

What is claimed is:

CLAIMS

1. A fusion protein comprising a human serum albumin (HSA) binding recombinantly engineered variant of stefin polypeptide and a PD-L1 binding polypeptide, wherein the HSA binding polypeptide binds to HSA with a K_d of $1 \times 10^{-6} M$ or less at pH 6.0 ~~and optionally a K_d for binding HSA at pH 7.4 that is at least half a log greater than the K_d for binding at pH 6.0, and wherein the PD-L1 binding polypeptide binds to PD-L1 with a K_d of $1 \times 10^{-6} M$.~~
2. A fusion protein comprising an HSA binding recombinantly engineered variant of stefin polypeptide that binds to HSA and a PD-L1 binding recombinantly engineered variant of stefin polypeptide that binds to PD-L1, wherein the protein has a circulating half-life in human subjects of at least 7 days.
3. The fusion protein of claim 1 ~~or 2~~, wherein the HSA binding polypeptide comprises a loop 2 sequences selected from any one of SEQ ID NOs: 86-138 and/or a loop 4 sequence selected from any one of SEQ ID NOs: 139-191.
4. The fusion protein of claim 1 ~~any one of claims 1-3~~, wherein the PD-L1 binding polypeptide comprises a loop 2 sequences selected from any one of SEQ ID NOs: 16-50 and/or a loop 4 sequence selected from any one of SEQ ID NOs: 51-85.
5. The fusion protein of any one of the preceding claims, wherein the fusion protein comprises the amino acid sequence of SEQ ID NO: 277, or an amino acid sequence having ~~at least 80%, at least 85%, at least 90%, or at least 95%~~ identity to the amino acid sequence of SEQ ID NO: 277, SEQ ID NO: 278, SEQ ID NO: 279, SEQ ID NO: 280, SEQ ID NO: 281, or SEQ ID NO: 282.
6. ~~The fusion protein of any one of claims 1-5, wherein the fusion protein comprises the amino acid sequence of SEQ ID NO: 278, or an amino acid sequence having at least 80%, at least 85%, at least 90%, or at least 95% identity to the amino acid sequence of SEQ ID NO: 278.~~

7. ~~— The fusion protein of any one of claims 1-5, wherein the fusion protein comprises the amino acid sequence of SEQ ID NO: 279, or an amino acid sequence having at least 80%, at least 85%, at least 90%, or at least 95% identity to the amino acid sequence of SEQ ID NO: 279.~~

8. ~~— The fusion protein of any one of claims 1-5, wherein the fusion protein comprises the amino acid sequence of SEQ ID NO: 280, or an amino acid sequence having at least 80%, at least 85%, at least 90%, or at least 95% identity to the amino acid sequence of SEQ ID NO: 280.~~

9. ~~— The fusion protein of any one of claims 1-5, wherein the fusion protein comprises the amino acid sequence of SEQ ID NO: 281, or an amino acid sequence having at least 80%, at least 85%, at least 90%, or at least 95% identity to the amino acid sequence of SEQ ID NO: 281.~~

10. ~~— The fusion protein of any one of claims 1-5, wherein the fusion protein comprises the amino acid sequence of SEQ ID NO: 282, or an amino acid sequence having at least 80%, at least 85%, at least 90%, or at least 95% identity to the amino acid sequence of SEQ ID NO: 282.~~

116. The fusion protein of claim 1 ~~any one of claims 1-10~~ further comprising a second a PD-L1 binding polypeptide, wherein the second PD-L1 binding polypeptide binds to PD-L1 with a K_d of $1 \times 10^{-6}M$.

127. An in-line fusion protein comprising a human serum albumin (HSA) binding recombinantly engineered variant of stefin, first a PD-L1 binding polypeptide, and a second a PD-L1 binding polypeptide, wherein the HSA binding polypeptide binds to HSA with a K_d of $1 \times 10^{-6}M$ or less at pH 6.0 ~~and optionally a K_d for binding HSA at pH 7.4 that is at least half a log greater than the K_d for binding at pH 6.0, and wherein the first and second PD-L1 binding polypeptides bind to PD-L1 with a K_d of $1 \times 10^{-6}M$.~~

138. The fusion protein of claim 1 ~~any one of the preceding claims~~, further comprising a linker.

149. The fusion protein of ~~claim 13~~ 8, wherein the linker is a rigid linker or a flexible linker.

~~1510.~~ The fusion protein of claim ~~14~~ 9, wherein the rigid linker comprises the sequence of SEQ ID NO: 294, or the flexible linker comprises the sequence of SEQ ID NO: 293.

~~1611.~~ The fusion protein of claim 8 ~~any one of claims 13-15~~, wherein the fusion protein comprises the amino acid sequence of SEQ ID NO: 283, or an amino acid sequence having at ~~least 80%, at least 85%, at least 90%, or at least 95%~~ identity to the amino acid sequence of SEQ ID NO: 283, SEQ ID NO: 284, SEQ ID NO: 285, SEQ ID NO: 286, SEQ ID NO: 287, SEQ ID NO: 290, SEQ ID NO: 291, or SEQ ID NO: 293.

~~17. The fusion protein of any one of claims 13-15, wherein the fusion protein comprises the amino acid sequence of SEQ ID NO: 284, or an amino acid sequence having at least 80%, at least 85%, at least 90%, or at least 95% identity to the amino acid sequence of SEQ ID NO: 284.~~

~~18. The fusion protein of any one of claims 13-15, wherein the fusion protein comprises the amino acid sequence of SEQ ID NO: 285, or an amino acid sequence having at least 80%, at least 85%, at least 90%, or at least 95% identity to the amino acid sequence of SEQ ID NO: 285.~~

~~19. The fusion protein of any one of claims 13-15, wherein the fusion protein comprises the amino acid sequence of SEQ ID NO: 286, or an amino acid sequence having at least 80%, at least 85%, at least 90%, or at least 95% identity to the amino acid sequence of SEQ ID NO: 286.~~

~~20. The fusion protein of any one of claims 13-15, wherein the fusion protein comprises the amino acid sequence of SEQ ID NO: 287, or an amino acid sequence having at least 80%, at least 85%, at least 90%, or at least 95% identity to the amino acid sequence of SEQ ID NO: 287.~~

~~21. The fusion protein of any one of claims 13-15, wherein the fusion protein comprises the amino acid sequence of SEQ ID NO: 290, or an amino acid sequence having at least 80%, at least 85%, at least 90%, or at least 95% identity to the amino acid sequence of SEQ ID NO: 290.~~

~~22. The fusion protein of any one of claims 13-15, wherein the fusion protein comprises the amino acid sequence of SEQ ID NO: 291, or an amino acid sequence having at least 80%, at least 85%, at least 90%, or at least 95% identity to the amino acid sequence of SEQ ID NO: 291.~~

~~23. The fusion protein of any one of claims 16-22, wherein the fusion protein further comprises a flexible linker, optionally wherein the flexible linker comprises the sequence of SEQ ID NO: 293.~~

~~24. A pharmaceutical composition suitable for therapeutic use in a human subject, comprising a fusion protein of any of any one of the preceding claims, and a pharmaceutically acceptable excipient.~~

~~2512. A polynucleotide comprising a sequence encoding the fusion protein of claim 1 any one of the claims 1-23.~~

~~26. The polynucleotide of claim 25, wherein the sequence encoding the fusion protein is operably linked to a transcriptional regulatory sequence.~~

~~2713. A viral vector, a plasmid, or a minicircle comprising the polynucleotide of claim 2512 or 26.~~

~~28. A plasmid or minicircle comprising the polynucleotide any of claim 25 or 26.~~

~~2914. A cell comprising the fusion protein of claim 1 any one of claims 1-23, the polynucleotide of claim 25 or 26, the viral vector of claim 27, or the plasmid or minicircle of claim 28.~~

~~3015. The fusion protein of claim 1 any one of claims 1-23, for use in a method for treating an infectious disease or cancer.~~

~~31. The fusion protein of any one of claims 1-23, for use in a method for treating cancer.~~

~~3216. The fusion protein of claim 1 any one of claims 1-23, further comprising one or more additional polypeptide sequences which may additional therapeutic activity and/or additional PK/ADME properties to the fusion protein.~~

~~33~~17. The fusion protein of claim ~~32~~ 16, wherein the additional polypeptide sequence is a receptor ligand, and wherein the receptor ligand is an immunostimulatory cytokine, such as IFN- α , IL-2, IL-15, IL-21, and IL-12, or a variant sequence thereof; or wherein the additional polypeptide sequence is a CD3 binding polypeptide sequence that directs the fusion protein to bind to CD3 on the surface of T-cells.

~~34.~~ The fusion protein of claim ~~33~~, wherein the receptor ligand is an immunostimulatory cytokine, such as IFN- α , IL-2, IL-15, IL-21, and IL-12, or a variant sequence thereof.

~~35~~18. The fusion protein of claim ~~33~~ 17, wherein the additional polypeptide sequence is a mutant IL-2 polypeptide comprising the amino acid sequence of SEQ ID NO: 1282, or a sequence at least ~~70%~~90% identical ~~thereof~~ thereto, having one or more amino acid substitutions relative to SEQ ID NO: 1282 that abolish or reduce affinity of the mutant IL-2 polypeptide to a high-affinity IL-2 receptor and preserve affinity of the mutant IL-2 polypeptide to an intermediate-affinity IL-2 receptor, as compared to a wild-type IL-2 polypeptide.

~~36.~~ The fusion protein of claim ~~35~~, wherein the additional polypeptide sequence is a mutant IL-2 polypeptide comprising the amino acid sequence of SEQ ID NO: 1282 but having one or more amino acid substitutions at positions selected from T3 (such as T3A), D20 (such as D20T), R38 (such as R38A and R38D), F42 (such as F42A, F42G, F42S, F42T, F42Q, F42E, F42N, F42D, F42R or F42K), K43 (such as K43E), E61 (such as E61R), E62 (such as E62A), Y45 (such as Y45A, Y45G, Y45S, Y45T, Y45Q, Y45E, Y45N, Y45D, Y45R or Y45K) and/or L72 (such as L72G, L72A, L72S, L72T, L72Q, L72E, L72N, L72D, L72R or L72K).

~~37~~19. The fusion protein of claim ~~33~~ 17, wherein the receptor ligand is for a co-stimulatory receptor and the fusion protein agonizes the co-stimulatory receptor upon binding.

~~38.~~ The fusion protein of claim ~~37~~, wherein the receptor ligand is selected from CD40L, CDB7.1, 4-1BBL, OX40L, GITRL or LIGHT.

~~39~~20. The fusion protein of claim ~~33~~ 17, further comprising a polypeptide sequences that induces dimerization or higher order multimerization of the fusion protein.

40. — The fusion protein of claim 33, wherein the additional polypeptide sequence is a CD3 binding polypeptide sequence that directs the fusion protein to bind to CD3 on the surface of T-cells.

