

MULTISPECIFIC PROTEINS

CROSS-REFERENCE TO RELATED APPLICATION

The present application claims the benefit of and priority from European patent application EP20174847 filed on 14 May 2020 with the European Patent Office and from European patent application EP20181498 filed on 22 June 2020 with the European Patent Office. The contents of European patent applications EP20174847 and EP20181498 are incorporated herein by reference in their entirety, including all tables, figures, and claims.

FIELD OF THE INVENTION

[1] The present invention relates to multispecific proteins comprising designed ankyrin repeat domains with binding specificity for different targets, such as, e.g. CD40 and FAP. In addition, the invention relates to nucleic acids encoding such multispecific proteins, pharmaceutical compositions comprising such multispecific proteins or nucleic acids, and the use of such binding proteins, nucleic acids or pharmaceutical compositions in methods for treating or diagnosing diseases, such as cancer, in a mammal, including a human.

BACKGROUND OF THE INVENTION

Tumor necrosis factor receptor (TNFR) superfamily member CD40 is a key co-stimulatory receptor, and when engaged by its ligand (CD40L) or by agonistic antibodies, it is involved in the regulation of a wide spectrum of molecular and cellular processes, including the initiation and progression of cellular and humoral adaptive immunity. For example, it has been demonstrated that CD40 engagement on the surface of dendritic cells promotes their cytokine production, induces the expression of costimulatory molecules on their surface, and facilitates the presentation of antigen. Overall, the impact of CD40 signaling 'licenses' dendritic cells to mature and achieve all of the necessary characteristics to effectively trigger T-cell activation and differentiation. CD40 signaling in B cells promotes germinal center formation, immunoglobulin (Ig) isotype switching, somatic hypermutation of the Ig to enhance affinity for antigen, and finally the formation of long-lived plasma cells and memory B cells. Moreover, it has been shown that the CD40 pathway is important for the survival of many cell types including germinal center B cells, dendritic cells, and endothelial cells under normal and inflammatory conditions. Deregulation of CD40 signaling has been observed in various autoimmune diseases. Together, this breadth of functions underlines the importance of the CD40 receptor for the generation of an acquired immune response.

CD40 was initially characterized on B cells and is also expressed on dendritic cells, monocytes, platelets, and macrophages as well as on non-hematopoietic cells such as myofibroblasts, fibroblasts, epithelial, and endothelial cells. The ligand of CD40, known as CD154 or CD40L, is expressed primarily by

activated T cells, as well as activated B cells and platelets, and under inflammatory conditions it is also induced in monocytic cells, natural killer cells, mast cells, and basophils.

Because CD40 can activate both the innate and adaptive immune system, it has been recognized as a suitable target for tumor immunotherapy. Several reports have confirmed that CD40 stimulation can enhance anti-tumor immune responses by means of dendritic cell maturation. Activation of dendritic cells with agonists of CD40 results in their increased survival, secretion of IL-1, IL-6, IL-8, IL-12, TNF- α , and macrophage inflammatory protein-1 α . Additionally, CD40 activation induces the upregulation of costimulatory molecules such as MHC class II, LFA-3, CD80, and CD86 and promotes antigen presentation, priming and cross-priming of T helper cells (Th) and cytotoxic T lymphocytes (CTL), respectively. Agonistic antibodies against CD40 have proved efficacious in preclinical murine tumor models. However, although their use in the clinic has shown some anti-tumor efficacy, clinical development of agonistic anti-CD40 antibodies has likely been hampered by dose-limiting toxicities and resulting low efficacies.

[2] Thus, there remains a need for new CD40-specific binding proteins, and for therapeutic and diagnostic approaches for the treatment and characterization of diseases, including cancer, benefitting from CD40-specific binding and activation. In particular, there is a need for new generation agonists that can effectively engage CD40 while avoiding undesired side effects.

SUMMARY OF THE INVENTION

[3] Based on the disclosure provided herein, those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following embodiments (E).

E1. A recombinant protein comprising a first ankyrin repeat domain that specifically binds fibroblast activation protein (FAP) and a second ankyrin repeat domain that specifically binds CD40.

E2. The recombinant protein of E1, further comprising a third ankyrin repeat domain that specifically binds CD40.

E3. The recombinant protein of E2, wherein said ankyrin repeat domains are arranged, from the N-terminus to C-terminus, according to the following formula: (FAP-binding domain) – (CD40 binding domain) – (CD40 binding domain).

E4. The recombinant protein of any one of E1-E3, further comprising a half-life extending moiety.

E5. The recombinant protein of E4, wherein said half-life extending moiety comprises a fourth ankyrin repeat domain that specifically binds serum albumin.

- E6. The recombinant protein of E5, wherein said ankyrin repeat domains are arranged, from the N-terminus to C-terminus, according to the following formula: (serum albumin binding domain) – (FAP-binding domain) – (CD40 binding domain) – (CD40 binding domain).
- E7. The recombinant protein of any one of E1-E6, further comprising a linker between any of said FAP-binding domain, said CD40 binding domain(s), and said half-life extending moiety.
- E8. The recombinant protein of any one of E1-E7, comprising the following formula, from the N-terminus to C-terminus: (FAP-binding domain) – (linker) – (CD40 binding domain) – (linker) – (CD40 binding domain).
- E9. The recombinant protein of any one of E1-E8, comprising the following formula, from the N-terminus to C-terminus: (serum albumin binding domain) – (linker) – (FAP-binding domain) – (linker) – (CD40 binding domain) – (linker) – (CD40 binding domain).
- E10. The recombinant protein of E4, wherein said half-life extending moiety comprises an immunoglobulin heavy chain constant domain.
- E11. The recombinant protein of E10, wherein said immunoglobulin domain is an Fc domain of an IgA1, IgA2, IgD, IgE, IgM, IgG1, IgG2, IgG3, or IgG4 immunoglobulin.
- E12. The recombinant protein of E11, wherein said Fc domain is the Fc domain of human IgG1 immunoglobulin.
- E13. The recombinant protein of E12, wherein said Fc domain is modified to reduce the effector function.
- E14. The recombinant protein of any one of E1-E13, wherein said FAP is human FAP.
- E15. The recombinant protein of any one of E1-E14, wherein said CD40 is human CD40.
- E16. The recombinant protein of any one of E5-E19, wherein said serum albumin is human serum albumin (HSA).
- E17. The recombinant protein of any one of E10-E13, wherein said immunoglobulin heavy chain constant domain is a human immunoglobulin heavy chain constant domain.
- E18. The recombinant protein of any one of E1-E17, wherein binding of said recombinant protein to FAP does not reduce the protease activity of FAP by more than 25%, more than 20%, more than 15%, more than 10%, or more than 5%.
- E19. The recombinant protein of any one of E1-E18, wherein said FAP-binding domain comprises an amino acid sequence that is at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least

92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% identical to SEQ ID NO: 2, and wherein optionally A at the second last position of SEQ ID NO: 2 is substituted with L and/or A at the last position of SEQ ID NO: 2 is substituted with N.

E20. The recombinant protein of E19, wherein said FAP-binding domain comprises the amino acid sequence of SEQ ID NO: 2.

E21. The recombinant protein of any one of E1-E18, wherein said FAP-binding domain comprises an amino acid sequence that is at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% identical to SEQ ID NO: 8, and wherein optionally A at the second last position of SEQ ID NO: 2 is substituted with L and/or A at the last position of SEQ ID NO: 8 is substituted with N.

E22. The recombinant protein of E21, wherein said FAP-binding domain comprises the amino acid sequence of SEQ ID NO: 8.

E23. The recombinant protein of any one of E1-E18, wherein said FAP-binding domain comprises an amino acid sequence that is at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% identical to any one of SEQ ID NOs: 9 and 28-38.

E24. The recombinant protein of E23, wherein said FAP-binding domain comprises the amino acid sequence of any one of SEQ ID NOs: 9 and 28-38.

E25. The recombinant protein of E23, wherein said FAP-binding domain comprises an amino acid sequence that is at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% identical to any one of SEQ ID NOs: 9, 28-31 and 38, and wherein optionally A at the second last position is substituted with L and/or A at the last position is substituted with N.

E26. The recombinant protein of E25, wherein said FAP-binding domain comprises an amino acid sequence that is at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% identical to SEQ ID NO: 28, and wherein optionally A at the second last position is substituted with L and/or A at the last position is substituted with N.

E27. The recombinant protein of E23, wherein said FAP-binding domain comprises an amino acid sequence that is at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least

93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% identical to any one of SEQ ID NOs: 32-37, and wherein optionally L at the second last position is substituted with A and/or N at the last position is substituted with A.

E28. The recombinant protein of E27, wherein said FAP-binding domain comprises an amino acid sequence that is at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% identical to SEQ ID NO: 34, and wherein optionally L at the second last position is substituted with A and/or N at the last position is substituted with A.

E29. The recombinant protein of any one of E1-E28, wherein said FAP-binding domain (i) comprises an amino acid sequence that is at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% identical to any one of SEQ ID NOs: 2, 8, 9 and 28-37, and (ii) further comprises at its N-terminus, a G, an S, or a GS.

E30. The recombinant protein of any one of E1-E29, wherein said FAP-binding domain comprises an amino acid sequence that is at least 90% identical to any one of SEQ ID NOs: 2, 8, 9 and 28-37, and further comprises at its N-terminus, a G, an S, or a GS.

E31. The recombinant protein of any one of E1-E30, wherein said CD40-binding domain or each of said CD40 binding domains independently comprises an amino acid sequence that is at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% identical to SEQ ID NO: 3, and wherein optionally A at the second last position of SEQ ID NO: 3 is substituted with L and/or A at the last position of SEQ ID NO: 3 is substituted with N.

E32. The recombinant protein of any one of E1-E31, wherein said CD40 binding domain or each of said CD40 binding domains comprises the amino acid sequence of SEQ ID NO: 3.

E33. The recombinant protein of any one of E1-E30, wherein said CD40-binding domain or each of said CD40 binding domains independently comprises an amino acid sequence that is at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% identical to any one of SEQ ID NOs: 10 and 43-50.

E34. The recombinant protein of any one of E1-E30 and E33, wherein said CD40-binding domain or each of said CD40 binding domains comprises the amino acid sequence of any one of SEQ ID NOs: 10 and 43-50.

E35. The recombinant protein of any one of E1-E30 and E33, wherein said CD40 binding domain or each of said CD40 binding domains independently comprises an amino acid sequence that is at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% identical to any one of SEQ ID NOs: 10, 43, 44, and 48-50, and wherein optionally A at the second last position is substituted with L and/or A at the last position is substituted with N.

E36. The recombinant protein of E35, wherein said CD40 binding domain or each of said CD40 binding domains independently comprises an amino acid sequence that is at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% identical to SEQ ID NO: 43, and wherein optionally A at the second last position is substituted with L and/or A at the last position is substituted with N.

E37. The recombinant protein of any one of E1-E30 and E33, wherein said CD40 binding domain or each of said CD40 binding domains independently comprises an amino acid sequence that is at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% identical to any one of SEQ ID NOs: 45-47, and wherein optionally L at the second last position is substituted with A and/or N at the last position is substituted with A.

E38. The recombinant protein of E37, wherein said CD40 binding domain or each of said CD40 binding domains independently comprises an amino acid sequence that is at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% identical to SEQ ID NO: 47, and wherein optionally L at the second last position is substituted with A and/or N at the last position is substituted with A.

E39. The recombinant protein of any one E1-E38, wherein said CD40 binding domain or each of said CD40 binding domains independently (i) comprises an amino acid sequence that is at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% identical to any one of SEQ ID NOs: 3, 10 and 43-49, and (ii) further comprises at its N-terminus, a G, an S, or a GS.

E40. The recombinant protein of any one E1-E39, wherein said CD40 binding domain or each of said CD40 binding domains independently comprises an amino acid sequence that is at least 90% identical to any one of SEQ ID NOs: 3, 10 and 43-49, and further comprises at its N-terminus, a G, an S, or a GS

E41. The recombinant protein of any one of E1-E40, wherein said CD40 binding domain or each of said CD40 binding domains independently comprises Q at position 8, L at position 15, R at position 143, and/or Q at position 147, wherein the position numbers correspond to the positions in SEQ ID NO: 3.

E42. The recombinant protein of any one of E1-E41, wherein said CD40 binding domain or each of said CD40 binding domains independently comprises Q at position 8, L at position 15, R at position 143, and Q at position 147, wherein the position numbers correspond to the positions in SEQ ID NO: 3.

E43. The recombinant protein of any one of E1-E42, wherein:

(a) said FAP binding domain comprises an amino acid sequence that is at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% identical to any one of SEQ ID NOs: 2, 8, 9 and 28-37; wherein its N-terminus optionally further comprises a G, an S, or a GS; and wherein the second last position can be L or A, and the last position can be N or A; and

(b) said CD40 binding domain or each of said CD40 binding domains independently comprises an amino acid sequence that is at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% identical to any one of SEQ ID NOs: 3, 10 and 43-49; wherein its N-terminus optionally further comprises a G, an S, or a GS; and wherein the second last position can be L or A, and the last position can be N or A.

E44. The recombinant protein of E43, wherein:

(a) said FAP binding domain comprises an amino acid sequence that is at least 90% identical to any one of SEQ ID NOs: 2, 8, 9 and 28-37; wherein its N-terminus optionally further comprises a G, an S, or a GS; and wherein the second last position can be L or A, and the last position can be N or A; and

(b) said CD40 binding domain or each of said CD40 binding domains independently comprises an amino acid sequence that is at least 90% identical to any one of SEQ ID NOs: 3, 10 and 43-49; wherein its N-terminus optionally further comprises a G, an S, or a GS; and wherein the second last position can be L or A, and the last position can be N or A.

E45. The recombinant protein of E43, wherein:

(a) said FAP binding domain comprises an amino acid sequence that is at least 95% identical to any one of SEQ ID NOs: 2, 8, 9 and 28-37; wherein its N-terminus optionally further comprises a G, an S, or a GS; and wherein the second last position can be L or A, and the last position can be N or A; and

(b) said CD40 binding domain or each of said CD40 binding domains independently comprises an amino acid sequence that is at least 95% identical to any one of SEQ ID NOs: 3, 10 and 43-49; wherein its N-terminus optionally further comprises a G, an S, or a GS; and wherein the second last position can be L or A, and the last position can be N or A.

E46. The recombinant protein of E43, wherein:

(a) said FAP binding domain comprises the amino acid sequence of any one of SEQ ID NOs: 2, 8, 9 and 28-37; wherein its N-terminus optionally further comprises a G, an S, or a GS; and wherein the second last position can be L or A, and the last position can be N or A; and

(b) said CD40 binding domain or each of said CD40 binding domains independently comprises the amino acid sequence of any one of SEQ ID NOs: 3, 10 and 43-49; wherein its N-terminus optionally further comprises a G, an S, or a GS; and wherein the second last position can be L or A, and the last position can be N or A.

E47. The recombinant protein of any one of E5-E9, E14-E16, and E18-E46, wherein said serum albumin binding domain comprises an amino acid sequence that is at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% identical to SEQ ID NO: 1, and wherein optionally A at the second last position is substituted with L and/or A at the last position is substituted with N.

E48. The recombinant protein of any one of E5-E9, E14-E16, and E18-E47, wherein said serum albumin binding domain comprises the amino acid sequence of SEQ ID NO: 1.

E49. The recombinant protein of any one of E5-E9, E14-E16, and E18-E46, wherein said serum albumin binding domain comprises an amino acid sequence that is at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96% at least 97%, at least 98%, at least 99%, or 100% identical to any one of SEQ ID NOs: 39-42, and wherein optionally A at the second last position of any one of SEQ ID NOs: 39-40 and 42 is substituted with L and/or A at the last position of any one of SEQ ID NOs: 39-40 and 42 is substituted with N, and wherein optionally L at the second last position of SEQ ID NO: 41 is substituted with A and/or N at the last position of SEQ ID NO: 41 is substituted with A.

E50. The recombinant protein of any one of E5-E9, E14-E16, E18-E45, and E49, wherein said serum albumin binding domain comprises the amino acid sequence of any one of SEQ ID NOs: 39-42.

E51. The recombinant protein of any one of E5-E9, E14-E16, and E18-E50, wherein said serum albumin binding domain (i) comprises an amino acid sequence that is at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% identical to any one of SEQ ID NOs: 1 and 39-41, or (ii) comprises

an amino acid sequence that is at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% identical to SEQ ID NO: 42 and further comprises at its N-terminus, a G, an S, or a GS.

E52. The recombinant protein of any one of E5-E9, E14-E16, and E18-E51, wherein said serum albumin binding domain comprises (i) an amino acid sequence that is at least 90% identical to any one of SEQ ID NOs: 1 and 39-41, or (ii) comprises an amino acid sequence that is at least 90% identical to SEQ ID NO: 42 and further comprises at its N-terminus, a G, an S, or a GS.

E53. The recombinant protein of any one of E5-E9, E14-E16, and E18-E52, wherein said serum albumin binding domain comprises (i) the amino acid sequence of any one of SEQ ID NOs: 1 and 39-41, or (ii) comprises the amino acid sequence of SEQ ID NO: 42 and further comprises at its N-terminus, a G, an S, or a GS.

E54. The recombinant protein of any one of E8-E53, wherein said linker comprises the amino acid sequence of SEQ ID NO: 4.

E55. The recombinant protein of any one of E1-E54, wherein said protein comprises exactly four ankyrin repeat domains.

E56. A recombinant protein comprising an amino acid sequence that is at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% identical to SEQ ID NO: 5 or SEQ ID NO: 6 or SEQ ID NO: 7, wherein said protein specifically binds FAP and CD40.

E57. The recombinant protein of E56 wherein said protein comprises an amino acid sequence that is at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% identical to SEQ ID NO: 5.

E58. The recombinant protein of any one of E56-E57, wherein said protein comprises an amino acid sequence that is at least 90% identical to SEQ ID NO: 5.

E59. The recombinant protein of any one of E56-E58, wherein said protein comprises the amino acid sequence of SEQ ID NO: 5.

E60. The recombinant protein of E56, wherein said protein comprises an amino acid sequence that is at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least

94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% identical to SEQ ID NO: 6.

E61. The recombinant protein of E56 or E60, wherein said protein comprises an amino acid sequence that is at least 90% identical to SEQ ID NO: 6.

E62. The recombinant protein of any one of E56 and E60-E61, wherein said protein comprises the amino acid sequence of SEQ ID NO: 6.

E63. The recombinant protein of any one of E56-E62, wherein said FAP is human FAP.

E64. The recombinant protein of any one of E56-E63, wherein said CD40 is human CD40.

E65. The recombinant protein of any one of E1-E64, wherein said FAP-binding domain binds human FAP with a KD value of or below: 100 nM, 90 nM, 80 nM, 75 nM, 70 nM, 60 nM, 50 nM, 40 nM, 30 nM, 20 nM, 10 nM, 5 nM, 2 nM, 1 nM, 900 pM, 800 pM, 700 pM, 600 pM, 500 pM, 400 pM, 300 pM, 250 pM, 200 pM, 150 pM, 140 pM, 130 pM, or 120 pM.

E66. The recombinant protein of any one of E1-E65, wherein said FAP-binding domain binds human FAP with a KD value of or below 100 nM.

E67. The recombinant protein of any one of E1-E66, wherein said FAP-binding domain binds human FAP with a KD value of or below 1 nM.

E68. The recombinant protein of any one of E1-E67, wherein said FAP-binding domain binds human FAP with a KD value of or below 120 pM.

E69. The recombinant protein of any one of E1-E68, wherein said CD40-binding domain or each of said CD40-binding domains independently binds human CD40 with a KD value of or below: 100 nM, 90 nM, 80 nM, or 75 nM.

E70. The recombinant protein of any one of E1-E69, wherein said CD40-binding domain or each of said CD40-binding domains independently binds human CD40 with a KD value of or below 100 nM.

E71. The recombinant protein of any one of E1-E70, wherein said CD40-binding domain or each of said CD40-binding domains independently binds human CD40 with a KD value of or below 75 nM.

E72. The recombinant protein of any one of E1-E71, wherein said serum albumin binding domain binds human serum albumin with a KD value of or below: 100 nM, 90 nM, 80 nM, 75 nM, 70 nM, 60 nM, 50 nM, 40 nM, or 35 nM.

E73. The recombinant protein of any one of E1-E72, wherein said serum albumin binding domain binds human serum albumin with a KD value of or below 100 nM.

E74. The recombinant protein of any one of E1-E73, wherein said serum albumin binding domain binds human serum albumin with a KD value of or below 50 nM.

E75. The recombinant protein of any one of E1-E74, wherein said serum albumin binding domain binds human serum albumin with a KD value of or below 35 nM.

E76. The recombinant protein of any one of E1-E75, wherein said recombinant protein binds human FAP with a KD value of or below: 100 nM, 90 nM, 80 nM, 75 nM, 70 nM, 60 nM, 50 nM, 40 nM, 30 nM, 20 nM, 10 nM, 5 nM, 2 nM, 1 nM, 900 pM, 800 pM, 700 pM, 600 pM, 500 pM, 400 pM, or 300 pM.

E77. The recombinant protein of any one of E1-E76, wherein said recombinant protein binds human FAP with a KD value of or below 100 nM.

E78. The recombinant protein of any one of E1-E77, wherein said recombinant protein binds human FAP with a KD value of or below 1 nM.

E79. The recombinant protein of any one of E1-E78, wherein said recombinant protein binds human FAP with a KD value of or below 500 pM.

E80. The recombinant protein of any one of E1-E79, wherein said recombinant protein binds human FAP with a KD value of or below 300 pM.

E81. The recombinant protein of any one of E1-E80, wherein said recombinant protein binds human CD40 with a KD value of or below: 100 nM, 90 nM, 80 nM, 75 nM, 70 nM, 60 nM, 50 nM, 40 nM, 30 nM, 20 nM, 10 nM, 5 nM, 2 nM, 1 nM, 900 pM, 800 pM, 700 pM, 600 pM, 500 pM, 400 pM, 300 pM, 250 pM, 200 pM, 150 pM, 140 pM, 130 pM, 120 pM, 115 pM, 110 pM, 105 pM or 100 pM.

E82. The recombinant protein of any one of E1-E81, wherein said recombinant protein binds human CD40 with a KD value of or below 100 nM.

E83. The recombinant protein of any one of E1-E82, wherein said recombinant protein binds human CD40 with a KD value of or below 1 nM.

E84. The recombinant protein of any one of E1-E83, wherein said recombinant protein binds human CD40 with a KD value of or below 500 pM.

E85. The recombinant protein of any one of E1-E84, wherein said recombinant protein binds human CD40 with a KD value of or below 100 pM.

E86. The recombinant protein of any one of E1-E85, wherein said recombinant protein binds human serum albumin with a KD value of or below: 100 nM, 90 nM, 80 nM, 75 nM, 70 nM, 60 nM, or 50 nM.

E87. The recombinant protein of any one of E1-E86, wherein said recombinant protein binds human serum albumin with a KD value of or below 100 nM.

E88. The recombinant protein of any one of E1-E87, wherein said recombinant protein binds human serum albumin with a KD value of or below 75 nM.

E89. The recombinant protein of any one of E1-E88, wherein said recombinant protein binds human serum albumin with a KD value of or below 50 nM.

E90. The recombinant protein of any one of E65-E89, wherein said KD is measured in PBS by surface plasmon resonance (SPR).

E91. The recombinant protein of E90, wherein said KD is measured using a Biacore T200 instrument.

E92. The recombinant protein of any one of E65-E89, wherein said KD is measured by bio-layer interferometry (BLI).

E93. The recombinant protein of E92, wherein said KD is measured using a ForteBio Octet instrument.

E94. The recombinant protein of any one of E1-E93, wherein said recombinant protein has a half maximal effective concentration (EC_{50}) of no more than about 100 nM, no more than about 75 nM, no more than about 65 nM, no more than about 55 nM, no more than about 45 nM, no more than about 35 nM, no more than about 25 nM, no more than about 15 nM, no more than about 10 nM, no more than about 5 nM, no more than about 4 nM, no more than about 3 nM, no more than about 2 nM, no more than about 1 nM, nor more than about 0.1 nM, from about 0.01 nM to about 50 nM, from about 0.01 nM to about 25 nM, from about 0.01 nM to about 10 nM, from about 0.01 nM to about 5 nM, from about 0.01 nM to about 1 nM, from about 0.01 nM to about 0.1 nM, from about 0.01 nM to about 0.07 nM, from about 0.04 nM to about 50 nM, from about 0.04 nM to about 25 nM, from about 0.04 nM to about 10 nM, from about 0.04 nM to about 5 nM, from about 0.04 nM to about 1 nM, from about 0.04 nM to about 0.1 nM, from about 0.04 nM to about 0.07 nM, from about 0.1 nM to about 50 nM, from about 0.1 nM to about 25 nM, from about 0.1 nM to about 10 nM, from about 0.1 nM to about 5 nM, from about 0.1 nM to about 1 nM, from about 0.1 nM to about 0.9 nM, from about 0.1 nM to about 0.85 nM, from about 0.18 nM to about 0.85 nM as assessed by an in vitro B cell activation assay.

E95. The recombinant protein of any one of E1-E94, wherein said recombinant protein has an EC_{50} of no more than about 10 nM, as assessed by an in vitro B cell activation assay.

E96. The recombinant protein of any one of E1-E95, wherein said recombinant protein has an EC_{50} of no more than about 1 nM, as assessed by an in vitro B cell activation assay.

E97. The recombinant protein of any one of E1-E96, wherein said recombinant protein has an EC_{50} of from about 0.1 nM to about 1 nM, preferably from about 0.18 nM to about 0.85 nM, as assessed by an in vitro B cell activation assay.

E98. The recombinant protein of any one of E1-E97, wherein said recombinant protein has an EC₅₀ of from about 0.01 nM to about 0.1 nM, preferably from about 0.04 nM to about 0.07 nM, as assessed by an in vitro B cell activation assay.

E99. The recombinant protein of any one of E94-E98, wherein said B cells activation assay is a human B cell activation assay.

E100. The recombinant protein of any one of E94-E99, wherein said EC₅₀ is measured using GraphPad Prism (version 8.1.2).

E101. A recombinant protein comprising:

a first ankyrin repeat domain that specifically binds serum albumin, a second ankyrin repeat domain that specifically binds fibroblast activation protein (FAP), a third ankyrin repeat domain that specifically binds CD40, and a fourth ankyrin repeat domain that specifically binds CD40, wherein said ankyrin repeat domains are arranged, from the N-terminus to the C-terminus, according to the following formula: (serum albumin binding domain) – (FAP-binding domain) – (CD40 binding domain) – (CD40 binding domain).

E102. The recombinant protein of E101, wherein said FAP binding domain comprises an amino acid sequence that is at least 90% identical to SEQ ID NO: 2, and binds human FAP with a K_D value of or below 100 nM, preferably of or below 1 nM, more preferably of or below 120 pM.

E103. The recombinant protein of E101 or E102, wherein said FAP binding domain comprises the amino acid sequence of SEQ ID NO: 2 or of SEQ ID NO: 8.

E104. The recombinant protein of any one of E101-E103, wherein each of said CD40 binding domains independently comprises an amino acid sequence that is at least 90% identical to SEQ ID NO: 3, and binds human CD40 with a K_D value of or below 100 nM, preferably of or below 75 nM.

E105. The recombinant protein of any one of E101-E104, wherein each of said CD40 binding domains independently comprises Q at position 8, L at position 15, R at position 143, and/or Q at position 147, wherein the position numbers correspond to the positions in SEQ ID NO: 3, preferably wherein each of said CD40 binding domains independently comprises Q at position 8, L at position 15, R at position 143, and Q at position 147, wherein the position numbers correspond to the positions in SEQ ID NO: 3.

E106. The recombinant protein of any one of E101-E105, wherein each of said CD40 binding domains comprises the amino acid sequence of SEQ ID NO: 3.

E107. The recombinant protein of any one of E101-E106, wherein said serum albumin binding domain comprises an amino acid sequence that is at least 90% identical to SEQ ID NO: 1, and binds human serum albumin with a K_D value of or below 100 nM, preferably of or below 50 nM, more preferably of or below 35 nM.

E108. The recombinant protein of any one of E101-E107, wherein the serum albumin domain comprises the amino acid sequence of SEQ ID NO: 1.

E109. The recombinant protein of any one of E101-E108, comprising the following formula, from the N-terminus to C-terminus: (serum albumin binding domain) – (linker) – (FAP-binding domain) – (linker) – (CD40 binding domain) – (linker) – (CD40 binding domain), wherein the linker comprises the amino acid sequence of SEQ ID NO: 4.

E110. The recombinant protein of any one of E101-E110, wherein said protein comprises exactly four ankyrin repeat domains.

E111. A recombinant protein comprising the amino acid sequence of SEQ ID NO: 5 or of SEQ ID NO: 6.

E112. A recombinant protein comprising the amino acid sequence of SEQ ID NO: 5.

E113. A recombinant protein comprising the amino acid sequence of SEQ ID NO: 6.

E114. A recombinant protein comprising an amino acid sequence that is at least 90% identical to SEQ ID NO: 5, and binds human FAP, human CD40, and human serum albumin with a K_D value of or below 100 nM.

E115. The recombinant protein of any one of E5-E114, wherein said protein is capable of binding to CD40, FAP, and serum albumin simultaneously.

E116. The recombinant protein of any one of E1-E115, wherein said protein has a half maximal effective concentration (EC_{50}) from about 0.1 nM to about 5 nM, as assessed by an in vitro human B cell activation assay.

E117. The recombinant protein of any one of E1-E116, wherein binding of said protein to FAP does not inhibit the prolyl endopeptidase activity of FAP by more than 25%.

E117a. The recombinant protein of any one of E1-E117, wherein said recombinant protein specifically binds to the N-terminal cysteine-rich domain 1 (CRD1) (amino acids 23-59 of SEQ ID NO: 51) of the CD40 receptor.

E118. A nucleic acid encoding the recombinant protein of any one of E1-E117 or an ankyrin repeat domain as defined in any one of E1-E117.

E119. The nucleic acid of E118, comprising the nucleotide sequence of SEQ ID NO: 58.

E120. A recombinant protein comprising an amino acid sequence encoded by the nucleotide sequence of SEQ ID NO: 58.

- E121. A recombinant protein comprising an amino acid sequence encoded by a nucleotide sequence that is at least 85%, 90%, 95%, or 99% identical to the sequence of SEQ ID NO: 58.
- E122. A recombinant protein comprising an amino acid sequence encoded by a nucleotide sequence capable of hybridizing under highly stringent conditions to the nucleotide sequence of SEQ ID NO: 58.
- E123. A vector comprising the nucleic acid of E118 or E119.
- E124. A host cell comprising the nucleic acid of E118 or E119.
- E125. A host cell comprising the vector of E123.
- E126. The host cell of E124 or E125, wherein said host cell is a bacterial cell.
- E127. The host cell of any one of E124-E126, wherein said host cell is *E. coli*.
- E128. The host cell of E124 or E125, wherein said host cell is a eukaryotic cell.
- E129. A method of producing the recombinant protein of any one of E1-E117 and E120-E122, comprising culturing the host cell of any one of E124-E128 under conditions wherein said recombinant protein is expressed.
- E130. The method of E129, further comprising isolating said recombinant protein.
- E131. A pharmaceutical composition comprising the recombinant protein of any one of E1-E117 and E120-E122 or the nucleic acid of E118 or E119, and optionally a pharmaceutically acceptable carrier or excipient.
- E132. A method of localized activation of CD40 in CD40-expressing cells in a mammal, including a human, the method comprising the step of administering to said mammal the recombinant protein of any one of E1-E117 and E120-E122, the nucleic acid of E118 or E119, or the pharmaceutical composition of E131.
- E133. The method of E132, wherein said CD40-expressing cells are located in a tumor, preferably a solid tumor.
- E134. The method of E133, wherein said tumor comprises cells that express FAP.
- E135. A method of treating a medical condition, comprising administering to a subject in need thereof a therapeutically effective amount of the recombinant protein of any one of E1-E117 and E120-E122, the nucleic acid of E118 or E119, or the pharmaceutical composition of E131.
- E136. The method of E134, wherein said subject is a human.
- E137. The method of E134 or E135, wherein said medical condition is a cancer.

E138. The method of E136, wherein said cancer is a solid tumor.

E139. The method of E136 or E137, wherein said cancer comprises cells that express FAP.

E140. The method of any one of E137-E139, wherein said cancer is brain cancer, bladder cancer, breast cancer, clear cell kidney cancer, cervical cancer, colon cancer, rectal cancer, endometrial cancer, gastric cancer, head and neck cancer, head/neck squamous cell carcinoma, lip cancer, oral cancer, liver cancer, cervix cancer, lung squamous cell carcinoma, melanoma, mesothelioma, non-small-cell lung cancer (NSCLC), non-melanoma skin cancer, ovarian cancer, pancreatic cancer, prostate cancer, renal cell carcinoma, urothelial carcinoma, sarcoma, small-cell lung cancer (SCLC), Squamous Cell Carcinoma of the Head and Neck (SCCHN), triple negative breast cancer, or thyroid cancer.

E141. The method of any one of E137-E140, wherein said cancer is adrenocortical tumor, alveolar soft part sarcoma, carcinoma, chondrosarcoma, colorectal carcinoma, desmoid tumors, desmoplastic small round cell tumor, endocrine tumors, endodermal sinus tumor, epithelioid hemangioendothelioma, Ewing sarcoma, germ cell tumor, hepatoblastoma, hepatocellular carcinoma, melanoma, nephroma, neuroblastoma, non-rhabdomyosarcoma soft tissue sarcoma (NRSTS), osteosarcoma, paraspinal sarcoma, renal cell carcinoma, retinoblastoma, rhabdomyosarcoma, synovial sarcoma, or Wilms tumor.

E142. The method of E137, wherein the cancer is acute lymphoblastic leukemia (ALL), acute myeloid leukemia (AML), chronic lymphocytic leukemia (CLL), or chronic myeloid leukemia (CML).

E143. The method of E137, wherein the cancer is diffuse large B-cell lymphoma (DLBCL), follicular lymphoma, Hodgkin's lymphoma (HL), mantle cell lymphoma (MCL), multiple myeloma (MM), myelodysplastic syndrome (MDS), non-Hodgkin's lymphoma (NHL), or small lymphocytic lymphoma (SLL).

E144. The method of any one of E132-E143, wherein said recombinant protein, nucleic acid or pharmaceutical composition is administered intravenously.

E145. The method of any one of E132-E144, wherein said recombinant protein, nucleic acid or pharmaceutical composition is administered subcutaneously.

E146. The method of any one of E132-E145, wherein said recombinant protein, nucleic acid or pharmaceutical composition is administered about twice a week, once a week, once every two weeks, once every three weeks, once every four weeks, once every five weeks, once every six weeks, once every seven weeks, once every eight weeks, once every nine weeks, once every ten weeks, twice a month, once a month, once every two months, once every three months, or once every four months.

E147. The recombinant protein of any one of E1-E117 and E120-E122, the nucleic acid of E118 or E119, or the pharmaceutical composition of E131 for use as a medicament.

E148. The recombinant protein of any one of E1-E117 and E120-E122, or the nucleic acid of E118 or E119, or the pharmaceutical composition of E131 for use in the treatment of a medical condition in a subject.

E149. The recombinant protein for use of E148, wherein said medical condition is cancer.

E150. Use of the recombinant protein of any one of E1-E117 and E120-E122, the nucleic acid of E118 or E119, or the pharmaceutical composition of E131 in the manufacture of a medicament for treating cancer in a subject.

E151. Use of the recombinant protein of any one of E1-E117 and E120-E122, the nucleic acid of E118 or E119, or the pharmaceutical composition of E131 for treating a medical condition in a subject.

E152. The use of E151, wherein said medical condition is cancer.

E153. A kit comprising a container, a composition within the container comprising the recombinant protein of any one of E1-E117 and E120-E122, or the nucleic acid of E118 or E119, or the pharmaceutical composition of E131, and a package insert containing instructions for administering a therapeutically effective amount of the recombinant protein, the nucleic acid or the pharmaceutical composition for treatment of a patient in need thereof.

E154. A method of inducing an anti-tumor immunological memory in a mammal, including a human, the method comprising the step of administering to said mammal the recombinant protein of any one of E1-E117 and E120-E122, the nucleic acid of E118 or E119, or the pharmaceutical composition of E131.

E155. The method of E154, wherein the immunological memory is not limited to FAP-related antigens.

E156. The recombinant protein of any one of E1-E117 and E120-E122, wherein said recombinant protein is capable of preferentially localizing and/or accumulating in a tumor in a mammal, including a human.

E157. The recombinant protein of E156, wherein said tumor comprises cells that express FAP.

E158. The recombinant protein of any one of E1-E117, E120-E122 and E156-E157, wherein said recombinant protein is capable of inducing an anti-tumor immunological memory in a mammal, including a human.

E159. The recombinant protein of E158, wherein said immunological memory is not limited to FAP-related antigens.

[4] The use of section headings herein is merely for the convenience of reading, and not intended to be limiting per se. The entire document is intended to be viewed as a unified disclosure, and it should be understood that all combinations of features described herein are contemplated.

BRIEF DESCRIPTION OF THE FIGURES

[5] FIGURE 1. A cartoon depicting the *in vitro* B cell activation assay. The assay was performed using purified primary human B cells and FAP expressing (+FAP) or non-FAP expressing (-FAP) CHO cells.

[6] FIGURE 2. Overview of the gating strategy used to determine MFI and percentage of cells positive for CD86. The following settings were used: FSC: 200; SSC: 400; Acquisition: 200ul/min, 100.000 events. Abbreviations: FMO = Fluorescence minus one, SSC = Side scatter, FSC = Forward scatter, FSC – A = Forward scatter area, FSC – H = Forward scatter height.

[7] FIGURE 3. HSA-binding domain(s) impair potency and efficacy of a bispecific FAPxCD40 ankyrin repeat binding protein. Human B cells were co-cultured in presence of FAP-expressing CHO cells (full symbols) and treated with increasing concentrations of SMA014 (triangle pointing up), SMA087 (triangle pointing down), SMA095 (diamond) and agonist anti-CD40 mAb (square). As control, B cells were co-cultured in presence of FAP-negative CHO cells and treated only with the highest concentration of the respective constructs, depicted as empty symbols. Activation of human B cells was assessed in terms of upregulation of CD86 (measured as mean fluorescence intensity (MFI) and percentage of cells (%)) in absence A) and in presence B) of 600 μ M HSA. Each value depicts the average of duplicated measurements. The shown data are representative of two independent experiments. Error bars show $\pm$ SEM. EC50 and efficacy values (in nM) for all constructs in presence of FAP-expressing CHO cells are shown in the depicted tables in the graphs.

[8] FIGURE 4. CD40 bivalency strongly increases potency and efficacy of a bispecific FAPxCD40 ankyrin repeat binding protein. Human B cells were cultured in presence of FAP expressing CHO cells and treated with increasing concentrations of SMA014 (triangle pointing up), SMA104 (triangle pointing down), SMA105 (diamond) and agonist anti-CD40 mAb (square). As control, B cells were co-cultured in presence of FAP-negative CHO cells and treated only with the highest concentration of the respective constructs, depicted as empty symbols. Activation of human B cells was assessed in terms of upregulation of CD86 (measured as mean fluorescence intensity (MFI) and percentage of cells (%)) in absence of HSA. Each value depicts the average of duplicated measurements. The shown data are representative of two independent experiments. Error bars show $\pm$ SEM. EC50 and efficacy values (in nM) for all constructs in presence of FAP-expressing CHO cells are shown in the depicted tables in the graphs.

[9] FIGURE 5. CD40 bivalency rescues the inhibitory effect induced by a HSA binding domain. Human B cells were cultured in presence of FAP expressing CHO cells and treated with increasing concentrations of SMA014 (triangle pointing up), SMA104 (triangle pointing down), SMA091 (circle), SMA099 (diamond), AS579 (hexagon) and agonist anti-CD40 mAb (square). As control, B cells were co-cultured in presence of FAP-negative CHO cells and treated only with the highest concentration of the respective constructs, depicted as empty symbol. Activation of human B cells was assessed in terms of upregulation of CD86 (measured as mean fluorescence intensity (MFI) and percentage of cells (%)) in absence A) and in presence B) of HSA. Each value depicts the average of duplicated

measurements. The shown data are representative of two independent experiments. Error bars show $\pm$ SEM. EC50 and efficacy values (in nM) for all constructs in presence of FAP expressing CHO cells are shown in the depicted tables in the graphs.

[10] FIGURE 6. Analysis of Protein #5 (also called SMA136) by size exclusion chromatography (SEC) and multiangle light scattering (MALS). The graph shows the SEC profile as molar mass over time. The determined molecular weight of Protein #5 is indicated.

[11] FIGURE 7. Surface plasmon resonance (SPR) traces showing binding of Protein #5 (also called SMA136) to human CD40 (A), human FAP (B) and human serum albumin (C). The determined K_D values are indicated.

[12] FIGURE 8. Surface plasmon resonance (SPR) trace showing simultaneous binding of Protein #5 to hCD40, hFAP and HSA. The vertical lines (1, 2 and 3) indicate the three injections: (1) Binding of Protein #5 to immobilized bio-hCD40; (2) Binding of hFAP (diamond $\blacklozenge$, triangle $\blacktriangle$, and circle $\bullet$ symbols) or Protein #5 (control; cross $\times$ symbol) to the bio-hCD40/Protein #5 complex; (3) Binding of HSA (diamond $\blacklozenge$, triangle $\blacktriangle$ symbols) or hFAP (control; circle $\bullet$ symbol) to the bio-hCD40/Protein #5/hFAP complex, followed by a 600s dissociation phase. The injection scheme is depicted in Table 8 using the same symbols. The simultaneous binding of hCD40/Protein #5/hFAP/HSA is measured in duplicate on two different lanes (diamond and triangle symbols).

[13] FIGURE 9. Protein #5 activates human B cells via CD40 *in vitro*. Human B cells were cultured in presence of FAP expressing CHO cells and treated with increasing concentrations of Protein #5 (circle symbols) and anti-CD40 mAb (square symbols). Activation of human B cells was assessed in terms of upregulation of CD86 and CD69 (measured as mean fluorescence intensity (MFI) and percentage of cells (%)). Each value depicts the average of duplicated measurements. The shown data are representative of thirteen independent experiments. Error bars show $\pm$ SEM. The depicted tables show EC50 and efficacy values for Protein #5 (left column) and anti-CD40 mAb (right column).

[14] FIGURE 10. Activation of human B cells by Protein #5 *in vitro* is FAP-dependent. The data show that Protein #5 does not induce upregulation of CD86 and CD69 in human B cells in the absence of FAP expressing CHO cells *in vitro*. The experiment and data plotting were performed as described in Figure 9, i.e. with Protein #5 (circle symbols) and anti-CD40 mAb (square symbols), but in presence of FAP-negative CHO cells. The shown data are representative of thirteen independent experiments. Error bars show $\pm$ SEM. The depicted tables show EC50 and efficacy values for anti-CD40 mAb only.

[15] FIGURE 11. Schematic representation of a human monocyte-derived dendritic cell (MDDC) activation assay using *in vitro* differentiated MDDC and irradiated FAP expressing (+FAP) or non-FAP expressing (-FAP) CHO cells.

[16] FIGURE 12. Schematic representation of the experimental design of anti-tumor efficacy studies *in vivo*. Mice were inoculated subcutaneously with MC38-FAP colon carcinoma cells on day 0. Mice were randomized into treatment groups based on tumor size and on the days as indicated in Figs. 12A and B for the respective studies. Four different experiments with different schedules were performed:

(A) early time point termination: mice were sacrificed 4 days after the first treatment and tumors were analyzed by FACS analysis (studies PD1033 and PD1038); (B) Late time point termination: mice were euthanized 10-11 days after the first treatment, tumor size was measured over time for anti-tumor efficacy assessment and at the day of termination, tumors were investigated by FACS analysis (studies PD1032 and PD1035). Mice were treated i.p. with AS598, AS608 or anti-CD40 antibody at the indicated time points.

[17] FIGURE 13. Mouse body weight during the anti-tumor efficacy studies. Mice were treated as described in Figure 12. Mean body weights per treatment group ($\pm$ SEM, n=10) are shown for study PD1032 (A) and study PD1035 (B). Dotted lines indicate the time point of randomization and start of the treatment. The statistical analysis was done using Kruskal-Wallis with multiple comparison to vehicle. Results were considered significant when * $p < 0.05$, ** $p < 0.01$ *** $p < 0.001$.

[18] FIGURE 14. Mean tumor growth volume during the anti-tumor efficacy studies. Mice were treated as described in Figure 12 and the tumor volume was measured every 3-4 days. Mean tumor volume per treatment group ($\pm$ SEM, n=10) is shown for study PD1032 (A) and for study PD1035 (B). The dotted lines indicate the time point of randomization and start of the treatment. The arrows indicate the time points of treatment. The statistical analysis was done using Kruskal-Wallis with multiple comparison to vehicle, or to the negative control AS608 (in parentheses) at termination. Results were considered significant when ** $p < 0.01$ *** $p < 0.001$ **** $p < 0.0001$.

[19] FIGURE 15. Average FAP activity in presence of various FAP-specific recombinant binding proteins. Conversion of substrate Z-GLY-PRO-AMC into a fluorescence product by recombinant human FAP was measured in the presence or absence of various recombinant proteins. FAP activity after incubation for 95 minutes is shown. In comparison to the FAP activity in the absence of a test molecule (first control: hFAP and substrate), all tested recombinant proteins containing a FAP-binding domain (Molecules 1-4) showed no inhibitory effect on the FAP enzymatic activity. Partial inhibition of FAP activity was observed for Molecule No. 5 (used as an assay control). Average FAP activity and standard deviation are shown from quadruplet measurements.

[20] FIGURE 16. (A) A representative SPECT/CT image of a MC38-FAP tumor-bearing mouse 96 hours after having been injected with indium-111-labelled Protein #7. Maximum intensity projections (MIP) generated using a normalized intensity setting are shown. Labelled Protein #7 localized and accumulated preferentially in the tumor. (B) Detection of Protein #7 (upper image) or a control DARPIn® protein (lower image) by immunohistochemistry (IHC) in MC38-FAP tumors 24 hours post-injection. Size bars are shown in the right bottom corners of the images. (C) Time course of tissue distribution of indium-111-labeled DARPIn® molecules. Tissue distribution of control DARPIn® molecule (solid bars) and Protein #7 (striped bars) was analyzed in tumor (left graph) and muscle (right graph) at the indicated time points. Data are given in percentage of the injected dose of DARPIn® molecule per gram of tissue (% ID/g) and expressed as mean $\pm$ SD. N= 4 mice per time point.

[21] FIGURE 17. (A) Schematic representation of the experimental design of an anti-tumor efficacy study *in vivo*. (B) Mean tumor growth volume during the anti-tumor efficacy study. Mice were treated and tumor volume measured as described in Figure 17A and in Example 9. Mean tumor volume per

treatment group ($\pm$ SEM, n=10) is shown for vehicle (triangle symbols), AS598 (round symbols) and anti-CD40 antibody (square symbols). The arrow indicates the start of the treatment.

[22] FIGURE 18. Long-term effects in anti-tumor efficacy studies. Mice bearing MC38-FAP tumor were treated with vehicle (triangle symbols), AS598 (square symbols), and AS608 (negative control) (round symbols) as described in Example 10 and the tumor volume was measured every 3-4 days. The mean tumor volume per treatment group ($\pm$ SEM) is shown in (A) and Kaplan-Meier survival curves are shown in (B). Arrows represent the treatment time-points.

[23] FIGURE 19. Induction of anti-tumor immunological memory. (A) The experiment shown in Figure 18A was followed for a longer time period, with the mean tumor growth curves shown for MC38-FAP tumor bearing mice treated with vehicle (open triangles pointing up), AS598 (open circles), and AS608 (negative control) (open triangles pointing down). Short arrows represent the treatment time-points. At about 120 days, tumor-free mice previously treated with AS598 were re-challenged with MC38-WT (solid triangles pointing down) or MC38-FAP (solid triangles pointing up) tumor cells and monitored up to day 200. Mean tumor growth curves are shown of 8 mice per group. (B) Naïve control mice were challenged with MC38-WT (triangles pointing down) or MC38-FAP (triangles pointing up) tumor cells at about 120 days. Mean tumor growth curves are shown of 5 mice per group.

[24] FIGURE 20. Toxicity assessments. (A) Mice bearing MC38-FAP tumor were treated once with vehicle (n=10) (triangles pointing up), AS608 (negative control) (n=5) (triangles pointing down), AS598 (Protein #7) (n=10) (circles) or anti-mCD40 antibody (n=10) (squares) and after 24 hours serum cytokines were measured. Samples were collected from two independent experiments and analyzed together. (B) Mice bearing MC38-FAP tumor were treated once with vehicle (triangles pointing up), AS608 (negative control) (triangles pointing down), AS598 (Protein #7) (circles) or anti-mCD40 antibody (squares) (5 mice per group) and after 24 hours serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were measured. Samples were collected from two independent experiments and analyzed together. (C) Mice bearing MC38-FAP tumor were treated once with vehicle, AS608 (negative control) (not shown), AS598 (Protein #7) or anti-mCD40 antibody (n=5-10) and after 24 hours livers were harvested and analyzed for tissue damage by immunohistochemistry (IHC). Representative pictures of the different treatments are shown. NEC, necrosis; ICI, immune cell infiltration. Size bars are indicated in the right bottom corners of the pictures.

[25] FIGURE 21. Structure determination by X-ray crystallography of human tumor necrosis factor receptor superfamily member 5 (hCD40) in complex with a DARPIn® protein with the amino acid sequence of SEQ ID NO: 3.

DETAILED DESCRIPTION OF THE INVENTION

1. OVERVIEW

[26] Disclosed herein are recombinant proteins comprising designed ankyrin repeat domains with binding specificity for FAP and CD40. Also disclosed are nucleic acids encoding the binding proteins,

pharmaceutical compositions comprising the binding proteins or nucleic acids, and methods of using the binding proteins, nucleic acids, or pharmaceutical compositions. In one aspect, the materials and methods of the disclosure take advantage of FAP's expression in tumor-associated stroma, allowing, e.g., specific targeting of CD40-expressing cells in the tumor and selective activation of CD40 in those CD40-expressing cells.

[27] CD40 agonist antibodies have demonstrated efficacy in preclinical murine tumor models, and their use in the clinic has also shown some anti-tumor efficacy. However, clinical development of agonistic anti-CD40 antibodies has likely been hampered by dose-limiting toxicities and resulting low efficacies.

[28] The multispecific recombinant proteins described herein promote cancer target-mediated and tumor-localized clustering and activation of CD40, thereby addressing challenges associated with previous therapeutic approaches. In natural settings, clustering of CD40 is achieved by binding to trimeric CD40 ligand (CD40L, CD154), which is expressed as a membrane molecule on the surface of certain cells, e.g. activated CD4⁺ T cells. CD40 clustering in the cell membrane of a cell targeted, e.g., by CD40L is a prerequisite for activation of its signaling pathway. The multispecific recombinant proteins of the invention disclosed herein take advantage of this clustering effect; and the activation of CD40 is linked to the expression of the tumor antigen FAP.

[29] Fibroblast-activation protein α (FAP, also known as Seprase) is a type II membrane-bound glycoprotein abundantly expressed in the stroma of many solid tumors by cancer-associated fibroblasts. FAP is expressed selectively in reactive stromal fibroblasts of more than 90% of epithelial malignancies (primary and metastatic), including lung, colorectal, bladder, ovarian and breast carcinomas, and in malignant mesenchymal cells of bone and soft tissue sarcomas, while it is generally absent from normal adult tissues (Brennen et al., *Mol Cancer Ther.* 11: 257–266 (2012); Garin-Chesa et al., *Proc Natl Acad Sci USA* 87, 7235–7239 (1990); Rettig et al., *Cancer Res.* 53:3327–3335 (1993); Rettig et al., *Proc Natl Acad Sci USA* 85, 3110–3 114 (1988)). FAP is also expressed on certain malignant tumor cells.

[30] Although not wishing to be bound by a particular theory, in the absence of the tumor antigen FAP (normal, non-malignant, non-cancer associated cells), minimal clustering of CD40 will occur, and immune activation will be limited. In contrast, in cancer-associated fibroblasts, FAP is highly expressed, and therefore, through FAP-binding, the multispecific proteins of the invention promote CD40 clustering and activation in CD40 expressing immune cells, such as, e.g., B cells and antigen presenting cells. The advantages of this strategy are twofold: systemic toxicities should be limited because activation will be largely confined to tissue expressing FAP, and tumor-mediated CD40 clustering should drive potent agonism.

2. DEFINITIONS

[31] Unless otherwise defined herein, scientific and technical terms used in connection with the present invention shall have the meanings that are commonly understood by those of ordinary skill in the art. Further, unless otherwise required by context, singular terms shall include pluralities and plural terms shall include the singular. Generally, nomenclatures used in connection with, and techniques of,

cell and tissue culture, molecular biology, immunology, microbiology, genetics and protein and nucleic acid chemistry and hybridization described herein are those well-known and commonly used in the art.

[32] The terms "comprising," "having," "including," and "containing" are to be construed as open-ended terms unless otherwise noted. If aspects of the invention are described as "comprising" a feature, embodiments also are contemplated "consisting of" or "consisting essentially of" the feature. The use of any and all examples, or exemplary language (e.g., "such as") provided herein, is intended merely to better illustrate the disclosure and does not pose a limitation on the scope of the disclosure unless otherwise claimed. No language in the specification should be construed as indicating any non-claimed element as essential to the practice of the disclosure. Other than in the operating examples, or where otherwise indicated, all numbers expressing quantities of ingredients or reaction conditions used herein should be understood as modified in all instances by the term "about" as that term would be interpreted by the person skilled in the relevant art.

[33] Recitation of ranges of values herein are merely intended to serve as a shorthand method of referring individually to each separate value falling within the range and each endpoint, unless otherwise indicated herein, and each separate value and endpoint is incorporated into the specification as if it were individually recited herein.

[34] The term "polypeptide" relates to a molecule consisting of one or more chains of multiple, i.e. two or more, amino acids linked via peptide bonds. Preferably, a polypeptide consists of more than eight amino acids linked via peptide bonds. The term "polypeptide" also includes multiple chains of amino acids, linked together by S-S bridges of cysteines. Polypeptides are well-known to the person skilled in the art.

[35] The term "protein" refers to a molecule comprising a polypeptide, wherein at least part of the polypeptide has, or is able to acquire, a defined three-dimensional arrangement by forming secondary, tertiary, and/or quaternary structures within a single polypeptide chain and/or between multiple polypeptide chains. If a protein comprises two or more polypeptide chains, the individual polypeptide chains may be linked non-covalently or covalently, e.g. by a disulfide bond between two polypeptide chains. A part of a protein, which individually has, or is able to acquire, a defined three-dimensional arrangement by forming secondary and/or tertiary structures, is termed "protein domain". Such protein domains are well known to the practitioner skilled in the art.

[36] Patent application WO2002/020565 and Forrer et al., 2003 (Forrer, P., Stumpp, M.T., Binz, H.K., Plückthun, A., 2003. FEBS Letters 539, 2-6), contain a general description of repeat protein, repeat domain and repeat module features, techniques and applications.

[37] The term "repeat domain" refers to a protein domain comprising two or more consecutive repeat modules as structural units, wherein said repeat modules have structural and sequence homology. Preferably, a repeat domain also comprises an N-terminal and/or a C-terminal capping module. For clarity, a capping module can be a repeat module. Such repeat domains, repeat modules, and capping modules, sequence motives, as well as structural homology and sequence homology are well known to the practitioner in the art from examples of ankyrin repeat domains (Binz et al., J. Mol. Biol. 332, 489–

503, 2003; Binz et al., 2004, loc. cit.; WO2002/020565; WO2012/069655), leucine-rich repeat domains (WO2002/020565), tetratricopeptide repeat domains (Main, E.R., Xiong, Y., Cocco, M.J., D'Andrea, L., Regan, L., Structure 11(5), 497-508, 2003), and armadillo repeat domains (WO2009/040338). It is further well known to the practitioner in the art, that such repeat domains are different from proteins comprising repeated amino acid sequences, where every repeated amino acid sequence is able to form an individual domain (for example FN3 domains of Fibronectin).

[38] The term "ankyrin repeat domain" refers to a repeat domain comprising two or more consecutive ankyrin repeat modules as structural units, wherein said ankyrin repeat modules have structural and sequence homology.

[39] The term "repeat modules" refers to the repeated amino acid sequence and structural units of the designed repeat domains, which are originally derived from the repeat units of naturally occurring repeat proteins. Each repeat module comprised in a repeat domain is derived from one or more repeat units of a family or subfamily of naturally occurring repeat proteins, preferably the family of ankyrin repeat proteins. Accordingly, the term "ankyrin repeat module" refers to a repeat module, which is originally derived from the repeat units of naturally occurring ankyrin repeat proteins. Ankyrin repeat proteins are well known to the person skilled in the art. See, for example, International Patent Publication Nos. WO 2002/020565, WO 2010/060748, WO 2011/135067, WO 2012/069654, WO 2012/069655, WO 2014/001442, WO 2014/191574, WO 2014/083208, WO 2016/156596, and WO 2018/054971.

[40] Ankyrin repeat domains may be modularly assembled into larger ankyrin repeat proteins according to the present disclosure, optionally with half-life extension domains, using standard recombinant DNA technologies (see, e.g., Forrer, P., et al., FEBS letters 539, 2-6, 2003, WO2012/069655, WO 2002/020565).

[41] The term "designed" as used in designed repeat protein, designed repeat domain, designed ankyrin repeat domain, and the like refers to the property that such repeat proteins and repeat domains, respectively, are man-made and do not occur in nature.

[42] The term "recombinant" as used in recombinant protein, recombinant binding protein, recombinant polypeptide, and the like, means that said protein or polypeptide is produced by the use of recombinant DNA technologies well known to the practitioner skilled in the art. For example, a recombinant DNA molecule (e.g. produced by gene synthesis) encoding a polypeptide can be cloned into a bacterial expression plasmid (e.g. pQE30, QIAGEN), yeast expression plasmid, mammalian expression plasmid, or plant expression plasmid, or a DNA enabling in vitro expression. If, for example, such a recombinant bacterial expression plasmid is inserted into appropriate bacteria (e.g. *Escherichia coli*), these bacteria can produce the polypeptide(s) encoded by this recombinant DNA. The correspondingly produced polypeptide or protein is called a recombinant polypeptide or recombinant protein.

[43] In the context of the present invention, the term "binding protein" refers to a protein comprising a binding domain. A binding protein may also comprise two, three, four, five or more binding domains. Preferably, said binding protein is a recombinant binding protein.

[44] The term "binding domain" means a protein domain exhibiting binding specificity for a target. Preferably, said binding domain is a recombinant binding domain.

[45] The term "target" refers to an individual molecule such as a nucleic acid molecule, a peptide, polypeptide or protein, a carbohydrate, or any other naturally occurring molecule, including any part of such individual molecule, or to complexes of two or more of such molecules, or to a whole cell or a tissue sample, or to any non-natural compound. Preferably, a target is a naturally occurring or non-natural polypeptide or protein, or a polypeptide or protein containing chemical modifications, for example, naturally occurring or non-natural phosphorylation, acetylation, or methylation. For example, the target of each of the designed ankyrin repeat domains consisting of SEQ ID NOs: 39 to 42, is serum albumin.

[46] The term "has binding specificity for a target", "specifically binding to a target", "binding to a target with high specificity", "specific for a target" or "target specificity" and the like means that a binding protein or binding domain reacts or associates more frequently, more rapidly, with greater duration and/or with greater affinity with a particular target (e.g., cell or substance) than it does with alternative targets (e.g., cells or substances). For example, a binding domain that specifically binds FAP may be defined as a binding domain that binds in PBS to FAP with a lower dissociation constant (i.e. it binds with higher affinity) than it binds to an unrelated protein such as the *E. coli* maltose binding protein (MBP). Preferably, the dissociation constant (K_D) in PBS for the target is at least 10^2 ; more preferably, at least 10^3 ; even more preferably, at least 10^4 ; or most preferably, at least 10^5 times lower than the corresponding dissociation constant for MBP. Methods to determine dissociation constants of protein-protein interactions, such as surface plasmon resonance (SPR) based technologies (e.g. SPR equilibrium analysis) or isothermal titration calorimetry (ITC) are well known to the person skilled in the art. The measured K_D values of a particular protein-protein interaction can vary if measured under different conditions (e.g., salt concentration, pH). Thus, measurements of K_D values are preferably made with standardized solutions of protein and a standardized buffer, such as PBS. It is also understood by reading this definition that, for example, an ankyrin repeat domain which specifically binds to a first target may or may not specifically or preferentially bind to a second target. As such, "specific binding" does not necessarily require (although it can include) exclusive binding. In general, under designated assay conditions, an ankyrin repeat domain binds preferentially to a particular target molecule and does not bind in a significant amount to other components present in a test sample.

[47] A variety of assay formats may be used to select or characterize an ankyrin repeat domain that specifically binds a molecule of interest. For example, solid-phase ELISA immunoassay, immunoprecipitation, BIAcore™ (GE Healthcare, Piscataway, NJ), fluorescence-activated cell sorting (FACS), Octet™ (ForteBio, Inc., Menlo Park, CA) and Western blot analysis are among many assays that may be used to identify an ankyrin repeat domain that specifically reacts with a target. Typically, a specific or selective reaction will be at least twice background signal or noise and more typically more

than 10 times background. Even more specifically, an ankyrin repeat domain is said to "specifically bind" a target when the equilibrium dissociation constant (K_D) value is $< 1 \mu\text{M}$, such as $< 100 \text{ nM}$, $< 10 \text{ nM}$, $< 100 \text{ pM}$, $< 10 \text{ pM}$, or $< 1 \text{ pM}$.

[48] The K_D value is often referred to as binding affinity. Binding affinity measures the strength of the sum total of non-covalent interactions between contact residues of one binding partner (e.g., FAP or CD40 binding domains disclosed herein) and contact residues of its binding partner (e.g., FAP or CD40). Unless indicated otherwise, as used herein, binding affinity refers to binding affinity that reflects a 1:1 interaction between members of a binding pair or binding partners. In case of a binding protein comprising two binding domains for one binding partner, binding affinity may refer to binding affinity that reflects a 1:2 interaction between the binding protein and the binding partner.

[49] A variety of methods of measuring binding affinity are known in the art, any of which can be used for purposes of the present invention. For example, as exemplified herein, the binding affinity can be expressed as K_D value, which refers to the dissociation rate of a particular ankyrin repeat domain and its binding target. K_D is the ratio of the rate of dissociation, also called the "off-rate (K_{off})", to the association rate, or "on-rate (K_{on})". Thus, K_D equals $K_{\text{off}}/K_{\text{on}}$ and is expressed as a molar concentration (M), and the smaller the K_D , the stronger the affinity of binding.

[50] K_D values can be determined using any suitable method. One exemplary method for measuring K_D is surface plasmon resonance (SPR) (see, e.g., Nguyen et al. *Sensors* (Basel). 2015 May 5; 15(5):10481-510). K_D value may be measured by SPR using a biosensor system such as a BIAcore® system. BIAcore kinetic analysis comprises analyzing the binding and dissociation of an antigen from chips with immobilized molecules (e.g., molecules comprising epitope binding domains), on their surface. Another method for determining the K_D of a protein is by using Bio-Layer Interferometry (see, e.g., Shah et al. *J Vis Exp*. 2014; (84): 51383). K_D value may be measured using OCTET® technology (Octet QKe system, ForteBio). Alternatively or in addition, a KinExA® (Kinetic Exclusion Assay) assay, available from Sapidyn Instruments (Boise, Id.) can also be used. Any method suitable for assessing the binding affinity between two binding partners is encompassed herein. Preferably, K_D values are determined in PBS by SPR, e.g. as described in Example 2.

[51] The term "polypeptide tag" refers to an amino acid sequence attached to a polypeptide/protein, wherein said amino acid sequence is useful for the purification, detection, or targeting of said polypeptide/protein, or wherein said amino acid sequence improves the physicochemical behavior of the polypeptide/protein, or wherein said amino acid sequence possesses an effector function. The individual polypeptide tags, moieties and/or domains of a binding protein may be connected to each other directly or via polypeptide linkers. These polypeptide tags are all well known in the art and are fully available to the person skilled in the art. Examples of polypeptide tags are small polypeptide sequences, for example, His (e.g. the His-tag consisting of SEQ ID NO: 57), myc, FLAG, or Strep-tags or moieties such as enzymes (for example enzymes like alkaline phosphatase), which allow the detection of said polypeptide/protein, or moieties which can be used for targeting (such as immunoglobulins or fragments thereof) and/or as effector molecules.

[52] The term "polypeptide linker" refers to an amino acid sequence, which is able to link, for example, two protein domains, a polypeptide tag and a protein domain, a protein domain and a non-polypeptide moiety such as polyethylene glycol, or two polypeptide tags. Such additional domains, tags, non-polypeptide moieties and linkers are known to the person skilled in the relevant art. Examples of such polypeptide linkers are the linkers consisting of SEQ ID NOs: 4 and 56.

[53] The terms "nucleic acid" or "nucleic acid molecule" refer to a polynucleotide molecule, which may be a ribonucleic acid (RNA) or deoxyribonucleic acid (DNA) molecule, either single stranded or double stranded, and includes modified and artificial forms of DNA or RNA. A nucleic acid molecule may either be present in isolated form, or be comprised in recombinant nucleic acid molecules or vectors.

[54] In the context of the invention, the terms "medical condition", "disease" and "disorder" are used interchangeably and include but are not limited to autoimmune disorders, inflammatory disorders, retinopathies (particularly proliferative retinopathies), neurodegenerative disorders, infectious diseases, metabolic diseases, and neoplastic diseases. A "medical condition" may be one that is characterized by inappropriate cell proliferation. A medical condition may be a hyperproliferative condition. A medical condition may be a neoplastic disease. The term "neoplastic disease", as used herein, refers to an abnormal state or condition of cells or tissue characterized by rapidly proliferating cell growth or neoplasm. A medical condition may be a malignant neoplastic disease. A medical condition may be a cancer. The terms "cancer" and "cancerous" are used herein to refer to or describe the physiological condition in mammals that is typically characterized by unregulated cell growth. Cancer encompasses solid tumors and liquid tumors, as well as primary tumors and metastases. A "tumor" comprises one or more cancerous cells. Solid tumors typically also comprise tumor stroma. Examples of cancer include, but are not limited to, primary and metastatic carcinoma, lymphoma, blastoma, sarcoma, myeloma, melanoma and leukemia, and any other epithelial and blood cell malignancies. More particular examples of such cancers include brain cancer, bladder cancer, breast cancer, ovarian cancer, kidney cancer, colorectal cancer, gastric cancer, head and neck cancer, lung cancer, pancreatic cancer, prostate cancer, malignant melanoma, osteosarcoma, soft tissue sarcoma, carcinoma, squamous cell carcinoma, clear cell kidney cancer, head/neck squamous cell carcinoma, lung adenocarcinoma, lung squamous cell carcinoma, non-small-cell lung cancer (NSCLC), renal cell carcinoma, small-cell lung cancer (SCLC), triple negative breast cancer, acute lymphoblastic leukemia (ALL), acute myeloid leukemia (AML), chronic lymphocytic leukemia (CLL), chronic myeloid leukemia (CML), diffuse large B-cell lymphoma (DLBCL), follicular lymphoma, Hodgkin's lymphoma (HL), mantle cell lymphoma (MCL), multiple myeloma (MM), myelodysplastic syndrome (MDS), non-Hodgkin's lymphoma (NHL), Squamous Cell Carcinoma of the Head and Neck (SCCHN), chronic myelogenous leukemia (CML), small lymphocytic lymphoma (SLL), malignant mesothelioma, liposarcoma, neuroblastoma, or synovial sarcoma.

[55] The term "treat," as well as words related thereto, does not necessarily imply 100% or complete cure. Rather, there are varying degrees of treatment of which one of ordinary skill in the art recognizes as having a potential benefit or therapeutic effect. In this respect, the methods of treating cancer of the present disclosure can provide any amount or any level of treatment. Furthermore, the treatment

provided by the method of the present disclosure can include treatment of (i.e., relief from) one or more conditions or symptoms. Also, the treatment provided by the methods of the present disclosure can encompass slowing the progression of the cancer. For example, the methods can treat cancer by virtue of enhancing the T cell activity or an immune response against the cancer, reducing tumor or cancer growth or appearance of new lesions, reducing metastasis of tumor cells, increasing cell death of tumor or cancer cells, inhibition of tumor or cancer cell survival, and the like. In exemplary aspects, the methods treat by way of delaying the onset or recurrence of the cancer by 1 day, 2 days, 4 days, 6 days, 8 days, 10 days, 15 days, 30 days, two months, 4 months, 6 months, 1 year, 2 years, 4 years, or more. In exemplary aspects, the methods treat by way increasing the survival of the subject. The term "treatment" also includes prophylactic treatment.

[56] Therapeutic responses in any given disease or condition can be determined by standardized response criteria specific to that disease or condition. Tumor response can be assessed using screening techniques such as magnetic resonance imaging (MRI) scan, x-radiographic imaging, computed tomographic (CT) scan, positron emission tomography (PET) scan, bone scan, ultrasound, tumor biopsy sampling, counting of tumor cells in circulation, and/or measurement of tumor antigen (e.g., prostate specific antigen (PSA) and/or alphafetoprotein (AFP)). In addition to these therapeutic responses, the subject undergoing therapy may experience the beneficial effect of an improvement in the symptoms associated with the disease.

[57] The term "treatment" or "treating" refers to both therapeutic treatment and prophylactic or preventative measures. Those in need of treatment include those who have already the disorder as well as those in which the disorder is to be prevented.

[58] The term "therapeutically effective amount" refers to the amount sufficient to induce a desired biological, pharmacological, or therapeutic outcome in a subject. A therapeutically effective amount in the context of the invention means a sufficient amount of the binding protein to treat or prevent a disease or disorder at a reasonable benefit/risk ratio applicable to any medical treatment.

[59] The term "mammal" for purposes of treatment refers to any animal classified as a mammal, including human, domestic and farm animals, nonhuman primates, and zoo, sports, or pet animals, such as dogs, horses, cats, cows, etc.

[60] The term "incubation" refers to incubation at pH 7.4. In one embodiment, said incubation at pH 7.4 refers to an incubation in PBS.

[61] The term "PBS" means a phosphate buffered water solution containing 137 mM NaCl, 10 mM phosphate and 2.7 mM KCl and having a pH of 7.4.

[62] The term improved pharmacokinetic properties refers to an increased area under the curve, a reduced clearance, or an increased terminal half-life. These parameters of pharmacokinetic properties and ways to determine them are well known in the art (see, e.g., Mahmood, I., Methods to determine pharmacokinetic profiles of therapeutic proteins, Drug Discov Today: Technol (2009), doi:10.1016/j.ddtec.2008.12.001).

[63] In the context of the present invention, the term "any amino acids" preferably means any of the 20 most often naturally occurring amino acids, namely alanine (ala; A), arginine (arg; R), asparagine (asn, N), aspartic acid (asp, D), cysteine (cys, C), glutamine (gln, Q), glutamic acid (glu, E), glycine (gly, G), histidine (his, H), isoleucine (ile, I), leucine (leu, L), lysine (lys, K), methionine (met, M), phenylalanine (phe, F), proline (pro, P), serine (ser, S), threonine (thr, T), tryptophan (trp, W), tyrosine (tyr, Y), valine (val, V).

3. MULTISPECIFIC MOLECULES THAT TARGET FAP AND CD40

[64] Disclosed herein are multispecific molecules that target FAP and CD40. The molecules are useful for, e.g., treating cancer. According to the present invention, the herein provided multispecific molecules that target FAP and CD40 are preferably designed repeat proteins, more preferably designed ankyrin repeat proteins.

3.1. ANKYRIN REPEAT DOMAINS AND ANKYRIN REPEAT PROTEINS

[65] Designed ankyrin repeat proteins are a class of binding molecules which have the potential to overcome limitations of monoclonal antibodies, hence allowing novel therapeutic approaches. Such ankyrin repeat proteins may comprise a single designed ankyrin repeat domain, or may comprise a combination of two, three, four, five or more designed ankyrin repeat domains with the same or different target specificities (Stumpp et al., *Drug Discov. Today* 13, 695-701, 2008; U.S. Patent No. 9,458,211). Ankyrin repeat proteins comprising only a single designed ankyrin repeat domain are small proteins (14 kDa) which can be selected to bind a given target protein with high affinity and specificity. These characteristics, and the possibility of combining two, three, four, five or more designed ankyrin repeat domains in one protein, make designed ankyrin repeat proteins ideal agonistic, antagonistic and/or inhibitory drug candidates. Furthermore, such ankyrin repeat proteins can be engineered to carry various effector functions, e.g. cytotoxic agents or half-life extending agents, enabling completely new drug formats. Taken together, designed ankyrin repeat proteins are an example of the next generation of protein therapeutics with the potential to surpass existing antibody drugs.

[66] The designed ankyrin repeat domains described herein generally comprise one or more designed repeat modules, preferably ankyrin repeat modules, as structural units (thereafter also referred to as structural repeats or repeat units), wherein said repeat modules, preferably said ankyrin repeat modules, have structural and sequence homology. An ankyrin repeat module generally comprises two anti-parallel α -helices followed by a beta-bulge and beta-hairpin containing loop connecting it to the next repeat, each of which has about 28-33 residues.

[67] Recombinant proteins, or designed binding domains thereof, comprising designed ankyrin repeat modules are also referred herein as DARPin® proteins. See Stumpp et al., *Curr Opin Drug Discov Devel.* 10(2): 153-9 (2007); and Binz et al., *Nature Biotech.* 22(5): 575-582 (2004). DARPin® proteins can be considered as antibody mimetics with high specificity and high binding affinity to a target protein.

In general, a DARPin® protein comprises at least one ankyrin repeat module, for example, at least 2, 3, or more ankyrin repeat modules. DARPin® is a trademark owned by Molecular Partners AG, Switzerland.

[68] The ankyrin repeat domains described herein generally comprise a core scaffold that provides structure, and target binding residues that bind to a target. The structural core includes conserved amino acid residues, and the target binding surface includes amino acid residues that differ depending on the target. For example, an ankyrin repeat module can comprise the following sequence: xDxxGxTPLHLAxxxGxxxIVxVLLxxGADVNA (SEQ ID NO: 23), wherein "x" denotes any amino acid, preferably wherein "x" is not cysteine, glycine, or proline. As other examples, an ankyrin repeat module can comprise the amino acid sequence of any of SEQ ID NOs: 24 to 27.

[69] Designed repeat protein libraries, including designed ankyrin repeat protein libraries (WO2002/020565; Binz et al., Nat. Biotechnol. 22, 575-582, 2004; Stumpp et al., Drug Discov. Today 13, 695-701, 2008), can be used for the selection/screening of target-specific designed repeat domains that bind to their target with high affinity. Such target-specific designed repeat domains in turn can be used as valuable components of recombinant binding proteins for the treatment of diseases. Methods of making such libraries are known to the person skilled in the art (WO2002/020565).

[70] Multiple ankyrin repeat domains can be linked (either through a covalent bond or non-covalent association) to form bispecific or multi-specific molecules. Such multi-specific molecules are disclosed herein, including molecules in which one FAP-binding domain and two CD40-binding domains are linked. Such molecules may also include a half-life extending moiety at the N-terminus.

3.2. N-TERMINAL AND C-TERMINAL CAPPING MODULES

[71] The repeat domains, preferably ankyrin repeat domains, of the recombinant protein disclosed herein preferably comprise a N-terminal and/or a C-terminal capping module (thereafter also referred to as capping repeats or capping units). Capping modules are located at the N- and/or C-terminal end of an ankyrin repeat domain, typically forming tight tertiary interactions (i.e. tertiary structure interactions) with the ankyrin repeat module(s) in between, thereby providing a cap that shields the hydrophobic core of the ankyrin repeat domain at the side from exposure to the solvent.

[72] The N- and/or C-terminal capping modules may be derived from, a capping unit or other structural unit found in a naturally occurring repeat protein adjacent to a repeat unit. Examples of capping sequences are described in International Patent Publication Nos. WO 2002/020565 and WO 2012/069655, in U.S. Patent Publication No. US20130296221, and by Interlandi et al., J Mol Biol. 2008 Jan 18;375(3):837-54. Examples of N-terminal capping modules (i.e. N-terminal capping repeats) are SEQ ID NOs: 11-16 and examples of C-terminal capping modules (i.e. C-terminal capping repeats) are SEQ ID NOs: 18-21.

[73] In an exemplary embodiment, the N-terminal capping module comprises the amino acid sequence DLGKKLLEAARAGQDDEVRIILAAGADVNA (SEQ ID NO: 14) or DLGKKLLEAARAGQDDEVRELLKAGADVNA (SEQ ID NO: 15), wherein up to 9, up to 8, up to 7, up to 6, up to 5, up to 4, up to 3, up to 2 or up to 1 amino acids of SEQ ID NO: 14 or SEQ ID NO: 15 are optionally exchanged by any amino acids; and wherein SEQ ID NO: 14 or SEQ ID NO: 15 may optionally further comprise a "G," an "S," or a "GS" sequence at its N-terminus. In an exemplary embodiment, the C-terminal capping module comprises the amino acid sequence QDIFGKTPADIAADAGHEDIAEVLQKAA (SEQ ID NO: 19) or QDKSGKTPADLAADAGHEDIAEVLQKAA (SEQ ID NO: 20), wherein up to 9, up to 8, up to 7, up to 6, up to 5, up to 4, up to 3, up to 2 or up to 1 amino acids of SEQ ID NO: 19 or SEQ ID NO: 20 are optionally exchanged by any amino acids.

[74] Advantageously, in some embodiments, certain amino acid residues in the N-terminal capping module and/or the C-terminal capping module of the designed ankyrin repeat domain herein provided are altered, resulting in improved pharmacokinetic properties, including a prolonged terminal half-life, of the designed ankyrin repeat domain and of the recombinant binding proteins comprising the designed ankyrin repeat domain. The altered amino acid residues are mostly surface exposed residues. Preferably, the altered amino acids residues are the amino acid residues at positions 8 and 15 of an N-terminal capping module, wherein the position numbers correspond to the positions in SEQ ID NO: 11, and the amino acid residues at positions 14 and 18 of a C-terminal capping module, wherein the position numbers correspond to the positions in SEQ ID NO: 18.

[75] In one preferred embodiment, the designed ankyrin repeat domains provided herein comprise an N-terminal capping module having an amino acid sequence wherein the amino acid at position 8 is Q and/or the amino acid at position 15 is L. Examples of such N-terminal capping modules are SEQ ID NOs: 11, 12 and 13. In one embodiment, said designed ankyrin repeat domains comprise an N-terminal capping module having an amino acid sequence wherein the amino acid at position 4 is S, the amino acid at position 8 is Q, the amino acid at position 15 is L, the amino acid at position 17 is T, the amino acid at position 20 is T, and/or the amino acid at position 23 is Q. An example of such a N-terminal capping module is SEQ ID NO: 16. In a preferred embodiment, said N-terminal capping module comprises an amino acid sequence of 30 amino acids. In a further preferred embodiment, said N-terminal capping module consists of an amino acid sequence of 30 amino acids. Preferably, said position numbers of positions of the N-terminal capping module are determined by alignment to SEQ ID NO: 11 using the position numbers of SEQ ID NO: 11. Preferably, said alignment comprises no amino acid gaps. Sequence alignment generation is a procedure well known in the art. Any of said N-terminal capping modules may optionally further comprise a "G," an "S," or a "GS" sequence at its N-terminus.

[76] For example, an N-terminal capping module with altered amino acid residues can comprise the following sequence: DLGxxLLQAxxGQLDxVRxLxxxGADVNA (SEQ ID NO: 17), wherein "x" denotes any amino acid.

[77] In an exemplary embodiment, the N-terminal capping sequence comprises DLGKKLLQAARAGQLDEVRELLKAGADVNA (SEQ ID NO: 11), DLGKKLLQAARAGQLDEVRIILLKAGADVNA (SEQ ID NO: 12) or DLGKKLLQAARAGQLDEVRIILLAAAGADVNA (SEQ ID NO: 13), wherein up to 9, up to 8, up to 7, up to 6, up to 5, up to 4, up to 3, up to 2 or up to 1 amino acids of SEQ ID NO: 11, SEQ ID NO: 12, or SEQ ID NO: 13 in positions other than positions 8 and 15 are optionally exchanged by any amino acids; and wherein SEQ ID NO: 11, SEQ ID NO: 12, or SEQ ID NO: 13 may optionally further comprise a "G," an "S," or a "GS" sequence at its N-terminus. Thus, in one embodiment, the designed repeat domain, preferably ankyrin repeat domain, of the invention comprises an N-terminal capping module having the amino acid sequence DLGKKLLQAARAGQLDEVRELLKAGADVNA (SEQ ID NO: 11), wherein up to 9, up to 8, up to 7, up to 6, up to 5, up to 4, up to 3, up to 2 or up to 1 amino acids of SEQ ID NO: 11 in positions other than positions 8 and 15 are optionally exchanged by any amino acids; and wherein SEQ ID NO: 11 may optionally further comprise a "G," an "S," or a "GS" sequence at its N-terminus.

[78] In another exemplary embodiment, the N-terminal capping sequence comprises DLGSKLLQAARAGQLDVTLLQAGADVNA (SEQ ID NO: 16), wherein up to 9, up to 8, up to 7, up to 6, up to 5, up to 4, up to 3, up to 2 or up to 1 amino acids of SEQ ID NO: 16 in positions other than positions 4, 8, 15, 17, 20 and 23 are optionally exchanged by any amino acids; and wherein SEQ ID NO: 16 may optionally further comprise a "G," an "S," or a "GS" sequence at its N-terminus.

[79] In a further preferred embodiment, the designed repeat domains, preferably ankyrin repeat domains, provided herein comprise a C-terminal capping module having an amino acid sequence wherein the amino acid at position 14 is R and/or the amino acid at position 18 is Q. Examples of such C-terminal capping modules are SEQ ID NOs: 18 and 19. In one embodiment, said designed ankyrin repeat domain comprises a C-terminal capping module having an amino acid sequence wherein the amino acid at position 3 is T, the amino acid at position 4 is Q, the amino acid at position 6 is T, the amino acid at position 14 is R, the amino acid at position 18 is Q, the amino acid at position 19 is Q, the amino acid at position 22 is S, and/or the amino acid at position 26 is Q. An example of such a C-terminal capping module is SEQ ID NO: 21. In a preferred embodiment, said C-terminal capping module comprises an amino acid sequence of 28 amino acids. In a further preferred embodiment, said C-terminal capping module consists of an amino acid sequence of 28 amino acids. Preferably, said position numbers of positions of the C-terminal capping module are determined by alignment to SEQ ID NO: 18 using the position numbers of SEQ ID NO: 18. Preferably, said alignment comprises no amino acid gaps.

[80] For example, a C-terminal capping module with altered amino acid residues can comprise the following sequence: xDxxGxTPADxAARxGHQxIAxVLQxAA (SEQ ID NO: 22), wherein "x" denotes any amino acid.

[81] In an exemplary embodiment, the C-terminal capping sequence comprises QDKSGKTPADLAARAGHQDIAEVLQKAA (SEQ ID NO: 18), wherein up to 9, up to 8, up to 7, up to 6, up to 5, up to 4, up to 3, up to 2 or up to 1 amino acids of SEQ ID NO: 18 in positions other than positions 14 and 18 are optionally exchanged by any amino acids.

[82] In another exemplary embodiment, the C-terminal capping sequence comprises QDTQGTTPADLAARAGHQQIASVLQQA (SEQ ID NO: 21), wherein up to 9, up to 8, up to 7, up to 6, up to 5, up to 4, up to 3, up to 2 or up to 1 amino acids of SEQ ID NO: 21 in positions other than positions 3, 4, 6, 14, 18, 19, 22 and 26 are optionally exchanged by any amino acids.

3.3. FAP-BINDING DOMAIN

[83] One attractive stromal cell target is the fibroblast activation protein (FAP), a transmembrane serine protease highly expressed in the cancer-associated stromal cells of virtually all epithelial cancers. FAP is also expressed during embryonic development, in tissues of healing wounds, and in chronic inflammatory and fibrotic conditions such as liver cirrhosis and idiopathic pulmonary fibrosis. However, FAP has not been detected by immunohistochemistry in benign tumors or in most normal quiescent adult stromal cells.

[84] The recombinant protein described herein comprises an ankyrin repeat domain that specifically binds FAP, also referred herein as "FAP binding domain".

[85] In some embodiments, the FAP binding domain described herein comprises an amino acid sequence that is at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% identical to SEQ ID NO: 2. In an exemplary embodiment, the FAP binding domain described herein comprises an amino acid sequence that is at least 90% identical to SEQ ID NO: 2. In a preferred embodiment, the FAP binding domain described herein comprises the amino acid sequence of SEQ ID NO: 2. In some embodiments, the FAP binding domain described herein comprises an amino acid sequence that is at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% identical to SEQ ID NO: 8. In an exemplary embodiment, the FAP binding domain described herein comprises an amino acid sequence that is at least 90% identical to SEQ ID NO: 8. In a preferred embodiment, the FAP binding domain described herein comprises the amino acid sequence of SEQ ID NO: 8. In some embodiments, the FAP binding domain described herein comprises an amino acid sequence that is at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% identical to SEQ ID NO: 9. In an exemplary embodiment, the FAP binding domain described herein comprises an amino acid sequence that is at least 90% identical to SEQ ID NO: 9. In another exemplary embodiment, the FAP binding domain described herein comprises the amino acid sequence of SEQ ID NO: 9. In some embodiments, the FAP binding domain described herein comprises an amino acid sequence that is at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at

least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% identical to any one of SEQ ID NOs: 28-38. In an exemplary embodiment, the FAP binding domain described herein comprises an amino acid sequence that is at least 90% identical to any one of SEQ ID NOs: 28-38. In another exemplary embodiment, the FAP binding domain described herein comprises the amino acid sequence of any one of SEQ ID NOs: 28-38.

[86] In some embodiments, no more than 10, no more than 9, no more than 8, no more than 7, no more than 6, no more than 5, no more than 4, no more than 3, no more than 2, or no more than 1 substitution is made relative to the sequence of SEQ ID NO: 2. In some embodiments, no more than 5 substitutions are made relative to the sequence of SEQ ID NO: 2. In some embodiments, no more than 4 substitutions are made relative to the sequence of SEQ ID NO: 2. In some embodiments, no more than 3 substitutions are made relative to the sequence of SEQ ID NO: 2. In some embodiments, no more than 2 substitutions are made relative to the sequence of SEQ ID NO: 2. In some embodiments, no more than 1 substitution is made relative to the sequence of SEQ ID NO: 2. In some embodiments, the substitution(s) do not change the K_D value by more than 1000-fold, more than 100-fold, or more than 10-fold, compared to the K_D value of the protein comprising the sequence of SEQ ID NO: 2. In some embodiments, no more than 10, no more than 9, no more than 8, no more than 7, no more than 6, no more than 5, no more than 4, no more than 3, no more than 2, or no more than 1 substitution is made relative to the sequence of SEQ ID NO: 8. In some embodiments, no more than 5 substitutions are made relative to the sequence of SEQ ID NO: 8. In some embodiments, no more than 4 substitutions are made relative to the sequence of SEQ ID NO: 8. In some embodiments, no more than 3 substitutions are made relative to the sequence of SEQ ID NO: 8. In some embodiments, no more than 2 substitutions are made relative to the sequence of SEQ ID NO: 8. In some embodiments, no more than 1 substitution is made relative to the sequence of SEQ ID NO: 8. In some embodiments, the substitution(s) do not change the K_D value by more than 1000-fold, more than 100-fold, or more than 10-fold, compared to the K_D value of the protein comprising the sequence of SEQ ID NO: 8. In certain embodiments, the substitution is a conservative substitution according to Table 1. In certain embodiments, the substitution is made outside the structural core residues of the ankyrin repeat domain, e.g. in the beta loops that connect the alpha-helices. In certain embodiments, the substitution is made within the structural core residues of the ankyrin repeat domain. For example, the ankyrin domain may comprise the consensus sequence: xDxxGxTPLHLAxxxGxxxIVxVLLxxGADVNA (SEQ ID NO: 23), wherein "x" denotes any amino acid, preferably not cysteine, glycine, or proline; or xDxxGxTPLHLAxxxGHLEIVEVLLKzGADVNA (SEQ ID NO: 24), wherein "x" denotes any amino acid, preferably not cysteine, glycine, or proline, and "z" is selected from the group consisting of asparagine, histidine, or tyrosine. In one embodiment, the substitution is made to residues designated as "x". In another embodiment, the substitution is made outside the residues designated as "x".

[87] In addition, the second last position can be "A" (see, e.g., SEQ ID NOs: 2, 8, 9, 28-31 and 38) or "L" (see, e.g., SEQ ID NOs: 32-37), and/or the last position can be "A" (see, e.g., SEQ ID NOs: 2, 8, 9, 28-31 and 38) or "N" (see, e.g., SEQ ID NOs: 32-37). Accordingly, in some embodiments, the FAP-

binding domain comprises an amino acid sequence that is at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% identical to any one of SEQ ID NOs: 2, 8, 9, 28-31 and 38, and wherein optionally A at the second last position is substituted with L and/or A at the last position is substituted with N. In an exemplary embodiment, the FAP-binding domain comprises an amino acid sequence that is at least 90% identical to any one of SEQ ID NOs: 2, 8, 9, 28-31 and 38, and wherein optionally A at the second last position is substituted with L and/or A at the last position is substituted with N. In some embodiments, the FAP-binding domain comprises an amino acid sequence that is at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% identical to SEQ ID NO: 2, and wherein optionally A at the second last position is substituted with L and/or A at the last position is substituted with N. In an exemplary embodiment, the FAP-binding domain comprises an amino acid sequence that is at least 90% identical to SEQ ID NO: 2, and wherein optionally A at the second last position is substituted with L and/or A at the last position is substituted with N. In a preferred embodiment, the FAP binding domain described herein comprises the amino acid sequence of SEQ ID NO: 2, and wherein optionally A at the second last position is substituted with L and/or A at the last position is substituted with N. In some embodiments, the FAP-binding domain comprises an amino acid sequence that is at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% identical to any one of SEQ ID NOs: 9, 28-31 and 38, and wherein optionally A at the second last position is substituted with L and/or A at the last position is substituted with N. In an exemplary embodiment, the FAP-binding domain comprises an amino acid sequence that is at least 90% identical to any one of SEQ ID NOs: 9, 28-31 and 38, and wherein optionally A at the second last position is substituted with L and/or A at the last position is substituted with N. In another exemplary embodiment, the FAP-binding domain comprises the amino acid sequence of any one of SEQ ID NOs: 9, 28-31 and 38, and wherein optionally A at the second last position is substituted with L and/or A at the last position is substituted with N. In some embodiments, the FAP-binding domain comprises an amino acid sequence that is at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% identical to any one of SEQ ID NOs: 32-37, and wherein optionally L at the second last position is substituted with A and/or N at the last position is substituted with A. In an exemplary embodiment, the FAP-binding domain comprises an amino acid sequence that is at least 90% identical to any one of SEQ ID NOs: 32-37, and wherein optionally L at the second last position is substituted with A and/or N at the last position is substituted with A. In another exemplary embodiment, the FAP-binding domain comprises the amino acid sequence of any one of SEQ ID NOs: 32-37, and wherein optionally L at the

second last position is substituted with A and/or N at the last position is substituted with A. The sequences may optionally comprise at its N-terminus, a G, an S, or a GS (see below).

[88] In addition, the FAP-binding domain may optionally further comprise a "G," an "S," or a "GS" sequence at its N-terminus (see, e.g., SEQ ID NO: 38 as compared with SEQ ID NO: 2). Accordingly, in some embodiments, the FAP-binding domain provided herein (i) comprises an amino acid sequence that is at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% identical to any one of SEQ ID NOs: 2, 8, 9, and 28-37, and (ii) further comprises at its N-terminus, a G, an S, or a GS. In an exemplary embodiment, the FAP-binding domain comprises an amino acid sequence that is at least 90% identical to any one of SEQ ID NOs: 2, 8, 9, and 28-37, and further comprises at its N-terminus, a G, an S, or a GS. In an exemplary embodiment, the FAP-binding domain comprises an amino acid sequence that is at least 95% identical to any one of SEQ ID NOs: 2, 8, 9, and 28-37, and further comprises at its N-terminus, a G, an S, or a GS. In an exemplary embodiment, the FAP-binding domain comprises the amino acid sequence of any one of SEQ ID NOs: 2, 8, 9, and 28-37, and further comprises at its N-terminus, a G, an S, or a GS. Also accordingly, in some embodiments, the FAP-binding domain provided herein comprises an amino acid sequence that is at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% identical to SEQ ID NO: 38, wherein G at position 1 and/or S at position 2 of SEQ ID NO: 38 are optionally missing.

[89] Accordingly, in one particular preferred embodiment, the FAP binding domain described herein comprises the amino acid sequence of SEQ ID NO: 2, wherein its N-terminus optionally further comprises a G, an S, or a GS, and wherein optionally A at the second last position is substituted with L and/or A at the last position is substituted with N.

[90] In certain embodiments, the affinity between the FAP binding domain or the recombinant protein comprising the FAP binding domain and its target (i.e., FAP) is described in terms of K_D . In exemplary embodiments, the K_D is about 10^{-1} M or less, about 10^{-2} M or less, about 10^{-3} M or less, about 10^{-4} M or less, about 10^{-5} M or less, about 10^{-6} M or less, about 10^{-7} M or less, about 10^{-8} M or less, about 10^{-9} M or less, about 10^{-10} M or less, about 10^{-11} M or less, about 10^{-12} M or less, about 10^{-13} M or less, about 10^{-14} M or less, from about 10^{-5} M to about 10^{-15} M, from about 10^{-6} M to about 10^{-15} M, from about 10^{-7} M to about 10^{-15} M, from about 10^{-8} M to about 10^{-15} M, from about 10^{-9} M to about 10^{-15} M, from about 10^{-10} M to about 10^{-15} M, from about 10^{-5} M to about 10^{-14} M, from about 10^{-6} M to about 10^{-14} M, from about 10^{-7} M to about 10^{-14} M, from about 10^{-8} M to about 10^{-14} M, from about 10^{-9} M to about 10^{-14} M, from about 10^{-10} M to about 10^{-14} M, from about 10^{-5} M to about 10^{-13} M, from about 10^{-6} M to about 10^{-13} M, from about 10^{-7} M to about 10^{-13} M, from about 10^{-8} M to about 10^{-13} M, from about 10^{-9} M to about 10^{-13} M, or from about 10^{-10} M to about 10^{-13} M.

[91] In exemplary embodiments, the FAP binding domain binds FAP with an K_D value of, or below: about 100nM, about 90 nM, about 80 nM, about 75nM, about 60 nM, about 50 nM, about 40 nM, about

30 nM, about 20 nM, about 10 nM, about 5 nM, about 2 nM, about 1 nM, about 900 pM, about 800 pM, about 700 pM, about 600 pM, about 500 pM, about 400 pM, about 300 pM, about 250 pM, about 200 pM, about 150 pM, about 100 pM, about 50 pM, about 40 pM, about 30 pM, about 25 pM, about 20 pM, about 15 pM, about 10 pM, about 5 pM, or about 1 pM, preferably with a K_D value of or below: 100 nM, 90 nM, 80 nM, 75 nM, 70 nM, 60 nM, 50 nM, 40 nM, 30 nM, 20 nM, 10 nM, 5 nM, 2 nM, 1 nM, 900 pM, 800 pM, 700 pM, 600 pM, 500 pM, 400 pM, 300 pM, 250 pM, 200 pM, 150 pM, 140 pM, 130 pM, or 120 pM. In one exemplary embodiment, the FAP binding domain binds FAP with a K_D value of or below about 100 nM. In another exemplary embodiment, the FAP binding domain binds FAP with a K_D value of or below about 10 nM. In another exemplary embodiment, the FAP binding domain binds FAP with a K_D value of or below about 1 nM. In one preferred embodiment, the FAP binding domain binds FAP with a K_D value of or below about 120 pM. Preferably, the FAP binding domain has the amino acid sequence of SEQ ID NO: 2.

[92] In exemplary embodiments, the recombinant protein comprising the FAP binding domain binds FAP with an K_D value of, or below: about 100nM, about 90 nM, about 80 nM, about 75nM, about 60 nM, about 50 nM, about 40 nM, about 30 nM, about 20 nM, about 10 nM, about 5 nM, about 2 nM, about 1 nM, about 900 pM, about 800 pM, about 700 pM, about 600 pM, about 500 pM, about 400 pM, about 300 pM, about 250 pM, about 200 pM, about 150 pM, about 100 pM, about 50 pM, about 40 pM, about 30 pM, about 25 pM, about 20 pM, about 15 pM, about 10 pM, about 5 pM, or about 1 pM, preferably with a K_D value of or below: 100 nM, 90 nM, 80 nM, 75 nM, 70 nM, 60 nM, 50 nM, 40 nM, 30 nM, 20 nM, 10 nM, 5 nM, 2 nM, 1 nM, 900 pM, 800 pM, 700 pM, 600 pM, 500 pM, 400 pM, or 300 pM. In one exemplary embodiment, the recombinant protein binds FAP with a K_D value of or below about 100 nM. In another exemplary embodiment, the FAP binding domain binds FAP with a K_D value of or below about 10 nM. In another exemplary embodiment, the recombinant protein binds FAP with a K_D value of or below about 1 nM. In another exemplary embodiment, the recombinant protein binds FAP with a K_D value of or below about 500 pM. In one preferred embodiment, the recombinant protein binds FAP with a K_D value of or below about 300 pM. Preferably, the recombinant protein has the amino acid sequence of SEQ ID NO: 5.

[93] In certain embodiments, the FAP is human FAP (SEQ ID NO: 52).

Table 1 Amino Acid Substitutions

Original Residue	Conservative Substitutions	Exemplary Substitutions
Ala (A)	Val	Val; Leu; Ile
Arg (R)	Lys	Lys; Gln; Asn
Asn (N)	Gln	Gln; His; Asp, Lys; Arg
Asp (D)	Glu	Glu; Asn
Cys (C)	Ser	Ser; Ala
Gln (Q)	Asn	Asn; Glu
Glu (E)	Asp	Asp; Gln
Gly (G)	Ala	Ala
His (H)	Arg	Asn; Gln; Lys; Arg
Ile (I)	Leu	Leu; Val; Met; Ala; Phe; Norleucine
Leu (L)	Ile	Norleucine; Ile; Val; Met; Ala; Phe
Lys (K)	Arg	Arg; Gln; Asn

Met (M)	Leu	Leu; Phe; Ile
Phe (F)	Tyr	Leu; Val; Ile; Ala; Tyr
Pro (P)	Ala	Ala
Ser (S)	Thr	Thr
Thr (T)	Ser	Ser
Trp (W)	Tyr	Tyr; Phe
Tyr(Y)	Phe	Trp; Phe; Thr; Ser
Val (V)	Leu	Ile; Leu; Met; Phe; Ala; Norleucine

3.4. CD40 BINDING DOMAINS

[94] The recombinant proteins disclosed herein also take advantage of the immune cell co-stimulatory activities induced by CD40.

[95] The recombinant protein described herein comprises an ankyrin repeat domain that specifically binds CD40, also referred herein as "CD40 binding domain". Like CD40 agonist antibodies, the CD40 binding domain activates the CD40/CD40L signaling pathway. The recombinant protein described herein may also comprise more than one CD40 binding domain, for example, two or three or more CD40 binding domains. Thus, the recombinant protein described herein may comprise a first and a second CD40 binding domain, or a first, a second and a third CD40 binding domain. The embodiments provided below describe such a first CD40 binding domain, second CD40 binding domain, and/or third CD40 binding domain. Preferably, the recombinant protein described herein comprises two CD40 binding domain, i.e. a first CD40 binding domain and a second CD40 binding domain.

[96] In some embodiments, the CD40 binding domain or each of said CD40 binding domains independently comprises an amino acid sequence that is at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% identical to SEQ ID NO: 3. In an exemplary embodiment, the CD40 binding domain or each of said CD40 binding domains independently comprises an amino acid sequence that is at least 90% identical to SEQ ID NO: 3. In a preferred embodiment, the CD40 binding domain or each of said CD40 binding domains comprises the amino acid sequence of SEQ ID NO: 3. In some embodiments, the CD40 binding domain or each of said CD40 binding domains independently comprises an amino acid sequence that is at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% identical to SEQ ID NO: 10. In an exemplary embodiment, the CD40 binding domain or each of said CD40 binding domains independently comprises an amino acid sequence that is at least 90% identical to SEQ ID NO: 10. In another exemplary embodiment, the CD40 binding domain or each of said CD40 binding domains comprises the amino acid sequence of SEQ ID NO: 10. In some embodiments, the CD40 binding domain or each of said CD40 binding domains independently comprises an amino acid sequence that is at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least

91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% identical to any one of SEQ ID NOs: 43-50. In an exemplary embodiment, the CD40 binding domain or each of said CD40 binding domains independently comprises an amino acid sequence that is at least 90% identical to any one of SEQ ID NOs: 43-50. In another exemplary embodiment, the CD40 binding domain or each of said CD40 binding domains independently comprises the amino acid sequence of any one of SEQ ID NOs: 43-50.

[97] In some embodiments, no more than 10, no more than 9, no more than 8, no more than 7, no more than 6, no more than 5, no more than 4, no more than 3, no more than 2, or no more than 1 substitution is made relative to the sequence of SEQ ID NO: 3. In some embodiments, no more than 5 substitutions are made relative to the sequence of SEQ ID NO: 3. In some embodiments, no more than 4 substitutions are made relative to the sequence of SEQ ID NO: 3. In some embodiments, no more than 3 substitutions are made relative to the sequence of SEQ ID NO: 3. In some embodiments, no more than 2 substitutions are made relative to the sequence of SEQ ID NO: 3. In some embodiments, no more than 1 substitution is made relative to the sequence of SEQ ID NO: 3. In some embodiments, the substitution(s) do not change the K_D value by more than 1000-fold, more than 100-fold, or more than 10-fold, compared to the K_D value of the protein comprising the sequence of SEQ ID NO: 3. In certain embodiments, the substitution is a conservative substitution according to Table 1. In certain embodiments, the substitution is made outside the structural core residues of the ankyrin repeat domain, e.g. in the beta loops that connect the alpha-helices. In certain embodiments, the substitution is made within the structural core residues of the ankyrin repeat domain. For example, the ankyrin domain or each the ankyrin binding domains may comprise the consensus sequence: xDxxGxTPLHLAxxxGxxxIVxVLLxxGADVNA (SEQ ID NO: 23), wherein "x" denotes any amino acid, preferably not cysteine, glycine, or proline; or xDxxGxTPLHLAxxxGHLEIVEVLLKzGADVNA (SEQ ID NO: 24), wherein "x" denotes any amino acid, preferably not cysteine, glycine, or proline, and "z" is selected from the group consisting of asparagine, histidine, or tyrosine. In one embodiment, the substitution is made to residues designated as "x". In another embodiment, the substitution is made outside the residues designated as "x".

[98] In addition, the second last position can be "A" (see, e.g., SEQ ID NOs: 3, 10, 43, 44, and 48-50) or "L" (see, e.g., SEQ ID NOs: 45-47), and/or the last position can be "A" (see, e.g., SEQ ID NOs: 3, 10, 43, 44, and 48-50) or "N" (see, e.g., SEQ ID NOs: 45-47). Accordingly, in some embodiments, the CD40-binding domain or each of said CD40 binding domains independently comprises an amino acid sequence that is at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% identical to any one of SEQ ID NOs: 3, 10, 43, 44, and 48-50, and wherein optionally A at the second last position is substituted with L and/or A at the last position is substituted with N. In an exemplary embodiment, the CD40-binding domain or each of said CD40 binding domains independently comprises an amino acid sequence that is at least 90% identical to any one of SEQ ID NOs: 3, 10, 43, 44, and 48-50, and wherein optionally A at the second last position is substituted with

L and/or A at the last position is substituted with N. In some embodiments, the CD40-binding domain or each of said CD40 binding domains independently comprises an amino acid sequence that is at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% identical to SEQ ID NO: 3, and wherein optionally A at the second last position is substituted with L and/or A at the last position is substituted with N. In an exemplary embodiment, the CD40-binding domain or each of said CD40 binding domains independently comprises an amino acid sequence that is at least 90% identical to SEQ ID NO: 3, and wherein optionally A at the second last position is substituted with L and/or A at the last position is substituted with N. In a preferred embodiment, the CD40 binding domain or each of said CD40 binding domains described herein comprises the amino acid sequence of SEQ ID NO: 3, and wherein optionally A at the second last position is substituted with L and/or A at the last position is substituted with N. In some embodiments, the CD40-binding domain or each of said CD40 binding domains independently comprises an amino acid sequence that is at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% identical to any one of SEQ ID NOs: 10, 43, 44, and 48-50, and wherein optionally A at the second last position is substituted with L and/or A at the last position is substituted with N. In an exemplary embodiment, the CD40-binding domain or each of said CD40 binding domains independently comprises an amino acid sequence that is at least 90% identical to any one of SEQ ID NOs: 10, 43, 44, and 48-50, and wherein optionally A at the second last position is substituted with L and/or A at the last position is substituted with N. In another exemplary embodiment, the CD40-binding domain or each of said CD40 binding domains independently comprises the amino acid sequence of any one of SEQ ID NOs: 10, 43, 44, and 48-50, and wherein optionally A at the second last position is substituted with L and/or A at the last position is substituted with N. The sequences may optionally comprise at its N-terminus, a G, an S, or a GS (see below).

[99] In addition, the CD40-binding domain or each of said CD40 binding domains may optionally further comprise a "G," an "S," or a "GS" sequence at its N-terminus (see, e.g., SEQ ID NO: 50 as compared with SEQ ID NO: 3). Accordingly, in some embodiments, the CD40-binding domain or each of said CD40 binding domains independently provided herein (i) comprises an amino acid sequence that is at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% identical to any one of SEQ ID NOs: 3, 10, and 43-49, and (ii) further comprises at its N-terminus, a G, an S, or a GS. In an exemplary embodiment, the CD40-binding domain or each of said CD40 binding domains independently comprises an amino acid sequence that is at least 90% identical to any one of SEQ ID NOs: 3, 10, and 43-49, and further comprises at its N-terminus, a G, an S, or a GS. In an exemplary embodiment, the CD40-binding domain or each of said CD40 binding domains independently comprises an amino acid sequence that is at least 95% identical to any one of SEQ ID NOs: 3, 10, and 43-49, and further comprises at its N-terminus, a G, an S, or a GS. In an exemplary embodiment, the

CD40-binding domain or each of said CD40 binding domains independently comprises the amino acid sequence of any one of SEQ ID NOs: 3, 10, and 43-49, and further comprises at its N-terminus, a G, an S, or a GS. Also accordingly, in some embodiments, the CD40-binding domain or each of said CD40 binding domains provided herein comprises an amino acid sequence that is at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% identical to SEQ ID NO: 50, wherein G at position 1 and/or S at position 2 of SEQ ID NO: 50 are optionally missing.

[100] Accordingly, in one particular preferred embodiment, the CD40 binding domain or each of said CD40 binding domains described herein comprises the amino acid sequence of SEQ ID NO: 3, wherein its N-terminus optionally further comprises a G, an S, or a GS, and wherein optionally A at the second last position is substituted with L and/or A at the last position is substituted with N.

[101] In some preferred embodiments, any of one of the CD40 binding domain described herein comprises Q at position 8, L at position 15, R at position 143, and/or Q at position 147, wherein the position numbers correspond to the positions in SEQ ID NO: 3. Accordingly, in some embodiments, the CD40 binding domain or each of said CD40 binding domains described herein comprises (1) Q at position 8 and (2) R at position 143 and/or Q at position 147, wherein the position numbers correspond to the positions in SEQ ID NO: 3. In some embodiments, the CD40 binding domain or each of said CD40 binding domains described herein comprises (1) L at position 15 and (2) R at position 143 and/or Q at position 147, wherein the position numbers correspond to the positions in SEQ ID NO: 3. In some embodiments, the CD40 binding domain or each of said CD40 binding domains described herein comprises (1) Q at position 8 and L at position 15 and (2) R at position 143 and/or Q at position 147, wherein the position numbers correspond to the positions in SEQ ID NO: 3. Also accordingly, in some embodiments, the CD40 binding domain or each of said CD40 binding domains described herein comprises (1) Q at position 8 and/or L at position 15 and (2) R at position 143, wherein the position numbers correspond to the positions in SEQ ID NO: 3. In some embodiments, the CD40 binding domain or each of said CD40 binding domains described herein comprises (1) Q at position 8 and/or L at position 15 and (2) Q at position 147, wherein the position numbers correspond to the positions in SEQ ID NO: 3. . In some embodiments, the CD40 binding domain or each of said CD40 binding domains described herein comprises (1) Q at position 8 and/or L at position 15 and (2) R at position 143 and Q at position 147, wherein the position numbers correspond to the positions in SEQ ID NO: 3. In some more preferred embodiments, the CD40 binding domain or each of said CD40 binding domains described herein comprises Q at position 8, L at position 15, R at position 143, and Q at position 147, wherein the position numbers correspond to the positions in SEQ ID NO: 3.

[102] Furthermore, in even more preferred embodiments, the CD40 binding domain or each of said CD40 binding domains described herein comprises Q at position 8, L at position 15, R at position 143, and Q at position 147, wherein the position numbers correspond to the positions in SEQ ID NO: 3, resulting in improved pharmacokinetic properties of said CD40 binding domain compared to a CD40 binding domain with an identical amino acid sequence except for the amino acids at positions 8, 15,

143 and 147, which are different than Q, L, R and Q, respectively, wherein the position numbers correspond to the positions in SEQ ID NO: 3.

[103] In certain embodiments, the affinity between the CD40 binding domain or each of said CD40 binding domains, or the recombinant protein comprising the CD40 binding domain(s), and its target (i.e., CD40) is described in terms of K_D . In exemplary embodiments, the K_D is about 10^{-1} M or less, about 10^{-2} M or less, about 10^{-3} M or less, about 10^{-4} M or less, about 10^{-5} M or less, about 10^{-6} M or less, about 10^{-7} M or less, about 10^{-8} M or less, about 10^{-9} M or less, about 10^{-10} M or less, about 10^{-11} M or less, about 10^{-12} M or less, about 10^{-13} M or less, about 10^{-14} M or less, from about 10^{-5} M to about 10^{-15} M, from about 10^{-6} M to about 10^{-15} M, from about 10^{-7} M to about 10^{-15} M, from about 10^{-8} M to about 10^{-15} M, from about 10^{-9} M to about 10^{-15} M, from about 10^{-10} M to about 10^{-15} M, from about 10^{-5} M to about 10^{-14} M, from about 10^{-6} M to about 10^{-14} M, from about 10^{-7} M to about 10^{-14} M, from about 10^{-8} M to about 10^{-14} M, from about 10^{-9} M to about 10^{-14} M, from about 10^{-10} M to about 10^{-14} M, from about 10^{-5} M to about 10^{-13} M, from about 10^{-6} M to about 10^{-13} M, from about 10^{-7} M to about 10^{-13} M, from about 10^{-8} M to about 10^{-13} M, from about 10^{-9} M to about 10^{-13} M, or from about 10^{-10} M to about 10^{-13} M.

[104] In exemplary embodiments, the CD40 binding domain or each of said CD40 binding domain independently binds CD40 with an K_D value of, or below: about 100 nM, about 90 nM, about 80 nM, about 75 nM, about 60 nM, about 50 nM, about 40 nM, about 30 nM, about 20 nM, about 10 nM, about 5 nM, about 2 nM, about 1 nM, about 900 pM, about 800 pM, about 700 pM, about 600 pM, about 500 pM, about 400 pM, about 300 pM, about 250 pM, about 200 pM, about 150 pM, about 100 pM, about 50 pM, about 40 pM, about 30 pM, about 25 pM, about 20 pM, about 15 pM, about 10 pM, about 5 pM, or about 1 pM, preferably with a K_D value of or below: 100 nM, 90 nM, 80 nM, or 75 nM. In one exemplary embodiment, the CD40 binding domain or each of said CD40 binding domain independently binds CD40 with a K_D value of or below about 100 nM. In one preferred embodiment, the CD40 binding domain or each of said CD40 binding domain independently binds CD40 with a K_D value of or below about 75 nM. Preferably, the CD40 binding domain or each of said CD40 binding domain has the amino acid sequence of SEQ ID NO: 3.

[105] In exemplary embodiments, the recombinant protein comprising two CD40 binding domains binds CD40 with an K_D value of, or below: about 100 nM, about 90 nM, about 80 nM, about 75 nM, about 60 nM, about 50 nM, about 40 nM, about 30 nM, about 20 nM, about 10 nM, about 5 nM, about 2 nM, about 1 nM, about 900 pM, about 800 pM, about 700 pM, about 600 pM, about 500 pM, about 400 pM, about 300 pM, about 250 pM, about 200 pM, about 150 pM, about 100 pM, about 50 pM, about 40 pM, about 30 pM, about 25 pM, about 20 pM, about 15 pM, about 10 pM, about 5 pM, or about 1 pM, preferably with a K_D value of or below: 100 nM, 90 nM, 80 nM, 75 nM, 70 nM, 60 nM, 50 nM, 40 nM, 30 nM, 20 nM, 10 nM, 5 nM, 2 nM, 1 nM, 900 pM, 800 pM, 700 pM, 600 pM, 500 pM, 400 pM, 300 pM, 250 pM, 200 pM, 150 pM, 140 pM, 130 pM, 120 pM, 115 pM, 110 pM, 105 pM or 100 pM. In one exemplary embodiment, the recombinant protein binds CD40 with a K_D value of or below about 100 nM. In another exemplary embodiment, the recombinant protein binds CD40 with a K_D value of or below about 1 nM. In another exemplary embodiment, the recombinant protein binds CD40 with a K_D value of or below about 500 pM. In one preferred embodiment, the recombinant protein binds CD40 with a K_D

value of or below about 100 pM. Preferably, the recombinant protein has the amino acid sequence of SEQ ID NO: 5.

[106] In some embodiments, two or more CD40 binding domains are preferred, to further promote CD40 clustering and immune cell co-stimulation. It has been reported that CD40 ligand binds to CD40 on immune cells as a trimer. However, trimerization alone is not sufficient to activate the CD40 signaling pathway. Higher order of clustering of CD40 is required for its activation. As described herein, through FAP-binding, the multispecific molecule already promotes CD40 clustering in the tumor environment. To further promote CD40 clustering, two or more CD40 binding domains can be used, to create a "cross-linking" effect on the cell surface. For example, as shown in FIG. 4, monovalent CD40 binder (FC) was sufficient to activate the CD40 pathway. Higher potency can be achieved by using two CD40 binding domains (FCC), or three CD40 binding domains (FCCC). FIG. 4 also shows that two CD40 binding domains are sufficient to activate the CD40 pathway with high potency, and it is not necessary to have three CD40 binding domains for efficient CD40 clustering.

[107] In certain embodiments, the CD40 is human CD40 (SEQ ID NO: 51).

3.5. HALF-LIFE EXTENDING MOIETIES

[108] The "half-life extending moiety" extends the serum half-life *in vivo* of the recombinant proteins described herein, compared to the same protein without the half-life extending moiety. Examples of half-life extending moieties include, but are not limited to, polyhistidine, Glu-Glu, glutathione S transferase (GST), thioredoxin, protein A, protein G, an immunoglobulin domain, maltose binding protein (MBP), human serum albumin (HSA) binding domain, or polyethylene glycol (PEG).

[109] In some embodiments, the recombinant multispecific protein described herein comprises an ankyrin repeat domain that specifically binds serum albumin, also referred herein as "serum albumin binding domain". The recombinant protein described herein may also comprise more than one serum albumin binding domain, for example, two or three or more serum albumin binding domains. Thus, the recombinant protein described herein may comprise a first and a second serum albumin binding domain, or a first, a second and a third serum albumin binding domain. The embodiments provided below describe such a first serum albumin binding domain, second serum albumin binding domain, and/or third serum albumin binding domain. Preferably, the recombinant protein described herein comprises only one serum albumin binding domain.

[110] In some embodiments, the serum albumin binding domain described herein comprises an amino acid sequence that is at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% identical to SEQ ID NO: 1. In an exemplary embodiment, the serum albumin binding domain described herein comprises an amino acid sequence that is at least 90% identical to SEQ ID NO: 1. In a preferred embodiment, the serum albumin binding domain described herein comprises the amino acid sequence

of SEQ ID NO: 1. In some embodiments, the serum albumin binding domain described herein comprises an amino acid sequence that is at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% identical to any one of SEQ ID NOs: 39-42. In an exemplary embodiment, the serum albumin binding domain described herein comprises an amino acid sequence that is at least 90% identical to any one of SEQ ID NOs: 39-42. In another exemplary embodiment, the serum albumin binding domain described herein comprises the amino acid sequence of any one of SEQ ID NOs: 39-42.

[111] In some embodiments, no more than 10, no more than 9, no more than 8, no more than 7, no more than 6, no more than 5, no more than 4, no more than 3, no more than 2, or no more than 1 substitution is made relative to the sequence of SEQ ID NO: 1. In some embodiments, no more than 5 substitutions are made relative to the sequence of SEQ ID NO: 1. In some embodiments, no more than 4 substitutions are made relative to the sequence of SEQ ID NO: 1. In some embodiments, no more than 3 substitutions are made relative to the sequence of SEQ ID NO: 1. In some embodiments, no more than 2 substitutions are made relative to the sequence of SEQ ID NO: 1. In some embodiments, no more than 1 substitution is made relative to the sequence of SEQ ID NO: 1. In some embodiments, the substitution(s) do not change the K_D value by more than 1000-fold, more than 100-fold, or more than 10-fold, compared to the K_D value of the protein comprising the sequence of SEQ ID NO: 1. In certain embodiments, the substitution is a conservative substitution according to Table 1. In certain embodiments, the substitution is made outside the structural core residues of the ankyrin repeat domain, e.g. in the beta loops that connect the alpha-helices. In certain embodiments, the substitution is made within the structural core residues of the ankyrin repeat domain. For example, the ankyrin domain may comprise the consensus sequence: xDxxGxTPLHLAxxxGxxxIVxVLLxxGADVNA (SEQ ID NO: 23), wherein "x" denotes any amino acid, preferably not cysteine, glycine, or proline; or xDxxGxTPLHLAxxxGHLEIVEVLLKzGADVNA (SEQ ID NO: 24), wherein "x" denotes any amino acid, preferably not cysteine, glycine, or proline, and "z" is selected from the group consisting of asparagine, histidine, or tyrosine. In one embodiment, the substitution is made to residues designated as "x". In another embodiment, the substitution is made outside the residues designated as "x".

[112] In addition, the second last position can be "A" (see, e.g., SEQ ID NOs: 1, 39, 40 and 42) or "L" (see, e.g., SEQ ID NO: 41), and/or the last position can be "A" (see, e.g., SEQ ID NOs: 1, 39, 40 and 42) or "N" (see, e.g., SEQ ID NO: 1). Accordingly, in some embodiments, the serum albumin binding domain comprises an amino acid sequence that is at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% identical to any one of SEQ ID NOs: 1, 39, 40 and 42, and wherein optionally A at the second last position is substituted with L and/or A at the last position is substituted with N. In an exemplary embodiment, the serum albumin binding domain comprises an amino acid sequence that is at least 90% identical to any one of SEQ ID NOs: 1, 39, 40 and 42, and wherein optionally A at the second last position is substituted with L and/or A at the last position is substituted with N. In some

embodiments, the serum albumin binding domain comprises an amino acid sequence that is at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% identical to SEQ ID NO: 1, and wherein optionally A at the second last position is substituted with L and/or A at the last position is substituted with N. In an exemplary embodiment, the serum albumin binding domain comprises an amino acid sequence that is at least 90% identical to SEQ ID NO: 1, and wherein optionally A at the second last position is substituted with L and/or A at the last position is substituted with N. In a preferred embodiment, the serum albumin binding domain described herein comprises the amino acid sequence of SEQ ID NO: 1, and wherein optionally A at the second last position is substituted with L and/or A at the last position is substituted with N. In some embodiments, the serum albumin binding domain comprises an amino acid sequence that is at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% identical to any one of SEQ ID NOs: 39, 40 and 42, and wherein optionally A at the second last position is substituted with L and/or A at the last position is substituted with N. In an exemplary embodiment, the serum albumin binding domain comprises an amino acid sequence that is at least 90% identical to any one of SEQ ID NOs: 39, 40 and 42, and wherein optionally A at the second last position is substituted with L and/or A at the last position is substituted with N. In another exemplary embodiment, the serum albumin binding domain comprises the amino acid sequence of any one of SEQ ID NOs: 39, 40 and 42, and wherein optionally A at the second last position is substituted with L and/or A at the last position is substituted with N. The sequences may optionally comprise at its N-terminus, a G, an S, or a GS (see below).

[113] In addition, the serum albumin binding domain may optionally further comprise a "G," an "S," or a "GS" sequence at its N-terminus (see, e.g., SEQ ID NO: 1 as compared with SEQ ID NO: 42). Accordingly, in some embodiments, the serum albumin binding domain provided herein (i) comprises an amino acid sequence that is at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% identical to SEQ ID NO: 42, and (ii) further comprises at its N-terminus, a G, an S, or a GS. In an exemplary embodiment, the serum albumin binding domain comprises an amino acid sequence that is at least 90% identical to SEQ ID NO: 42, and further comprises at its N-terminus, a G, an S, or a GS. In an exemplary embodiment, the serum albumin binding domain comprises an amino acid sequence that is at least 95% identical to SEQ ID NO: 42, and further comprises at its N-terminus, a G, an S, or a GS. In an exemplary embodiment, the serum albumin binding domain comprises the amino acid sequence of SEQ ID NO: 42, and further comprises at its N-terminus, a G, an S, or a GS. Also accordingly, in some embodiments, the serum albumin binding domain provided herein comprises an amino acid sequence that is at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%,

or 100% identical to any one of SEQ ID NOs: 1, and 39-41, wherein G at position 1 and/or S at position 2 of any one of SEQ ID NOs: 1, and 39-41 are optionally missing. In an exemplary embodiment, the serum albumin binding domain comprises an amino acid sequence that is at least 90% identical to any one of SEQ ID NOs: 1, and 39-41, wherein G at position 1 and/or S at position 2 of any one of SEQ ID NOs: 1, and 39-41 are optionally missing.

[114] Accordingly, in one particular preferred embodiment, the serum albumin binding domain described herein comprises the amino acid sequence of SEQ ID NO: 1, wherein G at position 1 and/or S at position 2 are optionally missing, and wherein optionally A at the second last position is substituted with L and/or A at the last position is substituted with N.

[115] In certain embodiments, the affinity between the serum albumin binding domain or the recombinant protein comprising the serum albumin binding domain and its target (i.e., serum albumin) is described in terms of K_D . In exemplary embodiments, the K_D is about 10^{-1} M or less, about 10^{-2} M or less, about 10^{-3} M or less, about 10^{-4} M or less, about 10^{-5} M or less, about 10^{-6} M or less, about 10^{-7} M or less, about 10^{-8} M or less, about 10^{-9} M or less, about 10^{-10} M or less, about 10^{-11} M or less, about 10^{-12} M or less, about 10^{-13} M or less, about 10^{-14} M or less, from about 10^{-5} M to about 10^{-15} M, from about 10^{-6} M to about 10^{-15} M, from about 10^{-7} M to about 10^{-15} M, from about 10^{-8} M to about 10^{-15} M, from about 10^{-9} M to about 10^{-15} M, from about 10^{-10} M to about 10^{-15} M, from about 10^{-5} M to about 10^{-14} M, from about 10^{-6} M to about 10^{-14} M, from about 10^{-7} M to about 10^{-14} M, from about 10^{-8} M to about 10^{-14} M, from about 10^{-9} M to about 10^{-14} M, from about 10^{-10} M to about 10^{-14} M, from about 10^{-5} M to about 10^{-13} M, from about 10^{-6} M to about 10^{-13} M, from about 10^{-7} M to about 10^{-13} M, from about 10^{-8} M to about 10^{-13} M, from about 10^{-9} M to about 10^{-13} M, or from about 10^{-10} M to about 10^{-13} M.

[116] In exemplary embodiments, the serum albumin binding domain binds serum albumin with an K_D value of, or below: about 100nM, about 90 nM, about 80 nM, about 75nM, about 60 nM, about 50 nM, about 40 nM, about 30 nM, about 20 nM, about 10 nM, about 5 nM, about 2 nM, about 1 nM, about 900 pM, about 800 pM, about 700 pM, about 600 pM, about 500 pM, about 400 pM, about 300 pM, about 250 pM, about 200 pM, about 150 pM, about 100 pM, about 50 pM, about 40 pM, about 30 pM, about 25 pM, about 20 pM, about 15 pM, about 10 pM, about 5 pM, or about 1 pM, preferably with a K_D value of or below: 100 nM, 90 nM, 80 nM, 75 nM, 70 nM, 60 nM, 50 nM, 40 nM, or 35 nM. In one exemplary embodiment, the serum albumin binding domain binds serum albumin with a K_D value of or below about 100 nM. In another exemplary embodiment, the serum albumin binding domain binds serum with a K_D value of or below about 50 nM. In one preferred embodiment, the serum albumin binding domain binds serum albumin with a K_D value of or below about 35 pM. Preferably, the serum albumin binding domain has the amino acid sequence of SEQ ID NO: 1.

[117] In exemplary embodiments, the recombinant protein comprising the serum albumin domain binds serum albumin with an K_D value of, or below: about 100nM, about 90 nM, about 80 nM, about 75nM, about 60 nM, about 50 nM, about 40 nM, about 30 nM, about 20 nM, about 10 nM, about 5 nM, about 2 nM, about 1 nM, about 900 pM, about 800 pM, about 700 pM, about 600 pM, about 500 pM, about 400 pM, about 300 pM, about 250 pM, about 200 pM, about 150 pM, about 100 pM, about 50 pM, about 40 pM, about 30 pM, about 25 pM, about 20 pM, about 15 pM, about 10 pM, about 5 pM, or about 1

pM, preferably with a K_D value of or below: 100 nM, 90 nM, 80 nM, 75 nM, 70 nM, 60 nM, or 50 nM. In one exemplary embodiment, the recombinant protein binds serum albumin with a K_D value of or below about 100 nM. In another exemplary embodiment, the recombinant protein binds serum albumin with a K_D value of or below about 75 nM. In one preferred embodiment, the recombinant protein binds serum albumin with a K_D value of or below about 50 nM. Preferably, the recombinant protein has the amino acid sequence of SEQ ID NO: 5.

[118] In certain embodiments, the serum albumin is human serum albumin (SEQ ID NO: 53).

[119] In some embodiments, the half-life extending moiety comprises an immunoglobulin domain. In some embodiments, the immunoglobulin domain comprises an Fc domain. In some embodiments, the Fc domain is derived from any one of the known heavy chain isotypes: IgG (γ), IgM (μ), IgD (δ), IgE (ϵ), or IgA (α). In some embodiments, the Fc domain is derived from any one of the known heavy chain isotypes or subtypes: IgG₁ (γ 1), IgG₂ (γ 2), IgG₃ (γ 3), IgG₄ (γ 4), IgA₁ (α 1), IgA₂ (α 2). In some embodiments, the Fc domain is the Fc domain of human IgG₁.

[120] In some embodiments, the Fc domain comprises an uninterrupted native sequence (i.e., wild type sequence) of an Fc domain. In some embodiments, the immunoglobulin Fc domain comprises a variant Fc domain resulting in altered biological activity. For example, at least one point mutation or deletion may be introduced into the Fc domain so as to reduce or eliminate the effector activity (e.g., International Patent Publication No. WO 2005/063815), and/or to increase the homogeneity during the production of the recombinant protein. In some embodiments, the Fc domain is the Fc domain of human IgG₁ and comprises one or more of the following effector-null substitutions: L234A, L235A, and G237A (Eu numbering). In some embodiments, the Fc domain does not comprise the lysine located at the C-terminal position of human IgG₁ (i.e., K447 by Eu numbering). The absence of the lysine may increase homogeneity during the production of the recombinant protein. In some embodiments, the Fc domain comprises the lysine located at the C-terminal position (K447, Eu numbering).

3.6. LINKERS

[121] The recombinant proteins described herein may comprise a linker. A "linker" is a molecule or group of molecules that binds two separate entities (e.g., FAP-binding domain and CD40 binding domain) to one another and can provide spacing and flexibility between the two entities such that they are able to achieve a conformation in which they, e.g., specifically bind their respective targets (e.g., FAP and CD40). Protein linkers are particularly preferred, and they may be expressed as a component of the recombinant protein using standard recombinant DNA techniques well-known in the art.

[122] The ankyrin repeat domains can be linked either covalently, for example, by a disulfide bond, a polypeptide bond or a crosslinking agent; or non-covalently, to produce a heterodimeric protein. The recombinant protein can comprise a linker between any of the binding domains, including FAP, and CD40 binding domains, and between any binding domain and the optional half-life extending moiety (which itself also can be a binding domain).

[123] In some embodiments, the linker is a peptidyl linker. In some embodiments, the peptidyl linker comprises about 1 to 30 amino acid residues. Exemplary linkers includes, e.g., a glycine rich peptide; a peptide comprising glycine and serine; a peptide having a sequence [Gly-Gly-Ser]_n, wherein n is 1, 2, 3, 4, 5, or 6; or a peptide having a sequence [Gly-Gly-Gly-Gly-Ser]_n (SEQ ID NO: 56), wherein n is 1, 2, 3, 4, 5, or 6. A glycine rich peptide linker comprises a peptide linker, wherein at least 25% of the residues are glycine. Glycine rich peptide linkers are well known in the art (e.g., Chichili et al. Protein Sci. 2013 February; 22(2): 153-167).

[124] In some embodiments, the peptidyl linker is a proline-threonine rich peptide linker. In an exemplary embodiment, the linker is the proline-threonine rich peptide linker of SEQ ID NO: 4.

[125] In some embodiments, the linker comprises the amino acid sequence of SEQ ID NO: 4.

3.7. FAP/CD40 DUAL TARGETING BISPECIFIC OR MULTISPECIFIC MOLECULES

[126] The multispecific molecules of the invention comprise any kind of combinations of the binding domains and optionally linkers described herein. That is, any one of the domains and linkers described in sections 3.3 to 3.6 above can be combined in the multispecific molecules of the invention. Furthermore, the binding domains of the multispecific molecules of the invention may comprise any of the N-terminal capping modules and/or C-terminal capping modules described in section 3.2 above.

[127] In some embodiments, the recombinant protein of the invention comprises, from the N-terminus to the C-terminus: (i) a first ankyrin repeat domain that specifically binds serum albumin, (ii) a second ankyrin repeat domain that specifically binds FAP, (iii) a third ankyrin repeat domain that specifically binds CD40, and (iv) a fourth ankyrin repeat domain that specifically binds CD40. Said first ankyrin repeat domain may be any one of the serum albumin binding domains as described in section 3.5 above, said second ankyrin repeat domain may be any one of the FAP binding domains as described in section 3.3 above, and said third and fourth ankyrin repeats domains may be any one of the CD40 binding domains as described in section 3.4 above. The third and fourth ankyrin repeat domains may have identical sequences, or may have different sequences.

[128] In some preferred embodiments, the multispecific recombinant protein of the invention comprises, from the N-terminus to the C-terminus: (serum albumin binding domain) – (linker) – (FAP binding domain) – (linker) – (CD40 binding domain) – (linker) – (CD40 binding domain), wherein the linker preferably comprises the amino sequence of SEQ ID NO: 4.

[129] In certain embodiments, the recombinant protein of the invention comprises an amino acid sequence that is at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% identical to SEQ ID NO: 5. In one preferred embodiment, the recombinant protein of the invention comprises an amino acid sequence that is at least 90% identical to SEQ ID NO: 5. In one more preferred

embodiment, the recombinant protein of the invention comprises the amino acid sequence of SEQ ID NO: 5.

[130] In certain embodiments, no more than 10, no more than 9, no more than 8, no more than 7, no more than 6, no more than 5, no more than 4, no more than 3, no more than 2, or no more than 1 substitution is made relative to any one of the four binding domains of SEQ ID NO: 5. In some embodiments, no more than 10 substitutions are made relative any one of the four binding domains of SEQ ID NO: 5. In some embodiments, no more than 5 substitutions are made relative to any one of the four binding domains of SEQ ID NO: 5. In some embodiments, no more than 4 substitutions are made relative to any one of the four binding domains of SEQ ID NO: 5. In some embodiments, no more than 3 substitutions are made relative any one of the four binding domains of SEQ ID NO: 5. In some embodiments, no more than 2 substitutions are made relative to any one of the four binding domains of SEQ ID NO: 5. In some embodiments, no more than 1 substitution is made relative to any one of the four binding domains of SEQ ID NO: 5. In some embodiments, the substitution(s) do not change the K_D value for FAP-binding or CD40 binding or serum albumin-binding by more than 1000-fold, more than 100-fold, or more than 10-fold, compared to the K_D value of the protein comprising the sequence of SEQ ID NO: 5. In certain embodiments, the substitution is a conservative substitution according to Table 1.

[131] In certain embodiments, the recombinant protein of the invention comprises an amino acid sequence that is at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% identical to SEQ ID NO: 6.

[132] In certain embodiments, the multispecific recombinant protein of the invention binds human CD40 in PBS with a dissociation constant (K_D) below $10^{-8}M$, below $10^{-9}M$, below $5 \times 10^{-10}M$, below $3 \times 10^{-10}M$, or below $2 \times 10^{-10}M$. In one preferred embodiment, the recombinant protein of the invention binds human CD40 in PBS with a dissociation constant (K_D) below $10^{-9}M$.

[133] In certain embodiments, the multispecific recombinant protein of the invention binds human FAP in PBS with a dissociation constant (K_D) below $10^{-8}M$, below $10^{-9}M$, below $5 \times 10^{-10}M$, or below $3 \times 10^{-10}M$. In one preferred embodiment, the recombinant protein of the invention binds human FAP in PBS with a dissociation constant (K_D) below $10^{-9}M$.

[134] In certain embodiments, the multispecific recombinant protein of the invention binds human serum albumin in PBS with a dissociation constant (K_D) below $10^{-7}M$, below $7 \times 10^{-8}M$, or below $5 \times 10^{-8}M$. In one preferred embodiment, the recombinant protein of the invention binds human serum albumin in PBS with a dissociation constant (K_D) below $10^{-7}M$.

[135] In certain embodiments, the multispecific recombinant protein of the invention binds human CD40 in PBS with a dissociation constant (K_D) below $10^{-8}M$, below $10^{-9}M$, below $5 \times 10^{-10}M$, below $3 \times 10^{-10}M$, or below $2 \times 10^{-10}M$, and/or the recombinant protein binds human FAP in PBS with a dissociation

constant (K_D) below $10^{-8}M$, below $10^{-9}M$, below $5 \times 10^{-10}M$, or below $3 \times 10^{-10}M$. In one preferred embodiment, the recombinant protein of the invention binds human CD40 in PBS with a dissociation constant (K_D) below $10^{-9}M$ and the recombinant protein binds human FAP in PBS with a dissociation constant (K_D) below $10^{-9}M$.

[136] In certain embodiments, the recombinant protein of the invention binds human CD40 in PBS with a dissociation constant (K_D) below $10^{-8}M$, below $10^{-9}M$, below $5 \times 10^{-10}M$, below $3 \times 10^{-10}M$, or below $2 \times 10^{-10}M$, and/or the recombinant protein binds human FAP in PBS with a dissociation constant (K_D) below $10^{-8}M$, below $10^{-9}M$, below $5 \times 10^{-10}M$, or below $3 \times 10^{-10}M$, and/or the recombinant protein binds human serum albumin in PBS with a dissociation constant (K_D) below $10^{-7}M$, below $7 \times 10^{-8}M$, or below $5 \times 10^{-8}M$. In one preferred embodiment, the recombinant protein of the invention binds human CD40 in PBS with a dissociation constant (K_D) below $10^{-9}M$ and the recombinant protein binds human FAP in PBS with a dissociation constant (K_D) below $10^{-9}M$. In one more preferred embodiment, the recombinant protein of the invention binds human CD40 in PBS with a dissociation constant (K_D) below $10^{-9}M$ and the recombinant protein binds human FAP in PBS with a dissociation constant (K_D) below $10^{-9}M$ and the recombinant protein binds human serum albumin in PBS with a dissociation constant (K_D) below $10^{-7}M$.

[137] In certain preferred embodiments, the recombinant binding protein of the invention is capable of binding FAP, CD40 and serum albumin simultaneously, wherein preferably said simultaneous binding is measured by surface plasmon resonance (SPR), further preferably as described in Example 3.

[138] In certain embodiments, the multispecific recombinant protein of the invention induces activation of B cells upon binding to FAP and CD40. In certain embodiments, the B cells are human B cells. In certain embodiments, the biological activity of the multispecific recombinant protein is assessed by an in vitro B cell activation assay that measures the expression of co-stimulatory molecules, such as CD86 and CD69. It has been reported that increased expression of these co-stimulatory molecules (CD86 and CD69) in B cells is indicative of CD40 activation.

[139] In certain embodiments, the multispecific recombinant protein of the invention activates human CD40 in CD40-expressing B cells in the presence of FAP-expressing CHO cells with an EC_{50} value of about $10^{-8}M$ or less, or about $10^{-9}M$ or less.

[140] In certain embodiments, the multispecific recombinant protein has a half maximal effective concentration (EC_{50}) of no more than about 100 nM, no more than about 75 nM, no more than about 65 nM, no more than about 55 nM, no more than about 45 nM, no more than about 35 nM, no more than about 25 nM, no more than about 15 nM, no more than about 10 nM, no more than about 5 nM, no more than about 4 nM, no more than about 3 nM, no more than about 2 nM, no more than about 1 nM, nor more than about 0.1 nM, from about 0.01 nM to about 50 nM, from about 0.01 nM to about 25 nM, from about 0.01 nM to about 10 nM, from about 0.01 nM to about 5 nM, from about 0.01 nM to about 1 nM, from about 0.01 nM to about 0.1 nM, from about 0.01 nM to about 0.07 nM, from about 0.04 nM to about 50 nM, from about 0.04 nM to about 25 nM, from about 0.04 nM to about 10 nM, from about 0.04 nM to about 5 nM, from about 0.04 nM to about 1 nM, from about 0.04 nM to about 0.1 nM,

from about 0.04 nM to about 0.07 nM, from about 0.1 nM to about 50 nM, from about 0.1 nM to about 25 nM, from about 0.1 nM to about 10 nM, from about 0.1 nM to about 5 nM, from about 0.1 nM to about 1 nM, from about 0.1 nM to about 0.9 nM, from about 0.1 nM to about 0.85 nM, from about 0.18 nM to about 0.85 nM, as assessed by an in vitro B cell activation assay.

[141] In an exemplary embodiment, the multispecific recombinant protein has an EC₅₀ of no more than about 10 nM, as assessed by an in vitro B cell activation assay. In another exemplary embodiment, the multispecific recombinant protein has an EC₅₀ of no more than about 1 nM, as assessed by an in vitro B cell activation assay. In another exemplary embodiment, the multispecific recombinant protein has an EC₅₀ of from about 0.01 nM to about 10 nM, preferably from about 0.1 nM to about 1.0 nM or from about 0.18 nM to about 0.85 nM, as assessed by an in vitro B cell activation assay. In another exemplary embodiment, the multispecific recombinant protein has an EC₅₀ of from about 0.01 nM to about 0.1 nM, preferably from about 0.04 nM to about 0.07 nM, as assessed by an in vitro B cell activation assay.

[142] In certain embodiments, the B cell activation assay is a human B cell activation assay. In an exemplary embodiment, EC₅₀ is measured using GraphPad Prism (version 8.1.2), according to the manufacturer's instructions. In an exemplary embodiment, EC₅₀ value is determined by fitting the data with the four-parameter logistical fit model using Graphpad Prism software. In an exemplary embodiment, EC₅₀ value is determined using the method described in the Examples.

[143] In certain embodiments, the multispecific recombinant protein has a terminal half-life in a mouse model of at least 10 hours, at least 20 hours, at least 30 hours, at least 40 hours, or about 44 hours. In certain embodiments, the multispecific recombinant protein has a terminal half-life in a cynomolgus monkey model of at least 1 day, at least 2 days, at least 3 days, at least 4 days, or about 2.8 days, or about 4.5 days.

[144] In certain embodiments, the multispecific recombinant protein of the invention is capable of inhibiting tumor growth in a FAP-expressing MC38 colon carcinoma mouse model. In one embodiment, the recombinant protein of the invention is capable of inhibiting tumor growth in a FAP-expressing MC38 colon carcinoma mouse model with treatment conditions as described in Example 6.

[145] In certain embodiments, the multispecific recombinant protein does not inhibit FAP protease activity. In certain embodiments, in the presence of the multispecific recombinant protein, FAP protease activity is reduced by no more than 25%, by no more than 20%, by no more than 15%, by no more than 10%, no more than 9%, no more than 8%, no more than 7%, no more than 6%, no more than 5%, no more than 4%, no more than 3%, or no more than 2%, as compared to a control (the control can be the FAP protease activity in the absence of the multispecific recombinant protein). In an exemplary embodiment, the FAP activity is measured using the method as exemplified in Example 7.

[146] In certain embodiments, the multispecific recombinant protein of the invention comprises two CD40 binding domains, wherein one of said CD40 binding domains or each of said CD40 binding domains independently comprises Q at position 8, L at position 15, R at position 143, and/or Q at position 147, wherein the position numbers correspond to the positions in SEQ ID NO: 3. In one

preferred embodiment, the multispecific recombinant protein of the invention comprises two CD40 binding domains, wherein each of said two CD40 binding domains comprises Q at position 8, L at position 15, R at position 143, and Q at position 147, wherein the position numbers correspond to the positions in SEQ ID NO: 3.

[147] In one embodiment, the multispecific recombinant protein of the invention comprises an amino acid sequence that is at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identical to SEQ ID NO: 5 and further has any one or any combination of the following properties: (i) the recombinant protein binds human CD40 in PBS with a dissociation constant (K_D) below $10^{-8}M$, below $10^{-9}M$, below $5 \times 10^{-10}M$, below $3 \times 10^{-10}M$, or below $2 \times 10^{-10}M$; (ii) the recombinant protein binds human FAP in PBS with a dissociation constant (K_D) below $10^{-8}M$, below $10^{-9}M$, below $5 \times 10^{-10}M$, or below $3 \times 10^{-10}M$; (iii) the recombinant protein binds human serum albumin in PBS with a dissociation constant (K_D) below $10^{-7}M$, below $7 \times 10^{-8}M$, or below $5 \times 10^{-8}M$; (iv) the recombinant protein activates human CD40 in CD40-expressing B cells in the presence of FAP-expressing CHO cells with an EC₅₀ value of about $10^{-8}M$ or less, or about $10^{-9}M$ or less; (v) the recombinant binding protein is capable of binding FAP, CD40 and serum albumin simultaneously; (vi) the recombinant protein does not inhibit FAP protease activity, or the reduction in FAP protease activity in the presence of the recombinant protein is no more than 25%, no more than 20%, no more than 15%, or no more than 10%; (vii) the recombinant protein has a terminal half-life in a mouse model of at least 10 hours, at least 20 hours, at least 30 hours, at least 40 hours, or about 44 hours; (viii) the recombinant protein has a terminal half-life in a cynomolgus monkey model of at least 1 day, at least 2 days, at least 3 days, at least 4 days, or about 2.8 days, or about 4.5 days; (ix) the recombinant protein is capable of inhibiting tumor growth in a FAP-expressing MC38 colon carcinoma mouse model; and (x) one of the two CD40 binding domains or each of said two CD40 binding domains independently comprises Q at position 8, L at position 15, R at position 143, and/or Q at position 147, wherein the position numbers correspond to the positions in SEQ ID NO: 3, or more preferably each of said two CD40 binding domains comprises Q at position 8, L at position 15, R at position 143, and Q at position 147, wherein the position numbers correspond to the positions in SEQ ID NO: 3.

[148] In certain embodiments, the multispecific recombinant protein of the invention comprises an amino acid sequence that is at least 90% identical to SEQ ID NO: 5, wherein said recombinant protein binds human CD40 in PBS with a dissociation constant (K_D) below $10^{-9}M$, and/or binds human FAP in PBS with a dissociation constant (K_D) below $10^{-9}M$.

[149] In certain embodiments, the multispecific recombinant protein of the invention comprises an amino acid sequence that is at least 90% identical to SEQ ID NO: 5, wherein said recombinant protein binds human CD40 in PBS with a dissociation constant (K_D) below $10^{-9}M$, binds human FAP in PBS with a dissociation constant (K_D) below $10^{-9}M$, and binds human serum albumin in PBS with a dissociation constant (K_D) below $10^{-7}M$.

[150] In certain embodiments, the multispecific recombinant protein of the invention comprises an amino acid sequence that is at least 90% identical to SEQ ID NO: 5 and activates human CD40 in

CD40-expressing B cells in the presence of FAP-expressing CHO cells with an EC₅₀ value of about 10⁻⁸M or less, or about 10⁻⁹M or less.

[151] In certain embodiments, the multispecific recombinant protein of the invention comprises an amino acid sequence that is at least 90% identical to SEQ ID NO: 5 and is capable of binding simultaneously to human CD40, human FAP and human serum albumin; wherein preferably said simultaneous binding is measured by surface plasmon resonance (SPR), further preferably as described in Example 3.

[152] In certain embodiments, the multispecific recombinant protein of the invention comprises an amino acid sequence that is at least 90% identical to SEQ ID NO: 5, wherein said recombinant protein does not inhibit FAP protease activity, or the reduction in FAP protease activity in the presence of the recombinant protein is no more than 25%, no more than 20%, no more than 15%, or no more than 10%.

[153] In certain embodiments, the multispecific recombinant protein of the invention comprises an amino acid sequence that is at least 90% identical to SEQ ID NO: 5 and has a terminal half-life in a mouse model of at least 10 hours, at least 20 hours, at least 30 hours, at least 40 hours, or about 44 hours.

[154] In certain embodiments, the multispecific recombinant protein of the invention comprises an amino acid sequence that is at least 90% identical to SEQ ID NO: 5 and has a terminal half-life in a cynomolgus monkey model of at least 1 day, at least 2 days, at least 3 days, at least 4 days, or about 2.8 days, or about 4.5 days.

[155] In certain embodiments, the multispecific recombinant protein of the invention comprises an amino acid sequence that is at least 90% identical to SEQ ID NO: 5, wherein said recombinant protein is capable of inhibiting tumor growth in a FAP-expressing MC38 colon carcinoma mouse model; wherein preferably said tumor inhibition is measured as described in Example 6.

[156] In certain embodiments, the multispecific recombinant protein of the invention comprises an amino acid sequence that is at least 90% identical to SEQ ID NO: 5, wherein one of the two CD40 binding domains or each of said two CD40 binding domains independently comprises Q at position 8, L at position 15, R at position 143, and/or Q at position 147, wherein the position numbers correspond to the positions in SEQ ID NO: 3. In certain preferred embodiments, the multispecific recombinant protein of the invention comprises an amino acid sequence that is at least 90% identical to SEQ ID NO: 5, wherein each of said two CD40 binding domains comprises Q at position 8, L at position 15, R at position 143, and Q at position 147, wherein the position numbers correspond to the positions in SEQ ID NO: 3.

[157] In one embodiment, the recombinant protein of the invention comprises an amino acid sequence that is at least 90% identical to SEQ ID NO: 5, wherein said recombinant protein binds human FAP, human CD40, and human serum albumin with a K_D value of or below 100 nM, and wherein said

recombinant protein has a terminal half-life in a cynomolgus monkey model of at least 1 day, at least 2 days, at least 3 days, at least 4 days, or about 2.8 days, or about 4.5 days.

[158] In one embodiment, the recombinant protein of the invention comprises an amino acid sequence that is at least 90% identical to SEQ ID NO: 5, wherein said recombinant protein binds human FAP, human CD40, and human serum albumin with a K_D value of or below 100 nM, and wherein in the presence of said recombinant protein, FAP protease activity is reduced by no more than 25%, no more than 20%, no more than 15%, no more than 10%, no more than 9%, no more than 8%, no more than 7%, no more than 6%, no more than 5%, no more than 4%, no more than 3%, or no more than 2%, as compared to a control, wherein typically and preferably said control is the FAP protease activity in the absence of said recombinant protein, and wherein further typically and preferably said FAP protease activity is measured as described in Example 7.

[159] In one embodiment, the recombinant protein of the invention comprises an amino acid sequence that is at least 90% identical to SEQ ID NO: 5, wherein said recombinant protein binds human FAP, human CD40, and human serum albumin with a K_D value of or below 100 nM, and wherein said recombinant protein has a terminal half-life in a cynomolgus monkey model of at least 1 day, at least 2 days, at least 3 days, at least 4 days, or about 2.8 days, or about 4.5 days, and wherein in the presence of said recombinant protein, FAP protease activity is reduced by no more than 25%, no more than 20%, no more than 15%, no more than 10%, no more than 9%, no more than 8%, no more than 7%, no more than 6%, no more than 5%, no more than 4%, no more than 3%, or no more than 2%, as compared to a control, wherein typically and preferably said control is the FAP protease activity in the absence of said recombinant protein, and wherein further typically and preferably said FAP protease activity is measured as described in Example 7.

[160] In certain embodiments, the multispecific recombinant protein of the invention comprises an amino acid sequence that is at least 90% identical to SEQ ID NO: 5; wherein said protein binds human CD40 in PBS with a dissociation constant (K_D) below 10^{-9} M; wherein said protein binds human FAP in PBS with a dissociation constant (K_D) below 10^{-9} M; wherein said protein activates human CD40 in CD40-expressing B cells in the presence of FAP-expressing CHO cells with an EC_{50} value of about 10^{-8} M or less; wherein said protein is capable of binding FAP and CD40 simultaneously; wherein said protein does not inhibit FAP protease activity, or the reduction in FAP protease activity in the presence of the recombinant protein is no more than 25%; wherein said protein has a terminal half-life in a mouse model of at least 10 hours.

In certain embodiments, the recombinant protein described here comprises a first ankyrin repeat domain that specifically binds serum albumin, a second ankyrin repeat domain that specifically binds fibroblast activation protein (FAP), a third ankyrin repeat domain that specifically binds CD40, and a fourth ankyrin repeat domain that specifically binds CD40, wherein said ankyrin repeat domains are arranged, from the N-terminus to C-terminus, according to the following formula: (serum albumin binding domain) – (linker) – (FAP-binding domain) – (linker) – (CD40 binding domain) – (linker) – (CD40 binding domain), and wherein said recombinant protein specifically binds human FAP in PBS with a dissociation constant (K_D) below 10^{-9} M, and wherein said recombinant protein specifically binds human

CD40 in PBS with a dissociation constant (K_D) below 10^{-6} M, and wherein said recombinant protein specifically binds human serum albumin in PBS with a dissociation constant (K_D) below 10^{-7} M, and wherein said FAP binding domain is any one of the FAP binding domains described in section 3.3, and wherein each of said two CD40 binding domains are independently any one of the CD40 binding domains described in section 3.4, and wherein said serum albumin binding domain is any one of the serum albumin binding domains described in section 3.5, and wherein the linker is any one of the linkers described in section 3.6. In certain embodiments, said multispecific recombinant protein is capable of simultaneously binding to human CD40, human FAP and human serum albumin; wherein preferably said simultaneous binding is measured by surface plasmon resonance (SPR), further preferably as described in Example 3. In certain embodiments, the recombinant protein activates human CD40 in CD40-expressing B cells in the presence of FAP-expressing CHO cells with an EC50 value of about 10^{-8} M or less. In certain embodiments, said multispecific recombinant protein does not inhibit FAP protease activity, or the reduction in FAP protease activity in the presence of the recombinant protein is no more than 25%. In certain embodiments, said multispecific recombinant protein has a terminal half-life in a mouse model of at least 20 hours. In certain embodiments, said multispecific recombinant protein said protein has a terminal half-life in a cynomolgus monkey model of at least 3 days. In certain embodiments, said multispecific recombinant protein is capable of inhibiting tumor growth in a FAP-expressing MC38 colon carcinoma mouse model; wherein said tumor growth inhibition is preferably measured as described in Example 6. In certain embodiments, one of said two CD40 binding domains or each of said two CD40 binding domains of said multispecific recombinant protein independently comprises Q at position 8, L at position 15, R at position 143, and/or Q at position 147, wherein the position numbers correspond to the positions in SEQ ID NO: 3. In certain embodiments, each of said two CD40 binding domains of said multispecific recombinant protein comprises Q at position 8, L at position 15, R at position 143, and Q at position 147, wherein the position numbers correspond to the positions in SEQ ID NO: 3. In certain embodiments, said multispecific recombinant protein comprises an amino acid sequence that is at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identical to SEQ ID NO: 5. In certain embodiments, said multispecific recombinant protein comprises an amino acid sequence that is at least 90% identical to SEQ ID NO: 5. In certain embodiments, said multispecific recombinant protein comprises a polypeptide, wherein said polypeptide has an amino acid sequence that is at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identical to SEQ ID NO: 5. In certain embodiments, said multispecific recombinant protein comprises a polypeptide, wherein said polypeptide has an amino acid sequence that is at least 90% identical to SEQ ID NO: 5.

3.8. NUCLEIC ACIDS AND METHODS OF PRODUCING MULTISPECIFIC PROTEINS

[161] The disclosure also provides polynucleotides encoding the recombinant proteins described herein. The disclosure also provides a method of producing any of the polynucleotides described herein. The disclosure also provides recombinant proteins obtained by said method. Polynucleotides can be generated and expressed by procedures known in the art.

[162] In one aspect, the disclosure provides polynucleotides or compositions comprising polynucleotides encoding a recombinant multispecific protein, wherein said protein comprises a first ankyrin repeat domain that specifically binds fibroblast activation protein (FAP), and a second ankyrin repeat domain that specifically binds CD40, and optionally, a half-life extending moiety.

[163] In one aspect, the disclosure provides polynucleotides or compositions comprising polynucleotides comprising a nucleic acid sequence encoding a recombinant protein comprising SEQ ID NO: 1, 2, 3, and/or 4. In one aspect, the disclosure provides polynucleotides or compositions comprising polynucleotides comprising a nucleic acid sequence encoding a recombinant protein comprising SEQ ID NO: 5 or 6. In one embodiment, the disclosure provides a nucleic acid comprising the nucleic acid sequence of SEQ ID NO: 58.

[164] In another aspect, the disclosure provides polynucleotides and variants thereof encoding a recombinant protein, wherein such variant polynucleotides share at least 70%, at least 75%, at least 80%, at least 85%, at least 87%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% sequence identity to any nucleic acid disclosed herein, such as a nucleic acid comprising the nucleic acid sequence of SEQ ID NO: 58.

[165] In another aspect, the disclosure provides polynucleotides and variants thereof encoding a recombinant protein, wherein such variant polynucleotides are capable of hybridizing under highly stringent conditions to the sequence of SEQ ID NO: 58. "Highly stringent conditions" includes those that: (1) employ low ionic strength and high temperature for washing, for example 0.015 M sodium chloride/0.0015 M sodium citrate/0.1% sodium dodecyl sulfate at 50° C; (2) employ a denaturing agent during hybridization, such as formamide, for example, 50% (v/v) formamide with 0.1% bovine serum albumin/0.1% Ficoll/0.1% polyvinylpyrrolidone/50 mM sodium phosphate buffer at pH 6.5 with 750 mM sodium chloride, 75 mM sodium citrate at 42°C; or (3) employ 50% formamide, 5×SSC (0.75 M NaCl, 0.075 M sodium citrate), 50 mM sodium phosphate (pH 6.8), 0.1% sodium pyrophosphate, 5×Denhardt's solution, sonicated salmon sperm DNA (50 pg/mL), 0.1% SDS, and 10% dextran sulfate at 42°C, with washes at 42°C in 0.2×SSC (sodium chloride/sodium citrate) and 50% formamide at 55°C, followed by a high-stringency wash consisting of 0.1×SSC containing EDTA at 55°C.

[166] Polynucleotides complementary to any such sequences are also encompassed by the present disclosure. Polynucleotides may be single-stranded (coding or antisense) or double-stranded, and may be DNA (recombinant, cDNA or synthetic) or RNA molecules. RNA molecules include hnRNA molecules, which contain introns and correspond to a DNA molecule in a one-to-one manner, and mRNA molecules, which do not contain introns. Additional coding or non-coding sequences may, but need not, be present within a polynucleotide of the present disclosure, and a polynucleotide may, but need not, be linked to other molecules and/or support materials.

[167] It will be appreciated by those of ordinary skill in the art that, as a result of the degeneracy of the genetic code, there are many nucleotide sequences that encode a recombinant protein (or its individual

domains) comprising an amino acid sequence as described herein. Some of these polynucleotides bear minimal homology to the nucleotide sequence of any native gene. Polynucleotides that vary due to differences in codon usage are specifically contemplated by the present disclosure.

[168] The present disclosure also includes codon-optimized polynucleotides wherein the nucleic acid sequence has been optimized to maximize expression in a particular cell. In general, codon optimization refers to a process of modifying a nucleic acid sequence for enhanced expression in the host cells of interest by replacing at least one codon (e.g., about or more than about 1, 2, 3, 4, 5, 10, 15, 20, 25, 50, or more codons) of the original sequence with codons that are more frequently or most frequently used in the genes of that host cell while maintaining the original amino acid sequence. Various species exhibit particular bias for certain codons of a particular amino acid. Codon bias (differences in codon usage between organisms) often correlates with the efficiency of translation of messenger RNA (mRNA), which is in turn believed to be dependent on, among other things, the properties of the codons being translated and the availability of particular transfer RNA (tRNA) molecules. The predominance of selected tRNAs in a cell is generally a reflection of the codons used most frequently in peptide synthesis. Accordingly, genes can be tailored for optimal gene expression in a given organism based on codon optimization. Codon usage tables are readily available, and these tables can be adapted in a number of ways (e.g., Nakamura, Y., et al. "Codon usage tabulated from the international DNA sequence databases: status for the year 2000" Nucl. Acids Res. 28:292 (2000)). Computer algorithms for codon optimizing a particular sequence for expression in a particular host cell are also available, such as Gene Forge (Aptagen; Jacobus, Pa.), are also available. In some embodiments, one or more codons (e.g., 1, 2, 3, 4, 5, 10, 15, 20, 25, 50, or more, or all codons) in a sequence encoding a recombinant protein correspond to the most frequently used codon for a particular amino acid.

[169] Suitable cloning vectors may be constructed according to standard techniques, or may be selected from a large number of cloning vectors available in the art. While the cloning vector selected may vary according to the host cell intended to be used, useful cloning vectors will generally have the ability to self-replicate, may possess a single target for a particular restriction endonuclease, and/or may carry genes for a marker that can be used in selecting clones containing the vector. Suitable examples include plasmids and bacterial viruses, e.g., pUC18, pUC19, Bluescript (e.g., pBS SK+) and its derivatives, mp18, mp19, pBR322, pMB9, ColE1, pCR1, RP4, phage DNAs, and shuttle vectors such as pSA3 and pAT28. These and many other cloning vectors are available from commercial vendors such as BioRad, Stratagene, and Invitrogen.

[170] Expression vectors are further provided. Expression vectors generally are replicable polynucleotide constructs that contain a polynucleotide according to the disclosure. It is implied that an expression vector must be replicable in the host cells either as episomes or as an integral part of the chromosomal DNA. Suitable expression vectors include but are not limited to plasmids, viral vectors, including adenoviruses, adeno-associated viruses, retroviruses, cosmids, and expression vector(s) disclosed in PCT Publication No. WO 87/04462. Vector components may generally include, but are not limited to, one or more of the following: a signal sequence; an origin of replication; one or more marker

genes; suitable transcriptional controlling elements (such as promoters, enhancers and terminator). For expression (i.e., translation), one or more translational controlling elements are also usually required, such as ribosome binding sites, translation initiation sites, and stop codons.

[171] The vectors containing the polynucleotides of interest and/or the polynucleotides themselves, can be introduced into the host cell by any of a number of appropriate means, including electroporation, transfection employing calcium chloride, rubidium chloride, calcium phosphate, DEAE-dextran, or other substances; microprojectile bombardment; lipofection; and infection (e.g., where the vector is an infectious agent such as vaccinia virus). The choice of introducing vectors or polynucleotides will often depend on features of the host cell.

[172] Exemplary host cells include an *E. coli* cell, a yeast cell, an insect cell, a simian COS cell, a Chinese hamster ovary (CHO) cell, or a myeloma cell. Preferred host cells include an *E. coli* cell, a CHO cell, a Human embryonic kidney (HEK) 293 cell, or a Sp2.0 cell, among many cells well-known in the art.

4. METHODS OF TREATMENT

[173] The recombinant proteins described herein can be used to, e.g., treat a subject that has a medical condition such as, e.g., cancer.

[174] The disclosure provides a method of treating a medical condition, comprising administering to a subject in need thereof a therapeutically effective amount of a recombinant protein, a nucleic acid or a pharmaceutical composition described herein. In certain embodiments, the subject is a human. In preferred embodiments, the medical condition is cancer. In certain embodiments, the cancer is a solid tumor. In certain embodiments, the cancer cell expresses FAP. In certain embodiments, tumor stromal cells express FAP.

[175] In some embodiments, the cancer is brain cancer, bladder cancer, breast cancer, clear cell kidney cancer, cervical cancer, colon and rectal cancer, endometrial cancer, gastric cancer, head and neck cancer, head/neck squamous cell carcinoma, lip cancer, oral cancer, liver cancer, cervix cancer, lung squamous cell carcinoma, melanoma, mesothelioma, non-small-cell lung cancer (NSCLC), non-melanoma skin cancer, ovarian cancer, pancreatic cancer, prostate cancer, renal cell carcinoma, urothelial carcinoma, sarcoma, small-cell lung cancer (SCLC), Squamous Cell Carcinoma of the Head and Neck (SCCHN), triple negative breast cancer, or thyroid cancer.

[176] In some embodiments, the cancer is cancer is adrenocortical tumor, alveolar soft part sarcoma, carcinoma, chondrosarcoma, colorectal carcinoma, desmoid tumors, desmoplastic small round cell tumor, endocrine tumors, endodermal sinus tumor, epithelioid hemangioendothelioma, Ewing sarcoma, germ cell tumor, hepatoblastoma, hepatocellular carcinoma, melanoma, nephroma, neuroblastoma, non-rhabdomyosarcoma soft tissue sarcoma (NRSTS), osteosarcoma, paraspinal sarcoma, renal cell carcinoma, retinoblastoma, rhabdomyosarcoma, synovial sarcoma, or Wilms tumor.

[177] In some embodiments, the cancer is acute lymphoblastic leukemia (ALL), acute myeloid leukemia (AML), chronic lymphocytic leukemia (CLL), or chronic myeloid leukemia (CML).

[178] In some embodiments, the cancer is diffuse large B-cell lymphoma (DLBCL), follicular lymphoma, Hodgkin's lymphoma (HL), mantle cell lymphoma (MCL), multiple myeloma (MM), myelodysplastic syndrome (MDS), non-Hodgkin's lymphoma (NHL), or small lymphocytic lymphoma (SLL).

[179] Indeed, cancers that can be treated include, but are not limited to, alveolar rhabdomyosarcoma, bone cancer, cancer of the anus, anal canal, or anorectum, cancer of the eye, cancer of the intrahepatic bile duct, cancer of the joints, cancer of the neck, gallbladder, or pleura, cancer of the nose, nasal cavity, or middle ear, cancer of the oral cavity, cancer of the vulva, esophageal cancer, gastrointestinal carcinoid tumor, hypopharynx cancer, larynx cancer, nasopharynx cancer, peritoneum, omentum, and mesentery cancer, pharynx cancer, small intestine cancer, soft tissue cancer, stomach cancer, testicular cancer, ureter cancer, and urinary bladder cancer.

[180] In particular aspects, the cancer is selected from the group consisting of: head and neck, ovarian, cervical, bladder and oesophageal cancers, pancreatic, gastrointestinal cancer, gastric, breast, endometrial and colorectal cancers, hepatocellular carcinoma, glioblastoma, bladder, lung cancer, and bronchioloalveolar carcinoma.

[181] In certain embodiments, the cancer is non-small cell lung cancer (NSCLC), head and neck cancer, renal cancer, triple negative breast cancer, or gastric cancer. In certain embodiments, the cancer is non-small cell lung cancer (NSCLC), small cell lung cancer (SCLC), head and neck cancer, renal cancer, breast cancer, melanoma, ovarian cancer, liver cancer, pancreatic cancer, colon cancer, prostate cancer, gastric cancer, lymphoma or leukemia. In certain embodiments, the cancer is brain cancer.

[182] The recombinant proteins described herein may be used prior to or following surgery to remove a tumor and may be used prior to, during or after radiation therapy. The recombinant protein may be used to treat a tumor that is large enough to be found by palpation or by imaging techniques well known in the art, such as MRI, ultrasound, or CAT scan. In some embodiments, the recombinant protein is used to treat an advanced stage tumor having dimensions of at least about 200 mm³, 300 mm³, 400 mm³, 500 mm³, 750 mm³, or up to 1000 mm³.

5. PHARMACEUTICAL COMPOSITIONS AND ADMINISTRATION

[183] In another aspect, the disclosure also provides pharmaceutical compositions comprising the recombinant multispecific proteins described herein.

[184] The pharmaceutical compositions may comprise a pharmaceutically acceptable carrier, diluent, or excipient. Standard pharmaceutical carriers include a phosphate buffered saline solution, water, emulsions such as an oil/water or water/oil emulsion, and various types of wetting agents.

[185] The pharmaceutical compositions can comprise any pharmaceutically acceptable ingredients, including, for example, acidifying agents, additives, adsorbents, aerosol propellants, air displacement agents, alkalizing agents, anticaking agents, anticoagulants, antimicrobial preservatives, antioxidants, antiseptics, bases, binders, buffering agents, chelating agents, coating agents, coloring agents, desiccants, detergents, diluents, disinfectants, disintegrants, dispersing agents, dissolution enhancing agents, dyes, emollients, emulsifying agents, emulsion stabilizers, fillers, film forming agents, flavor enhancers, flavoring agents, flow enhancers, gelling agents, granulating agents, humectants, lubricants, mucoadhesives, ointment bases, ointments, oleaginous vehicles, organic bases, pastille bases, pigments, plasticizers, polishing agents, preservatives, sequestering agents, skin penetrants, solubilizing agents, solvents, stabilizing agents, suppository bases, surface active agents, surfactants, suspending agents, sweetening agents, therapeutic agents, thickening agents, tonicity agents, toxicity agents, viscosity-increasing agents, water-absorbing agents, water-miscible cosolvents, water softeners, or wetting agents. See, e.g., the *Handbook of Pharmaceutical Excipients*, Third Edition, A. H. Kibbe (Pharmaceutical Press, London, UK, 2000). *Remington's Pharmaceutical Sciences*, Sixteenth Edition, E. W. Martin (Mack Publishing Co., Easton, Pa., 1980).

[186] The pharmaceutical compositions can be formulated to achieve a physiologically compatible pH. In some embodiments, the pH of the pharmaceutical composition can be, for example, between about 4 or about 5 and about 8.0, or between about 4.5 and about 7.5, or between about 5.0 and about 7.5. In exemplary embodiments, the pH of the pharmaceutical composition is between 5.5 and 7.5.

[187] The recombinant multispecific proteins described herein can be administered to the subject via any suitable route of administration, such as parenteral, nasal, oral, pulmonary, topical, vaginal, or rectal administration. Formulations suitable for parenteral administration include aqueous and non-aqueous, isotonic sterile injection solutions, which can contain anti-oxidants, buffers, bacteriostats, and solutes that render the formulation isotonic with the blood of the intended recipient, and aqueous and non-aqueous sterile suspensions that can include suspending agents, solubilizers, thickening agents, stabilizers, and preservatives. For additional details, see *Pharmaceutics and Pharmacy Practice*, J. B. Lippincott Company, Philadelphia, PA, Banker and Chalmers, eds., pages 238-250 (1982), and *ASHP Handbook on Injectable Drugs*, Toissel, 4th ed., pages 622-630 (1986)).

[188] The dose of the active agent of the present disclosure administered over the course of a therapeutic regimen should be sufficient to treat cancer in a clinically-acceptable time frame (e.g., 1 to 4 weeks or longer (such as 5 to 20 or more weeks)) from the time of administration. In certain embodiments, the time period could be even longer. The dose will be determined by the efficacy of the particular active agent and the condition of the animal (e.g., human), as well as, at times, the body weight of the animal (e.g., human) to be treated. The extent to which cancer is treated upon administration of a certain dose can be represented by, for example, the cytotoxicity of the active agent or the extent of tumor regression achieved with the active agent. Methods of measuring cytotoxicity of the recombinant multispecific proteins and methods of assaying tumor regression are known in the art. By way of example and not intending to limit the present disclosure, the dose of the active agent of the

present disclosure can be about 0.0001 to about 1 g/kg body weight of the subject being treated/day, from about 0.0001 to about 0.001 g/kg body weight/day, or about 0.01 mg to about 1 g/kg body weight/day. Dosage units may be also expressed in rag/m^2 , which refer to the quantity in milligrams per square meter of body surface area.

[189] The recombinant multispecific proteins described herein may be used in combination with another therapeutic agent, such as another anti-cancer agent. Each therapeutic agent may be administered simultaneously (e.g., in the same medicament or at the same time), concurrently (i.e., in separate medicaments administered one right after the other in any order) or sequentially in any order. Sequential administration may be useful when the therapeutic agents in the combination therapy are in different dosage forms (e.g., one agent is a tablet or capsule and another agent is a sterile liquid) and/or are administered on different dosing schedules, e.g., a chemotherapeutic that is administered at least daily and a biotherapeutic that is administered less frequently, such as once weekly, once every two weeks, or once every three weeks.

EXAMPLES

Example 1 – Design of Multispecific Binding Proteins

[190] Multispecific binding proteins in various formats were generated and their FAP-specificity, efficacy and potency of CD40 activation were determined. These multispecific proteins all comprised a FAP-specific binding domain and a CD40-specific binding domain. The impact of (i) adding human serum albumin (HSA)-binding domain(s), (ii) increasing valency by adding further CD40-binding domain(s), and (iii) changing the order of the binding domains within the protein were evaluated.

[191] To compare the different formats, an *in vitro* assay was set up measuring the upregulation of the co-stimulatory receptor, CD86, expressed on human B cells upon CD40 triggering. This cellular assay used primary human B cells in the presence or absence of FAP-expressing cells. The upregulation of CD86 co-stimulatory molecule was evaluated as a marker of B cell activation. An anti-CD40 monoclonal antibody, whose mechanism of action is independent on FAP-mediated cross-linking, was used as reference material.

[192] *Multispecific proteins of different formats.* Starting with a parental molecule (SEQ ID NO: 59; SMA014) comprising one FAP-specific binding domain and one CD40-specific binding domain, several multispecific protein formats were generated, as summarized in **Table 2**.

Table 2. Multispecific proteins in various domain formats

SEQ ID NO / Construct Name	Format
SEQ ID NO: 59 / SMA014	FC
SEQ ID NO: 60 / SMA087	HFC
SEQ ID NO: 61 / SMA095	HFCH
SEQ ID NO: 62 / SMA104	FCC

SEQ ID NO: 63 / SMA091	HFCC
SEQ ID NO: 64 / SMA099	HFCH
SEQ ID NO: 65 / AS579	HHFCC
SEQ ID NO: 66 / SMA105	FCCC

[193] "C", "F", and "H" in Table 2 indicate ankyrin repeat domains specifically binding CD40, FAP and HSA, respectively. The order of the different domains as indicated in Table 2 reflects the actual sequence of the domains from N-terminus to C-terminus in the molecular structure of the proteins. All the proteins additionally had a His-tag (SEQ ID NO: 57) at the N-terminus for ease of purification.

Materials and Methods

As a reference, a CD40 monoclonal antibody was used. Binding of this CD40 mAb (an IgG2 mAb) to CD40 leads to the activation of antigen presenting cells independently of FAP. The anti-CD40 mAb corresponds to sequence 21.4.1 of US 7,338,660 B2.

CHO cells were cultured at 37°C, 5% CO₂ in DMEM media containing 10% FBS and splitted every 2-3 days using accutase to detach cells.

The FAP-expressing CHO cell line is a stably transfected clonal cell line expressing human FAP on the cell surface. A plasmid containing a GFP-fusion of the ORF of human FAP was obtained from OriGene Technologies (#RG204692). The cDNA coding for human FAP (without GFP) was sub-cloned using standard molecular biology techniques. This plasmid was then transfected into CHO cells to produce stable transfectants overexpressing human FAP using Lipofectamine. Selection pressure was applied using different concentrations of Geneticin G-418 (Promega, V8091). Expression of FAP was analyzed by flow cytometry using an anti-FAP antibody corresponding to ESC11 (WO2011/040972). The population of FAP-CHO transfectants from condition 1.9 mg/mL G-418 (FAP-CHO-1.9) showed a lower expression level of FAP and those from condition 1.7mg/mL (FAP-CHO-1.7) showed a higher expression level of FAP. The data in this Example were generated using FAP-CHO-1.7.

In vitro B cell activation assay. The design of the *in vitro* B cell activation assay is schematically shown in **Figure 1**. Buffy coats were obtained from the Zurich blood donation center and diluted with PBS. Peripheral blood mononuclear cells (PBMCs) were then isolated by density centrifugation using Leucosep tubes. After several washing steps, human CD19⁺ B cells were enriched from PBMCs using a positive selection (human CD19 MicroBeads Kit) according to the manufacturer's recommendations. CD19⁺ B cells at 1x10⁵/well and FAP-expressing CHO cells or CHO wildtype (WT-CHO) cells at 5x10⁴ cells/well were seeded together in RPMI 1640 media + 10% FBS with or without 600µM HSA into 96-well plates together with dose titrations (400, 200, 40, 8, 5, 1.6, 0.3, 0 nM) of the indicated molecules. Cultures were incubated for 24 hours at 37°C, 5% CO₂ and the upregulation of CD86 and CD69 on CD20⁺ B cells was assessed by flow cytometry using AttuneNXT.

FACS staining, flow cytometer settings and antibody dilutions. Cells were first washed with 150 µl PBS and then incubated for 20 minutes at room temperature (RT) with 100 µl of BD human Fc-Block diluted

(1:100) in PBS. After Fc-blocking incubation, the cells were incubated with 100 μ l of directly labelled antibodies diluted (see Table 3 below for the dilution factors) in FACS buffer and incubated for another 20 minutes at 4°C in the dark. Cells were washed with PBS, resuspended in 100 μ l Live/Dead staining diluted (1:1000) in PBS and incubated for 20 minutes at 4°C in the dark. One hundred μ l of FACS buffer containing FBS reaction was added to stop the Live/Dead staining reaction. Cells were washed again with PBS and fixed using BD Cell Fix solution diluted (1:10) in water according to the manufacturer's recommendations. Dilutions of antibodies and FACS settings are summarized in **Table 3** below. Compensation of the FACS machine was done with compensation beads according to the manufacturer's recommendations (ThermoFisher; AbC™ Total Antibody Compensation Bead Kit). Raw_fcs files were analyzed using FlowJo software (version 10.0.3). Cells were gated on live cells using Live-Dead discriminating dye followed by gating on CD20 positive cells as shown in **Figure 2** for CD86. The MFI and percentage of positive cells for CD86 were exported and plotted using GraphPad prism software, version 8.1.2.

Table 3

FSC: 200

SSC: 400

Acquisition: 200ul/min, 100.000 events

Target	Fluorochrome	Dilution	Dilution media	Channel	Voltages
CD20	APC-Cy7	1:100	FACS buffer	BL2	400
CD86	PE	1:200	FACS buffer	YL1	380
CD69	APC	1:200	FACS buffer	RL-1	400
Live/Dead	Aqua	1:1000	PBS	VL2	400

EC50 Determinations. EC50 values were determined using GraphPad Prism version 7.02 by converting the x values (concentrations) in a log mode and fitting in a non-linear mode log (agonist) vs. response with a variable slope (three parameter) equation for determination of EC50 values.

Efficacy Determination. Efficacy values were determined using GraphPad Prism version 7.02 by calculating the average of duplicates for the MFI values at the highest concentration (400nM).

Results

HSA binding domain(s) impair potency and efficacy of a bispecific FAPxCD40 ankyrin repeat binding protein (F-C format). A bispecific FAPxCD40 DARPin® molecule in the F-C format, SMA014, was cloned in different formats adding one (H-F-C, SMA087) or two (H-F-C-H, SMA095) HSA-binding ankyrin repeat domains and tested in the *in vitro* B cell activation assay. As shown in **Figure 3**, the HSA binding domain(s) impaired both the potency and the efficacy of the original bispecific binding protein in the F-C format and the level of impairment correlated with the number of HSA binding domains added to the binding protein. Importantly, the inhibition was more pronounced in presence of 600 μ M of albumin mimicking the physiological concentration of albumin in the human serum. It is plausible to hypothesize that the complex HSA binder/albumin could be a steric impairment for the binding, and

consequently the activity, of the CD40 and/or FAP domain(s). As expected, the DARPin® proteins and the anti-CD40 mAb upregulated CD86 in a dose dependent manner and the DARPin® proteins were active only in presence of FAP-expressing CHO cells (**Figure 3**). In absence and presence of HSA, HSA binding domains did not influence the FAP specific mode of action. As expected, the agonistic anti-CD40 mAb induced activation of human B-cells independently of FAP expression, activating B-cells in the presence of either FAP-CHO or WT-CHO cells. EC50 (potency) and maximum MFI (efficacy) mean values of two independent experiments are summarized in **Table 4** and **Table 5** for FAP-CHO and in **Table 6** for WT-CHO, respectively.

In summary, the addition of half-life extending HSA-binding domain(s) impaired the functionality of the bispecific FAPxCD40 binding protein (F-C format), the inhibition increased with the number of added HSA binding domains, and the inhibition was more pronounced in presence of physiological concentration of albumin.

Bivalency for CD40 increases potency and efficacy and rescues the inhibitory effect induced by HSA-binding domain. Applicant then investigated how CD40 valency affects the performance of the bispecific FAPxCD40 DARPin® molecule. The bispecific FAPxCD40 DARPin® molecule in the F-C format, SMA014, was cloned in different formats adding one (F-C-C, SMA104) or two (F-C-C-C, SMA105) CD40-binding DARPin domains, and tested in the *in vitro* B-cell activation assay. As shown in **Figure 4**, the bivalent and trivalent formats induced a stronger upregulation of CD86, indicating that valency contributes favorably to the performance of the molecule. Specifically, CD40 bivalency (SMA104) strongly increased potency (20x) and efficacy (2x) of the molecule in presence of FAP expressing CHO cells. CD40 trivalency (SMA105) only caused slightly increased potency of the molecule compared to CD40 bivalency, but did not further impact the efficacy. In absence of FAP, the bivalent CD40 format (SMA104) did not induce upregulation of CD86 on human B cells, while the trivalent CD40 format (SMA105) showed slight activation also in absence of FAP at the highest concentration, suggesting a possible FAP-independent activation induced by the trivalent CD40 binder. In view of these results, the bivalent CD40 format was selected for further characterization. In particular, Applicant tested if the CD40 bivalency could rescue the inhibitory effect of the HSA binding domain. To address this question, the bivalent CD40 construct SMA104 was cloned with additional HSA binding domain(s) in different positions (clones SMA091, SMA099 and AS579; see Table 2 above for information on their domain formats). All the tested formats showed improved potency and efficacy compared to SMA014 (**Figure 5A**). Importantly, in a more physiological condition in presence of HSA, activity of all formats with HSA binding domain was reduced, but SMA091 still showed improved activity compared to SMA014 (**Figure 5B**). None of the ankyrin repeat binding proteins enhanced expression of CD86 on the B cells in absence of FAP even at highest concentration (**Figure 5A and B**). As expected, the agonistic anti-CD40 mAb induced activation of human B-cells independently on FAP expression, activating B-cells either with FAP-CHO or WT-CHO. EC50 (potency) and maximum MFI (efficacy) mean values of two independent experiments are summarized in Table 4 and Table 5 for FAP-CHO and in Table 6 for WT-CHO, respectively.

Table 4

	Protein	EC50 [nM]			
		MFI CD86		% CD86	
		w/o HSA	w HSA	w/o HSA	w HSA
	SMA014	8.46	7.7	4.21	6.15
	SMA087	28.92	52.01	11.44	72.34
	SMA095	287.55	-	89.84	-
	CD40 mAb	1.13	0.51	1.9	0.88
	SMA091	3.93	8.05	0.76	2.98
	SMA099	18.23	19.41	4.68	7.5
	SMA104	0.74		0.23	
	SMA105	0.33		0.11	
	AS579	10.01	23.1	2.27	10.25
	CD40 mAb	4.73	2.05	0.51	0.3

Table 5

	Protein	Efficacy [MFI at highest dose]			
		MFI CD86		% CD86	
		w/o HSA	w HSA	w/o HSA	w HSA
	SMA014	2436.25	2189.75	48.4	47
	SMA087	1643.25	799.25	43.4	27.01
	SMA095	773	232.25	28.7	-
	CD40 mAb	1339.5	1528.5	38.6	45.05
	SMA091	6230.25	2983.25	78.15	75.63
	SMA099	4954	1718.75	84.4	71.68
	SMA104	6270.75		86.8	
	SMA105	5558.5		87.15	
	AS579	4864.5	1781.75	82.98	71.9
	CD40 mAb	2064.25	1867.25	78.63	76.63

Table 6

	Protein	Efficacy [MFI at highest dose]			
		MFI CD86		% CD86	
		w/o HSA	w HSA	w/o HSA	w HSA
	SMA014	293.75	310	13.15	13.29
	SMA087	291	299	12.53	13.73
	SMA095	299	306.5	12.48	13.68
	CD40 mAb	1389.5	1473	38.83	40.05
	SMA091	289.5	295.5	47.78	43.13
	SMA099	289.25	273.75	47.38	42.53
	SMA104	251.75		46.05	
	SMA105	672.5		69.25	
	AS579	252.5	242.5	42.05	40.13

CD40 mAb	2053.75	1902	79	77.83
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[194] *Conclusions:* The bispecific FAPxCD40 ankyrin repeat protein in the F-C format showed good biological activity in functional cell assays and good physical properties. However, this binding protein may require a half-life extension domain to allow its clinical development. Thus, different formats were analyzed to determine if and how the number and location of half-life extension HSA-binding domains impacts the activity of the molecule. It was observed that a half-life extension domain had a detrimental effect on the activity of the molecule and that this effect increased with the number of half-extension domains and the presence of HSA. Furthermore, it was then surprisingly found that CD40 bivalency (by having two CD40 binding domains) strongly increased the potency (20x) and the efficacy (2x) of the binding protein and maintained a stringent FAP-specific mechanism of action, but that CD40 trivalency (by having three CD40 binding domains) increased only slightly the potency compared to CD40 bivalency and did not show any further impact on the efficacy. In addition, the trivalent CD40 binding protein showed slight activation also in absence of FAP at the highest concentration, suggesting a partial loss of FAP-specific mode of action. CD40 bivalency was further found to be able to rescue the inhibitory effect of one half-life extension domain, by increasing the potency and the efficacy of the binding protein. Specifically, at physiological concentration of HSA, the binding protein in the H-F-C-C format retained an activity and FAP-specificity comparable to the binding protein in the F-C format. In conclusion, by adding a second CD40 binding domain we could prevent the detrimental effect of a HSA binding half-life extension domain and generate a molecule with similar functional properties to the parental binding protein in the F-C format but equipped with the half-life extension domain that will facilitate its clinical development. For all these reasons, H-F-C-C domain format was chosen for further investigations. This format is used in the binding proteins of the invention comprising the amino acid sequence of SEQ ID NO: 5 (DARPin® Protein #5 or just "Protein #5"), SEQ ID NO: 6 (DARPin® Protein #6 or just "Protein #6") or SEQ ID NO: 7 (DARPin® Protein #7 or just "Protein #7") described in the following Examples.

Example 2 – Biophysical Properties, Binding Affinities, and Binding Specificities of a Multi-Specific Binding Protein of the Invention

This Example describes experiments which were conducted to determine (1) biophysical properties, such as aggregate formation, and (2) binding affinity to the various target proteins (i.e. FAP, CD40 and serum albumin) and species cross-reactivity, of a multi-specific binding protein of the invention.

Aggregate Formation

Protein #5 (also sometimes called SMA136 herein) was analyzed by size exclusion chromatography (SEC) and multiangle light scattering (MALS). **Figure 6** shows the results of this analysis. This SEC profile demonstrates that Protein #5 is monomeric and monodisperse in solution and does not form aggregates.

Binding Affinity to Target Proteins

Summary. The binding of DARPin® Protein #5 ("Protein #5") to CD40, FAP and serum albumin from human, cynomolgus monkey and mouse was analyzed by surface plasmon resonance (SPR). The results showed that Protein #5 binds specifically to (i) CD40 of human and cynomolgus origin, (ii) serum albumin of human, mouse and cynomolgus origin, and (iii) FAP of human and cynomolgus origin. No specific binding was detected to mouse CD40 and mouse FAP. Kinetic parameters of Protein #5 are summarized in **Table 7**. SPR traces of binding of Protein #5 to human CD40, human FAP and human serum albumin are shown in **Figure 7**.

Table 7. Kinetic parameters of Protein #5 binding to CD40, serum albumin and FAP of different species (human, mouse or cynomolgus).

Protein Name	K_{on} [M ⁻¹ s ⁻¹]	K_{off} [s ⁻¹]	K_d [nM]	STDEV K_d [nM]	Rmax [RU]	Chi ² /Rmax [†] [%]
Bio-hCD40 *	1.01E+07	1.03E-03	0.103	0.012	95	7.5
Bio-cCD40 *	8.25E+06	8.24E-04	0.101	0.011	116	9.6
Bio-mCD40 *	No specific binding detected					
HSA	3.58E+05	1.79E-02	50	n.d.	126	2.3
CSA	5.32E+05	2.17E-01	409	n.d.	134	7.6
MSA	3.15E+05 [!]	1.26E-02 [!]	40 [!]	n.d.	86	13.1 [†]
hFAP**	4.09E+05	1.26E-04	0.307	0.019	154	5
cFAP	4.32E+05	1.46E-04	0.339	n.d.	110	4
mFAP *	No specific binding detected					

* The values represent the average of a duplicate measurement

** The values represent the average of a triplicate measurement

[†] Chi²/Rmax>10% defined as inaccurate fit

[!] K_D of MSA binding on Protein #5 is overestimated due to the strong nonspecific binding of MSA on the chip

n.d.: Standard deviation not determined as no replicates were performed

Materials and Methods.

All SPR measurements were performed using a ProteOn XPR36 instrument (BioRad) and a running buffer of PBS pH 7.4 containing 0.005% Tween 20® (PBST). The 1:1 Langmuir model was used for the fitting of SPR traces.

ProteOn setup for Protein #5 binding to CD40 of different species. CD40 proteins (Acro Biosystem) of different species (human, cynomolgus, mouse) were biotinylated with standard methods known in the art, resulting in bio-hCD40, bio-cCD40 and bio-mCD40 proteins. The bio-hCD40, bio-cCD40 and bio-mCD40 proteins were immobilized on an NLC chip (BioRad) to a level of respectively 300 RU, 250 RU and 300 RU. The interaction of Protein #5 with CD40 was measured by injecting Protein #5 in a serial dilution of 3, 1.5, 0.75, 0.38 and 0.19 nM with an association of 120 s and dissociation of 900 s using a constant flow of 100 µl/min. The measurement was repeated two or three times and the targets were regenerated between the individual measurements using 10 mM glycine pH 2 for 18 s at a flow of 100 µl/min. The signals were double referenced against the running buffer (PBST) treated control lanes.

ProteOn setup for Protein #5 binding to serum albumin of different species. First, human FAP (hFAP) was coated on a GLC chip (BioRad) to a level of 700 RU before 50 nM Protein #5 was immobilized as analyte for 120 s (dissociation of 0 s) at constant flow of 100 μ l/min to a level of 120 RU. The binding of the serum albumin to Protein #5 was measured by injecting human serum albumin (HSA), cynomolgus serum albumin (CSA) and mouse serum albumin (MSA) in a serial dilution of 100, 33, 11 and 3.7 nM with an association of 120 s and a dissociation of 600 s at a constant flow of 100 μ l/min. HSA, CSA and MSA binding was measured serially and the hFAP/ Protein #5 complex was regenerated each time with 10 mM glycine pH2 for 18 s at a flow of 100 μ l/min. Thus, Protein #5 had to be re-immobilized on hFAP after each regeneration step. The signals were double referenced against the control lanes ((a) PBST running buffer; (b) coated with a non-relevant target to which Protein #5 does not bind). The kinetic was calculated on the first 300 s of dissociation.

ProteOn setup for Protein #5 binding to FAP of different species. hFAP, cynomolgus FAP (cFAP) and mouse FAP (mFAP) were immobilized on a GLC chip (BioRad) in 10mM Na Acetate buffer pH5.3 to a level of 1200 RU, 800 RU and 2000 RU, respectively. The interaction of FAP and Protein #5 was measured by applying Protein #5 in a serial dilution of 50, 25, 12, 6.5 and 3.13 nM with an association of 120 s and dissociation of 1800 s using a constant flow of 100 μ l/min. The targets were regenerated using 10 mM glycine pH 2 and 124 mM H_3PO_4 . The signals were double referenced against the PBST treated control lanes.

Results and Conclusions

Surface plasmon resonance measurements showed that Protein #5 binds tightly to human and cynomolgus CD40 with essentially identical binding affinities (K_D) of 103 ± 12 pM and 101 ± 11 pM, respectively. Protein #5 is not cross-reactive to mouse CD40, potentially due to low sequence identity of the extracellular domain of only 57.5%. Protein #5 showed binding to human serum albumin with a binding affinity (K_D) of 50 nM. Furthermore, Protein #5 showed binding to human and cynomolgus FAP with similar binding affinities (K_D) of 0.307 nM and 0.339 nM, respectively, while no cross-reactivity could be detected for mouse FAP.

Example 3 - Simultaneous binding of Protein #5 to CD40, FAP and serum albumin analyzed by surface plasmon resonance

[195] The following experiment describes a surface plasmon resonance experiment that was performed to analyze the simultaneous binding of a multi-specific protein comprising SEQ ID NO:5 to human CD40, human FAP and human serum albumin, respectively.

[196] *Materials and Methods.* SPR measurements were performed using a ProteOn XPR36 instrument (BioRad). The running buffer was PBS pH 7.4 containing 0.005% Tween 20® (PBST). Biotinylated human CD40 (bio-hCD40-Fc) was immobilized on a NeutrAvidin coated NLC sensor chip to a level of 550 RUs. In a first analyte step (analyte 1) 25 nM of Protein #5 was immobilized on bio-hCD40 with an association of 120 s and dissociation of 0 s. This first step was directly followed by a second analyte step (analyte 2) during which either PBST, 25 nM Protein #5 or 50 nM hFAP were injected with an

association of 120 s and dissociation of 0 s. In a third step (analyte 3), either PBST, 50 nM hFAP or 50 nM HSA were applied with an association of 120 s and dissociation of 600 s (injection scheme: see **Table 8**). The entire experiment was performed at a constant flow of 100 μ l/min. The setup allowed binding of hFAP and HSA only if Protein #5 (analyte 1) was already bound to CD40 (immobilized on the chip). The signals were double referenced to the PBST treated control lane of L6 and A6.

[197] **Table 8.** Injection scheme of the SPR measurement

Analysis	Symbol	Immobilization	Analyte 1	Analyte 2	Analyte 3
A1	diamond ◆	Bio-hCD40-Fc	Protein #5	hFAP	HSA
A2	triangle ▲	Bio-hCD40-Fc	Protein #5	hFAP	HSA
A3	cross X	Bio-hCD40-Fc	Protein #5	Protein #5	PBST
A4	circle ●	Bio-hCD40-Fc	Protein #5	hFAP	hFAP
A6	not depicted (used for referencing)	Bio-hCD40-Fc	PBST	PBST	PBST

[198] **Results.** SPR traces of simultaneous binding of Protein #5 to hCD40, hFAP and HSA are depicted in **Figure 8**. Briefly, 550 RUs of biotinylated human CD40 were immobilized on a neutravidin chip. In a first association step, Protein #5 was bound to saturation to hCD40, reaching a total signal of 200 RUs as depicted in Figure 8, injection 1. In a second association step (Figure 8, injection 2), hFAP was bound to the complex Protein #5/CD40, resulting in an increase of 200 RUs. It has to be noted that Protein #5 already started to dissociate from hCD40 during the interval of time between injection 1 and injection 2 (loss of 75 RUs). A third association step (Figure 8, injection 3) resulted in the binding of human serum albumin to the complex hCD40/Protein #5/hFAP (increase of 80 RUs), indicating that simultaneous binding of Protein #5 to all three targets occurred.

[199] In conclusion, the results of the surface plasmon resonance study show that Protein #5 is capable of binding to CD40, FAP and serum albumin simultaneously.

Example 4 – Activation of Human B Cells via CD40

[200] The aim of the study was to assess the biological activity and the FAP-specific mechanism of action of multispecific binding proteins, Protein #5, comprising SEQ ID NO: 5, and Protein #6, comprising SEQ ID NO: 6. Protein #5 and Protein #6 were tested in a cell assay using primary human B cells in presence or absence of FAP-expressing cells. The upregulation of costimulatory molecules, in particular CD86 and CD69, was evaluated as markers of B cell activation mediated by CD40 signaling. An anti-CD40 monoclonal antibody, which has a mechanism of action that is independent on FAP-mediated cross-linking, was used as reference material. Potency, efficacy and FAP-specificity of Protein #5 or Protein #6, in comparison to the reference material, were evaluated.

[201] The *in vitro* data obtained in the human B cell activation assay showed that Protein #5 and Protein #6 can activate B cells via CD40 activation, as reflected by the upregulation of CD86 and CD69, and that Protein #5 and Protein #6 can activate B cells only in the presence of FAP-positive cells, but not in

the presence of FAP-negative cells, confirming a mechanism of action that is strictly dependent on FAP-mediated cross-linking.

Materials and Methods

The *in vitro* B cell activation assay, FACS staining, flow cytometer settings and antibody dilutions, EC50 determinations and efficacy determinations were performed essentially as described in Example 1.

The anti-CD40 antibody used as a reference as well as the FAP expressing and FAP non-expressing CHO cells were as described in Example 1.

Results

Protein #5 and Protein #6 induce upregulation of co-stimulatory molecules in human B cells with a FAP-dependent mechanism of action. Protein #5 and Protein #6 were assessed for their ability to activate human B cells via CD40 in the presence of FAP-expressing CHO cells. Protein #5 induced upregulation of co-stimulatory molecules CD86 and CD69 in human B cells co-cultured with FAP-expressing CHO cells with an EC50 of 0.04 – 0.07 nM (**Figure 9**). Conversely, Protein #5 did not activate human B cells in presence of non-FAP expressing CHO cells, wild type (WT)-CHO cells (**Figure 10**). As expected, the agonistic anti-CD40 mAb induced activation of human B cells independently on FAP expression, activating B cells either with FAP-CHO or WT-CHO. Similar results as for Protein #5 were obtained for Protein #6.

Conclusions

Protein #5 and Protein #6 induced upregulation of two different co-stimulatory molecules, CD86 and CD69, in primary human B cells only in the presence of FAP-positive CHO cells, but not in the presence of FAP-negative CHO cells, confirming a mechanism of action that is strictly dependent on FAP-mediated cross-linking. Protein #5, in presence of FAP, showed similar potency and efficacy as the comparator anti-CD40 monoclonal antibody. Protein #5 induced the upregulation of co-stimulatory molecules in a dose-dependent manner with an EC50 of 0.04 – 0.07 nM, in the presence of FAP expressing CHO cells. Similar results were obtained for Protein #6.

Example 5 – Activation of Human Dendritic Cells via CD40

A conceptually similar study as the one described for B cells in Example 4 was performed with human monocyte-derived dendritic cell (MDDC) (see schematic representation in **Figure 11**).

[202] The aim of the study was to assess the biological activity and the FAP-specific mechanism of action of multispecific binding proteins, Protein #5, comprising SEQ ID NO: 5, and Protein #6, comprising SEQ ID NO: 6, on MDDC. Protein #5 and Protein #6 were tested in a cell assay using human MDDC in presence or absence of FAP-expressing cells. The upregulation of costimulatory molecules, in particular CD86, CD83 and CD80, and the secretion of IL-12 were evaluated as markers of MDDC activation mediated by CD40 signaling. An anti-CD40 monoclonal antibody, which has a mechanism of action that is independent on FAP-mediated cross-linking, was used as reference material. Potency,

efficacy and FAP-specificity of Protein #5 or Protein #6, in comparison to the reference material, were evaluated.

[203] The *in vitro* data obtained in the human MDDC activation assay showed that Protein #5 and Protein #6 can activate MDDC via CD40 activation, as reflected by the upregulation of the costimulatory molecules and the secretion of IL-12, and that Protein #5 and Protein #6 can activate MDDC only in the presence of FAP-positive cells, but not in the presence of FAP-negative cells, confirming a mechanism of action that is strictly dependent on FAP-mediated cross-linking. Protein #5 was able to induce the upregulation of co-stimulatory molecules and the secretion of IL-12 in a dose-dependent manner with an EC₅₀ of 0.03 – 7.67 nM and 0.83 – 7.63 nM, respectively, in the presence of FAP expressing CHO cells, but not in the presence of FAP negative CHO cells. Similar results were obtained with Protein #6.

[204] In conclusion, this study showed that Protein #5 and Protein #6 are able to activate human MDDC via CD40 *in vitro*, in a FAP-dependent manner.

Example 6 – Protein #7 demonstrated anti-tumor activity *in vivo*

[205] The following Example evaluated the dose-dependent *in vivo* efficacy of repeated doses of the multi-specific binding protein, DARPin® Protein #7, in a murine MC38 colon carcinoma model. Protein #7 is a mouse surrogate for Protein #5 or Protein #6 comprising a FAP binding domain that binds mouse FAP and CD40 binding domains that bind mouse CD40. The MC38 carcinoma model has previously been shown to be susceptible to CD40 agonist treatment. Since syngeneic mouse tumors such as MC38 have generally been found to express very low levels of stromal FAP compared to the human tumor stroma, the MC38 cell line was transfected to express FAP, thereby mimicking better the FAP expression observed in human tumors.

[206] In the described studies PD1032, PD1033, PD1035 and PD1038, Protein #7 (with a N-terminal His-tag (SEQ ID NO: 57); also called AS598) was tested at dose of 2.5 mg/kg, and in studies PD1032 and PD1033 it was also tested at dose of 12.5mg/kg. The commercially available anti-CD40 antibody (FGK45; BioXell), which binds mouse CD40, was used as a positive control. A non-FAP binding variant of Protein #7 (called AS608; SEQ ID NO: 67 with a N-terminal His-tag (SEQ ID NO: 57)), in which the FAP binding domain was replaced by a non-binding ankyrin repeat domain, was used as a negative control molecule to demonstrate dependence of the pharmacological activity of Protein #7 on binding to mFAP.

[207] The multi-specific binding proteins of the invention, such as, e.g., Protein #7, are intended to activate CD40 locally in the tumor tissue in order to reduce systemic toxicity. Therefore, to assess the safety profile of Protein #7, in comparison to anti-mCD40 antibody, which is known to induce liver toxicity, several parameters of systemic toxicity, including weight loss, serum cytokine and transaminase elevation and liver tissue damage, were determined in addition to tumor growth and inhibition.

Materials and Methods:

[208] *Tumor experiment:* The tumor experiments were performed as shown schematically in **Figure 12**. Syngeneic mice (C57BL/6JRj) were inoculated subcutaneously into the right flank region with MC38-mFAP polyclonal tumor cells (day 0). Mice were randomized into treatment groups and treated the same day (PD1032 at day 25, PD1033 at day 39, PD1035 at day 26, PD1038 at day 37). The test articles (AS598, AS608, FGK45) were then administered to the tumor-bearing mice according to the predetermined regimen as shown in **Table 9** (see also Figure 12). Tumor growth was monitored every 3 to 4 days by caliper measurement until day 36, 43, 36 and 40 after inoculation, respectively. On day 36 (PD1032 and PD1035) of the experiment, mice were sacrificed, tumors removed, and immune-phenotyping was performed by flow cytometry. On day 43 (PD1033) and on day 40 (PD1038) of the experiment, mice were sacrificed, tumors removed, and immune-phenotyping was performed by flow cytometry.

[209] PD1032 and PD1035 were two independent main studies of anti-tumor efficacy, while PD1033 and PD1038 were two adjacent studies for tumor environment analysis by FACS. In the main studies, AS598 was administered at doses of 2.5 mg/kg (studies PD1032, PD1035) and 12.5 mg/kg (study PD1032) three times every four days. The non-FAP targeted control AS608 was administered at 2.5 mg/kg three times every four days (study PD1035). In the early termination studies for FACS analysis, AS598 was administered at doses of 2.5 mg/kg (studies PD1033, PD1038) and 12.5 mg/kg (study PD1033) two times with three days in between. The non-FAP targeted control AS608 was similarly administered at dose 2.5 mg/kg two times with three days in between (study PD1038). The anti-CD40 antibody FGK45 was used as positive control in all studies and administered at 5 mg/kg (equal molar dose to AS598 at 2.5mg/kg) at the same schedule as the DARPin® proteins.

Table 9. Study design - experimental groups

PD1032 and PD1035:

Group	N	Inoculum (s.c.)	Treatment	Dose (mg/kg)	Dosing Route	Treatment Schedule	Termination/ FACS
1	10	MC38_mFAP_ polyclonal 9x10 ⁶ cells	Vehicle		i.p.	D0-4-8	Spleen and Tumor
2	10		AS598	2.5mg/kg	i.p.	D0-4-8	Spleen and Tumor
3	10		AS598 (PD1032) or AS608 (PD1035)	2.5mg/kg (AS608) or 12.5mg/kg (AS598)	i.p.	D0-4-8	Spleen and Tumor
4	10		FGK45 (anti-CD40)	5 mg/kg	i.p.	D0-4-8	Spleen and Tumor

PD1033 and PD1038:

Group	N/N	Inoculum (s.c.)	Treatment	Dose (mg/kg)	Dosing Route	Treatment Schedule	Termination/ FACS
1	5/6	MC38_mFAP_ polyclonal 9x10 ⁶ cells	Vehicle		i.p.	D0-3	Spleen and Tumor
2	5/6		AS598	2.5mg/kg	i.p.	D0-3	Spleen and Tumor
3	5/6		AS598 (PD1033) or AS608 (PD1038)	2.5mg/kg (AS608) or 12.5mg/kg (AS598)	i.p.	D0-3	Spleen and Tumor
4	5/6		FGK45 (anti-CD40)	5 mg/kg	i.p.	D0-3	Spleen and Tumor

Note: N: animal number; Number of mice/group in PD10033 was different from PD1038 (N/N)

Tumor inoculation: Under standard isoflurane anesthesia, 90 (PD1032 + PD1033) or 105 (PD1035 + PD1038) female C57BL6 mice were inoculated subcutaneously into the right hind flank/back region with MC38-mFAP polyclonal tumor cells (9x10⁶) in 0.2 mL of PBS for tumor development.

Tumor measurement: Tumor measurement was performed twice a week from day 15 (PD1032 and PD1033) or day 14 (PD1035 and PD1038) post tumor inoculation. The length and the width of the tumor was measured with calipers. The tumor volume was calculated with the formula: (length x (width)² x π) / 6.

Randomization: Group randomization based on tumor volume was performed on day 25, 39, 26 and 37 post tumor inoculation. From the original 90 (PD1032 and PD1033) mice engrafted with tumor, 40 were randomized respectively into 4 sub-groups of 10 animals 25 days after tumor inoculation (PD1032). The 50 mice left were randomized respectively into 4 sub-groups of 5 animals 39 days after tumor inoculation (PD1033). From the original 105 (PD1035 and PD1038) mice engrafted with tumor, 40 were randomized respectively into 4 sub-groups of 10 animals 26 days after tumor inoculation (PD1035). The 65 mice left were randomized respectively into 4 sub-groups of 6 animals 37 days after tumor inoculation (PD1038).

Observations and Data Collection: After group randomization, the animals were checked twice weekly, during which body weight and tumor measurements were performed. The animals were also checked for any effects of tumor growth and treatments on normal behavior such as mobility, visual estimation of food and water consumption, body weight gain/loss, eye/hair matting and any other abnormal effect. Death and observed clinical signs were recorded on the basis of the numbers of animals within each subset.

Sampling: Tumors were removed, weighed and used for FACS and remaining material fixed in Formalin. Spleens were removed and one half used for FACS and the other half fixed in Formalin. Livers were removed and fixed in Formalin. Blood samples were taken at day 24, 26 and 36 for PD1032, day 43 for PD1033, day 27 and 36 for PD1035 and day 41 for PD1038 in Multivette 600Z Gel (Sarstedt

#15.1674) and centrifuged at 15,000 rpm for 5 minutes. The serum was harvested and stored at minus 80°C for possible later analysis.

Statistical analysis: Statistical analysis was performed with the Prism 8.2.0 software (GraphPad Software). All statistic with multiple comparisons were performed by using Kruskal-Wallis non-parametric test followed by Dunn's multiple comparisons test for all groups compared to vehicle. For comparison between two groups for difference, non-parametric Mann-Whitney 2-tail analysis was performed.

[210] *Liver enzymes.* In murine models as well as in clinical trials in humans, agonistic antibodies against CD40 have been demonstrated to increase significantly, but transiently, liver enzymes such as aspartate aminotransferase (AST) and alanine aminotransferase (ALT). In humans, AST is found in a variety of tissues, including the liver, brain, pancreas, heart, kidneys, lungs, and skeletal muscles. If any of these tissues are damaged, AST will be released into the bloodstream. While increased AST levels are indicative of a tissue injury, it is not specific to the liver per se. By contrast, ALT is found primarily in the liver. Any elevation of the ALT is a direct indication of a liver injury.

[211] Therefore, the levels of AST and ALT were determined in these experiments as a measure of liver toxicity. Measurements were performed at 24-hour time-points after treatment. The 24-hour time point after treatment was the most appropriate based on the literature and in-house time-titration experiments. ALT and AST analyses were performed using the kits MAK052 and MAK055 (Sigma-Aldrich), respectively, according to the manufacturer's guidelines.

[212] *Cytokine levels.* Blood from MC38-FAP colon carcinoma tumor bearing mice treated according to Figure 12 was taken 24 hours after the first injection (main studies = PD1032 and PD1035), 24 hours after the second injection (adjacent studies = PD1033 and PD1038) and at study end (main studies = PD1032 and PD1035). 11 different cytokines (TNF-alpha, IL-6, IFN-gamma, IL12p70, MCP-1, MIP-1 alpha, MIP-1 beta, IP-10, IL-10, IL-2 and IL-1 beta) were analyzed using Luminex assays (R&D Systems) according to manufacturer's recommendations.

[213] *Immunohistochemistry (IHC) analysis of liver tissue.* Livers were harvested 24 h after the first injection, washed in PBS, immediately fixed in formalin and embedded in paraffin blocks. Hematoxylin/Eosin staining was performed on sections of the paraffined-embedded livers and analyzed by a pathologist in blinded manner.

Results:

Overall health and weight. During the study duration, no signs of negative health effects or weight decrease of the mice were observed with AS598 treatment (**Figures 13A and B**). In contrast, and consistent with previous reports, FGK45 treatment resulted in significant, but transient, weight loss after the first injection (Figures 13A and B).

Tumor growth. Tumor growth was measured every 3-4 days over the duration of the study. The treatment in studies PD1032 and PD1035 was started at days 25 and 26, respectively, when the mean tumor volume exceeded 300mm³. The mean tumor growth curves of different treatment groups in both studies are shown in **Figures 14A and B**.

AS598 demonstrated statistically significant anti-tumor efficacy compared to vehicle group at both doses tested (Figures 14A and B). The anti-tumor efficacy was similar to the one observed with the positive control anti-CD40 antibody FGK45. The non-FAP-binding control AS608 used in study PD1035 did not have any anti-tumor efficacy, demonstrating that the anti-tumor efficacy observed in this tumor model was FAP-dependent.

In addition to tumor volume measurements, the tumors were dissected from the mice at the end of the study and the tumor weight was determined. The results and conclusions from the tumor weight measurements were consistent with those from the tumor volume measurements (data not shown).

Furthermore, results obtained in the studies PD1033 and PD1038 were consistent with those obtained in the studies PD1032 and PD1035 (data not shown).

Blood cytokine levels. FGK45 significantly increased blood levels of eight of the measured cytokines, namely of TNF-alpha, IL-6, IFN-gamma, IL12p70, MCP-1, MIP-1 alpha, MIP-1 beta, and IP-10 (**Figure 20A** and data not shown). In contrast, AS598 (2.5 mg/kg or 12.5 mg/kg), AS608 or vehicle did not increase blood levels of any of the measured cytokines (Figure 20A and data not shown).

Liver enzymes and injury. As expected, FGK45 induced a significant increase of the ALT levels, which was detected 24 hours after the first injection but not after subsequent injections (**Figure 20B**). This was in accordance with the literature and with previous studies in-house. In contrast to FGK45, AS598 did not induce any increases of ALT levels (Figure 20B). In addition to ALT, also AST was increased 24 hours after the first injection in the FGK45 treatment group, but not in the AS598 or control treated animals (Figure 20B).

IHC of liver tissue. Histological analysis revealed a diffused tissue damage characterized by vascular-centered multifocal inflammation with aggregates of mononuclear leukocytes, thrombosis and necrosis when mice were treated with anti-mCD40 antibody (**Figure 20C**). In contrast, IHC analysis of livers from mice treated with Protein #7 did not show evidence of hepatocellular toxicity, having a similar histological profile to the one observed in vehicle-treated livers (Figure 20C).

Taken together, in contrast to anti-mCD40 antibody, Protein #7 did not show signs of systemic toxicity in terms of weight loss, elevation of proinflammatory cytokines (e.g. IL-6, TNF α , IFN γ and IL-12p70), increased levels of transaminases (AST and ALT) or liver tissue damage.

Conclusions:

[214] A surrogate mouse-specific binding protein, Protein #7, with binding specificity for mouse FAP and mouse CD40 in addition to serum albumin, was generated. Protein #7 tested in murine cell-based *in vitro* assays showed comparable results as Protein #5 and Protein #6 in human cell-based *in vitro* assays (see, e.g., Example 4), displaying a strict FAP-dependent activation of CD40 (data not shown). As shown in this Example, Protein #7 (with a N-terminal His-tag; AS598) was also active *in vivo* and inhibited substantially the progression of FAP-positive tumors. Moreover, in contrast to an anti-mouse CD40 antibody (FGK45), the antitumor activity of Protein #7 was neither associated with elevated blood cytokine levels nor with the tested indicators of hepatotoxicity. Elevated blood cytokine levels and hepatotoxicity manifest as dose limiting toxicities of some of the clinical CD40 activating antibodies. The

presented data support a mode of action, both *in vitro* and *in vivo*, that is dependent on FAP-mediated crosslinking of CD40 receptor, resulting in a tumor-localized CD40 activation without peripheral or off-tumor organ toxicity.

[215] In conclusion, a tumor-targeted, CD40 agonistic, multi-specific DARPIn® protein has been generated, which is able to activate the CD40 receptor locally in FAP-positive tumors and to cause substantial antitumor activity in the absence of systemic toxicity.

Example 7: Protease activity of FAP in the presence or absence of multi-specific binding proteins of the invention

[216] This example describes a FAP activity assay that was performed in the presence or absence of various multi-specific binding proteins of the invention to determine whether the intrinsic FAP enzymatic activity is inhibited upon binding of the multispecific recombinant proteins.

[217] FAP is a type II single transmembrane serine protease whose expression is highly upregulated on sites of tissue remodeling like tumors (e.g. expressed at the surface of stromal fibroblasts in >90% of epithelial cancers), wound healing, embryonic tissue and sites of inflammations (e.g. atherosclerosis / arthritis), while FAP expression is difficult to detect in non-diseased adult organs. This atypical serine protease has both dipeptidyl peptidase (exopeptidase) and endopeptidase activities, cleaving substrates at a post-proline bond. Structurally, FAP consists of a short cytoplasmic N-terminal sequence (4 aa), a single transmembrane helix (21 aa) and an extracellular domain (735 aa) which forms an eight bladed β -propeller and a α/β -hydrolase domain. FAP is active as a homodimer. The catalytic triad, essential for FAP protease activity, is composed of residues Ser624, Asp702 and His734. The active site is accessible either through the central hole of the beta-propeller or through a cavity at the interface of the beta-propeller and the hydrolase domain.

[218] The protease activity of FAP produces cleavage of a variety of substrates, including neuropeptide Y, type I collagen and $\alpha 2$ -antiplasmin but also the substrate Z-GLY-PRO-AMC, which can be cleaved by both the exopeptidase or endopeptidase activity into a product that can be measured with a fluorescence reader.

[219] Molecules tested in the FAP activity assay are summarized in **Table 10**.

Table 10. Recombinant proteins used in the assay

Molecule No	Name	Format and Description
1	Multi-specific binding protein (SEQ ID NO: 5)	HFCC
2	Multi-specific binding protein (SEQ ID NO: 7 with N-terminal His-tag)	HF*C*C*
3	FAP-binding domain only (SEQ ID NO: 2 with N-terminal His-tag)	F
4	FAP-binding domain only (SEQ ID NO: 9 with N-terminal His-tag)	F*
5	Alternative FAP-binding domain (SEQ ID NO: 59 with N-terminal His-tag); This FAP-binding domain served as an assay control	Con

H Albumin binding domain

F	FAP-binding domain comprised in SEQ ID NO: 5
F*	FAP-binding domain comprised in SEQ ID NO: 7
C	CD40 binding domain comprised in SEQ ID NO: 5
C*	CD40-binding domain comprised in SEQ ID NO: 7
Con	A FAP-binding domain which partially inhibited FAP activity and served as assay control

[220] *FAP Activity Assay.* The human FAP (hFAP) target was diluted to 0.67 nM in the assay buffer (50 mM Tris, 1 M NaCl, 1 mg/ml BSA, pH 7.5) and 45 µl per well was added to a 96 well plate (leading to a final hFAP concentration of 0.3 nM in the activity assay). Molecules 1-5, as shown in Table 10, were applied at a 500-fold molar excess by adding 5 µl of 3 µM molecule to the target sample (final concentration 150 nM). Finally, 50 µl of 100 µM Z-GLY-PRO-AMC substrate (final concentration 50 µM) was added to obtain a total volume of 100 µl in each well. Molecule No. 5 was used as control to show partial inhibition of the FAP activity.

[221] Prior to measurement, the plate was centrifuged for 2 min at 4000 rpm to remove any assay interfering bubbles. The fluorescence was measured every 5 min over a period of 95 min at 380 nm excitation and 460 nm emission using a fluorescence reader with a manual gain set at 105%. Quadruplet measurements were performed and illustrated as mean and standard deviation.

[222] *Results.* In a first step, dose response curves were measured using FAP concentrations from 0.01 nM up to 1.2 nM at fixed substrate concentration of 50 µM. A linear time-dependent signal increase was observed at an hFAP target concentration of 0.3 nM over a time period of 95 min (measured every 5 minutes with an R^2 of 0.999). To determine the effect of protein binding on the enzymatic activity of FAP, the assay was conducted under FAP saturating conditions by adding the FAP-binding-molecules (e.g. Molecule No. 1 (SEQ ID NO: 5)) in a 500-fold molar (150 nM) excess over FAP (0.3 nM). This concentration of Molecule No. 1 (SEQ ID NO: 5) was 500-fold above the binding affinity of Molecule No. 1 (SEQ ID NO: 5) against human FAP (K_d = 0.3 nM).

[223] As summarized in **Figure 15**, Molecules Nos. 1-4 did not inhibit the intrinsic dipeptidyl FAP enzymatic activity. Partial FAP activity inhibition was observed by an alternative FAP-binding molecule (Molecule No. 5), which was used as an assay control.

[224] *Conclusion.* Binding of multi-specific binding proteins of the invention, such as Molecule No.1 (HFCC) or Molecule No. 2 (HF*C*C*), or of their FAP-binding domains (F or F*), to FAP did not affect the protease activity of FAP, as measured by its ability to cleave the fluorogenic substrate Z-GLY-PRO-AMC.

Example 8: Preferential localization and accumulation of a multi-specific binding protein of the invention in tumor tissue

[225] This Example describes experiments that were performed to investigate if multi-specific binding proteins of the invention can preferentially localize and accumulate in tumor tissue, presumably via binding to FAP expressed in the tumor tissue. For this purpose, the syngeneic MC38-FAP mouse model described in Example 6 was used as a suitable experimental system. Protein #7, also described in Example 6, was used as a representative multi-specific binding protein of the invention.

[226] Three different methodologies were used to investigate localization and accumulation of Protein #7 in the tumor tissue, namely SPECT/CT imaging, immunohistochemistry (IHC), and *in vivo* tissue distribution analysis.

Materials and Methods

Tumor inoculation and treatments: As previously described, mice were subcutaneously inoculated into the right shoulder flank with 9×10^6 of MC38-FAP mouse colon carcinoma cells. When tumors reached the size of 500 mm³, mice were injected with approximately 150 KBq of radio-conjugated molecules (Protein #7 or a control DARPin® protein, which has a corresponding four ankyrin repeat domain structure and binds to HSA, but does not bind to FAP or CD40) into the tail vein corresponding to 2.5 mg/kg = 50 µg/mouse.

Indium-111 labeling: To label the DARPin® molecules with indium-111, maleimide-DTPA (Chematech, Cat Number: C107), a bifunctional chelator with a specific reactivity toward sulfhydryl (SH) groups, was used to conjugate the radio label to the free SH-group of a cysteine added at the C-terminal end of DARPin® molecules. Molecules were stirred in metal-free PBS (pH 7.4, PSI grade) and 0.05 mM EDTA for 1 hour at room temperature (RT). A 10-fold molar excess of maleimide-DTPA in DMSO (Sigma-Aldrich) over the amount of protein was added and incubated for 1 hour at RT. The reaction solution was transferred into ultrafiltration tubes (Amicon Amicon Ultra 15, 10kDa MW cut-off) and 4 mL of metal-free PBS was added. Tubes were centrifuged for 6 min at 4000 x g at RT. After the spinning, flow-through was discarded, another 4 mL of metal-free PBS was added and the tubes were centrifuged again as described above. Filter supernatant was transferred to a low protein binding Eppendorf reaction tube and concentration was determined. Site-specific conjugation was determined using electrospray ionization time of flight mass spectrometry (ESI-TOF-MS). The final conjugated products were stored at 4°.

SPECT/CT imaging study: Single Photon emission computed tomography (SPECT)/X-Ray computed tomography (CT) images were performed with the NanoSPECT/CTPlus camera (version 1.2, Bioscan). Acquisitions of SPECT and CT were performed with the software Nucline (version 1.02). The CT was reconstructed with the software Nucline, whereas for the SPECT the software HISPECT was used (version 1.4.3049, Scivis GmbH). Fusions of SPECT and CT-data were analyzed with the VivoQuant™ post-processing software (Version 3.5, Invivo, USA). Whole-body activity was measured in a gamma counter tube prior to imaging acquisition. SPECT/CT *in vivo* images were taken from anesthetized mice (by inhalation of a 2% isoflurane/oxygen mixture) at time points 4, 24, 48, 72, 96 hours post injection of ¹¹¹In-DARPin® molecules. The image taken 96 hours post-injection is shown in Figure 16A. Every SPECT projection took 20 sec to 60 sec per frame, resulting in scan time of 15 min to 45 min per image. CT scans were performed with a tube voltage of 55 kVp and a tube current of 145 µA and an exposure time of 1000 msec per projection.

IHC study: Formalin-fixed paraffin embedded (FFPE)-tumor tissue slides were initially deparaffined by 3 cycles of 8 minutes at 70 °C followed by a cycle of 95 °C for 48 minutes at pH 7.4 (EDTA based solution, CC1 condition on Ventana auto-stainer) for antigen retrieval step. Slides were then incubated for 2 hours at 37°C with rabbit anti-DARPin® antibody conjugate (produced in house, working

concentration: 2.0 µg/mL). The anti-DARPin® antibody complex was detected by the HRP system using the Omni Rabbit HRP auto dispenser (Roche Diagnostics) for 20 minutes at 37°C. Finally, the slides were stained with hematoxylin, dehydrated in a gradient of ethanol (70%>90%>100%&100%, 1 minute each step), washed in xylene for 2 minutes and coverslips were applied to the slides with cytooseal mounting medium. The slides were scanned with Vectra Polaris and assessed qualitatively for the DARPin® protein accumulation.

In vivo tissue distribution study: MC38-FAP tumor bearing mice were treated as described above (*Tumor inoculation and treatments*), and euthanized 4, 24, 48, 72, or 96 hours post-injection. Organs of interest were dissected, weighed and the radioactivity was determined as counts per minute (CPM) with a γ-counter (Packard Cobra II Gamma D5010). CPM values per analyzed organ were then converted to µg DARPin® protein per organ (µg DARPin® protein/ organ) using the total amount of DARPin® protein per mouse as reference value. µg DARPin® protein/ organ was converted to µg DARPin® protein per gram tissue (µg DARPin® protein/ g tissue) and plotted in the graphs as percentage of the injected dose per gram of tissue (% ID/g) and expressed as mean ± SD, using 4 mice per time point.

Results

[227] The SPECT/CT imaging experiment revealed a preferential localization and accumulation of the multi-specific binding protein of the invention, Protein #7, in tumor tissue (**Figure 16A**). Some off-tumor uptake was observed mainly in the spleen, probably because of the on-target distribution induced by the CD40-specific domains of Protein #7.

[228] MC38-FAP tumor-bearing mice were sacrificed and tumors analyzed for the presence of DARPin® molecules by IHC. IHC analysis showed a strong presence of the multi-specific binding protein of the invention, Protein #7, in the tumor tissue (**Figure 16B**, upper panel). In contrast, the negative control DARPin® molecule, which binds HSA but not FAP or CD40, was not detected at significant levels in the tumor tissue (**Figure 16B**, lower panel). These data confirmed that tumor localization and accumulation of Protein #7 were mediated by the FAP- and/or CD40-specific binding domains. Any minor signal observed in tumors treated with the negative control was likely caused by the presence of albumin in the tumor microenvironment and binding of the negative control DARPin® molecule to it or was simply due to tissue permeability leading to molecule diffusion.

[229] The *in vivo* tissue distribution study, in which radiolabeled Protein #7 or control DARPin® protein was injected in MC38-FAP tumor-bearing mice, showed an accumulation of the multi-specific binding protein of the invention, Protein #7, specifically in tumor tissue (**Figure 16C**, left panel), but not in other organs, such as, e.g., muscle (**Figure 16C**, right panel), bone marrow, liver and kidney (data not shown). Consistent with the SPECT/CT study, some off-tumor accumulation was only observed in the spleen, most likely because of the expression of CD40 in this lymphoid organ (data not shown). Importantly, the radiolabeled negative control DARPin® protein, which binds HSA but neither FAP nor CD40, did not accumulate in tumor or any other tissue. This confirmed that the tumor localization and accumulation of Protein #7 occurred via a mechanism mediated by FAP- and/or CD40-specific binding.

Conclusions

[230] The data presented in this Example provide experimental evidence that a multi-specific binding protein of the invention can preferentially localize and accumulate in tumor tissue in a FAP- and/or CD40-dependent manner. These results are consistent with findings that a FAP-specific ankyrin repeat domain can effectively mediate tumor localization and accumulation of a multi-specific binding protein via binding to tumor-expressed FAP.

Example 9: Tumor inhibition by a multi-specific binding protein of the invention is dependent on FAP expression in the tumor

Example 6 showed that Protein #7 (with a N-terminal His-tag; AS598) was active *in vivo* and inhibited substantially the progression of FAP-positive tumors. In this Example, a syngeneic mouse model with a low FAP expression was used to provide further evidence of the FAP-dependent mechanism of action of Protein #7. Specifically, the non-transfected MC38-WT cell line, which generates tumors with very low expression levels of FAP *in vivo*, was used for performing the efficacy study.

Materials and Methods:

Tumor experiment: The tumor experiment was performed as shown schematically in **Figure 17A**. Female syngeneic mice (C57BL/6J) were inoculated subcutaneously into the flank region with MC38-WT colon carcinoma tumor cells (day 0). Mice were randomized into treatment groups based on the tumor size of approximately 77 mm³ on the day 7. The test articles (Protein #7 (with a N-terminal His-tag; AS598) and anti-CD40 antibody) were administered to the tumor-bearing mice intraperitoneally (i.p.) according to the predetermined regimen as shown in **Table 11**. The test articles were administered every 4 days and at equimolar concentrations to each other. Tumor growth was monitored every 3 to 4 days by caliper measurement until the individual mice were terminated due to the ethical tumor volume limit.

Table 11. Study design - experimental groups

Group	N	Inoculum (s.c.)	Treatment	Dose (mg/kg)	Dosing Route	Treatment Schedule
1	10	MC38-WT	Vehicle		i.p.	DR0-4-8-12-16
2	10		AS598	2.5 mg/kg	i.p.	DR0-4-8-12-16
3	10		anti-CD40 Ab	5 mg/kg	i.p.	DR0-4-8

N: animal number

Tumor inoculation: Under standard isoflurane anesthesia, mice were inoculated subcutaneously into the flank with 1x10⁶ MC38-WT tumor cells in 0.1 mL of PBS.

Tumor measurement: Tumor measurement was performed twice a week post-tumor inoculation. The length and the width of the tumor was measured with calipers. The tumor volume was calculated with the formula: $V = (L \times W \times W)/2$

Randomization: Randomization was performed based on "Matched distribution" randomization method (StudyDirector™ software) on day 7.

Observations and Data Collection: After tumor cell inoculation, the animals were checked daily for morbidity and mortality. At the time of routine monitoring, the animals were checked for any effects of tumor growth and treatments on behavior such as mobility, food and water consumption, body weight gain/loss and any other abnormalities. Mortality and observed clinical signs were recorded for individual animals.

Statistical analysis. The statistical analysis was done using Kruskal-Wallis non-parametric test followed by Dunn's multiple comparisons test for all groups compared to vehicle. Results were considered significant when * $p < 0.05$ ** $p < 0.01$ *** $p < 0.001$.

Results and Conclusions:

As shown in **Figure 17B**, Protein #7 did not show any statistically significant anti-tumor efficacy compared to vehicle in this FAP^{LOW} tumor model, thus providing further evidence of the FAP-dependent mechanism of action of Protein #7. Differently, the anti-CD40 antibody, whose *in vivo* activity is not related to the presence of FAP in the tumor microenvironment, inhibited tumor progression as expected, thus confirming the susceptibility of the MC38-WT tumor cell line to CD40 agonist.

Example 10: Long-term anti-tumor effects of a multi-specific binding protein of the invention

This Example describes a study in which MC38-FAP tumor bearing mice were treated and then followed for a longer time period in order to further investigate the full therapeutic potential of a multi-specific binding protein of the invention.

Materials and Methods

Tumor experiment: The tumor experiment was essentially performed as previously described in Example 6 for Study PD1035 and Figure 14B, but in this case the mice were monitored over time and sacrificed only when either they showed signs of distress, as defined in the government-approved animal protocol, or when tumors exceeded the endpoint defined by a predetermined tumor size of 2000mm³. The test articles (Protein #7 (with a N-terminal His-tag; AS598) and a non-FAP binding variant of Protein #7 (AS608; SEQ ID NO: 67 with a N-terminal His-tag (SEQ ID NO: 57)) as a negative control molecule) were administered to the tumor-bearing mice according to the predetermined regimen as shown in **Table 12**. Tumor growth was monitored every 3 to 4 days by caliper measurement until the individual mice were terminated due to the ethical tumor volume limit.

Table 12. Study design - experimental groups

Group	N	Inoculum (s.c.)	Treatment	Dose (mg/kg)	Dosing Route	Treatment Schedule
1	8	MC38-FAP	Vehicle		i.p.	DR0-4-8
2	5		AS608	5 mg/kg	i.p.	DR0-4-8
3	8		AS598	5 mg/kg	i.p.	DR0-4-8

N: animal number

Tumor inoculation and randomization: Under standard isoflurane anesthesia, mice were inoculated subcutaneously into the flank with 9×10^6 MC38-FAP tumor cells in 0.2 mL of PBS. When tumors reached the average size of approximately 300 mm³, mice were randomized as previously described.

Tumor measurement: Tumor measurement was performed twice a week post tumor inoculation. The length and the width of the tumor was measured with calipers. The tumor volume was calculated with the formula: $V = (L \times W \times W \times \pi) / 6$

Observations and Data Collection: At the time of routine monitoring, the animals were checked for any effects of tumor growth and treatments on behavior such as mobility, food and water consumption, body weight gain/loss and any other abnormalities. Mortality and observed clinical signs were recorded for individual animals.

Results and Conclusions

The results provide evidence that Protein #7 was able to inhibit and reject MC38-FAP tumors and to confer long-term protection against tumor relapse. MC38-FAP tumors were no longer detectable approximately 20 days after the last treatment with Protein #7 (**Figure 18A**). Furthermore, the mice treated with Protein #7 survived for at least 200 days post-inoculation (i.e. the entire measurement period), demonstrating that the anti-tumor effect of Protein #7 was durable and long-term (**Figure 18B**). In contrast, MC38-FAP tumors treated with the negative control, which binds CD40 target, but not FAP target, progressed similarly to the vehicle group (**Figure 18A**) and the mice died or had to be sacrificed at a similar rate as in the vehicle group (**Figure 18B**). These data confirmed the FAP-dependency of the anti-tumor mechanism of action of Protein #7 in vivo. All together, these data provided evidence that a multi-specific binding protein of the invention can elicit strong and durable anti-tumor activity in a FAP-dependent manner in the MC38-FAP tumor mouse model.

Example 11: Induction of protective anti-tumor immunological memory by a multi-specific binding protein of the invention

Mice bearing well-established MC38-FAP tumors were able to reject tumors to undetectable size after treatment with Protein #7, as shown in Figure 18. The same tumor free mice were re-challenged with MC38-FAP tumor cells in order to study possible anti-tumor immunological memory induced by Protein #7.

Materials and Methods

Tumor experiment: The tumor experiment and treatments were performed essentially as previously described in Example 6 for Study PD1035 and Figure 14B and in Example 10 for Figure 18B, and mice treated with Protein #7 were monitored for about 120 days before being re-challenged with MC38-WT or MC38-FAP tumor cells (1 or 9 x10⁶ cell/mouse, respectively, in 0,2 mL of PBS). In parallel, naïve mice were challenged with exactly the same tumor cells. Tumor growth was monitored every 3 to 4 days by caliper measurement until the individual mice were terminated due to the ethical tumor volume limit.

Results and Conclusions

After an initial and weak tumor growth, all mice previously treated with Protein #7 rejected completely the re-challenged MC38-FAP tumors (**Figure 19A**), suggesting the presence of an immunological anti-tumor memory response. Interestingly, the same phenomenon was observed in mice re-challenged with MC38-WT tumor cells (Figure 19A), suggesting that Protein #7 contributed to the establishment of an immunological memory, which was not limited to the FAP-related antigens, but was more broadly directed to tumor antigens. Furthermore, the naïve control groups, inoculated either with MC38-WT or MC38-FAP tumor cells, showed clearly the tumorigenic capacity of the used tumor cells (**Figure 19B**), thus confirming that the absence of tumor growth in the re-challenged mice shown in Figure 19A was due to an immune-mediated anti-tumor response. In conclusion, experimental evidence was obtained suggesting that a multi-specific binding protein of the invention has the capacity to induce protective anti-tumor immunological memory and furthermore that this immunological memory is not limited to the FAP-related antigens in the MC38-FAP tumor model.

Example 12: X-ray structure analysis of a complex of human tumor necrosis factor receptor superfamily member 5 (hCD40) bound by a CD40-specific binding protein

[231] The aim of this study was to generate and analyze complexes of recombinant hCD40 bound by a CD40-specific binding protein of the invention using X-ray crystallography. The CD40-specific binding protein used for this structural analysis was the DARPin® protein with the amino acid sequence of SEQ ID NO: 3.

Materials and Methods

Protein production. hCD40 was expressed in Hi5 cells in the presence of tunicamycin to block glycosylation. Protein from culture supernatant was purified via HIS-Trap, THB-digest, negative HIS-Trap and SEC. Purified hCD40 was mixed in a 1:1.2 ratio with CD40-specific DARPin® protein (SEQ ID NO: 3). Excess of DARPin® protein was removed from the hCD40 : DARPin® protein complex via SEC in 10 mM HEPES/NaOH pH 7, 150 mM NaCl. Sample was concentrated to 36.7 mg/ml. This procedure yielded homogenous protein with a purity greater than 95% as judged from Coomassie stained SDS-PAGE.

Crystallisation. The purified protein was used in crystallisation trials employing both, a standard screen with approximately 1200 different conditions, as well as crystallisation conditions identified using literature data. Conditions initially obtained were optimised using standard strategies, systemically varying parameters critically influencing crystallisation, such as temperature, protein concentration,

drop ratio, and others. These conditions were also refined by systematically varying pH or precipitant concentrations.

Final crystallisation condition:

30 %w/v PEG 4K

0.24 M LiSO₄

0.1 M Tris pH=8.50

0.35 M NaBr

[232] *Data collection and processing.* Crystals were flash-frozen and measured at a temperature of 100 K. The X-ray diffraction data were collected from complex crystals of hCD40 bound to the ligand DARPin® protein (SEQ ID NO: 3) at the SWISS LIGHT SOURCE (SLS, Villigen, Switzerland) using cryogenic conditions. The crystals belong to space group C 2. Data were processed using the programmes autoPROC, XDS and autoPROC, AIMLESS. The data collection and processing statistics for the DARPin® protein are listed in **Table 13** below.

Table 13

Ligand	DARPin hC23
X-ray source	PXII/X10SA (SLS ¹)
Wavelength [Å]	0.9998
Detector	EIGER
Temperature [K]	100
Space group	C 2
Cell: a; b; c; [Å]	193.67; 59.56; 81.84
α; β; γ; [°]	90.0; 107.0; 90.0
Resolution [Å]	2.29 (2.33-2.29)
Unique reflections	39800 (1932)
Multiplicity	4.3 (4.4)
Completeness [%]	97.8 (97.3)
R _{pim} [%] ⁶	4.3 (61.8)
R _{sym} [%] ³	7.8 (114.9)
R _{meas} [%] ⁴	8.9 (130.9)
CC1/2 [%]	99.70 (43.00)
Mean(I)/sd ⁵	10.1 (1.3)

¹ SWISS LIGHT SOURCE (SLS, Villigen, Switzerland)² values in parenthesis refer to the highest resolution bin.

$$^3 R_{sym} = \frac{\sum_h \sum_i^{n_h} |\hat{I}_h - I_{h,i}|}{\sum_h \sum_i^{n_h} I_{h,i}} \text{ with } \hat{I}_h = \frac{1}{n_h} \sum_i^{n_h} I_{h,i}$$

where $I_{h,i}$ is the intensity value of the i th measurement of h

$$^4 R_{meas} = \frac{\sum_h \sqrt{\frac{n_h}{n_h-1}} \sum_i^{n_h} |\hat{I}_h - I_{h,i}|}{\sum_h \sum_i^{n_h} I_{h,i}} \text{ with } \hat{I}_h = \frac{1}{n_h} \sum_i^{n_h} I_{h,i}$$

where $I_{h,i}$ is the intensity value of the i th measurement of h ⁵ calculated from independent reflections

$$^6 \text{ Precision-indicating } R_{pim} = \frac{\sum_h \sqrt{\frac{1}{(N-1)}} |I_{hi} - \langle I_h \rangle|}{\sum_h \langle I_h \rangle}$$

Structure modelling and refinement. The phase information necessary to determine and analyse the structure was obtained by molecular replacement. A previously solved structure of hCD40 was used as a search model. Subsequent model building and refinement was performed according to standard protocols with COOT and the software package CCP4, respectively. For calculation of the free R-factor, a measure to cross-validate the correctness of the final model, about 4.9% of measured reflections were excluded from the refinement procedure (see **Table 14** below). TLS refinement (using REFMAC5, CCP4) was carried out, which resulted in lower R-factors and higher quality of the electron density map.

Automatically generated local NCS restraints were applied (keyword "ncsr local" of newer REFMAC5 versions). The ligand parameterisation and generation of the corresponding library files were carried out with GRADE (Global Phasing Limited). The water model was built with the "Find waters"-algorithm of COOT by putting water molecules in peaks of the $F_o - F_c$ map contoured at 3.0 with REFMAC5 and checking all waters with validation tool of COOT. The criteria for the list of suspicious waters were: B-factor greater $80 \text{ \AA}^2$, $2F_o - F_c$ map less than 1.2σ , distance to closest contact less than $2.3 \text{ \AA}$, or more than $3.5 \text{ \AA}$. The suspicious water molecules and those in the ligand binding site (distance to ligand less than $10 \text{ \AA}$) were checked manually. The Ramachandran Plot of the final model shows 92.2 % of all residues in the most favoured region, 7.8 % in the additionally allowed region, and 0.0 % in the generously allowed region. No residues are found in the disallowed region (**Table 14**). Statistics of the final structure and the refinement process are listed in **Table 14** below.

Table 14¹

Ligand	DARPin hc23
Resolution [$\text{\AA}$]	92.61-2.29
Number of reflections (working /test)	37835 / 1962
R_{cryst} [%]	21.9
R_{free} [%] ²	25.1
Total number of atoms:	
Protein	4894
Water	208
Sodium	2
Deviation from ideal geometry: ³	
Bond lengths [$\text{\AA}$]	0.014
Bond angles [$^\circ$]	1.59
Bonded B's [$\text{\AA}^2$] ⁴	2.9
Ramachandran plot: ⁵	
Most favoured regions [%]	92.2
Additional allowed regions [%]	7.8
Generously allowed regions [%]	0.0
Disallowed regions [%]	0.0

¹ Values as defined in REFMAC5, without sigma cut-off

² Test-set contains 4.9% of measured reflections

³ Root mean square deviations from geometric target values

⁴ Calculated with MOLEMAN

⁵ Calculated with PROCHECK

[233] Results

The structure was solved and refined to a final resolution of $2.29 \text{ \AA}$. The structure analyses using X-ray crystallography revealed that the DARPin® protein (SEQ ID NO: 3) bound to cysteine-rich domain (CRD) 1 (amino acids 23-59) of the CD40 receptor (SEQ ID NO: 51) and that it bound to the CD40 receptor at one side opposite to the binding site of the CD40 ligand (CD40L), indicating the absence of direct binding site competition between the DARPin protein and the CD40L (Fig. 21A and 21B). The CRD1 domain of the CD40 receptor is located distant from the cell membrane.

[234] It has been reported that potent CD40 agonist antibodies bind membrane distal epitopes of the CD40 receptor (Yu *et al.*, *Cancer Cell* **33**, 664-675 e664 (2018)). Similarly, the X-ray crystallography study described in this Example showed that the CD40-specific binding protein (SEQ ID NO: 3) interacts with the CRD1 of CD40 receptor, distant from the cell membrane. As it has already been suggested for CD40 agonist antibodies, a more cell membrane-distant epitope may lead to less steric hindrance, allowing better access to a CD40-specific binding protein comprising a localizer molecule (such as a recombinant protein comprising the amino acid sequence of SEQ ID NO: 5 or SEQ ID NO: 6) and a more efficient clustering, and consequently a more efficient activation, of the CD40 receptor. Moreover, the region of interaction between the CD40-specific binding protein (SEQ ID NO: 3) and CRD1 was shown to be opposite to the binding site of CD40L, suggesting the absence of direct binding competition between the binding protein of the invention and the CD40L. A compound that does not compete for CD40L may have an additive or synergistic effect with the ligand, resulting in a better activation of the receptor (see, e.g., Yu *et al.*, *loc. cit.*; Challa *et al.*, *Allergy* **54**, 576-583 (1999); Pound *et al.*, *Int Immunol* **11**, 11-20 (1999)).

[235] The specification is most thoroughly understood in light of the teachings of the references cited within the specification. The embodiments within the specification provide an illustration of embodiments of the invention and should not be construed to limit the scope of the invention. The skilled artisan readily recognizes that many other embodiments are encompassed by the invention. All publications, patents, and GenBank sequences cited in this disclosure are incorporated by reference in their entirety. To the extent the material incorporated by reference contradicts or is inconsistent with this specification, the specification will supersede any such material. The citation of any references herein is not an admission that such references are prior art to the present invention.

[236] Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following embodiments.

CLAIMS

1. A recombinant protein comprising:
a first ankyrin repeat domain that specifically binds serum albumin, a second ankyrin repeat domain that specifically binds fibroblast activation protein (FAP), a third ankyrin repeat domain that specifically binds CD40, and a fourth ankyrin repeat domain that specifically binds CD40,
wherein said ankyrin repeat domains are arranged, from the N-terminus to the C-terminus, according to the following formula: (serum albumin binding domain) – (FAP-binding domain) – (CD40 binding domain) – (CD40 binding domain).
2. The recombinant protein of claim 1, wherein said FAP binding domain comprises an amino acid sequence that is at least 90% identical to SEQ ID NO: 2, preferably wherein said FAP binding domain comprises the amino acid sequence of SEQ ID NO: 2 or of SEQ ID NO: 8.
3. The recombinant protein of claim 1 or 2, wherein each of said CD40 binding domains independently comprises an amino acid sequence that is at least 90% identical to SEQ ID NO: 3, preferably wherein each of said CD40 binding domains comprises the amino acid sequence of SEQ ID NO: 3.
4. The recombinant protein of any one of claims 1 to 3, wherein each of said CD40 binding domains independently comprises Q at position 8, L at position 15, R at position 143, and Q at position 147, wherein the position numbers correspond to the positions in SEQ ID NO: 3.
5. The recombinant protein of any one of claims 1 to 4, wherein said serum albumin binding domain comprises an amino acid sequence that is at least 90% identical to SEQ ID NO: 1, preferably wherein said serum albumin binding domain comprises the amino acid sequence of SEQ ID NO: 1.
6. The recombinant protein of any one of claims 1 to 5, comprising the following formula, from the N-terminus to C-terminus: (serum albumin binding domain) – (linker) – (FAP-binding domain) – (linker) – (CD40 binding domain) – (linker) – (CD40 binding domain), wherein the linker comprises the amino acid sequence of SEQ ID NO: 4.
7. The recombinant protein of any one of claims 1 to 6, wherein said FAP binding domain binds human FAP with a K_D value of or below 100 nM, and/or wherein each of said CD40 binding domains binds human CD40 with a K_D value of or below 100 nM, and/or wherein said serum albumin binding domain binds human serum albumin with a K_D value of or below 100 nM,
8. A recombinant protein comprising an amino acid sequence that is at least 90% identical to SEQ ID NO: 5, wherein said recombinant protein binds human FAP, human CD40, and human serum albumin with a K_D value of or below 100 nM, preferably wherein said recombinant protein comprises the amino acid sequence of SEQ ID NO: 5 or SEQ ID NO: 6.

9. The recombinant protein of any one of claims 1 to 8, wherein said protein has a half maximal effective concentration (EC_{50}) of from about 0.01 nM to about 10 nM, as assessed by an in vitro human B cell activation assay.
10. The recombinant protein of any one of claims 1 to 9, wherein binding of said protein to FAP does not inhibit the prolyl endopeptidase activity of FAP by more than 25%.
11. A nucleic acid encoding the recombinant protein of any one of claims 1 to 10 or an ankyrin repeat domain as defined in any one of claims 1 to 10.
12. A pharmaceutical composition comprising the recombinant protein of any one of claims 1 to 10 or the nucleic acid of claim 11, and optionally a pharmaceutically acceptable carrier or excipient.
13. A host cell comprising the nucleic acid of claim 11.
14. A method for producing the recombinant protein of any one of claims 1 to 10, comprising the step of culturing the host cell of claim 13 under conditions wherein said recombinant protein is expressed.
15. A method of treating a medical condition, the method comprising the step of administering to a subject, preferably a human, in need thereof a therapeutically effective amount of the recombinant protein of any one of claims 1 to 10, the nucleic acid of claim 11, or the pharmaceutical composition of claim 12, preferably wherein said medical condition is cancer, and preferably wherein said cancer is a solid tumor.

FIG. 1

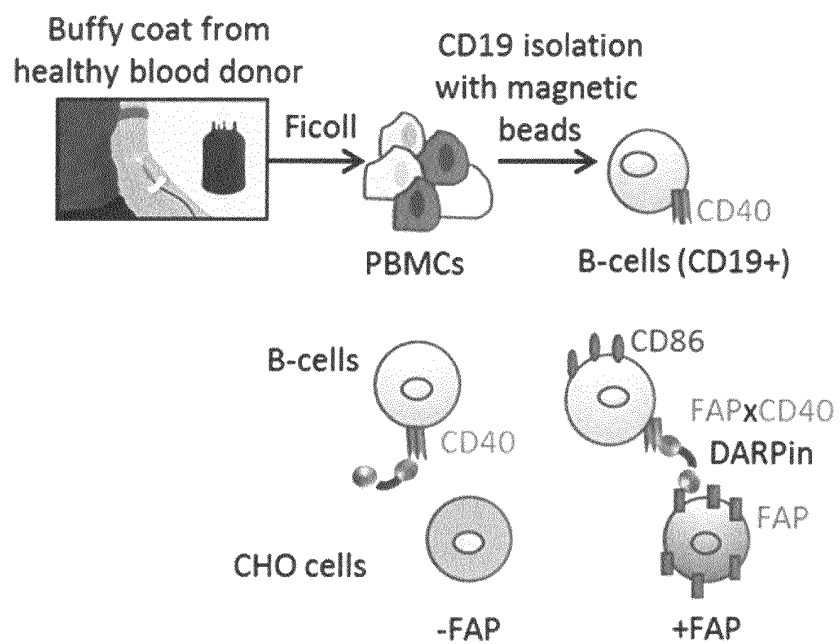


FIG. 2

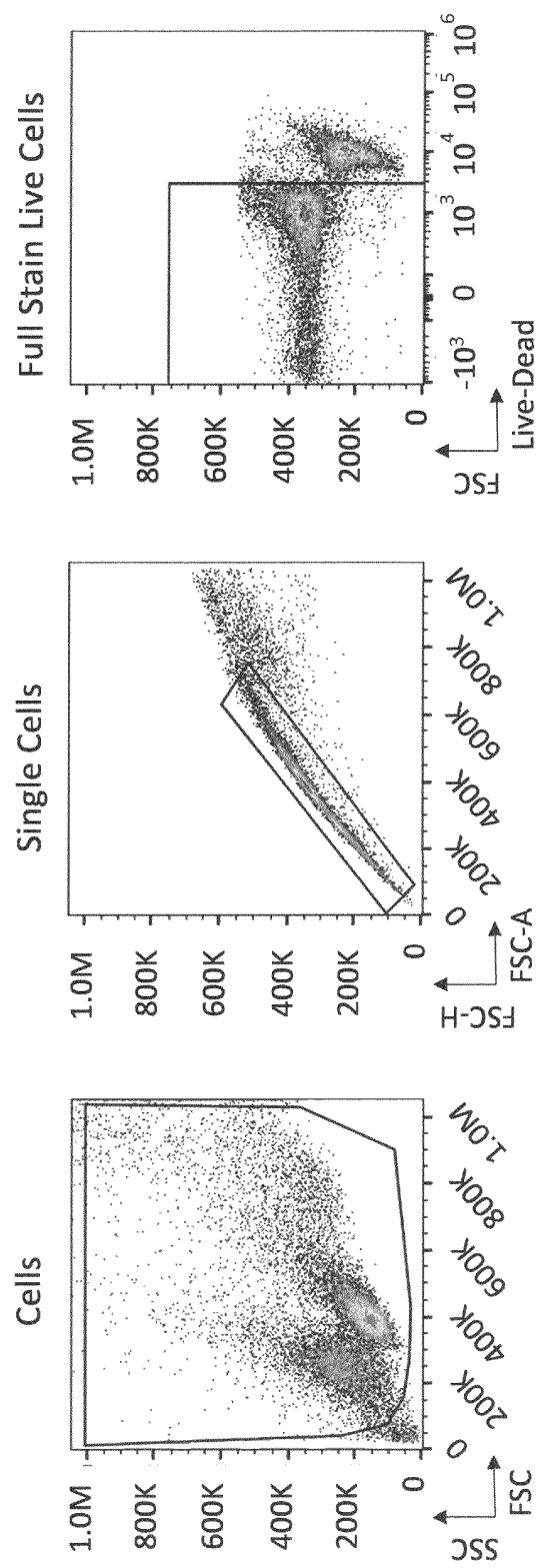


FIG. 2 (cont.)

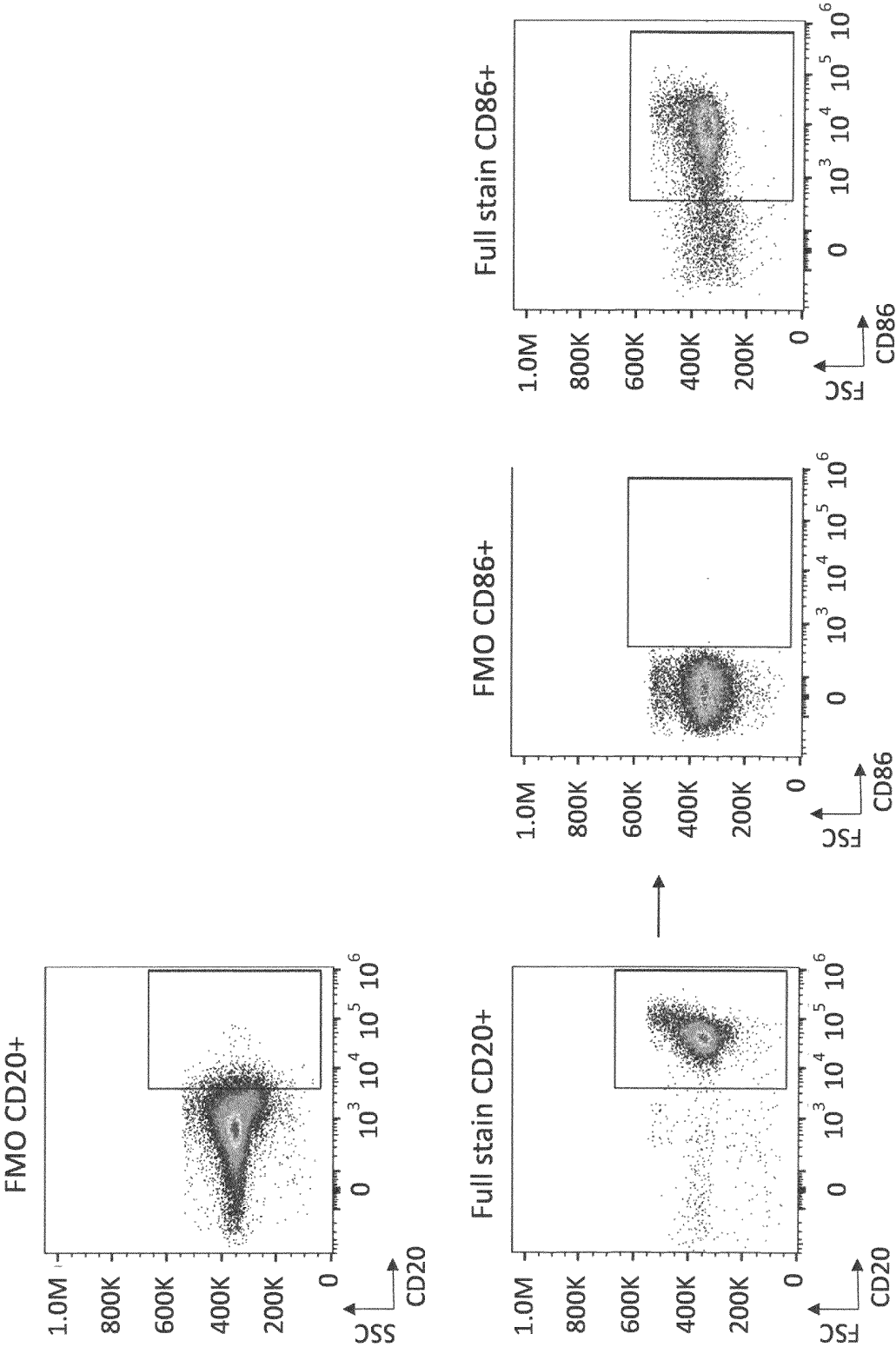


FIG. 3

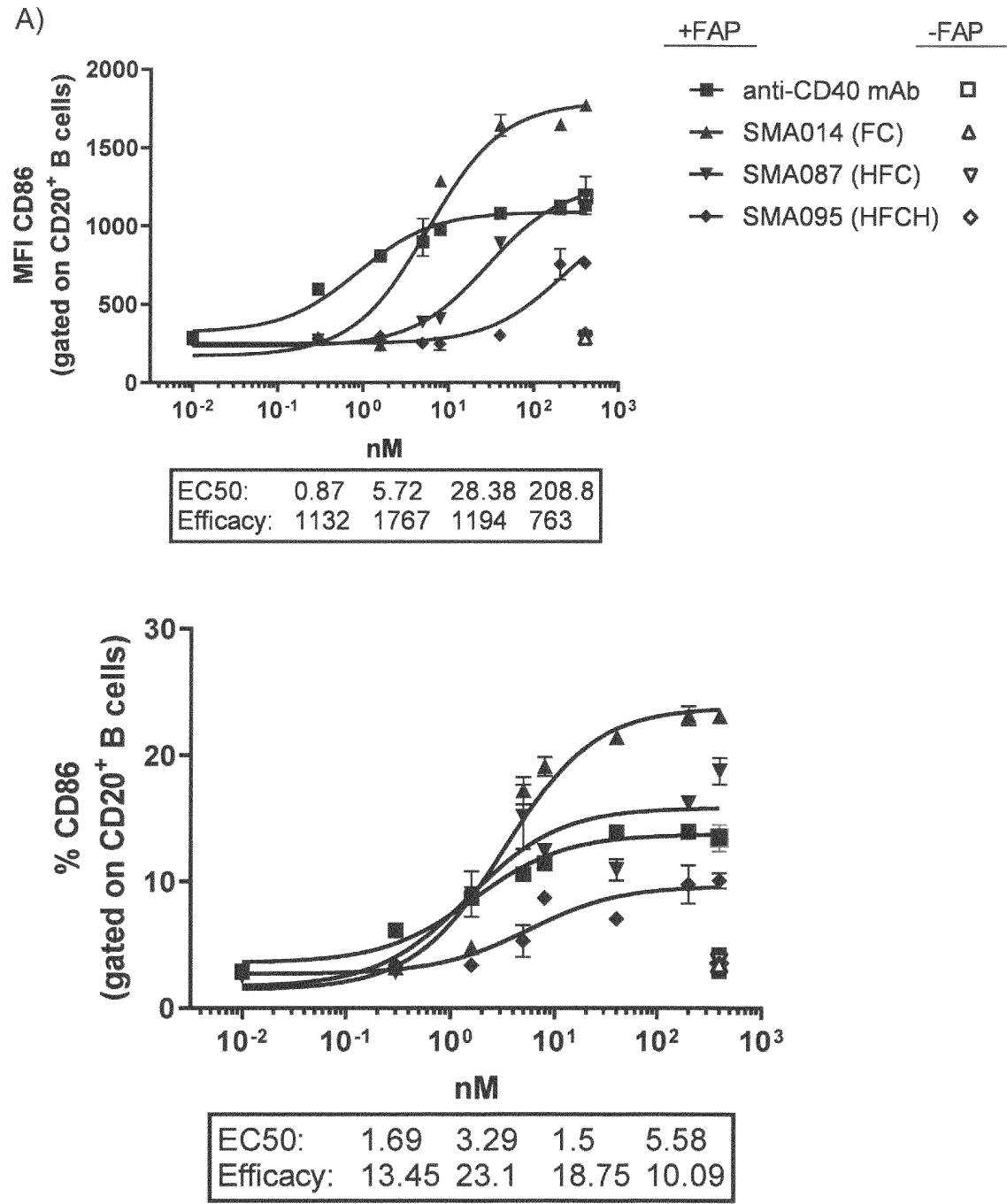


FIG. 3 (cont.)

B)

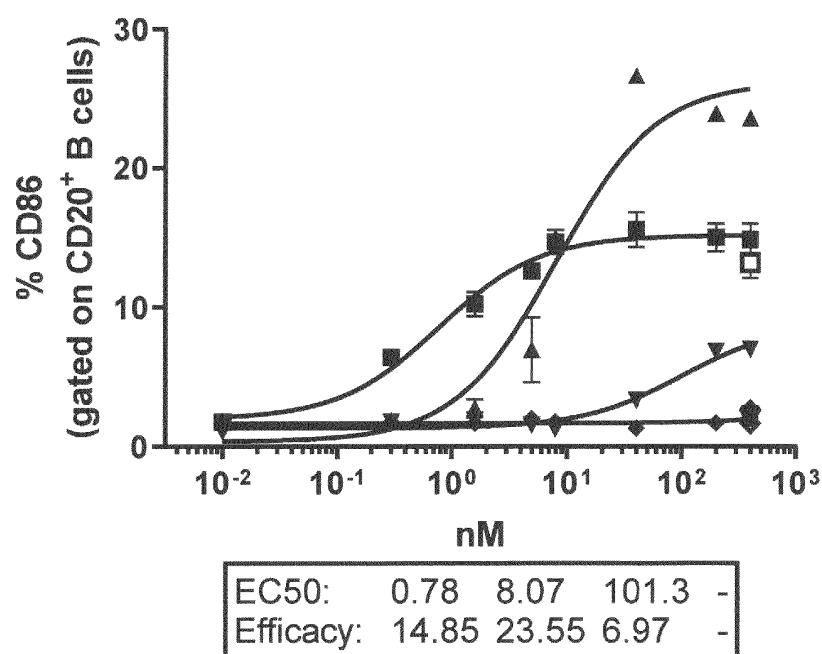
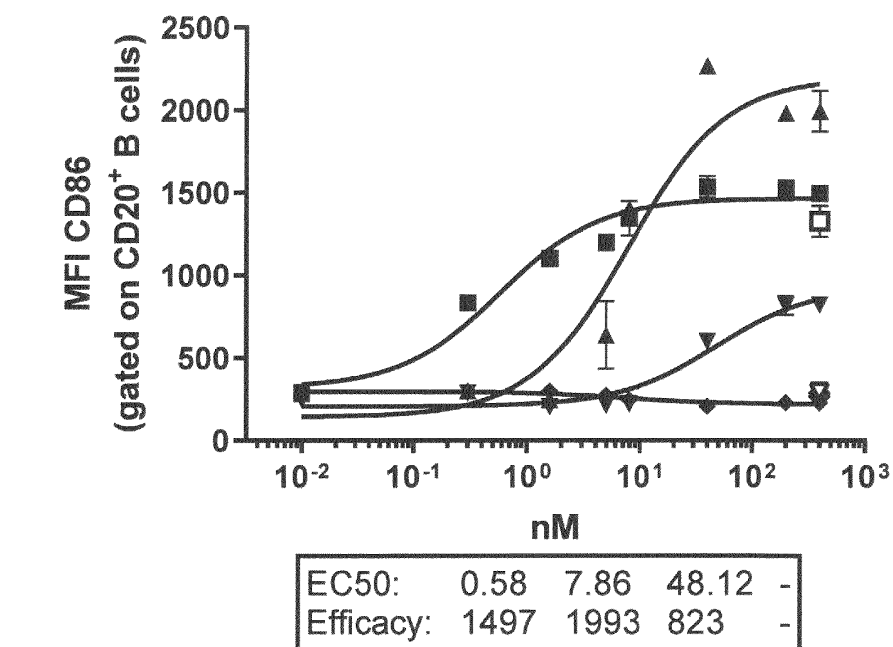


FIG. 4

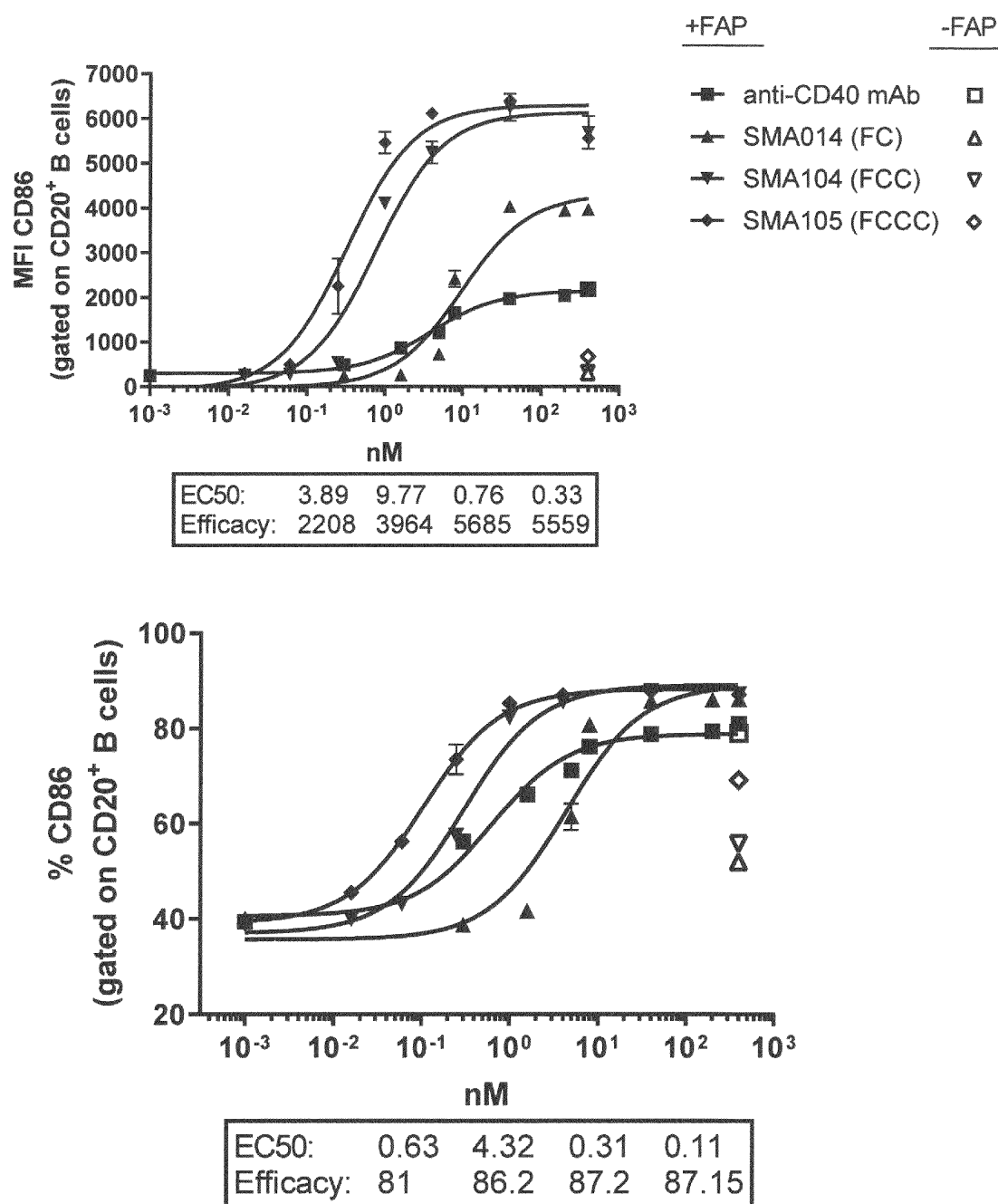


FIG. 5

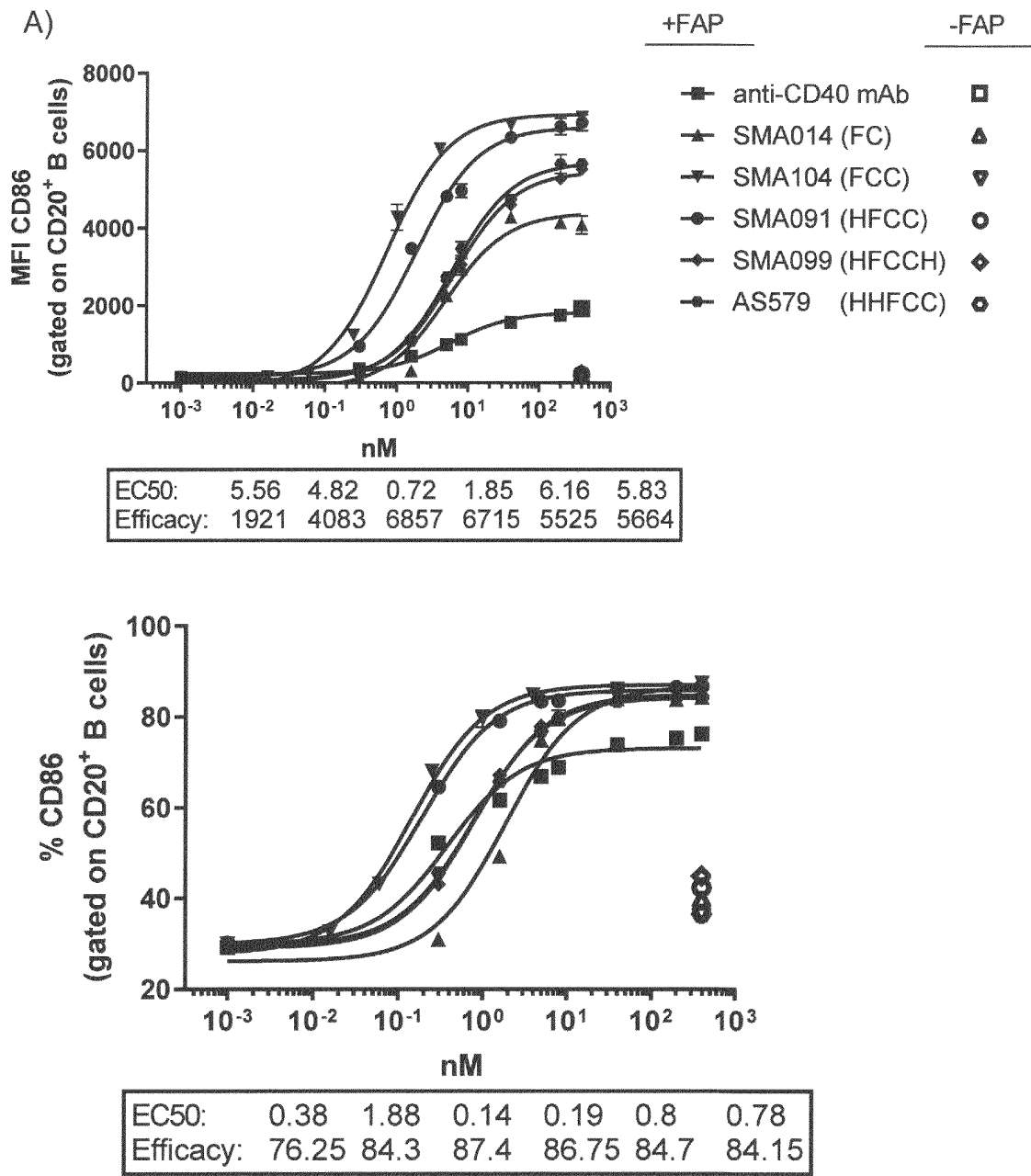


FIG. 5 (cont.)

B)

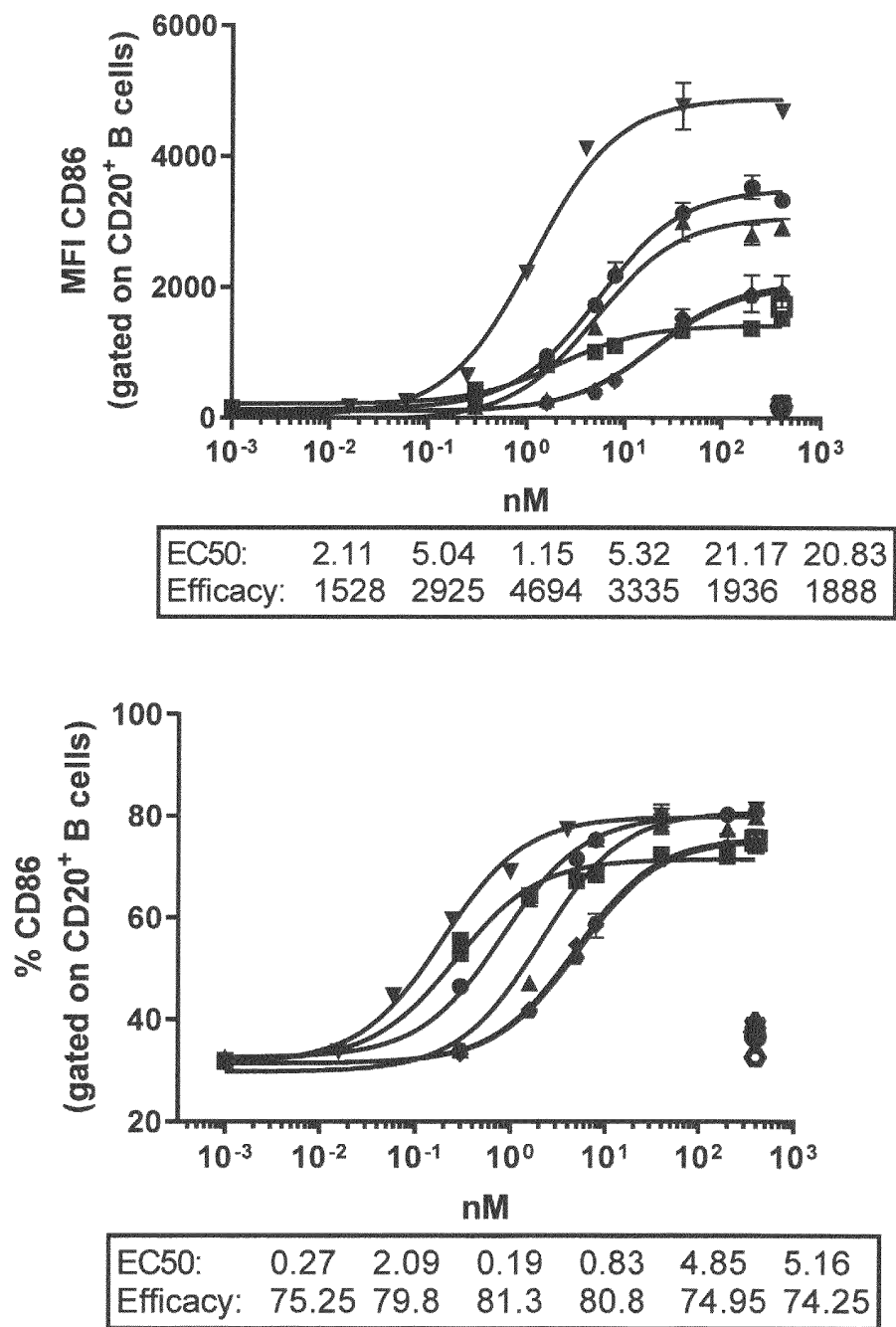


FIG. 6

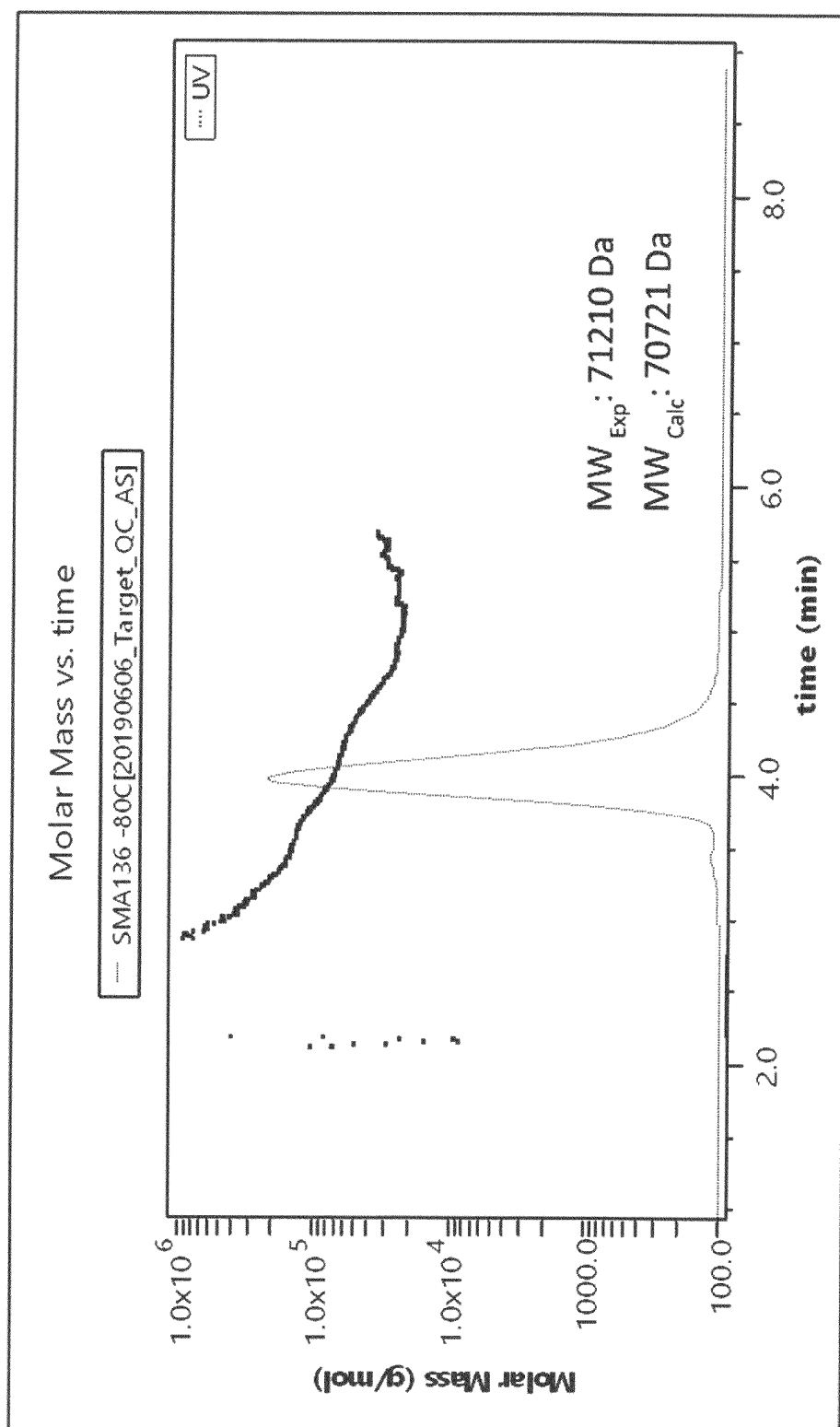


FIG. 7

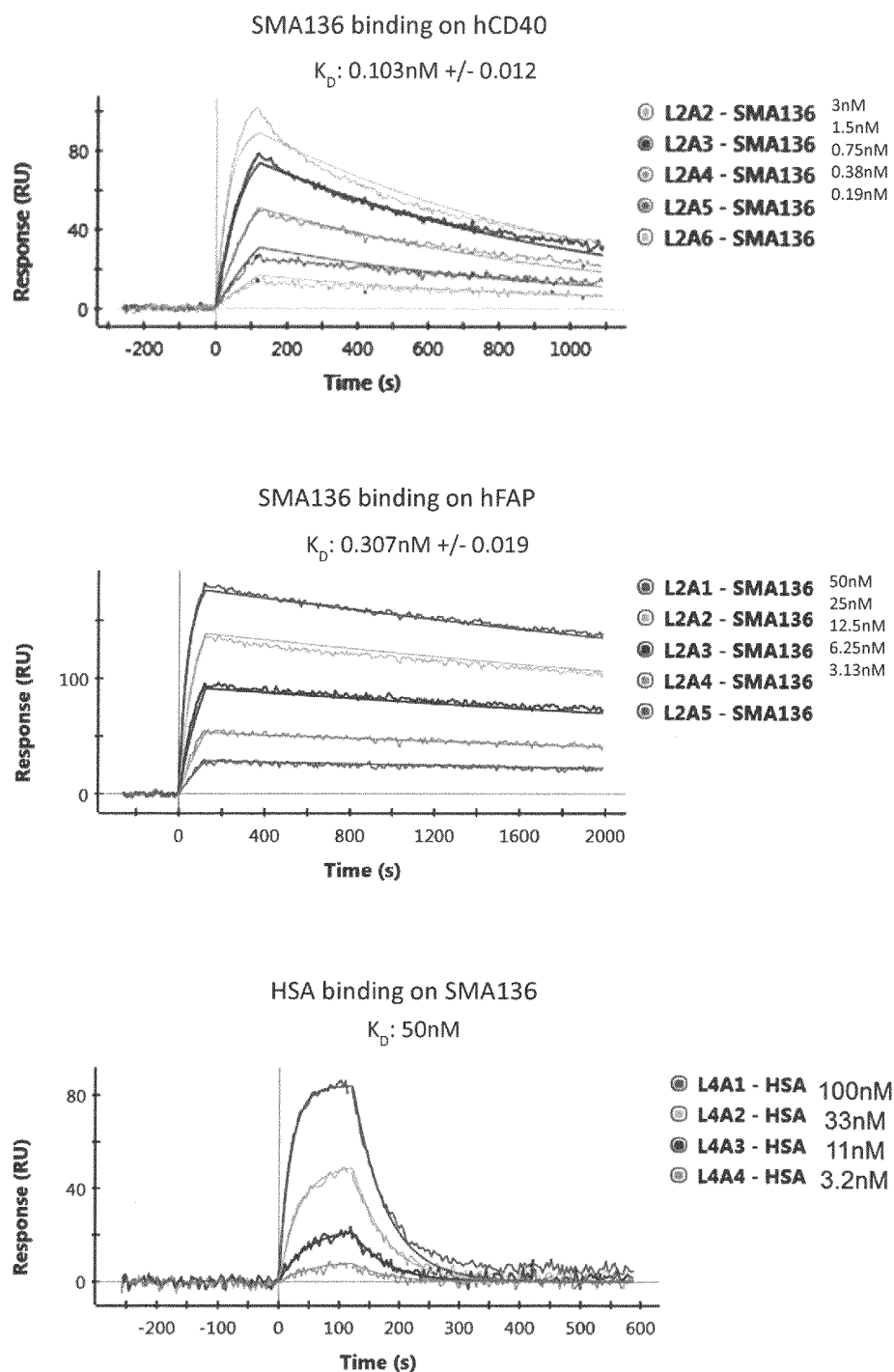


FIG. 8

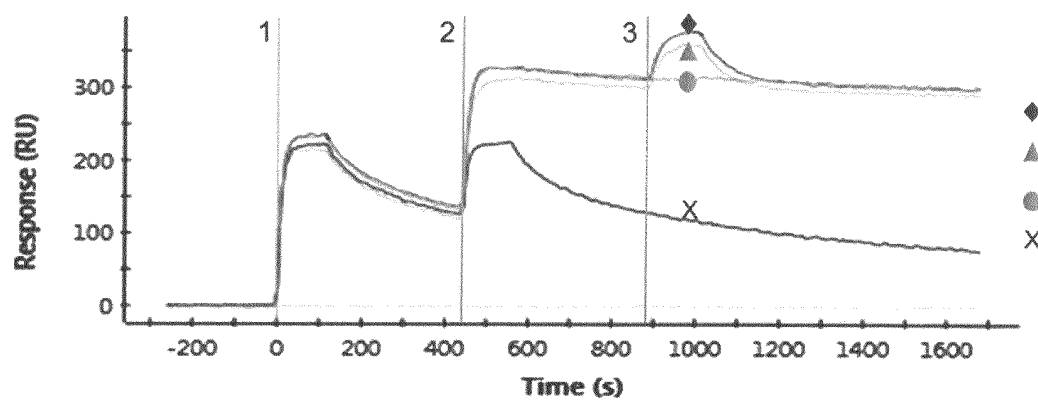


FIG. 9

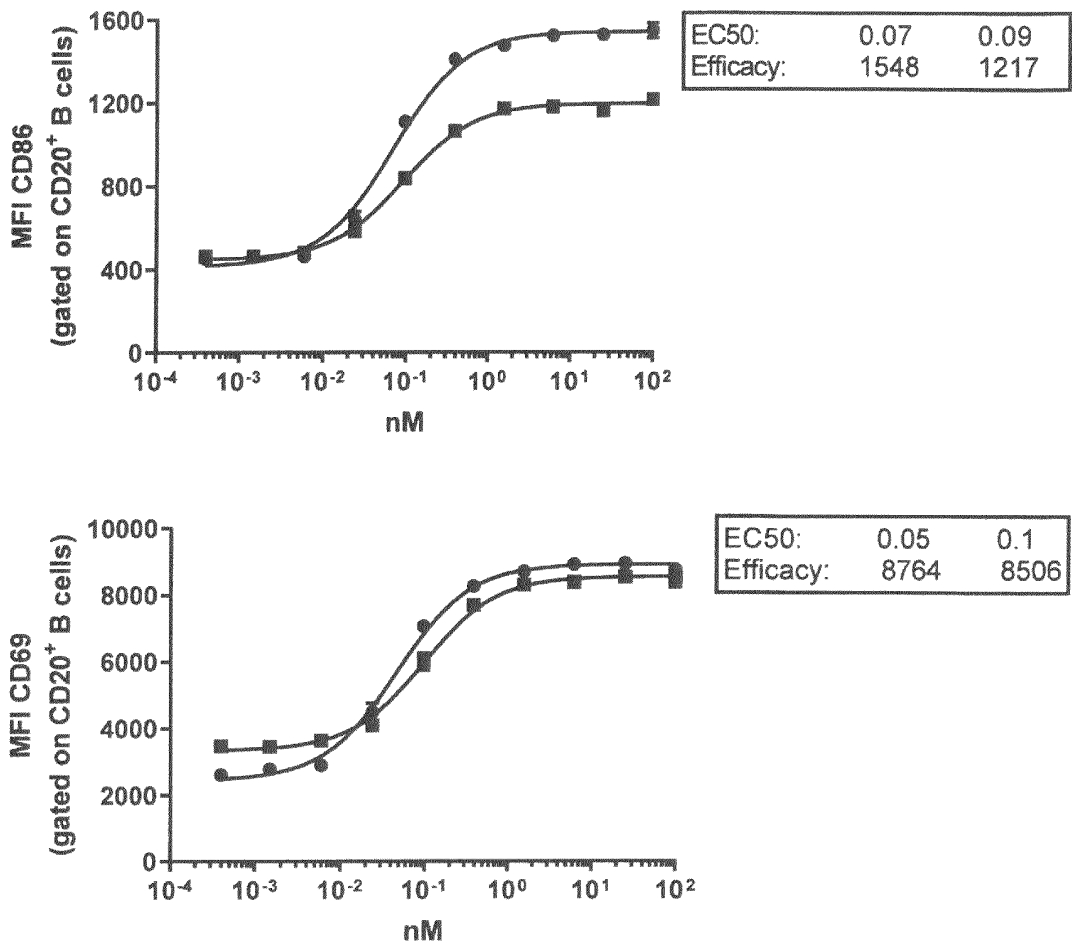


FIG. 9 (cont.)

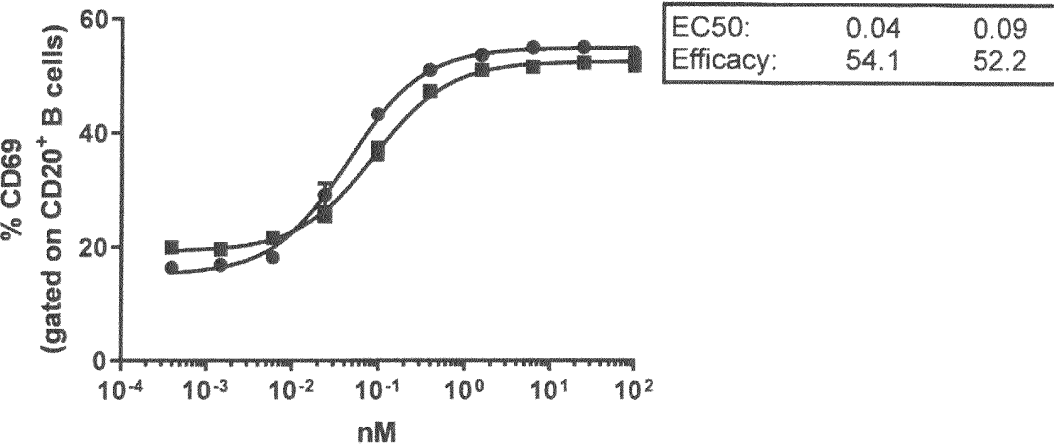
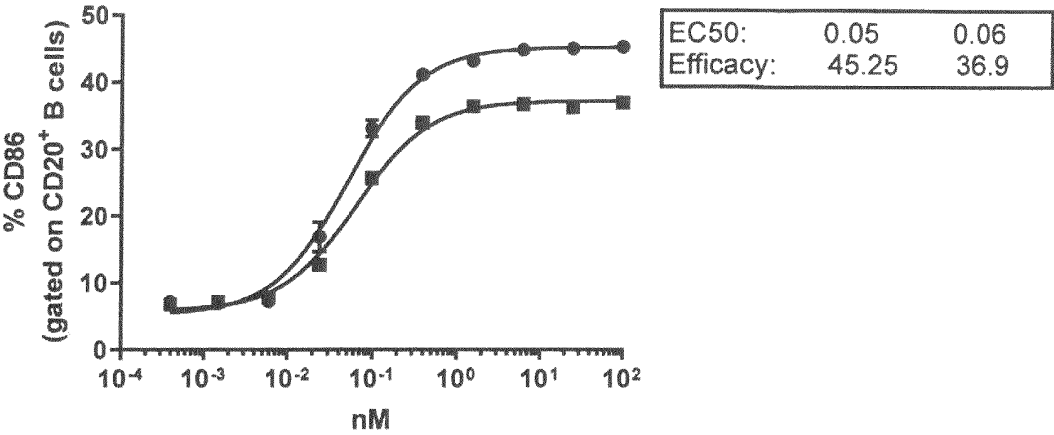


FIG. 10

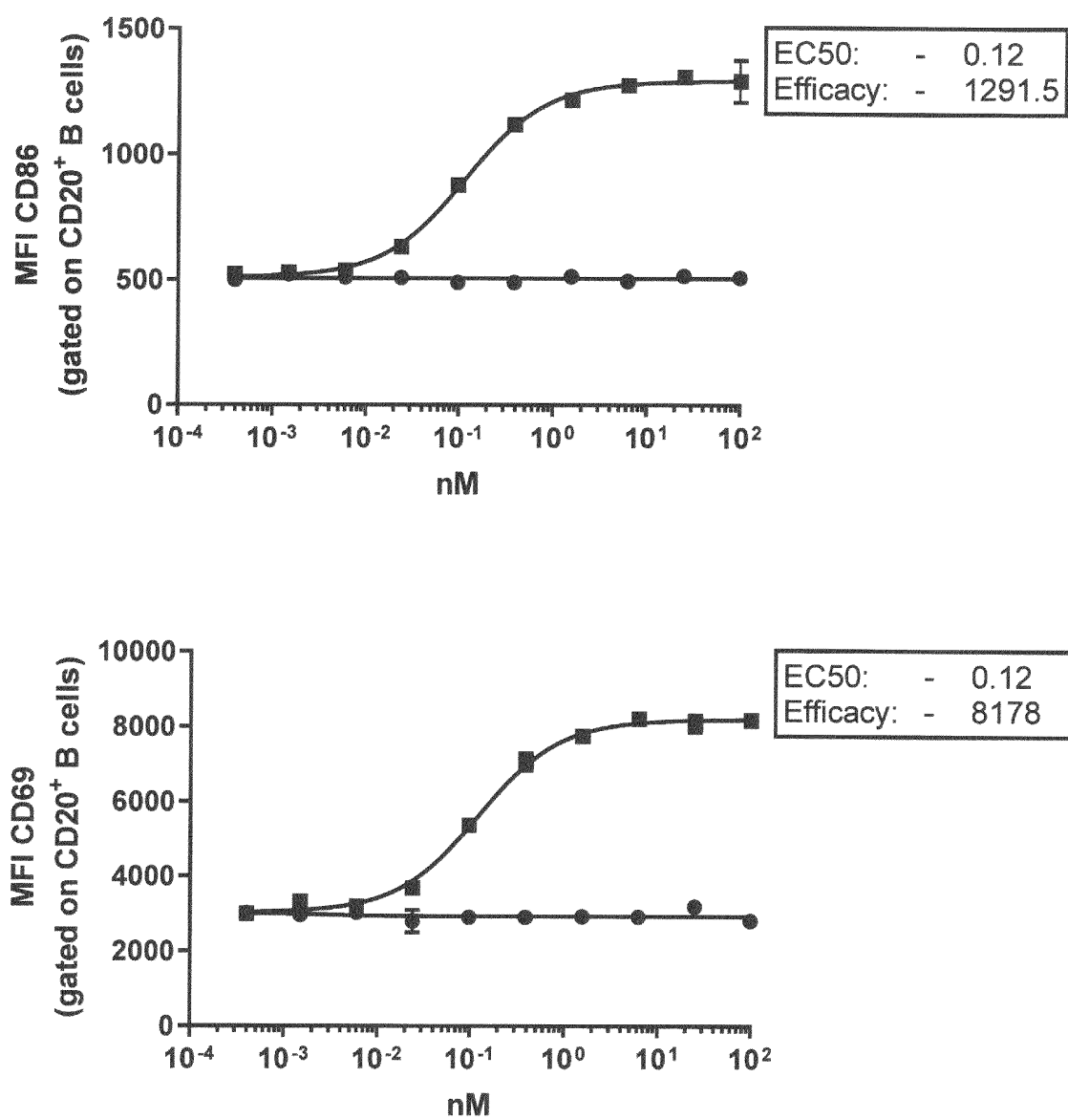


FIG. 10 (cont.)

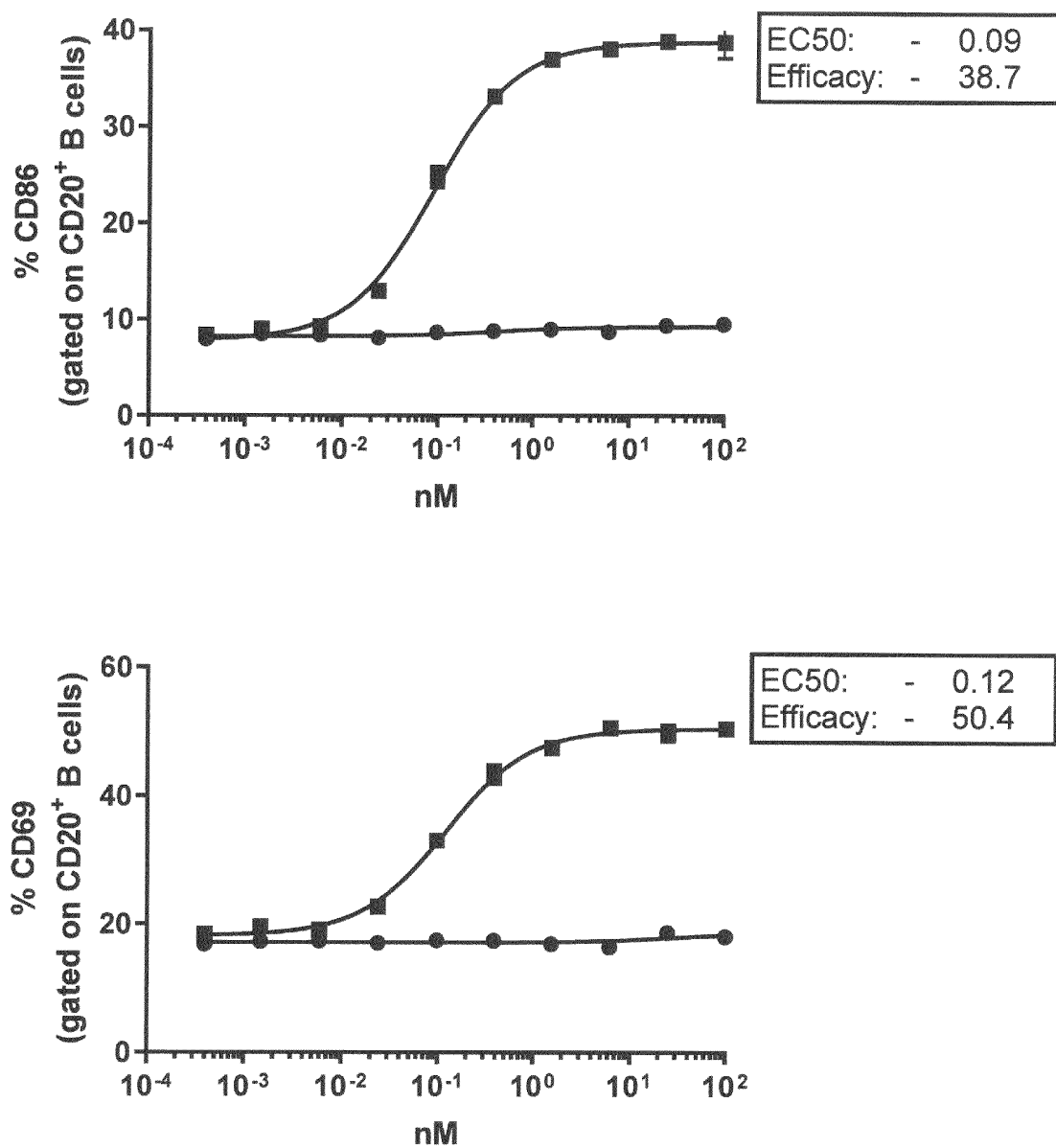


FIG. 11

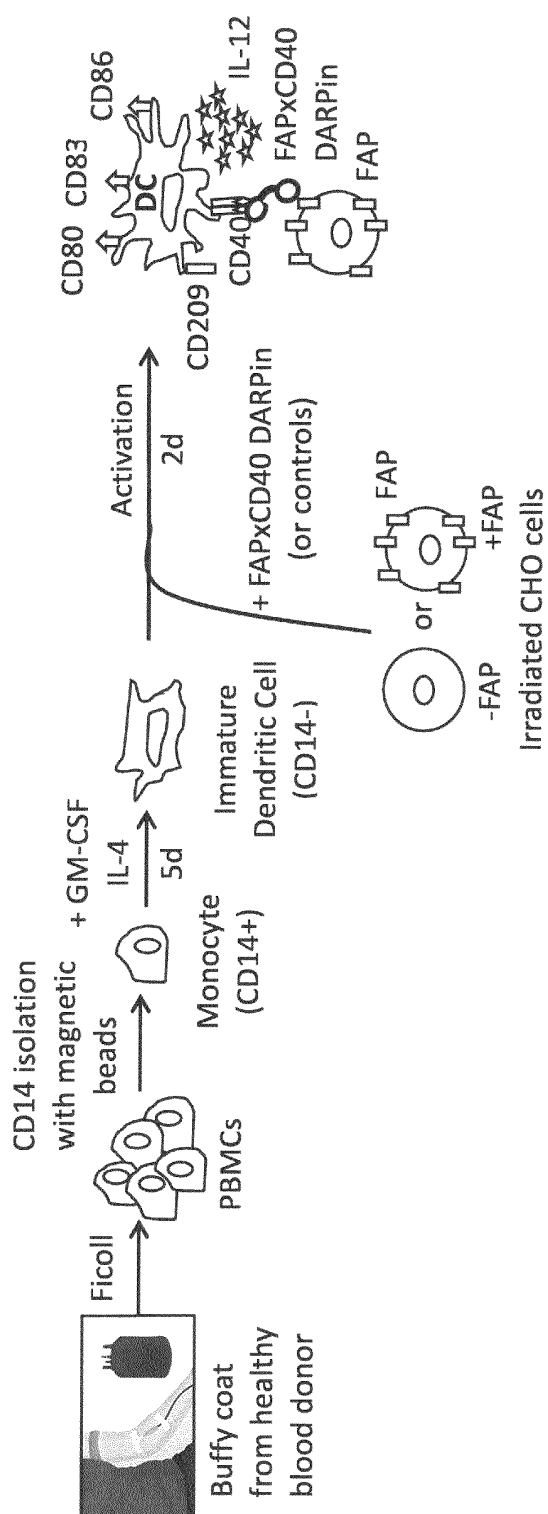
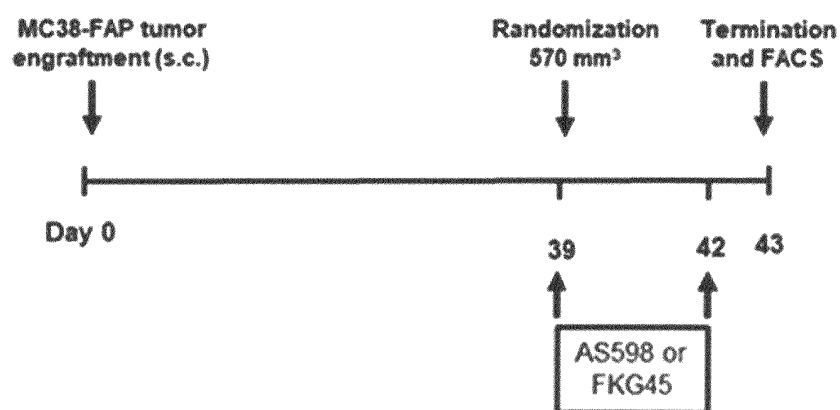


FIG. 12

A Early time point termination for FACS analysis

Study PD1033



Study PD1038

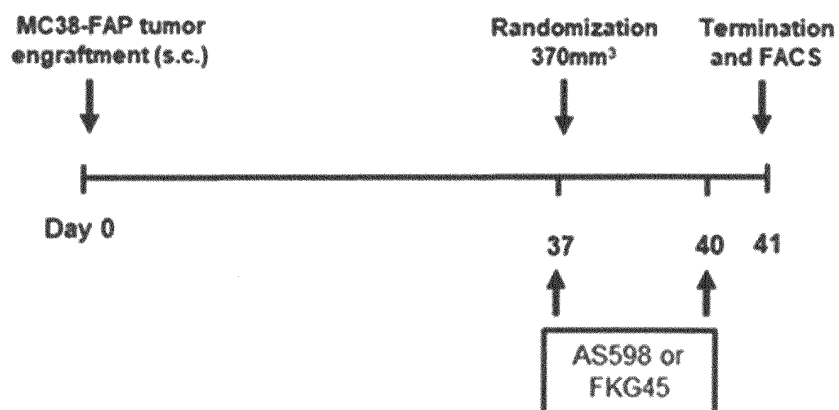
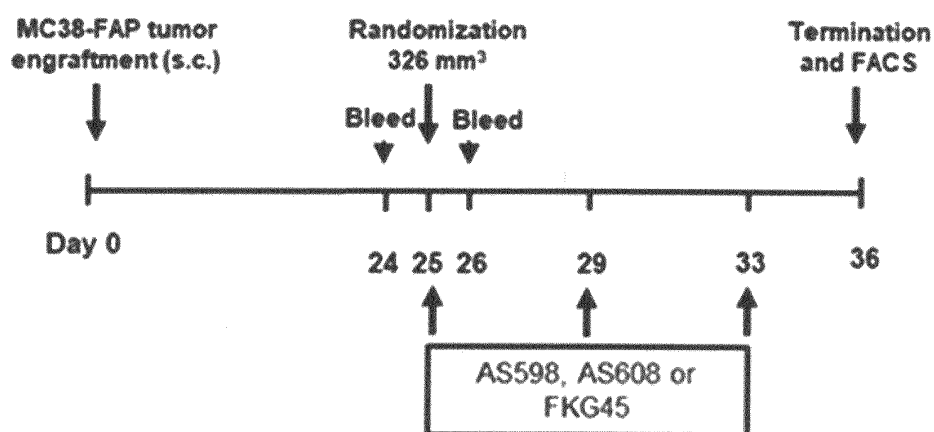


FIG. 12 (cont.)

B Tumor efficacy studies+ termination FACS

Study PD1032



Study PD1035

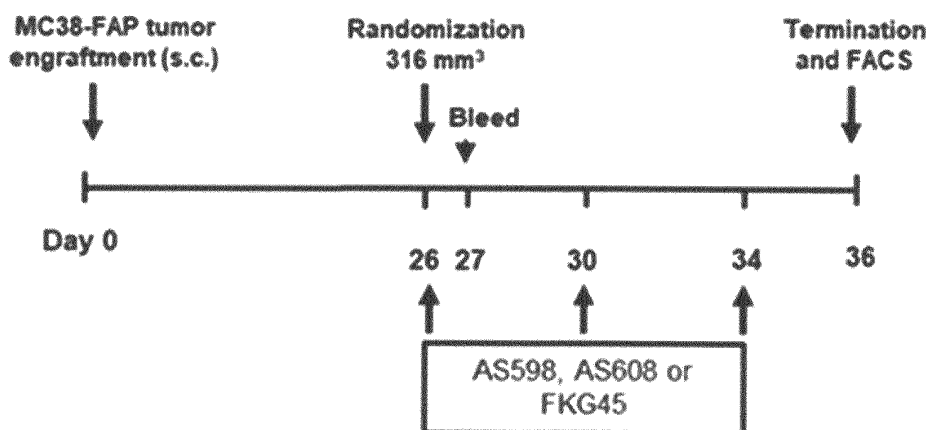


FIG. 13

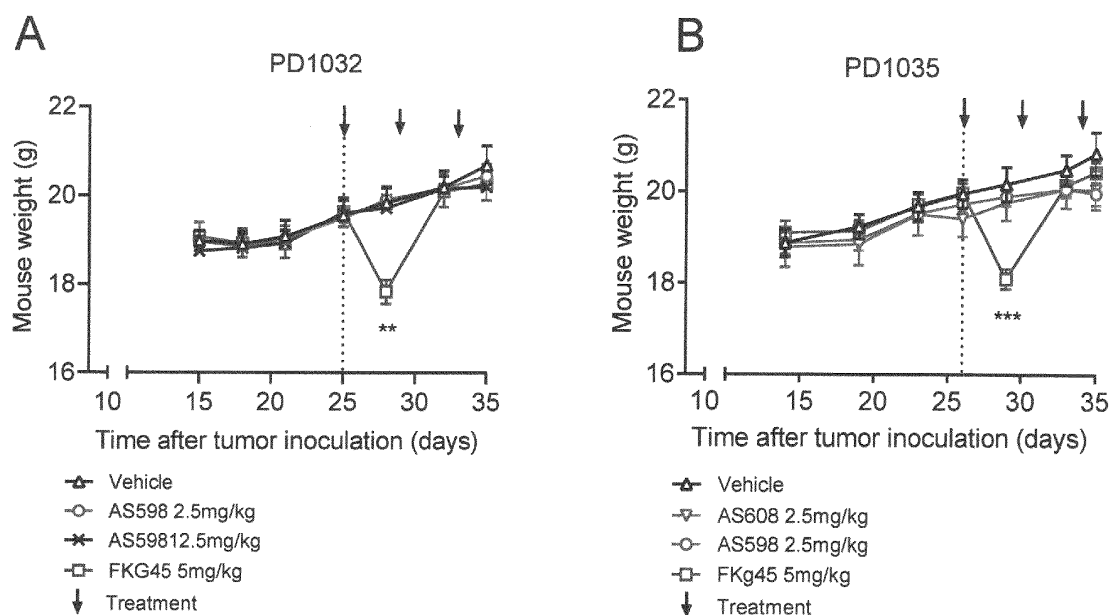


FIG. 14

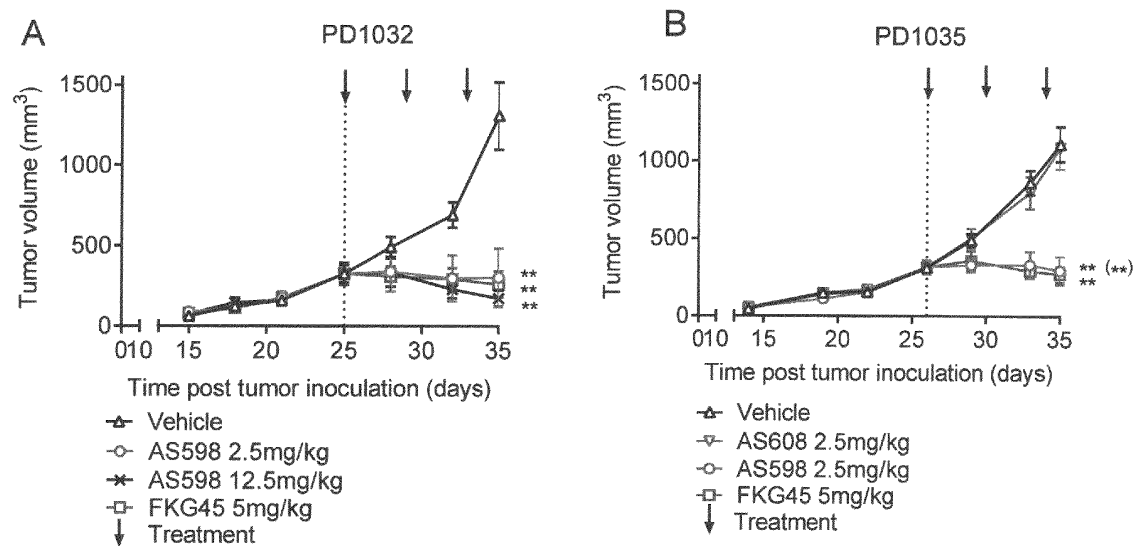


FIG. 15

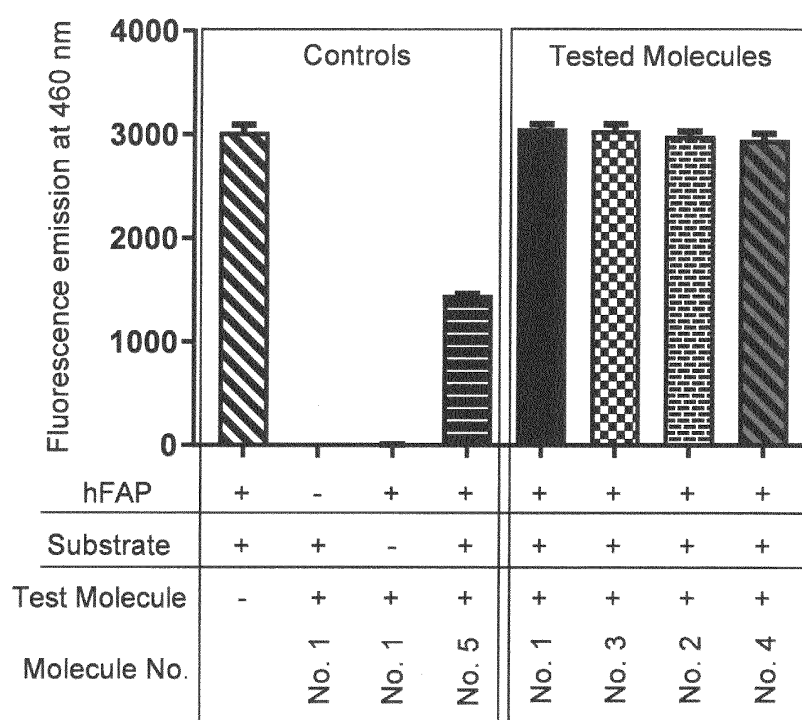


FIG. 16

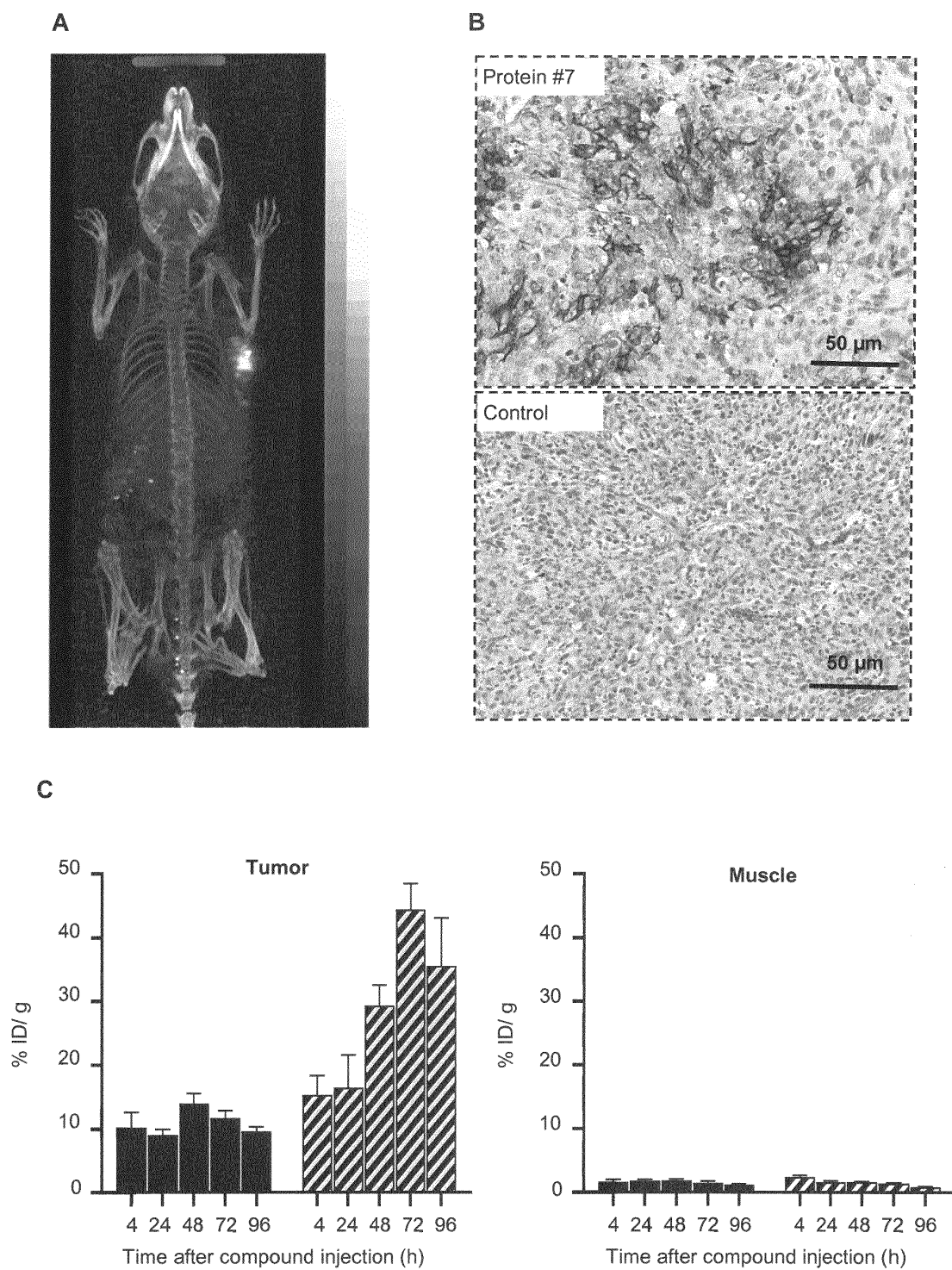


FIG. 17

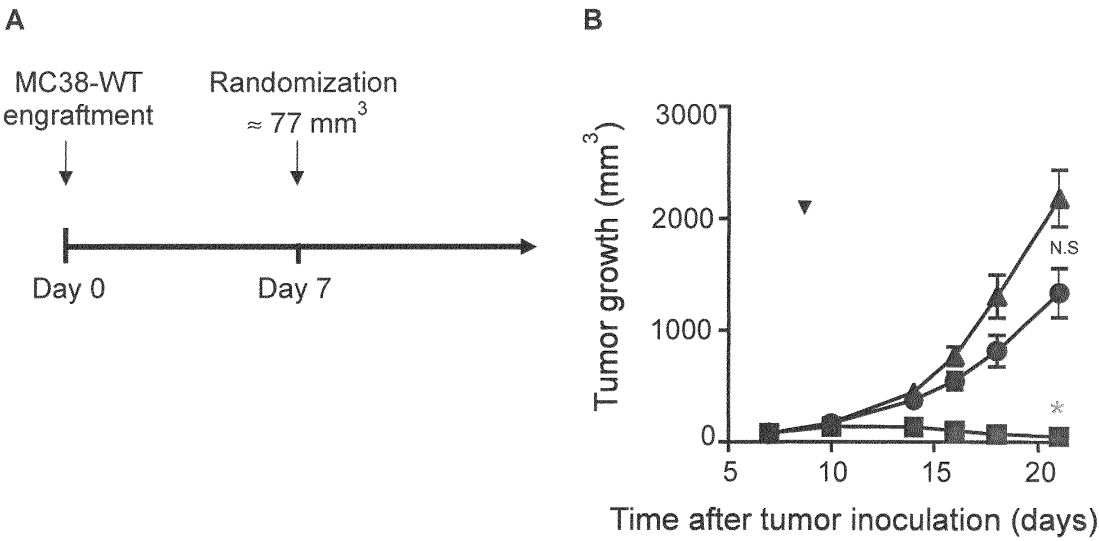


FIG. 18

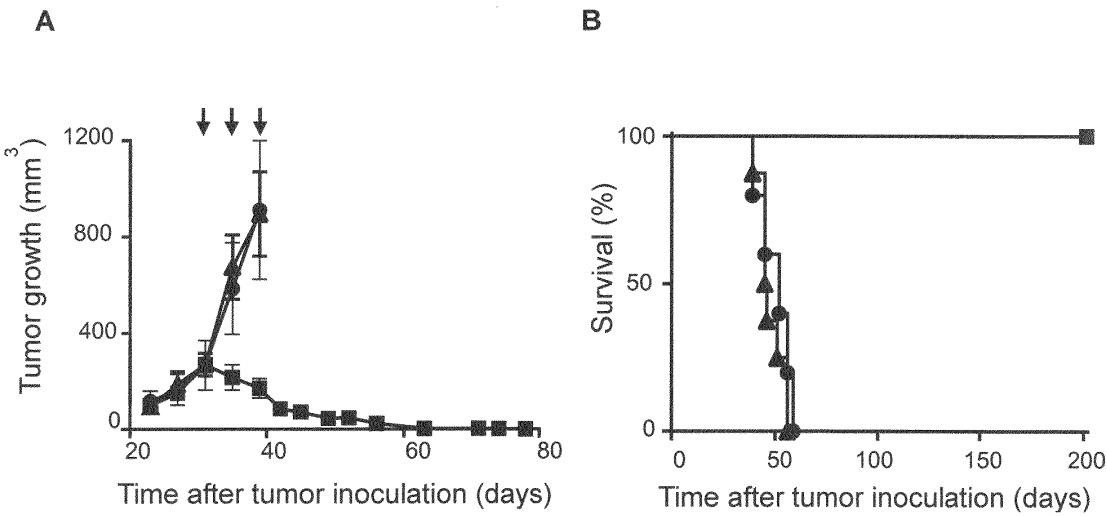
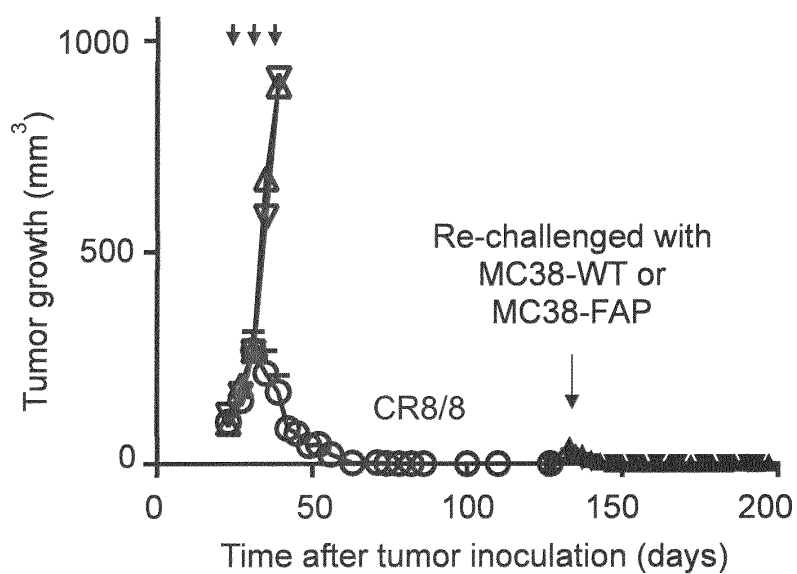


FIG. 19

A



B

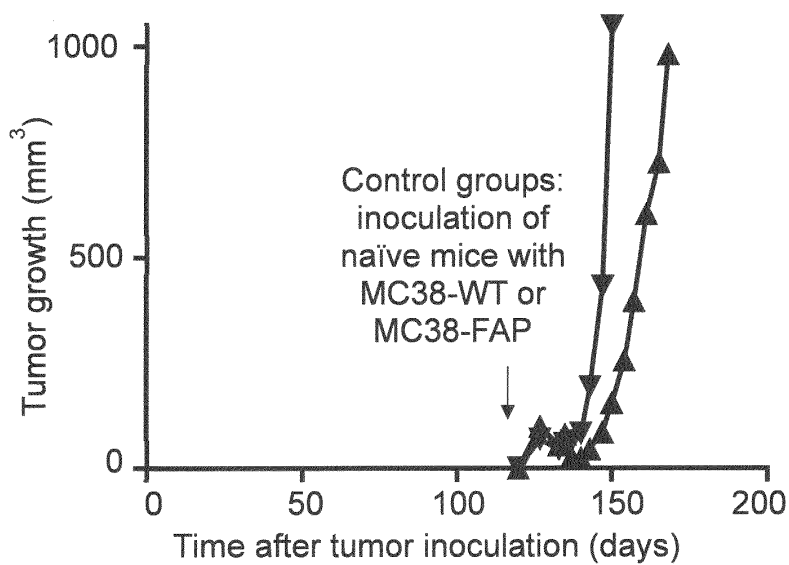


FIG. 20

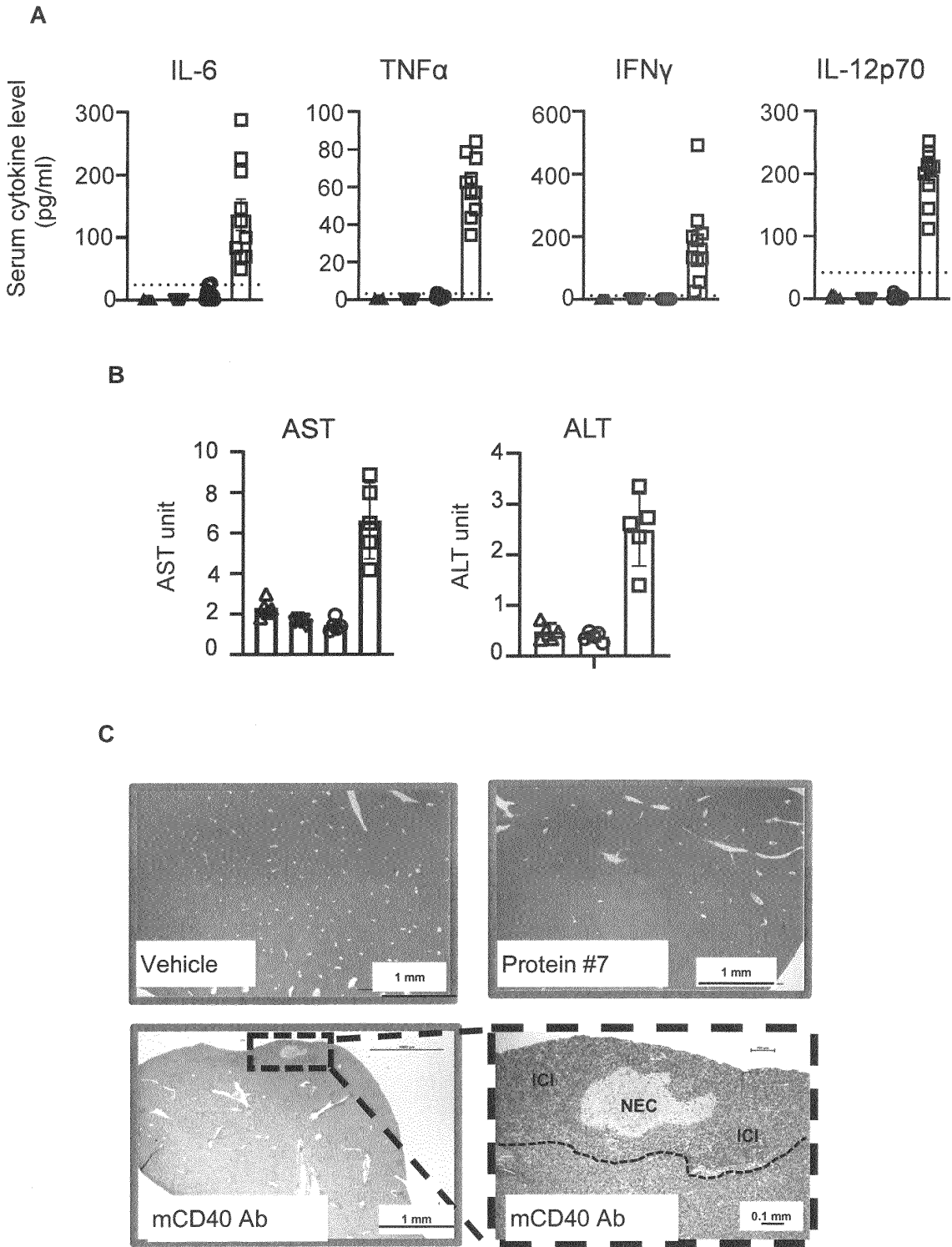


FIG. 21

