

CLAIMS:

1. An agent comprising at least two moieties separated by a linker, wherein a first moiety binds membranal CD28 (mCD28) on a surface of a cell and inhibits proteolytic cleavage of said mCD28 and wherein a second moiety increases stability of said first moiety.
2. The agent of claim 1, wherein said first moiety, said second moiety, or both are smaller than 100 kilodaltons (kDa).
3. The agent of claim 2, wherein said agent is smaller than 100 kDa.
4. The agent of any one of claims 1 to 3, wherein said first moiety, is selected from an antigen binding fragment of an antibody, a Fab fragment, a single chain antibody, a single domain antibody, a small molecule and a peptide that specifically binds to CD28.
5. The agent of any one of claims 1 to 4, wherein said first moiety comprises or consists of a single domain antibody.
6. The agent of claim 5, wherein said single domain antibody is a camelid or shark antibody.
7. The agent of any one of claims 4 to 6, wherein said first moiety comprises three CDRs wherein:

CDR1 comprises the amino acid sequence set forth in SEQ ID NO: 1 (INAMG), CDR2 comprises the amino acid sequence as set forth in SEQ ID NO: 2 (AISGGGDTYYADSVKG), CDR3 comprises the amino acid sequence as set forth in SEQ ID NO: 3 (DLYGSDYWD);

CDR1 comprises the amino acid sequence set forth in SEQ ID NO: 4 (INAMA), CDR2 comprises the amino acid sequence as set forth in SEQ ID NO: 5 (AITSSGSTNYANSVKG), CDR3 comprises the amino acid sequence as set forth in SEQ ID NO: 6 (DEYGSDYWI); or

CDR1 comprises the amino acid sequence set forth in SEQ ID NO: 1 (INAMG), CDR2 comprises the amino acid sequence as set forth in SEQ ID NO: 7 (AITSGGSTNYADSVKG), CDR3 comprises the amino acid sequence as set forth in SEQ ID NO: 8 (DLYGEDYWI).
8. The agent of claim 7, wherein said first moiety comprises a sequence selected from a group consisting of:

- a. EVQLVESGGGLVQAGESLRLSCAASGSIASINAMGWYRQAPGSQRELVA AISGG
GDTYYADSVKGRFTISRDN AKTTVY LQMNSLRPEDTAVYYCVVDLYGSDYWD
WGQGTQVTVSS (SEQ ID NO: 9);
 - b. EVQLVESGGGLVQAGGSLRLSCAASGSLFSINAMAWYRQAPGKQRELVA AITSS
GSTNYANSVKGRFTVSRDN AKNTMYLQMNSLKPEDTAVYYCVVDEYGS DYWI
WGQGTQVTVSS (SEQ ID NO: 10); and
 - c. QVQLVESGGGLVQAGGSLRLSCAASGSIFSINAMGWYRQAPGKQRERVA AITSG
GSTNYADSVKGRFTISRDN AKNTVY LQMNNLEPRDAGVYYCVVDLYGEDYWI
WGQGTQVTVSS (SEQ ID NO: 11).
9. The agent of any one of claims 1 to 4, wherein said first moiety comprises three heavy chain CDRs (CDR-H) and three light chain CDRs (CDR-L), wherein:
- CDR-H1 comprises the amino acid sequence set forth in SEQ ID NO: 12 (GFTFSSYYMS), CDR-H2 comprises the amino acid sequence as set forth in SEQ ID NO: 13 (TISDGGDNTYYAGTVTG), CDR-H3 comprises the amino acid sequence as set forth in SEQ ID NO: 14 (IHWPYYFDS), CDR-L1 comprises the amino acid sequence as set forth in SEQ ID NO: 15 (RASSSVSYMN), CDR-L2 comprises the amino acid sequence as set forth in SEQ ID NO: 16 (ATSDLAS), and CDR-L3 comprises the amino acid sequence as set forth in SEQ ID NO: 17 (QQWSSHPPT).
10. The agent of any one of claims 1 to 9, wherein said agent is not a CD28 agonist.
11. The agent of any one of claims 1 to 10, wherein said agent is not a CD28 antagonist.
12. The agent of any one of claims 1 to 11, wherein said increased stability of said first moiety comprises increasing stability in blood.
13. The agent of claim 12, wherein increasing stability in blood comprises reducing clearance of said first moiety from blood.
14. The agent of claim 13, wherein reducing clearance from blood comprises reducing renal filtration, reducing lysosomal degradation or both.
15. The agent any one of claims 1 to 14, wherein said linker is a rigid peptide linker.
16. The agent of claim 15, wherein said rigid peptide linker comprises a helix motif.
17. The agent of claim 15 or 16, wherein said rigid peptide linker comprises at least 15 amino acids.

18. The agent of any one of claims 15 to 17, wherein said rigid peptide linker comprises at most 30 amino acids.
19. The agent of any one of claims 1 to 18, wherein said linker comprises at least one cysteine residue.
20. The agent of claim 19, wherein said at least one cysteine is a C-terminal cysteine.
21. The agent of claim 19 or 20, wherein said second moiety is polyethylene glycol (PEG) and is attached to said cysteine via a thiol linkage.
22. The agent of any one of claims 1 to 21, wherein said second moiety is PEG and said PEG is conjugated within 10 amino acids of a C-terminus of said first moiety.
23. The agent of claim 22, wherein said linker is a peptide linker and PEG is conjugated to an amino acid of said peptide linker.
24. The agent of any one of claims 21 to 23, wherein said PEG is linear or branched PEG comprising a size from 2000-40,000 Daltons (Da).
25. The agent of any one of claims 1 to 24, wherein said second moiety comprises or consists of a human serum albumin (HSA) polypeptide.
26. The agent of any one of claims 1 to 24, wherein said second moiety comprises or consists of an HSA binding polypeptide.
27. The agent of claim 26, wherein said HSA binding polypeptide is a single domain antibody.
28. The agent of claim 27, wherein said single domain antibody comprises or consists of the sequence:
EVQLVESGGGLVQPGNSLRRLSCAASGFTFSSFGMSWVRQAPGKGLEWVSSISGS
GSDTLYADSVKGRFTISRDNAAKTTLYLQMNSLRPEDTAVYYCTIGGSLRSSQGT
LVTVSSAAA (SEQ ID NO: 19).
29. The agent of any one of claims 1 to 28, wherein a C-terminus of said first moiety is linked to said linker and an N-terminus of said second moiety is linked to said linker.
30. The agent of claim 29, wherein a C-terminus of said first moiety is linked to an N-terminus of said linker and an N-terminus of said second moiety is linked to a C-terminus of said linker.
31. The agent of any one of claims 1 to 30, wherein said agent comprises or consist of an amino acid sequence selected from SEQ ID NO: 63 and 64.

32. The agent of any one of claims 1 to 31, wherein said agent comprises an amino acid sequence of SEQ ID NO: 36 and comprises irreversible conjugating a PEG moiety to an amino acid residue within 10 amino acids of a C-terminus of said first moiety.
33. The agent of any one of claims 1 to 32, wherein said agent comprises an amino acid sequence of SEQ ID NO: 36 and comprises reversible conjugating a PEG moiety to an amino acid residue within 10 amino acids of a C-terminus of said first moiety.
34. The agent of claim 32 or 33, wherein said PEG is linear or branched PEG comprising a size from 2000-40,000 Daltons (Da).
35. A nucleic acid molecule encoding the agent of any one of claims 1 to 34.
36. An expression vector comprising the nucleic acid molecule of claim 35.
37. A method of generating an agent of any one of claims 1 to 34, the method comprising at least one of:
- a. obtaining a first moiety that binds mCD28 on a cell surface and inhibits proteolytic cleavage of said mCD28;
 - b. linking said first moiety to a second moiety by a linker to produce a linked agent and testing binding of said linked agent to mCD28 on a surface of a cell and inhibition of proteolytic cleavage of said mCD28; and
 - c. selecting a linked agent that binds mCD28 on a cell surface and inhibits proteolytic cleavage of said mCD28;
- and
- d. culturing a host cell comprising one or more vectors comprising a nucleic acid sequence encoding an agent, wherein the nucleic acid sequence is that of an agent that was selected by:
 - i. obtaining a first moiety that binds mCD28 on a cell surface and inhibits proteolytic cleavage of said mCD28;
 - ii. linking said first moiety to a second moiety by a linker to produce a linked agent and testing binding of said linked agent to mCD28 on a surface of a cell and inhibition of proteolytic cleavage of said mCD28; and
 - iii. selecting a linked agent that binds mCD28 on a cell surface and inhibits proteolytic cleavage of said mCD28;

thereby generating an agent of any one of claims 1 to 34.

38. The method of claim 37, further comprising assaying mCD28 downstream signaling in the presence of said linked agent and selecting at least one linked agent that neither substantially agonizes nor substantially antagonizes mCD28 signaling.
39. The method of claim 37 or 38, further comprising assaying stability of said linked agent in blood and selecting at least one linked agent comprising an increased stability as compared to said first moiety in blood.
40. An agent produced by the method of any one of claims 37 to 39.
41. A pharmaceutical composition comprising an agent of any one of claims 1 to 23 and 40, and a pharmaceutically acceptable carrier, excipient or adjuvant.
42. A method of decreasing soluble CD28 (sCD28) levels in a subject in need thereof, the method comprising administering an agent of any one of claims 1 to 34 and 40 or a pharmaceutical composition of claim 41, thereby decreasing sCD28 in a subject.
43. A method of treating and/or preventing cancer in a subject in need thereof, the method comprising administering an agent of any one of claims 1 to 34 and 40 or a pharmaceutical composition of claim 41, thereby treating and/or preventing cancer in a subject.
44. A method of improving PD-1 and/or PD-L1 based immunotherapy in a subject in need thereof, the method comprising administering an agent of any one of claims 1 to 34 and 40 or a pharmaceutical composition of claim 39, thereby improving PD-1 and/or PD-L1 based immunotherapy in a subject.
45. The method of claim 42 or 44, wherein said subject suffers from cancer.
46. The method of any one of claims 42 to 45, wherein said method does not degrade mCD28, decrease mCD28-mediated immune cell activation, or activate mCD28-mediated immune cell activation.