

CTLA-4 is upregulated and competes with CD28 for CD80 and CD86 binding on APCs but with significantly higher affinity, therefore downregulating—or deactivating—the T cell (Fig. 1). CTLA-4, therefore, downregulates T-cell responses and APC function, resulting in a decreased immune response to tumor-associated antigens and immune tolerance.

5 The mechanisms whereby CTLA4 and PD1 exert their inhibitory effects on T-cell activation are multifaceted. CTLA4 functions primarily to limit T-cell activation and clonal expansion, whereas PD1 functions primarily to limit effector T-cell function in the peripheral tissues. Their distinct molecular structures, regulation, signaling pathways, ligand distribution, and function on Tregs and other immune cells suggest that combined therapeutic blockade of
10 CTLA4 and PD1 could synergize to mediate anti-tumor immunity. Intlekofer & Thompson, J. Leuko. Biol. 94(1): 25-39 (2013); Hurwitz *et al.* Proc. Natl. Acad. Sci. USA 95: 10067–10071 (1998); Parry *et al.* Mol. Cell. Biol. 25(21): 9543-9553 (2005); Callahan *et al.* Front. Oncol. Vol 4, Art. 385 (2015).

 One method by which to inhibit CTLA4-mediated downregulation is by interfering
15 with its interaction with its ligands by binding it with a Nanobody. The possibility exists that Nanobodies, originating in llamas, could cause an unwanted anti-drug immune response, *e.g.*, by binding of the Nanobodies by pre-existing antibodies in the patient's serum. Thus, novel methods by which to humanize Nanobodies so as to decrease or eliminate such a response are particularly valuable as are Nanobodies that are created by such methods.

SUMMARY

 The present disclosure provides a multispecific immunoglobulin single variable domain (ISVD) such as a Nanobody that binds to human CTLA4 by contacting human CTLA4 at one or more of the following residues VRVTVL (amino acids 33 – 38 of SEQ ID NO:
25 110), ADSQVTEVC (amino acids 41-49 of SEQ ID NO: 110) and CKVELMYPPPYLG (amino acids 93-106 of SEQ ID NO: 110), *e.g.*, all all three sites. For example, the binder protects the residues from hydrogen-deuterium exchange in the presence of a deuterium source such as D₂O. In an embodiment, the

that comprises the amino acid sequence FYGMG (amino acids 6-10 of SEQ ID NO: 2) or GGTFsfYGMG (SEQ ID NO: 2); CDR2 that comprises the amino acid sequence DIRTSAGRTYYADSVKG (SEQ ID NO: 3) or DIRTSAGRTY (amino acids 1-10 of SEQ ID NO: 3); CDR3 that comprises the amino acid sequence EXSGISGWDY (SEQ ID NO: 4); optionally, wherein the ISVD comprises a mutation at residues 11 and 89 (*e.g.*, L11V and/or V89L, for example (E1D, L11V, A14P, Q45R, A74S, K83R, V89L, M96P, Q108L) wherein said residue numbers are Kabat residue numbers.

The present disclosure provides a CTLA4 binder (*e.g.*, an ISVD, *e.g.*, a Nanobody) comprising CDR1, CDR2 and CDR3 of an immunoglobulin comprising amino acid sequence set forth in SEQ ID NO: 1 wherein said CTLA4 binder comprises at least one mutation with respect to the amino acid sequence set forth in SEQ ID NO: 1 wherein said at least one mutation is at a position selected from the group consisting of 11, 89, 110 and 112, wherein said positions are numbered according to Kabat; and optionally including any number of additional mutations that are set forth herein or otherwise, *e.g.* up to 10 (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10) additional mutations (*e.g.*, point mutations, substitutions, deletions, insertions). For example, in an embodiment, CDR1 comprises the amino acid sequence: FYGMG (SEQ ID NO: 2) or GGTFsfYGMG (SEQ ID NO: 5); CDR2 comprises the amino acid sequence: DIRTSAGRTYYADSVKG (SEQ ID NO: 3) or DIRTSAGRTY (SEQ ID NO: 6); and CDR3 comprises the amino acid sequence: EMSGISGWDY (SEQ ID NO: 4). For example, in an embodiment, the CTLA4 binder has a mutation relative to SEQ ID NO: 1 wherein the amino acid residue at position 11 is chosen from L or V; the amino acid residue at position 89 is suitably chosen from T, V or L; the amino acid residue at position 110 is suitably chosen from T, K or Q; and/or the amino acid residue at position 112 is suitably chosen from S, K or Q; *e.g.*, wherein the mutation is 89T; 89L in combination with 11V; 89L in combination with 110K or 110Q; 89L in combination with 112K or 112Q; 89L in combination with 11V and 110K or 110Q; 89L in combination with 11V and 112K or 112Q; 11V in combination with 110K or 110Q; and/or 11V in combination with 112K or 112Q. In

WFRQAPGKEREFFVADIRTSAGRTYYADSVKGRFTISRDN SKNTVYLQMNSLRPEDTALYY
CAAEPGSGISGWDYWGQGLTVTVSSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSE
VQLVESGGGVVQPGGSLRLSCAASGGTFSFYGMGWFRQAPGKEREFFVADIRTSAGRTYYA
DSVKGRFTISRDN SKNTVYLQMNSLRPEDTALYYCAAEPGSGISGWDYWGQGLTVTVSSGG
5 GGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSEVQLVESGGGVVQPGNSLRLSCAASGF
TFSSFGMSWVRQAPGKGLEWVSSISGSGSDTLYADSVKGRFTISRDN AKTTLYLQMNSLR
PEDTALYYCTIGGSLSRSSQGLTVTVSSA (SEQ ID NO: 62); or
DVQLVESGGGVVQPGGSLRLSCAASGGTFSFYGMG

WFRQAPGKEREFFVADIRTSAGRTYYADSVKGRFTISRDN SKNTVYLQMNSLRPEDTALYY
10 CAAEPGSGISGWDYWGQGLTVTVSSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSE
VQLVESGGGVVQPGNSLRLSCAASGFTFSSFGMSWVRQAPGKGLEWVSSISGSGSDTLYA
DSVKGRFTISRDN AKTTLYLQMNSLRPEDTALYYCTIGGSLSRSSQGLTVTVSSA (SEQ ID NO: 64).

In an aspect, there is provided a CTLA4 binder comprising one or more
immunoglobulin single variable domains (ISVDs) that bind to human CTLA4, comprising the
15 amino acid sequence:

DVQLVESGGGVVQPGGSLRLSCAASGGTFSFYGMGWFRQAPGKEREFFVADIRTSAGRTYYADSVKGRFTISRDN
SKNTVYLQMNSLRPEDTALYYCAAEPGSGISGWDYWGQGLTVTVSSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSG
GGGSEVQLVESGGGVVQPGGSLRLSCAASGGTFSFYGMGWFRQAPGKEREFFVADIRTSAGRTYYADSVKGRFTIS
RDNSKNTVYLQMNSLRPEDTALYYCAAEPGSGISGWDYWGQGLTVTVSSGGGGSGGGGSGGGGSGGGGSGGGGSGG
20 GGGGGGSEVQLVESGGGVVQPGNSLRLSCAASGFTFSSFGMSWVRQAPGKGLEWVSSISGSGSDTLYADSVKGR
FTISRDN AKTTLYLQMNSLRPEDTALYYCTIGGSLSRSSQGLTVTVSSA (SEQ ID NO: 62).

In an aspect, there is provided a CTLA4 binder comprising one or more
immunoglobulin single variable domains (ISVDs) that bind to human CTLA4, comprising the
amino acid sequence:

25 DVQLVESGGGVVQPGGSLRLSCAASGGTFSFYGMGWFRQAPGKEREFFVADIRTSAGRTYYADSVKGRFTISRDN
SKNTVYLQMNSLRPEDTALYYCAAEPGSGISGWDYWGQGLTVTVSSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSG
GGGSEVQLVESGGGVVQPGNSLRLSCAASGFTFSSFGMSWVRQAPGKGLEWVSSISGSGSDTLYA
DSVKGRFTISRDN AKTTLYLQMNSLRPEDTALYYCTIGGSLSRSSQGLTVTVSSA (SEQ ID NO: 64).

The present disclosure also provides a binder (*e.g.*, an antibody) that cross-blocks a
30 CTLA4 binder set forth herein from binding to CTLA4. In another aspect, there is provided a
binder that cross-blocks a CTLA4 binder as disclosed herein from binding to human CTLA4.

The present disclosure, in another aspect, provides an injection device or vessel that comprises any CTLA4 binder set forth herein (*e.g.*, comprising the amino acid sequence set forth in SEQ ID NO: 62 or 64) optionally in association with a further therapeutic agent.

The present disclosure, in another aspect, provides a polynucleotide (*e.g.*, DNA) encoding any CTLA4 binder set forth herein (*e.g.*, comprising the amino acid sequence set forth in SEQ ID NO: 62 or 64); *e.g.*, comprising the nucleotide sequence of SEQ ID NO: 61 or 63; or, in another aspect, a vector comprising such a polynucleotide; or, in another aspect, a host cell comprising such a polynucleotide or vector.

The present disclosure, in another aspect, provides a method for making the CTLA4 binder set forth herein (*e.g.*, comprising the amino acid sequence set forth in SEQ ID NO: 62 or 64) comprising introducing a polynucleotide encoding the CTLA4 binder into a host cell (*e.g.*, a CHO cell or *Pichia* cell) and culturing the host cell in a medium under conditions favorable to expression of said CTLA4 binder from said polynucleotide and, optionally, purifying the CTLA4 binder from said host cell and/or said medium. The present disclosure, in another aspect, also provides for a CTLA4 binder produced by such a method.

The present disclosure also provides a method for preventing CTLA4 from binding to CD80 or CD86 (*e.g.*, in the body of a subject) comprising contacting said CTLA4 with the CTLA4 binder (*e.g.*, comprising the amino acid sequence set forth in SEQ ID NO: 62 or 64) optionally in association with a further therapeutic agent; as well as a method for enhancing an immune response in the body of a subject (*e.g.*, a human) comprising administering an effective amount of the CTLA4 binder (*e.g.*, comprising the amino acid sequence set forth in SEQ ID NO: 62 or 64) to the subject optionally in association with a further therapeutic agent (*e.g.*, pembrolizumab). In another aspect, there is provided an *in vitro* method for preventing CTLA4 from binding to CD80 or CD86 comprising contacting CTLA4 with the CTLA4 binder as defined herein optionally in association with a further therapeutic agent. In another aspect, there is provided a CTLA4 binder as disclosed herein for use in enhancing an immune response in the body of a subject optionally in association with a further therapeutic agent. In addition, the present disclosure provides a method for treating or preventing cancer or an infectious disease in the body of a subject comprising administering an effective amount of CTLA4 binder (*e.g.*, comprising the amino acid sequence set forth in SEQ ID NO: 62 or 64) optionally in association with a further therapeutic agent (*e.g.*, pembrolizumab) to the subject. In another aspect, there is provided use of a CTLA4 binder as disclosed herein in the manufacture of a medicament for the treatment or prevention of cancer or an infectious disease, wherein optionally the

medicament is to be administered with a further therapeutic agent. In an embodiment, the cancer is metastatic cancer, a solid tumor, a hematologic cancer, leukemia, lymphoma, osteosarcoma, rhabdomyosarcoma, neuroblastoma, kidney cancer, leukemia, renal transitional cell cancer, bladder cancer, Wilm's cancer, ovarian cancer, pancreatic cancer, breast cancer, 5 prostate cancer, bone cancer, lung cancer, non-small cell lung cancer, gastric cancer, colorectal cancer, cervical cancer, synovial sarcoma, head and neck cancer, squamous cell carcinoma, multiple myeloma, renal cell cancer, retinoblastoma, hepatoblastoma, hepatocellular carcinoma, melanoma, rhabdoid tumor of the kidney, Ewing's

We claim:

1. A CTLA4 binder comprising one or more immunoglobulin single variable domains (ISVDs) that bind to human CTLA4, wherein the ISVD comprises the amino acid sequence:

5 XVQLVESGGGVVQPGGSLRLSCAASGGTFSFYGMG
WFRQAPGKEREFVADIRTSAGRTYYADSVKGRFTISRDN SKNTVY LQMNSLRPEDTALYYCAAEP SGISGWDYWG
QGTLVTVSS, wherein X is D or E (SEQ ID NO: 60); optionally comprising a half-life extender
and/or a C-terminal extender.

10 2. A CTLA4 binder comprising one or more immunoglobulin single variable domains
(ISVDs) that bind to human CTLA4, wherein the ISVD comprises the amino acid sequence
selected from SEQ ID NOs: 93-109; optionally lacking the signal sequence (amino acids
AAADYKDHDGDYKDHDIDYKDDDDKGA AHHHHHH (amino acids 120-153 of SEQ ID NO: 93))
thereof.

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3. The CTLA4 binder of claim 1 or 2 fused to a half-life extender.

4. The CTLA4 binder of claim 2, wherein the half-life extender is an ISVD that binds to
human serum albumin.

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5. The CTLA4 binder of claim 4 wherein the ISVD that binds to human serum albumin
comprises the amino acid sequence SEQ ID NO: 66.

6. A CTLA4 binder of claim 1 or 2 comprising:

- 25 • an ISVD that binds to CTLA4 comprising the amino acid sequence set forth in SEQ
ID NO: 60 wherein X is D or E;
- a peptide linker;
- an ISVD that binds to CTLA4 comprising the amino acid sequence set forth in SEQ
ID NO: 60 wherein X is D or E;
- 30 • a peptide linker;
- a half-life extender; and, optionally,
- a C-terminal extension alanine.

7. A CTLA4 binder of claim 1 or 2 comprising:

- an ISVD that binds to CTLA4 comprising the amino acid sequence set forth in SEQ ID NO: 60 wherein X is D or E;
- a peptide linker;
- a half-life extender; and, optionally,
- a C-terminal extension alanine.

8. The CTLA4 binder of claim 6 or 7 wherein the peptide linker comprises the amino acid sequence GGGGSGGGSGGGSGGGSGGGSGGGSGGGSGGGSGGGG (SEQ ID NO: 65).

9. A CTLA4 binder comprising one or more immunoglobulin single variable domains (ISVDs) that bind to human CTLA4, comprising the amino acid sequence:

DVQLVESGGGVVQPGGSLRLSCAASGGTFSFYGMG
WFRQAPGKEREADVADIRTSAGRTYYADSVKGRFTISRDNKNTVYLMNSLRPEDTALYY
CAAEPGSGISGWDYWGQGLTVTVSSGGGSGGGSGGGSGGGSGGGSGGGSGGGSGGGSGGGSGGGGSE
VQLVESGGGVVQPGGSLRLSCAASGGTFSFYGMGWFRQAPGKEREADVADIRTSAGRTYYA
DSVKGRFTISRDNKNTVYLMNSLRPEDTALYYCAAEPGSGISGWDYWGQGLTVTVSSGG
GGSGGGSGGGSGGGSGGGSGGGSGGGSGGGSGGGSGGGSEVQLVESGGGVVQPGNSLRLSCAASGF
TFSSFGMSWVRQAPGKLEWVSSISGSGSDTLYADSVKGRFTISRDNKNTLYLMNSLR
PEDTALYYCTIGGSLSRSSQGLTVTVSSA (SEQ ID NO: 62).

10. A CTLA4 binder comprising one or more immunoglobulin single variable domains (ISVDs) that bind to human CTLA4, comprising the amino acid sequence:

DVQLVESGGGVVQPGGSLRLSCAASGGTFSFYGMG
WFRQAPGKEREADVADIRTSAGRTYYADSVKGRFTISRDNKNTVYLMNSLRPEDTALYY
CAAEPGSGISGWDYWGQGLTVTVSSGGGSGGGSGGGSGGGSGGGSGGGSGGGSGGGSGGGSGGGGSE
VQLVESGGGVVQPGNSLRLSCAASGFTFSSFGMSWVRQAPGKLEWVSSISGSGSDTLYA
DSVKGRFTISRDNKNTLYLMNSLRPEDTALYYCTIGGSLSRSSQGLTVTVSSA (SEQ ID NO: 64).

11. The CTLA4 binder of claims 1 or 2 which is multivalent.

12. A binder that cross-blocks a CTLA4 binder of any one of claims 1-11 from binding to human CTLA4.

13. An injection device or vessel that comprises the CTLA4 binder of any one of claims 1-12 optionally in association with a further therapeutic agent.

14. A polynucleotide encoding the CTLA4 binder of any one of claims 1-12.

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15. The polynucleotide of claim 14 which comprises the nucleotide sequence of SEQ ID NO: 61 or 63.

16. A vector comprising the polynucleotide of claim 14 or 15.

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17. A host cell comprising the polynucleotide or vector of any one of claims 14-16.

18. A method for making the CTLA4 binder of any one of claims 1-12 comprising introducing a polynucleotide encoding the CTLA4 binder into a host cell and culturing the host cell in a medium under conditions favorable to expression of said CTLA4 binder from said polynucleotide and, optionally, purifying the CTLA4 binder from said host cell and/or said medium.

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19. A CTLA4 binder produced by the method of claim 18.

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20. An *in vitro* method for preventing CTLA4 from binding to CD80 or CD86 comprising contacting CTLA4 with the CTLA4 binder of any one of claims 1-12 optionally in association with a further therapeutic agent.

21. A CTLA4 binder of any one of claims 1-12 for use in enhancing an immune response in the body of a subject optionally in association with a further therapeutic agent.

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22. Use of a CTLA4 binder of any one of claims 1-12 in the manufacture of a medicament for the treatment or prevention of cancer or an infectious disease, wherein optionally the medicament is to be administered with a further therapeutic agent.

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23. The use of claim 22 wherein the cancer is metastatic cancer, a solid tumor, a hematologic cancer, leukemia, lymphoma, osteosarcoma, rhabdomyosarcoma, neuroblastoma, kidney cancer, leukemia, renal transitional cell cancer, bladder cancer, Wilm's cancer, ovarian cancer,

pancreatic cancer, breast cancer, prostate cancer, bone cancer, lung cancer, non-small cell lung cancer, gastric cancer, colorectal cancer, cervical cancer, synovial sarcoma, head and neck cancer, squamous cell carcinoma, multiple myeloma, renal cell cancer, retinoblastoma, hepatoblastoma, hepatocellular carcinoma, melanoma, rhabdoid tumor of the kidney, Ewing's sarcoma, chondrosarcoma, brain cancer, glioblastoma, meningioma, pituitary adenoma, vestibular schwannoma, a primitive neuroectodermal tumor, medulloblastoma, astrocytoma, anaplastic astrocytoma, oligodendroglioma, ependymoma, choroid plexus papilloma, polycythemia vera, thrombocythemia, idiopathic myelofibrosis, soft tissue sarcoma, thyroid cancer, endometrial cancer, carcinoid cancer or liver cancer, breast cancer or gastric cancer.

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24. The use of claim 22 wherein the infectious disease is a bacterial infection, a viral infection or a fungal infection.

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25. The use of any one of claims 22-24 wherein the medicament is to be administered with a further therapeutic agent or a therapeutic procedure.