

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;
 - (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,
wherein the peptide ligand is selected from the group comprising 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).
- ~~2. The fusion protein of claim 1, wherein the peptide ligand is a stabilizing peptide.~~
- ~~32. The fusion protein of claim 1 or 2, wherein the first linker polypeptide comprises preferably 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.~~
- ~~43. The fusion protein of any one of claims 1 to 32, wherein the first and second linker polypeptides comprise at least about 80 percent glycine, alanine and/or serine residues.~~
- ~~54. The fusion protein of any one of claims 1 to 43, 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:
 - 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),~~

~~SEQ ID NO: 5 (AVFDRKSDAK), SEQ ID NO: 6 (KILGRVFFV), SEQ ID NO: 7 (KLAEAIFKL)~~
~~and SEQ ID NO: 8 (YAETAAFAY).~~

85. The fusion protein of any one of claims 1 to ~~74~~, wherein the β 2m polypeptide comprises the amino acid sequence set forth in SEQ ID NO: 9.

96. The fusion protein of any one of claims 1 to ~~85~~, 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.

407. The fusion protein of any one of claims 1 to ~~96~~, 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.

448. The fusion protein of any one of claims 1 to ~~407~~, 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.

429. The fusion protein of any one of claims 1 to ~~448~~, 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.

4310. 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.

4411. The complexed multimer of claim ~~4310~~, comprising a dimer, trimer, tetramer or pentamer of said single chain fusion protein.

4512. The complexed multimer of claim ~~4310~~ or ~~4411~~, wherein said single chain fusion proteins are complexed with, for example, streptavidin or other complexing agent.

4613. An isolated recombinant DNA molecule comprising a DNA sequence encoding a single chain fusion protein of any one of claims 1 to ~~429~~.

4714. The recombinant DNA molecule of claim ~~4613~~, 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.

4815. The recombinant DNA molecule of claim ~~4714~~, 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.

~~49~~16. The recombinant DNA molecule of claim ~~17-14~~ or ~~48~~15, 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~~17. The recombinant DNA molecule of any one of claims ~~46-13~~ to ~~49~~16, 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.

~~24~~18. The recombinant DNA molecule of any one of claims ~~46-13~~ to ~~20~~17, when dependent from claim 8 or claim 9, 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~~19. The recombinant DNA molecule of any one of claims ~~46-13~~ to ~~24~~18, 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~~20. An expression vector comprising the recombinant DNA molecule defined in any one of claims ~~16-13~~ to ~~22~~19.

~~24~~21. Use of an expression vector according to claim ~~23-20~~ for the recombinant production of secreted fusion proteins.

~~25~~22. A method for the production of recombinant secreted fusion proteins defined in any one of claims 1 to ~~42-9~~ 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~~23. The method according to claim ~~25-22~~, wherein the cell is a eukaryotic cell and has been infected with a recombinant baculovirus expressing said expression vector.

~~27~~24. The method according to claim ~~25-22~~ or ~~26~~23, 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~~25. A method of peptide exchange comprising the steps:

- i) providing a fusion protein of any one of claims 1 to ~~44-9~~ or a complexed multimer of any one of claims ~~43-10~~ to ~~45~~12;

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.

3026. The method of claim 2925, wherein 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.

3427. Use of an enzyme-cleavable fusion protein, for peptide exchange, of any one of claims 1 to 12-9 or a complexed multimer of any one of claims 1310-15-12 to detect or isolate antigen-specific CD8⁺ T cells.