

models. The lack of activity in humans is hypothesized to be due to insufficient antibody crosslinking. This may be due to differences in serum IgG, FcγR and or TRAIL concentrations between immune compromised mice and human cancer patients.

[0007] The present disclosure provides multivalent fusion proteins targeting DR5 that are capable of potently agonizing DR5 signaling mediating direct cell death. The fusion proteins of the present disclosure can be bivalent, trivalent, tetravalent, pentavalent, or hexavalent. Importantly, the fusion proteins of the present disclosure are capable of eliciting apoptosis of DR5 expressing cells independently of exogenous crosslinking agents.

[0008] In some embodiments, the fusion proteins of the present disclosure incorporate a binding domain (DR5BD) that binds DR5.

[0008a] In a first aspect, there is provided an isolated polypeptide that binds at least death receptor 5 (DR5) and comprises a plurality of DR5 binding domains (DR5BDs), wherein each DR5BD is a VHH, wherein each VHH comprises an amino acid sequence selected from the group consisting of SEQ ID NOs: 87, 89, and 17-20.

[0008b] In preferred embodiments, the DR5 binding DR5BD does not bind DR4, decoy R1, decoy R2, Osteopontin, or any other TNFRSF member. In preferred embodiments the DR5 binding DR5BD binds human and cynomolgus monkey DR5. In some embodiments, the DR5 binding DR5BD blocks the interaction of DR5 and its ligand TRAIL. In other embodiments, the DR5 binding DR5BD does not block the interaction of DR5 and its ligand TRAIL. In some embodiments, the fusion protein of the present disclosure incorporates multiple DR5 binding DR5BDs that recognize distinct epitopes on DR5. In some embodiments, the fusion protein of the present disclosure incorporates multiple DR5 binding DR5BDs, wherein some DR5BDs block the DR5-TRAIL interaction and other do not block the DR5-TRAIL interaction. In preferred embodiments, DR5 targeting fusion proteins of the present disclosure induce direct cell death of tumor cells. The DR5 targeting fusion proteins of the present disclosure have utility in treating tumors both hematologic and solid in nature.

[0009] The present disclosure provides multivalent DR5 binding fusion proteins, which comprise 2 or more DR5 binding domains (DR5BDs). In some embodiments, the fusion proteins of the present disclosure have utility in treating neoplasms. In some embodiments, the fusion proteins of the present disclosure bind DR5 expressed on a tumor cell. In some embodiments, the fusion protein contains two or more different DR5BDs, where each DR5BD binds DR5. In some embodiments, the fusion protein contains multiple copies of a DR5BD that binds DR5. For example, in some embodiments, the fusion protein contains at least two copies of a DR5BD that binds DR5. In some embodiments, the fusion protein contains at least three copies of a DR5BD that binds DR5. In some embodiments, the fusion protein contains at least four copies of a DR5BD that binds DR5. In some embodiments, the fusion protein contains at least five copies of a DR5BD that binds DR5.

Chemother Pharmacol. 2015 May;75(5):887-95. doi: 10.1007/s00280-015-2712-0. Epub 2015 Feb 27.). In support of this idea, it has been well-documented that aggregated forms of DR5 agonists induce hepatotoxicity whereas non-aggregated forms do not (J Lemke, S von Karstedt, J Zinngrebe and H Walczak. Getting TRAIL back on track for cancer therapy. Cell Death and Differentiation (2014) 21, 1350–1364).

[0012a] Disclosed is an isolated polypeptide that binds at least death receptor 5 (DR5), wherein the isolated polypeptide is not recognized by one or more human anti-VH auto-antibodies (HAVH).

[0012b] Disclosed is an isolated polypeptide that binds at least death receptor 5 (DR5) and comprises two or more copies of an amino acid sequence selected from the group consisting of SEQ ID NOs: 15-91 or two or more copies of an amino acid sequence comprising a complementarity determining region 1 (CDR1) comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 31, 128, 134, 138, 141, 142, 159, 162, 163, 168, 173, 176, 178, 181, and 188; a complementarity determining region 2 (CDR2) comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 28, 129, 131-133, 135, 137, 139, 143, 160, 164, 166, 167, 169, 171, 172, 174, 177, 179, 182, 184, 185, and 189; and a complementarity determining region 3 (CDR3) comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 130, 136, 140, 144-158, 161, 165, 170, 175, 180, 183, 186, 187, and 190.

[0013] In some embodiments, the fusion protein contains at least one DR5BD that comprises an amino acid sequence selected from the group consisting of SEQ ID NO: 15-91. In some embodiments, the fusion protein contains two or more copies of a DR5BD that comprises an amino acid sequence selected from the group consisting of SEQ ID NO: 15-91. In some embodiments, the fusion protein contains three or more copies of a DR5BD that comprises an amino acid sequence selected from the group consisting of SEQ ID NO: 15-91. In some embodiments, the fusion protein contains four or more copies of a DR5BD that comprises an amino acid sequence selected from the group consisting of SEQ ID NO: 15-91. In some embodiments, the fusion protein contains five or more copies of a DR5BD that comprises an amino acid sequence selected from the group consisting of SEQ ID NO: 15-91. In some embodiments, the fusion protein contains six or more copies of a DR5BD that comprises an amino acid sequence selected from the group consisting of SEQ ID NO: 15-91.

[0014] In some embodiments, the fusion protein contains at least one DR5BD that comprises a complementarity determining region 1 (CDR1) comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 31, 128, 134, 138, 141, 142, 159, 162, 163, 168, 173, 176, 178, 181, and 188; a complementarity determining region 2 (CDR2) comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 28, 129, 131-133, 135, 137, 139, 143, 160, 164, 166, 167, 169, 171, 172, 174, 177, 179, 182, 184, 185, and 189; and a complementarity determining region 3 (CDR3) comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 130, 136, 140, 144-158, 161, 165, 170, 175, 180, 183, 186, 187, and 190. In some embodiments, the fusion protein contains two or more copies of a DR5BD that comprises a CDR1 comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 31, 128, 134, 138, 141, 142, 159, 162, 163, 168, 173, 176, 178, 181, and 188; a CDR2 comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 28, 129, 131-133, 135, 137, 139, 143, 160, 164, 166, 167, 169, 171, 172, 174, 177, 179, 182, 184, 185, and 189; and a CDR3 comprising an amino acid sequence selected from the group

some embodiments, the fusion protein contains six or more copies of a DR5BD that comprises a CDR1 comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 31, 128, 134, 138, 141, 142, 159, 162, 163, 168, 173, 176, 178, 181, and 188; a CDR2 comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 28, 129, 131-133, 135, 137, 139, 143, 160, 164, 166, 167, 169, 171, 172, 174, 177, 179, 182, 184, 185, and 189; and a CDR3 comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 130, 136, 140, 144-158, 161, 165, 170, 175, 180, 183, 186, 187, and 190; and at least one immunoglobulin Fc region polypeptide comprising an amino acid sequence selected from the group consisting of SEQ ID NOs: 1-5 or 127.

[0017] In some embodiments, the fusion protein comprises an amino acid sequence selected from the group consisting of SEQ ID NOs: 92-124.

[0017a] Disclosed is an isolated polypeptide that binds at least death receptor 5 (DR5) and comprises an amino acid sequence selected from the group consisting of SEQ ID NOs: 92-124.

[0017b] In some embodiments, the fusion protein comprises an amino acid sequence selected from the group consisting of SEQ ID NOs: 92-118. In some embodiments, the fusion protein comprises an amino acid sequence selected from the group consisting of SEQ ID NOs: 119-124.

[0018] The fusion proteins of the present disclosure are capable of enhanced clustering of TNFRSF members compared to non-cross-linked bivalent antibodies. The enhanced clustered of TNFRSF members mediated by the fusion proteins of the present disclosure induce enhanced TNFRSF-dependent signaling compared to non-cross-linked bivalent antibodies. In most embodiments, the fusion protein will incorporate more than 2 DR5BDs, for example, three, four, five, or six. In some embodiments the fusion protein will incorporate DR5BDs and a binding domain directed toward non-TNFRSF member antigen. In these embodiments, the interaction of the non-TNFRSF antigen is capable of providing the additional crosslinking function and TNFRSF activation is achieved with only one or two DR5BDs. In these embodiments, the fusion protein is multispecific, binding two distinct antigens. In other embodiments, the fusion protein incorporates three or more DR5BDs and a binding domain directed toward an antigen other than DR5, wherein the interaction with this additional antigen does not enhance DR5 clustering beyond what is achieved by the DR5BD containing portion alone, but rather provides a biodistribution advantage, focusing the DR5 agonistic activity of the fusion protein to a specific site within a subject. For example, a tetravalent DR5 binding fusion protein of the present disclosure may include an additional antigen binding domain that focuses activity to a specific site, yet does not enhance the agonistic activity beyond that achieved by a tetravalent DR5 binding fusion protein lacking this additional antigen binding domain.

following structure: DR5BD-Linker- DR5BD-Linker- DR5BD-Linker-Hinge-Fc, where the DR5BD is a humanized or fully human VHH sequence.

[0058] In some embodiments, the multivalent fusion proteins targeting DR5 of the present disclosure are operably linked via amino acid linkers. In some embodiments, these linkers are composed predominately of the amino acids Glycine and Serine, denoted as GS-linkers herein. The GS-linkers of the fusion proteins of the present disclosure can be of various lengths, for example 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 amino acids in length.

[0059] In some embodiments, the GS-linker comprises an amino acid sequence selected from the group consisting of GGSGGS, i.e., (GGG)₂ (SEQ ID NO: 11); GGSGGSGGS, i.e., (GGG)₃ (SEQ ID NO: 12); GGSGGSGGSGGS, i.e., (GGG)₄ (SEQ ID NO: 13); and GGSGGSGGSGGSGGS, i.e., (GGG)₅ (SEQ ID NO: 14).

[0060] In some embodiments, the multivalent DR5 binding fusion protein is tetravalent. In some embodiments, the tetravalent DR5 binding fusion protein has the following structure: VHH-Linker-VHH-Linker-Hinge-Fc, where the VHH is a humanized or fully human VHH sequence. In some embodiments, the VHH sequence is selected from the group consisting of SEQ ID NO: 15-91. In some embodiments, the tetravalent DR5 binding fusion protein comprises an amino acid sequence selected from the group consisting of SEQ ID NOs: 92-118.

[0061] In some embodiments, the multivalent DR5 binding fusion protein is hexavalent. In some embodiments, the hexavalent DR5 binding fusion protein has the following structure: VHH-Linker-VHH-Linker-VHH-Linker-Hinge-Fc, where the VHH is a humanized or fully human VHH sequence. In some embodiments, the VHH sequence is selected from the group consisting of SEQ ID NO: 15-91. In some embodiments, the hexavalent DR5 binding fusion protein comprises an amino acid sequence selected from the group consisting of SEQ ID NOs: 119-124.

[0061a] In a second aspect, there is provided use of the polypeptide as described herein in the manufacture of a medicament for treating neoplasms.

[0061b] In a third aspect, there is provided use of the polypeptide as described herein in the manufacture of a medicament for modulating immune cells to enhance tumor destruction.

[0061c] In a fourth aspect, there is provided a pharmaceutical composition comprising any one of the polypeptides as described herein and a pharmaceutically acceptable carrier.

[0061d] In a fifth aspect, there is provided an isolated nucleic acid that encodes any one of the polypeptides as described herein.

[0061e] In a sixth aspect, there is provided a vector comprising the nucleic acid as described herein.

[0061f] In a seventh aspect, there is provided a host cell comprising the nucleic acid as described herein or the vector as described herein.

[0061g] In an eighth aspect, there is provided a host cell that expresses the polypeptide as described herein.

[0061h] In a ninth aspect, there is provided a method of producing any one of the polypeptides as described herein comprising incubating the host cell as described herein under conditions suitable for expression of the polypeptide.

BRIEF DESCRIPTION OF FIGURES

[0062] Figure 1 is schematic of exemplary multivalent and multispecific fusion proteins of the present disclosure.

[0063] Figures 2A, 2B, 2C, 2D, 2E, 2F, and 2G are a series of graphs demonstrating the binding of representative DR5 VHHs or humanized variants thereof to either human DR5 (Figures 2A, 2B, 2E, 2F, and 2G) and cyno DR5 (Figures 2C and 2D). Figures 2A, 2B,

What is claimed is:

1. An isolated polypeptide that binds at least death receptor 5 (DR5) and comprises a plurality of DR5 binding domains (DR5BDs), wherein each DR5BD is a VHH, wherein each VHH comprises an amino acid sequence selected from the group consisting of SEQ ID NOs: 87, 89, and 17-20.
2. The isolated polypeptide of claim 1, wherein the isolated polypeptide is monospecific.
3. The isolated polypeptide of claim 1, wherein the isolated polypeptide is multispecific or bispecific; or wherein the polypeptide comprises at least a second binding domain (BD2) that binds a second antigen.
4. The isolated polypeptide of any one of claims 1 to 3, wherein the plurality of DR5BDs comprises at least two DR5BDs, at least four DR5BDs, or at least six DR5BDs.
5. The isolated polypeptide of any one of claims 1 to 4, wherein each of the DR5BDs in the plurality of DR5BDs comprises the same amino acid sequence selected from the group consisting of SEQ ID NOs: 87, 89, and 17-20.
6. The isolated polypeptide of any one of claims 1 to 5, wherein each of the DR5BDs in the plurality of DR5BDs comprises the amino acid sequence of SEQ ID NO: 87.
7. The isolated polypeptide of any one of claims 1 to 6, wherein the isolated polypeptide comprises an immunoglobulin Fc region polypeptide.
8. The isolated polypeptide of claim 7, wherein the immunoglobulin Fc region polypeptide is an IgG1 Fc region polypeptide, an IgG2 Fc region polypeptide, an IgG3 Fc region polypeptide, or an IgG4 Fc region polypeptide.
9. The isolated polypeptide of claim 7, wherein the immunoglobulin Fc region polypeptide comprises an amino acid sequence selected from the group consisting of SEQ ID NOs: 1-5 or 127.

10. The isolated polypeptide of claim 7, wherein the immunoglobulin Fc region polypeptide comprises the amino acid sequence of SEQ ID NO: 2.
11. The isolated polypeptide of any one of claims 1 to 10, wherein the VHH is a humanized VHH.
12. The isolated polypeptide of any one of claims 1, 2, or 4 to 11, wherein the polypeptide is tetravalent and comprises the structure: DR5BD-Linker-DR5BD-Linker-Hinge-Fc, wherein each DR5BD is a humanized VHH sequence.
13. The isolated polypeptide of any one of claims 1 to 12, wherein the polypeptide comprises two or more copies of the amino acid sequence of SEQ ID NO: 87.
14. The isolated polypeptide of any one of claims 1 to 12, wherein the polypeptide comprises an amino acid sequence selected from the group consisting of SEQ ID NOs: 113 and 115.
15. The isolated polypeptide of any one of claims 1 to 12, wherein the polypeptide comprises the amino acid sequence of SEQ ID NO: 113.
16. The isolated polypeptide of any one of claims 1, 2, or 4 to 11, wherein the polypeptide is hexavalent and comprises the structure: DR5BD-Linker-DR5BD-Linker-DR5BD-Linker-Hinge-Fc, wherein each DR5BD is a humanized VHH sequence.
17. The isolated polypeptide of any one of claims 1 to 11 or 16, wherein the polypeptide comprises three or more copies of the amino acid sequence of SEQ ID NO: 87.
18. Use of the polypeptide of any one of claims 1 to 17 in the manufacture of a medicament for treating neoplasms.
19. The use of claim 18, wherein the neoplasm is cancer.
20. Use of the polypeptide of any one of claims 1 to 17 in the manufacture of a medicament for modulating immune cells to enhance tumor destruction.

21. A pharmaceutical composition comprising the polypeptide of any one of claims 1 to 17 and a pharmaceutically acceptable carrier.
22. An isolated nucleic acid that encodes the polypeptide of any one of claims 1 to 17.
23. A vector comprising the nucleic acid of claim 22.
24. A host cell comprising the nucleic acid of claim 22 or the vector of claim 23.
25. A host cell that expresses the polypeptide of any one of claims 1 to 17.
26. The host cell of claim 24 or 25, which secretes the polypeptide of any one of claims 1 to 17.
27. A method of producing the polypeptide of any one of claims 1 to 17 comprising incubating the host cell of any one of claims 24 to 26 under conditions suitable for expression of the polypeptide.
28. The method of claim 27, further comprising isolating the polypeptide.