant co-receptor is a chimeric CD8 comprising:

a) a chimeric CD8α chain comprising an extracellular region of human CD8α and transmembrane and cytoplasmic regions of mouse CD8α, and

b) a chimeric CD8β chain comprising an extracellular region of human CD8β and transmembrane and cytoplasmic regions of mouse CD8β.

29. An isolated cell derived from a mouse lymphocyte, the cell comprising:

a) a first polynucleotide encoding a chimeric CD8α chain comprising an extracellular region of human CD8α and transmembrane and cytoplasmic regions of mouse CD8α, and

b) a second polynucleotide encoding a chimeric CD8β chain comprising an extracellular region of human CD8β and transmembrane and cytoplasmic regions of mouse CD8β,

wherein the cell expresses CD3 but does not express endogenous TCR on the cell surface.

30. The cell of claim 28 or 29, wherein the chimeric CD8α chain comprises the amino acid sequence of SEQ ID NO: 1, and the chimeric CD8β chain comprises the amino acid sequence of SEQ ID NO: 2.

31. The cell of claim 30, wherein the amino acid sequence of the chimeric CD8α chain consists of the amino acid sequence of SEQ ID NO: 1, and the amino acid sequence of the chimeric CD8β chain consists of the amino acid sequence of SEQ ID NO: 2.

32. The cell of any one of claims 1-31, wherein the surface expression of the recombinant co-receptor or chain thereof is regulatable.

33. The cell of claim 32, wherein the first polynucleotide is flanked by a first pair of compatible site-specific recombination sequences.

34. The cell of claim 32, wherein the second polynucleotide is flanked by a second pair of compatible site-specific recombination sequences.

35. The cell of claim 32, wherein a portion of the first polynucleotide, the portion encoding a transmembrane region and a cytoplasmic region together, is flanked by a first pair of compatible site-specific recombination sequences.

36. The cell of claim 32, wherein a portion of the second polynucleotide, the portion encoding a transmembrane region and a cytoplasmic region together, is flanked by a second pair of compatible site-specific recombination sequences.

37. The cell of any one of claims 33-36, wherein the first pair of compatible site-specific recombination sequences does not cross-react with the second pair of compatible site-specific recombination sequences.

38. The cell of any one of claims 33-37, wherein the surface expression of the recombinant co-receptor, or chain thereof, can be attenuated by contacting the first polynucleotide and/or the second polynucleotide with a cognate site-specific recombinase that recognizes the first pair of compatible site-specific recombination sequences of the first polynucleotide and/or the second pair of compatible site-specific recombination sequences of the second polynucleotide.

39. The cell of any one of claims 33-37, wherein the surface expression of the recombinant co-receptor, or chain thereof, can be abrogated by contacting the first

polynucleotide and/or the second polynucleotide with a cognate site-specific recombinase that recognizes the first pair of compatible site-specific recombination sequences of the first polynucleotide and/or the second pair of compatible site-specific recombination sequences of the second polynucleotide.

40. The cell of any one of claims 33-39, wherein the cell further comprises a third polynucleotide encoding a cognate site-specific recombinase that recognizes the first and/or the second pair of compatible site-specific recombination sequences.

41. The cell of claim 40, wherein the transcription of the third polynucleotide is regulated by an inducible promoter.

42. The cell of any one of claims 38-41, wherein the first and second pairs of site-specific recombination sequences are compatible pairs of lox sites and the cognate site-specific recombinase is a Cre recombinase.

43. The cell of claim 42, wherein the compatible pairs of lox sites are compatible pairs of loxP sites.

44. The cell of any one of claims 32-43, wherein the recombinant co-receptor is CD8.

45. The cell of claim 44, wherein the first polynucleotide comprises the polynucleotide sequence of residues 1-675 of SEQ ID NO: 3.

46. The cell of claim 44, wherein the first polynucleotide comprises the polynucleotide sequence of SEQ ID NO: 3.

47. The cell of claim 44, wherein the second polynucleotide comprises the polynucleotide sequence of residues 1-600 of SEQ ID NO: 4.

48. The cell of claim 44, wherein the second polynucleotide comprises the polynucleotide sequence of SEQ ID NO: 4.

49. The cell of claim 44, wherein the first chain of the recombinant co-receptor comprises the amino acid sequence of SEQ ID NO: 25.

50. The cell of claim 49, wherein the first chain of the recombinant co-receptor comprises the amino acid sequence of SEQ ID NO: 5.

51. The cell of claim 44, wherein the second chain of the recombinant co-receptor comprises the amino acid sequence of SEQ ID NO: 26.

52. The cell of claim 51, wherein the second chain of the recombinant co-receptor comprises the amino acid sequence of SEQ ID NO: 6.

53. The cell of claim 32, wherein the transcription of the first polynucleotide is regulated by an inducible promoter.

54. The cell of claim 32 or 53, wherein the transcription of the second polynucleotide is regulated by an inducible promoter.

55. The cell of any one of claims 1-54, wherein the cell further comprises one or more reporter systems.

56. The cell of claim 55, wherein the one or more reporter systems provide an indication of one or more activities of the cell selected from the group consisting of nuclear factor of activated T-cell (NFAT) signaling, IL-2 signaling, activator protein-1 (AP1) signaling, NFκB/Rel signaling, and calcium flux.

57. The cell of claim 55 or 56, wherein the one or more reporter systems comprise a polynucleotide comprising a promoter region operably linked to a polynucleotide sequence encoding a detectable marker.

58. The cell of claim 57, wherein the promoter region comprises one or more transcriptional response elements selected from the group consisting of nuclear factor of activated T-cell (NFAT) response elements, activator protein-1 (AP1) response elements, and NFκB/Rel response elements.

59. The cell of claim 57 or 58, wherein the detectable marker is selected from the group consisting of a fluorescent protein, a cell surface marker, and an antibiotic selection marker.

60. The cell of claim 57, wherein the promoter region is an IL-2 promoter.

61. The cell of claim 60, wherein the IL-2 promoter is a minimal IL-2 promoter.

62. The cell of claim 60, wherein the IL-2 promoter comprises one or more NFAT binding sites.

63. The cell of claim 62, wherein the IL-2 promoter comprises three NFAT binding sites.

64. The cell of any one of claims 60-63, wherein the detectable marker is enhanced green fluorescent protein (EGFP).

65. The cell of any one of claims 1-62, wherein the cell is derived from a single cell and wherein when the cell is expanded to a plurality of cells of at least 10^5 and transduced or transfected with separate polynucleotide vectors encoding a TCR α chain and a TCR β chain to express a recombinant TCR comprising the TCR α chain and the TCR β chain under conditions that maximize transduction or transfection efficiency, at least 20% of the plurality of cells express the recombinant TCR on the cell surface.

66. The cell of claim 65, wherein the cell has previously been subjected to one or more rounds of transduction or transfection.

67. The cell of any one of claims 1-66, wherein the lymphocyte is a T cell, B cell, or an NK cell.

68. The cell of claim 67, wherein the lymphocyte is a T cell that is selected from the group consisting of a $CD8^+$ cytotoxic T cell, a $CD8^+$ regulatory T cell, a $CD4^+$ cytotoxic T cell, a $CD4^+$ helper T cell, and a $CD4^+$ regulatory T cell.

69. The cell of any one of claims 1-68, wherein the lymphocyte is a mammalian lymphocyte.

70. The cell of claim 69, wherein the lymphocyte is a murine lymphocyte.

71. The cell of claim 70, wherein the lymphocyte is selected from the group consisting of a RAG1 or RAG2 knockout murine cell, an immature murine T cell, a mouse DO-11.10.7 58 α- β- (58-/-) cell, and a mouse Bw5147 cell.

72. The cell of claim 70 or 71, wherein the lymphocyte is a mouse lymphocyte.

73. The cell of any one of claims 1-72, wherein the cell further expresses a recombinant TCR on the cell surface, the recombinant TCR comprising a TCR α chain and a TCR β chain, wherein the TCR α chain and the TCR β chain each comprises a variable region and a non-variable region, each non-variable region comprising a constant region, a transmembrane region, and a cytoplasmic region, wherein for each chain, the variable region is linked to the constant region, the constant region is linked to the transmembrane region, and the transmembrane region is linked to the cytoplasmic region.

74. The cell of claim 73, wherein, for each of the TCR α chain and the TCR β chain, the amino acid sequence of the cytoplasmic region is at least 60% identical, optionally 70, 80, 90, or 100% identical, to the amino acid sequence of a corresponding cytoplasmic region of a naturally occurring TCR chain from the same species as the lymphocyte.

75. The cell of claim 73, wherein, for each of the TCR α chain and the TCR β chain, the amino acid sequence of the transmembrane region and the cytoplasmic region, together, is at least 90% identical, optionally 91, 92, 93, 94, 95, 96, 97, 98, 99, or 100% identical, to the amino acid sequence of a corresponding region of a naturally occurring TCR chain from the same species as the lymphocyte.

76. The cell of claim 75, wherein the amino acid sequence of the transmembrane region and the cytoplasmic region of the TCR α chain, together, comprises the amino acid sequence of SEQ ID NO: 14.

77. The cell of claim 75 or 76, wherein the amino acid sequence of the transmembrane region and the cytoplasmic region of the TCR β chain, together, comprises the amino acid sequence selected from the group consisting of SEQ ID NOs: 16, 18, and 20.

78. The cell of any one of claims 73-75, wherein, for each of the TCR α chain and the TCR β chain, the amino acid sequence of the non-variable region is at least 90% identical, optionally 91, 92, 93, 94, 95, 96, 97, 98, 99, or 100% identical, to the amino acid sequence of a corresponding non-variable region of a naturally occurring TCR chain from the same species as the lymphocyte.

79. The cell of claim 78, wherein the non-variable region of the TCR α chain comprises the amino acid sequence of SEQ ID NO: 13.

80. The cell of claim 78 or 79, wherein the non-variable region of the TCR β chain comprises the amino acid sequence selected from the group consisting of SEQ ID NOs: 15, 17, and 19.

81. The cell of any one of claims 73-80, wherein the amino acid sequence of each variable region is derived from a corresponding variable region of a TCR chain from a different species than the lymphocyte.

82. The cell of any one of claims 73-75, wherein, for each of the TCR α chain and the TCR β chain, the amino acid sequence of the variable region and the constant region, together, is derived from a corresponding region of a TCR chain from a different species than the lymphocyte.

83. The cell of claim 81 or 82, wherein the TCR from a different species than the lymphocyte is human.

84. The cell of any one of claims 73-83, wherein the recombinant TCR is a fusion molecule comprising a co-stimulatory signaling region.

85. The cell of claim 84, wherein the co-stimulatory signaling region is fused to the cytoplasmic region of the TCR α chain or the cytoplasmic region of the TCR β chain.

86. The cell of claim 84 or 85, wherein the co-stimulatory signaling region comprises the signaling region of a co-stimulatory molecule selected from the group consisting of CD27, CD28, 4-1BB, OX40, CD30, CD40, ICOS, lymphocyte function-associated antigen-1 (LFA-1), CD2, CD7, LIGHT, NKG2C, NKG2D, B7-H3, and a ligand that specifically binds CD83.

87. The cell of claim 86, wherein the co-stimulatory signaling region comprises the signaling region of CD28, the signaling region of 4-1BB, or a combination thereof.

88. The cell of any one of claims 84-87, wherein the fusion molecule further comprises a CD3ζ signaling region.

89. The cell of any one of claims 73-88, wherein the expression of the recombinant TCR is coupled to the expression of

(1) at least one antibiotic selection marker;

(2) at least one screening marker; or

(3) a combination thereof.

90. The cell of claim 89, wherein the at least one screening marker is a fluorescent protein or a cell surface marker.

91. The cell of any one of claims 73-90, wherein the recombinant TCR is derived from a source selected from the group consisting of fetal thymus, cord blood, tumor infiltrating lymphocytes (TILs) isolated from a cancer patient, a vaccinated subject, a subject naturally exposed to an antigen of interest, a healthy subject, and a synthetic library.

92. The cell of claim 91, wherein the vaccinated subject is a human who has received an HPV vaccine, an EBV vaccine, or a cancer vaccine.

93. The cell of claim 91, wherein the vaccinated subject is a transgenic mouse expressing human major histocompatibility complex (MHC) that has been immunized with an antigen of interest.

94. The cell of claim 93, wherein the vaccinated subject is a transgenic mouse expressing HLA-A2 that has been immunized with an antigen of interest.

95. The cell of any one of claims 73-94, wherein the cell expresses (1) a recombinant TCR α chain and a recombinant TCR β chain, (2) a recombinant TCR α chain and multiple different recombinant TCR β chains, (3) a recombinant TCR β chain and multiple different recombinant TCR α chains, or (4) multiple different recombinant TCR α chains and multiple different recombinant TCR β chains.

96. A cellular library comprising a plurality of cells of any one of claims 73-95.

97. The cellular library of claim 96, wherein the cellular library has a TCR diversity of at least 10^7, 10^8, or 10^9.

98. A method for the isolation and identification of a TCR with a desired characteristic comprising subjecting a plurality of cells of any one of claims 73-95 or the cellular library of claim 96 or 97 to a selection process comprising selecting for the desired characteristic in the presence of a desired peptide-MHC complex, isolating a cell that expresses a TCR, and determining that the TCR of the isolated cell has the desired characteristic.

99. A method for the identification of at least one polynucleotide encoding a TCR with a desired characteristic, comprising:

(a) providing a plurality of cells of any one of claims 1-72,

(b) transducing or transfecting the cells with a plurality of expression constructs encoding a plurality of TCRs,

(c) expressing the TCRs on the surface of the cells,

(d) selecting cells expressing the TCRs for the desired characteristic in the presence of a desired peptide-MHC complex,

(e) isolating the at least one polynucleotide encoding the TCR with the desired characteristic from the selected cells, and

(f) identifying the at least one polynucleotide encoding the TCR with the desired characteristic isolated in step (e).

100. A method for the identification of at least one polynucleotide encoding a TCR with a desired characteristic, comprising:

(a) providing a plurality of cells of any one of claims 1-72;

(b) transducing or transfecting the cells with a plurality of expression constructs encoding a plurality of TCRs,

(c) expressing the TCRs on the surface of the cells,

(d) selecting cells expressing the TCRs for the desired characteristic in the presence of a desired peptide-MHC complex,

(e) modulating an activity of the recombinant co-receptor or chain thereof in the cells selected in step (d),

(f) further selecting cells expressing the TCRs produced by step (e) for the desired characteristic in the presence of the desired peptide-MHC complex,

(g) isolating the at least one polynucleotide encoding the TCR with the desired characteristic from the cells selected in step (f), and

(h) identifying the at least one polynucleotide encoding the TCR with the desired characteristic isolated in step (g).

101. The method of claim 100, wherein step (d) is performed in the presence of surface expression of the recombinant co-receptor or chain thereof, and wherein step (e) comprises abrogation of cell surface expression of the recombinant co-receptor or chain thereof.

102. The method of claim 101, wherein the plurality of cells of step (a) comprise the cells of any one of claims 33-40, and wherein in step (e), the cell surface expression of the recombinant co-receptor or chain thereof is abrogated by contacting the site-specific recombination sequences in the first and/or second polynucleotide with a cognate site-specific recombinase.

103. The method of claim 102, wherein the cognate site-specific recombinase is expressed in the cells under the control of an inducible promoter.

104. The method of claim 102, wherein the cognate site-specific recombinase is introduced into the cells selected in step (d) prior to step (e).

105. The method of claim 104, wherein the cognate site-specific recombinase is introduced by transfecting or transducing the cells selected in step (d) with an expression construct encoding the site-specific recombinase.

106. The method of claim 104, wherein the cognate site-specific recombinase is introduced as a protein.

107. The method of any one of claims 102-106, wherein the site-specific recombination sequences are lox sites and the cognate site-specific recombinase is a Cre recombinase.

108. The method of claim 107, wherein the site-specific recombination sequences are lox sites and the cognate site-specific recombinase is a Cre recombinase fusion protein comprising a protein transduction domain.

109. The method of claim 108, wherein the protein transduction domain is from HIV Tat.

110. A method for the identification of at least one polynucleotide encoding a TCR with a desired characteristic, comprising:

(a) providing a plurality of cells of any one of claims 1-72;

(b) transducing or transfecting the cells with a plurality of expression constructs encoding a plurality of TCRs,

(c) expressing the TCRs on the surface of the cells,

(d) selecting cells expressing the TCRs for the desired characteristic in the presence of an MHC molecule complexed with a peptide of interest, wherein the MHC molecule is a variant of a wild type MHC molecule, wherein the variant MHC molecule binds to a co-receptor with reduced affinity compared to the affinity of the wild type MHC molecule for the co-receptor,

(e) isolating the at least one polynucleotide encoding the TCR with the desired characteristic from the cells selected in step (d), and

(f) identifying the at least one polynucleotide encoding the TCR with the desired characteristic isolated in step (e).

111. The method of claim 110, wherein the co-receptor is CD8 and wherein the variant MHC molecule is a variant MHC class I molecule comprising an α chain that is a variant of a wild type MHC class I α chain.

112. The method of claim 111, wherein the co-receptor is human CD8 and wherein the variant MHC class I molecule is a variant HLA-A2 comprising an α chain that is a variant of wild type HLA-A2 α chain.

113. The method of claim 112, wherein the variant HLA-A2 α chain comprises an A245V mutation or a D227K/T228A mutation, numbered according to the mature protein sequence.

114. The method of any one of claims 98-113, wherein the desired characteristic is selected from the group consisting of high selectivity for the desired peptide-MHC complex, high affinity for the desired peptide-MHC complex, and demonstration of a positive functional readout in the presence of the desired peptide-MHC complex.

115. The method of claim 114, wherein the functional readout is selected from the group consisting of expression of activation marker, triggering of signaling pathway, production of cytokine, calcium flux, proliferation, and activation of reporter system.

116. The method of claim 115, wherein the activation marker is CD69 or CD137.

117. The method of claim 115 or 116, wherein the plurality of cells comprise the cells of any one of claims 55-64 and wherein the functional readout is the activation of the one or more reporter systems.

118. The method of any one of claims 99-117, wherein the plurality of expression constructs encode (i) a TCR α chain and a TCR β chain, (ii) a TCR α chain and multiple different TCR β chains, (iii) a TCR β chain and multiple different TCR α chains, or (iv) multiple different TCR α chains and multiple different TCR β chains.

119. The method of any one of claims 98-118, wherein the desired peptide-MHC complex comprises an MHC class I or MHC class II molecule.

120. The method of any one of claims 98-119, wherein the desired peptide-MHC complex is a cell free peptide-MHC complex.

121. The method of claim 120, wherein the desired peptide-MHC complex comprises a dimer, a tetramer, or a multimer of MHC molecules.

122. The method of any one of claims 98-119, wherein the desired peptide-MHC complex is displayed on the surface of a cell.

123. The method of claim 122, wherein the cell is a mammalian cell, a yeast cell, or an insect cell.

124. The method of claim 122, wherein the cell is a T2 cell or an antigen-presenting cell.

125. The method of any one of claims 98-124, wherein the peptide is a phosphopeptide or a phosphopeptide mimetic.

126. The method of any one of claims 99-125, wherein in step (b), the plurality of expression constructs encoding a plurality of TCRs is transduced into the cells using retroviral vector particles comprising an envelope of PVC-211 murine leukemia virus.

127. The method of any one of claims 99-125, wherein each TCR comprises a TCR α chain and a TCR β chain, wherein the TCR α chain and the TCR β chain are encoded by separate expression constructs.

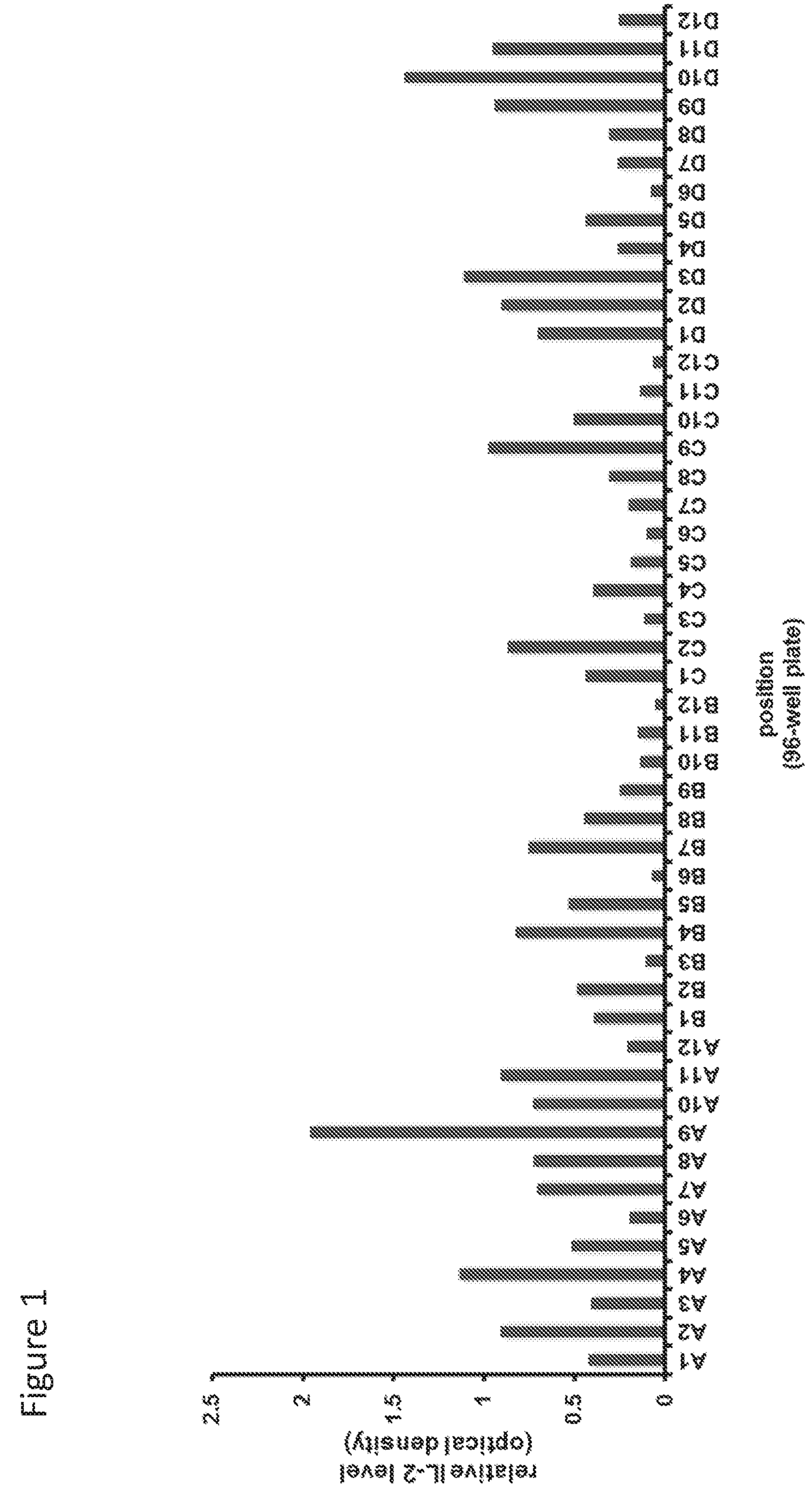

relative IL-2 level
(optical density)
0
0.5
1
1.5
2
2.5
position
(96-well plate)
A1
A2
A3
A4
A5
A6
A7
A8
A9
A10
A11
A12
B1
B2
B3
B4
B5
B6
B7
B8
B9
B10
B11
B12
C1
C2
C3
C4
C5
C6
C7
C8
C9
C10
C11
C12
D1
D2
D3
D4
D5
D6
D7
D8
D9
D10
D11
D12

Figure 1

Figure 2A

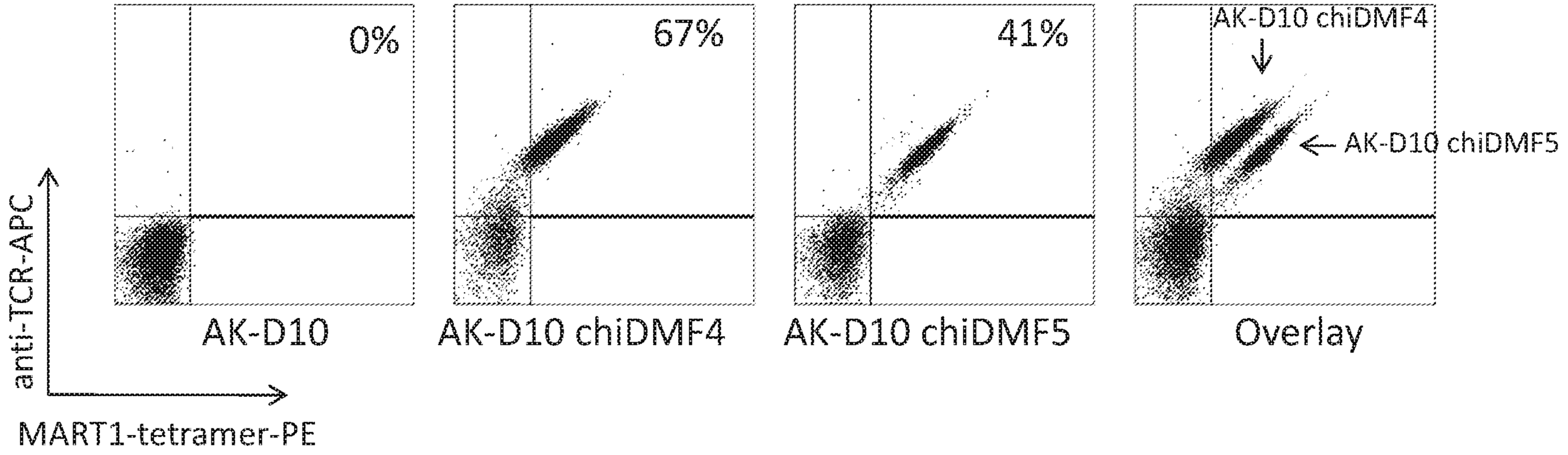


Figure 2B

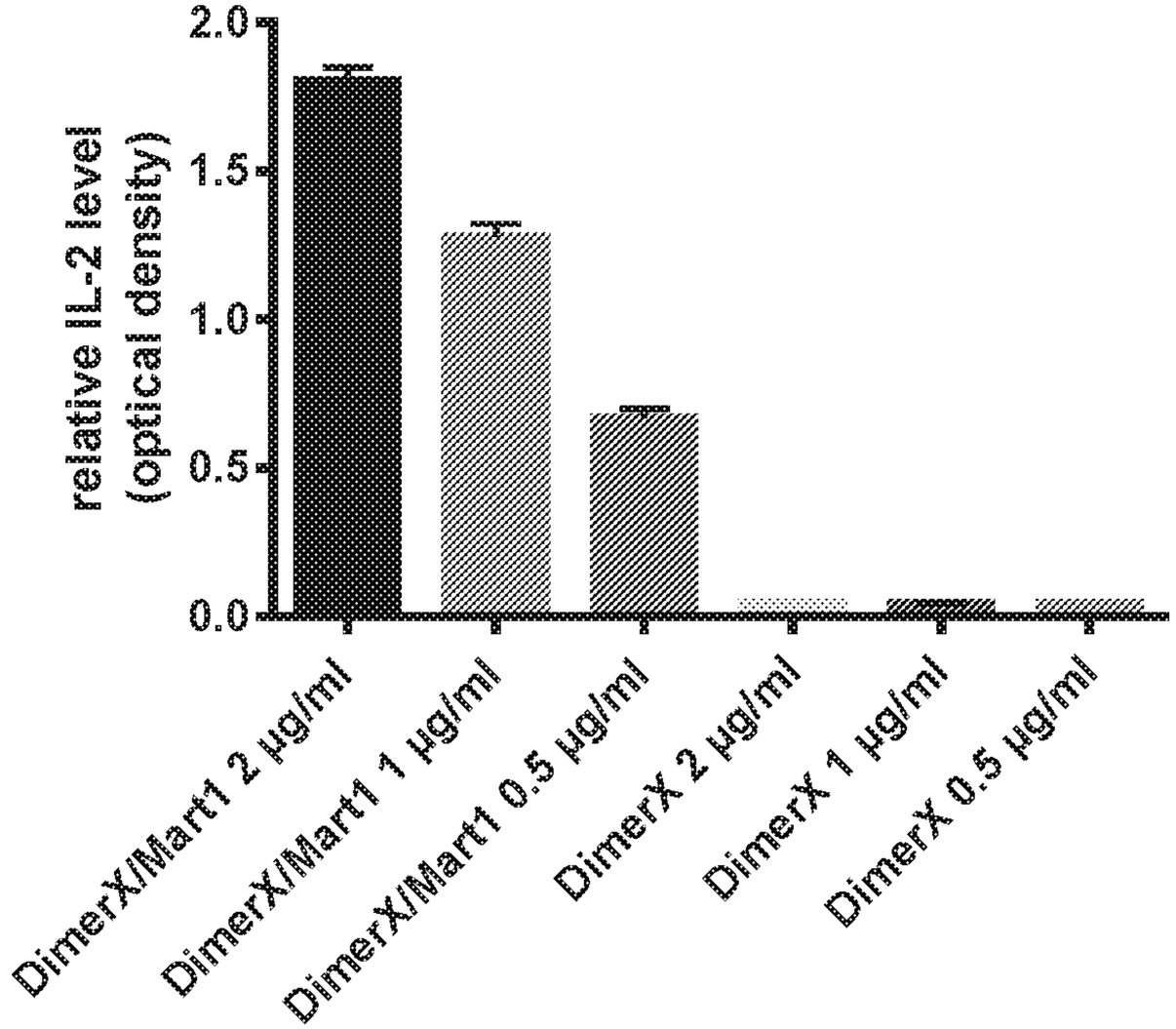
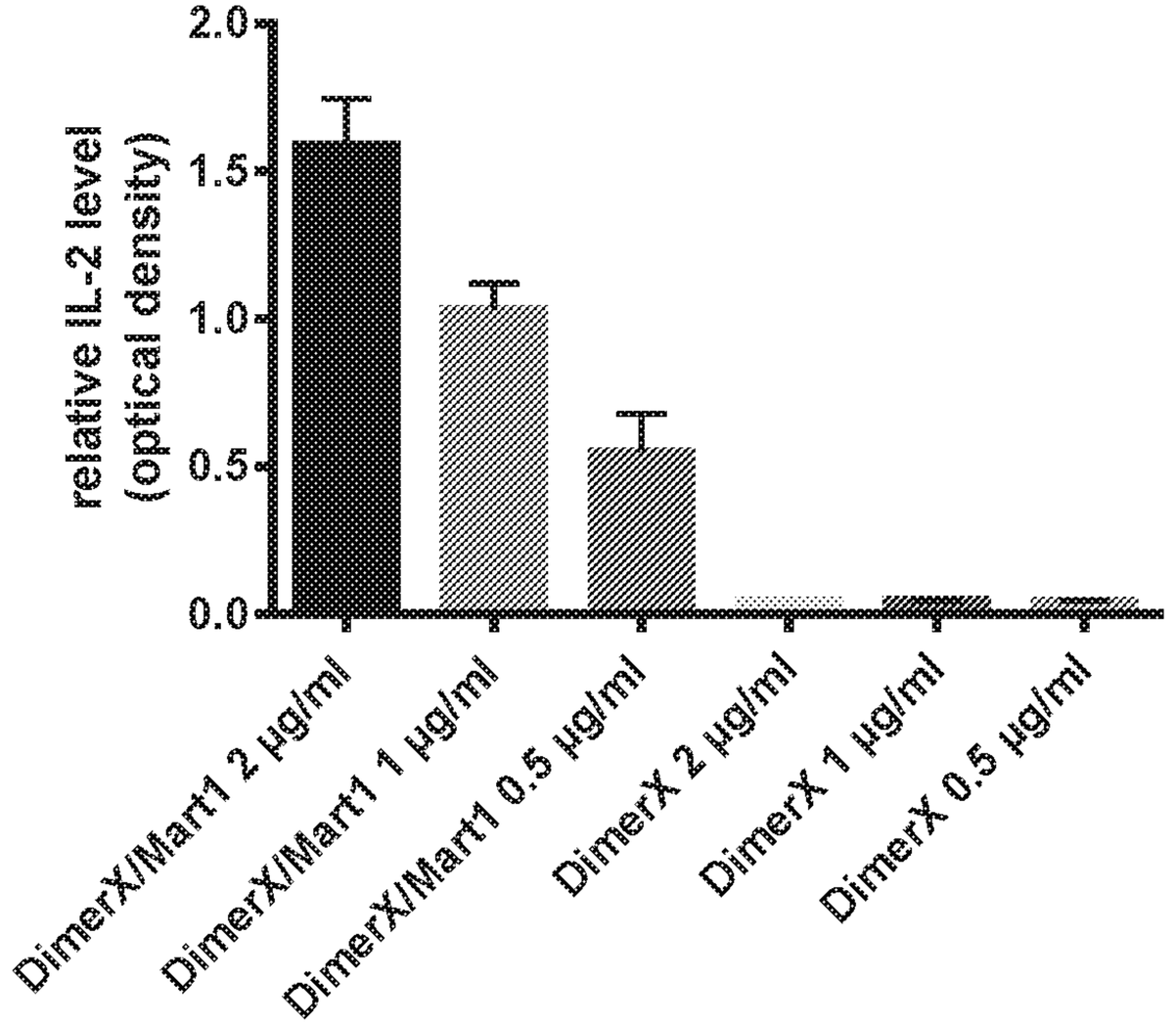


Figure 2C



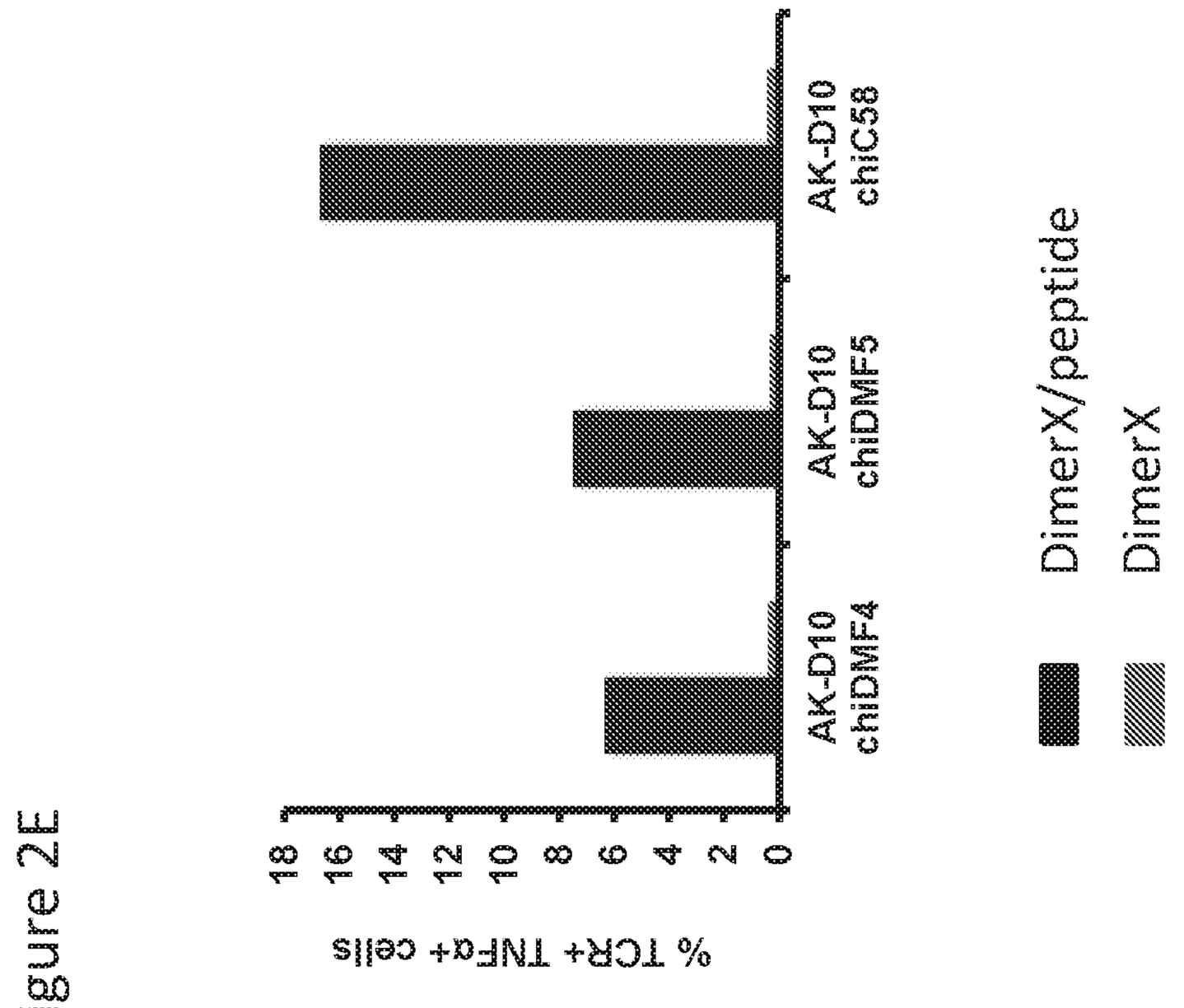


Figure 2E

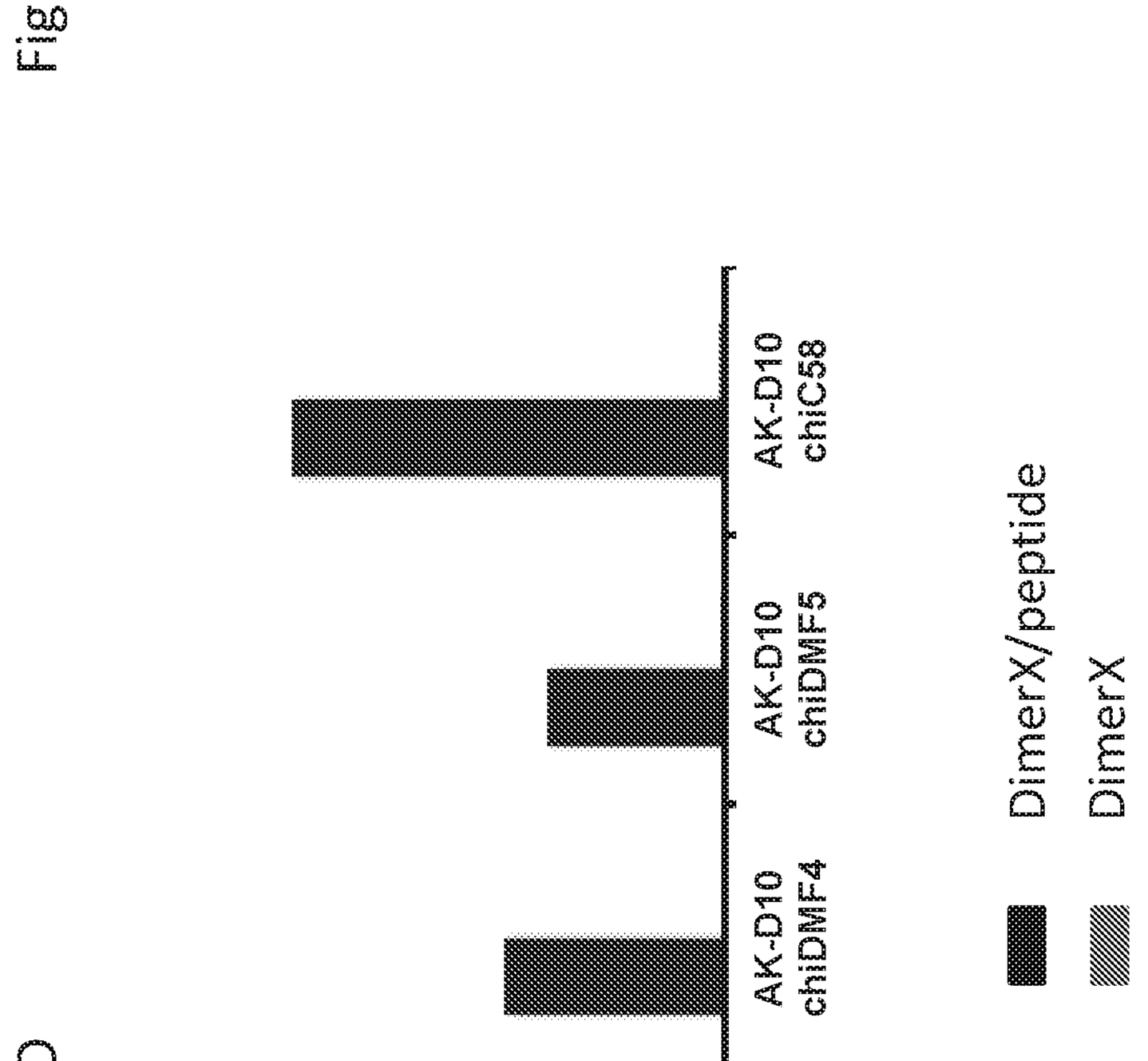


Figure 2D

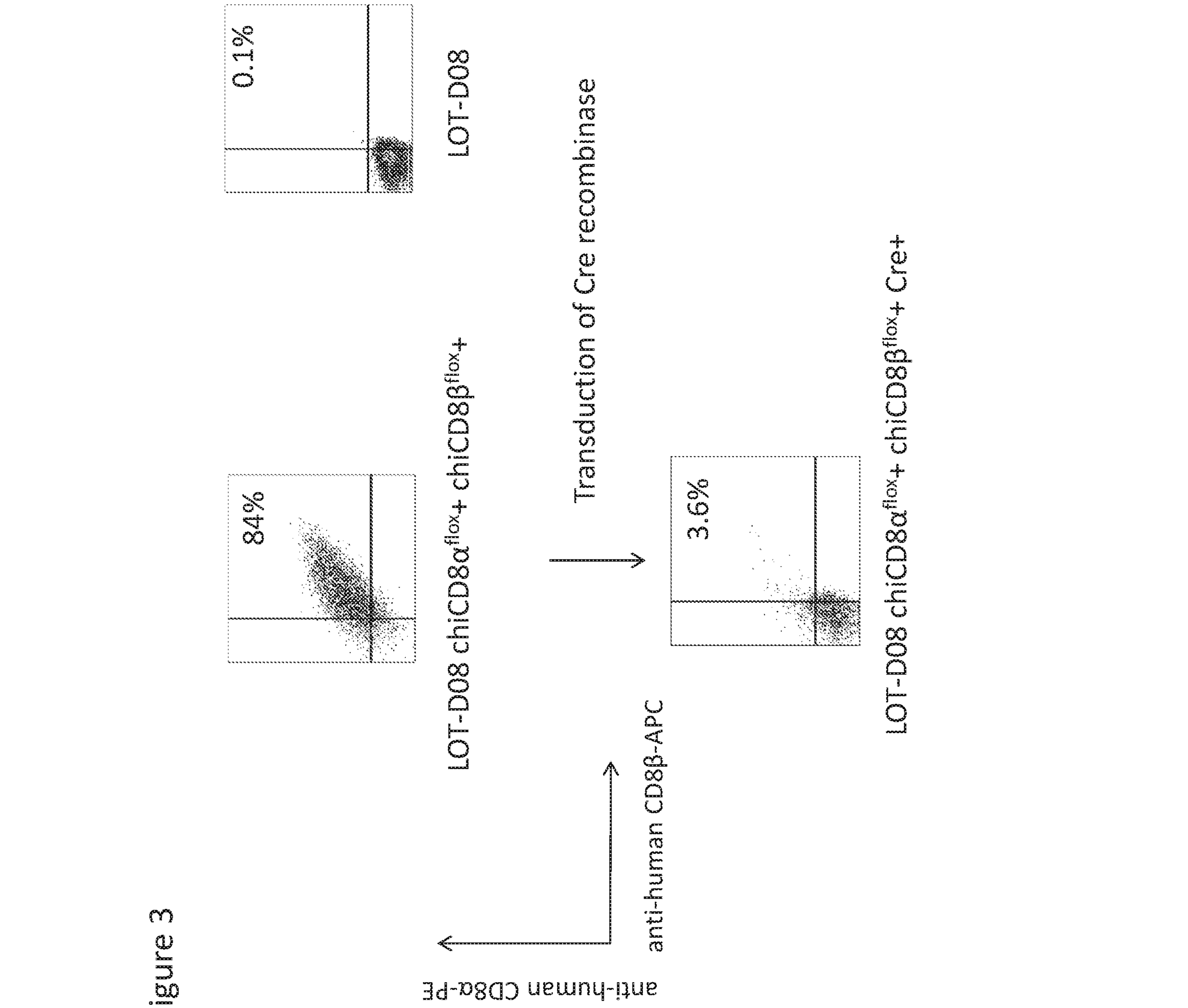

anti-human CD8α-PE
anti-human CD8β-APC
LOT-D08 chiCD8α^{flox}+ chiCD8β^{flox}+
84%
Transduction of Cre recombinase
LOT-D08 chiCD8α^{flox}+ chiCD8β^{flox}+ Cre+
3.6%
LOT-D08
0.1%

Figure 3

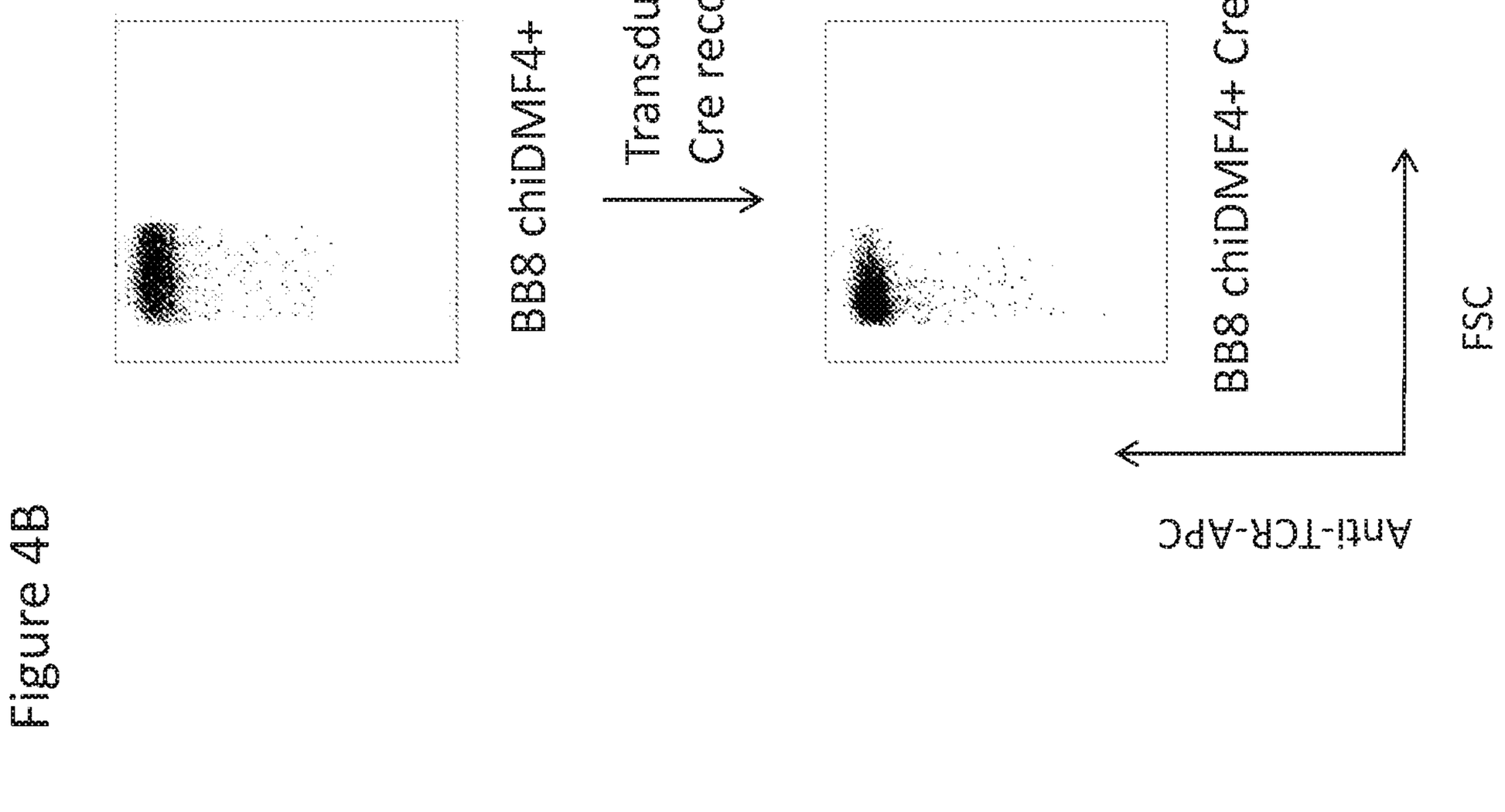


Figure 4B

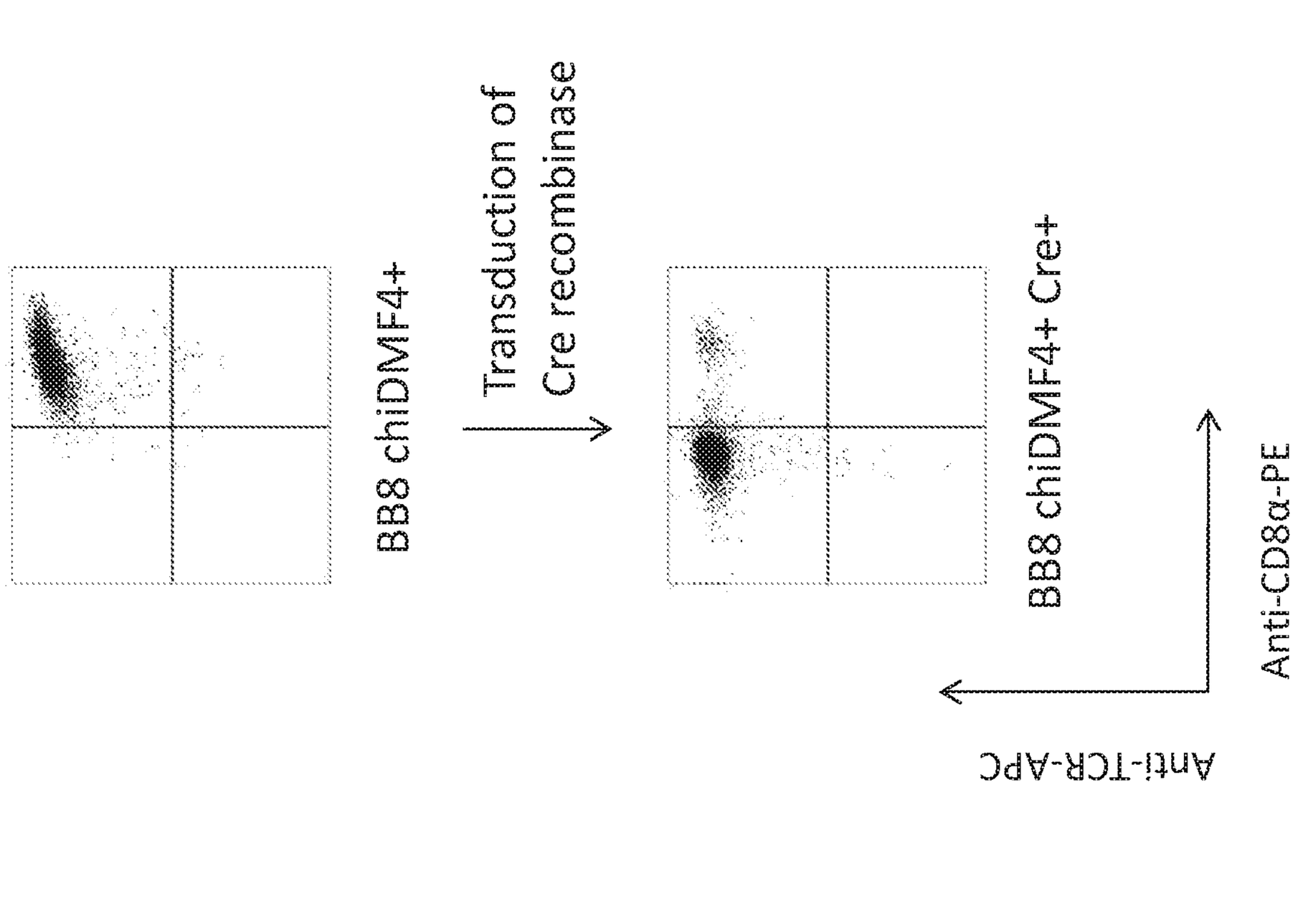


Figure 4A

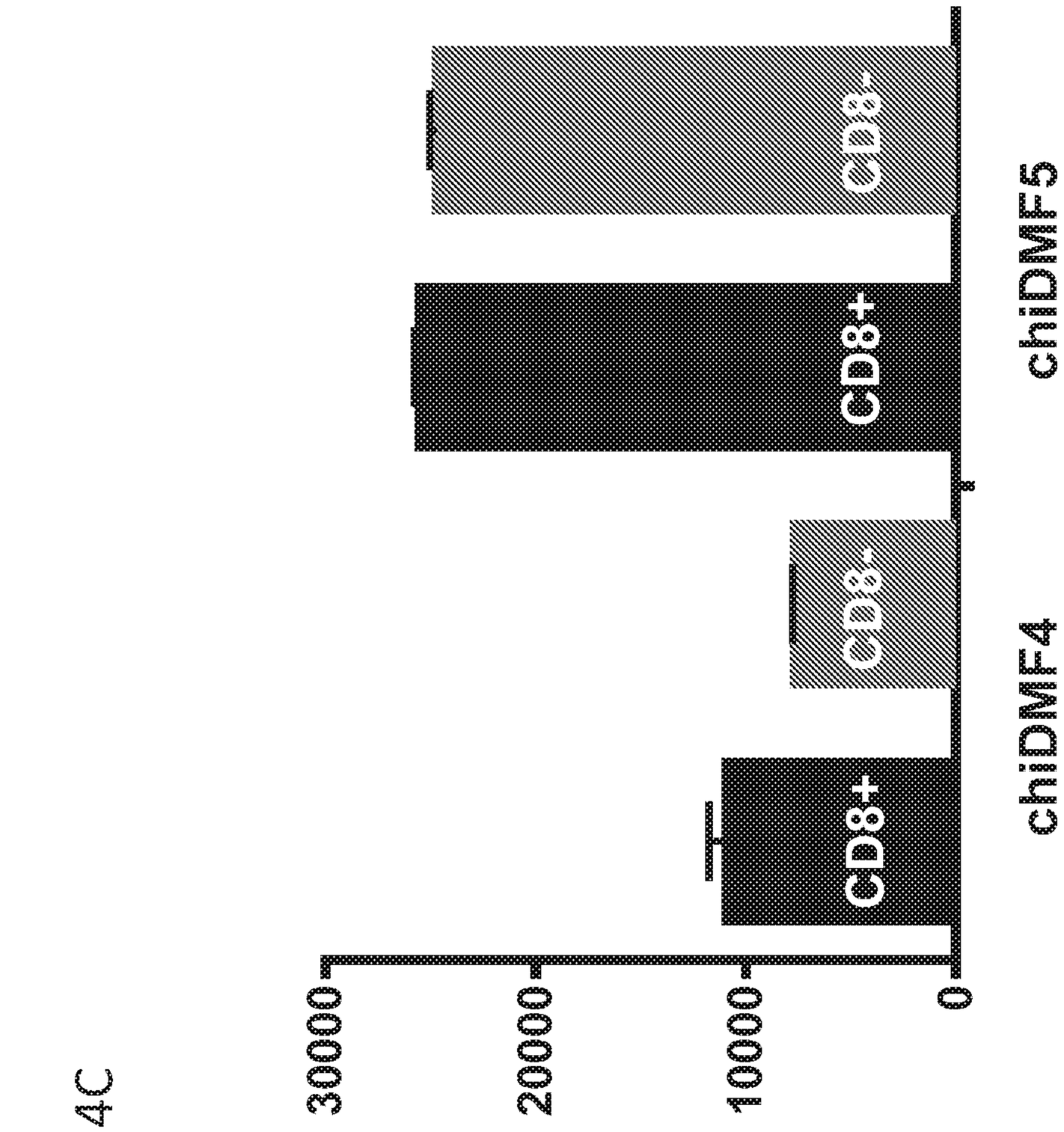

CD8-
CD8+
CD8-
CD8+
chiDMF4
chiDMF5
MFI tetramer
30000
20000
10000
0

Figure 4C

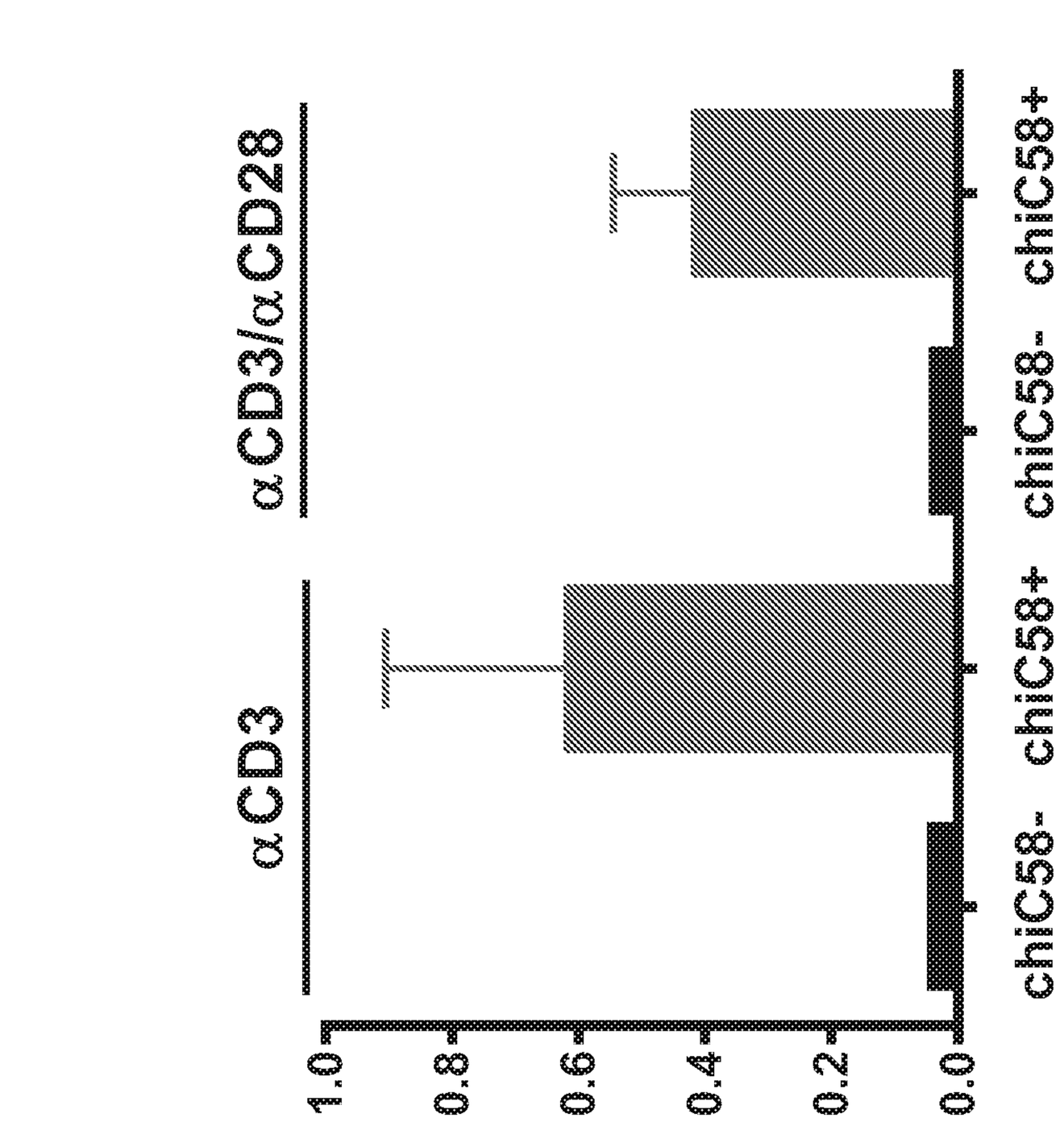
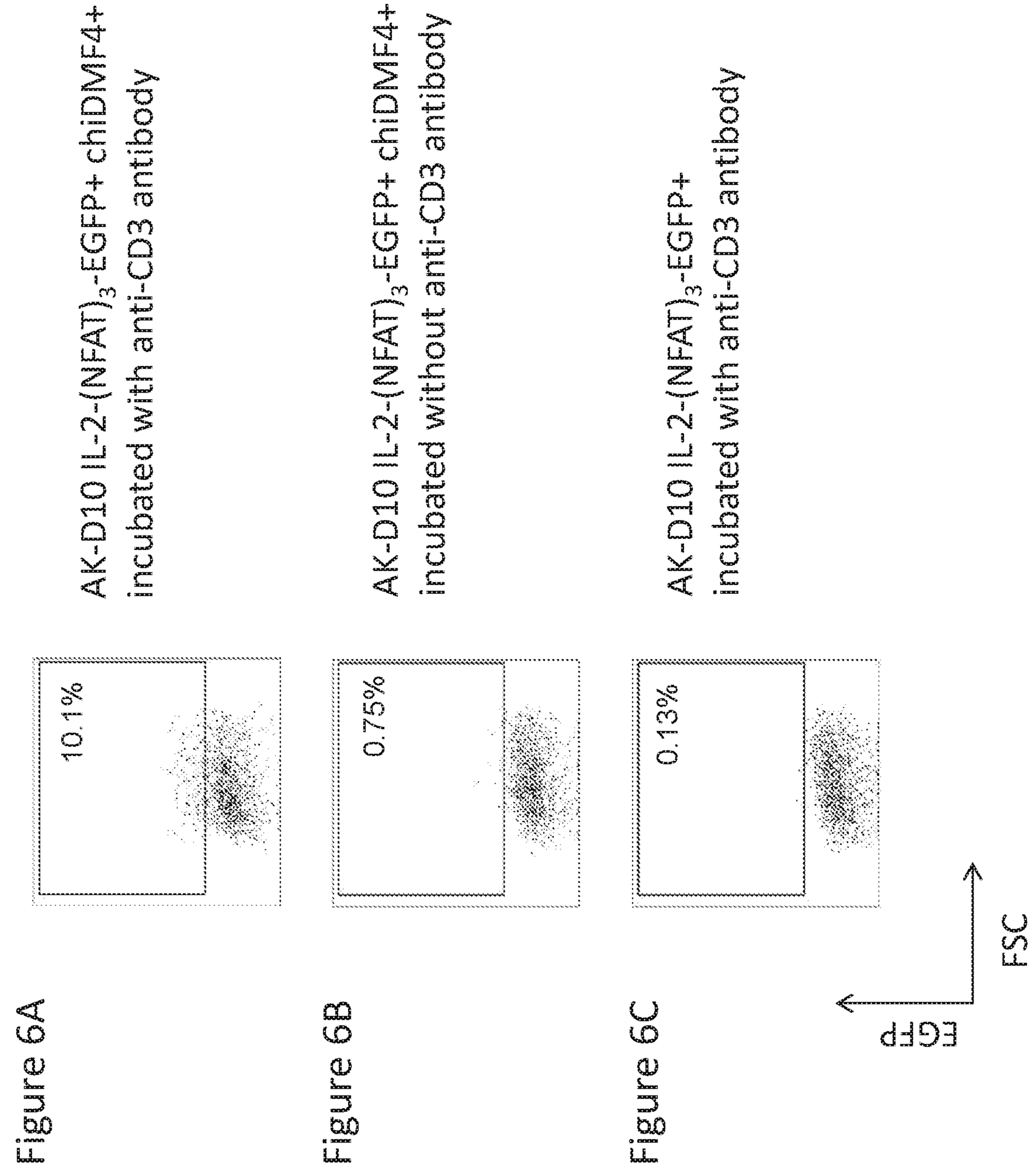
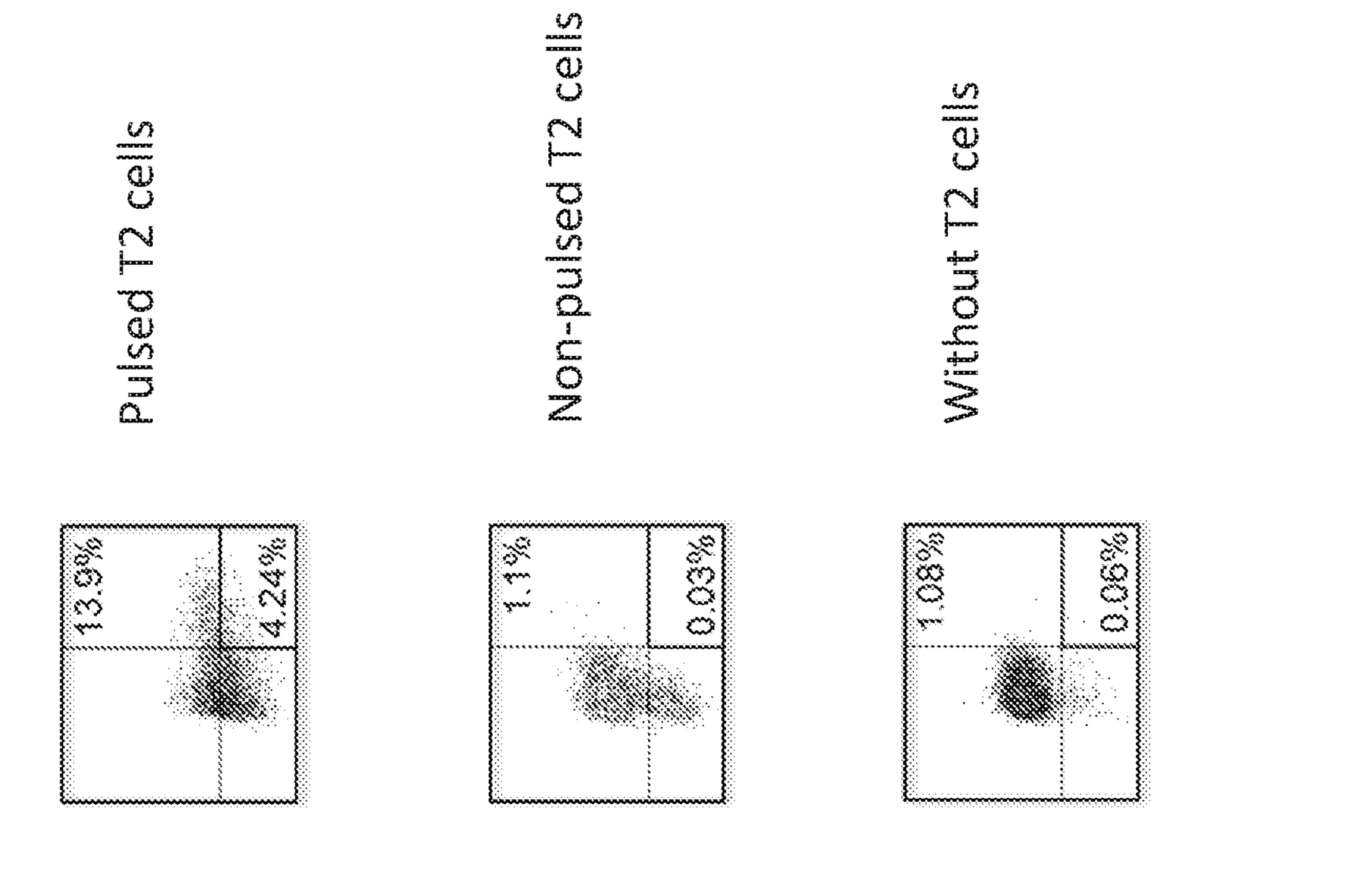

αCD3
αCD3/αCD28
chiC58+
chiC58-
chiC58+
chiC58-
1.0
0.8
0.6
0.4
0.2
0.0
relative IL-2 level
(optical density)

Figure 5


AK-D10 IL-2-(NFAT)3-EGFP+ chiDMF4+
incubated with anti-CD3 antibody
10.1%
FIG. 6A
AK-D10 IL-2-(NFAT)3-EGFP+ chiDMF4+
incubated without anti-CD3 antibody
0.75%
FIG. 6B
AK-D10 IL-2-(NFAT)3-EGFP+
incubated with anti-CD3 antibody
0.13%
FIG. 6C
EGFP
FSC


Figure 7A
Figure 7B
Figure 7C
Pulsed T2 cells
Non-pulsed T2 cells
Without T2 cells
15%
3.12%
0.94%
0.05%
0.85%
0.01%
13.9%
4.24%
1.1%
0.03%
1.08%
0.06%
TCR chic259
EGFP

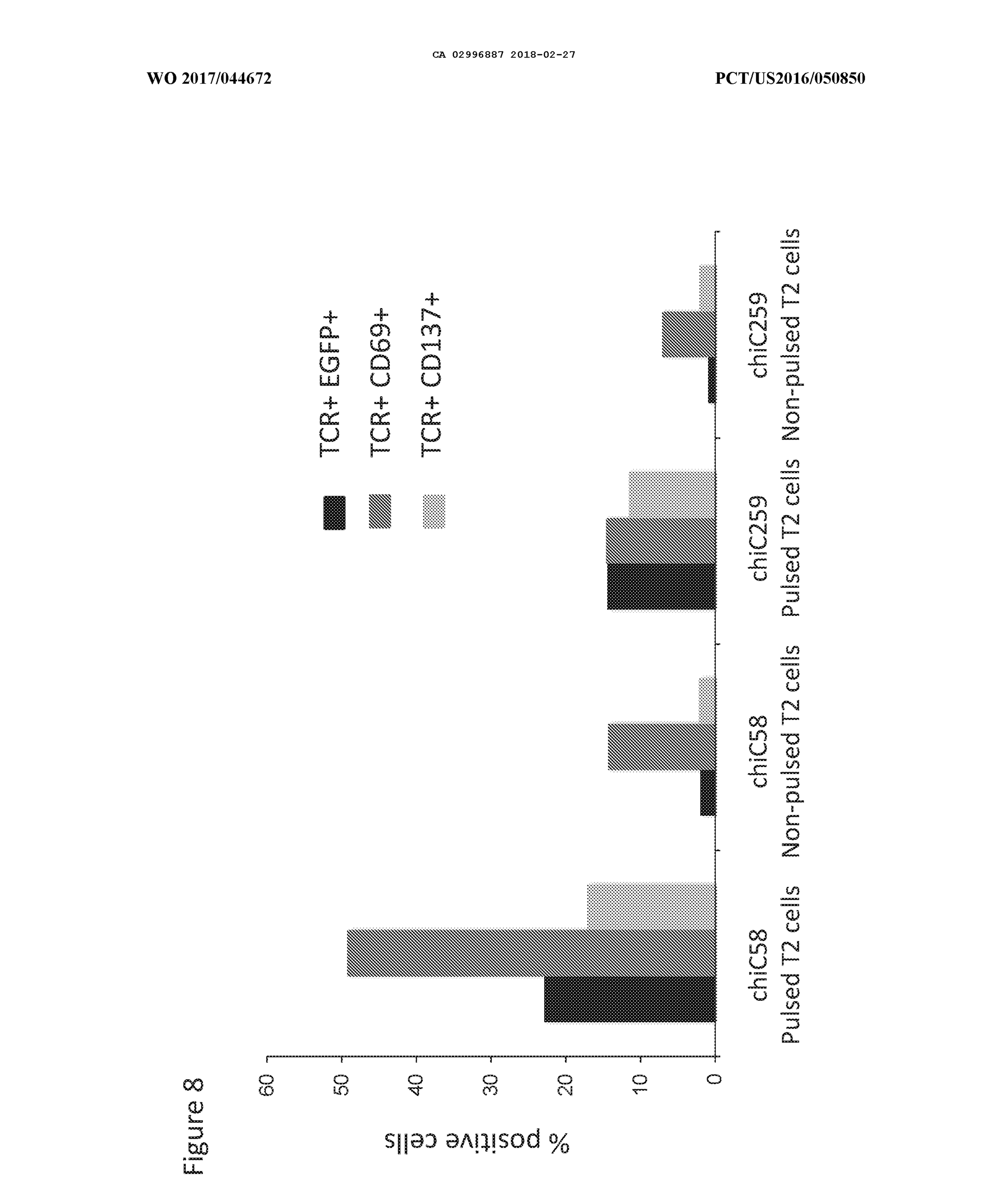

TCR+ EGFP+
TCR+ CD69+
TCR+ CD137+
chiC59
Non-pulsed T2 cells
Pulsed T2 cells
chiC58
Non-pulsed T2 cells
Pulsed T2 cells
chiC58
% positive cells
60
50
40
30
20
10
0

Figure 8

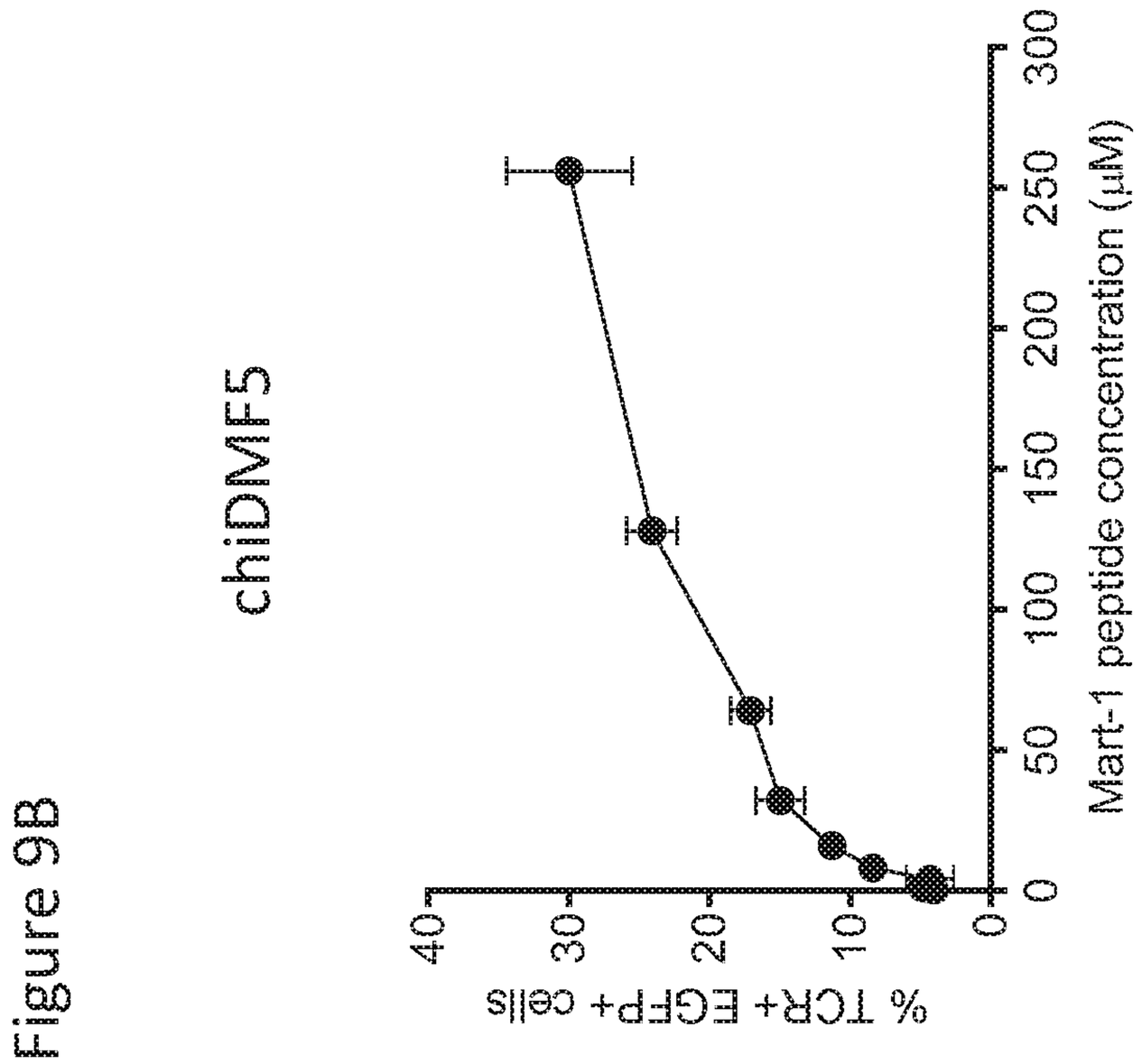


Figure 9B

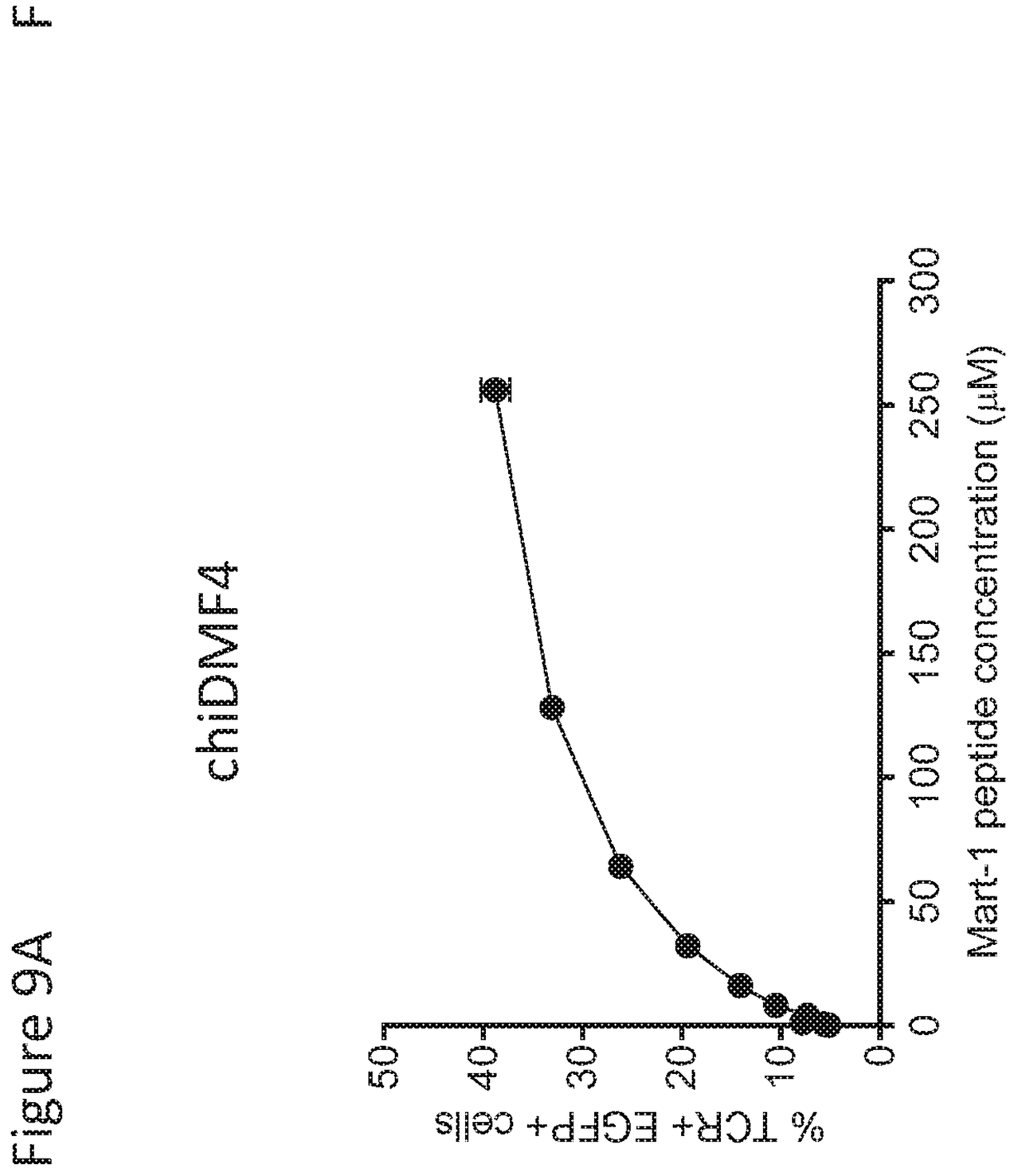


Figure 9A

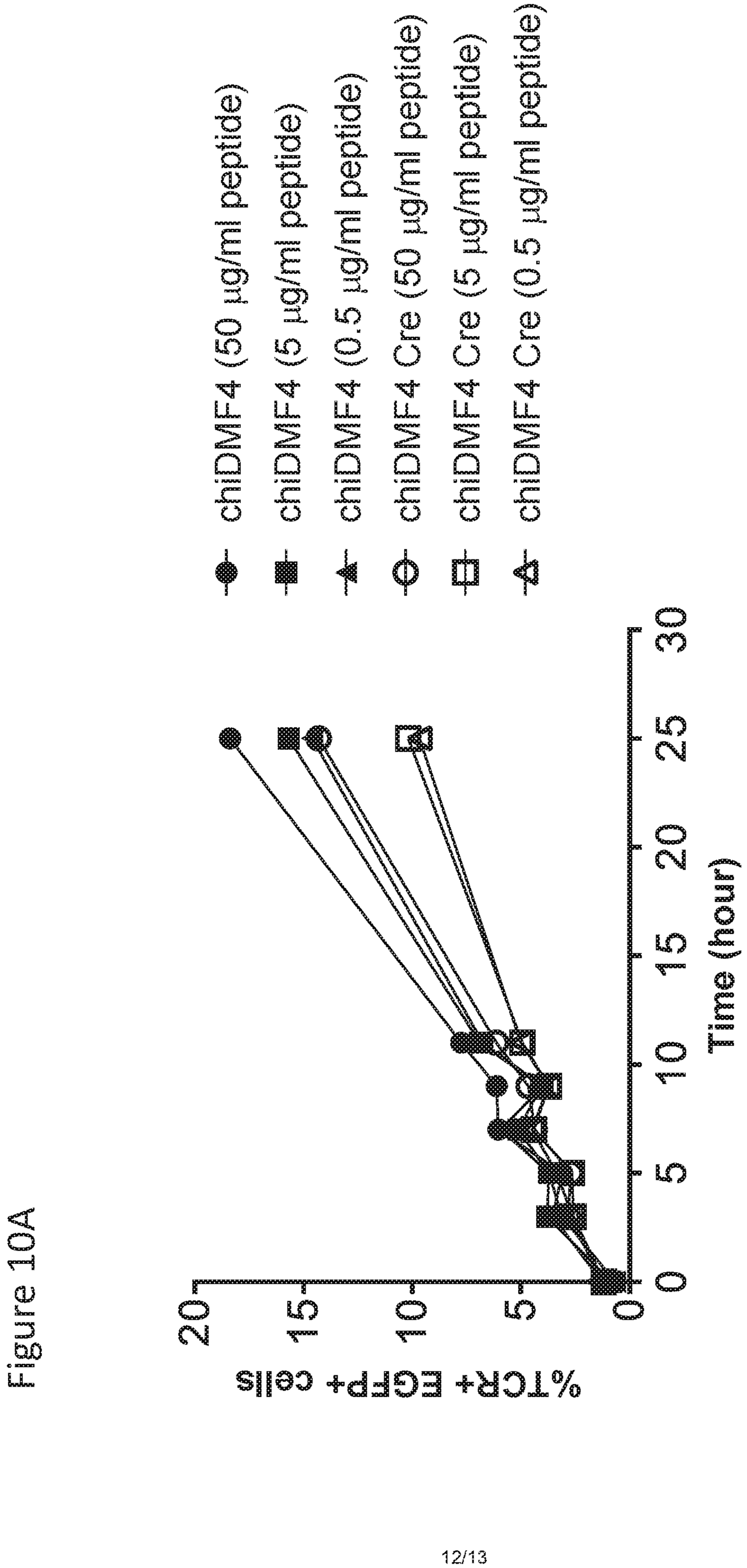

chiDMF4 (50 µg/ml peptide)
chiDMF4 (5 µg/ml peptide)
chiDMF4 (0.5 µg/ml peptide)
chiDMF4 Cre (50 µg/ml peptide)
chiDMF4 Cre (5 µg/ml peptide)
chiDMF4 Cre (0.5 µg/ml peptide)
%TCR+ EGFP+ cells
0
5
10
15
20
25
30
Time (hour)

Fig. 10A

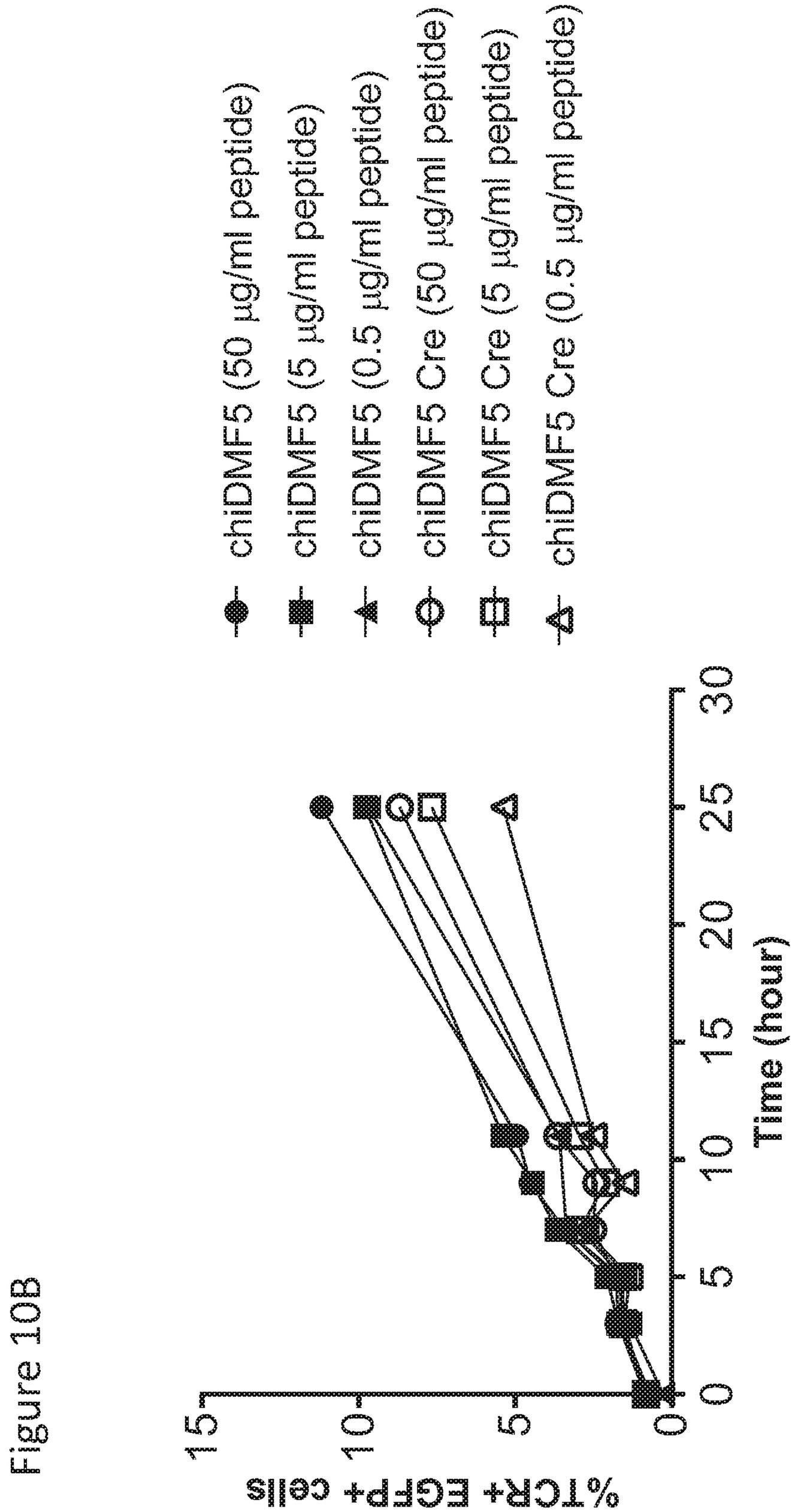

chiDMF5 (50 µg/ml peptide)
chiDMF5 (5 µg/ml peptide)
chiDMF5 (0.5 µg/ml peptide)
chiDMF5 Cre (50 µg/ml peptide)
chiDMF5 Cre (5 µg/ml peptide)
chiDMF5 Cre (0.5 µg/ml peptide)
%TCR+ EGFP+ cells
50
40
30
20
10
0
0
5
10
15
20
25
Time (hour)

Figure 10B