proteins by fusing the bacterially-derived albumin-binding peptide to a therapeutic protein. The fusion protein is intended to bind serum albumin and thus enhance the half-life. For example, in one case, a recombinant fusion of the albumin-binding domain from streptococcal protein G to human complement receptor type I increased its half-life 3-fold to 5 h in rats (Makrides, S. et al. (1996) *J. Pharmacol. Exp. Ther.* 277, 534–542); in another case, fusion to this domain enhanced the immunological response directed to peptide antigens (Sjolander, A et al. (1997) *J. Immunol. Methods* 201, 115–123). Additional albumin-binding protein sequences are provided in, *e.g.*, PCT Publication No. WO 91/19741, PCT Publication No. WO 05/097202, PCT Publication No. WO 01/45746 and U.S. Patent Publication No. 2004/0001827. Fusion products, however, are prone to misfolding and the fused region can create highly immunogenic sites, resulting in a strong immunological reaction to the construct upon administration to a subject. The fusion molecule also may be less soluble than the therapeutic molecule alone.

Accordingly, there remains a need in the art for strategies of improving the bioavailability, stability, and/or serum half-life of molecules to allow for more effective therapeutics, without exacerbating immunogenicity of the therapeutic in the subject and/or without excessively reducing solubility. The instant invention provides compositions and techniques directed to addresing these and other needs.

The foregoing discussion is presented solely to provide a better understanding of the nature of the problems confronting the art and should not be construed in any way as an admission as to prior art nor should the citation of any reference herein be construed as an admission that such reference constitutes "prior art" to the instant application.

SUMMARY ~~OF THE INVENTION~~

[0013] The present ~~invention~~disclosure provides compounds, compositions, and methods of using modified albumin-binding domains, that are de-immunized and/or modified to improve solubility. Specifically, de-immunized albumin-binding domains of *Streptococcus zooepidemicus*, as well as albumin-binding fragments or derivatives thereof, are provided, in which one or more T_H epitopes of the albumin-binding domain is reduced or eliminated to decrease immunogenicity of the peptide. When linked to a therapeutic molecule, the modified albumin-binding domains can enhance the pharmaceutical potential of the molecule, by increasing half-life but not exacerbating immunogenicity and/or not excessively decreasing solubility, while maintaining the therapeutic properties of the molecule.

[0014] Accordingly, in one aspect, ~~of the invention relates to~~there is provided an agent comprising a therapeutic molecule linked to at least one albumin-binding domain, or an albumin-binding fragment or derivative thereof, where the albumin-binding domain comprises an amino acid sequence corresponding to SEQ ID NO:1 (PEP) and where the domain is modified by at least one amino acid substitution selected from the group consisting of E12D, T29H-K35D, and A45D, the substitutions referring to amino acid positions in SEQ ID NO:1; ~~such that~~and wherein the agent has an increased serum half life as compared to the therapeutic molecule.

[0015] In some particular embodiments, the albumin-binding domain comprises the amino acid substitution E12D, the substitution referring to an amino acid position in SEQ ID NO:1. In some particular embodiments, the albumin-binding domain comprises the amino acid substitution T29H-K35D, the substitution referring to an amino acid position in SEQ ID NO:1. In some particular embodiments, the albumin-binding domain comprises the amino acid substitution A45D, the substitution referring to an amino acid position in SEQ ID NO:1. In some particular embodiments, the albumin-binding domain comprises an amino acid sequence corresponding to SEQ ID NO:31.

[0016] In some preferred embodiments, the serum half life of the agent is increased by at least about 5 fold compared to the therapeutic molecule without the albumin-binding domain. In some preferred embodiments, serum half life is increased by at least about 8 fold or by at least about 10 fold. In some preferred embodiments, the serum half life of the agent is at least about 30 hours, or at least about 40 hours.

[0017] In some preferred embodiments, the agent also has increased solubility compared to the therapeutic molecule. For example, in some preferred embodiments, the solubility is increased by at least about 2 fold, by at least about 5 fold, by at least about 10 fold, or by at least about 15 fold.

[0018] In some particular embodiments, the linkage between the therapeutic molecule and albumin-binding domain is via a linker. In some preferred embodiments, the linker is a peptide linker, *e.g.*, a peptide linker comprising an amino acid sequence corresponding to SEQ ID NO:30. In some particularly preferred embodiments, the therapeutic molecule is a therapeutic polypeptide or peptide and in some further embodiments, the therapeutic polypeptide or peptide is linked to the albumin-binding domain as a fusion. The therapeutic molecule may be selected from the group consisting of protamine, gp60, gp30, gp18, protein A, a G protein, a protein transduction domain, toxins, cytotoxins, radionuclides, and macrocyclic chelators. In some particularly preferred embodiments, the therapeutic molecule

substitutions referring to amino acid positions in SEQ ID NO:3. In some preferred embodiments, the dimer comprises at least one light chain variable domain comprising an amino acid sequence corresponding to SEQ ID NOs:20-24, SEQ ID NOs: 25-29, or a TNF-alpha-binding fragment or derivative thereof. In some preferred embodiments, the dimer comprises at least one amino acid sequence selected from the group consisting of SEQ ID NOs: 35-44 (VL18-3L-VL11/PEP variants), and a TNF-alpha-binding fragment or derivative thereof.

[0022a] In another aspect, there is provided the use of an albumin-binding domain, or an albumin-binding fragment or derivative thereof, for the preparation of a medicament for enhancing efficacy of a therapeutic molecule in a subject, comprising:

linking said therapeutic molecule to said albumin-binding domain, or said albumin-binding fragment or derivative thereof, wherein said albumin-binding domain comprises an amino acid sequence corresponding to SEQ ID NO:1 (PEP) wherein said domain is modified by at least one amino acid substitution selected from the group consisting of E12D, T29H-K35D, and A45D, said substitutions referring to amino acid positions in SEQ ID NO:1.

[0023] Another ~~aspect~~embodiment of the instant ~~invention~~disclosure relates to a method of enhancing the efficacy of a therapeutic molecule in a subject, comprising: providing an agent comprising the therapeutic molecule linked at least one albumin-binding domain, or an albumin-binding fragment or derivative thereof, where the albumin-binding domain comprises an amino acid sequence corresponding to SEQ ID NO:1 (PEP) where the domain is modified by at least one amino acid substitution selected from the group consisting of E12D, T29H-K35D, and A45D, the substitutions referring to amino acid positions in SEQ ID NO:1; and administering the agent to the subject.

[0023a] In another aspect, there is provided a pharmaceutical composition comprising the agent as defined above and a pharmaceutically acceptable carrier.

[0023b] In another aspect, there is provided a nucleic acid comprising a nucleotide sequence encoding the agent as defined above, a vector comprising the nucleic acid, or a host cell compring the vector.

[0024] Yet another ~~aspect~~embodiment of the ~~invention~~disclosure relates to a pharmaceutical composition comprising the agent according to the ~~invention~~disclosure, and/or or a nucleic acid comprising a nucleotide sequence encoding an agent, and a pharmaceutically acceptable carrier. Still yet another ~~aspect~~embodiment of the ~~invention~~disclosure relates to a nucleic acid comprising a nucleotide sequence encoding an agent according to the ~~invention~~disclosure, as well as vectors and/or host cells and/or pharmaceutical compositions comprising same.

[0025] ~~Still~~In yet another aspect, ~~of the invention relates to~~there is provided a method of making an agent ~~according to the invention~~as defined above, comprising (i) providing a host cell comprising a vector encoding the agent; (ii) culturing the cell under conditions allowing expression of the agent; and (iii) recovering the agent from the culture.

BRIEF DESCRIPTION OF THE FIGURES

[0026] **FIG.1** illustrate proposed positions for substitutions (highlighted in gray) in an albumin binding domain in accordance with the invention, using Kabat and Ordinal numbering

[0027] **FIGs.2A-B** illustrate proposed positions for substitutions (highlighted in gray) in two VL single domain antibodies (A-B) for fusion with an albumin-binding domain of the invention, using Kabat and Ordinal numbering; CDRs are indicated by x.

CLAIMS

1. An agent comprising a therapeutic molecule linked to at least one albumin-binding domain, or an albumin-binding fragment or derivative thereof,

wherein said albumin-binding domain comprises an amino acid sequence corresponding to SEQ ID NO:1 (PEP), wherein said domain is modified by at least one amino acid substitution selected from the group consisting of E12D, T29H-K35D, and A45D, said substitutions referring to amino acid positions in SEQ ID NO:1; and

wherein said agent has an increased serum half life as compared to the therapeutic molecule.

2. The agent of claim 1 wherein said albumin-binding domain comprises the amino acid substitution E12D, said substitution referring to an amino acid position in SEQ ID NO:1 (PEP).

3. The agent of claim 1 wherein said albumin-binding domain comprises the amino acid substitution T29H-K35D, said substitution referring to an amino acid position in SEQ ID NO:1 (PEP).

4. The agent of claim 1 wherein said albumin-binding domain comprises the amino acid substitution A45D, said substitution referring to an amino acid position in SEQ ID NO:1 (PEP).

5. The agent of claim 1 wherein said albumin-binding domain comprises an amino acid sequence corresponding to SEQ ID NO:31.

6. The agent of any one of the above claims wherein the serum half life is increased by at least about 5 fold.

7. The agent of any one of the above claims wherein the serum half life is increased by at least about 8 fold.

8. The agent of any one of the above claims wherein the serum half life is increased by at least about 10 fold.

~~9. The agent of any one of the above claims wherein the serum half life is at least about~~ 30 hours.

10. The agent of any one of the above claims wherein the serum half life is at least about 40 hours.

11. The agent of any one of the above claims wherein said agent also has increased solubility compared to the therapeutic molecule.

12. The agent of claim 11 wherein the solubility is increased by at least about 2 fold.

13. The agent of claim 11 wherein the solubility is increased by at least about 5 fold.

14. The agent of claim 11 wherein the solubility is increased by at least about 10 fold.

15. The agent of claim 11 wherein the solubility is increased by at least about 15 fold.

16. The agent of any one of the above claims wherein the linkage is via a linker.

17. The agent of any one of the above claims wherein the linker is a peptide linker.

18. The agent of claim 17 wherein said peptide linker comprises an amino acid sequence corresponding to SEQ ID NO:30.

19. The agent of any one of the above claims wherein the therapeutic molecule is a therapeutic polypeptide or peptide.

20. The agent of claim 19 wherein said therapeutic polypeptide or peptide is linked to said albumin-binding domain as a fusion.

21. The agent of claim 19 or 20 wherein the therapeutic molecule is selected from the group consisting of protamine, gp60, gp30, gp18, protein A, a G protein, a protein transduction domain, toxins, cytotoxins, radionuclides, and macrocyclic chelators.

22. The agent of claim 19 or 20 wherein the therapeutic molecule is an antibody or antibody fragment.

23. The agent of claim 22 wherein the therapeutic antibody or antibody fragment is selected from the group consisting of a monoclonal antibody, multispecific antibody, humanized antibody, synthetic antibody, chimeric antibody, polyclonal antibody, single-~~chain Fv (scFv), single chain antibody, anti-idiotypic (anti-Id) antibody, diabody, minibody,~~

nanobody, single domain antibody, Fab fragment, F(ab') fragment, disulfide-linked bispecific Fv (sdFv), and intrabody.

24. The agent of claim 23 wherein the therapeutic molecule comprises a dimer of two antibody single domains or antigen-binding fragments thereof, wherein said domains comprise light chain variable domains.

25. The agent of claim 24 wherein said dimer binds TNF-alpha.

26. The agent of claim 25 wherein said dimer comprises at least one light chain variable domain comprising an amino acid sequence corresponding to SEQ ID NO:2 (VL18), SEQ ID NO:3 (VL11), SEQ ID NOs:4-19, or a TNF-alpha-binding fragment or derivative thereof.

27. The agent of claim 25 wherein said dimer comprises two light chain variable domains comprising an amino acid sequence corresponding SEQ ID NO:32 (VL18-3L-VL11), or a TNF-alpha-binding fragment or derivative thereof.

28. The agent according to any one of claims 25-27 wherein said at least one variable domain antagonizes binding of human TNF-alpha to a TNF-alpha receptor.

29. The agent according to claim 28 wherein said at least one variable domain further cross-reacts with at least one other mammalian TNF-alpha, wherein said mammal is not a primate.

30. The agent according to claim 29 wherein said variable domain cross-reacts with TNF-alpha of at least two other mammals, said at least two other mammals being a rodent and a non-rodent species.

31. The agent according to any one of claims 24-30 wherein said dimer is further de-immunized by eliminating at least one T_H epitope of in at least one of said variable domains.

32. The agent according to claim 31 wherein said at least one variable domain comprises an amino acid sequence corresponding to SEQ ID NO:2 (VL18), which is also de-immunized by at least one amino acid substitution selected from the group consisting of T7Q, V15P, (A51V-L54R/A51V-L54E), K63S, E79K, (C80S), T91A, and L111K, said substitutions referring to amino acid positions in SEQ ID NO:2.

33. The agent according to claim 31 wherein said at least one variable domain comprises an amino acid sequence corresponding to SEQ ID NO:3 (VL11), which is also de-

immunized by at least one amino acid substitution selected from the group consisting of T7Q, V15P, R31S, (A51V-54R /A51V-L54E), K63S, E79K, (C80S), T91A, A100S, and E106K, said substitutions referring to amino acid positions in SEQ ID NO:3.

34. The agent according to claim 31 wherein said dimer comprises at least one amino acid sequence selected from the group consisting of SEQ ID NOs:20-24, SEQ ID NOs: 25-29, SEQ ID NOs: 34-44 (VL18-3L-VL11/PEP variants), and a TNF-alpha-binding fragment or derivative thereof.

35. A method of enhancing the efficacy of a therapeutic molecule in a subject, comprising:

providing an agent comprising said therapeutic molecule linked at least one albumin-binding domain, or an albumin-binding fragment or derivative thereof, wherein said albumin-binding domain comprises an amino acid sequence corresponding to SEQ ID NO:1 (PEP) wherein said domain is modified by at least one amino acid substitution selected from the group consisting of E12D, T29H-K35D, and A45D, said substitutions referring to amino acid positions in SEQ ID NO:1; and

administering said agent to said subject.

36. The method of claim 35 wherein said agent comprises the amino acid substitution E12D, said substitution referring to an amino acid position in SEQ ID NO:1.

37. The method of claim 35 wherein said agent comprises the amino acid substitution T29H-K35D, said substitution referring to an amino acid position in SEQ ID NO:1.

38. The method of claim 35 wherein said agent comprises the amino acid substitution A45D, said substitution referring to an amino acid position in SEQ ID NO:1.

39. The method of claim 35 wherein the agent has an increased serum half life as compared to the therapeutic molecule.

40. The method of any of claims 35-39 wherein the linkage is via a linker.

41. The method of claim 40 wherein the linker is a peptide linker.

42. The method of any one of claims 35-41 wherein the therapeutic molecule is a therapeutic polypeptide or peptide.

43. The method of claim 42 wherein said therapeutic polypeptide or peptide is linked to said albumin-binding domain as a fusion.

44. The method of claim 42 or 43 wherein the therapeutic molecule is selected from the group consisting of protamine, gp60, gp30, gp18, protein A, a G protein, a protein transduction domain, toxins, cytotoxins, radionuclides, and macrocyclic chelators.

45. The method of claim 42 or 43 wherein the therapeutic molecule is an antibody or antibody fragment.

46. The method of claim 45 wherein the therapeutic antibody or antibody fragment is selected from the group consisting of a monoclonal antibody, multispecific antibody, humanized antibody, synthetic antibody, chimeric antibody, polyclonal antibody, single-chain Fv (scFv), single chain antibody, anti-idiotypic (anti-Id) antibody, diabody, minibody, nanobody, single domain antibody, Fab fragment, F(ab') fragment, disulfide-linked bispecific Fv (sdFv), and intrabody.

47. The method of claim 46 wherein the therapeutic molecule comprises a dimer of two antibody single domains or antigen-binding fragments thereof, wherein said domains comprise light chain variable domains.

48. A pharmaceutical composition comprising the agent according to any of claims 1-34 and a pharmaceutically acceptable carrier.

49. A nucleic acid comprising a nucleotide sequence encoding the agent according to any one of claims 19-34.

50. A vector comprising the nucleic acid according to claim 49.

51. A host cell compring the vector according to claim 50.

52. A method of making the agent according to any one of claims 19-34 comprising:

(i) providing a host cell comprising a vector encoding said agent;
(ii) culturing said cell under conditions allowing expression of said agent; and
(iii) recovering said agent from said culture.

CLAIMS

1. An agent comprising a therapeutic molecule linked to at least one albumin-binding domain, or an albumin-binding fragment or derivative thereof,

wherein said albumin-binding domain comprises an amino acid sequence corresponding to SEQ ID NO:1 (PEP), wherein said domain is modified by at least one amino acid substitution selected from the group consisting of E12D, T29H-K35D, and A45D, said substitutions referring to amino acid positions in SEQ ID NO:1; and

wherein said agent has an increased serum half life as compared to the therapeutic molecule.

2. The agent of claim 1 wherein said albumin-binding domain comprises:

(i) the amino acid substitution E12D, said substitution referring to an amino acid position in SEQ ID NO:1 (PEP),

(ii) the amino acid substitution T29H-K35D, said substitution referring to an amino acid position in SEQ ID NO:1 (PEP);

(iii) the amino acid substitution A45D, said substitution referring to an amino acid position in SEQ ID NO:1 (PEP); or

(iv) an amino acid sequence corresponding to SEQ ID NO:31.

3. The agent of any one of the above claims wherein the serum half life is increased by at least about 5 fold, preferably by at least about 8 fold, or preferably by at least about 10 fold; and/or

wherein the serum half life is at least about 30 hours, preferably at least about 40 hours.

4. The agent of any one of the above claims wherein said agent also has increased solubility compared to the therapeutic molecule,

preferably wherein the solubility is increased by at least about 2 fold, by at least about 5 fold, by at least about 10 fold, or by at least about 15 fold.

5. The agent of any one of the above claims wherein the linkage is via a linker, preferably wherein the linker is a peptide linker, preferably comprising an amino acid sequence corresponding to SEQ ID NO:30.

6. The agent of any one of the above claims wherein the therapeutic molecule is a therapeutic polypeptide or peptide, preferably wherein said therapeutic polypeptide or peptide is linked to said albumin-binding domain as a fusion; and/or

wherein the therapeutic molecule is selected from the group consisting of protamine, gp60, gp30, gp18, protein A, a G protein, a protein transduction domain, toxins, cytotoxins, radionuclides, and macrocyclic chelators; and/or

wherein the therapeutic molecule is an antibody or antibody fragment, preferably the therapeutic antibody or antibody fragment being selected from the group consisting of a monoclonal antibody, multispecific antibody, humanized antibody, synthetic antibody, chimeric antibody, polyclonal antibody, single-chain Fv (scFv), single chain antibody, anti-idiotypic (anti-Id) antibody, diabody, minibody, nanobody, single domain antibody, Fab fragment, F(ab') fragment, disulfide-linked bispecific Fv (sdFv), and intrabody.

7. The agent of any one of the above claims wherein the therapeutic molecule is a therapeutic polypeptide or peptide, preferably wherein said therapeutic polypeptide or peptide is linked to said albumin-binding domain as a fusion; and/or

wherein the therapeutic molecule comprises a dimer of two antibody single domains or antigen-binding fragments thereof, wherein said domains comprise light chain variable domains,

preferably wherein said dimer binds TNF-alpha,

preferably wherein said dimer comprises at least one light chain variable domain comprising an amino acid sequence corresponding to SEQ ID NO:2 (VL18), SEQ ID NO:3 (VL11), SEQ ID NOs:4-19, or a TNF-alpha-binding fragment or derivative thereof, or

preferably wherein said dimer comprises two light chain variable domains comprising an amino acid sequence corresponding SEQ ID NO:32 (VL18-3L-VL11), or a TNF-alpha-binding fragment or derivative thereof.

8. The agent according to claim 7 wherein said at least one variable domain antagonizes binding of human TNF-alpha to a TNF-alpha receptor,

preferably wherein said at least one variable domain further cross-reacts with at least one other mammalian TNF-alpha, wherein said mammal is not a primate,

preferably wherein said variable domain cross-reacts with TNF-alpha of at least two other mammals, said at least two other mammals being a rodent and a non-rodent species.

9. The agent according to claim 7 or 8 wherein said dimer is further de-immunized by eliminating at least one T_H epitope of in at least one of said variable domains,

preferably wherein said at least one variable domain comprises an amino acid sequence corresponding to SEQ ID NO:2 (VL18), which is also de-immunized by at least one amino acid substitution selected from the group consisting of T7Q, V15P, (A51V-L54R/A51V-L54E), K63S, E79K, (C80S), T91A, and L111K, said substitutions referring to amino acid positions in SEQ ID NO:2, or

preferably wherein said at least one variable domain comprises an amino acid sequence corresponding to SEQ ID NO:3 (VL11), which is also de-immunized by at least one amino acid substitution selected from the group consisting of T7Q, V15P, R31S, (A51V-54R /A51V-L54E), K63S, E79K, (C80S), T91A, A100S, and E106K, said substitutions referring to amino acid positions in SEQ ID NO:3, or

preferably wherein said dimer comprises at least one amino acid sequence selected from the group consisting of SEQ ID NOs:20-24, SEQ ID NOs: 25-29, SEQ ID NOs: 34-44 (VL18-3L-VL11/PEP variants), and a TNF-alpha-binding fragment or derivative thereof.

10. Use of an albumin-binding domain, or an albumin-binding fragment or derivative thereof, for the preparation of a medicament for enhancing efficacy of a therapeutic molecule in a subject, comprising:

linking said therapeutic molecule to said albumin-binding domain, or said albumin-binding fragment or derivative thereof,

wherein said albumin-binding domain comprises an amino acid sequence corresponding to SEQ ID NO:1 (PEP) wherein said domain is modified by at least one amino acid substitution selected from the group consisting of E12D, T29H-K35D, and A45D, said substitutions referring to amino acid positions in SEQ ID NO:1.

11. The use of claim 10 wherein said albumin-binding domain further comprises

(i) the amino acid substitution E12D, said substitution referring to an amino acid position in SEQ ID NO:1,

(ii) the amino acid substitution T29H-K35D, said substitution referring to an amino acid position in SEQ ID NO:1, or

(iii) the amino acid substitution A45D, said substitution referring to an amino acid position in SEQ ID NO:1, or

wherein said agent has an increased serum half life as compared to the therapeutic molecule.

12. The use of claim 10 or 11 wherein the linkage is via a linker, preferably wherein the linker is a peptide linker; and/or

wherein the therapeutic molecule is a therapeutic polypeptide or peptide, preferably wherein said therapeutic polypeptide or peptide is linked to said albumin-binding domain as a fusion; and/or

wherein the therapeutic molecule is selected from the group consisting of protamine, gp60, gp30, gp18, protein A, a G protein, a protein transduction domain, toxins, cytotoxins, radionuclides, and macrocyclic chelators; or

wherein the therapeutic molecule is an antibody or antibody fragment, preferably wherein the therapeutic antibody or antibody fragment is selected from the group consisting of a monoclonal antibody, multispecific antibody, humanized antibody, synthetic antibody, chimeric antibody, polyclonal antibody, single-chain Fv (scFv), single chain antibody, anti-idiotypic (anti-Id) antibody, diabody, minibody, nanobody, single domain antibody, Fab fragment, F(ab') fragment, disulfide-linked bispecific Fv (sdFv), and intrabody, and

preferably wherein the therapeutic molecule comprises a dimer of two antibody single domains or antigen-binding fragments thereof, wherein said domains comprise light chain variable domains.

13. A pharmaceutical composition comprising the agent according to any of claims 1-12 and a pharmaceutically acceptable carrier.

14. A nucleic acid comprising a nucleotide sequence encoding the agent according to any one of claims 6-9,

a vector comprising the nucleic acid, or

a host cell compring the vector.

15. A method of making the agent according to any one of claims 6-9 comprising:

(i) providing a host cell comprising a vector encoding said agent;

(ii) culturing said cell under conditions allowing expression of said agent; and

(iii) recovering said agent from said culture.