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RNI, Neeraja; c/o ImmunoSCAPE Pte. Ltd., 1 Scotts Road, #24-10, Singapore 228208 (SG).

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(74) Agent: DOWSING, Bruce John; Marks & Clerk Singapore LLP, Tanjong Pagar Post Office, P.O. Box 636, Singapore 910816 (SG).

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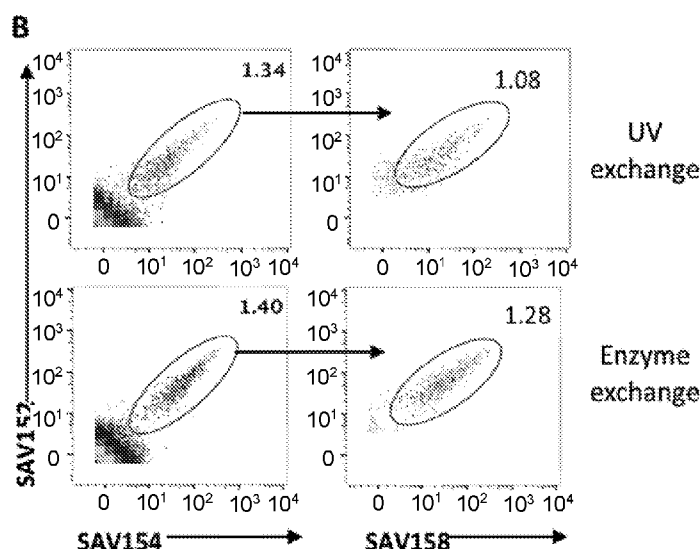
(71) Applicants: AGENCY FOR SCIENCE, TECHNOLOGY AND RESEARCH [SG/SG]; 1 Fusionopolis Way, #20-10, Connexis North Tower, Singapore 138632 (SG). IMMUNOSCAPE PTE. LTD. [SG/SG]; 1 Scotts Road, #24-10, Singapore 228208 (SG).

(72) Inventors: NEWELL, Evan; c/o Singapore Immunology Network, 8a Biomedical Grove, Level 3 Immunos Building, Singapore 138648 (SG). LIM, Jack Wee; c/o Singapore Immunology Network, 8a Biomedical Grove, Level 3 Immunos Building, Singapore 138648 (SG). KULKA-

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(54) Title: HIGH YIELD PRODUCTION AND USE OF ENZYMATIC-EXCHANGEABLE PEPTIDE MAJOR HISTOCOMPATIBILITY COMPLEX CLASS I SINGLE CHAIN TRIMER TETRAMER

Figure 5



(57) Abstract: The present invention relates to methods to produce an enzymatic-exchangeable peptide Major Histocompatibility Complex Class I (MHC-I) Single Chain Trimer (SCT), or tetramer thereof, constructs encoding same and uses thereof, such as detection or isolation of antigen-specific CD8⁺ T cells. Said SCT comprises, in order from N-terminus to C-terminus, (i) a peptide ligand, (ii) a first linker polypeptide comprising an enzyme-cleavable portion, (iii) a β -2 microglobulin (β 2m) polypeptide, (iv) a second linker polypeptide, and (v) a mature MHC-I heavy chain polypeptide. In addition, a method of defining a peptide ligand suitable for successful production of a single fusion protein for peptide exchange is claimed.

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HIGH YIELD PRODUCTION AND USE OF ENZYMATIC-EXCHANGEABLE PEPTIDE MAJOR HISTOCOMPATIBILITY COMPLEX CLASS I SINGLE CHAIN TRIMER TETRAMER

FIELD OF THE INVENTION

5 The present invention relates to methods to produce an enzymatic-exchangeable peptide Major Histocompatibility Complex Class I (MHC-I) Single Chain Trimer, or tetramer thereof, constructs encoding same and uses thereof, such as the detection of antigen-specific CD8⁺ T cells.

BACKGROUND OF THE INVENTION

10 Conditional ligand class I MHC tetramers have been used for the purpose of generating antigen specific peptide-MHC molecules for the detection of antigen-specific CD8⁺ T cells, whereby conditional UV-cleavable class I MHC could be generated for HLA-A2, -A1, -A3, -A11 and -B7 and that such peptide-exchange strategies would permit high-throughput generation of peptide MHC complexes [Bakker AH, et al., *Proc Natl Acad Sci USA*, 105(10):
15 3825-30 (2008)]. A limitation of this method is observed UV incompatibility with fluorescent labels, the production of reactive nitroso species and photodamage of MHC-I protein.

 Others have independently expressed the soluble heavy chain of the MHC class I and β -2 -microglobulin (β 2m) in *Escherichia coli* [Garboczi DN, et al., *Proc. Natl Acad. Sci. USA* 89(8): 3429–3433 (1992)]. The class I MHC α -chain has the transmembrane region removed,
20 only expressing the extracellular domain and is typically engineered to contain the biotin 15 amino acid recognition tag on the C-terminus. In such preparations, the subunits will be expressed as inclusion bodies within the bacteria. The heavy chain and the light chain, along with the peptide of interest, are refolded, and the enzyme BirA is used to specifically biotinylate the lysine residue within the 15 amino acid recognition sequence. A limitation of this method
25 is often the laborious screening of different refolding conditions using synthetic peptides. It is understood that the closed peptide binding groove in MHC-I protein limits the optimal peptide size, requiring a 10-15 amino acid first linker separating the peptide and the β 2m [WO 2015/195531].

 There is a need to further improve methods of producing MHC-I single chain trimers
30 bearing and exchangeable peptide within the functional pMHC-I complex recognized by CD8⁺ T cells.

SUMMARY OF THE INVENTION

The present invention provides methods to overexpress large amounts (mg/L) of secreted pMHC-I protein as single chain trimer (SCT) bearing an exchangeable peptide (> 20-mer) within the functional pMHC-I complex recognized by CD8⁺ T cells.

5 Using a Baculovirus expression vector system, the inventors provide in a preferred embodiment a nucleic acid construct that encodes a MHC-I SCT consisting of (a) a secretion leader upstream of a peptide bearing an enterokinase cleavable linker to the conserved human $\beta 2m$ and a GS-linker to the polymorphic heavy chain bearing a BirA motif for streptavidin tetramerization, and (b) a nucleic acid region bearing unique BamHI and XbaI sites, encoding
10 56 amino acids (flanking the peptide ligand) for peptide-screening to optimize high yield pMHC-I protein secretion without the need of refolding. In the present invention, the nucleic acid construct not only promotes the secretion of large amounts of pMHC-I protein into the growth media for rapid recovery but also allows peptide reloading upon enterokinase treatment of the pMHC-I SCT protein. The novel constructs are functional as demonstrated by efficient
15 and specific detection of antigen-specific T cells after reloading with relevant peptide antigen(s) and use to stain PBMC from healthy donors with a pre-existing T cell response.

According to a first aspect of the present invention, there is a single chain fusion protein comprising, in order from N-terminus to C-terminus of the fusion protein;

- (i) a peptide ligand that stabilizes the MHC-I protein prior to enzyme cleavage;
- 20 (ii) a first linker polypeptide comprising an enzyme-cleavable portion;
- (iii) a $\beta 2m$ polypeptide;
- (iv) a second linker polypeptide; and
- (v) a mature MHC-I heavy chain polypeptide.

In some embodiments the peptide ligand is a stabilizing peptide.

25 In some embodiments the first linker polypeptide is cleavable by an enzyme selected from the group comprising or consisting of Enterokinase, Thrombin, Caspase 1, Caspase 2, Caspase 3, Caspase 4, Caspase 5, Caspase 6, Caspase 7, Caspase 8, Caspase 9, Caspase 10, Factor Xa and Granzyme B.

30 In some embodiments the first linker polypeptide comprises or consists of about 15, 16, 17, 18, 19, 20 or 21 or more amino acid residues, more preferably about 21 amino acid residues.

In some embodiments the first and second polypeptide linkers comprise at least about 80 percent glycine, alanine and/or serine residues. In some embodiments the first and second polypeptide linkers consist of at least 80 percent glycine, alanine and/or serine residues.

5 In some embodiments the first linker polypeptide comprises or consists of the amino acid sequence set forth in SEQ ID NO: 2.

In some embodiments the second linker polypeptide comprises or consists of about 15, 16, 17, 18, 19 or 20 or more amino acid residues, more preferably about 20 amino acid residues.

10 In some embodiments the second linker polypeptide comprises or consists of the amino acid sequence set forth in SEQ ID NO: 3.

In some embodiments the peptide ligand comprises from about 4 to 30 amino acid residues. In some embodiments the peptide ligand consists of 4 to 30 amino acid residues.

15 In some embodiments the peptide ligand comprises from about 6 to 20 amino acid residues, preferably about 8 to 15 amino acid residues. In some embodiments the peptide ligand consists of 8 to 15 amino acid residues.

20 In some embodiments the peptide ligand is selected from the group comprising or consisting of the amino acid sequence set forth in SEQ ID NO: 4 (AVFAAASDAK), SEQ ID NO: 5 (AVFDRKSDAK), SEQ ID NO: 6 (KILGRVFFV), SEQ ID NO: 7 (KLAEAIFKL) and SEQ ID NO: 8 (YAETAAFAY). It is understood that the stabilizing peptide may be antigenic or non-antigenic and any antigenicity of the peptide ligand may be abolished by the enzyme cleaved linker, which weakens its binding affinity to the MHC-I protein.

In some embodiments the β 2m polypeptide comprises or consists of the amino acid sequence set forth in SEQ ID NO: 9.

25 In some embodiments the class I heavy chain polypeptide is comprised or consists of an HLA-A, HLA-B, HLA-C, 1a, 1b, H-2-K, H-2-Dd or H-2-Ld heavy chain.

In some embodiments the MHC-I heavy chain polypeptide comprises or consists of the amino acid sequence set forth in SEQ ID NO: 10, SEQ ID NO: 11 or SEQ ID NO: 12.

30 In some embodiments the fusion protein further comprises a GS-linker having the amino acid sequence PGS preceding a BirA motif having the amino acid sequence set forth in SEQ ID NO: 13 at the C-terminal end of the MHC-I heavy chain polypeptide, for streptavidin tetramerization and, preferably, also a His6x peptide having the amino acid sequence set forth in SEQ ID NO: 14.

In some embodiments the fusion protein comprises or consists of an amino acid sequence selected from the group comprising or consisting of SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, SEQ ID NO: 18 and SEQ ID NO: 19.

5 According to a second aspect of the present invention, there is provided a complexed multimer of the single chain fusion protein of any aspect of the invention, wherein the multimer comprises or consists of a plurality of said single chain fusion protein.

In some embodiments the complexed multimer comprises or consists of a dimer, trimer, tetramer or pentamer of said single chain fusion protein.

10 In some embodiments said single chain fusion proteins are complexed with, for example, streptavidin or other complexing agent. In preferred embodiments the single chain fusion proteins are complexed with streptavidin.

According to a third aspect of the present invention, there is provided an isolated recombinant DNA molecule comprising or consisting of a DNA sequence encoding a single chain fusion protein of any one of claims 1 to 17.

15 In some embodiments the DNA sequence further encodes a secretion leader polypeptide, preferably a secretion leader polypeptide such as Mellitin comprising or consisting of the amino acid sequence set forth in SEQ ID NO: 1.

It would be understood that due to the redundancy in the genetic code, a nucleic acid sequence may have less than 100% identity and still encode the same amino acid sequence.

20 In some embodiments the DNA sequence encoding the secretion leader of Mellitin has, due to redundancy in the genetic code, at least 85%, at least 90%, at least 95% or 100% nucleic acid sequence identity to the nucleic acid sequence set forth in SEQ ID NO: 20.

In some embodiments the DNA sequence encodes for a secretion leader polypeptide, a peptide ligand and a first linker polypeptide with a combined length of about 60 amino acids or less, preferably of about 55 amino acids or less, more preferably of about 50 amino acids. In some embodiments the combined length is 50 amino acids.

In some embodiments the DNA sequence encoding the first linker has, due to redundancy in the genetic code, at least 85%, at least 90%, at least 95% or 100% nucleic acid sequence identity to the nucleic acid sequence set forth in SEQ ID NO: 21.

30 In some embodiments the DNA sequence encoding the second linker has, due to redundancy in the genetic code, at least 85%, at least 90%, at least 95% or 100% nucleic acid sequence identity to the nucleic acid sequence set forth in SEQ ID NO: 22.

In some embodiments the DNA sequence encoding the peptide ligand has, due to redundancy in the genetic code, at least 85%, at least 90%, at least 95% or 100% nucleic acid sequence identity to the nucleic acid sequence set forth in SEQ ID NO: 23, SEQ ID NO: 24, SEQ ID NO: 25, SEQ ID NO: 26 or SEQ ID NO: 27.

5 In some embodiments the DNA sequence encoding the β 2m polypeptide has, due to redundancy in the genetic code, at least 85%, at least 90%, at least 95% or 100% nucleic acid sequence identity to the nucleic acid sequence set forth in SEQ ID NO: 28.

10 In some embodiments the DNA sequence encoding the MHC-I heavy chain polypeptide has, due to redundancy in the genetic code, at least 85%, at least 90%, at least 95% or 100% nucleic acid sequence identity to the nucleic acid sequence set forth in SEQ ID NO: 29, SEQ ID NO: 30 or SEQ ID NO: 31.

15 In some embodiments the DNA sequence encoding the third GS-linker has, due to redundancy in the genetic code, at least 85%, at least 90%, at least 95% or 100% nucleic acid sequence identity to the nucleic acid sequence CCGGGTAGT and/ or the DNA sequence encoding the BirA motif has, due to redundancy in the genetic code, at least 85%, at least 90%, at least 95% or 100% nucleic acid sequence identity to the nucleic acid sequence set forth in SEQ ID NO: 32 and/or the DNA sequence encoding the His6x peptide has, due to redundancy in the genetic code, at least 85%, at least 90%, at least 95% or 100% nucleic acid sequence identity to the nucleic acid sequence set forth in SEQ ID NO: 33.

20 In some embodiments the DNA sequence encoding the fusion protein has, due to redundancy in the genetic code, at least 85%, at least 90%, at least 95% or 100% nucleic acid sequence identity to the nucleic acid sequence set forth in SEQ ID NO: 34, SEQ ID NO: 35, SEQ ID NO: 36, SEQ ID NO: 37 or SEQ ID NO: 38.

25 According to a fourth aspect of the present invention, there is provided an expression vector comprising the recombinant DNA molecule defined in any aspect of the invention.

According to a fifth aspect of the present invention, there is provided use of an expression vector according to the third aspect for the recombinant production of secreted fusion proteins.

30 According to a sixth aspect of the present invention, there is provided a method for the production of recombinant secreted fusion proteins, wherein the fusion proteins are defined in the first aspect, comprising the steps:

(i) cultivating a eukaryotic or prokaryotic cell that has been transfected with a recombinant DNA molecule as defined in the second aspect, or an expression vector defined in the third aspect in a cultivation medium and

(ii) recovering the recombinant secreted fusion proteins from the cell or the cultivation medium.

In some embodiments the cell is a eukaryotic cell and has been infected with a recombinant baculovirus expressing said expression vector. In some embodiments the eukaryotic cell is a mammalian cell. In some embodiments the mammalian cell is Chinese hamster ovary cell or human kidney cell.

In some embodiments the eukaryotic cell is an insect cell. In some embodiments the insect cell is preferably a Sf9 or Sf21 cell from *Spodoptera frugiperda* or Hi5 cell from *Trichoplusia ni*.

According to a seventh aspect of the present invention, there is provided a method of defining a peptide ligand suitable for successful production of a single chain fusion protein for peptide exchange according to the first aspect, comprising the steps:

i) identify an antigenic or non-antigenic peptide, known to bind to a MHC-I protein of the single chain fusion protein, of from about 4 to 30 amino acid residues, from about 6 to 20 amino acid residues or preferably from about 8 to 15 amino acid residues in length, and its amino acid sequence;

(ii) modify one or more of the amino acids of the peptide that are not interacting with a HLA binding groove of the MHC-1 protein until the peptide sequence, while retaining a HLA binding motif, does not map to any known antigen and/or is not recognized by T cells anymore,

wherein preferably the modified amino acids are mutated to or substituted by neutral non-polar residues such as alanine and are exposed out of the HLA binding groove;

(iii) produce monomers of the single chain fusion protein and verify proper binding of peptide ligand to MHC-I based on monomer secretion.

In some embodiments the peptide ligand is a stabilizing ligand.

According to an eighth aspect of the present invention, there is provided a method of peptide exchange comprising the steps:

i) providing a fusion protein of the first aspect or a complexed multimer of the second aspect;

ii) co-incubating, for a period of time, the fusion protein or complexed multimer with a peptide of interest and a cleavage enzyme that will cleave said first linker polypeptide, independent of the order of the components added,

wherein said cleavage results in exchange of the peptide ligand with the peptide of interest, thereby generating a distinctively-labeled, soluble MHC-I monomer or complexed multimer loaded with the peptide of interest.

In some embodiments the peptide of interest is a peptide corresponding to viral-derived epitope.

In some embodiments step ii) is performed in an exchange buffer between about pH 5 and about pH 8.0, for a period of about 1 h to about 16 h, at a temperature of between about 15°C to about 37°C to release the peptide ligand and allow rescue peptide binding.

According to a ninth aspect of the present invention, there is provided use of a fusion protein of the first aspect or a complexed multimer of the second aspect to detect, isolate or manipulate antigen-specific CD8⁺ T cells.

In some embodiments the complexed multimer is a tetramer.

In some embodiments the antigen-specific CD8⁺ T cells are specific for a particular antigenic peptide derived from viruses, bacteria and self-antigen or tumours.

BRIEF DESCRIPTION OF THE FIGURES

Figures 1A-B show the experimental design and results for the validation of enzyme exchangeable HLA-A*11:01 with exchangeable peptide AVFAAASDAK (SEQ ID NO: 4). Figure 1A shows a table of the experimental design for the validation of enzyme exchangeable HLA-A*11:01 with exchangeable peptide AVFAAASDAK (SEQ ID NO: 4) for tetramer construction and binding to antigen-specific CD8⁺ T cells. Figure 1B shows the results. Peptides corresponding to viral-derived epitopes were loaded into the new HLA construct after enzyme-mediated cleavage of the stabilizing peptide; they were also loaded into classical HLA monomer constructs using standard UV exchange protocols. The two types of monomers were then tetramerized and tested on PBMC from healthy donor for detection of peptide-specific CD8⁺ T cells and also level of background staining. The donor PBMC contains peptide 4 and peptide 5-specific CD8⁺ T cells. Data shown above are gated on live CD45⁺CD3⁺CD8⁺ cells. The new HLA-A*11:01 construct with exchangeable peptide AVFAAASDAK (SEQ ID NO: 4) allows an efficient peptide exchange after enzymatic cleavage of the stabilizing peptide, as the level of detection of peptide-specific T cells is comparable to that obtained with the UV-exchanged construct (0.49-0.75% versus 0.5-0.76% of CD8⁺ T cells, respectively).

Figure 2 shows the experimental design and results for the validation of enzyme exchangeable HLA-A*11:01 with exchangeable peptide AVFAAASDAK (SEQ ID NO: 4) for multiplexed tetramer staining on mass cytometer. **(A)** UV exchangeable and enzyme exchangeable HLA-A*11:01 were loaded with peptides shown in (A). Triple coded tetramers were formed. Streptavidin codes are as shown in the table (A). Healthy HLA-A*11:01 specific PBMCs were stained with a cocktail of multiplexed-triple coded HLA-A*11:01 tetramer, anti-CD45, anti-CD3, anti-CD8 and live-dead staining followed by acquiring on mass cytometer. **(B)** Data shown are gated on Live⁺CD45⁺CD3⁺CD8⁺ cells. Dot plot indicates EBV peptide IVTDFSVIK (SEQ ID NO: 39)-specific CD45⁺CD3⁺CD8⁺ cells. **(C)** Data shown are gated on Live⁺CD45⁺CD3⁺CD8⁺ cells. Dot plot indicates EBV peptide NTLEQTVKK (SEQ ID NO: 40)-specific CD45⁺CD3⁺CD8⁺ cells. **(D)** Data shown are gated on Live⁺CD45⁺CD3⁺CD8⁺ cells. Dot plot indicates Influenza peptide AVFDRKSDAK (SEQ ID NO: 5) specific CD45⁺CD3⁺CD8⁺ cells. Overall, New HLA-A*11:01 construct with exchangeable peptide AVFAAASDAK (SEQ ID NO: 4) allows an efficient peptide exchange after enzymatic cleavage of the stabilizing peptide, as well as allow triple coded-multiplexed tetramer staining. The level of detection of EBV peptide 1, EBV peptide 2 and Influenza Peptide 1 specific T cells is comparable to that obtained with the UV-exchanged construct. (0.74% versus 0.66% of CD8⁺ T cells for EBV peptide 1, 0.027% versus 0.03% of CD8⁺ T cells for EBV Peptide 2 and 0.68% versus 0.5% of CD8⁺ T cells for Influenza Peptide 1, respectively)

Figures 3A-B show the experimental design and results for the validation of enzyme exchangeable HLA-A*02:01 with exchangeable peptide KILGRVFFV (SEQ ID NO: 6) for tetramer construction and binding to antigen-specific CD8⁺ T cells. Peptide 1, 2, 3 and 15 corresponding to viral-derived epitopes were loaded into the new HLA construct after enzyme-mediated cleavage of the stabilizing peptide using 4 different exchange protocols as indicated; Peptides were also loaded into classical HLA monomer constructs using standard UV exchange protocol. The various monomers were then tetramerized and tested on PBMC from healthy donor for detection of peptide-specific CD8⁺ T cells and also level of background staining. The donor PBMC contains peptide 1-specific CD8⁺ T cells. Data shown are gated on live CD45⁺CD3⁺CD8⁺ cells. Overall, the new construct allows an efficient peptide exchange after enzymatic cleavage of the stabilizing peptide; the best protocols for enzymatic exchange are pH 6.2 cacodylate buffer at RT and pH 8 TRIS buffer at RT, as they allow for high resolution of the peptide-specific CD8⁺ T cell population. The level of detection of peptide 1-specific CD8⁺ T cells is comparable to that obtained with the UV-exchanged tetramers (1.14-1.34% versus 0.98-1.21% of CD8⁺ T cells, respectively).

Figures 4A-B show the experimental design and results for the validation of enzyme exchangeable HLA-A*02:01 with exchangeable peptide KLAEAIFKL (SEQ ID NO: 7) for

tetramer construction and binding to antigen-specific CD8⁺ T cells. Peptide 1, 2, and 11 corresponding to viral-derived epitopes were loaded into the new HLA construct after enzyme-mediated cleavage of the stabilizing peptide using 2 different exchange protocols as indicated; Peptides were also loaded into classical HLA monomer constructs using standard UV exchange protocol. The various monomers were then tetramerized and tested on PBMC from healthy donor for detection of peptide-specific CD8⁺ T cells and also level of background staining. The donor PBMC contains peptide 1-specific CD8⁺ T cells. Data shown are gated on live CD45⁺CD3⁺CD8⁺ cells. Overall, the new HLA-A*02:01 construct with exchangeable peptide KLAEAIFKL (SEQ ID NO: 7) allows efficient peptide exchange after enzymatic cleavage of the stabilizing peptide; the best protocols for enzymatic exchange are pH 6.2 cacodylate buffer at RT and pH 8 TRIS buffer at RT, as they allow for high resolution of the peptide-specific CD8⁺ T cell population. The level of detection of peptide 1-specific CD8⁺ T cells is comparable to that obtained with the UV-exchanged tetramers (0.92-1.38% versus 1.14-1.26% of CD8⁺ T cells, respectively).

Figure 5 shows the experimental design and results for the validation of enzyme exchangeable HLA-A*02:01 with exchangeable peptide KLAEAIFKL (SEQ ID NO: 7) for multiplexed tetramer staining on mass cytometer. UV exchangeable and enzyme exchangeable HLA-A*02:01 were loaded with different peptides as shown in figure (A). Triple coded tetramers were formed. Streptavidin codes are as shown in the table (A). Healthy HLA-A*02:01 PBMCs were stained with a cocktail of multiplexed-triple coded HLA-A*02:01 tetramers, anti-CD45, anti-CD3, anti-CD8⁺ and live-dead staining followed by acquiring on mass cytometer **(B)** Data shown are gated on Live⁺CD45⁺CD3⁺CD8⁺ cells. Dot plot indicates CMV peptide NLVPMVATV (SEQ ID NO: 41)-specific CD45⁺CD3⁺CD8⁺ cells. Overall, New HLA-A*02:01 construct with exchangeable peptide KLAEAIFKL (SEQ ID NO: 7) allows an efficient peptide exchange after enzymatic cleavage of the stabilizing peptide, as well as allow triple coded-multiplexed tetramer staining. The level of detection of CMV peptide 1 specific T cells is comparable to that obtained with the UV-exchanged construct (1.28% versus 1.08% for CMV peptide 1).

Figure 6 shows results for the validation of enzyme exchangeable HLA-A*01:01 with exchangeable peptide YAETAAFAY (SEQ ID NO: 8) for tetramer construction and binding to antigen-specific CD8⁺ T cells. CMV-derived peptide VETEHDITLLY (SEQ ID NO: 42) was loaded into the new HLA construct after enzyme-mediated cleavage of the stabilizing peptide YAETAAFAY (SEQ ID NO: 8); it was also loaded into classical HLA monomer constructs using standard UV exchange protocols. The two types of monomers were then tetramerized with streptavidin and tested on PBMC from healthy donor for detection of CMV peptide VETEHDITLLY (SEQ ID NO: 42)-specific CD8⁺ T cells and also level of background staining.

Data shown above are gated on live CD45⁺CD3⁺ cells. The new HLA-A*01:01 construct with exchangeable peptide YAETAAFAY (SEQ ID NO: 8) allows an efficient peptide exchange after enzymatic cleavage of the stabilizing peptide, as the level of detection of peptide-specific T cells is comparable to that obtained with the UV-exchanged construct (1.25% versus 1.5% of CD8⁺ T cells, respectively).

Definitions

Certain terms employed in the specification, examples and appended claims are collected here for convenience.

As used in the specification and the appended claims, the singular forms “a,” “an” and “the” include plural referents unless the context clearly dictates otherwise.

As used herein, ranges can be expressed as from “about” one particular value, and/or to “about” another particular value. When such a range is expressed, another embodiment includes from the one particular value and/or to the other particular value. Similarly, when values are expressed as approximations, by use of the antecedent “about,” it will be understood that the particular value forms another embodiment. It will be further understood that the endpoints of each of the ranges are significant both in relation to the other endpoint, and independently of the other endpoint. It is also understood that there are a number of values disclosed herein, and that each value is also herein disclosed as “about” that particular value in addition to the value itself. For example, if the value “10” is disclosed, then “about 10” is also disclosed. It is also understood that when a value is disclosed that “less than or equal to” the value, “greater than or equal to the value” and possible ranges between values are also disclosed, as appropriately understood by the skilled artisan. For example, if the value “10” is disclosed the “less than or equal to 10” as well as “greater than or equal to 10” is also disclosed. It is also understood that each unit between two particular units are also disclosed. For example, if 10 and 15 are disclosed, then 11, 12, 13, and 14 are also disclosed.

The terms “amino acid” or “amino acid sequence,” as used herein, refer to an oligopeptide, peptide, polypeptide, or protein sequence, or a fragment of any of these, and to naturally occurring or synthetic molecules. Where “amino acid sequence” is recited herein to refer to an amino acid sequence of a naturally occurring protein molecule, “amino acid sequence” and like terms are not meant to limit the amino acid sequence to the complete native amino acid sequence associated with the recited protein molecule.

As used herein, the terms “polypeptide”, “peptide” or “protein” refer to one or more chains of amino acids, wherein each chain comprises amino acids covalently linked by peptide bonds, and wherein said polypeptide or peptide can comprise a plurality of chains non-covalently and/or covalently linked together by peptide bonds, having the sequence of native proteins, that is, proteins produced by naturally-occurring and specifically non-recombinant

cells, or genetically-engineered or recombinant cells, and comprise molecules having the amino acid sequence of the native protein, or molecules having deletions from, additions to, and/or substitutions of one or more amino acids of the native sequence. A "polypeptide", "peptide" or "protein" can comprise one (termed "a monomer") or a plurality (termed "a multimer") of amino acid chains.

As used herein, the term "stabilizing polypeptide" refers to an inert polypeptide which binds and/or associates with the antigen binding pocket of a MHC-I protein, thereby ensuring the conformation of the MHC-I protein until the stabilizing peptide can be cleaved off and replaced by a peptide of interest. It is understood that the stabilizing peptide may be antigenic or non-antigenic and any antigenicity of the peptide ligand may be abolished by the enzyme cleaved linker, which weakens its binding affinity to the MHC-I protein. In one example, the stabiliser protein may be cleaved off and replaced or exchanged by a peptide corresponding to viral-derived epitope.

As used herein, the term "comprising" or "including" is to be interpreted as specifying the presence of the stated features, integers, steps or components as referred to, but does not preclude the presence or addition of one or more features, integers, steps or components, or groups thereof. However, in context with the present disclosure, the term "comprising" or "including" also includes "consisting of". The variations of the word "comprising", such as "comprise" and "comprises", and "including", such as "include" and "includes", have correspondingly varied meanings.

DETAILED DESCRIPTION OF THE INVENTION

Bibliographic references mentioned in the present specification are for convenience listed at the end of the examples. The whole content of such bibliographic references is herein incorporated by reference.

EXAMPLES

A person skilled in the art will appreciate that the present invention may be practiced without undue experimentation according to the methods given herein. The methods, techniques and chemicals are as described in the references given or from protocols in standard biotechnology and molecular biology text books. Standard molecular biology techniques known in the art and not specifically described were generally followed as described in Sambrook and Russel, Molecular Cloning: A Laboratory Manual, Cold Springs Harbor Laboratory, New York (2001). Briefly, a melittin-leader bearing baculovirus expression vector is used to make the pMHC-I single chain trimer protein. In the single chain trimer design, the single chain module is separated by the first and second linkers, where the N-terminus

stabilizing peptide bears an additional cleavable DDDD|K peptide sequence (SEQ ID NO: 43) to aid peptide dissociation and exchange. Following standard bacmid preparation and infection of insect cells, different single chain pMHC-I proteins are overexpressed as secreted proteins in Sf9 (*Spodoptera frugiperda*) or High Five (*Trichoplusia ni*) cells. The secreted single chain pMHC-I proteins are purified, biotinylated and validated for tetramer staining of CD8⁺ T cells using flow and mass cytometry.

Materials and Methods

Engineering, production and purification of MHC-I single chain protein

A melittin-leader bearing baculovirus expression vector such as the PFASTBAC™1 vector (ThermoFisher) is used to make the secreted peptide-MHC (pMHC)-I single chain trimer protein. Following standard molecular cloning techniques, the gene encoding the single chain trimer is subcloned into the baculovirus expression vector. In the single chain trimer design in the order of peptide, β 2m and MHC-I heavy chain polypeptide, the single chain module is separated by the first and second linkers, where the N-terminus stabilizing peptide bears an additional cleavable DDDD|K peptide sequence (SEQ ID NO: 43) to aid peptide dissociation and exchange upon enterokinase addition. Following standard bacmid preparation and infection of insect cells, different single chain pMHC-I proteins are overexpressed as secreted proteins in Sf9 (*Spodoptera frugiperda*) or HIGH FIVE™ (*Trichoplusia ni*; BTI-Tn-5B1-4) cells using the BAC-TO-BAC™ Baculovirus Expression System (ThermoFisher). The secreted single chain pMHC-I proteins are purified from the culture media using the standard Ni-NTA resin purification in 20 mM Tris pH 8.0 and 150 mM NaCl buffer, eluted with imidazole and biotinylated with BirA enzyme as previously described (Fairhead M. and Howarth M. *Methods Mol Biol.* 1266: 171-84 (2015); incorporated herein by reference). The biotinylated protein is further purified in size exclusion chromatography using the SUPERDEX™ S200 gel filtration column in 20 mM Tris pH 8.0 and 150 mM NaCl buffer.

Peptide exchange

UV-cleavable pMHC molecules are produced in house as previously described (Newell EW, et al., *Nat Biotechnol.* (7): 623-9 (2013); Leong ML, and Newell EW. *Methods Mol Biol.* 1346: 115-31 (2015); incorporated herein by reference). For UV-mediated peptide exchange, UV cleavable pMHC-I are exposed to 365 nm UV irradiation for 15-min in the presence of a single peptide of interest. Peptide exchange reactions are set up in 96 well plates at least 12 h before tetramerization. For enzyme mediated peptide exchange, 0.17 μ l of enterokinase (16,000 U/ μ l) (NEB cat no. P8070S) is added to 10 μ l of 0.1 mg/ml enzyme-cleavable MHC-I single chain protein. Enzyme reaction is set at either pH 6 or pH 8 for at least

16 h at 37 °C or at room temperature (RT). To quench the enzyme reaction, a triple volume of 1 X PBS is added followed by 1 h incubation in ice.

Tetramerization

APC, PE and APC-Cy7-labelled streptavidins for flow cytometry staining are purchased from BioLegend (San Diego, CA, USA). For mass cytometry, streptavidin is produced in-house as described in (Newell EW, et al., *Nat Biotechnol.* (7): 623-9 (2013); incorporated herein by reference) and conjugated with heavy metals using DN3 polymer linker as reported previously (Leong ML, and Newell EW. *Methods Mol Biol.* 1346: 115-31 (2015); incorporated herein by reference). To produce pMHC tetramers, fluorescently or heavy metal labelled streptavidin or streptavidin mixtures are added to the respective pMHC complexes in three repeated steps (10 min incubation at RT per addition) so to achieve a total final molar ratio of 1 (streptavidin): 4 (pMHC). 10 µM free biotin (Sigma) is added to each reaction to quench free streptavidin molecules. In case of multiplexing experiments, tetramerized pMHC complexes are pulled together and concentrated by using a 10 kDa cut-off filter (Merck Millipore), buffer is exchanged with cytometry buffer (PBS, 2 % v/v FCS, 2 mM EDTA, 0.05 % v/v sodium azide), filtered through a 1 µm centrifugal filter (Merck Millipore) and used to stain the cells.

Cell staining

For pMHC tetramer staining experiments, PBMC samples from HLA-A*02:01, HLA-A*11:01 and HLA-A*01:01 positive healthy donors are used. For mass cytometry and flow cytometry, cells are stained with tetramers and surface antibodies as described earlier (Fehlings M, et al., *J Immunol Methods.* 453: 30-36 (2018); Simoni Y, et al., *Methods Mol Biol.* 1989: 147-158 (2019), incorporated herein by reference). Briefly, for mass cytometry, purified antibodies are tagged with heavy metal loaded maleimide conjugated DN3 MAXPAR® chelating polymers (Fluidigm) according to manufacturer's recommendations. Three to 5 million cells are transferred to 96 well plates and stained with 200 µM cisplatin (Sigma) for live/dead discrimination. 100 µl of tetramer cocktail is added to cisplatin-stained cells and incubated for 1 hour at RT followed by staining with 50 µl of heavy metal-labeled antibody cocktail for 30 min on ice. Stained cells are fixed overnight in 2 % v/v paraformaldehyde (Electron Microscopy Sciences) at 4 °C followed by labelling with 250 nM (1:2000) iridium DNA nucleic acid intercalator (Fluidigm) in 2 % v/v PFA in 1 X PBS at room temperature for 20 min. Finally, cells are washed and resuspended in distilled water at 0.5 million cells/ml cell concentration. For flow cytometry, 0.5 to 1 million cells are transferred to 96 well plates and stained with 100 µl of tetramer cocktail for 1 hour at RT, followed by staining with a cocktail of BV421 labelled anti-human CD45 (clone HI30), Alexa Fluor 700 labelled anti-human CD3

(clone OKT3), BV605 or FITC labelled anti-human CD8 (clone SK1) and LIVE/DEAD® fixable Aqua Dead Cell Stain (ThermoFisher) for the live/dead discrimination for 30 min on ice. Stained cells are resuspended in 1X PBS.

5 Data analysis

Fluorescently labelled samples are acquired by BD LSRFORTESSA™ and heavy metal labelled samples are acquired on a Fluidigm HELIOS™ mass cytometer. Mass cytometry data is converted to .fcs format by Fluidigm acquisition software. The signal of each parameter is normalized based on the EQ beads (Fluidigm) as described previously (Finck
10 R, et al., *Cytometry A*. 83(5): 483-94 (2013), incorporated herein by reference). FLOWJO™ software is used for flow as well as mass cytometry data analysis. For Flow data, CD8⁺ T cells are selected by manually gating on Live⁺CD45⁺CD3⁺CD8⁺ cells. For mass cytometry data, CD8⁺ T cells are selected by manually gating on cisplatin⁻ CD45⁺CD3⁺CD8⁺. Tetramer positive cell populations are then identified based on the fluorescently- or heavy metal-labelled
15 streptavidin assigned to the pMHC tetramer.

EXAMPLE 1

Peptides corresponding to viral-derived epitopes were loaded into the new HLA construct after enzyme-mediated cleavage of the stabilizing peptide; they were also loaded
20 into classical HLA monomer constructs using standard UV exchange protocols described above. The two types of monomers were then tetramerized and tested on PBMC from healthy donor for detection of peptide-specific CD8⁺ T cells and also level of background staining. The donor PBMC contains peptide 4 and peptide 5-specific CD8⁺ T cells. Figures 1A and 1B show the experimental design and results for the validation of enzyme exchangeable HLA-A*11:01
25 with exchangeable peptide AVFAAASDAK (SEQ ID NO: 4). Figure 1A shows a table of the experimental design for the validation of enzyme exchangeable HLA-A*11:01 with exchangeable peptide AVFAAASDAK (SEQ ID NO: 4) for tetramer construction and binding to antigen-specific CD8⁺ T cells. Figure 1B shows the results. Data shown above are gated on live CD45⁺CD3⁺CD8⁺ cells. The new HLA-A*11:01 construct with exchangeable peptide
30 AVFAAASDAK (SEQ ID NO: 4) allows an efficient peptide exchange after enzymatic cleavage of the stabilizing peptide, as the level of detection of peptide-specific T cells is comparable to that obtained with the UV-exchanged construct (0.49-0.75% versus 0.5-0.76% of CD8⁺ T cells, respectively).

EXAMPLE 2

The experimental design and results for validation of enzyme exchangeable HLA-A*11:01 with exchangeable peptide AVFAAASDAK (SEQ ID NO: 4) for multiplexed tetramer staining on mass cytometer are shown in Figure 2. Methods for exchange are described above. UV exchangeable and enzyme exchangeable HLA-A*11:01 were loaded with peptides shown in (A). Triple coded tetramers were formed. Streptavidin codes are as shown in the table. Healthy HLA-A*11:01 specific PBMCs were stained with a cocktail of multiplexed-triple coded HLA-A*11:01 tetramer, anti-CD45, anti-CD3, anti-CD8 and live-dead staining followed by acquiring on mass cytometer using methods described above. The Figure 2B data shown are gated on Live⁺CD45⁺CD3⁺CD8⁺ cells, and the Dot plot indicates EBV peptide IVTDFSVIK (SEQ ID NO: 39)-specific CD45⁺CD3⁺CD8⁺ cells. The Figure 2C data shown are gated on Live⁺CD45⁺CD3⁺CD8⁺ cells, and the Dot plot indicates EBV peptide NTLEQTVKK (SEQ ID NO: 40)-specific CD45⁺CD3⁺CD8⁺ cells. The Figure 2B data shown are gated on Live⁺CD45⁺CD3⁺CD8⁺ cells, and the Dot plot indicates Influenza peptide AVFDRKSDAK (SEQ ID NO: 5) specific CD45⁺CD3⁺CD8⁺ cells. Overall, the new HLA-A*11:01 construct with exchangeable peptide AVFAAASDAK (SEQ ID NO: 4) allows an efficient peptide exchange after enzymatic cleavage of the stabilizing peptide, as well as allows triple coded-multiplexed tetramer staining. The level of detection of EBV peptide 1, EBV peptide 2 and Influenza Peptide 1 specific T cells is comparable to that obtained with the UV-exchanged construct. (0.74% versus 0.66% of CD8⁺ T cells for EBV peptide 1, 0.027% versus 0.03% of CD8⁺ T cells for EBV Peptide 2 and 0.68% versus 0.5% of CD8⁺ T cells for Influenza Peptide 1, respectively)

EXAMPLE 3

The experimental design and results for validation of enzyme exchangeable HLA-A*02:01 with exchangeable peptide KILGRVFFV (SEQ ID NO: 6) for tetramer construction and binding to antigen-specific CD8⁺ T cells are shown in Figure 3. Peptides 1, 2, 3 and 15, corresponding to viral-derived epitopes, were loaded into the new HLA construct after enzyme-mediated cleavage of the stabilizing peptide using 4 different exchange protocols as indicated; Peptides were also loaded into classical HLA monomer constructs using standard UV exchange protocol described above. The various monomers were then tetramerized as described above and tested on PBMC from healthy donor for detection of peptide-specific CD8⁺ T cells and also level of background staining. The donor PBMC contains peptide 1-specific CD8⁺ T cells. The data shown in Figure 3B are gated on live CD45⁺CD3⁺CD8⁺ cells. Overall, the new construct allows an efficient peptide exchange after enzymatic cleavage of the stabilizing peptide; the best protocols for enzymatic exchange are pH 6.2 cacodylate buffer

at RT and pH 8 TRIS buffer at RT, as they allow for high resolution of the peptide-specific CD8⁺ T cell population. The level of detection of peptide 1-specific CD8⁺ T cells is comparable to that obtained with the UV-exchanged tetramers (1.14-1.34% versus 0.98-1.21% of CD8⁺ T cells, respectively).

5 EXAMPLE 4

The experimental design and results for validation of enzyme exchangeable HLA-A*02:01 with exchangeable peptide KLAEAIFKL (SEQ ID NO: 7) for tetramer construction and binding to antigen-specific CD8⁺ T cells are shown in Figure 4. Peptides 1, 2, and 11 corresponding to viral-derived epitopes were loaded into the new HLA construct after enzyme-mediated cleavage of the stabilizing peptide using 2 different exchange protocols as indicated; Peptides were also loaded into classical HLA monomer constructs using standard UV exchange protocol described above. The various monomers were then tetramerized as described above and tested on PBMC from healthy donor for detection of peptide-specific CD8⁺ T cells and also level of background staining. The donor PBMC contains peptide 1-specific CD8⁺ T cells. The data shown in Figure 4B are gated on live CD45⁺CD3⁺CD8⁺ cells. Overall, the new HLA-A*02:01 construct with exchangeable peptide KLAEAIFKL (SEQ ID NO: 7) allows efficient peptide exchange after enzymatic cleavage of the stabilizing peptide; the best protocols for enzymatic exchange are pH 6.2 cacodylate buffer at RT and pH 8 TRIS buffer at RT, as they allow for high resolution of the peptide-specific CD8⁺ T cell population. The level of detection of peptide 1-specific CD8⁺ T cells is comparable to that obtained with the UV-exchanged tetramers (0.92-1.38% versus 1.14-1.26% of CD8⁺ T cells, respectively).

EXAMPLE 5

The experimental design and results for the validation of enzyme exchangeable HLA-A*02:01 with exchangeable peptide KLAEAIFKL (SEQ ID NO: 7) for multiplexed tetramer staining on mass cytometer are shown in Figure 5. UV exchangeable and enzyme exchangeable HLA-A*02:01 were loaded with different peptides as per the method described above, and as shown in Figure 5A. Triple coded tetramers were formed. Streptavidin codes are as shown in the table. Healthy HLA-A*02:01 PBMCs were stained with a cocktail of multiplexed-triple coded HLA-A*02:01 tetramers, anti-CD45, anti-CD3, anti-CD8⁺ and live-dead staining followed by acquiring on mass cytometer, as described above. Data shown are gated on Live⁺CD45⁺CD3⁺CD8⁺ cells. Dot plot indicates CMV peptide NLVPMVATV (SEQ ID NO: 41)-specific CD45⁺CD3⁺CD8⁺ cells (Figure 5B). Overall, New HLA-A*02:01 construct with exchangeable peptide KLAEAIFKL (SEQ ID NO: 7) allows an efficient peptide exchange after enzymatic cleavage of the stabilizing peptide, as well as allow triple coded-multiplexed

tetramer staining. The level of detection of CMV peptide 1 specific T cells is comparable to that obtained with the UV-exchanged construct (1.28% versus 1.08% for CMV peptide 1).

EXAMPLE 6

Validation of enzyme exchangeable HLA-A*01:01 with exchangeable peptide
5 YAETAAFAY (SEQ ID NO: 8) for tetramer construction and binding to antigen-specific CD8⁺
T cells is shown in Figure 6. CMV-derived peptide VETEHDTLLY (SEQ ID NO: 42) was loaded
into the new HLA construct after enzyme-mediated cleavage of the stabilizing peptide
YAETAAFAY (SEQ ID NO: 8); it was also loaded into classical HLA monomer constructs using
10 standard UV exchange protocols described above. The two types of monomers were then
tetramerized with streptavidin as previously described, and tested on PBMC from healthy
donor for detection of CMV peptide VETEHDTLLY (SEQ ID NO: 42)-specific CD8⁺ T cells and
also level of background staining. Data shown are gated on live CD45⁺CD3⁺ cells. The new
HLA-A*01:01 construct with exchangeable peptide YAETAAFAY (SEQ ID NO: 8) allows an
efficient peptide exchange after enzymatic cleavage of the stabilizing peptide, as the level of
15 detection of peptide-specific T cells is comparable to that obtained with the UV-exchanged
construct (1.25% versus 1.5% of CD8⁺ T cells, respectively).

References

Any listing or discussion of an apparently prior-published document in this specification
should not necessarily be taken as an acknowledgement that such document is part of the
20 state of the art or is common general knowledge.

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CLAIMS

1. A single chain fusion protein comprising, in order from N-terminus to C-terminus of the fusion protein;
 - (i) a peptide ligand;
 - (ii) a first linker polypeptide comprising an enzyme-cleavable portion;
 - 5 (iii) a β -2 microglobulin (β 2m) polypeptide;
 - (iv) a second linker polypeptide; and
 - (v) a mature Major Histocompatibility Complex Class I (MHC-I) heavy chain polypeptide.
2. The fusion protein of claim 1, wherein the peptide ligand is a stabilizing peptide.
3. The fusion protein of claim 1 or 2, wherein the first linker polypeptide comprises preferably
10 about 15, 16, 17, 18, 19, 20 or 21 or more amino acid residues, more preferably about 21 amino acid residues and/or the second linker polypeptide comprises preferably about 15, 16, 17, 18, 19 or 20 or more amino acid residues, more preferably about 20 amino acid residues.
4. The fusion protein of any one of claims 1 to 3, wherein the first and second linker
15 polypeptides comprise at least about 80 percent glycine, alanine and/or serine residues.
5. The fusion protein of any one of claims 1 to 4, wherein the first linker polypeptide comprises the amino acid sequence set forth in SEQ ID NO: 2 and/or the second linker polypeptide comprises the amino acid sequence set forth in SEQ ID NO: 3.
6. The fusion protein of any one of claims 1 to 5, wherein the peptide ligand comprises:
20
 - i) from about 4 to 30 amino acid residues; or
 - ii) from about 6 to 20 amino acid residues; or
 - iii) from about 8 to 15 amino acid residues.
7. The fusion protein of any one of claims 1 to 6, wherein the peptide ligand is selected from the group comprising the amino acid sequence set forth in SEQ ID NO: 4 (AVFAAASDAK),
25 SEQ ID NO: 5 (AVFDRKSDAK), SEQ ID NO: 6 (KILGRVFFV), SEQ ID NO: 7 (KLAEAIFKL) and SEQ ID NO: 8 (YAETAAFAY).
8. The fusion protein of any one of claims 1 to 7, wherein the β 2m polypeptide comprises the amino acid sequence set forth in SEQ ID NO: 9.

9. The fusion protein of any one of claims 1 to 8, wherein the class I heavy chain polypeptide is comprised of an HLA-A, HLA-B, HLA-C, 1a, 1b, H-2-K, H-2-Dd or H-2-Ld heavy chain.
10. The fusion protein of any one of claims 1 to 9, wherein the MHC-I heavy chain polypeptide comprises the amino acid sequence set forth in SEQ ID NO: 10, SEQ ID NO: 11 or SEQ ID NO: 12.
11. The fusion protein of any one of claims 1 to 10, further comprising a GS-linker polypeptide having the amino acid sequence PGS preceding a BirA motif having the amino acid sequence set forth in SEQ ID NO: 13 at the C-terminal end of the MHC-I heavy chain polypeptide, for streptavidin tetramerization and, preferably, also a His6x peptide having the amino acid sequence set forth in SEQ ID NO: 14.
12. The fusion protein of any one of claims 1 to 11, wherein the fusion protein comprises the amino acid sequence set forth in SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, SEQ ID NO: 18 or SEQ ID NO: 19.
13. A complexed multimer of the single chain fusion protein of any one of the preceding claims, wherein the multimer comprises a plurality of said single chain fusion protein.
14. The complexed multimer of claim 13, comprising a dimer, trimer, tetramer or pentamer of said single chain fusion protein.
15. The complexed multimer of claim 13 or 14, wherein said single chain fusion proteins are complexed with, for example, streptavidin or other complexing agent.
16. An isolated recombinant DNA molecule comprising a DNA sequence encoding a single chain fusion protein of any one of claims 1 to 12.
17. The recombinant DNA molecule of claim 16, wherein the DNA sequence further encodes a secretion leader polypeptide, preferably a secretion leader polypeptide such as Mellitin comprising the amino acid sequence set forth in SEQ ID NO: 1.
18. The recombinant DNA molecule of claim 17, wherein the DNA sequence encoding the secretion leader of Mellitin has, due to redundancy in the genetic code, at least 85%, at least 90%, at least 95% or 100% nucleic acid sequence identity to the nucleic acid sequence set forth in SEQ ID NO: 20.
19. The recombinant DNA molecule of claim 17 or 18, wherein the DNA sequence encodes for a secretion leader polypeptide, a peptide ligand and a first linker polypeptide with a combined length of about 60 amino acids or less, preferably of about 55 amino acids or less, more preferably of about 50 amino acids.
20. The recombinant DNA molecule of any one of claims 16 to 19, wherein:

the DNA sequence encoding the first linker has, due to redundancy in the genetic code, at least 85%, at least 90%, at least 95% or 100% nucleic acid sequence identity to the nucleic acid sequence set forth in SEQ ID NO: 21; and/or

the DNA sequence encoding the second linker has, due to redundancy in the genetic code, at least 85%, at least 90%, at least 95% or 100% nucleic acid sequence identity to the nucleic acid sequence set forth in SEQ ID NO: 22; and/or

the DNA sequence encoding the peptide ligand has, due to redundancy in the genetic code, at least 85%, at least 90%, at least 95% or 100% nucleic acid sequence identity to the nucleic acid sequence set forth in SEQ ID NO: 23, SEQ ID NO: 24, SEQ ID NO: 25, SEQ ID NO: 26 or SEQ ID NO: 27; and/or

the DNA sequence encoding the β 2m polypeptide has, due to redundancy in the genetic code, at least 85%, at least 90%, at least 95% or 100% nucleic acid sequence identity to the nucleic acid sequence set forth in SEQ ID NO: 28; and/or

the DNA sequence encoding the MHC-I heavy chain polypeptide has, due to redundancy in the genetic code, at least 85%, at least 90%, at least 95% or 100% nucleic acid sequence identity to the nucleic acid sequence set forth in SEQ ID NO: 29, SEQ ID NO: 30 or SEQ ID NO: 31.

21. The recombinant DNA molecule of any one of claims 16 to 20, wherein the DNA sequence encoding the GS-linker has, due to redundancy in the genetic code, at least 85%, at least 90%, at least 95% or 100% nucleic acid sequence identity to CCGGGTAGT and/ or the DNA sequence encoding the BirA motif has, due to redundancy in the genetic code, at least 85%, at least 90%, at least 95% or 100% nucleic acid sequence identity to the nucleic acid sequence set forth in SEQ ID NO: 32 and/or the DNA sequence encoding the His₆x peptide has, due to redundancy in the genetic code, at least 85%, at least 90%, at least 95% or 100% nucleic acid sequence identity to the nucleic acid sequence set forth in SEQ ID NO: 33.

22. The recombinant DNA molecule of any one of claims 16 to 21, wherein the DNA sequence encoding the fusion protein has, due to redundancy in the genetic code, at least 85%, at least 90%, at least 95% or 100% nucleic acid sequence identity to the nucleic acid sequence set forth in SEQ ID NO: 34, SEQ ID NO: 35, SEQ ID NO: 36, SEQ ID NO: 37 or SEQ ID NO: 38.

23. An expression vector comprising the recombinant DNA molecule defined in any one of claims 16 to 22.

24. Use of an expression vector according to claim 23 for the recombinant production of secreted fusion proteins.

25. A method for the production of recombinant secreted fusion proteins defined in any one of claims 1 to 12 comprising the steps:

- i) cultivating a eukaryotic or prokaryotic cell that has been transfected with a recombinant DNA molecule as defined in any one of claims 16 to 22, or an expression vector of claim 23 in a cultivation medium and
- ii) recovering the recombinant secreted fusion proteins from the cell or the cultivation medium.

26. The method according to claim 25, wherein the cell is a eukaryotic cell and has been infected with a recombinant baculovirus expressing said expression vector.

27. The method according to claim 25 or 26, wherein the eukaryotic cell is a mammalian cell, preferably a Chinese hamster ovary cell or human kidney cell, or the eukaryotic cell is an insect cell, preferably a Sf9 or Sf21 cell from *Spodoptera frugiperda* or Hi5 cell from *Trichoplusia ni*.

28. A method of defining a peptide ligand suitable for successful production of a single chain fusion protein for peptide exchange comprising the steps:

- i) identify an antigenic or non-antigenic peptide, known to bind to a MHC-I protein of the single chain fusion protein, of claim 6, and its amino acid sequence;
- ii) modify one or more of the amino acids of the peptide that are not interacting with a HLA binding groove of the MHC-I protein until the peptide sequence, while retaining a HLA binding motif, does not map to any known antigen and/or is not recognized by T cells anymore, wherein preferably the one or more modified amino acids are mutated to or substituted by neutral non-polar residues such as alanine and are exposed out of the HLA binding groove;
- iii) produce monomers of the single chain fusion protein and verify proper binding of said peptide ligand to MHC-I based on monomer secretion.

29. A method of peptide exchange comprising the steps:

- i) providing a fusion protein of any one of claims 1 to 14 or a complexed multimer of any one of claims 13 to 15;
- ii) co-incubating, for a period of time, the fusion protein or complexed multimer with a peptide of interest and a cleavage enzyme that will cleave said first linker polypeptide, independent of the order of the components added,

wherein said cleavage results in exchange of the peptide ligand with the peptide of interest, thereby generating a distinctively-labeled, soluble MHC monomer or complexed multimer loaded with the peptide of interest.

30. The method of claim 29, wherein step ii) is performed in an exchange buffer between about
5 pH 5 and about pH 8.0, for a period of about 1 h to about 16 h, at a temperature of between about 15°C to about 37°C to release the peptide ligand and allow rescue peptide binding.

31. Use of an enzyme-cleavable fusion protein, for peptide exchange, of any one of claims 1 to 12 or a complexed multimer of any one of claims 13-15 to detect or isolate antigen-specific CD8⁺ T cells.

Figure 1A

		UV-exchange				
		1	2	3	4	5
PE	Peptide 4	Peptide 4	Peptide 4	Peptide 4	Peptide 4	Peptide 4
APC-Cy7	Naïve	Digested Un-exchanged	Peptide 5	Dengue 1	Dengue 2	
		6	7	8	9	10
PE	Peptide 4	Peptide 4	Peptide 4	Peptide 4	Peptide 4	Peptide 4
APC-Cy7	Naïve	Digested Un-exchanged	Peptide 5	Dengue 1	Dengue 2	
		11	12	13	14	15
PE	Peptide 4	Peptide 4	Peptide 4	Peptide 4	Peptide 4	Peptide 4
APC-Cy7	Naïve	Digested Un-exchanged	Peptide 5	Dengue 1	Dengue 2	
		Enzyme exchange Citrate buffer PH 6.2 RT for 16 hours				

Positive control:

Peptide 4: AVFDRKSDAK SEQ ID NO: 5 Dengue 1: GTSGSPIVNR SEQ ID NO: 44
Peptide 5: IVTDFSVIK SEQ ID NO: 39 Dengue 2: ATYGWNLVK SEQ ID NO: 45

Negative controls:

Figure 1B

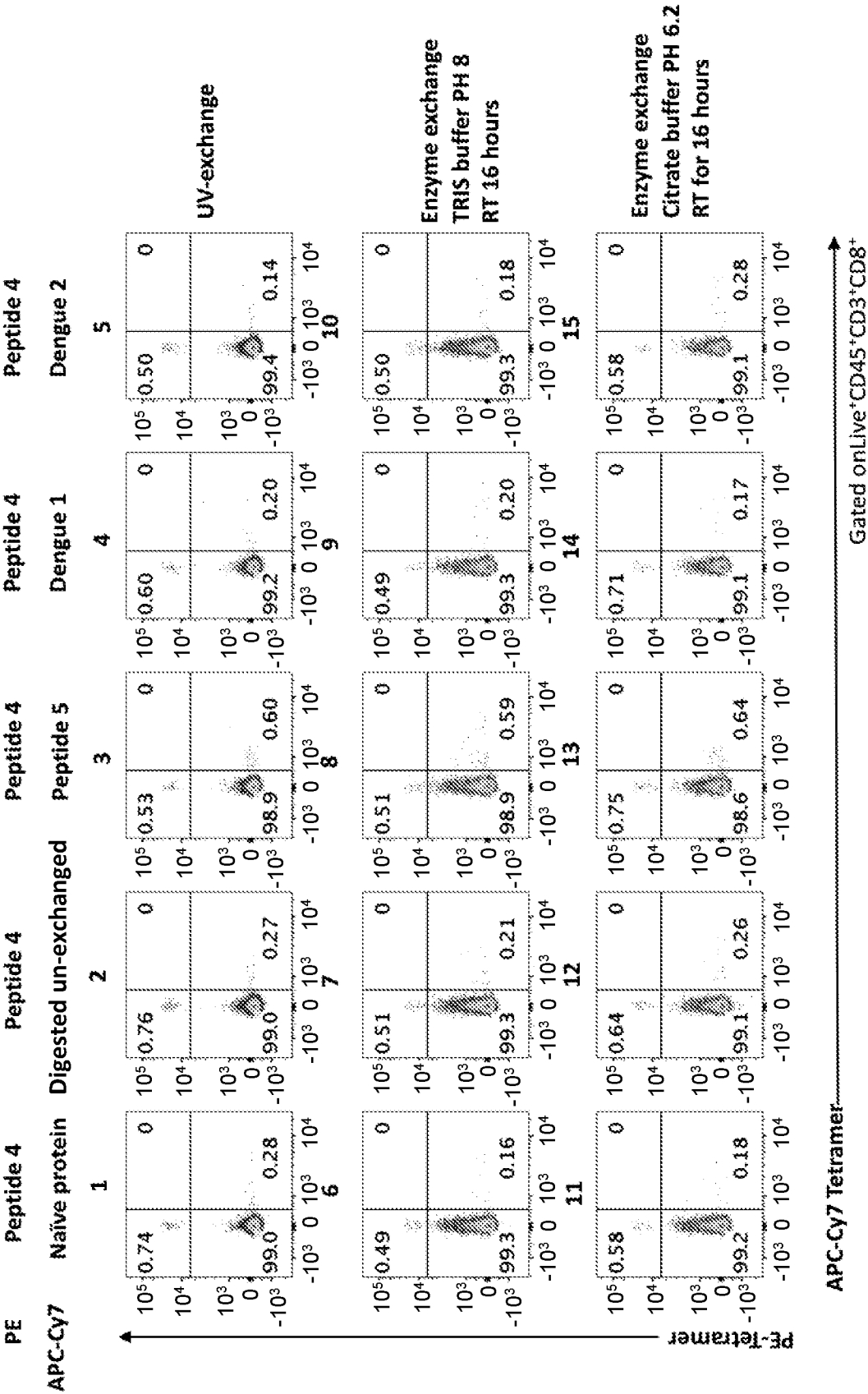


Figure 2

A

No.	HLA-A*11:01 Control peptides Sequence	SAV 1	SAV 2	SAV 3	
1	SIIPSGPLK	152	166	175	SEQ ID NO: 46
2	RMVLASTTAK	166	174	175	SEQ ID NO: 47
3	KSMREEYRK	152	174	175	SEQ ID NO: 48
4	AVFDRKSDAK (influenza 1)	164	166	174	SEQ ID NO: 5
5	IVTDFSVIK (EBV 1)	152	154	166	SEQ ID NO: 39
6	NTLEQTVKK (EBV 2)	152	164	166	SEQ ID NO: 40
7	ATIGTAMYK	154	164	166	SEQ ID NO: 49
8	SSCSCPLSK	152	166	174	SEQ ID NO: 50
9	YVNVNMGLK	154	164	175	SEQ ID NO: 51
10	NAIVE	154	166	174	
11	Digested un-exchanged	152	164	174	

B EBV peptide 1 (IVTDFSVIK): 166-152-154

SEQ ID NO: 39

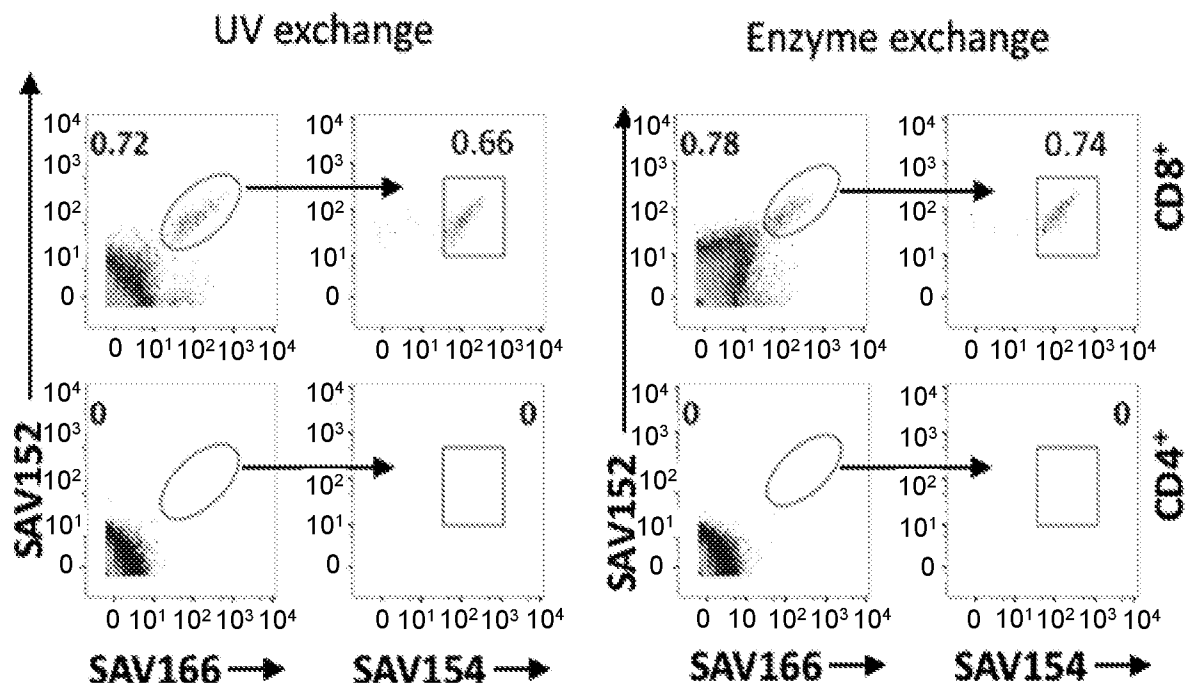
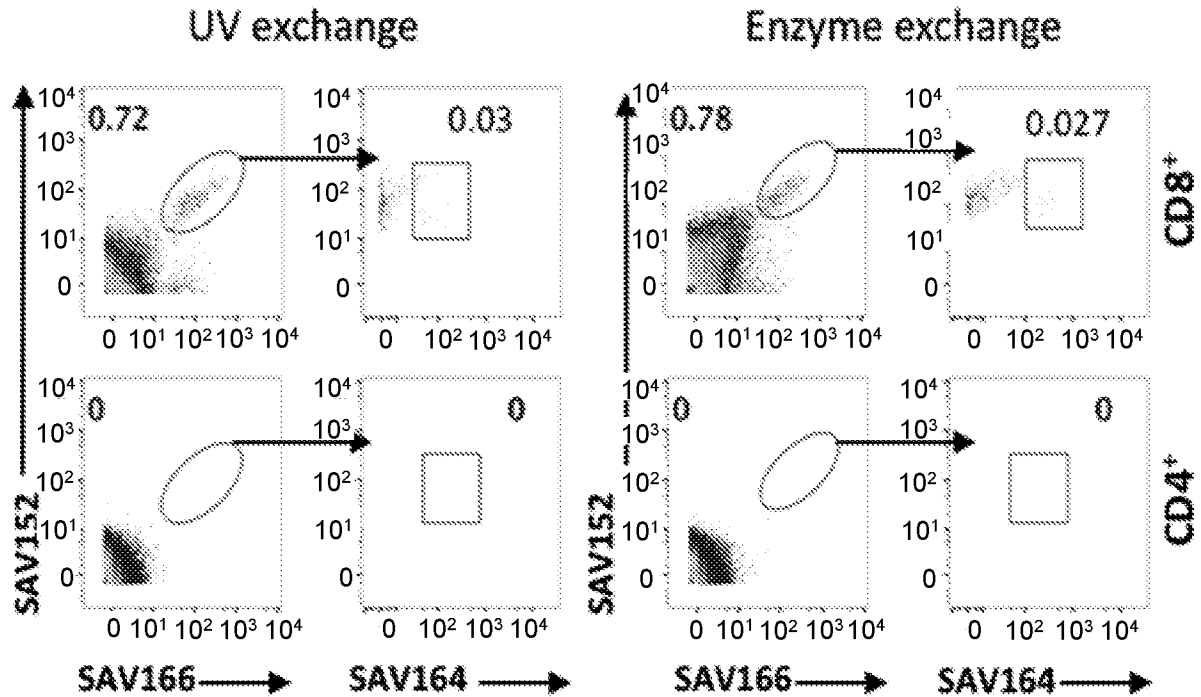


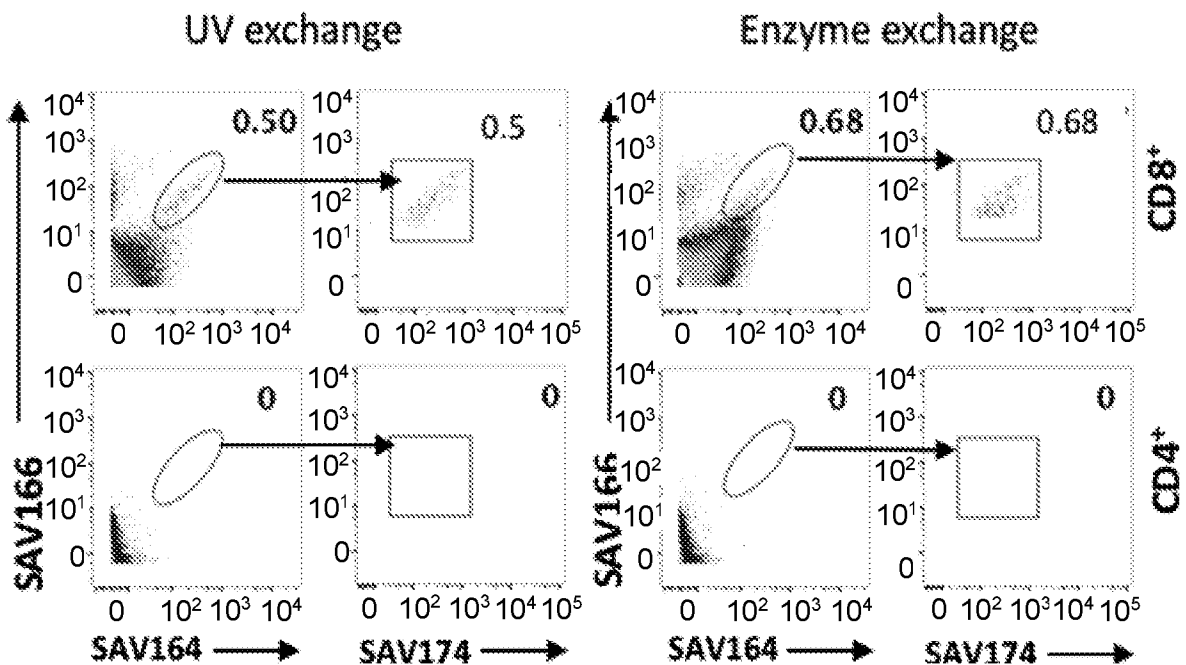
Figure 2 (continued)

C EBV peptide 2 (NTLEQTVKK): 166-152-164

SEQ ID NO: 40

**D Influenza peptide 1 (AVFDRKSDAK): 166-164-174**

SEQ ID NO: 5



5/11

Figure 3A

PE	Peptide 1	Peptide 1	Peptide 1	Peptide 1	Peptide 1	UV-exchange (1.2mg/ml)
APC	Naive	Digested	Peptide 2	Peptide 3	Peptide 15	
Unexchanged						
PE	Peptide 1	Peptide 1	Peptide 1	Peptide 1	Peptide 1	UV-exchange (5mg/ml)
APC	Naive	Digested	Peptide 2	Peptide 3	Peptide 15	
Unexchanged						
PE	Peptide 1	Peptide 1	Peptide 1	Peptide 1	Peptide 1	Enzyme exchange TRIS buffer PH 8 RT 16 hours
APC	Naive	Digested	Peptide 2	Peptide 3	Peptide 15	
Unexchanged						
PE	Peptide 1		Peptide 1	Peptide 1	Peptide 1	Enzyme exchange TRIS Buffer PH 8 37°C 16 hours
APC	Naive		Peptide 2	Peptide 3	Peptide 15	
PE	Peptide 1		Peptide 1	Peptide 1	Peptide 1	Enzyme exchange Citrate buffer PH 6.2 RT for 16 hours
APC	Naive		Peptide 2	Peptide 3	Peptide 15	
PE	Peptide 1		Peptide 1	Peptide 1	Peptide 1	Enzyme exchange Citrate buffer PH 6.2 37°C for 16 hours
APC	Naive		Peptide 2	Peptide 3	Peptide 15	

Positive control:

Peptide 1: NLVPMVATV SEQ ID NO: 41

Negative controls:

Peptide 2: VLEETSVML SEQ ID NO: 52

Peptide 3: GTSGSPIVNR SEQ ID NO: 44

Peptide 15: FLDKGTYTL SEQ ID NO: 53

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Figure 3B

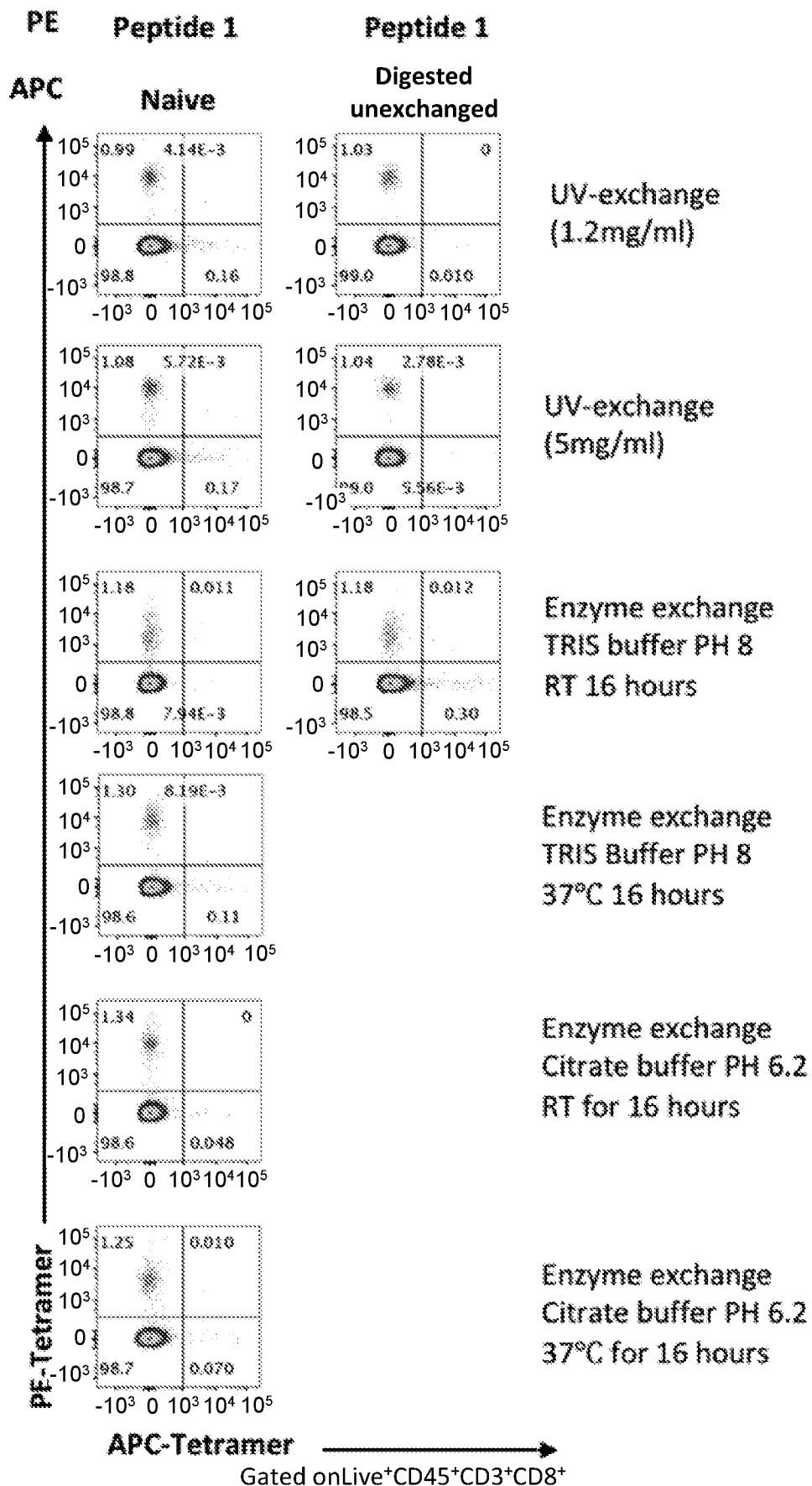
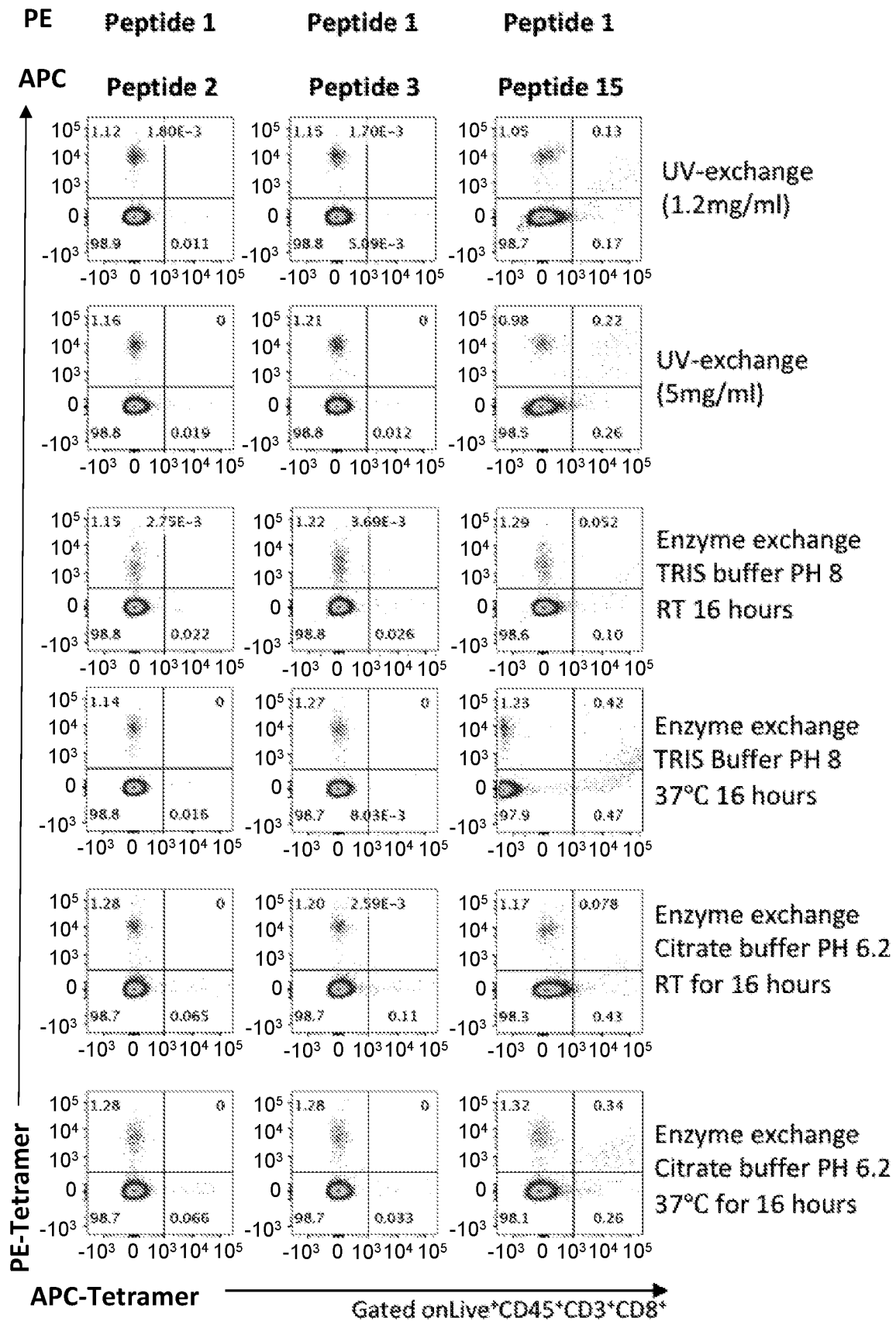


Figure 3B (continued)



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Figure 4A

PE	Peptide 1	Peptide 1	Peptide 1	Peptide 1
APC	Naive	Digested unexchanged	Peptide 2	Peptide 11

UV-exchange

PE	Peptide 1	Peptide 1	Peptide 1	Peptide 1
APC	Naive	Digested unexchanged	Peptide 2	Peptide 11

**Enzyme exchange
Cacodylate buffer PH 6.2
RT for 16 hours**

PE	Peptide 1	Peptide 1	Peptide 1	Peptide 1
APC	Naive	Digested unexchanged	Peptide 2	Peptide 11

**Enzyme exchange
TRIS buffer PH 8
RT 16 hours****Positive control:**

Peptide 1: NLVPMVATV SEQ ID NO: 41

Negative controls:

Peptide 2: VLEETSVML SEQ ID NO: 52

Peptide 11: FLYALALL SEQ ID NO: 54

Figure 5

A

No.	Peptide	SAV Codes	
1	NLVPMVATV (CMV)	152, 154, 158	SEQ ID NO: 41
2	VLEETSVML	164, 166, 171	SEQ ID NO: 52
3	FLDKGTYTL	166,171, 175	SEQ ID NO: 53
4	FLYALALLL	164, 166, 175	SEQ ID NO: 54
5	Digested-Unexchanged	164, 173, 175	
6	Naïve Protein	171, 173, 175	

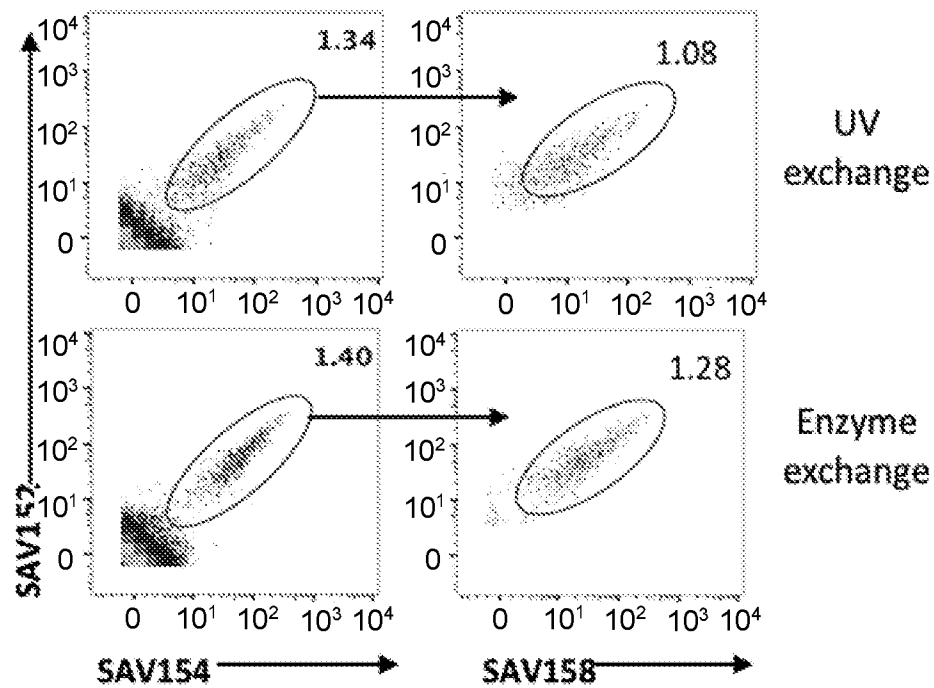
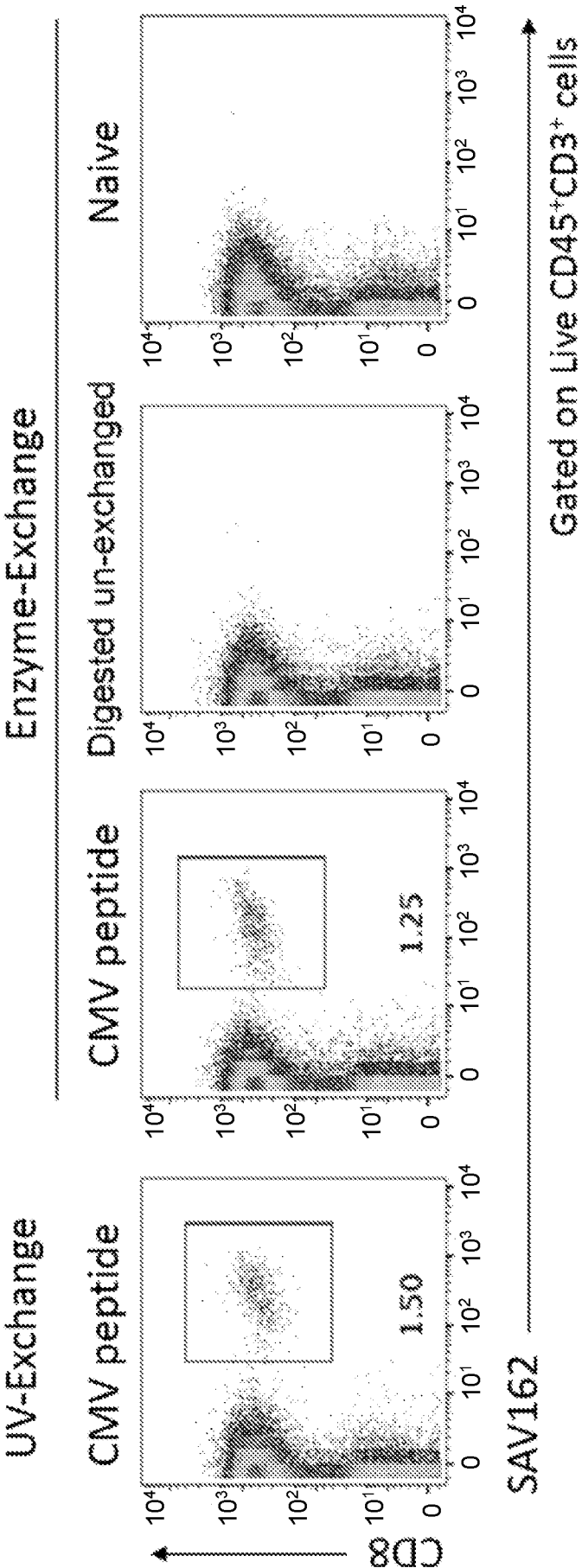
B

Figure 6



INTERNATIONAL SEARCH REPORT

International application No.

PCT/SG2020/050264

A. CLASSIFICATION OF SUBJECT MATTER

C07K 14/705 (2006.01) G01N 33/68 (2006.01)

According to International Patent Classification (IPC)

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C07K, G01N

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

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

EPODOC/WPI/CAPLUS/MEDLINE/BIOSIS/EMBASE: Major histocompatibility complex class I (MHC-I), MHC peptide, β -2 microglobulin, enzymatic, protease, cleavable, liable, protease-activatable and similar terms.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	HANSEN, T.H. ET AL., Translational and basic applications of peptide-MHCI single chain trimers. <i>Trends Immunol</i> , 9 September 2010, Vol. 31, No. 10, pages 363-369. NIH Public Access Manuscript. [Retrieved on 2020-08-13 from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2949479/] <DOI: 10.1016/J.IT.2010.07.003> Whole document, particularly Figure 1; Text Box 1.	1-27 & 29-31
A	HUANG, C.-H. ET AL., Cancer immunotherapy using a DNA vaccine encoding a single-chain trimer of MHC class I linked to an HPV-16 E6 immunodominant CTL epitope. <i>Gene Therapy</i> , 31 March 2005, Vol. 12, No. 15, pages 1180-1186 [Retrieved on 2020-08-13] <DOI: 10.1038/SJ.GT.3302519> Whole document, particularly Figure 1.	1-27 & 29-31
A	SAMANTA, D. ET AL., Structural and functional characterization of a single-chain peptide-MHC molecule that modulates both naive and activated CD8+ T cells. <i>PNAS</i> , 16 August 2011, Vol. 108, No. 33, pages 13682-13687 [Retrieved on 2020-08-13] <DOI: 10.1073/PNAS.1110971108> Whole document, particularly Figure 1.	1-27 & 29-31

☒ Further documents are listed in the continuation of Box C.

☐ See patent family annex.

*Special categories of cited documents:

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

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

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

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

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

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

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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search

13/08/2020

(day/month/year)

Date of mailing of the international search report

17/08/2020

(day/month/year)

Name and mailing address of the ISA/SG

Intellectual Property Office of Singapore
1 Paya Lebar Link, #11-03
PLQ 1, Paya Lebar Quarter
Singapore 408533

Email: pct@ipos.gov.sg

Authorized officer

Teclise Ng (Dr)

IPOS Customer Service Tel. No.: (+65) 6339 8616

INTERNATIONAL SEARCH REPORT

International application No.

PCT/SG2020/050264

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	SYLVESTER-HVID, C. ET AL., A single-chain fusion molecule consisting of peptide, major histocompatibility gene complex class I heavy chain and β_2 -microglobulin can fold partially correctly, but binds peptide inefficiently. <i>Scand. J. Immunol</i> , 1 October 1999, Vol. 50, No. 4, pages 335-362 [Retrieved on 2020-08-13] <DOI: 10.1046/J.1365-3083.1999.00601.X> Whole document, particularly Figures 1 & 2.	1-27 & 29-31
A	BAKKER, A.H. ET AL., Conditional MHC class I ligands and peptide exchange technology for the human MHC gene products HLA-A1, -A3, -A11, and -B7. <i>PNAS</i> , 28 February 2008, Vol. 105, No. 10, pages 3825-3830 [Retrieved on 2020-08-13] <DOI: 10.1073/PNAS.0709717105> Whole document, particularly Figure 2.	1-27 & 29-31
A	LUIMSTRA, J.J. ET AL., A flexible MHC class I multimer loading system for large-scale detection of antigen-specific T cells. <i>Journal of Experimental Medicine</i> , 17 April 2018, Vol. 215, No. 5, pages 1493-1504 [Retrieved on 2020-08-13] <DOI: 10.1084/JEM.20180156> Whole document, particularly Figure 1.	1-27 & 29-31
A	DAVIS, M.M. ET AL., Interrogating the repertoire: broadening the scope of peptide-MHC multimer analysis. <i>Nat Rev Immunol</i> , 15 July 2011, Vol. 11, No. 8, pages 551-558 [Retrieved on 2020-08-13] <DOI: 10.1038/NRI3020> Whole document.	1-27 & 29-31
P,X	JUREWICZ, M.M. ET AL., MHC-I peptide binding activity assessed by exchange after cleavage of peptide covalently linked to β_2 -microglobulin. <i>Anal Biochem</i> , 13 June 2019, Vol. 584, pages 113328: 1-10 [Retrieved on 2020-08-13] <DOI: 10.1016/J.AB.2019.05.017> Abstract.	1-27 & 29-31

INTERNATIONAL SEARCH REPORT

International application No.

PCT/SG2020/050264

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:
 - a. ☐ forming part of the international application as filed:
 - ☐ in the form of an Annex C/ST.25 text file.
 - ☐ on paper or in the form of an image file.
 - b. ☐ furnished together with the international application under PCT Rule 13~~ter~~.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.
 - c. ☐ furnished subsequent to the international filing date for the purposes of international search only:
 - ☐ in the form of an Annex C/ST.25 text file (Rule 13~~ter~~.1(a)).
 - ☐ on paper or in the form of an image file (Rule 13~~ter~~.1(b) and Administrative Instructions, Section 713).
2. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

Although a sequence listing has been filed or furnished, it was not used for the purposes of this search.

Since only one version or copy of a sequence listing has been filed or furnished, the statements under item 2 are not required.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/SG2020/050264

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

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

1. ☐ Claims Nos.:

because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:

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

3. ☐ Claims Nos.:

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

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

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

Please refer to Supplemental Box (Continuation of Box No. III).

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Claims 1-27 & 29-31

Remark on Protest ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/SG2020/050264

Supplemental Box (Continuation of Box No. III)

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

Invention Group 1 (Claims 1-27 & 29-31):

A single fusion protein comprising, in the order of N-terminus to C-terminus of the fusion protein: (i) a peptide ligand, (ii) a first linker polypeptide comprising an enzyme-cleavable portion, (iii) a β -2 microglobulin (β 2m) polypeptide, (iv) a second linker polypeptide, and (v) a mature Major Histocompatibility Complex Class (I) heavy chain polypeptide; a complexed multimers; isolated recombinant DNA; expression vector; uses and methods thereof.

Invention Group 2 (Claim 28):

A method of defining a peptide ligand suitable for successful production of a single fusion protein for peptide exchange comprising, inter alia, a step of identifying an antigen or non-antigenic peptide, known to bind to a MHC-protein of the single chain fusion protein, of claim 6 and its amino acid sequence.

Please refer to **Box No. IV** of Written Opinion of The International Searching Authority (Form PCT/ISA/237) for detailed explanation.