

MATERIALS AND METHODS FOR IMPROVED SINGLE CHAIN VARIABLE FRAGMENTS

CROSS-REFERENCE TO RELATED APPLICATIONS

5 [0001] This application claims the benefit of U.S. Provisional Patent Application No. 62/946,897, filed December 11, 2019, U.S. Provisional Patent Application No. 62/946,886, filed December 11, 2019, U.S. Provisional Patent Application No. 62/946,882, filed December 11, 2019, U.S. Provisional Patent Application No. 62/946,877, filed December 11, 2019, U.S. Provisional Patent Application No. 62/946,865, filed December 11, 2019, U.S. Provisional Patent Application No. 62/887,529, filed August 15, 2019, U.S. Provisional Patent Application No. 62/887,527, filed August 15, 2019, U.S. Provisional Patent Application No. 62/887,524, filed August 15, 2019, U.S. Provisional Patent Application No. 62/887,519, filed August 15, 2019, and U.S. Provisional Patent Application No. 62/887,514, filed August 15, 2019, each of which is incorporated by reference herein in its entirety.

15 SEQUENCE LISTING

[0002] This application incorporates by reference a Sequence Listing submitted with this application as a text format, entitled "14620-227-228_SL.txt," created on August 5, 2020 having a size of 258,724 bytes.

1. TECHNICAL FIELD

20 [0003] Disclosed are materials and methods for improved single chain variable fragments.

2. BACKGROUND

[0004] Antigen binding single chain variable fragments (scFv) are modules that can be utilized broadly as therapeutics, imaging agents, diagnostic agents or as portions of heterologous molecules such as multispecific molecules. One of the challenges of scFvs is the low stability and tendencies to aggregate (reviewed in Worn and Pluckthun (2001) *J Mol Biol* 305: 989-1010; Rothlisberger *et al.*, (2005) *J Mol Biol* 347: 773-789; Gross *et al.*, (1989) *Transplant Proc* 21(1 Pt 1): 127-130, Porter *et al.*, (2011) *J Cancer* 2: 331-332; Porter *et al.*, (2011) *N Engl J Med* 365: 725-733). Therefore there is a need for improved scFv designs that may be optionally incorporated into multispecific molecules and heterologous molecules.

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3. SUMMARY

[0005] In one aspect, the disclosure provides an isolated single chain variable fragment (scFv) comprising a heavy chain variable region (VH), a linker (L) and a light chain variable region (VL), wherein the scFv comprises

- 5 a first disulfide bond between a structurally conserved surface exposed VH cysteine (Cys) and a first L Cys;
a second disulfide bond between a structurally conserved surface exposed VL Cys and a second L Cys; or
the first disulfide bond between the structurally conserved surface exposed VH Cys and the first
10 L Cys and the second disulfide bond between the structurally conserved surface exposed VL Cys and the second L Cys.

[0006] The disclosure also provides an isolated scFv comprising a VH, a L and a VL, wherein the VH comprises a VH Cys at a structurally conserved surface exposed VH framework residue position and the L comprises a first L Cys;

- 15 the VL comprises a VL Cys at a structurally conserved surface exposed VL framework residue position and the L comprises a second L Cys; or
the VH comprises the VH Cys at a structurally conserved surface exposed VH framework residue position, the VL comprises the VL Cys at a structurally conserved surface exposed VL framework residue position and the L comprises the first L Cys and the second L Cys, wherein
20 the VH Cys and the first L Cys are capable of forming a disulfide bond and the VL Cys and the second L Cys are capable of forming a disulfide bond.

[0007] The disclosure also provides an scFv comprising a VH, a L and a VL, wherein the VH comprises Cys at H105;
the VL comprises Cys at L42;

- 25 the L comprises an amino acid sequence of SEQ ID NOs: 2, 3, 4, 5, 6 or 7; and
the scFv is in the VL-L-VH orientation.

[0008] The disclosure also provides a scFv comprising a VH, a L and a VL, wherein the VH comprises Cys at H105;
the VL comprises Cys at L45;

- 30 the L comprises an amino acid sequence of SEQ ID NOs: 2, 3, 4, 5, 6 or 7; and
the scFv is in the VL-L-VH orientation.

[0009] The disclosure also provides a scFv comprising a VH, a L and a VL, wherein the VH comprises Cys at H105;
the VL comprises Cys at L39;
the L comprises an amino acid sequence of SEQ ID NOs: 2, 3, 4, 5, 6 or 7; and
5 the scFv is in the VL-L-VH orientation.

[0010] The disclosure also provides a scFv comprising a VH, a L and a VL, wherein the VH comprises Cys at H5;
the VL comprises Cys at L42;
the L comprises an amino acid sequence of SEQ ID NOs: 2, 3, 4, 5, 6 or 7; and
10 the scFv is in the VL-L-VH orientation.

[0011] The disclosure also provides a scFv comprising a VH, a L and a VL, wherein the VH comprises Cys at H5;
the VL comprises Cys at L45;
the L comprises an amino acid sequence of SEQ ID NOs: 2, 3, 4, 5, 6 or 7; and
15 the scFv is in the VL-L-VH orientation.

[0012] The disclosure also provides a scFv comprising a VH, a L and a VL, wherein the VH comprises Cys at H5;
the VL comprises Cys at L39;
the L comprises an amino acid sequence of SEQ ID NOs: 2, 3, 4, 5, 6 or 7; and
20 the scFv is in the VL-L-VH orientation.

[0013] The disclosure also provides a scFv comprising a VH, a L and a VL, wherein the VH comprises Cys at H3;
the VL comprises Cys at L42;
the L comprises an amino acid sequence of SEQ ID NOs: 2, 3, 4, 5, 6 or 7; and
25 the scFv is in the VL-L-VH orientation.

[0014] The disclosure also provides a scFv comprising a VH, a L and a VL, wherein the VH comprises Cys at H3;
the VL comprises Cys at L45;
the L comprises an amino acid sequence of SEQ ID NOs: 2, 3, 4, 5, 6 or 7; and
30 the scFv is in the VL-L-VH orientation.

[0015] The disclosure also provides a scFv comprising a VH, a L and a VL, wherein

the VH comprises Cys at H3;
the VL comprises Cys at L39;
the L comprises an amino acid sequence of SEQ ID NOs: 2, 3, 4, 5, 6 or 7; and
the scFv is in the VL-L-VH orientation.

- 5 **[0016]** The disclosure also provides a scFv comprising a VH, a L and a VL, wherein
the VH comprises Cys at H43;
the VL comprises Cys at L100;
the L comprises an amino acid sequence of SEQ ID NOs: 2, 3, 4, 5, 6 or 7; and
the scFv is in the VH-L-VL orientation.

- 10 **[0017]** The disclosure also provides a scFv comprising a VH, a L and a VL, wherein
the VH comprises Cys at H43;
the VL comprises Cys at L102;
the L comprises an amino acid sequence of SEQ ID NOs: 2, 3, 4, 5, 6 or 7; and
the scFv is in the VH-L-VL orientation.

- 15 **[0018]** The disclosure also provides a scFv comprising a VH, a L and a VL, wherein
the VH comprises Cys at H43;
the VL comprises Cys at L5;
the L comprises an amino acid sequence of SEQ ID NOs: 2, 3, 4, 5, 6 or 7; and
the scFv is in the VH-L-VL orientation.

- 20 **[0019]** The disclosure also provides a scFv comprising a VH, a L and a VL, wherein
the VH comprises Cys at H43;
the VL comprises Cys at L3;
the L comprises an amino acid sequence of SEQ ID NOs: 2, 3, 4, 5, 6 or 7; and
the scFv is in the VH-L-VL orientation.

- 25 **[0020]** The disclosure also provides a scFv comprising a VH, a L and a VL, wherein
the VH comprises Cys at H40;
the VL comprises Cys at L100;
the L comprises an amino acid sequence of SEQ ID NOs: 2, 3, 4, 5, 6 or 7; and
the scFv is in the VH-L-VL orientation.

- 30 **[0021]** The disclosure also provides a scFv comprising a VH, a L and a VL, wherein
the VH comprises Cys at H40;

the VL comprises Cys at L102;

the L comprises an amino acid sequence of SEQ ID NOs: 2, 3, 4, 5, 6 or 7; and

the scFv is in the VH-L-VL orientation.

[0022] The disclosure also provides a scFv comprising a VH, a L and a VL, wherein

5 the VH comprises Cys at H40;

the VL comprises Cys at L5;

the L comprises an amino acid sequence of SEQ ID NOs: 2, 3, 4, 5, 6 or 7; and

the scFv is in the VH-L-VL orientation.

[0023] The disclosure also provides a scFv comprising a VH, a L and a VL, wherein

10 the VH comprises Cys at H40;

the VL comprises Cys at L3;

the L comprises an amino acid sequence of SEQ ID NOs: 2, 3, 4, 5, 6 or 7; and

the scFv is in the VH-L-VL orientation.

[0024] The disclosure also provides a scFv comprising a VH, a L and a VL, wherein

15 the VH comprises Cys at H46;

the VL comprises Cys at L100;

the L comprises an amino acid sequence of SEQ ID NOs: 2, 3, 4, 5, 6 or 7; and

the scFv is in the VH-L-VL orientation.

[0025] The disclosure also provides a scFv comprising a VH, a L and a VL, wherein

20 the VH comprises Cys at H46;

the VL comprises Cys at L102;

the L comprises an amino acid sequence of SEQ ID NOs: 2, 3, 4, 5, 6 or 7; and

the scFv is in the VH-L-VL orientation.

[0026] The disclosure also provides a scFv comprising a VH, a L and a VL, wherein

25 the VH comprises Cys at H46;

the VL comprises Cys at L5;

the L comprises an amino acid sequence of SEQ ID NOs: 2, 3, 4, 5, 6 or 7; and

the scFv is in the VH-L-VL orientation.

[0027] The disclosure also provides a scFv comprising a VH, a L and a VL, wherein

30 the VH comprises Cys at H46;

the VL comprises Cys at L3;

the L comprises an amino acid sequence of SEQ ID NOs: 2, 3, 4, 5, 6 or 7; and
the scFv is in the VH-L-VL orientation.

[0028] The disclosure also provides a pharmaceutical composition comprising the spFv of the disclosure and a pharmaceutically acceptable carrier.

5 **[0029]** The disclosure also provides a polynucleotide encoding the spFv of the disclosure.

[0030] The disclosure also provides a vector comprising the polynucleotide of the disclosure.

[0031] The disclosure also provides a host cell comprising the vector of the disclosure.

[0032] The disclosure also provides a method of producing the spFv of the disclosure,
comprising culturing the host cell of the disclosure in conditions that the spFv is produced, and
10 purifying the spFv.

[0033] The disclosure also provides an anti-idiotypic antibody that binds to the spFv of the disclosure.

[0034] The disclosure also provides a kit comprising the spFv of the disclosure.

[0035] In another aspect, the disclosure provides a multispecific molecule comprising a single
15 chain variable fragment (scFv) comprising a heavy chain variable region (VH), a linker (L) and a
light chain variable region (VL), wherein the scFv comprises
a first disulfide bond between a structurally conserved surface exposed VH cysteine (Cys) and a
first L Cys;

a second disulfide bond between a structurally conserved surface exposed VL Cys and a second
20 L Cys; or

the first disulfide bond between the structurally conserved surface exposed VH Cys and the first
L Cys and the second disulfide bond between the structurally conserved surface exposed VL Cys
and the second L Cys.

[0036] The disclosure also provides a multispecific molecule comprising a scFv comprising a
25 VH, a L and a VL, wherein
the VH comprises a VH Cys at a structurally conserved surface exposed VH framework residue
position and the L comprises a first L Cys;

the VL comprises a VL Cys at a structurally conserved surface exposed VL framework residue
position and the L comprises a second L Cys; or

30 the VH comprises the VH Cys at a structurally conserved surface exposed VH framework
residue position, the VL comprises the VL Cys at a structurally conserved surface exposed VL

framework residue position and the L comprises the first L Cys and the second L Cys, wherein the VH Cys and the first L Cys are capable of forming a disulfide bond and the VL Cys and the second L Cys are capable of forming a disulfide bond.

[0037] The disclosure also provides a multispecific molecule comprising a scFv comprising a

5 VH, a L and a VL, wherein

the VH comprises Cys at H105;

the VL comprises Cys at L42;

the L comprises an amino acid sequence of SEQ ID NOs: 2, 3, 4, 5, 6 or 7; and

the scFv is in the VL-L-VH orientation.

10 **[0038]** The disclosure also provides a multispecific molecule comprising a scFv comprising a

VH, a L and a VL, wherein

the VH comprises Cys at H105;

the VL comprises Cys at L45;

the L comprises an amino acid sequence of SEQ ID NOs: 2, 3, 4, 5, 6 or 7; and

15 the scFv is in the VL-L-VH orientation.

[0039] The disclosure also provides a multispecific molecule comprising a scFv comprising a

VH, a L and a VL, wherein

the VH comprises Cys at H105;

the VL comprises Cys at L39;

20 the L comprises an amino acid sequence of SEQ ID NOs: 2, 3, 4, 5, 6 or 7; and

the scFv is in the VL-L-VH orientation.

[0040] The disclosure also provides a multispecific molecule comprising a scFv comprising a

VH, a L and a VL, wherein

the VH comprises Cys at H5;

25 the VL comprises Cys at L42;

the L comprises an amino acid sequence of SEQ ID NOs: 2, 3, 4, 5, 6 or 7; and

the scFv is in the VL-L-VH orientation.

[0041] The disclosure also provides a multispecific molecule comprising a scFv comprising a

VH, a L and a VL, wherein

30 the VH comprises Cys at H5;

the VL comprises Cys at L45;

the L comprises an amino acid sequence of SEQ ID NOs: 2, 3, 4, 5, 6 or 7; and
the scFv is in the VL-L-VH orientation.

[0042] The disclosure also provides a multispecific molecule comprising a scFv comprising a
VH, a L and a VL, wherein

5 the VH comprises Cys at H5;

the VL comprises Cys at L39;

the L comprises an amino acid sequence of SEQ ID NOs: 2, 3, 4, 5, 6 or 7; and

the scFv is in the VL-L-VH orientation.

[0043] The disclosure also provides a multispecific molecule comprising a scFv comprising a
10 VH, a L and a VL, wherein

the VH comprises Cys at H3;

the VL comprises Cys at L42;

the L comprises an amino acid sequence of SEQ ID NOs: 2, 3, 4, 5, 6 or 7; and

the scFv is in the VL-L-VH orientation.

15 **[0044]** The disclosure also provides a multispecific molecule comprising a scFv comprising a
VH, a L and a VL, wherein

the VH comprises Cys at H3;

the VL comprises Cys at L45;

the L comprises an amino acid sequence of SEQ ID NOs: 2, 3, 4, 5, 6 or 7; and

20 the scFv is in the VL-L-VH orientation.

[0045] The disclosure also provides a multispecific molecule comprising a scFv comprising a
VH, a L and a VL, wherein

the VH comprises Cys at H3;

the VL comprises Cys at L39;

25 the L comprises an amino acid sequence of SEQ ID NOs: 2, 3, 4, 5, 6 or 7; and

the scFv is in the VL-L-VH orientation.

[0046] The disclosure also provides a multispecific molecule comprising a scFv comprising a
VH, a L and a VL, wherein

the VH comprises Cys at H43;

30 the VL comprises Cys at L100;

the L comprises an amino acid sequence of SEQ ID NOs: 2, 3, 4, 5, 6 or 7; and

the scFv is in the VH-L-VL orientation.

[0047] The disclosure also provides a multispecific molecule comprising a scFv comprising a VH, a L and a VL, wherein

the VH comprises Cys at H43;

5 the VL comprises Cys at L102;

the L comprises an amino acid sequence of SEQ ID NOs: 2, 3, 4, 5, 6 or 7; and

the scFv is in the VH-L-VL orientation.

[0048] The disclosure also provides a multispecific molecule comprising a scFv comprising a VH, a L and a VL, wherein

10 the VH comprises Cys at H43;

the VL comprises Cys at L5;

the L comprises an amino acid sequence of SEQ ID NOs: 2, 3, 4, 5, 6 or 7; and

the scFv is in the VH-L-VL orientation.

[0049] The disclosure also provides a multispecific molecule comprising a scFv comprising a VH, a L and a VL, wherein

15 the VH comprises Cys at H43;

the VL comprises Cys at L3;

the L comprises an amino acid sequence of SEQ ID NOs: 2, 3, 4, 5, 6 or 7; and

the scFv is in the VH-L-VL orientation.

20 **[0050]** The disclosure also provides a multispecific molecule comprising a scFv comprising a VH, a L and a VL, wherein

the VH comprises Cys at H40;

the VL comprises Cys at L100;

the L comprises an amino acid sequence of SEQ ID NOs: 2, 3, 4, 5, 6 or 7; and

25 the scFv is in the VH-L-VL orientation.

[0051] The disclosure also provides a multispecific molecule comprising a scFv comprising a VH, a L and a VL, wherein

the VH comprises Cys at H40;

the VL comprises Cys at L102;

30 the L comprises an amino acid sequence of SEQ ID NOs: 2, 3, 4, 5, 6 or 7; and

the scFv is in the VH-L-VL orientation.

[0052] The disclosure also provides a multispecific molecule comprising a scFv comprising a VH, a L and a VL, wherein
the VH comprises Cys at H40;
the VL comprises Cys at L5;
5 the L comprises an amino acid sequence of SEQ ID NOs: 2, 3, 4, 5, 6 or 7; and
the scFv is in the VH-L-VL orientation.

[0053] The disclosure also provides a multispecific molecule comprising a scFv comprising a VH, a L and a VL, wherein
the VH comprises Cys at H40;
10 the VL comprises Cys at L3;
the L comprises an amino acid sequence of SEQ ID NOs: 2, 3, 4, 5, 6 or 7; and
the scFv is in the VH-L-VL orientation.

[0054] The disclosure also provides a multispecific molecule comprising a scFv comprising a VH, a L and a VL, wherein
15 the VH comprises Cys at H46;
the VL comprises Cys at L100;
the L comprises an amino acid sequence of SEQ ID NOs: 2, 3, 4, 5, 6 or 7; and
the scFv is in the VH-L-VL orientation.

[0055] The disclosure also provides a multispecific molecule comprising a scFv comprising a
20 VH, a L and a VL, wherein
the VH comprises Cys at H46;
the VL comprises Cys at L102;
the L comprises an amino acid sequence of SEQ ID NOs: 2, 3, 4, 5, 6 or 7; and
the scFv is in the VH-L-VL orientation.

[0056] The disclosure also provides a multispecific molecule comprising a scFv comprising a
25 VH, a L and a VL, wherein
the VH comprises Cys at H46;
the VL comprises Cys at L5;
the L comprises an amino acid sequence of SEQ ID NOs: 2, 3, 4, 5, 6 or 7; and
30 the scFv is in the VH-L-VL orientation.

[0057] The disclosure also provides a multispecific molecule comprising a scFv comprising a VH, a L and a VL, wherein
the VH comprises Cys at H46;
the VL comprises Cys at L3;
5 the L comprises an amino acid sequence of SEQ ID NOs: 2, 3, 4, 5, 6 or 7; and
the scFv is in the VH-L-VL orientation.

[0058] The disclosure also provides a pharmaceutical composition comprising the multispecific molecule provided herein and a pharmaceutically acceptable carrier.

[0059] In yet another aspect, the disclosure provides a heterologous molecule comprising a
10 single chain variable fragment (scFv) comprising a heavy chain variable region (VH), a linker (L) and a light chain variable region (VL), wherein the scFv comprises
a first disulfide bond between a structurally conserved surface exposed VH cysteine (Cys) and a first L Cys;
a second disulfide bond between a structurally conserved surface exposed VL Cys and a second
15 L Cys; or
the first disulfide bond between the structurally conserved surface exposed VH Cys and the first L Cys and the second disulfide bond between the structurally conserved surface exposed VL Cys and the second L Cys.

[0060] The disclosure also provides a heterologous molecule comprising a scFv comprising a
20 VH, a L and a VL, wherein
the VH comprises a VH Cys at a structurally conserved surface exposed VH framework residue position and the L comprises a first L Cys;
the VL comprises a VL Cys at a structurally conserved surface exposed VL framework residue position and the L comprises a second L Cys; or
25 the VH comprises the VH Cys at a structurally conserved surface exposed VH framework residue position, the VL comprises the VL Cys at a structurally conserved surface exposed VL framework residue position and the L comprises the first L Cys and the second L Cys, wherein the VH Cys and the first L Cys are capable of forming a disulfide bond and the VL Cys and the second L Cys are capable of forming a disulfide bond.

30 [0061] The disclosure also provides a heterologous molecule comprising a scFv comprising a VH, a L and a VL, wherein

the VH comprises Cys at H105;
the VL comprises Cys at L42;
the L comprises an amino acid sequence of SEQ ID NOs: 2, 3, 4, 5, 6 or 7; and
the scFv is in the VL-L-VH orientation.

- 5 **[0062]** The disclosure also provides a heterologous molecule comprising a scFv comprising a VH, a L and a VL, wherein
the VH comprises Cys at H105;
the VL comprises Cys at L45;
the L comprises an amino acid sequence of SEQ ID NOs: 2, 3, 4, 5, 6 or 7; and
10 the scFv is in the VL-L-VH orientation.

[0063] The disclosure also provides a heterologous molecule comprising a scFv comprising a VH, a L and a VL, wherein
the VH comprises Cys at H105;
the VL comprises Cys at L39;
15 the L comprises an amino acid sequence of SEQ ID NOs: 2, 3, 4, 5, 6 or 7; and
the scFv is in the VL-L-VH orientation.

- [0064]** The disclosure also provides a heterologous molecule comprising a scFv comprising a VH, a L and a VL, wherein
the VH comprises Cys at H5;
20 the VL comprises Cys at L42;
the L comprises an amino acid sequence of SEQ ID NOs: 2, 3, 4, 5, 6 or 7; and
the scFv is in the VL-L-VH orientation.

- [0065]** The disclosure also provides a heterologous molecule comprising a scFv comprising a VH, a L and a VL, wherein
25 the VH comprises Cys at H5;
the VL comprises Cys at L45;
the L comprises an amino acid sequence of SEQ ID NOs: 2, 3, 4, 5, 6 or 7; and
the scFv is in the VL-L-VH orientation.

- [0066]** The disclosure also provides a heterologous molecule comprising a scFv comprising a
30 VH, a L and a VL, wherein
the VH comprises Cys at H5;

the VL comprises Cys at L39;

the L comprises an amino acid sequence of SEQ ID NOs: 2, 3, 4, 5, 6 or 7; and

the scFv is in the VL-L-VH orientation.

5 **[0067]** The disclosure also provides a heterologous molecule comprising a scFv comprising a
VH, a L and a VL, wherein
the VH comprises Cys at H3;
the VL comprises Cys at L42;
the L comprises an amino acid sequence of SEQ ID NOs: 2, 3, 4, 5, 6 or 7; and
the scFv is in the VL-L-VH orientation.

10 **[0068]** The disclosure also provides a heterologous molecule comprising a scFv comprising a
VH, a L and a VL, wherein
the VH comprises Cys at H3;
the VL comprises Cys at L45;
the L comprises an amino acid sequence of SEQ ID NOs: 2, 3, 4, 5, 6 or 7; and
15 the scFv is in the VL-L-VH orientation.

[0069] The disclosure also provides a heterologous molecule comprising a scFv comprising a
VH, a L and a VL, wherein
the VH comprises Cys at H3;
the VL comprises Cys at L39;
20 the L comprises an amino acid sequence of SEQ ID NOs: 2, 3, 4, 5, 6 or 7; and
the scFv is in the VL-L-VH orientation.

[0070] The disclosure also provides a heterologous molecule comprising a scFv comprising a
VH, a L and a VL, wherein
the VH comprises Cys at H43;
25 the VL comprises Cys at L100;
the L comprises an amino acid sequence of SEQ ID NOs: 2, 3, 4, 5, 6 or 7; and
the scFv is in the VH-L-VL orientation.

[0071] The disclosure also provides a heterologous molecule comprising a scFv comprising a
VH, a L and a VL, wherein
30 the VH comprises Cys at H43;
the VL comprises Cys at L102;

the L comprises an amino acid sequence of SEQ ID NOs: 2, 3, 4, 5, 6 or 7; and
the scFv is in the VH-L-VL orientation.

[0072] The disclosure also provides a heterologous molecule comprising a scFv comprising a
VH, a L and a VL, wherein

5 the VH comprises Cys at H43;

the VL comprises Cys at L5;

the L comprises an amino acid sequence of SEQ ID NOs: 2, 3, 4, 5, 6 or 7; and

the scFv is in the VH-L-VL orientation.

[0073] The disclosure also provides a heterologous molecule comprising a scFv comprising a
10 VH, a L and a VL, wherein

the VH comprises Cys at H43;

the VL comprises Cys at L3;

the L comprises an amino acid sequence of SEQ ID NOs: 2, 3, 4, 5, 6 or 7; and

the scFv is in the VH-L-VL orientation.

15 **[0074]** The disclosure also provides a heterologous molecule comprising a scFv comprising a
VH, a L and a VL, wherein

the VH comprises Cys at H40;

the VL comprises Cys at L100;

the L comprises an amino acid sequence of SEQ ID NOs: 2, 3, 4, 5, 6 or 7; and

20 the scFv is in the VH-L-VL orientation.

[0075] The disclosure also provides a heterologous molecule comprising a scFv comprising a
VH, a L and a VL, wherein

the VH comprises Cys at H40;

the VL comprises Cys at L102;

25 the L comprises an amino acid sequence of SEQ ID NOs: 2, 3, 4, 5, 6 or 7; and

the scFv is in the VH-L-VL orientation.

[0076] The disclosure also provides a heterologous molecule comprising a scFv comprising a
VH, a L and a VL, wherein

the VH comprises Cys at H40;

30 the VL comprises Cys at L5;

the L comprises an amino acid sequence of SEQ ID NOs: 2, 3, 4, 5, 6 or 7; and

the scFv is in the VH-L-VL orientation.

[0077] The disclosure also provides a heterologous molecule comprising a scFv comprising a VH, a L and a VL, wherein

the VH comprises Cys at H40;

5 the VL comprises Cys at L3;

the L comprises an amino acid sequence of SEQ ID NOs: 2, 3, 4, 5, 6 or 7; and

the scFv is in the VH-L-VL orientation.

[0078] The disclosure also provides a heterologous molecule comprising a scFv comprising a VH, a L and a VL, wherein

10 the VH comprises Cys at H46;

the VL comprises Cys at L100;

the L comprises an amino acid sequence of SEQ ID NOs: 2, 3, 4, 5, 6 or 7; and

the scFv is in the VH-L-VL orientation.

[0079] The disclosure also provides a heterologous molecule comprising a scFv comprising a VH, a L and a VL, wherein

15 the VH comprises Cys at H46;

the VL comprises Cys at L102;

the L comprises an amino acid sequence of SEQ ID NOs: 2, 3, 4, 5, 6 or 7; and

the scFv is in the VH-L-VL orientation.

20 **[0080]** The disclosure also provides a heterologous molecule comprising a scFv comprising a VH, a L and a VL, wherein

the VH comprises Cys at H46;

the VL comprises Cys at L5;

the L comprises an amino acid sequence of SEQ ID NOs: 2, 3, 4, 5, 6 or 7; and

25 the scFv is in the VH-L-VL orientation.

[0081] The disclosure also provides a heterologous molecule comprising a scFv comprising a VH, a L and a VL, wherein

the VH comprises Cys at H46;

the VL comprises Cys at L3;

30 the L comprises an amino acid sequence of SEQ ID NOs: 2, 3, 4, 5, 6 or 7; and

the scFv is in the VH-L-VL orientation.

[0082] The disclosure also provides a pharmaceutical composition comprising the heterologous molecule of the disclosure and a pharmaceutically acceptable carrier.

[0083] In yet another aspect, the disclosure provides a process for preparing a stabilized scFv, comprising:

- 5 providing a heavy chain variable region (VH) and a light chain variable region (VL) that form an antigen binding domain;
- providing a linker (L) that comprises or is engineered to comprise a first L Cys;
- engineering the VH to comprise a VH Cys at a structurally conserved surface exposed VH framework residue position; and
- 10 forming a disulfide bond between the VH Cys and the first L Cys to prepare the stabilized scFv.

[0084] The disclosure also provides a process for preparing a stabilized scFv, comprising:

- providing a VH and a VL that form an antigen binding domain;
- providing a L that comprises or is engineered to comprise a second L Cys;
- engineering the VL to comprise a VL Cys at a structurally conserved surface exposed VL
- 15 framework residue position; and
- forming a disulfide bond between the VL Cys and the second L Cys to prepare the stabilized scFv.

[0085] The disclosure also provides a process for preparing a stabilized scFv, comprising:

- providing a VH and a VL that form an antigen binding domain;
- 20 providing a L that comprises or is engineered to comprise a first L Cys and a second L Cys;
- engineering the VH to comprise a VH Cys at a structurally conserved surface exposed VH framework residue position;
- engineering the VL to comprise a VL Cys at a structurally conserved surface exposed VL framework residue position; and
- 25 forming a disulfide bond between the VH Cys and the first L Cys and a disulfide bond between the VL Cys and the second L Cys to prepare the stabilized scFv.

[0086] The disclosure also provides a process for preparing a stabilized scFv, comprising

- providing a polynucleotide encoding a VH, a L and a VL, wherein
- the VH comprises Cys at H105 and the VL comprises Cys at L42;
- 30 the VH comprises Cys at H43 and the VL comprises Cys at a L100;
- the VH comprises Cys at H3 and the VL comprises Cys at L3;

- the VH comprises Cys at H3 and the VL comprises Cys at L5;
the VH comprises Cys at H3 and the VL comprises Cys at L39;
the VH comprises Cys at H3 and the VL comprises Cys at L42;
the VH comprises Cys at H3 and the VL comprises Cys at L45;
5 the VH comprises Cys at H3 and the VL comprises Cys at L100;
the VH comprises Cys at H3 and the VL comprises Cys at L102;
the VH comprises Cys at H5 and the VL comprises Cys at L3;
the VH comprises Cys at H5 and the VL comprises Cys at L5;
the VH comprises Cys at H5 and the VL comprises Cys at L39;
10 the VH comprises Cys at H5 and the VL comprises Cys at L42;
the VH comprises Cys at H5 and the VL comprises Cys at L45;
the VH comprises Cys at H5 and the VL comprises Cys at L100;
the VH comprises Cys at H5 and the VL comprises Cys at L102;
the VH comprises Cys at H40 and the VL comprises Cys at L3;
15 the VH comprises Cys at H40 and the VL comprises Cys at L5;
the VH comprises Cys at H40 and the VL comprises Cys at L39;
the VH comprises Cys at H40 and the VL comprises Cys at L42;
the VH comprises Cys at H40 and the VL comprises Cys at L45;
the VH comprises Cys at H40 and the VL comprises Cys at L100;
20 the VH comprises Cys at H40 and the VL comprises Cys at L102;
the VH comprises Cys at H43 and the VL comprises Cys at L3;
the VH comprises Cys at H43 and the VL comprises Cys at L5;
the VH comprises Cys at H43 and the VL comprises Cys at L39;
the VH comprises Cys at H43 and the VL comprises Cys at L42;
25 the VH comprises Cys at H43 and the VL comprises Cys at L45;
the VH comprises Cys at H43 and the VL comprises Cys at L102;
the VH comprises Cys at H46 and the VL comprises Cys at L3;
the VH comprises Cys at H46 and the VL comprises Cys at L5;
the VH comprises Cys at H46 and the VL comprises Cys at L39;
30 the VH comprises Cys at H46 and the VL comprises Cys at L42;
the VH comprises Cys at H46 and the VL comprises Cys at L45;

- the VH comprises Cys at H46 and the VL comprises Cys at L100;
the VH comprises Cys at H46 and the VL comprises Cys at L102;
the VH comprises Cys at H105 and the VL comprises Cys at L3;
the VH comprises Cys at H105 and the VL comprises Cys at L5;
5 the VH comprises Cys at H105 and the VL comprises Cys at L39;
the VH comprises Cys at H105 and the VL comprises Cys at L45;
the VH comprises Cys at H105 and the VL comprises Cys at L100; or
the VH comprises Cys at H105 and the VL comprises Cys at L102, wherein residue numbering
is according to Chothia.
- 10 the L comprises an amino acid sequence of SEQ ID NOs: 2, 3, 4, 5, 6 or 7; and
expressing the polynucleotide in a host cell to produce the stabilized scFv.
- [0087]** In yet another aspect, the disclosure provides an isolated single chain variable fragment
(scFv) comprising a heavy chain variable region (VH), a means for linking (L) and a light chain
variable region (VL), wherein the scFv comprises:
- 15 a first disulfide bond between a structurally conserved surface exposed VH cysteine (Cys) and a
first L Cys;
a second disulfide bond between a structurally conserved surface exposed VL Cys and a second
L Cys; or
the first disulfide bond between the structurally conserved surface exposed VH Cys and the first
20 L Cys and the second disulfide bond between the structurally conserved surface exposed VL Cys
and the second L Cys.
- [0088]** The disclosure also provides an isolated single chain variable fragment (scFv)
comprising a means for antigen binding, a linker (L) and a light chain variable region (VL),
wherein the scFv comprises
- 25 a first disulfide bond between a structurally conserved surface exposed antigen binding means
cysteine (Cys) and a first L Cys;
a second disulfide bond between a structurally conserved surface exposed VL Cys and a second
L Cys; or
the first disulfide bond between the structurally conserved surface exposed antigen binding
30 means Cys and the first L Cys and the second disulfide bond between the structurally conserved
surface exposed VL Cys and the second L Cys.

[0089] The disclosure also provides an isolated single chain variable fragment (scFv) comprising a heavy chain variable region (VH), a linker (L) and a means for antigen binding, wherein the scFv comprises
a first disulfide bond between a structurally conserved surface exposed VH cysteine (Cys) and a
5 first L Cys;
a second disulfide bond between a structurally conserved surface exposed antigen binding means Cys and a second L Cys; or
the first disulfide bond between the structurally conserved surface exposed VH Cys and the first L Cys and the second disulfide bond between the structurally conserved surface exposed antigen
10 binding means Cys and the second L Cys.

[0090] The disclosure also provides a multispecific molecule comprising a single chain variable fragment (scFv) comprising a heavy chain variable region (VH), a means for linking (L) and a light chain variable region (VL), wherein the scFv comprises
a first disulfide bond between a structurally conserved surface exposed VH cysteine (Cys) and a
15 first L Cys;
a second disulfide bond between a structurally conserved surface exposed VL Cys and a second L Cys; or
the first disulfide bond between the structurally conserved surface exposed VH Cys and the first L Cys and the second disulfide bond between the structurally conserved surface exposed VL Cys
20 and the second L Cys.

[0091] The disclosure also provides a multispecific molecule comprising a single chain variable fragment (scFv) comprising a means for antigen binding, a linker (L) and a light chain variable region (VL), wherein the scFv comprises
a first disulfide bond between a structurally conserved surface exposed antigen binding means
25 cysteine (Cys) and a first L Cys;
a second disulfide bond between a structurally conserved surface exposed VL Cys and a second L Cys; or
the first disulfide bond between the structurally conserved surface exposed antigen binding means Cys and the first L Cys and the second disulfide bond between the structurally conserved
30 surface exposed VL Cys and the second L Cys.

[0092] The disclosure also provides a multispecific molecule comprising a single chain variable fragment (scFv) comprising a heavy chain variable region (VH), a linker (L) and a means for antigen binding (VL), wherein the scFv comprises
a first disulfide bond between a structurally conserved surface exposed VH cysteine (Cys) and a
5 first L Cys;
a second disulfide bond between a structurally conserved surface exposed antigen binding means
Cys and a second L Cys; or
the first disulfide bond between the structurally conserved surface exposed VH Cys and the first
L Cys and the second disulfide bond between the structurally conserved surface exposed antigen
10 binding means Cys and the second L Cys.

[0093] The disclosure also provides a heterologous molecule comprising a single chain variable fragment (scFv) comprising a heavy chain variable region (VH), a means for linking (L) and a light chain variable region (VL), wherein the scFv comprises
a first disulfide bond between a structurally conserved surface exposed VH cysteine (Cys) and a
15 first L Cys;
a second disulfide bond between a structurally conserved surface exposed VL Cys and a second
L Cys; or
the first disulfide bond between the structurally conserved surface exposed VH Cys and the first
L Cys and the second disulfide bond between the structurally conserved surface exposed VL Cys
20 and the second L Cys.

[0094] The disclosure also provides a heterologous molecule comprising a single chain variable fragment (scFv) comprising a means for antigen binding, a linker (L) and a light chain variable region (VL), wherein the scFv comprises
a first disulfide bond between a structurally conserved surface exposed antigen binding means
25 cysteine (Cys) and a first L Cys;
a second disulfide bond between a structurally conserved surface exposed VL Cys and a second
L Cys; or
the first disulfide bond between the structurally conserved surface exposed antigen binding
means Cys and the first L Cys and the second disulfide bond between the structurally conserved
30 surface exposed VL Cys and the second L Cys.

[0095] The disclosure also provides a heterologous molecule comprising a single chain variable fragment (scFv) comprising a heavy chain variable region (VH), a linker (L) and a means for antigen binding, wherein the scFv comprises a first disulfide bond between a structurally conserved surface exposed VH cysteine (Cys) and a first L Cys;
5 a second disulfide bond between a structurally conserved surface exposed antigen binding means Cys and a second L Cys; or
the first disulfide bond between the structurally conserved surface exposed VH Cys and the first L Cys and the second disulfide bond between the structurally conserved surface exposed antigen
10 binding means Cys and the second L Cys.

[0096] The disclosure also provides a means for encoding the scFv provided herein.

[0097] The disclosure also provides an means for replicating the vector provided herein.

[0098] The disclosure also provides a composition comprising a means for stabilizing a scFv.

[0099] The disclosure also provides a composition comprising a means for increasing
15 thermostability of a scFv.

[00100] The disclosure also provides a multispecific molecule comprising a means for stabilizing a scFv.

[00101] The disclosure also provides a multispecific molecule comprising a means for increasing thermostability of a scFv.

[00102] The disclosure also provides a heterologous molecule comprising a means for
20 stabilizing a scFv.

[00103] The disclosure also provides a heterologous molecule comprising a means for increasing thermostability of a scFv.

4. BRIEF DESCRIPTION OF THE DRAWINGS

[00104] FIG. 1 shows an exemplary design of the stabilized scFv (spFv). The VL and the VH are connected by a flexible linker shown as a dashed line in the Figure containing a staple sequence CPPC (SEQ ID NO: 1) "SS" indicates disulfide bonds between the staple sequence in the linker and anchor points.

[00105] FIG. 2 shows a graphical illustration of anchor point selection for spFv in the VL-linker-VH orientation. Fv of the germline human antibody (pdb id 5I19, GLk1) was used for graphics and illustrative distance measurements. Distances shown in dashed lines are between
30

C β atoms of the residues in Å. Structurally conserved framework positions with desired distances were chosen as anchor points for mutation into Cys. Anchor points for VL-linker-VH orientation were Chothia position 42 for VL (K42 in the Figure) and position 105 for VH (Q105 in the Figure). The C-terminal VL residue (K107) and the N-terminal VH residue (Q1) are also shown.

[00106] FIG. 3 shows a graphical illustration of anchor point selection for spFv in the VH-linker-VL orientation. Fv of the germline human antibody (pdb id 5I19, GLk1) was used for graphics and illustrative distance measurements. Distances shown in dashed lines are between C β atoms of the residues in Å. Structurally conserved framework positions with desired distances were chosen as anchor points for mutation into Cys. Anchor points for VH-linker-VL orientation were Chothia position 43 for VH (K43 in the Figure) and position 100 for VL (Q100 in the Figure). The C-terminal VH residue (S114) and the N-terminal VL residue (D1) are also shown.

[00107] FIG. 4 shows a graphical illustration of C β (Cys1)-C β (Cys2) distance between the two Cys residues in the mouse two heavy chain IgG2a (pdb id 1igt) hinge CPPC (SEQ ID NO: 1). The distances are shown in Ångstrom in the Figure.

[00108] FIG. 5 shows a graphical illustration of C β (Cys1)-C β (Cys2) distance between the two Cys residues in the two heavy chains of human IgG (pdb id 5dk3) hinge CPPC (SEQ ID NO: 1). The distances are shown in Ångstrom in the Figure.

[00109] FIG. 6 shows the chosen VL anchor points highlighted in grey and numbered as 1 and 2 below the amino acid alignments. The VL sequences are numbered according to the Chothia numbering scheme. The VL anchor point 1 (Chothia position 42) was used in spFv in the VL-linker-VH orientation and the VL anchor point 2 (Chothia position 100) was used in spFv in the VH-linker-VL orientation. GLk1VL: SEQ ID NO: 56, GLk2VL: SEQ ID NO: 57, CAT2200VL: SEQ ID NO: 58; CAT2200bVL: SEQ ID NO: 59.

[00110] FIG. 7 shows the chosen VH anchor points highlighted in grey and numbered as 1 and 2 below the amino acid alignments. The VH sequences are numbered according to the Chothia numbering scheme. The VH anchor point 1 (Chothia position 105) was used in spFv in the VL-linker-VH orientation and the VH anchor point 2 (Chothia position 43) was used in spFv in the VH-linker-VL orientation. GLk1VH: SEQ ID NO: 60; GLk2VH: SEQ ID NO: 61, CAT2200aVH: SEQ ID NO: 62.

[00111] FIG. 8 shows the structure of GLk1 spFv VL-VH. The formation of the staple between the VH and VL anchor points and the linker is evident from the structure.

[00112] FIG. 9 shows the structure of GLk1 spFv VH-VL. The formation of the staple between the VH and VL anchor points and the linker is evident from the structure.

5 [00113] FIG. 10 shows the structure of GLk2 spFv VH-VL. The formation of the staple between the VH and VL anchor points and the linker is evident from the structure.

[00114] FIG. 11 shows the structure of CAT2200b spFv VH-VL. The formation of the staple between the VH and VL anchor points and the linker is evident from the structure.

[00115] FIG. 12 shows the comparison of the unbound CAT2200b spFv VH-VL (top) compared to CAT2200a scFv VL-VH bound to IL-17A (bottom).

[00116] FIG. 13 shows the comparison of the front views of the structures of unbound CAT2200b spFv VH-VL (top) compared to CAT2200a spFv VL-VH bound to IL-17A (bottom).

[00117] FIG. 14 shows the comparison of the back views of the structures of unbound CAT2200b spFv VH-VL (top) compared to CAT2200a spFv VL-VH bound to IL-17A (bottom).

15 [00118] FIGS. 15A-15M show schematic representations of antibodies fused to stapled scFv. FIGS. 15A-15D show 2:1 heterodimers, isotype control antibody fused to stapled scFv derived from LTBRmAb1. FIGS. 15E-15H show 2:1 heterodimers, EDBmAb1 fused to stapled scFv derived from LTBRmAb1. FIGS. 15I-15J show 2:1 heterodimers, EDBmAb1 fused to stapled scFv derived from lower affinity variants of LTBRmAb1. FIGS. 15K-15L show 2:1 heterodimers, EDBmAb1 or B21M fused to stapled scFv derived from LTBRmAb1, without protein A mutations in the Fc region. FIG. 15M shows a 2:1 heterodimer, MSLNmAb1 fused to stapled scFv derived from LTBRmAb1.

[00119] FIGS. 16A-16C show graphs demonstrating the results of A549 NF-κB reporter assays using 2:1 bispecific antibodies. FIG. 16A: Comparison of TAA-dependent LTBR activation by COVA1456 with COVA1482, their respective control molecules COVA1462 and COVA1486, and recombinant human LIGHT; FIG. 16B: Comparison of TAA-dependent LTBR activation by COVA1482 and bispecifics containing lower affinity variants of LTBRmAb1 COVA14107 and COVA14108, and COVA1486; FIG. 16C: Comparison of TAA-dependent LTBR activation by COVA1482 and COVA14133 (construct without protein A mutations), and their respective control molecules COVA1486 and COVA14136.

[00120] FIG. 17 shows the results of flow cytometry staining of ICAM-1 on A375 cells after co-culture experiment. COVA1482 and its control molecule COVA1486 are compared to recombinant human LIGHT.

[00121] FIGS. 18A-18D show graphs demonstrating measurements of cytokines in supernatants of co-cultures treated with anti-EDB/anti-LTBR bispecific antibodies COVA14133 compared to COVA14136 and COVA1440. Assays are performed using the MSD platform.

FIG. 18A: Concentrations of human RANTES; FIG. 18B: Concentrations of human IL-6; FIG. 18C: Concentrations of human IL-8; FIG. 18D: Concentrations of human MIP-3b.

[00122] FIGS. 19A-19B show LTBR activation by a MSLN/LTBR bispecific in A549 NF-κB reporter/CHOK1MSLN or A549 NF-κB reporter/H226 co-culture cell assays. FIG. 19A:

Activation of LTBR in A549 NF-κB reporter/H226 co-culture assay. COVA14146 (2:1 MSLNmAb1 x LTBRmAb1) compared to LIGHT and to the isotype control 2:1 constructs COVA1486; FIG. 19B: Concentration of secreted RANTES upon activation of LTBR in A549 NF-κB reporter/H226 co-culture assay. COVA14146 (2:1 MSLNmAb1 x LTBRmAb1)

compared to LIGHT and to the isotype control 2:1 constructs COVA1486.

5. DETAILED DESCRIPTION

[00123] The disclosed methods may be understood more readily by reference to the following detailed description taken in connection with the accompanying Figures, which form a part of this disclosure. It is to be understood that the disclosed methods are not limited to the specific methods described and/or shown herein, and that the terminology used herein is for the purpose of describing particular embodiments by way of example only and is not intended to be limiting.

[00124] All patents, published patent applications and publications cited herein are incorporated by reference as if set forth fully herein.

[00125] When a list is presented, unless stated otherwise, it is to be understood that each individual element of that list, and every combination of that list, is a separate embodiment. For example, a list of embodiments presented as “A, B, or C” is to be interpreted as including the embodiments, “A,” “B,” “C,” “A or B,” “A or C,” “B or C,” or “A, B, or C.”

[00126] As used in this specification and the appended claims, the singular forms “a,” “an,” and “the” include plural referents unless the content clearly dictates otherwise. Thus, for example, reference to “a cell” includes a combination of two or more cells, and the like.

[00127] The transitional terms “**comprising**,” “**consisting essentially of**,” and “**consisting of**” are intended to connote their generally accepted meanings in the patent vernacular; that is, (i) “comprising,” which is synonymous with “including,” “containing,” or “characterized by,” is inclusive or open-ended and does not exclude additional, unrecited elements or method steps; (ii) 5 “consisting of” excludes any element, step, or ingredient not specified in the claim; and (iii) “consisting essentially of” limits the scope of a claim to the specified materials or steps “and those that do not materially affect the basic and novel characteristic(s)” of the claimed invention. Embodiments described in terms of the phrase “comprising” (or its equivalents) also provide as embodiments those independently described in terms of “consisting of” and “consisting 10 essentially of.”

[00128] “**About**” means within an acceptable error range for the particular value as determined by one of ordinary skill in the art, which will depend in part on how the value is measured or determined, *i.e.*, the limitations of the measurement system. Unless explicitly stated otherwise within the Examples or elsewhere in the Specification in the context of a particular assay, result 15 or embodiment, “about” means within one standard deviation per the practice in the art, or a range of up to 5%, whichever is larger.

[00129] “**Alternative scaffold**” refers to a single chain protein framework that contains a structured core associated with variable domains of high conformational tolerance. The variable domains tolerate variation to be introduced without compromising scaffold integrity, and hence 20 the variable domains can be engineered and selected for binding to a specific antigen.

[00130] “**Antibody-dependent cellular cytotoxicity**,” “**antibody-dependent cell-mediated cytotoxicity**” or “**ADCC**” refers to the mechanism of inducing cell death that depends upon the interaction of antibody-coated target cells with effector cells possessing lytic activity, such as natural killer cells (NK), monocytes, macrophages and neutrophils via Fc gamma receptors 25 (FcγR) expressed on effector cells.

[00131] “**Antibody-dependent cellular phagocytosis**” or “**ADCP**” refers to the mechanism of elimination of antibody-coated target cells by internalization by phagocytic cells, such as macrophages or dendritic cells.

[00132] “**Antigen**” refers to any molecule (*e.g.*, protein, peptide, polysaccharide, glycoprotein, 30 glycolipid, nucleic acid, portions thereof, or combinations thereof) that is capable of mediating

an immune response. Exemplary immune responses include antibody production and activation of immune cells, such as T cells, B cells or NK cells.

[00133] “**Antigen binding fragment**” or “**antigen binding domain**” refers to a portion of a protein that binds the antigen. Antigen binding fragments may be synthetic, enzymatically obtainable or genetically engineered polypeptides and include portions of an immunoglobulin that bind an antigen, such as a VH, a VL, the VH and the VL, Fab, Fab’, F(ab’)₂, Fd and Fv fragments, domain antibodies (dAb) consisting of one VH domain or one VL domain, camelized VH domains, VHH domains, minimal recognition units consisting of the amino acid residues that mimic the CDRs of an antibody, such as FR3-CDR3-FR4 portions, the HCDR1, the HCDR2 and/or the HCDR3 and the LCDR1, the LCDR2 and/or the LCDR3, alternative scaffolds that bind an antigen, and multispecific proteins comprising the antigen binding fragments. Antigen binding fragments (such as the VH and the VL) may be linked together via a synthetic linker to form various types of single antibody designs in which the VH/VL domains may pair intramolecularly, or intermolecularly in those cases when the VH and the VL domains are expressed by separate single chains, to form a monovalent antigen binding domain, such as single chain Fv (scFv) or diabody. Antigen binding fragments may also be conjugated to other antibodies, proteins, antigen binding fragments or alternative scaffolds which may be monospecific or multispecific to engineer bispecific and multispecific proteins.

[00134] “**Antibodies**” is meant in a broad sense and includes immunoglobulin molecules including monoclonal antibodies including murine, human, humanized and chimeric monoclonal antibodies, antigen binding fragments, multispecific antibodies, such as bispecific, trispecific, tetraspecific, *etc.*, dimeric, tetrameric or multimeric antibodies, single chain antibodies, domain antibodies and any other modified configuration of the immunoglobulin molecule that comprises an antigen binding site of the required specificity. “Full length antibodies” are comprised of two heavy chains (HC) and two light chains (LC) inter-connected by disulfide bonds as well as multimers thereof (*e.g.*, IgM). Each heavy chain is comprised of a heavy chain variable region (VH) and a heavy chain constant region (comprised of domains CH1, hinge, CH2 and CH3). Each light chain is comprised of a light chain variable region (VL) and a light chain constant region (CL). The VH and the VL regions may be further subdivided into regions of hypervariability, termed complementarity determining regions (CDR), interspersed with framework regions (FR). Each VH and VL is composed of three CDRs and four FR segments,

arranged from amino-to-carboxy-terminus in the following order: FR1, CDR1, FR2, CDR2, FR3, CDR3 and FR4. Immunoglobulins may be assigned to five major classes, IgA, IgD, IgE, IgG and IgM, depending on the heavy chain constant domain amino acid sequence. IgA and IgG are further sub-classified as the isotypes IgA1, IgA2, IgG1, IgG2, IgG3 and IgG4. Antibody light chains of any vertebrate species may be assigned to one of two clearly distinct types, namely kappa (κ) and lambda (λ), based on the amino acid sequences of their constant domains.

[00135] “Bispecific” refers to a molecule (such as an antibody) that specifically binds two distinct antigens or two distinct epitopes within the same antigen. The bispecific molecule may have cross-reactivity to other related antigens, for example to the same antigen from other species (homologs), such as human or monkey, for example *Macaca cynomolgus* (cynomolgus, cyno) or *Pan troglodytes*, or may bind an epitope that is shared between two or more distinct antigens.

[00136] “Chimeric antigen receptor” or “**CAR**” refers to engineered T cell receptors which graft a ligand or antigen specificity onto T cells (for example naïve T cells central memory T cells effector memory T cells or combinations thereof). CARs are also known as artificial T- cell receptors, chimeric T-cell receptors or chimeric immunoreceptors. CARs comprise an extracellular domain capable of binding to an antigen, a transmembrane domain and at least one intracellular domain. CAR intracellular domain comprises a polypeptide known to function as a domain that transmits a signal to cause activation or inhibition of a biological process in a cell. The transmembrane domain comprises any peptide or polypeptide known to span the cell membrane and that can function to link the extracellular and signaling domains. A chimeric antigen receptor may optionally comprise a hinge domain which serves as a linker between the extracellular and transmembrane domains.

[00137] “Complement-dependent cytotoxicity” or “**CDC**”, refers to the mechanism of inducing cell death in which the Fc effector domain of a target-bound protein binds and activates complement component C1q which in turn activates the complement cascade leading to target cell death. Activation of complement may also result in deposition of complement components on the target cell surface that facilitate CDC by binding complement receptors (*e.g.*, CR3) on leukocytes.

[00138] “Complementarity determining regions” (CDR) are antibody regions that bind an antigen. There are three CDRs in the VH (HCDR1, HCDR2, HCDR3) and three CDRs in the

VL (LCDR1, LCDR2, LCDR3). CDRs may be defined using various delineations such as Kabat (Wu *et al.*, (1970) *J Exp Med* 132: 211-250; Kabat *et al.*, Sequences of Proteins of Immunological Interest, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, Md., 1991), Chothia (Chothia *et al.*, (1987) *J Mol Biol* 196: 901-17), IMGT (Lefranc *et al.*, (2003) *Dev Comp Immunol* 27: 55-77) and AbM (Martin and Thornton (1996) *J Biol Chem* 271: 800-815). The correspondence between the various delineations and variable region numbering is described (see *e.g.*, Lefranc *et al.* (2003) *Dev Comp Immunol* 27: 55-77; Honegger and Pluckthun, *J Mol Biol* (2001) 309:657-670; International ImMunoGeneTics (IMGT) database; Web resources, <http://www.imgt.org>). Available programs such as abYsis by UCL Business PLC may be used to delineate CDRs. The term “CDR”, “HCDR1”, “HCDR2”, “HCDR3”, “LCDR1”, “LCDR2” and “LCDR3” as used herein includes CDRs defined by any of the methods described supra, Kabat, Chothia, IMGT or AbM, unless otherwise explicitly stated in the specification.

[00139] “**Decrease**,” “**lower**” or “**reduce**,” refers generally to the ability of a test molecule to mediate a reduced response (*i.e.*, downstream effect) when compared to the response mediated by a control or a vehicle. Exemplary responses include binding of a protein to its antigen or receptor, enhanced binding to FcγR or enhanced Fc effector functions such as enhanced ADCC, CDC and/or ADCP. Decrease may be a statistically significant difference in the measured response between the test molecule and the control (or the vehicle), or a decrease in the measured response, such as a decrease of about 1.1, 1.2, 1.5, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20 or 30 fold or more, such as 500, 600, 700, 800, 900 or 1000 fold or more.

[00140] “**Enhance**,” “**promote**” or “**increase**,” refers generally to the ability of the test molecule to mediate a greater response (*i.e.*, downstream effect) when compared to the response mediated by a control or a vehicle. Exemplary responses are binding of a protein to its antigen or receptor, enhanced binding to FcγR or enhanced Fc effector functions such as enhanced ADCC, CDC and/or ADCP. Enhance may be a statistically significant difference in the measured response between the test molecule and control (or vehicle), or an increase in the measured response, such as an increase of about 1.1, 1.2, 1.5, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20 or 30 fold or more, such as 500, 600, 700, 800, 900 or 1000 fold or more.

[00141] “**Expression vector**” refers to a vector that can be utilized in a biological system or in a reconstituted biological system to direct the translation of a polypeptide encoded by a polynucleotide sequence present in the expression vector.

5 [00142] “**Heterologous**” refers to a polypeptide or a polynucleotide that comprises two or more polypeptides or two or more polynucleotides which are not found in the same relationship to each other in nature.

[00143] “**Heterologous polynucleotide**” refers to a polynucleotide that comprises two or more polynucleotides which are not found in the same relationship to each other in nature.

10 [00144] “**Heterologous polypeptide**” refers to a polypeptide that comprises two or more polypeptides which are not found in the same relationship to each other in nature.

[00145] “**Human antibody**” refers to an antibody that is optimized to have minimal immune response when administered to a human subject. Variable regions of human antibody are derived from human immunoglobulin sequences. If human antibody contains a constant region or a portion of the constant region, the constant region is also derived from human immunoglobulin sequences. Human antibody comprises heavy and light chain variable regions that are “derived from” sequences of human origin if the variable regions of the human antibody are obtained from a system that uses human germline immunoglobulin or rearranged immunoglobulin genes. Such exemplary systems are human immunoglobulin gene libraries displayed on phage, and transgenic non-human animals such as mice or rats carrying human immunoglobulin loci. “Human antibody” typically contains amino acid differences when compared to the immunoglobulins expressed in humans due to differences between the systems used to obtain the human antibody and human immunoglobulin loci, introduction of somatic mutations or intentional introduction of substitutions into the frameworks or CDRs, or both. Typically, “human antibody” is at least about 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 25 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% identical in amino acid sequence to an amino acid sequence encoded by human germline immunoglobulin or rearranged immunoglobulin genes. In some cases, “human antibody” may contain consensus framework sequences derived from human framework sequence analyses, for example as described in Knappik *et al.*, (2000) *J Mol Biol* 296:57-86, or a synthetic HCDR3 incorporated into human immunoglobulin gene libraries displayed on phage, for example as described in Shi *et al.*, (2010) 30 *J Mol Biol* 397:385-396, and in Int. Patent Publ. No. WO2009/085462. Antibodies in which at

least one CDR is derived from a non-human species are not included in the definition of “human antibody”.

[00146] “**Humanized antibody**” refers to an antibody in which at least one CDR is derived from non-human species and at least one framework is derived from human immunoglobulin sequences. Humanized antibody may include substitutions in the frameworks so that the frameworks may not be exact copies of expressed human immunoglobulin or human immunoglobulin germline gene sequences.

[00147] “**Isolated**” refers to a homogenous population of molecules (such as scFv of the disclosure or heterologous proteins comprising the scFv of the disclosure) which have been substantially separated and/or purified away from other components of the system the molecules are produced in, such as a recombinant cell, as well as a protein that has been subjected to at least one purification or isolation step. “Isolated” refers to a molecule that is substantially free of other cellular material and/or chemicals and encompasses molecules that are isolated to a higher purity, such as to 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% purity.

[00148] “**Modulate**” refers to either enhanced or decreased ability of a test molecule to mediate an enhanced or a reduced response (*i.e.*, downstream effect) when compared to the response mediated by a control or a vehicle.

[00149] “**Monoclonal antibody**” refers to an antibody obtained from a substantially homogenous population of antibody molecules, *i.e.*, the individual antibodies comprising the population are identical except for possible well-known alterations such as removal of C-terminal lysine from the antibody heavy chain or post-translational modifications such as amino acid isomerization or deamidation, methionine oxidation or asparagine or glutamine deamidation. Monoclonal antibodies typically bind one antigenic epitope. A bispecific monoclonal antibody binds two distinct antigenic epitopes. Monoclonal antibodies may have heterogeneous glycosylation within the antibody population. Monoclonal antibody may be monospecific or multispecific such as bispecific, monovalent, bivalent or multivalent.

[00150] “**Multispecific**” refers to a molecule that binds two or more distinct antigens or two or more distinct epitopes within the same antigen. Multispecific molecule may have cross-reactivity to other related antigens, for example to the same antigen from other species

(homologs), such as human or monkey, for example *Macaca fascicularis* (cynomolgus, cyno) or *Pan troglodytes*, or may bind an epitope that is shared between two or more distinct antigens.

5 [00151] “**Polynucleotide**” refers to a molecule comprising a chain of nucleotides covalently linked by a sugar-phosphate backbone or other equivalent covalent chemistry. cDNA is a typical example of a polynucleotide.

[00152] “**Protein**” or “**polypeptide**” are used interchangeably herein and refers to a molecule that comprises one or more polypeptides each comprised of at least two amino acid residues linked by a peptide bond. Protein may be a monomer, or may be a protein complex of two or more subunits, the subunits being identical or distinct. Small polypeptides of less than 50 amino acids may be referred to as “peptides”. Protein may be a heterologous fusion protein, a glycoprotein, or a protein modified by post-translational modifications such as phosphorylation, acetylation, myristoylation, palmitoylation, glycosylation, oxidation, formylation, amidation, citrullination, polyglutamylation, ADP-ribosylation, pegylation or biotinylation.

15 [00153] “**Recombinant**” refers to polynucleotides, polypeptides, vectors, viruses and other macromolecules that are prepared, expressed, created or isolated by recombinant means.

[00154] “**Single chain Fv**” or “**scFv**” refers to a single chain protein comprising a VH, a VL and a linker between the VH and the VL. The scFv may have the VL and VH variable regions in either orientation, e.g., with respect to the N- to C-terminal order of the VH and the VL. The scFv may thus be in the orientation VL-linker-VH or VH-linker-VL. scFv may be engineered to

20 [00155] “**Specifically binds**,” “**specific binding**,” “**specifically binding**” or “**binds**” refers to a protein such as a scFv binding to an antigen or an epitope within the antigen with greater affinity than for other antigens. Typically, the protein, such as the scFv binds to the antigen or the epitope within the antigen with an equilibrium dissociation constant (K_D) of about 1×10^{-6} M or less, about 1×10^{-7} M or less, about 5×10^{-8} M or less, about 1×10^{-8} M or less, about 1×10^{-9} M or less, about 1×10^{-10} M or less, about 1×10^{-11} M or less, or about 1×10^{-12} M or less, typically with the K_D that is at least one hundred fold less than its K_D for binding to a non-specific antigen (e.g., BSA, casein).

30 [00156] “**Stapled single chain Fv**” or “**spFv**” refers to a scFv that comprises one or more disulfide bonds between the VH and the linker or the VL and the linker. Typically the spFv may comprise one disulfide bond between the VH and the linker, one disulfide bond between the VL

and the linker, or two disulfide bonds between the VH and the linker and the VL and the linker. scFv molecules which comprise disulfide bonds between the VH and the VL are excluded from the term “spFv”.

5 [00157] “**Subject**” includes any human or nonhuman animal. “Nonhuman animal” includes all vertebrates, *e.g.*, mammals and non-mammals, such as nonhuman primates, sheep, dogs, cats, horses, cows, chickens, amphibians, reptiles, *etc.* The terms “subject” and “patient” can be used interchangeably herein.

[00158] “**Therapeutically effective amount**” refers to an amount effective, at dosages and for periods of time necessary, to achieve a desired therapeutic result. A therapeutically effective
10 amount may vary according to factors such as the disease state, age, sex, and weight of the individual, and the ability of a therapeutic or a combination of therapeutics to elicit a desired response in the individual.

[00159] “**Treat**,” “**treating**” or “**treatment**” of a disease or disorder refers to accomplishing one or more of the following: reducing the severity and/or duration of the disorder, inhibiting
15 worsening of symptoms characteristic of the disorder being treated, limiting or preventing recurrence of the disorder in subjects that have previously had the disorder, or limiting or preventing recurrence of symptoms in subjects that were previously symptomatic for the disorder.

[00160] “**Trispecific**” refers to a molecule (such as an antibody) that specifically binds three
20 distinct antigens or three distinct epitopes within the same antigen. The trispecific molecule may have cross-reactivity to other related antigens, for example to the same antigen from other species (homologs), such as human or monkey, for example *Macaca cynomolgus* (cynomolgus, cyno) or *Pan troglodytes*, or may bind an epitope that is shared between three or more distinct antigens.

25 [00161] “**Variant**,” “**mutant**” or “**altered**” refers to a polypeptide or a polynucleotide that differs from a reference polypeptide or a reference polynucleotide by one or more modifications, for example one or more substitutions, insertions or deletions.

[00162] The numbering of amino acid residues of the antibody constant region throughout the specification is according to the EU index as described in Kabat *et al.*, Sequences of Proteins of
30 Immunological Interest, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, MD. (1991), unless otherwise explicitly stated.

[00163] Mutations in the Ig constant regions are referred to as follows: L351Y_F405A_Y407V refers to L351Y, F405A and Y407V mutations in one immunoglobulin constant region.

L351Y_F405A_Y407V/T394W refers to L351Y, F405A and Y407V mutations in the first Ig constant region and T394W mutation in the second Ig constant region present in the molecule.

5 [00164] The numbering of the variable regions is according to Chothia unless otherwise explicitly stated.

[00165] “**VH Cysteine**” or “**VH Cys**” refers to a Cys residue that resides in the VH framework.

[00166] “**VL Cysteine**” or “**VL Cys**” refers to a Cys residue that resides in the VL framework.

10 [00167] “**Stabilized**” refers to a The scFvs retaining comparable binding to hK2 when compared to a non-heated scFv sample are referred to as being thermostable.

[00168] “**Improved stability**” refers to the spFv of the disclosure having an elevated melting point (T_m) when compared to a parent scFv that is devoid of disulfide bonds and Cys residues introduced into the spFv. The elevated T_m may be an elevation of 2°C or more, such as 3°C, 4°C, 5°C, 6°C, 7°C, 8°C, 9°C, 10°C, 11°C, 12°C, 13°C, 14°C or 15°C.

15 [00169] “**Anchor point**” refers to a scFv VH or a VL framework Cys residue that can be mutagenized to Cys without adverse effect to the overall scFv structure and is capable of forming a disulfide bond with a Cys residing in the scFv linker.

[00170] “**Staple**” refers to the scFv linker that contains one or two Cys residues which are 20 capable of forming a disulfide bond with the anchor point Cys.

[00171] “**Surface exposed**” refers to an amino acid residue that is at least partially exposed to a surface of a protein and accessible to solvent, such as accessible to deuteration. Algorithms are well-known in the art for predicting surface accessibility of residues based on primary sequence or a protein. Alternatively, surface exposed residues may be identified from a crystal 25 structure of the protein.

[00172] “**LTBR**” refers to a polypeptide that is a cell surface receptor for lymphotoxin involved in apoptosis and cytokine release, which is a member of the tumor necrosis factor receptor superfamily. LTBR can also be referred to as “tumor necrosis factor receptor superfamily member 3 (TNFRSF3).” LTBR is expressed on the surface of many cell types, 30 including cells of epithelial and myeloid lineages. LTBR can specifically bind the lymphotoxin membrane form (a complex of lymphotoxin-alpha and lymphotoxin-beta). Activation of LTBR

can trigger apoptosis via TRAF3 and TRAF5 and can lead to the release of interleukin 8. Unless noted, preferably the LTBR is a human LTBR. A human LTBR amino acid sequence is provided by UniProt number P36941.

5 [00173] “**EDB**” or “**extra domain B**” refers to a domain of fibronectin that can be included in fibronectin molecules based on the splicing pattern of the fibronectin pre-mRNA. Extra domain B is a complete fibronectin (FN) type III repeat that comprises 91 amino acid residues. Generally, EDB is undetectable in normal adult tissues, but exhibits greater expression in fetal and tumor tissues in the extracellular matrix, and accumulates around neovasculature during angiogenic processes, thus making EDB a potential marker and target of angiogenesis. Unless
10 noted, preferably EDB is a human EDB. A human EDB containing fibronectin isoform amino acid sequence is provided by UniProt number P02751.

[00174] “**Fibronectin**” refers to a polypeptide that is a high molecular weight glycoprotein of the extracellular matrix. Fibronectin can bind to membrane-spanning receptor proteins, referred to as integrins. Fibronectin can also bind other extracellular matrix proteins, such as collagen,
15 fibrin, and heparan sulfate proteoglycans. Fibronectin can exist as a protein dimer, consisting of two nearly identical monomers linked by a pair of disulfide bonds. Fibronectin is produced from a single gene, but alternative splicing of the fibronectin pre-mRNA molecule leads to the creation of several isoforms of fibronectin, one of which is EDB fibronectin. Fibronectin can play a role in cell adhesion, growth, migration, and differentiation, and it can be important for
20 processes such as wound healing and embryonic development. A human fibronectin amino acid sequence is provided by UniProt number P02751, which contains extra domain B, and NCBI Accession Numbers NP_001263337 (isoform B), NP_001263338 (isoform c), NP_001263339 (isoform d), NP_001263340 (isoform e), and NP_001263341 (isoform f), NP_001293058 (isoform 8), NP_001293059 (isoform 9), NP_001293060 (isoform 10), NP_001293061 (isoform
25 11), and NP_002017 (isoform 3).

5.1 Compositions of matter

[00175] The disclosure provides stabilized scFv molecules (herein referred to as spFv (stapled Fv), heterologous and multispecific molecules comprising the spFv, polynucleotides encoding them, vectors, host cells and methods of making and using them. The disclosure is based, at
30 least in part, on the identification of suitable residue positions in the VH and/or the VL (herein referred to as VH anchor point or VL anchor point) and in the flexible linker (herein referred to

as staple) which may be engineered to cysteine residues resulting in formation of disulfide bonds between the linker and the variable domains in the scFv. The “stapling” strategy described herein is widely applicable to all VH/VL domains and pre-existing scFv molecules providing structural identity to scFv with improved stability. The spFv described herein may be conjugated
5 into any heterologous protein, bispecific or multispecific format, including chimeric antigen receptors (CAR), T cell redirection molecules, bi- and multispecific molecules and may be used as therapeutic, diagnostic and detection molecules.

spFv of the disclosure

[00176] The disclosure provides an isolated single chain variable fragment (scFv) comprising a
10 heavy chain variable region (VH), a linker (L) and a light chain variable region (VL), wherein the scFv comprises

a first disulfide bond between a structurally conserved surface exposed VH cysteine (Cys) and a first L Cys;

a second disulfide bond between a structurally conserved surface exposed VL Cys and a second
15 L Cys; or

the first disulfide bond between the structurally conserved surface exposed VH Cys and the first L Cys and the second disulfide bond between the structurally conserved surface exposed VL Cys and the second L Cys.

[00177] The disclosure also provides an isolated scFv comprising a VH, a L and a VL, wherein
20 the VH comprises a VH Cys at a structurally conserved surface exposed VH framework residue position and the L comprises a first L Cys;

the VL comprises a VL Cys at a structurally conserved surface exposed VL framework residue position and the L comprises a second L Cys; or

the VH comprises the VH Cys at a structurally conserved surface exposed VH framework
25 residue position, the VL comprises the VL Cys at a structurally conserved surface exposed VL framework residue position and the L comprises the first L Cys and the second L Cys, wherein the VH Cys and the first L Cys are capable of forming a disulfide bond and the VL Cys and the second L Cys are capable of forming a disulfide bond. The disulfide bond typically forms during expression of the scFv of the disclosure.

[00178] While the specific examples disclose spFv with two disulfide bonds, it is readily
30 envisioned that spFv with one disulfide bond, formed between the linker Cys and either the VH

Cys or the VL Cys can be made and utilized, generating “half-anchored” molecules. The anchor positions are the same in spFv having one or two disulfide bonds. The linker Cys position may vary in the half-anchored molecule as long as it satisfies distance and geometry requirements for disulfide bond formation with the anchor point. It is expected that the half-anchored spFv will
5 restrain VL/VH relative movement similar to the VL/VH pair stabilized with two disulfide bonds, and thus will also be stabilizing.

[00179] The spFv of the disclosure exhibited increased thermal stability when compared to the parent scFv devoid of the disulfide bonds. In general, the T_m of the spFv was about 10°C higher when compared to the parent scFv devoid of the disulfide bonds regardless of the T_m of the
10 parent scFv. Stability in general may be thermal stability or mechanical stability.

Thermostability may be evaluated using differential thermal calorimetry (DSC), in which DSC scans are performed using heated protein samples (such as samples heated to 60°C) followed by analyses of the resulting thermal melting profiles using 2-state or non-2-state transitions. For non-2-state transitions, two transitions (T_{m1} and T_{m2}) are recorded which correspond to the
15 melting T_m of the VL and the VH domains, respectively.

[00180] In some embodiments, the distance between the VH Cys and the VL Cys is from about 7 Å to about 9 Å. In some embodiments, the distance between the VH Cys and the VL Cys is about 7 Å. In some embodiments, the distance between the VH Cys and the VL Cys is about 8 Å. In some embodiments, the distance between the VH Cys and the VL Cys is about 9 Å.

[00181] In some embodiments, the VH Cys is at H3, H5, H40, H43, H46 or H105, wherein residue numbering is according to Chothia.

[00182] In some embodiments, the VH Cys is at H3.

[00183] In some embodiments, the VH Cys is at H5.

[00184] In some embodiments, the VH Cys is at H40.

[00185] In some embodiments, the VH Cys is at H43.

[00186] In some embodiments, the VH Cys is at H46.

[00187] In some embodiments, the VH Cys is at H105.

[00188] In some embodiments, the VL Cys is at L3, L5, L39, L42, L45, L100 or L102, wherein residue numbering is according to Chothia.

[00189] In some embodiments, the VL Cys is at L3.

[00190] In some embodiments, the VL Cys is at L5.

- [00191] In some embodiments, the VL Cys is at L39.
- [00192] In some embodiments, the VL Cys is at L42.
- [00193] In some embodiments, the VL Cys is at L45.
- [00194] In some embodiments, the VL Cys is at L100.
- 5 [00195] In some embodiments, the VL Cys is at L102.
- [00196] In some embodiments, the VH Cys is at H105 and the VL Cys is at L42.
- [00197] In some embodiments, the VH Cys is at H43 and the VL Cys is at L100.
- [00198] In some embodiments, the VH Cys is at H3 and the VL Cys is at L3.
- [00199] In some embodiments, the VH Cys is at H3 and the VL Cys is at L5.
- 10 [00200] In some embodiments, the VH Cys is at H3 and the VL Cys is at L39.
- [00201] In some embodiments, the VH Cys is at H3 and the VL Cys is at L42.
- [00202] In some embodiments, the VH Cys is at H3 and the VL Cys is at L45.
- [00203] In some embodiments, the VH Cys is at H3 and the VL Cys is at L100.
- [00204] In some embodiments, the VH Cys is at H3 and the VL Cys is at L102.
- 15 [00205] In some embodiments, the VH Cys is at H5 and the VL Cys is at L3.
- [00206] In some embodiments, the VH Cys is at H5 and the VL Cys is at L5.
- [00207] In some embodiments, the VH Cys is at H5 and the VL Cys is at L39.
- [00208] In some embodiments, the VH Cys is at H5 and the VL Cys is at L42.
- [00209] In some embodiments, the VH Cys is at H5 and the VL Cys is at L45.
- 20 [00210] In some embodiments, the VH Cys is at H5 and the VL Cys is at L100.
- [00211] In some embodiments, the VH Cys is at H5 and the VL Cys is at L102.
- [00212] In some embodiments, the VH Cys is at H40 and the VL Cys is at L3.
- [00213] In some embodiments, the VH Cys is at H40 and the VL Cys is at L5.
- [00214] In some embodiments, the VH Cys is at H40 and the VL Cys is at L39.
- 25 [00215] In some embodiments, the VH Cys is at H40 and the VL Cys is at L42.
- [00216] In some embodiments, the VH Cys is at H40 and the VL Cys is at L45.
- [00217] In some embodiments, the VH Cys is at H40 and the VL Cys is at L100.
- [00218] In some embodiments, the VH Cys is at H40 and the VL Cys is at L102.
- [00219] In some embodiments, the VH Cys is at H43 and the VL Cys is at L3.
- 30 [00220] In some embodiments, the VH Cys is at H43 and the VL Cys is at L5.
- [00221] In some embodiments, the VH Cys is at H43 and the VL Cys is at L39.

- [00222] In some embodiments, the VH Cys is at H43 and the VL Cys is at L42.
- [00223] In some embodiments, the VH Cys is at H43 and the VL Cys is at L45.
- [00224] In some embodiments, the VH Cys is at H43 and the VL Cys is at L102.
- [00225] In some embodiments, the VH Cys is at H46 and the VL Cys is at L3.
- 5 [00226] In some embodiments, the VH Cys is at H46 and the VL Cys is at L5.
- [00227] In some embodiments, the VH Cys is at H46 and the VL Cys is at L39.
- [00228] In some embodiments, the VH Cys is at H46 and the VL Cys is at L42.
- [00229] In some embodiments, the VH Cys is at H46 and the VL Cys is at L45.
- [00230] In some embodiments, the VH Cys is at H46 and the VL Cys is at L100.
- 10 [00231] In some embodiments, the VH Cys is at H46 and the VL Cys is at L102.
- [00232] In some embodiments, the VH Cys is at H105 and the VL Cys is at L3.
- [00233] In some embodiments, the VH Cys is at H105 and the VL Cys is at L5.
- [00234] In some embodiments, the VH Cys is at H105 and the VL Cys is at L39.
- [00235] In some embodiments, the VH Cys is at H105 and the VL Cys is at L45.
- 15 [00236] In some embodiments, the VH Cys is at H105 and the VL Cys is at L100.
- [00237] In some embodiments, the VH Cys is at H105 and the VL Cys is at L102.
- [00238] The residue numbering of the VH and the VL regions are according to Chothia.
- [00239] Chothia numbering is well known. Other numbering systems, such as Kabat or IMGT numbering, or sequential numbering may be used to number the VH and the VL residue
- 20 positions. **Table 1** shows the correspondence between Chothia, Kabat and sequential numbering for an exemplary VH, GLk1 VH (**SEQ ID NO: 60**). **Table 2** shows the correspondence between Chothia, Kabat and sequential numbering for an exemplary VL, GLk1 VL (**SEQ ID NO: 56**).

Table 1.

Chothia numbering	Kabat numbering	Sequential numbering	Amino acid residue at position	Chothia numbering	Kabat numbering	Sequential numbering	Amino acid residue at position
H1	H1	1	E	H60	H60	61	A
H2	H2	2	V	H61	H61	62	D
H3	H3	3	Q	H62	H62	63	S
H4	H4	4	L	H63	H63	64	V
H5	H5	5	L	H64	H64	65	K

Chothia numbering	Kabat numbering	Sequential numbering	Amino acid residue at position	Chothia numbering	Kabat numbering	Sequential numbering	Amino acid residue at position
H6	H6	6	E	H65	H65	66	G
H7	H7	7	S	H66	H66	67	R
H8	H8	8	G	H67	H67	68	F
H9	H9	9	G	H68	H68	69	T
H10	H10	10	G	H69	H69	70	I
H11	H11	11	L	H70	H70	71	S
H12	H12	12	V	H71	H71	72	R
H13	H13	13	Q	H72	H72	73	D
H14	H14	14	P	H73	H73	74	N
H15	H15	15	G	H74	H74	75	S
H16	H16	16	G	H75	H75	76	K
H17	H17	17	S	H76	H76	77	N
H18	H18	18	L	H77	H77	78	T
H19	H19	19	R	H78	H78	79	L
H20	H20	20	L	H79	H79	80	Y
H21	H21	21	S	H80	H80	81	L
H22	H22	22	C	H81	H81	82	Q
H23	H23	23	A	H82	H82	83	M
H24	H24	24	A	H82A	H82A	84	N
H25	H25	25	S	H82B	H82B	85	S
H26	H26	26	G	H82C	H82C	86	L
H27	H27	27	F	H83	H83	87	R
H28	H28	28	T	H84	H84	88	A
H29	H29	29	F	H85	H85	89	E
H30	H30	30	S	H86	H86	90	D
H31	H31	31	S	H87	H87	91	T
H32	H32	32	Y	H88	H88	92	A
H33	H33	33	A	H89	H89	93	V
H34	H34	34	M	H90	H90	94	Y

Chothia numbering	Kabat numbering	Sequential numbering	Amino acid residue at position	Chothia numbering	Kabat numbering	Sequential numbering	Amino acid residue at position
H35	H35	35	S	H91	H91	95	Y
H36	H36	36	W	H92	H92	96	C
H37	H37	37	V	H93	H93	97	A
H38	H38	38	R	H94	H94	98	K
H39	H39	39	Q	H95	H95	99	Y
H40	H40	40	A	H96	H96	100	D
H41	H41	41	P	H97	H97	101	G
H42	H42	42	G	H98	H98	102	I
H43	H43	43	K	H99	H99	103	Y
H44	H44	44	G	H100	H100	104	G
H45	H45	45	L	H100A	H100A	105	E
H46	H46	46	E	H100B	H100B	106	L
H47	H47	47	W	H101	H101	107	D
H48	H48	48	V	H102	H102	108	F
H49	H49	49	S	H103	H103	109	W
H50	H50	50	A	H104	H104	110	G
H51	H51	51	I	H105	H105	111	Q
H52	H52	52	S	H106	H106	112	G
H52A	H52A	53	G	H107	H107	113	T
H53	H53	54	S	H108	H108	114	L
H54	H54	55	G	H109	H109	115	V
H55	H55	56	G	H110	H110	116	T
H56	H56	57	S	H111	H111	117	V
H57	H57	58	T	H112	H112	118	S
H58	H58	59	Y	H113	H113	119	S
H59	H59	60	Y				

Table 2.

Chothia numbering	Kabat numbering	Sequential numbering	Amino acid residue at position	Chothia numbering	Kabat numbering	Sequential numbering	Amino acid residue at position
L1	L1	1	D	L55	L55	55	Q
L2	L2	2	I	L56	L56	56	S
L3	L3	3	Q	L57	L57	57	G
L4	L4	4	M	L58	L58	58	V
L5	L5	5	T	L59	L59	59	P
L6	L6	6	Q	L60	L60	60	S
L7	L7	7	S	L61	L61	61	R
L8	L8	8	P	L62	L62	62	F
L9	L9	9	S	L63	L63	63	S
L10	L10	10	S	L64	L64	64	G
L11	L11	11	L	L65	L65	65	S
L12	L12	12	S	L66	L66	66	G
L13	L13	13	A	L67	L67	67	S
L14	L14	14	S	L68	L68	68	G
L15	L15	15	V	L69	L69	69	T
L16	L16	16	G	L70	L70	70	D
L17	L17	17	D	L71	L71	71	F
L18	L18	18	R	L72	L72	72	T
L19	L19	19	V	L73	L73	73	L
L20	L20	20	T	L74	L74	74	T
L21	L21	21	I	L75	L75	75	I
L22	L22	22	T	L76	L76	76	S
L23	L23	23	C	L77	L77	77	S
L24	L24	24	R	L78	L78	78	L
L25	L25	25	A	L79	L79	79	Q
L26	L26	26	S	L80	L80	80	P
L27	L27	27	Q	L81	L81	81	E
L28	L28	28	S	L82	L82	82	D

Chothia numbering	Kabat numbering	Sequential numbering	Amino acid residue at position	Chothia numbering	Kabat numbering	Sequential numbering	Amino acid residue at position
L29	L29	29	I	L83	L83	83	F
L30	L30	30	S	L84	L84	84	A
L31	L31	31	S	L85	L85	85	T
L32	L32	32	Y	L86	L86	86	Y
L33	L33	33	L	L87	L87	87	Y
L34	L34	34	N	L88	L88	88	C
L35	L35	35	W	L89	L89	89	Q
L36	L36	36	Y	L90	L90	90	Q
L37	L37	37	Q	L91	L91	91	S
L38	L38	38	Q	L92	L92	92	Y
L39	L39	39	K	L93	L93	93	S
L40	L40	40	P	L94	L94	94	T
L41	L41	41	G	L95	L95	95	P
L42	L42	42	K	L96	L96	96	L
L43	L43	43	A	L97	L97	97	T
L44	L44	44	P	L98	L98	98	F
L45	L45	45	K	L99	L99	99	G
L46	L46	46	L	L100	L100	100	Q
L47	L47	47	L	L101	L101	101	G
L48	L48	48	I	L102	L102	102	T
L49	L49	49	Y	L103	L103	103	K
L50	L50	50	A	L104	L104	104	V
L51	L51	51	A	L105	L105	105	E
L52	L52	52	S	L106	L106	106	I
L53	L53	53	S	L107	L107	107	K
L54	L54	54	L	L108	L108	108	R

[00240] In some embodiments, the L comprises a contiguous amino acid sequence derived from an immunoglobulin (Ig) hinge region.

[00241] In some embodiments, the Ig hinge region is derived from a human or a non-human Ig hinge region. Exemplary non-human Ig hinge regions are those from mouse, rat, dog, chicken and non-human primates, such as monkeys.

[00242] In some embodiments, the Ig hinge region is derived from the human Ig hinge region.

5 [00243] In some embodiments, the human Ig hinge region is an IgG1, IgG2, IgG3, IgG4, IgM, IgA or IgE isotype.

[00244] The Ig hinge region is in general defined as including residue 216 and terminating at residue 230 of human IgG, wherein residue numbering is according to the EU Index. In some instances, a lower hinge region from about residue 231 to about residue 237 may also be
10 included into the hinge. An IgG1 hinge region therefore may comprise the amino acid sequence EPKSCDKTHTCPPCP (SEQ ID NO: 63), or when the lower hinge is included, the amino acid sequence EPKSCDKTHTCPPCPAPPELLGG (SEQ ID NO: 64). The hinge regions of other Ig isotypes are well known and their amino acid sequences may be obtained for example at ImMunoGeneTics web site. For example, IgG2 hinge comprises the amino acid sequence
15 ERKCCVECP (SEQ ID NO: 65).

[00245] The L comprises the contiguous amino acid sequence which is “**derived from**” the Ig hinge region in those instances when the L comprises at least a portion of the Ig hinge region amino acid sequence or at least a portion of an engineered Ig hinge region. Engineered Ig hinge region comprises one or more mutations when compared to the wild-type Ig hinge. Exemplary
20 mutations that may be introduced are substitutions of Cys residues to reduce the number of Cys in the L to one or two, substitution of Pro residues, or any conservative modifications, such as conservative substitutions.

[00246] “**Conservative modifications**” refer to amino acid modifications that do not significantly affect or alter the binding characteristics of the antibody containing the amino acid
25 modifications. Conservative modifications include amino acid substitutions, additions and deletions. Conservative amino acid substitutions are those in which the amino acid is replaced with an amino acid residue having a similar side chain. The families of amino acid residues having similar side chains are well defined and include amino acids with acidic side chains (*e.g.*, aspartic acid, glutamic acid), basic side chains (*e.g.*, lysine, arginine, histidine), nonpolar side
30 chains (*e.g.*, alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine), uncharged polar side chains (*e.g.*, glycine, asparagine, glutamine, cysteine, serine, threonine, tyrosine,

tryptophan), aromatic side chains (*e.g.*, phenylalanine, tryptophan, histidine, tyrosine), aliphatic side chains (*e.g.*, glycine, alanine, valine, leucine, isoleucine, serine, threonine), amide (*e.g.*, asparagine, glutamine), beta-branched side chains (*e.g.*, threonine, valine, isoleucine) and sulfur-containing side chains (cysteine, methionine). Furthermore, any native residue in the

5 polypeptide may also be substituted with alanine, as has been previously described for alanine scanning mutagenesis (MacLennan *et al.*, (1988) *Acta Physiol Scand Suppl* 643:55-67; Sasaki *et al.*, (1988) *Adv Biophys* 35:1-24). Amino acid substitutions to may be made by known methods for example by PCR mutagenesis (US Pat. No. 4,683,195). The resulting variant hinges may be incorporated into the spFv constructs of the disclosure and tested for their characteristics such as

10 stability and binding to an antigen using known assays and assays described herein.

[00247] In some embodiments, the L comprises an amino acid sequence C(X)_yC (**SEQ ID NO: 23**), wherein X is glycine (Gly), serine (Ser), proline (Pro), alanine (Ala), arginine (Arg), asparagine (Asn), aspartic acid (Asp), glutamic acid (Glu), glutamine (Gln), histidine (His), isoleucine (Ile), leucine (Leu), lysine (Lys), phenylalanine (Phe), threonine (Thr), tryptophan

15 (Trp) or tyrosine (Tyr), and y is an integer from 1 to 3. Pro may be included into the linker to provide rigidity. Gly may be included into the linker to allow maximum flexibility. Any other amino acid may also be used in the L except for Cys and Met.

[00248] In some embodiments, the L comprises the amino acid sequence C(X)_yC (**SEQ ID NO: 24**), wherein X is Gly, Ser or Pro, and y is an integer from 1 to 3.

20 **[00249]** In some embodiments, the L comprises the amino acid sequence CPC, CGC, CSC, CPPC (**SEQ ID NO: 1**), CGPC (**SEQ ID NO: 28**), CPGC (**SEQ ID NO: 29**), CGGC (**SEQ ID NO: 30**), CSPG (**SEQ ID NO: 31**), CPSC (**SEQ ID NO: 32**), CSSC (**SEQ ID NO: 33**), CGSC (**SEQ ID NO: 34**), CSGC (**SEQ ID NO: 35**), CPPPC (**SEQ ID NO: 36**), CGPPC (**SEQ ID NO: 37**), CPGPC (**SEQ ID NO: 38**), CPPGC (**SEQ ID NO: 39**), CGGPC (**SEQ ID NO: 40**),

25 CPGGC (**SEQ ID NO: 41**), CGGGC (**SEQ ID NO: 42**), CSPPC (**SEQ ID NO: 43**), CPSPC (**SEQ ID NO: 44**), CPPSC (**SEQ ID NO: 45**), CSSPC (**SEQ ID NO: 46**), CPSSC (**SEQ ID NO: 47**), CSSSC (**SEQ ID NO: 48**), CGSPC (**SEQ ID NO: 49**), CPGSC (**SEQ ID NO: 50**), CSGPC (**SEQ ID NO: 51**) or CPSGC (**SEQ ID NO: 52**).

[00250] In some embodiments, the L comprises the amino acid sequence CPC.

30 **[00251]** In some embodiments, the L comprises the amino acid sequence CGC.

[00252] In some embodiments, the L comprises the amino acid sequence CSC.

- [00253] In some embodiments, the L comprises the amino acid sequence CPPC (SEQ ID NO: 1).
- [00254] In some embodiments, the L comprises the amino acid sequence CGPC (SEQ ID NO: 28).
- 5 [00255] In some embodiments, the L comprises the amino acid sequence CPGC (SEQ ID NO: 29).
- [00256] In some embodiments, the L comprises the amino acid sequence CGGC (SEQ ID NO: 30).
- [00257] In some embodiments, the L comprises the amino acid sequence CSPG (SEQ ID NO: 31).
- 10 [00258] In some embodiments, the L comprises the amino acid sequence CPSC (SEQ ID NO: 32).
- [00259] In some embodiments, the L comprises the amino acid sequence CSSC (SEQ ID NO: 33).
- 15 [00260] In some embodiments, the L comprises the amino acid sequence CGSC (SEQ ID NO: 34).
- [00261] In some embodiments, the L comprises the amino acid sequence CSGC (SEQ ID NO: 35).
- [00262] In some embodiments, the L comprises the amino acid sequence CPPPC (SEQ ID NO: 36).
- 20 [00263] In some embodiments, the L comprises the amino acid sequence CGPPC (SEQ ID NO: 37).
- [00264] In some embodiments, the L comprises the amino acid sequence CPGPC (SEQ ID NO: 38).
- 25 [00265] In some embodiments, the L comprises the amino acid sequence CPPGC (SEQ ID NO: 39).
- [00266] In some embodiments, the L comprises the amino acid sequence CGGPC (SEQ ID NO: 40).
- [00267] In some embodiments, the L comprises the amino acid sequence CPGGC (SEQ ID NO: 41).
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- [00268] In some embodiments, the L comprises the amino acid sequence CGGGC (SEQ ID NO: 42).
- [00269] In some embodiments, the L comprises the amino acid sequence CSPPC (SEQ ID NO: 43).
- 5 [00270] In some embodiments, the L comprises the amino acid sequence CPSPC (SEQ ID NO: 44).
- [00271] In some embodiments, the L comprises the amino acid sequence CPPSC (SEQ ID NO: 45).
- [00272] In some embodiments, the L comprises the amino acid sequence CSSPC (SEQ ID NO: 46).
- 10 [00273] In some embodiments, the L comprises the amino acid sequence CPSSC (SEQ ID NO: 47).
- [00274] In some embodiments, the L comprises the amino acid sequence CSSSC (SEQ ID NO: 48).
- 15 [00275] In some embodiments, the L comprises the amino acid sequence CGSPC (SEQ ID NO: 49).
- [00276] In some embodiments, the L comprises the amino acid sequence CPGSC (SEQ ID NO: 50).
- [00277] In some embodiments, the L comprises the amino acid sequence CSGPC (SEQ ID NO: 51).
- 20 [00278] In some embodiments, the L comprises the amino acid sequence CPSGC (SEQ ID NO: 52).
- [00279] In some embodiments, the L comprises from about 14 to about 19 amino acids.
- [00280] In some embodiments, the L comprises about 14 amino acids.
- 25 [00281] In some embodiments, the L comprises about 15 amino acids.
- [00282] In some embodiments, the L comprises about 16 amino acids.
- [00283] In some embodiments, the L comprises about 17 amino acids.
- [00284] In some embodiments, the L comprises about 18 amino acids.
- [00285] In some embodiments, the L comprises about 19 amino acids.
- 30 [00286] In some embodiments, the L comprises the amino acid sequence $(X)_mC(X)_yC(X)_n$ (SEQ ID NO: 25), wherein X is Gly, Ser, Pro, Ala, Arg, Asn, Asp, Glu, Gln, His, Ile, leu, Lys,

Phe Thr, Trp or Tyr, m is an integer from 6 to 9, y is an integer from 1 to 3 and n is an integer from 4 to 6.

[00287] In some embodiments, the L comprises the amino acid sequence $(X)_mC(X)_yC(X)_n$ (**SEQ ID NO: 26**), wherein X is Gly, Ser, Pro, Ala, Arg, Asn, Asp, Glu, Gln, His, Ile, Leu, Lys, Thr or Tyr, m is an integer from 6 to 9, y is an integer from 1 to 3 and n is an integer from 4 to 6.

[00288] In some embodiments, the L comprises the amino acid sequence $(X)_mC(X)_yC(X)_n$ (**SEQ ID NO: 27**); wherein X is Gly or Pro, m is an integer from 6 to 9, y is an integer from 1 to 3 and n is an integer from 4 to 6.

[00289] In some embodiments, the L comprises the amino acid sequence of **SEQ ID NOs: 2, 3, 4, 5, 6 or 7**.

[00290] In some embodiments, the L comprises the amino acid sequence of **SEQ ID NO: 2**.

[00291] In some embodiments, the L comprises the amino acid sequence of **SEQ ID NO: 3**.

[00292] In some embodiments, the L comprises the amino acid sequence of **SEQ ID NO: 4**.

[00293] In some embodiments, the L comprises the amino acid sequence of **SEQ ID NO: 5**.

[00294] In some embodiments, the L comprises the amino acid sequence of **SEQ ID NO: 6**.

[00295] In some embodiments, the L comprises the amino acid sequence of **SEQ ID NO: 7**.

[00296] In some embodiments, the spFv of the disclosure is in the VL-L-VH orientation.

[00297] In some embodiments, the spFv of the disclosure is in the VH-L-VL orientation.

[00298] The disclosure also provides a scFv comprising a VH, a L and a VL, wherein the VH comprises Cys at H105; the VL comprises Cys at L42; the L comprises an amino acid sequence of **SEQ ID NOs: 2, 3, 4, 5, 6 or 7**; and the scFv is in the VL-L-VH orientation.

[00299] The disclosure also provides a scFv comprising a VH, a L and a VL, wherein the VH comprises Cys at H105; the VL comprises Cys at L45; the L comprises an amino acid sequence of **SEQ ID NOs: 2, 3, 4, 5, 6 or 7**; and the scFv is in the VL-L-VH orientation.

[00300] The disclosure also provides a scFv comprising a VH, a L and a VL, wherein the VH comprises Cys at H105; the VL comprises Cys at L39;

the L comprises an amino acid sequence of SEQ ID NOs: 2, 3, 4, 5, 6 or 7; and
the scFv is in the VL-L-VH orientation.

[00301] The disclosure also provides a scFv comprising a VH, a L and a VL, wherein
the VH comprises Cys at H5;

5 the VL comprises Cys at L42;

the L comprises an amino acid sequence of SEQ ID NOs: 2, 3, 4, 5, 6 or 7; and
the scFv is in the VL-L-VH orientation.

[00302] The disclosure also provides a scFv comprising a VH, a L and a VL, wherein
the VH comprises Cys at H5;

10 the VL comprises Cys at L45;

the L comprises an amino acid sequence of SEQ ID NOs: 2, 3, 4, 5, 6 or 7; and
the scFv is in the VL-L-VH orientation.

[00303] The disclosure also provides a scFv comprising a VH, a L and a VL, wherein
the VH comprises Cys at H5;

15 the VL comprises Cys at L39;

the L comprises an amino acid sequence of SEQ ID NOs: 2, 3, 4, 5, 6 or 7; and
the scFv is in the VL-L-VH orientation.

[00304] The disclosure also provides a scFv comprising a VH, a L and a VL, wherein
the VH comprises Cys at H3;

20 the VL comprises Cys at L42;

the L comprises an amino acid sequence of SEQ ID NOs: 2, 3, 4, 5, 6 or 7; and
the scFv is in the VL-L-VH orientation.

[00305] The disclosure also provides a scFv comprising a VH, a L and a VL, wherein
the VH comprises Cys at H3;

25 the VL comprises Cys at L45;

the L comprises an amino acid sequence of SEQ ID NOs: 2, 3, 4, 5, 6 or 7; and
the scFv is in the VL-L-VH orientation.

[00306] The disclosure also provides a scFv comprising a VH, a L and a VL, wherein
the VH comprises Cys at H3;

30 the VL comprises Cys at L39;

the L comprises an amino acid sequence of SEQ ID NOs: 2, 3, 4, 5, 6 or 7; and

the scFv is in the VL-L-VH orientation.

[00307] The disclosure also provides a scFv comprising a VH, a L and a VL, wherein the VH comprises Cys at H43;
the VL comprises Cys at L100;

- 5 the L comprises an amino acid sequence of SEQ ID NOs: 2, 3, 4, 5, 6 or 7; and
the scFv is in the VH-L-VL orientation.

[00308] The disclosure also provides a scFv comprising a VH, a L and a VL, wherein the VH comprises Cys at H43;
the VL comprises Cys at L102;

- 10 the L comprises an amino acid sequence of SEQ ID NOs: 2, 3, 4, 5, 6 or 7; and
the scFv is in the VH-L-VL orientation.

[00309] The disclosure also provides a scFv comprising a VH, a L and a VL, wherein the VH comprises Cys at H43;
the VL comprises Cys at L5;

- 15 the L comprises an amino acid sequence of SEQ ID NOs: 2, 3, 4, 5, 6 or 7; and
the scFv is in the VH-L-VL orientation.

[00310] The disclosure also provides a scFv comprising a VH, a L and a VL, wherein the VH comprises Cys at H43;
the VL comprises Cys at L3;

- 20 the L comprises an amino acid sequence of SEQ ID NOs: 2, 3, 4, 5, 6 or 7; and
the scFv is in the VH-L-VL orientation.

[00311] The disclosure also provides a scFv comprising a VH, a L and a VL, wherein the VH comprises Cys at H40;
the VL comprises Cys at L100;

- 25 the L comprises an amino acid sequence of SEQ ID NOs: 2, 3, 4, 5, 6 or 7; and
the scFv is in the VH-L-VL orientation.

[00312] The disclosure also provides a scFv comprising a VH, a L and a VL, wherein the VH comprises Cys at H40;
the VL comprises Cys at L102;

- 30 the L comprises an amino acid sequence of SEQ ID NOs: 2, 3, 4, 5, 6 or 7; and
the scFv is in the VH-L-VL orientation.

[00313] The disclosure also provides a scFv comprising a VH, a L and a VL, wherein
the VH comprises Cys at H40;
the VL comprises Cys at L5;
the L comprises an amino acid sequence of SEQ ID NOs: 2, 3, 4, 5, 6 or 7; and
5 the scFv is in the VH-L-VL orientation.

[00314] The disclosure also provides a scFv comprising a VH, a L and a VL, wherein
the VH comprises Cys at H40;
the VL comprises Cys at L3;
the L comprises an amino acid sequence of SEQ ID NOs: 2, 3, 4, 5, 6 or 7; and
10 the scFv is in the VH-L-VL orientation.

[00315] The disclosure also provides a scFv comprising a VH, a L and a VL, wherein
the VH comprises Cys at H46;
the VL comprises Cys at L100;
the L comprises an amino acid sequence of SEQ ID NOs: 2, 3, 4, 5, 6 or 7; and
15 the scFv is in the VH-L-VL orientation.

[00316] The disclosure also provides a scFv comprising a VH, a L and a VL, wherein
the VH comprises Cys at H46;
the VL comprises Cys at L102;
the L comprises an amino acid sequence of SEQ ID NOs: 2, 3, 4, 5, 6 or 7; and
20 the scFv is in the VH-L-VL orientation.

[00317] The disclosure also provides a scFv comprising a VH, a L and a VL, wherein
the VH comprises Cys at H46;
the VL comprises Cys at L5;
the L comprises an amino acid sequence of SEQ ID NOs: 2, 3, 4, 5, 6 or 7; and
25 the scFv is in the VH-L-VL orientation.

[00318] The disclosure also provides a scFv comprising a VH, a L and a VL, wherein
the VH comprises Cys at H46;
the VL comprises Cys at L3;
the L comprises an amino acid sequence of SEQ ID NOs: 2, 3, 4, 5, 6 or 7; and
30 the scFv is in the VH-L-VL orientation.

[00319] In some embodiments, the L comprises the amino acid sequence of **SEQ ID NO: 3**.

[00320] In some embodiments, the L comprises the amino acid sequence of **SEQ ID NO: 4**.

[00321] In some embodiments, the L comprises the amino acid sequence of **SEQ ID NO: 5**.

[00322] In some embodiments, the L comprises the amino acid sequence of **SEQ ID NO: 6**.

[00323] In some embodiments, the L comprises the amino acid sequence of **SEQ ID NO: 7**.

5 **Heterologous molecules comprising the spFv of the disclosure**

[00324] The spFv of the disclosure may be conjugated to a second molecule similarly to non-stabilized scFv devoid of disulfide bonds as known in the art. Exemplary second molecules are disclosed herein and include half-life extending moieties, imaging agents, therapeutic agents, antibodies comprising various antibody formats and fragments thereof, antigen binding domains, Fc regions, immunoglobulin heavy/light chains or fragments thereof, multispecific molecules and chimeric antigen receptors (CAR).

[00325] The disclosure also provides a heterologous molecule comprising a single chain variable fragment (scFv) comprising a heavy chain variable region (VH), a linker (L) and a light chain variable region (VL), wherein the scFv comprises

15 a first disulfide bond between a structurally conserved surface exposed VH cysteine (Cys) and a first L Cys;

a second disulfide bond between a structurally conserved surface exposed VL Cys and a second L Cys; or

the first disulfide bond between the structurally conserved surface exposed VH Cys and the first

20 L Cys and the second disulfide bond between the structurally conserved surface exposed VL Cys and the second L Cys.

[00326] The disclosure also provides a heterologous molecule comprising a scFv comprising a VH, a L and a VL, wherein

25 the VH comprises a VH Cys at a structurally conserved surface exposed VH framework residue position and the L comprises a first L Cys;

the VL comprises a VL Cys at a structurally conserved surface exposed VL framework residue position and the L comprises a second L Cys; or

the VH comprises the VH Cys at a structurally conserved surface exposed VH framework residue position, the VL comprises the VL Cys at a structurally conserved surface exposed VL

30 framework residue position and the L comprises the first L Cys and the second L Cys, wherein

the VH Cys and the first L Cys are capable of forming a disulfide bond and the VL Cys and the second L Cys are capable of forming a disulfide bond.

[00327] In some embodiments, the distance between the VH Cys and the VL Cys is from about 7 Å to about 9 Å.

5 **[00328]** In some embodiments, the VH Cys is at H3, H5, H40, H43, H46 or H105, wherein residue numbering is according to Chothia.

[00329] In some embodiments, the VL Cys is at L3, L5, L39, L42, L45, L100 or L102, wherein residue numbering is according to Chothia.

[00330] In some embodiments, the VH Cys is at H105 and the VL Cys is at L42.

10 **[00331]** In some embodiments, the VH Cys is at H43 and the VL Cys is at a L100.

[00332] In some embodiments, the VH Cys is at H3 and the VL Cys is at L3.

[00333] In some embodiments, the VH Cys is at H3 and the VL Cys is at L5.

[00334] In some embodiments, the VH Cys is at H3 and the VL Cys is at L39.

[00335] In some embodiments, the VH Cys is at H3 and the VL Cys is at L42.

15 **[00336]** In some embodiments, the VH Cys is at H3 and the VL Cys is at L45.

[00337] In some embodiments, the VH Cys is at H3 and the VL Cys is at L100.

[00338] In some embodiments, the VH Cys is at H3 and the VL Cys is at L102.

[00339] In some embodiments, the VH Cys is at H5 and the VL Cys is at L3.

[00340] In some embodiments, the VH Cys is at H5 and the VL Cys is at L5.

20 **[00341]** In some embodiments, the VH Cys is at H5 and the VL Cys is at L39.

[00342] In some embodiments, the VH Cys is at H5 and the VL Cys is at L42.

[00343] In some embodiments, the VH Cys is at H5 and the VL Cys is at L45.

[00344] In some embodiments, the VH Cys is at H5 and the VL Cys is at L100.

[00345] In some embodiments, the VH Cys is at H5 and the VL Cys is at L102.

25 **[00346]** In some embodiments, the VH Cys is at H40 and the VL Cys is at L3.

[00347] In some embodiments, the VH Cys is at H40 and the VL Cys is at L5.

[00348] In some embodiments, the VH Cys is at H40 and the VL Cys is at L39.

[00349] In some embodiments, the VH Cys is at H40 and the VL Cys is at L42.

[00350] In some embodiments, the VH Cys is at H40 and the VL Cys is at L45.

30 **[00351]** In some embodiments, the VH Cys is at H40 and the VL Cys is at L100.

[00352] In some embodiments, the VH Cys is at H40 and the VL Cys is at L102.

- [00353] In some embodiments, the VH Cys is at H43 and the VL Cys is at L3.
- [00354] In some embodiments, the VH Cys is at H43 and the VL Cys is at L5.
- [00355] In some embodiments, the VH Cys is at H43 and the VL Cys is at L39.
- [00356] In some embodiments, the VH Cys is at H43 and the VL Cys is at L42.
- 5 [00357] In some embodiments, the VH Cys is at H43 and the VL Cys is at L45.
- [00358] In some embodiments, the VH Cys is at H43 and the VL Cys is at L100.
- [00359] In some embodiments, the VH Cys is at H43 and the VL Cys is at L102.
- [00360] In some embodiments, the VH Cys is at H46 and the VL Cys is at L3.
- [00361] In some embodiments, the VH Cys is at H46 and the VL Cys is at L5.
- 10 [00362] In some embodiments, the VH Cys is at H46 and the VL Cys is at L39.
- [00363] In some embodiments, the VH Cys is at H46 and the VL Cys is at L42.
- [00364] In some embodiments, the VH Cys is at H46 and the VL Cys is at L45.
- [00365] In some embodiments, the VH Cys is at H46 and the VL Cys is at L100.
- [00366] In some embodiments, the VH Cys is at H46 and the VL Cys is at L102.
- 15 [00367] In some embodiments, the VH Cys is at H105 and the VL Cys is at L3.
- [00368] In some embodiments, the VH Cys is at H105 and the VL Cys is at L5.
- [00369] In some embodiments, the VH Cys is at H105 and the VL Cys is at L39.
- [00370] In some embodiments, the VH Cys is at H105 and the VL Cys is at L42.
- [00371] In some embodiments, the VH Cys is at H105 and the VL Cys is at L45.
- 20 [00372] In some embodiments, the VH Cys is at H105 and the VL Cys is at L100.
- [00373] In some embodiments, the VH Cys is at H105 and the VL Cys is at L102.
- [00374] The residue numbering of the VH and the VL regions are according to Chothia.
- [00375] In some embodiments, the L comprises a contiguous amino acid sequence derived from an immunoglobulin (Ig) hinge region.
- 25 [00376] In some embodiments, the Ig hinge region is derived from a human or a non-human Ig hinge region. Exemplary non-human Ig hinge regions are those from mouse, rat, dog, chicken and non-human primates, such as monkeys.
- [00377] In some embodiments, the Ig hinge region is derived from the human Ig hinge region.
- [00378] In some embodiments, the human Ig hinge region is an IgG1, IgG2, IgG3, IgG4, IgM,
- 30 IgA or IgE isotype.

[00379] In some embodiments, the L comprises an amino acid sequence $C(X)_yC$ (**SEQ ID NO: 23**), wherein X is Gly, Ser, Pro, Ala, Arg, Asn, Asp, Glu, Gln, His, Ile, Leu, Lys, Phe, Thr, Trp or Tyr, and y is an integer from 1 to 3. Pro may be included into the linker to provide rigidity. Gly may be included into the linker to allow maximum flexibility. Any other amino acid may also be used in the L except for Cys and Met.

[00380] In some embodiments, the L comprises the amino acid sequence $C(X)_yC$ (**SEQ ID NO: 24**), wherein X is Gly, Ser or Pro, and y is an integer from 1 to 3.

[00381] In some embodiments, the L comprises the amino acid sequence CPC, CGC, CSC, CPPC (**SEQ ID NO: 1**), CGPC (**SEQ ID NO: 28**), CPGC (**SEQ ID NO: 29**), CGGC (**SEQ ID NO: 30**), CSPG (**SEQ ID NO: 31**), CPSC (**SEQ ID NO: 32**), CSSC (**SEQ ID NO: 33**), CGSC (**SEQ ID NO: 34**), CSGC (**SEQ ID NO: 35**), CPPPC (**SEQ ID NO: 36**), CGPPC (**SEQ ID NO: 37**), CPGPC (**SEQ ID NO: 38**), CPPGC (**SEQ ID NO: 39**), CGGPC (**SEQ ID NO: 40**), CPGGC (**SEQ ID NO: 41**), CGGGC (**SEQ ID NO: 42**), CSPPC (**SEQ ID NO: 43**), CPSPC (**SEQ ID NO: 44**), CPPSC (**SEQ ID NO: 45**), CSSPC (**SEQ ID NO: 46**), CPSSC (**SEQ ID NO: 47**), CSSSC (**SEQ ID NO: 48**), CGSPC (**SEQ ID NO: 49**), CPGSC (**SEQ ID NO: 50**), CSGPC (**SEQ ID NO: 51**) or CPSGC (**SEQ ID NO: 52**).

[00382] In some embodiments, the L comprises from about 14 to about 19 amino acids.

[00383] In some embodiments, the L comprises about 14 amino acids.

[00384] In some embodiments, the L comprises about 15 amino acids.

[00385] In some embodiments, the L comprises about 16 amino acids.

[00386] In some embodiments, the L comprises about 17 amino acids.

[00387] In some embodiments, the L comprises about 18 amino acids.

[00388] In some embodiments, the L comprises about 19 amino acids.

[00389] In some embodiments, the L comprises the amino acid sequence $(X)_mC(X)_yC(X)_n$ (**SEQ ID NO: 25**); wherein X is Gly, Ser, Pro, Ala, Arg, Asn, Asp, Glu, Gln, His, Ile, leu, Lys, Phe Thr, Trp or Tyr, m is an integer from 6 to 9, y is an integer from 1 to 3 and n is an integer from 4 to 6.

[00390] In some embodiments, the L comprises the amino acid sequence $(X)_mC(X)_yC(X)_n$ (**SEQ ID NO: 26**); wherein X is Gly, Ser or Pro, Ala, Arg, Asn, Asp, Glu, Gln, His, Ile, Leu, Lys, Thr or Tyr, m is an integer from 6 to 9, y is an integer from 1 to 3 and n is an integer from 4 to 6.

[00391] In some embodiments, the L comprises the amino acid sequence $(X)_mC(X)_yC(X)_n$ (**SEQ ID NO: 27**); wherein X is Gly or Pro, m is an integer from 6 to 9, y is an integer from 1 to 3 and n is an integer from 4 to 6.

5 **[00392]** In some embodiments, the L comprises the amino acid sequence of SEQ ID NOs: 2, 3, 4, 5, 6 or 7.

[00393] In some embodiments, the spFv of the disclosure is in the VL-L-VH orientation.

[00394] In some embodiments, the spFv of the disclosure is in the VH-L-VL orientation.

[00395] The disclosure also provides a heterologous molecule comprising a scFv comprising a VH, a L and a VL, wherein

10 the VH comprises Cys at H105;

the VL comprises Cys at L42;

the L comprises an amino acid sequence of SEQ ID NOs: 2, 3, 4, 5, 6 or 7; and

the scFv is in the VL-L-VH orientation.

[00396] The disclosure also provides a heterologous molecule comprising a scFv comprising a

15 VH, a L and a VL, wherein

the VH comprises Cys at H105;

the VL comprises Cys at L45;

the L comprises an amino acid sequence of SEQ ID NOs: 2, 3, 4, 5, 6 or 7; and

the scFv is in the VL-L-VH orientation.

20 **[00397]** The disclosure also provides a heterologous molecule comprising a scFv comprising a

VH, a L and a VL, wherein

the VH comprises Cys at H105;

the VL comprises Cys at L39;

the L comprises an amino acid sequence of SEQ ID NOs: 2, 3, 4, 5, 6 or 7; and

25 the scFv is in the VL-L-VH orientation.

[00398] The disclosure also provides a heterologous molecule comprising a scFv comprising a

VH, a L and a VL, wherein

the VH comprises Cys at H5;

the VL comprises Cys at L42;

30 the L comprises an amino acid sequence of SEQ ID NOs: 2, 3, 4, 5, 6 or 7; and

the scFv is in the VL-L-VH orientation.

[00399] The disclosure also provides a heterologous molecule comprising a scFv comprising a VH, a L and a VL, wherein

the VH comprises Cys at H5;

the VL comprises Cys at L45;

5 the L comprises an amino acid sequence of SEQ ID NOs: 2, 3, 4, 5, 6 or 7; and

the scFv is in the VL-L-VH orientation.

[00400] The disclosure also provides a heterologous molecule comprising a scFv comprising a VH, a L and a VL, wherein

the VH comprises Cys at H5;

10 the VL comprises Cys at L39;

the L comprises an amino acid sequence of SEQ ID NOs: 2, 3, 4, 5, 6 or 7; and

the scFv is in the VL-L-VH orientation.

[00401] The disclosure also provides a heterologous molecule comprising a scFv comprising a VH, a L and a VL, wherein

15 the VH comprises Cys at H3;

the VL comprises Cys at L42;

the L comprises an amino acid sequence of SEQ ID NOs: 2, 3, 4, 5, 6 or 7; and

the scFv is in the VL-L-VH orientation.

[00402] The disclosure also provides a heterologous molecule comprising a scFv comprising a VH, a L and a VL, wherein

20 the VH comprises Cys at H3;

the VL comprises Cys at L45;

the L comprises an amino acid sequence of SEQ ID NOs: 2, 3, 4, 5, 6 or 7; and

the scFv is in the VL-L-VH orientation.

25 **[00403]** The disclosure also provides a heterologous molecule comprising a scFv comprising a VH, a L and a VL, wherein

the VH comprises Cys at H3;

the VL comprises Cys at L39;

the L comprises an amino acid sequence of SEQ ID NOs: 2, 3, 4, 5, 6 or 7; and

30 the scFv is in the VL-L-VH orientation.

[00404] The disclosure also provides a heterologous molecule comprising a scFv comprising a VH, a L and a VL, wherein

the VH comprises Cys at H43;

the VL comprises Cys at L100;

- 5 the L comprises an amino acid sequence of SEQ ID NOs: 2, 3, 4, 5, 6 or 7; and
the scFv is in the VH-L-VL orientation.

[00405] The disclosure also provides a heterologous molecule comprising a scFv comprising a VH, a L and a VL, wherein

the VH comprises Cys at H43;

- 10 the VL comprises Cys at L102;

the L comprises an amino acid sequence of SEQ ID NOs: 2, 3, 4, 5, 6 or 7; and
the scFv is in the VH-L-VL orientation.

[00406] The disclosure also provides a heterologous molecule comprising a scFv comprising a VH, a L and a VL, wherein

- 15 the VH comprises Cys at H43;

the VL comprises Cys at L5;

the L comprises an amino acid sequence of SEQ ID NOs: 2, 3, 4, 5, 6 or 7; and
the scFv is in the VH-L-VL orientation.

[00407] The disclosure also provides a heterologous molecule comprising a scFv comprising a VH, a L and a VL, wherein

- 20 the VH comprises Cys at H43;

the VL comprises Cys at L3;

the L comprises an amino acid sequence of SEQ ID NOs: 2, 3, 4, 5, 6 or 7; and
the scFv is in the VH-L-VL orientation.

- 25 **[00408]** The disclosure also provides a heterologous molecule comprising a scFv comprising a VH, a L and a VL, wherein

the VH comprises Cys at H40;

the VL comprises Cys at L100;

the L comprises an amino acid sequence of SEQ ID NOs: 2, 3, 4, 5, 6 or 7; and
30 the scFv is in the VH-L-VL orientation.

[00409] The disclosure also provides a heterologous molecule comprising a scFv comprising a VH, a L and a VL, wherein

the VH comprises Cys at H40;

the VL comprises Cys at L102;

- 5 the L comprises an amino acid sequence of SEQ ID NOs: 2, 3, 4, 5, 6 or 7; and
the scFv is in the VH-L-VL orientation.

[00410] The disclosure also provides a heterologous molecule comprising a scFv comprising a VH, a L and a VL, wherein

the VH comprises Cys at H40;

- 10 the VL comprises Cys at L5;

the L comprises an amino acid sequence of SEQ ID NOs: 2, 3, 4, 5, 6 or 7; and
the scFv is in the VH-L-VL orientation.

[00411] The disclosure also provides a heterologous molecule comprising a scFv comprising a VH, a L and a VL, wherein

- 15 the VH comprises Cys at H40;

the VL comprises Cys at L3;

the L comprises an amino acid sequence of SEQ ID NOs: 2, 3, 4, 5, 6 or 7; and
the scFv is in the VH-L-VL orientation.

[00412] The disclosure also provides a heterologous molecule comprising a scFv comprising a VH, a L and a VL, wherein

- 20 the VH comprises Cys at H46;

the VL comprises Cys at L100;

the L comprises an amino acid sequence of SEQ ID NOs: 2, 3, 4, 5, 6 or 7; and
the scFv is in the VH-L-VL orientation.

- 25 **[00413]** The disclosure also provides a heterologous molecule comprising a scFv comprising a VH, a L and a VL, wherein

the VH comprises Cys at H46;

the VL comprises Cys at L102;

- the L comprises an amino acid sequence of SEQ ID NOs: 2, 3, 4, 5, 6 or 7; and
30 the scFv is in the VH-L-VL orientation.

[00414] The disclosure also provides a heterologous molecule comprising a scFv comprising a VH, a L and a VL, wherein

the VH comprises Cys at H46;

the VL comprises Cys at L5;

- 5 the L comprises an amino acid sequence of SEQ ID NOs: 2, 3, 4, 5, 6 or 7; and
the scFv is in the VH-L-VL orientation.

[00415] The disclosure also provides a heterologous molecule comprising a scFv comprising a VH, a L and a VL, wherein

the VH comprises Cys at H46;

- 10 the VL comprises Cys at L3;

the L comprises an amino acid sequence of SEQ ID NOs: 2, 3, 4, 5, 6 or 7; and
the scFv is in the VH-L-VL orientation.

[00416] In some embodiments, the L comprises the amino acid sequence of **SEQ ID NO: 3**.

[00417] In some embodiments, the L comprises the amino acid sequence of **SEQ ID NO: 4**.

- 15 [00418] In some embodiments, the L comprises the amino acid sequence of **SEQ ID NO: 5**.

[00419] In some embodiments, the L comprises the amino acid sequence of **SEQ ID NO: 6**.

[00420] In some embodiments, the L comprises the amino acid sequence of **SEQ ID NO: 7**.

[00421] In some embodiments, the scFv of the disclosure is conjugated to a second protein, a polynucleotide, a therapeutic agent, a cytotoxic agent or a detectable label.

- 20 [00422] In some embodiments, the second protein is a half-life extending moiety.

[00423] In some embodiments, the second protein is an antibody or a fragment thereof.

[00424] In some embodiments, the second protein is an antigen binding fragment.

[00425] In some embodiments, the second protein is a therapeutic molecule.

Heterologous molecules comprising the spFv of the disclosure and half-life extending moiety

- 25 [00426] In some embodiments, the spFv of the disclosure is conjugated to a half-life extending moiety.

- [00427] Exemplary half-life extending moieties are an immunoglobulin (Ig), a fragment of the Ig, an Ig constant region, a fragment of the Ig constant region, a Fc region, transferrin, albumin,
30 albumin variants, an albumin binding domain or polyethylene glycol. Amino acid sequences of the human Ig are well known, and include IgG1, IgG2, IgG3, IgG4, IgM, IgA and IgE.

[00428] In some embodiments, the spFv of the disclosure is conjugated to the Ig or the fragment of the Ig.

[00429] In some embodiments, the spFv of the disclosure is conjugated to the Fc region.

[00430] In some embodiments, the spFv of the disclosure is conjugated to transferrin.

5 [00431] In some embodiments, the spFv of the disclosure is conjugated to albumin.

[00432] In some embodiments, the spFv of the disclosure is conjugated to albumin binding protein.

[00433] In some embodiments, the spFv of the disclosure is conjugated to polyethylene glycol (PEG). Exemplary PEG molecules are PEG5000 or PEG20,000.

10 [00434] In some embodiments, the spFv of the disclosure is conjugated to a fatty acid or a fatty acid ester. Exemplary fatty acids and fatty acid esters are laurate, myristate, stearate, arachidate, behenate, oleate, arachidonate, octanedioic acid, tetradecanedioic acid, octadecanedioic acid, docosanedioic acid, and the like, polylysine, octane, carbohydrates (dextran, cellulose, oligo- or polysaccharides) for desired properties.

15 [00435] The half-life extending moiety may be a direct fusion with the spFv of the disclosure and may be generated by standard cloning and expression techniques. Alternatively, well-known chemical coupling methods may be used to attach the moieties to recombinantly produced spFvs of the disclosure.

Heterologous molecules comprising the spFv of the disclosure and a cytotoxic agent or a detectable label

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[00436] The disclosure also provides a heterologous molecule comprising the spFv of the disclosure, wherein the spFv of the disclosure is conjugated to a second protein, a polynucleotide, a therapeutic agent, a cytotoxic agent or a detectable label.

[00437] The heterologous molecule comprising the spFv of the disclosure may be used to direct therapeutics, mediate killing, visualize, identify or purify cells that express the antigen the spFv binds to, *in vitro* or *in vivo*.

[00438] In some embodiments, the detectable label is also a cytotoxic agent.

[00439] Detectable label includes compositions that, when conjugated to the spFv of the disclosure, renders the latter detectable, via spectroscopic, photochemical, biochemical, immunochemical, or chemical means.

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[00440] Exemplary detectable labels include radioactive isotopes, magnetic beads, metallic beads, colloidal particles, fluorescent dyes, electron-dense reagents, enzymes (for example, as commonly used in an ELISA), biotin, digoxigenin, haptens, luminescent molecules, chemiluminescent molecules, fluorochromes, fluorophores, fluorescent quenching agents,

5 colored molecules, radioactive isotopes, scintillates, avidin, streptavidin, protein A, protein G, antibodies or fragments thereof, polyhistidine, Ni^{2+} , Flag tags, myc tags, heavy metals, enzymes, alkaline phosphatase, peroxidase, luciferase, electron donors/acceptors, acridinium esters, and colorimetric substrates.

[00441] A detectable label may emit a signal spontaneously, such as when the detectable label is a radioactive isotope. In other cases, the detectable label emits a signal as a result of being stimulated by an external field.

[00442] Exemplary radioactive isotopes may be γ -emitting, Auger-emitting, β -emitting, an alpha-emitting or positron-emitting radioactive isotope. Exemplary radioactive isotopes include ^3H , ^{11}C , ^{13}C , ^{15}N , ^{18}F , ^{19}F , ^{55}Co , ^{57}Co , ^{60}Co , ^{61}Cu , ^{62}Cu , ^{64}Cu , ^{67}Cu , ^{68}Ga , ^{72}As , ^{75}Br , ^{86}Y , ^{89}Zr , ^{90}Sr , $^{94\text{m}}\text{Tc}$, $^{99\text{m}}\text{Tc}$, ^{115}In , ^{123}I , ^{124}I , ^{125}I , ^{131}I , ^{211}At , ^{212}Bi , ^{213}Bi , ^{223}Ra , ^{226}Ra , ^{225}Ac and ^{227}Ac .

[00443] Exemplary metal atoms are metals with an atomic number greater than 20, such as calcium, scandium, titanium, vanadium, chromium, manganese, iron, cobalt, nickel, copper, zinc, gallium, germanium, arsenic, selenium, bromine, krypton, rubidium, strontium, yttrium, zirconium, niobium, molybdenum, technetium, ruthenium, rhodium, palladium, silver, cadmium, indium, tin, antimony, tellurium, iodine, xenon, cesium, barium, lanthanum, hafnium, tantalum, tungsten, rhenium, osmium, iridium, platinum, gold, mercury, thallium, lead, bismuth, francium, radium, actinium, cerium, praseodymium, neodymium, promethium, samarium, europium, gadolinium, terbium, dysprosium, holmium, erbium, thulium, ytterbium, lutetium, thorium, protactinium, uranium, neptunium, plutonium, americium, curium, berkelium, californium, einsteinium, fermium, mendelevium, nobelium, or lawrencium atoms.

[00444] In some embodiments, the metal atoms may be alkaline earth metals with an atomic number greater than twenty.

[00445] In some embodiments, the metal atoms may be lanthanides.

[00446] In some embodiments, the metal atoms may be actinides.

[00447] In some embodiments, the metal atoms may be transition metals.

[00448] In some embodiments, the metal atoms may be poor metals.

- [00449]** In some embodiments, the metal atoms may be gold atoms, bismuth atoms, tantalum atoms, and gadolinium atoms.
- [00450]** In some embodiments, the metal atoms may be metals with an atomic number of 53 (*i.e.*, iodine) to 83 (*i.e.*, bismuth).
- 5 **[00451]** In some embodiments, the metal atoms may be atoms suitable for magnetic resonance imaging.
- [00452]** The metal atoms may be metal ions in the form of +1, +2, or +3 oxidation states, such as Ba²⁺, Bi³⁺, Cs⁺, Ca²⁺, Cr²⁺, Cr³⁺, Cr⁶⁺, Co²⁺, Co³⁺, Cu⁺, Cu²⁺, Cu³⁺, Ga³⁺, Gd³⁺, Au⁺, Au³⁺, Fe²⁺, Fe³⁺, F³⁺, Pb²⁺, Mn²⁺, Mn³⁺, Mn⁴⁺, Mn⁷⁺, Hg²⁺, Ni²⁺, Ni³⁺, Ag⁺, Sr²⁺, Sn²⁺, Sn⁴⁺, and Zn²⁺.
- 10 The metal atoms may comprise a metal oxide, such as iron oxide, manganese oxide, or gadolinium oxide.
- [00453]** Suitable dyes include any commercially available dyes such as, for example, 5(6)-carboxyfluorescein, IRDye 680RD maleimide or IRDye 800CW, ruthenium polypyridyl dyes, and the like.
- 15 **[00454]** Suitable fluorophores are fluorescein isothiocyanate (FITC), fluorescein thiosemicarbazide, rhodamine, Texas Red, CyDyes (*e.g.*, Cy3, Cy5, Cy5.5), Alexa Fluors (*e.g.*, Alexa488, Alexa555, Alexa594; Alexa647), near infrared (NIR) (700-900 nm) fluorescent dyes, and carbocyanine and aminostyryl dyes.
- [00455]** The heterologous molecule comprising the scFv of the disclosure conjugated to a
- 20 detectable label may be used as an imaging agent.
- [00456]** In some embodiments, the cytotoxic agent is a chemotherapeutic agent, a drug, a growth inhibitory agent, a toxin (*e.g.*, an enzymatically active toxin of bacterial, fungal, plant, or animal origin, or fragments thereof), or a radioactive isotope (*i.e.*, a radioconjugate).
- [00457]** In some embodiments, the cytotoxic agent is daunomycin, doxorubicin, methotrexate,
- 25 vindesine, bacterial toxins such as diphtheria toxin, ricin, geldanamycin, maytansinoids or calicheamicin. The cytotoxic agent may elicit their cytotoxic and cytostatic effects by mechanisms including tubulin binding, DNA binding, or topoisomerase inhibition.
- [00458]** In some embodiments, the cytotoxic agent is an enzymatically active toxin such as diphtheria A chain, nonbinding active fragments of diphtheria toxin, exotoxin A chain (from
- 30 *Pseudomonas aeruginosa*), ricin A chain, abrin A chain, modeccin A chain, alpha-sarcin, *Aleurites fordii* proteins, dianthin proteins, *Phytolacca americana* proteins (PAPI, PAPII, and

PAP-S), *momordica charantia* inhibitor, curcin, crotin, *sapaonaria officinalis* inhibitor, gelonin, mitogellin, restrictocin, phenomycin, enomycin, and the tricothecenes.

[00459] In some embodiments, the cytotoxic agent is a radionuclide, such as ^{212}Bi , ^{131}I , ^{131}In , ^{90}Y , and ^{186}Re .

5 **[00460]** In some embodiments, the cytotoxic agent is dolastatins or dolostatin peptidic analogs and derivatives, auristatin or monomethyl auristatin phenylalanine. Exemplary molecules are disclosed in U.S. Pat No. 5,635,483 and 5,780,588. Dolastatins and auristatins have been shown to interfere with microtubule dynamics, GTP hydrolysis, and nuclear and cellular division and have anticancer and antifungal activity. The dolastatin or auristatin drug moiety may be attached
10 to the antibody of the invention through the N (amino) terminus or the C (carboxyl) terminus of the peptidic drug moiety (see *e.g.*, WO02/088172), or via any cysteine engineered into a protein

[00461] Conjugation to a detectable label may be done using known methods.

[00462] In some embodiments, the detectable label is complexed with a chelating agent.

[00463] In some embodiments, the detectable label is conjugated to the spFv of the disclosure
15 via a linker.

[00464] The detectable label or the cytotoxic moiety may be linked directly, or indirectly, to the spFv of the disclosure using known methods. Suitable linkers are known in the art and include, for example, prosthetic groups, non-phenolic linkers (derivatives of N-succinimidyl-benzoates; dodecaborate), chelating moieties of both macrocyclics and acyclic chelators, such as
20 derivatives of 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA), derivatives of diethylenetriaminepentaacetic acid (DTPA), derivatives of S-2-(4-Isothiocyanatobenzyl)-1,4,7-triazacyclononane-1,4,7-triacetic acid (NOTA) and derivatives of 1,4,8,11-tetraazacyclododecan-1,4,8,11-tetraacetic acid (TETA), N-succinimidyl-3-(2-pyridyldithiol) propionate (SPDP), iminothiolane (IT), bifunctional derivatives of imidoesters (such as dimethyl adipimidate HCl),
25 active esters (such as disuccinimidyl suberate), aldehydes (such as glutaraldehyde), bis-azido compounds (such as bis(p-azidobenzoyl)hexanediamine), bis-diazonium derivatives (such as bis-(p-diazoniumbenzoyl)-ethylenediamine), diisocyanates (such as toluene 2,6-diisocyanate), and bis-active fluorine compounds (such as 1,5-difluoro-2,4-dinitrobenzene) and other chelating moieties. Suitable peptide linkers are well known.

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Heterologous molecules comprising the spFv of the disclosure and immunoglobulin (Ig) constant region or fragments thereof

- 5 [00465] The spFv of the disclosure may be conjugated to an Ig constant region or a fragment of the Ig constant region to impart antibody-like properties, including Fc effector functions C1q binding, complement dependent cytotoxicity (CDC), Fc receptor binding, antibody-dependent cell-mediated cytotoxicity (ADCC), phagocytosis or down regulation of cell surface receptors (*e.g.*, B cell receptor, BCR). The Ig constant region or the fragment of the Ig constant region functions also as a half-life extending moiety as described herein. The spFv of the disclosure may also be engineered into full length antibodies using standard methods. The full length antibodies comprising the spFv of the disclosure may further be engineered as described herein.
- 10 [00466] Immunoglobulin heavy chain constant region is comprised of subdomains CH1, hinge, CH2 and CH3. The CH1 domain spans residues 118-215, the CH2 domain residues 231-340 and the CH3 domain residues 341-447 on the heavy chain, residue numbering according to the EU Index. In some instances residue 341 is referred as a CH2 domain residue. Hinge is generally defined as including residue 216 and terminating at 230 of human IgG1 but may also include a lower hinge region from about residue 231 to about residue 237 as described herein. Ig Fc region comprises at least the CH2 and the CH3 domains of the Ig constant region, and therefore comprises at least a region from about 231 to 447 of Ig heavy chain constant region.
- 15 [00467] The invention also provides a spFv of the disclosure conjugated to an immunoglobulin (Ig) constant region or a fragment of the Ig constant region.
- 20 [00468] In some embodiments, the Ig constant region is a heavy chain constant region
- [00469] In some embodiments, the Ig constant region is a light chain constant region.
- [00470] In some embodiments, the fragment of the Ig constant region comprises a Fc region.
- [00471] In some embodiments, the fragment of the Ig constant region comprises a CH2 domain.
- 25 [00472] In some embodiments, the fragment of the Ig constant region comprises a CH3 domain.
- [00473] In some embodiments, the fragment of the Ig constant region comprises the CH2 domain and the CH3 domain.

[00474] In some embodiments, the fragment of the Ig constant region comprises at least portion of a hinge, the CH2 domain and the CH3 domain. Portion of the hinge refers to one or more amino acid residues of the Ig hinge.

5 [00475] In some embodiments, the fragment of the Ig constant region comprises the hinge, the CH2 domain and the CH3 domain.

[00476] In some embodiments, the spFv of the disclosure is conjugated to the N-terminus of the Ig constant region or the fragment of the Ig constant region.

[00477] In some embodiments, the spFv of the disclosure is conjugated to the C-terminus of the Ig constant region or the fragment of the Ig constant region.

10 [00478] The spFv of the disclosure conjugated to Ig constant region or the fragment of the Ig constant region may be assessed for their functionality using several known assays. Binding to target antigen may be assessed using methods described herein. Altered properties imparted by the Ig constant domain or the fragment of the Ig constant region such as Fc region may be assayed in Fc receptor binding assays using soluble forms of the receptors, such as FcγRI,
15 FcγRII, FcγRIII or FcRn, or using cell-based assays measuring for example ADCC, CDC or ADCP.

[00479] ADCC may be assessed using an *in vitro* assay using cells that express the antigen to which the spFv of the disclosure binds to as target cells and NK cells as effector cells. Cytolysis may be detected by the release of label (*e.g.*, radioactive substrates, fluorescent dyes or natural
20 intracellular proteins) from the lysed cells. In an exemplary assay, target cells are used with a ratio of 1 target cell to 4 effector cells. Target cells are pre-labeled with BATDA and combined with effector cells and the test antibody. The samples are incubated for 2 hours and cell lysis measured by measuring released BATDA into the supernatant. Data is normalized to maximal cytotoxicity with 0.67% Triton X-100 (Sigma Aldrich) and minimal control determined by
25 spontaneous release of BATDA from target cells in the absence of any antibody.

[00480] ADCP may be evaluated by using monocyte-derived macrophages as effector cells and any cell that express the antigen to which the spFv of the disclosure binds to as target cells which are engineered to express GFP or other labeled molecule. In an exemplary assay, effector:target cell ratio may be for example 4:1. Effector cells may be incubated with target cells
30 for 4 hours with or without the antibody of the invention. After incubation, cells may be detached using accutase. Macrophages may be identified with anti-CD11b and anti-CD14

antibodies coupled to a fluorescent label, and percent phagocytosis may be determined based on % GFP fluorescence in the CD11⁺CD14⁺ macrophages using standard methods.

5 **[00481]** CDC of cells may be measured for example by plating Daudi cells at 1×10^5 cells/well (50 μ L/well) in RPMI-B (RPMI supplemented with 1% BSA), adding 50 μ L of test protein to the wells at final concentration between 0-100 μ g/mL, incubating the reaction for 15 min at room temperature, adding 11 μ L of pooled human serum to the wells, and incubation the reaction for 45 min at 37° C. Percentage (%) lysed cells may be detected as % propidium iodide stained cells in FACS assay using standard methods.

10 **Heterologous molecules comprising the spFv of the disclosure and a chimeric antigen receptor (CAR) or a fragment thereof**

[00482] The spFv of the disclosure may be conjugated to a chimeric antigen receptor (CAR) or to a fragment of the CAR. The CAR comprising the spFv of the disclosure may hence be monospecific or multispecific, comprising, as its extracellular domain, one or more scFv molecules of the disclosure.

15 **[00483]** Chimeric antigen receptors (CARs) are genetically engineered receptors. These engineered receptors can be readily inserted into and expressed by immune cells, including T cells in accordance with techniques known in the art. With a CAR, a single receptor can be programmed to both recognize a specific antigen and, when bound to that antigen, activate the immune cell to attack and destroy the cell bearing that antigen. When these antigens exist on target cells, an immune cell that expresses the CAR can target and kill the target cell.

20 **[00484]** CAR typically comprises an extracellular domain that binds the antigen ad an optional linker, a transmembrane domain, and a cytosolic domain comprising a costimulatory domain and/or a signaling domain.

25 **[00485]** The extracellular domain of CAR may contain any polypeptide that binds the desired antigen, such as the scFv of the disclosure. CARs may also be engineered to bind two or more desired antigens that may be arranged in tandem and separated by linker sequences. For example, one or more scFvs of the disclosure, domain antibodies, llama VHH antibodies or other VH only antibody fragments may be organized in tandem via a linker to generate bispecific or multispecific CARs.

30 **[00486]** The transmembrane domain of CAR may be derived from the transmembrane domain of CD8, an alpha, beta or zeta chain of a T-cell receptor, CD28, CD3 epsilon, CD45, CD4, CD5,

CD8, CD9, CD16, CD22, CD33, CD37, CD64, CD80, CD86, CD134, CD137, CD154, KIRDS2, OX40, CD2, CD27, LFA-1 (CD11a, CD18), ICOS (CD278), 4-1 BB (CD137), 4-1 BBL, GITR, CD40, BAFR, HVEM (LIGHT), SLAMF7, NKp80 (KLRFI), CD160, CD119, IL2R beta, IL2R gamma, IL7R alpha, ITGA1, VLA1, CD49a, ITGA4, IA4, CD49D, ITGA6, VLA-6, CD49f, ITGAD, CD11d, ITGAE, CD103, ITGAL, CD11a, LFA-1, ITGAM, CD11b, ITGAX, CD11c, ITGB1, CD29, ITGB2, CD118, LFA-1, ITGB7, TNFR2, DNAM1 (CD226), SLAMF4 (CD244, 2B4), CD84, CD96 (Tactile), CEACAM1, CRTAM, Ly9 (CD229), CD160 (BY55), PSGL1, CD100 (SEMA4D), SLAMF6 (NTB-A, Lyl08), SLAM (SLAMF1, CD150, IPO-3), BLAME (SLAMF8), SELPLG (CD162), LTBR, PAG/Cbp, NKp44, NKp30, NKp46, NKG2D, and/or NKG2C.

[00487] The intracellular costimulatory domain of CAR may be derived from the intracellular domains of one or more co-stimulatory molecules. Co-stimulatory molecules are well-known cell surface molecules other than antigen receptors or Fc receptors that provide a second signal required for efficient activation and function of T lymphocytes upon binding to antigen. Exemplary co-stimulatory domains that can be used in CARs are intracellular domains of 4-1BB, CD2, CD7, CD27, CD28, CD30, CD40, CD54 (ICAM), CD83, CD134 (OX40), CD150 (SLAMF1), CD152 (CTLA4), CD223 (LAG3), CD270 (HVEM), CD278 (ICOS), DAP10, LAT, NKD2C SLP76, TRIM, and ZAP70.

[00488] The intracellular signaling domain of CAR may be derived from the signaling domains of for example CD3ζ, CD3ε, CD22, CD79a, CD66d or CD39. “**Intracellular signaling domain**” refers to the part of a CAR polypeptide that participates in transducing the message of effective CAR binding to a target antigen into the interior of the immune effector cell to elicit effector cell function, *e.g.*, activation, cytokine production, proliferation and cytotoxic activity, including the release of cytotoxic factors to the CAR-bound target cell, or other cellular responses elicited following antigen binding to the extracellular CAR domain.

[00489] The optional linker within CAR positioned between the extracellular domain and the transmembrane domain may be a polypeptide of about 2 to 100 amino acids in length. The linker may include or be composed of flexible residues such as glycine and serine so that the adjacent protein domains are free to move relative to one another. Longer linkers may be used when it is desirable to ensure that two adjacent domains do not sterically interfere with one another. Linkers may be cleavable or non-cleavable. Exemplary cleavable linkers include 2A.

[00490] An exemplary CAR comprises the scFv of the disclosure, CD8 transmembrane domain and CD3 ζ signaling domain. Another exemplary CAR comprise the scFv of the disclosure, CD8 or CD28 transmembrane domain, CD28, 41BB or OX40 costimulatory domain and CD3 ζ signaling domain.

5 [00491] CARs are generated by standard molecular biology techniques.

[00492] The spFv of the disclosure may be conjugated directly to the second molecule or via a linker. Exemplary linkers include portions of immunoglobulin hinge area, CL or CH1 derived from any immunoglobulin heavy or light chain isotype, Gly rich linkers, Gly and Ser containing linkers, Gly and Ala containing linkers, Ala and Ser containing linkers and Pro containing
10 linkers. Exemplary amino acids that may be included into the linker are Gly, Ser Pro, Thr, Glu, Lys, Arg, Ile, Leu and His. Alternatively, a variety of non-proteinaceous polymers, including polyethylene glycol (PEG), polypropylene glycol, polyoxyalkylenes, or copolymers of polyethylene glycol and polypropylene glycol, may find use as linkers. Exemplary linkers are described for example in Int. Pat. Publ. No. WO2019/060695.

15 [00493] In some embodiments, the heterologous molecule is monospecific.

[00494] In some embodiments, the heterologous molecule is multispecific.

[00495] In some embodiments, the heterologous molecule is bispecific.

[00496] In some embodiments, the heterologous molecule is trispecific.

[00497] In some embodiments, the heterologous molecule is tetraspecific.

20 **Multispecific molecules comprising the spFv of the disclosure**

[00498] The disclosure also provides a multispecific molecule comprising a single chain variable fragment (scFv) comprising a heavy chain variable region (VH), a linker (L) and a light chain variable region (VL), wherein the scFv comprises
25 a first disulfide bond between a structurally conserved surface exposed VH cysteine (Cys) and a first L Cys;
a second disulfide bond between a structurally conserved surface exposed VL Cys and a second L Cys; or
the first disulfide bond between the structurally conserved surface exposed VH Cys and the first L Cys and the second disulfide bond between the structurally conserved surface exposed VL Cys
30 and the second L Cys.

[00499] The disclosure also provides a multispecific molecule comprising a scFv comprising a VH, a L and a VL, wherein

the VH comprises a VH Cys at a structurally conserved surface exposed VH framework residue position and the L comprises a first L Cys;

5 the VL comprises a VL Cys at a structurally conserved surface exposed VL framework residue position and the L comprises a second L Cys; or

the VH comprises the VH Cys at a structurally conserved surface exposed VH framework residue position, the VL comprises the VL Cys at a structurally conserved surface exposed VL framework residue position and the L comprises the first L Cys and the second L Cys, wherein

10 the VH Cys and the first L Cys are capable of forming a disulfide bond and the VL Cys and the second L Cys are capable of forming a disulfide bond.

[00500] In some embodiments, the distance between the VH Cys and the VL Cys is from about 7 Å to about 9 Å.

[00501] In some embodiments, the VH Cys is at H3, H5, H40, H43, H46 or H105, wherein
15 residue numbering is according to Chothia.

[00502] In some embodiments, the VL Cys is at L3, L5, L39, L42, L45, L100 or L102, wherein residue numbering is according to Chothia.

[00503] In some embodiments,

the VH Cys is at H105 and the VL Cys is at L42;

20 the VH Cys is at H43 and the VL Cys is at a L100;

the VH Cys is at H3 and the VL Cys is at L3;

the VH Cys is at H3 and the VL Cys is at L5;

the VH Cys is at H3 and the VL Cys is at L39;

the VH Cys is at H3 and the VL Cys is at L42;

25 the VH Cys is at H3 and the VL Cys is at L45;

the VH Cys is at H3 and the VL Cys is at L100;

the VH Cys is at H3 and the VL Cys is at L102;

the VH Cys is at H5 and the VL Cys is at L3;

the VH Cys is at H5 and the VL Cys is at L5;

30 the VH Cys is at H5 and the VL Cys is at L39;

the VH Cys is at H5 and the VL Cys is at L42;

- the VH Cys is at H5 and the VL Cys is at L45;
the VH Cys is at H5 and the VL Cys is at L100;
the VH Cys is at H5 and the VL Cys is at L102;
the VH Cys is at H40 and the VL Cys is at L3;
5 the VH Cys is at H40 and the VL Cys is at L5;
the VH Cys is at H40 and the VL Cys is at L39;
the VH Cys is at H40 and the VL Cys is at L42;
the VH Cys is at H40 and the VL Cys is at L45;
the VH Cys is at H40 and the VL Cys is at L100;
10 the VH Cys is at H40 and the VL Cys is at L102;
the VH Cys is at H43 and the VL Cys is at L3;
the VH Cys is at H43 and the VL Cys is at L5;
the VH Cys is at H43 and the VL Cys is at L39;
the VH Cys is at H43 and the VL Cys is at L42;
15 the VH Cys is at H43 and the VL Cys is at L45;
the VH Cys is at H43 and the VL Cys is at L102;
the VH Cys is at H46 and the VL Cys is at L3;
the VH Cys is at H46 and the VL Cys is at L5;
the VH Cys is at H46 and the VL Cys is at L39;
20 the VH Cys is at H46 and the VL Cys is at L42;
the VH Cys is at H46 and the VL Cys is at L45;
the VH Cys is at H46 and the VL Cys is at L100;
the VH Cys is at H46 and the VL Cys is at L102;
the VH Cys is at H105 and the VL Cys is at L3;
25 the VH Cys is at H105 and the VL Cys is at L5;
the VH Cys is at H105 and the VL Cys is at L39;
the VH Cys is at H105 and the VL Cys is at L45;
the VH Cys is at H105 and the VL Cys is at L100; or
the VH Cys is at H105 and the VL Cys is at L102;
30 herein residue numbering is according to Chothia.

[00504] In some embodiments, the L comprises a contiguous amino acid sequence derived from an immunoglobulin (Ig) hinge region.

[00505] In some embodiments, the Ig hinge region is derived from a human or a non-human Ig hinge region.

5 **[00506]** In some embodiments, the Ig hinge region is derived from the human Ig hinge region.

[00507] In some embodiments, the human Ig hinge region is an IgG1, IgG2, IgG3 or IgG4 isotype.

[00508] In some embodiments, the L comprises an amino acid sequence C(X)_yC (**SEQ ID NO: 23**), wherein X is Gly, Ser, Pro, Ala, Arg, Asn, Asp, Glu, Gln, His, Ile, Leu, Lys, Phe, Thr, Trp or Tyr, and y is an integer from 1 to 3.

[00509] In some embodiments, the L comprises the amino acid sequence C(X)_yC (**SEQ ID NO: 24**), wherein X is Gly, Ser or Pro, and y is an integer from 1 to 3.

[00510] In some embodiments, the L comprises the amino acid sequence CPC, CGC, CSC, CPPC (**SEQ ID NO: 1**), CGPC (**SEQ ID NO: 28**), CPGC (**SEQ ID NO: 29**), CGGC (**SEQ ID NO: 30**), CSPG (**SEQ ID NO: 31**), CPSC (**SEQ ID NO: 32**), CSSC (**SEQ ID NO: 33**), CGSC (**SEQ ID NO: 34**), CSGC (**SEQ ID NO: 35**), CPPPC (**SEQ ID NO: 36**), CGPPC (**SEQ ID NO: 37**), CPGPC (**SEQ ID NO: 38**), CPPGC (**SEQ ID NO: 39**), CGGPC (**SEQ ID NO: 40**), CPGGC (**SEQ ID NO: 41**), CGGGC (**SEQ ID NO: 42**), CSPPC (**SEQ ID NO: 43**), CPSPC (**SEQ ID NO: 44**), CPPSC (**SEQ ID NO: 45**), CSSPC (**SEQ ID NO: 46**), CPSSC (**SEQ ID NO: 47**), CSSSC (**SEQ ID NO: 48**), CGSPC (**SEQ ID NO: 49**), CPGSC (**SEQ ID NO: 50**), CSGPC (**SEQ ID NO: 51**) or CPSGC (**SEQ ID NO: 52**).

[00511] In some embodiments, the L comprises from about 14 to about 19 amino acids, such as about 14, about 15, about 16, about 17, about 18 or about 19 amino acids.

[00512] In some embodiments, the L comprises the amino acid sequence (X)_mC(X)_yC(X)_n (**SEQ ID NO: 25**); wherein X is Gly, Ser, Pro, Ala, Arg, Asn, Asp, Glu, Gln, His, Ile, leu, Lys, Phe Thr, Trp or Tyr, m is an integer from 6 to 9, y is an integer from 1 to 3 and n is an integer from 4 to 6.

[00513] In some embodiments, the L comprises the amino acid sequence (X)_mC(X)_yC(X)_n (**SEQ ID NO: 26**); wherein X is Gly, Ser, Pro, Ala, Arg, Asn, Asp, Glu, Gln, His, Ile, Leu, Lys, Thr or Tyr, m is an integer from 6 to 9, y is an integer from 1 to 3 and n is an integer from 4 to 6.

[00514] In some embodiments, the L comprises the amino acid sequence $(X)_mC(X)_yC(X)_n$ (**SEQ ID NO: 27**); wherein X is Gly or Pro, m is an integer from 6 to 9, y is an integer from 1 to 3 and n is an integer from 4 to 6.

5 **[00515]** In some embodiments, the L comprises the amino acid sequence of SEQ ID NOs: 2, 3, 4, 5, 6 or 7.

[00516] In some embodiments, the spFv of the disclosure is in the VL-L-VH orientation.

[00517] In some embodiments, the spFv of the disclosure is in the VH-L-VL orientation.

[00518] The disclosure also provides a multispecific molecule comprising a scFv comprising a VH, a L and a VL, wherein

10 the VH comprises Cys at H105;

the VL comprises Cys at L42;

the L comprises an amino acid sequence of SEQ ID NOs: 2, 3, 4, 5, 6 or 7; and

the scFv is in the VL-L-VH orientation.

[00519] The disclosure also provides a multispecific molecule comprising a scFv comprising a

15 VH, a L and a VL, wherein

the VH comprises Cys at H105;

the VL comprises Cys at L45;

the L comprises an amino acid sequence of SEQ ID NOs: 2, 3, 4, 5, 6 or 7; and

the scFv is in the VL-L-VH orientation.

20 **[00520]** The disclosure also provides a multispecific molecule comprising a scFv comprising a

VH, a L and a VL, wherein

the VH comprises Cys at H105;

the VL comprises Cys at L39;

the L comprises an amino acid sequence of SEQ ID NOs: 2, 3, 4, 5, 6 or 7; and

25 the scFv is in the VL-L-VH orientation.

[00521] The disclosure also provides a multispecific molecule comprising a scFv comprising a VH, a L and a VL, wherein

the VH comprises Cys at H5;

the VL comprises Cys at L42;

30 the L comprises an amino acid sequence of SEQ ID NOs: 2, 3, 4, 5, 6 or 7; and

the scFv is in the VL-L-VH orientation.

[00522] The disclosure also provides a multispecific molecule comprising a scFv comprising a VH, a L and a VL, wherein
the VH comprises Cys at H5;
the VL comprises Cys at L45;
5 the L comprises an amino acid sequence of SEQ ID NOs: 2, 3, 4, 5, 6 or 7; and
the scFv is in the VL-L-VH orientation.

[00523] The disclosure also provides a multispecific molecule comprising a scFv comprising a VH, a L and a VL, wherein
the VH comprises Cys at H5;
10 the VL comprises Cys at L39;
the L comprises an amino acid sequence of SEQ ID NOs: 2, 3, 4, 5, 6 or 7; and
the scFv is in the VL-L-VH orientation.

[00524] The disclosure also provides a multispecific molecule comprising a scFv comprising a VH, a L and a VL, wherein
15 the VH comprises Cys at H3;
the VL comprises Cys at L42;
the L comprises an amino acid sequence of SEQ ID NOs: 2, 3, 4, 5, 6 or 7; and
the scFv is in the VL-L-VH orientation.

[00525] The disclosure also provides a multispecific molecule comprising a scFv comprising a
20 VH, a L and a VL, wherein
the VH comprises Cys at H3;
the VL comprises Cys at L45;
the L comprises an amino acid sequence of SEQ ID NOs: 2, 3, 4, 5, 6 or 7; and
the scFv is in the VL-L-VH orientation.

[00526] The disclosure also provides a multispecific molecule comprising a scFv comprising a
25 VH, a L and a VL, wherein
the VH comprises Cys at H3;
the VL comprises Cys at L39;
the L comprises an amino acid sequence of SEQ ID NOs: 2, 3, 4, 5, 6 or 7; and
30 the scFv is in the VL-L-VH orientation.

[00527] The disclosure also provides a multispecific molecule comprising a scFv comprising a VH, a L and a VL, wherein

the VH comprises Cys at H43;

the VL comprises Cys at L100;

- 5 the L comprises an amino acid sequence of SEQ ID NOs: 2, 3, 4, 5, 6 or 7; and
the scFv is in the VH-L-VL orientation.

[00528] The disclosure also provides a multispecific molecule comprising a scFv comprising a VH, a L and a VL, wherein

the VH comprises Cys at H43;

- 10 the VL comprises Cys at L102;

the L comprises an amino acid sequence of SEQ ID NOs: 2, 3, 4, 5, 6 or 7; and
the scFv is in the VH-L-VL orientation.

[00529] The disclosure also provides a multispecific molecule comprising a scFv comprising a VH, a L and a VL, wherein

- 15 the VH comprises Cys at H43;

the VL comprises Cys at L5;

the L comprises an amino acid sequence of SEQ ID NOs: 2, 3, 4, 5, 6 or 7; and
the scFv is in the VH-L-VL orientation.

[00530] The disclosure also provides a multispecific molecule comprising a scFv comprising a VH, a L and a VL, wherein

- 20 the VH comprises Cys at H43;

the VL comprises Cys at L3;

the L comprises an amino acid sequence of SEQ ID NOs: 2, 3, 4, 5, 6 or 7; and
the scFv is in the VH-L-VL orientation.

- 25 **[00531]** The disclosure also provides a multispecific molecule comprising a scFv comprising a VH, a L and a VL, wherein

the VH comprises Cys at H40;

the VL comprises Cys at L100;

- the L comprises an amino acid sequence of SEQ ID NOs: 2, 3, 4, 5, 6 or 7; and
30 the scFv is in the VH-L-VL orientation.

[00532] The disclosure also provides a multispecific molecule comprising a scFv comprising a VH, a L and a VL, wherein

the VH comprises Cys at H40;

the VL comprises Cys at L102;

- 5 the L comprises an amino acid sequence of SEQ ID NOs: 2, 3, 4, 5, 6 or 7; and
the scFv is in the VH-L-VL orientation.

[00533] The disclosure also provides a multispecific molecule comprising a scFv comprising a VH, a L and a VL, wherein

the VH comprises Cys at H40;

- 10 the VL comprises Cys at L5;

the L comprises an amino acid sequence of SEQ ID NOs: 2, 3, 4, 5, 6 or 7; and
the scFv is in the VH-L-VL orientation.

[00534] The disclosure also provides a multispecific molecule comprising a scFv comprising a VH, a L and a VL, wherein

- 15 the VH comprises Cys at H40;

the VL comprises Cys at L3;

the L comprises an amino acid sequence of SEQ ID NOs: 2, 3, 4, 5, 6 or 7; and
the scFv is in the VH-L-VL orientation.

[00535] The disclosure also provides a multispecific molecule comprising a scFv comprising a VH, a L and a VL, wherein

- 20 the VH comprises Cys at H46;

the VL comprises Cys at L100;

the L comprises an amino acid sequence of SEQ ID NOs: 2, 3, 4, 5, 6 or 7; and
the scFv is in the VH-L-VL orientation.

- 25 **[00536]** The disclosure also provides a multispecific molecule comprising a scFv comprising a VH, a L and a VL, wherein

the VH comprises Cys at H46;

the VL comprises Cys at L102;

the L comprises an amino acid sequence of SEQ ID NOs: 2, 3, 4, 5, 6 or 7; and

- 30 the scFv is in the VH-L-VL orientation.

[00537] The disclosure also provides a multispecific molecule comprising a scFv comprising a VH, a L and a VL, wherein
the VH comprises Cys at H46;
the VL comprises Cys at L5;
5 the L comprises an amino acid sequence of SEQ ID NOs: 2, 3, 4, 5, 6 or 7; and
the scFv is in the VH-L-VL orientation.

[00538] The disclosure also provides a multispecific molecule comprising a scFv comprising a VH, a L and a VL, wherein
the VH comprises Cys at H46;
10 the VL comprises Cys at L3;
the L comprises an amino acid sequence of SEQ ID NOs: 2, 3, 4, 5, 6 or 7; and
the scFv is in the VH-L-VL orientation.

[00539] In some embodiments, the L comprises the amino acid sequence of **SEQ ID NO: 3**.

[00540] In some embodiments, the L comprises the amino acid sequence of **SEQ ID NO: 6**.

15 **[00541]** In some embodiments, the L comprises the amino acid sequence of **SEQ ID NO: 7**.

[00542] In some embodiments, the multispecific molecule comprises an antibody or an antibody fragment.

[00543] In some embodiments, the multispecific protein comprises an Ig constant region or a fragment of the Ig constant region.

20 **[00544]** In some embodiments, the Ig constant region comprises a Fc region.

[00545] In some embodiments, the Ig constant region comprises a CH2 domain.

[00546] In some embodiments, the fragment of the Ig constant region comprises a CH3 domain.

25 **[00547]** In some embodiments, the fragment of the Ig constant region comprises the CH2 domain and the CH3 domain.

[00548] In some embodiments, the fragment of the Ig constant region comprises at least a portion of a hinge, the CH2 domain and the CH3 domain.

[00549] In some embodiments, the fragment of the Ig constant region comprises the hinge, the CH2 domain and the CH3 domain.

30 **[00550]** In some embodiments, the spFv of the disclosure is conjugated to the N-terminus of the Ig constant region or to the N-terminus of the fragment of the Ig constant region.

[00551] In some embodiments, the spFv of the disclosure is conjugated to the C-terminus of the Ig constant region or to the C-terminus of the fragment of the Ig constant region.

[00552] In some embodiments, the Ig constant region or the fragment of the Ig constant region is an IgG1, an IgG2, and IgG3 or an IgG4 isotype.

5 **[00553]** In some embodiments, the Ig constant region or the fragment of the Ig constant region comprises at least one mutation that results in reduced binding of the multispecific molecule to FcγR.

[00554] In some embodiments, the at least one mutation that results in reduced binding of the multispecific molecule to FcγR is selected from the group consisting of F234A/L235A,
10 L234A/L235A, L234A/L235A/D265S, V234A/G237A/ P238S/H268A/V309L/A330S/P331S, F234A/L235A, S228P/F234A/ L235A, N297A, V234A/G237A, K214T/E233P/ L234V/L235A/G236-deleted/A327G/P331A/D365E/L358M, H268Q/V309L/A330S/P331S, S267E/L328F, L234F/L235E/D265A, L234A/L235A/G237A/P238S/H268A/A330S/P331S, S228P/F234A/L235A/G237A/P238S and S228P/F234A/L235A/G236-deleted/G237A/P238S,
15 wherein residue numbering is according to the EU index.

[00555] In some embodiments, the Ig constant region or the fragment of the Ig constant region comprises at least one mutation that results in enhanced binding of the multispecific molecule to FcγR.

[00556] In some embodiments, the at least one mutation that results in enhanced binding of the
20 multispecific molecule to FcγR is selected from the group consisting of S239D/I332E, S298A/E333A/K334A, F243L/R292P/Y300L, F243L/R292P/Y300L/P396L, F243L/R292P/Y300L/V305I/P396L and G236A/S239D/I332E, wherein residue numbering is according to the EU index.

[00557] In some embodiments, FcγR is FcγRI, FcγRIIA, FcγRIIB or FcγRIII.

25 **[00558]** In some embodiments, the Ig constant region or fragment of the Ig constant region comprises at least one mutation that modulates a half-life of the multispecific molecule.

[00559] In some embodiments, the at least one mutation that modulates the half-life of the multispecific molecule is selected from the group consisting of H435A, P257I/N434H, D376V/N434H, M252Y/S254T/T256E/H433K/N434F, T308P/N434A and H435R, wherein
30 residue numbering is according to the EU index.

[00560] In some embodiments, the Ig constant region or fragment of the Ig constant region comprises at least one mutation in the CH3 domain.

[00561] In some embodiments, the at least one mutation in the CH3 domain is selected from the group consisting of T350V, L351Y, F405A, Y407V, T366Y, T366W, F405W, T394W,

5 T394S, Y407T, Y407A, T366S/L368A/Y407V, L351Y/F405A/Y407V, T366I/K392M/T394W, F405A/Y407V, T366L/K392M/T394W, L351Y/Y407A, T366A/K409F, L351Y/Y407A, T366V/K409F, T366A/K409F, T350V/L351Y/F405A/Y407V and T350V/T366L/K392L/T394W, wherein residue numbering is according to the EU index.

[00562] In some embodiments, the multispecific molecule is bispecific.

10 [00563] In some embodiments, the multispecific molecule is trispecific.

[00564] In some embodiments, the multispecific molecule is tetraspecific.

5.2 Generation of multispecific proteins that comprise the spFv of the disclosure

[00565] The spFv of the disclosure may be engineered into multispecific molecules of any known format using known recombinant technologies, expression and purification protocols.

15 [00566] The spFv of the disclosure may be engineered into full length multispecific antibodies having one or more mutations in the CH3 domain which promoter stability of the two half molecules. These multispecific antibodies may be generated *in vitro* using Fab arm exchange or by co-expression of the various chains. For *in vitro* Fab arm exchange, two monospecific bivalent antibodies are engineered to have the one or more substitutions in the CH3 domain, the
20 antibodies are incubated together under reducing conditions sufficient to allow the cysteines in the hinge region to undergo disulfide bond isomerization; thereby generating the bispecific antibody by Fab arm exchange. The incubation conditions may optimally be restored to non-reducing. Exemplary reducing agents that may be used are 2- mercaptoethylamine (2-MEA), dithiothreitol (DTT), dithioerythritol (DTE), glutathione, tris(2-carboxyethyl)phosphine (TCEP),
25 L-cysteine and beta-mercaptoethanol, preferably a reducing agent selected from the group consisting of: 2- mercaptoethylamine, dithiothreitol and tris(2-carboxyethyl)phosphine. For example, incubation for at least 90 min at a temperature of at least 20°C in the presence of at least 25 mM 2-MEA or in the presence of at least 0.5 mM dithiothreitol at a pH of from 5-8, for example at pH of 7.0 or at pH of 7.4 may be used.

30 [00567] CH3 mutations that may be used include technologies such as Knob-in-Hole mutations (Genentech), electrostatically-matched mutations (Chugai, Amgen, NovoNordisk, Oncomed), the

Strand Exchange Engineered Domain body (SEEDbody) (EMD Serono), Duobody® mutations (Genmab), and other asymmetric mutations (*e.g.*, Zymeworks).

[00568] Knob-in-hole mutations are disclosed for example in WO1996/027011 and include mutations on the interface of CH3 region in which an amino acid with a small side chain (hole) is introduced into the first CH3 region and an amino acid with a large side chain (knob) is introduced into the second CH3 region, resulting in preferential interaction between the first CH3 region and the second CH3 region. Exemplary CH3 region mutations forming a knob and a hole are T366Y/F405A, T366W/F405W, F405W/Y407A, T394W/Y407T, T394S/Y407A, T366W/T394S, F405W/T394S and T366W/T366S_L368A_Y407V.

[00569] Heavy chain heterodimer formation may be promoted by using electrostatic interactions by substituting positively charged residues on the first CH3 region and negatively charged residues on the second CH3 region as described in US2010/0015133, US2009/0182127, US2010/028637 or US2011/0123532.

[00570] Other asymmetric mutations that can be used to promote heavy chain

heterodimerization are L351Y_F405A_Y407V/T394W, T366I_K392M_T394W/F405A_Y407V, T366L_K392M_T394W/F405A_Y407V, L351Y_Y407A/T366A_K409F, L351Y_Y407A/T366V_K409F, Y407A/T366A_K409F, or T350V_L351Y_F405A_Y407V/T350V_T366L_K392L_T394W as described in US2012/0149876 or US2013/0195849 (Zymeworks).

[00571] SEEDbody mutations involve substituting select IgG residues with IgA residues to promote heavy chain heterodimerization as described in US20070287170.

[00572] Other exemplary mutations that may be used are R409D_K370E/D399K_E357K, S354C_T366W/Y349C_T366S_L368A_Y407V,

Y349C_T366W/S354C_T366S_L368A_Y407V, T366K/L351D, L351K/Y349E,

L351K/Y349D, L351K/L368E, L351Y_Y407A/T366A_K409F, L351Y_Y407A/T366V_K409F, K392D/D399K, K392D/E356K, K253E_D282K_K322D/D239K_E240K_K292D, K392D_K409D/D356K_D399K as described in WO2007/147901, WO 2011/143545, WO2013/157954, WO2013/096291 and US2018/0118849.

[00573] Duobody® mutations (Genmab) are disclosed for example in US9150663 and

US2014/0303356 and include mutations F405L/K409R, wild-type/F405L_R409K, T350I_K370T_F405L/K409R, K370W/K409R, D399AFGHILMNRSTVWY/K409R,

T366ADEFHILMQVY/K409R, L368ADEGHNRSTVQ/K409AGRH,
D399FHKRQ/K409AGRH, F405IKLSTVW/K409AGRH and Y407LWQ/K409AGRH.

- [00574]** Additional bispecific or multispecific structures into which the spFv of the disclosure may be incorporated include Dual Variable Domain Immunoglobulins (DVD) (Int. Pat. Publ. No. WO2009/134776; DVDs are full length antibodies comprising the heavy chain having a structure VH1-linker-VH2-CH and the light chain having the structure VL1-linker-VL2-CL; linker being optional), structures that include various dimerization domains to connect the two antibody arms with different specificity, such as leucine zipper or collagen dimerization domains (Int. Pat. Publ. No. WO2012/022811, U.S. Pat. No. 5,932,448; U.S. Pat. No. 6,833,441), two or more domain antibodies (dAbs) conjugated together, diabodies, heavy chain only antibodies such as camelid antibodies and engineered camelid antibodies, Dual Targeting (DT)-Ig (GSK/Domantis), Two-in-one Antibody (Genentech), Cross-linked Mabs (Karmanos Cancer Center), mAb2 (F-Star) and CovX-body (CovX/Pfizer), IgG-like Bispecific (InnClone/Eli Lilly), Ts2Ab (MedImmune/AZ) and BsAb (Zymogenetics), HERCULES (Biogen Idec) and TvAb (Roche), ScFv/Fc Fusions (Academic Institution), SCORPION (Emergent BioSolutions/Trubion, Zymogenetics/BMS), Dual Affinity Retargeting Technology (Fc-DART) (MacroGenics) and Dual(ScFv)₂-Fab (National Research Center for Antibody Medicine--China), Dual-Action or Bis-Fab (Genentech), Dock-and-Lock (DNL) (ImmunoMedics), Bivalent Bispecific (Biotecnol) and Fab-Fv (UCB-Celltech). ScFv-, diabody-based, and domain antibodies, include but are not limited to, Bispecific T Cell Engager (BiTE) (Micromet), Tandem Diabody (Tandab) (Affimed), Dual Affinity Retargeting Technology (DART) (MacroGenics), Single-chain Diabody (Academic), TCR-like Antibodies (AIT, ReceptorLogics), Human Serum Albumin ScFv Fusion (Merrimack) and COMBODY (Epigen Biotech), dual targeting nanobodies (Ablynx), dual targeting heavy chain only domain antibodies.
- [00575]** The scFv of the disclosure may also be engineered into multispecific protein which comprises three polypeptide chains. In such designs, at least one antigen binding domain is in the form of a scFv of the disclosure. Exemplary designs include (in which “1” indicates the first antigen binding domain, “2” indicates the second antigen binding domain and “3” indicates the third antigen binding domain:
- Design 1: Chain A) scFv1- CH2-CH3; Chain B) VL2-CL; Chain C) VH2-CH1-hinge-CH2-CH3

Design 2: Chain A) scFv1- hinge- CH2-CH3; Chain B) VL2-CL; Chain C) VH2-CH1-hinge-CH2-CH3

Design 3: Chain A) scFv1- CH1-hinge- CH2-CH3; Chain B) VL2-CL; Chain C) VH2-CH1-hinge-CH2-CH3

5 Design 4: Chain A) CH2-CH3-scFv1; Chain B) VL2-CL; Chain C) VH2-CH1-hinge-CH2-CH3

[00576] CH3 engineering may be incorporated to the Designs 1-4, such as mutations

L351Y_F405A_Y407V/T394W, T366I_K392M_T394W/F405A_Y407V,

T366L_K392M_T394W/F405A_Y407V, L351Y_Y407A/T366A_K409F,

L351Y_Y407A/T366V_K409F, Y407A/T366A_K409F, or

10 T350V_L351Y_F405A_Y407V/T350V_T366L_K392L_T394W as described in US2012/0149876 or US2013/0195849 (Zymeworks).

5.3 Isotypes, allotypes and Fc engineering

[00577] The Ig constant region or the fragment of the Ig constant region, such as the Fc region present in the multispecific molecules or in the heterologous molecules of the disclosure may be
15 of any allotype or isotype.

[00578] In some embodiments, the Ig constant region or the fragment of the Ig constant region is an IgG1 isotype.

[00579] In some embodiments, the Ig constant region or the fragment of the Ig constant region is an IgG2 isotype.

20 **[00580]** In some embodiments, the Ig constant region or the fragment of the Ig constant region is an IgG3 isotype.

[00581] In some embodiments, the Ig constant region or the fragment of the Ig constant region is an IgG4 isotype.

[00582] The Ig constant region or the fragment of the Ig constant region may be of any
25 allotype. It is expected that allotype has no influence on properties of the Ig constant region, such as binding or Fc-mediated effector functions. Immunogenicity of therapeutic proteins comprising Ig constant regions of fragments thereof is associated with increased risk of infusion reactions and decreased duration of therapeutic response (Baert *et al.*, (2003) *N Engl J Med* 348:602-608). The extent to which therapeutic proteins comprising Ig constant regions of
30 fragments thereof induce an immune response in the host may be determined in part by the allotype of the Ig constant region (Stickler *et al.*, (2011) *Genes and Immunity* 12:213-221). Ig

constant region allotype is related to amino acid sequence variations at specific locations in the constant region sequences of the antibody. **Table 3** shows select IgG1, IgG2 and IgG4 allotypes.

Table 3.

Allotype	Amino acid residue at position of diversity (residue numbering: EU Index)							
	IgG2		IgG4		IgG1			
	189	282	309	422	214	356	358	431
G2m(n)	T	M						
G2m(n-)	P	V						
G2m(n)/(n-)	T	V						
nG4m(a)			L	R				
G1m(17)					K	E	M	A
G1m(17,1)					K	D	L	A

5 **[00583]** C-terminal lysine (CTL) may be removed from the Ig constant region by endogenous circulating carboxypeptidases in the blood stream (Cai *et al.*, (2011) *Biotechnol Bioeng* 108:404-412). During manufacturing, CTL removal may be controlled to less than the maximum level by control of concentration of extracellular Zn^{2+} , EDTA or EDTA – Fe^{3+} as described in U.S. Patent Publ. No. US2014/0273092. CTL content of proteins may be measured using known methods.

10 **[00584]** In some embodiments, the spFv of the disclosure conjugated to the Ig constant region has a C-terminal lysine content from about 10% to about 90%. In some embodiments, the C-terminal lysine content is from about 20% to about 80%. In some embodiments, the C-terminal lysine content is from about 40% to about 70%. In some embodiments, the C-terminal lysine content is from about 55% to about 70%. In some embodiments, the C-terminal lysine content is about 60%.

15 **[00585]** Fc region mutations may be made to the multispecific molecules or the heterologous molecules of the disclosure comprising the Ig constant region or the fragment of the Ig constant region to modulate their effector functions such as ADCC, ADCP and/or ADCP and/or pharmacokinetic properties. This may be achieved by introducing mutation(s) into the Fc that modulate binding of the mutated Fc to activating $Fc\gamma Rs$ ($Fc\gamma RI$, $Fc\gamma RIIa$, $Fc\gamma RIII$), inhibitory $Fc\gamma RI Ib$ and/or to $FcRn$.

20 **[00586]** In some embodiments, the multispecific molecule or the heterologous molecule of the disclosure comprises at least one mutation in the Ig constant region or in the fragment of the Ig constant region.

[00587] In some embodiments, the at least one mutation is in the Fc region.

[00588] In some embodiments, the multispecific molecule or the heterologous molecule of the disclosure comprises at least one, two, three, four, five, six, seven, eight, nine, ten, eleven, twelve, thirteen, fourteen or fifteen mutations in the Fc region.

5 [00589] In some embodiments, the multispecific molecule or the heterologous molecule of the disclosure comprises at least one mutation in the Fc region that modulates binding of the antibody to FcRn.

[00590] Fc positions that may be mutated to modulate half-life (*e.g.*, binding to FcRn) include positions 250, 252, 253, 254, 256, 257, 307, 376, 380, 428, 434 and 435. Exemplary mutations
10 that may be made singularly or in combination are mutations T250Q, M252Y, I253A, S254T, T256E, P257I, T307A, D376V, E380A, M428L, H433K, N434S, N434A, N434H, N434F, H435A and H435R. Exemplary singular or combination mutations that may be made to increase the half-life are mutations M428L/N434S, M252Y/S254T/T256E, T250Q/M428L, N434A and T307A/E380A/N434A. Exemplary singular or combination mutations that may be made to
15 reduce the half-life are mutations H435A, P257I/N434H, D376V/N434H, M252Y/S254T/T256E/H433K/N434F, T308P/N434A and H435R.

[00591] In some embodiments, the multispecific molecule or the heterologous molecule of the disclosure comprises M252Y/S254T/T256E mutation in the Fc region.

[00592] In some embodiments, the multispecific molecule or the heterologous molecule of the disclosure comprises at least one mutation in the Fc region that reduces binding of the protein to
20 an activating Fcγ receptor (FcγR) and/or reduces Fc effector functions such as C1q binding, complement dependent cytotoxicity (CDC), antibody-dependent cell-mediated cytotoxicity (ADCC) or phagocytosis (ADCP).

[00593] Fc positions that may be mutated to reduce binding of the multispecific molecule or
25 the heterologous molecule of the disclosure to the activating FcγR and subsequently to reduce effector function include positions 214, 233, 234, 235, 236, 237, 238, 265, 267, 268, 270, 295, 297, 309, 327, 328, 329, 330, 331 and 365. Exemplary mutations that may be made singularly or in combination are mutations K214T, E233P, L234V, L234A, deletion of G236, V234A, F234A, L235A, G237A, P238A, P238S, D265A, D265S, S267E, H268A, H268Q, Q268A, N297A,
30 A327Q, P329A, D270A, Q295A, V309L, A327S, L328F, A330S and P331S in IgG1, IgG2, IgG3 or IgG4. Exemplary combination mutations that result in the multispecific molecule or the

heterologous molecule of the disclosure with reduced ADCC are mutations L234A/L235A on IgG1, L234A/L235A/D265S on IgG1, V234A/G237A/ P238S/H268A/V309L/A330S/P331S on IgG2, F234A/L235A on IgG4, S228P/F234A/ L235A on IgG4, N297A on all Ig isotypes, V234A/G237A on IgG2, K214T/E233P/ L234V/L235A/G236-

5 deleted/A327G/P331A/D365E/L358M on IgG1, H268Q/V309L/A330S/P331S on IgG2, S267E/L328F on IgG1, L234F/L235E/D265A on IgG1, L234A/L235A/G237A/P238S/H268A/A330S/P331S on IgG1, S228P/F234A/L235A/G237A/P238S on IgG4, and S228P/F234A/L235A/G236-
10 deleted/G237A/P238S on IgG4. Hybrid IgG2/4 Fc domains may also be used, such as Fc with residues 117-260 from IgG2 and residues 261-447 from IgG4.

[00594] Exemplary mutation that results in the multispecific molecule or the heterologous molecule of the disclosure with reduced CDC is a K322A mutation.

[00595] Well-known S228P mutation may be made in IgG4 to enhance IgG4 stability.

[00596] In some embodiments, the multispecific molecule or the heterologous molecule of the disclosure comprises at least one mutation in the Fc region selected from the group consisting of
15 K214T, E233P, L234V, L234A, deletion of G236, V234A, F234A, L235A, G237A, P238A, P238S, D265A, S267E, H268A, H268Q, Q268A, N297A, A327Q, P329A, D270A, Q295A, V309L, A327S, L328F, A330S and P331S.

[00597] In some embodiments, the multispecific molecule or the heterologous molecule of the disclosure comprises L234A/L235A/D265S mutation in the Fc region.
20

[00598] In some embodiments, the multispecific molecule or the heterologous molecule of the disclosure comprises L234A/L235A mutation in the Fc region.

[00599] In some embodiments, the multispecific molecule or the heterologous molecule of the disclosure comprises at least one mutation in the Fc region that enhances binding of the
25 multispecific molecule or the heterologous molecule of the disclosure to FcγR and/or enhances Fc effector functions such as C1q binding, complement dependent cytotoxicity (CDC), antibody-dependent cell-mediated cytotoxicity (ADCC) and/or phagocytosis (ADCP).

[00600] Fc positions that may be mutated to increase binding of the multispecific molecule or the heterologous molecule of the disclosure to the activating FcγR and/or enhance Fc effector
30 functions include positions 236, 239, 243, 256, 290, 292, 298, 300, 305, 312, 326, 330, 332, 333, 334, 345, 360, 339, 378, 396 or 430 (residue numbering according to the EU index). Exemplary

mutations that may be made singularly or in combination are G236A, S239D, F243L, T256A, K290A, R292P, S298A, Y300L, V305L, K326A, A330K, I332E, E333A, K334A, A339T and P396L. Exemplary combination mutations that result in proteins with increased ADCC or ADCP are a S239D/I332E, S298A/E333A/K334A, F243L/R292P/Y300L,

5 F243L/R292P/Y300L/P396L, F243L/R292P/Y300L/V305I/P396L and G236A/S239D/I332E.

[00601] Fc positions that may be mutated to enhance CDC include positions 267, 268, 324, 326, 333, 345 and 430. Exemplary mutations that may be made singularly or in combination are S267E, F1268F, S324T, K326A, K326W, E333A, E345K, E345Q, E345R, E345Y, E430S, E430F and E430T. Exemplary combination mutations that result in the multispecific molecule or the heterologous molecule of the disclosure with increased CDC are K326A/E333A,

10 K326W/E333A, H268F/S324T, S267E/H268F, S267E/S324T and S267E/H268F/S324T.

[00602] The specific mutations described herein are mutations when compared to the IgG1, IgG2 and IgG4 wild-type amino acid sequences of SEQ ID NOs: 66, 67 and 68, respectively.

15 **SEQ ID NO: 66**, wild-type IgG1

ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSS
GLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPELLGG
PSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQY
NSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSR
20 DELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTTPVLDSDGSFFLYSKLTVDK
SRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK

SEQ ID NO: 67, wild-type IgG2

ASTKGPSVFPLAPCSRSTSESTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSS
25 GLYSLSSVVTVPSSNFGTQTYTCNVNHDHKPSNTKVDKTVERKCCVECPAPPVAGPSV
FLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVQFNWYVDGVEVHNAKTKPREEQFNST
FRVSVSVLTVVHQDWLNGKEYKCKVSNKGLPAPIEKTISKTKGQPREPQVYTLPPSREEM
TKNQVSLTCLVKGFYPSDISVEWESNGQPENNYKTTTPMLDSDGSFFLYSKLTVDKSRW
QQGNVFSCSVMHEALHNHYTQKSLSLSPGK

30

SEQ ID NO: 68, wild-type IgG4

ASTKGPSVFPLAPCSRSTSESTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSS
 GLYSLSSVVTVPSSSLGKTYTCNVDPKPSNTKVDKRVESKYGPPCPSCPAPEFLGGPSV
 FLFPPKPKDTLMISRTPEVTCVVVDVSQEDPEVQFNWYVDGVEVHNAKTKPREEQFNST
 YRVVSVLTVLHQDWLNGKEYKCKVSNKGLPSSIEKTISKAKGQPREPQVYTLPPSQEEM
 5 TKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSRLTVDKSRW
 QEGNVFSCSVMHEALHNHYTQKSLSLGLK

[00603] Binding of the multispecific molecule or the heterologous molecule of the disclosure to FcγR or FcRn may be assessed on cells engineered to express each receptor using flow
 10 cytometry. In an exemplary binding assay, 2×10^5 cells per well are seeded in 96-well plate and blocked in BSA Stain Buffer (BD Biosciences, San Jose, USA) for 30 min at 4°C. Cells are incubated with a test multispecific molecule or a test heterologous molecule of the disclosure on ice for 1.5 hour at 4°C. After being washed twice with BSA stain buffer, the cells are incubated with R-PE labeled anti-human IgG secondary antibody (Jackson ImmunoResearch Laboratories)
 15 for 45 min at 4°C. The cells are washed twice in stain buffer and then resuspended in 150 μL of Stain Buffer containing 1:200 diluted DRAQ7 live/dead stain (Cell Signaling Technology, Danvers, USA). PE and DRAQ7 signals of the stained cells are detected by Miltenyi MACSQuant flow cytometer (Miltenyi Biotec, Auburn, USA) using B2 and B4 channel respectively. Live cells are gated on DRAQ7 exclusion and the geometric mean
 20 fluorescence signals are determined for at least 10,000 live events collected. FlowJo software (Tree Star) is used for analysis. Data is plotted as the logarithm of antibody concentration versus mean fluorescence signals. Nonlinear regression analysis is performed.

5.4 Glycoengineering

[00604] The ability of the multispecific molecule or the heterologous molecule of the
 25 disclosure conjugated to the Ig constant region or to the fragment of the Ig constant region to mediate ADCC can be enhanced by engineering the Ig constant region or the fragment of the Ig constant region oligosaccharide component. Human IgG1 or IgG3 are N-glycosylated at Asn297 with the majority of the glycans in the well-known biantennary G0, G0F, G1, G1F, G2 or G2F forms. Ig constant region containing proteins may be produced by non-engineered CHO cells
 30 typically have a glycan fucose content of about at least 85%. The removal of the core fucose from the biantennary complex-type oligosaccharides attached to the Ig constant region or to the

fragment of the Ig constant region enhances ADCC of the multispecific molecule or the heterologous molecule of the disclosure via improved FcγRIIIa binding without altering antigen binding or CDC activity. Such multispecific molecules or heterologous molecules can be achieved using different methods reported to lead to the successful expression of relatively high defucosylated immunoglobulins bearing the biantennary complex-type of Fc oligosaccharides such as control of culture osmolality (Konno *et al.*, (2012) *Cytotechnology* 64:249-265), application of a variant CHO line Lec13 as the host cell line (Shields *et al.*, (2002) *J Biol Chem* 277:26733-26740), application of a variant CHO line EB66 as the host cell line (Olivier *et al.*, (2010) *MAbs*;2: 405-415), application of a rat hybridoma cell line YB2/0 as the host cell line (Shinkawa *et al.*, (2003) *J Biol Chem* 278:3466-3473), introduction of small interfering RNA specifically against the α-1,6-fucosyltransferase (*FUT8*) gene (Mori *et al.*, (2004) *Biotechnol Bioeng* 88:901-908), or coexpression of β-1,4-*N*-acetylglucosaminyltransferase III and Golgi α-mannosidase II or a potent alpha-mannosidase I inhibitor, kifunensine (Ferrara *et al.*, (2006) *J Biol Chem* 281:5032-5036).

[00605] In some embodiments, the multispecific molecule or the heterologous molecule of the disclosure comprising the Ig constant region or to the fragment of the Ig constant region has a biantennary glycan structure with fucose content of about between 1% to about 15%, for example about 15%, 14%, 13%, 12%, 11%, 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2% or 1%. In some embodiments, the multispecific molecule or the heterologous molecule of the disclosure comprising the Ig constant region or to the fragment of the Ig constant region has a glycan structure with fucose content of about 50%, 40%, 45%, 40%, 35%, 30%, 25%, or 20%.

[00606] “**Fucose content**” refers to the amount of the fucose monosaccharide within the sugar chain at Asn297. The relative amount of fucose is the percentage of fucose-containing structures related to all glycostructures. These may be characterized and quantified by multiple methods, for example: 1) using MALDI-TOF of N-glycosidase F treated sample (*e.g.*, complex, hybrid and oligo- and high-mannose structures) as described in Int Pat. Publ. No. WO2008/077546; 2) by enzymatic release of the Asn297 glycans with subsequent derivatization and detection/quantitation by HPLC (UPLC) with fluorescence detection and/or HPLC-MS (UPLC-MS); 3) intact protein analysis of the native or reduced mAb, with or without treatment of the Asn297 glycans with Endo S or other enzyme that cleaves between the first and the second GlcNAc monosaccharides, leaving the fucose attached to the first GlcNAc; 4) digestion of the mAb to

constituent peptides by enzymatic digestion (*e.g.*, trypsin or endopeptidase Lys-C), and subsequent separation, detection and quantitation by HPLC-MS (UPLC-MS); 5) Separation of the mAb oligosaccharides from the mAb protein by specific enzymatic deglycosylation with PNGase F at Asn 297. The oligosaccharides thus released can be labeled with a fluorophore, separated and identified by various complementary techniques which allow: fine characterization of the glycan structures by matrix-assisted laser desorption ionization (MALDI) mass spectrometry by comparison of the experimental masses with the theoretical masses, determination of the degree of sialylation by ion exchange HPLC (GlycoSep C), separation and quantification of the oligosaccharide forms according to hydrophilicity criteria by normal-phase HPLC (GlycoSep N), and separation and quantification of the oligosaccharides by high performance capillary electrophoresis-laser induced fluorescence (HPCE-LIF).

- [00607] “**Low fucose**” or “**low fucose content**” refers to the multispecific molecule or the heterologous molecule of the disclosure comprising the Ig constant region or to the fragment of the Ig constant region with fucose content of about between 1%-15%.
- 15 [00608] “**Normal fucose**” or “**normal fucose content**” refers to the multispecific molecule or the heterologous molecule of the disclosure comprising the Ig constant region or to the fragment of the Ig constant region with fucose content of about over 50%, typically about over 80% or over 85%.

5.5 Anti-idiotypic antibodies

- 20 [00609] Anti-idiotypic antibodies are antibodies that specifically bind to the spFv of the disclosure.
- [00610] The invention also provides an anti-idiotypic antibody that specifically binds to the spFv of the disclosure.
- [00611] In some embodiments, the anti-idiotypic antibody binds to the disulfide bond in the spFv of the disclosure.
- 25 [00612] In some embodiments, the anti-idiotypic antibody binds to the antigen binding domain of the spFv of the disclosure.

5.6 Polynucleotides, vectors, host cells

- [00613] The disclosure also provides an isolated polynucleotide encoding the spFv of the disclosure.
- 30 [00614] The disclosure also provides a vector comprising the polynucleotide of the disclosure.

[00615] In some embodiments, the vector is an expression vector. Expression vectors may be plasmid vectors, viral vectors, vectors for baculovirus expression, vectors for prokaryotic expression, vectors for eukaryotic expression, transposon based vectors or any other vector suitable for introduction of the polynucleotide of the disclosure into a given cell or organism.

5 The polynucleotide encoding the spFv of the disclosure may be operably linked to control sequences in the expression vector that facilitate the expression of the spFv. Such regulatory elements may include a transcriptional promoter, sequences encoding suitable mRNA ribosomal binding sites, and sequences that control the termination of transcription and translation. Expression vectors may also include one or more nontranscribed elements such as an origin of

10 replication, other 5' or 3' flanking nontranscribed sequences, 5' or 3' nontranslated sequences (such as necessary ribosome binding sites), splice donor and acceptor sites, or selection markers. The polynucleotide may be a cDNA. The promoter driving spFv expression may be strong, weak, tissue-specific, inducible or developmental-specific promoter. Exemplary promoters that may be used are hypoxanthine phosphoribosyl transferase (HPRT), adenosine deaminase,

15 pyruvate kinase, beta-actin, human myosin, human hemoglobin, human muscle creatine, and others. In addition, many viral promoters function constitutively in eukaryotic cells and are suitable for use with the described embodiments. Such viral promoters include Cytomegalovirus (CMV) immediate early promoter, the early and late promoters of SV40, the Mouse Mammary Tumor Virus (MMTV) promoter, the long terminal repeats (LTRs) of Moloney leukemia virus,

20 Human Immunodeficiency Virus (HIV), Epstein Barr Virus (EBV), Rous Sarcoma Virus (RSV), and other retroviruses, and the thymidine kinase promoter of Herpes Simplex Virus. Inducible promoters such as the metallothionein promoter, tetracycline-inducible promoter, doxycycline-inducible promoter, promoters that contain one or more interferon-stimulated response elements (ISRE) such as protein kinase R 2',5'-oligoadenylate synthetases, Mx genes and ADAR1.

25 Vectors of the disclosure may also contain one or more Internal Ribosome Entry Site(s) (IRES). Inclusion of an IRES sequence into fusion vectors may be beneficial for enhancing expression of some proteins. Vectors of the disclosure may be circular or linear. They may be prepared to contain a replication system functional in a prokaryotic or eukaryotic host cell. Replication systems can be derived, *e.g.*, from ColE1, SV40, 2 μ plasmid, λ , bovine papilloma virus, and the

30 like. The expression vectors can be designed for either transient expression, for stable

expression, or for both. The expression vectors can be made for constitutive expression or for inducible expression.

[00616] Exemplary vectors that may be used are Bacterial: pBs, phagescript, PsiX174, pBluescript SK, pBs KS, pNH8a, pNH16a, pNH18a, pNH46a (Stratagene, La Jolla, Calif., USA); pTrc99A, pKK223-3, pKK233-3, pDR540, and pRIT5 (Pharmacia, Uppsala, Sweden). Eukaryotic: pWLneo, pSV2cat, pOG44, PXR1, pSG (Stratagene) pSVK3, pBPV, pMSG and pSVL (Pharmacia), pEE6.4 (Lonza) and pEE12.4 (Lonza). Additional vectors include the pUC series (Fermentas Life Sciences, Glen Burnie, Md.), the pBluescript series (Stratagene, LaJolla, Calif.), the pET series (Novagen, Madison, Wis.), the pGEX series (Pharmacia Biotech, Uppsala, Sweden), and the pEX series (Clontech, Palo Alto, Calif.). Bacteriophage vectors, such as λGT10, λGT11, λEMBL4, and λNM1149, λZapII (Stratagene) can be used. Exemplary plant expression vectors include pBI01, pBI01.2, pBI121, pBI101.3, and pBIN19 (Clontech). Exemplary animal expression vectors include pEUK-C1, pMAM, and pMAMneo (Clontech). The expression vector may be a viral vector, *e.g.*, a retroviral vector, *e.g.*, a gamma retroviral vector.

[00617] The disclosure also provides a host cell comprising the vector of the disclosure.

[00618] “Host cell” refers to a cell into which a vector has been introduced. It is understood that the term host cell is intended to refer not only to the particular subject cell but to the progeny of such a cell, and also to a stable cell line generated from the particular subject cell. Because certain modifications may occur in succeeding generations due to either mutation or environmental influences, such progeny may not be identical to the parent cell, but are still included within the scope of the term “host cell” as used herein. Such host cells may be eukaryotic cells, prokaryotic cells, plant cells or archeal cells. *Escherichia coli*, bacilli, such as *Bacillus subtilis*, and other enterobacteriaceae, such as *Salmonella*, *Serratia*, and various *Pseudomonas* species are examples of prokaryotic host cells. Other microbes, such as yeast, are also useful for expression. *Saccharomyces* (*e.g.*, *S. cerevisiae*) and *Pichia* are examples of suitable yeast host cells. Exemplary eukaryotic cells may be of mammalian, insect, avian or other animal origins. Mammalian eukaryotic cells include immortalized cell lines such as hybridomas or myeloma cell lines such as SP2/0 (American Type Culture Collection (ATCC), Manassas, VA, CRL-1581), NS0 (European Collection of Cell Cultures (ECACC), Salisbury, Wiltshire, UK, ECACC No. 85110503), FO (ATCC CRL-1646) and Ag653 (ATCC CRL-1580)

murine cell lines. An exemplary human myeloma cell line is U266 (ATTC CRL-TIB-196).

Other useful cell lines include those derived from Chinese Hamster Ovary (CHO) cells such as CHO-K1SV (Lonza Biologics, Walkersville, MD), CHO-K1 (ATCC CRL-61) or DG44.

- [00619]** The disclosure also provides a method of producing the spFv of the disclosure, comprising culturing the host cell of the disclosure in conditions that the spFv is produced, and recovering the spFv produced by the host cell. Methods of making scFvs and purifying them are known. Once synthesized (either chemically or recombinantly), the spFv of the disclosure may be purified according to standard procedures, including ammonium sulfate precipitation, affinity columns, column chromatography, high performance liquid chromatography (HPLC) purification, gel electrophoresis, and the like (see generally Scopes, Protein Purification (Springer- Verlag, N.Y., (1982)). The scFv of the disclosure may be substantially pure, *e.g.*, at least about 80% to 85% pure, at least about 85% to 90% pure, at least about 90% to 95% pure, or at least about 98% to 99%, or more, pure, *e.g.*, free from contaminants such as cell debris, macromolecules, *etc.* other than the subject protein
- [00620]** The polynucleotides encoding the scFv of the disclosure may be incorporated into vectors using standard molecular biology methods. Host cell transformation, culture, antibody expression and purification are done using well known methods.

5.7 Pharmaceutical compositions and Administration

- [00621]** The disclosure also provides a pharmaceutical composition comprising the spFv, the heterologous molecule comprising the spFv or the multispecific molecule comprising the spFv of the disclosure and a pharmaceutically acceptable carrier. For therapeutic use, the spFv, the heterologous molecule comprising the spFv or the multispecific molecule comprising the spFv of the disclosure may be prepared as pharmaceutical compositions containing an effective amount of the spFv, the heterologous molecule comprising the spFv or the multispecific molecule comprising the spFv of the disclosure as an active ingredient in a pharmaceutically acceptable carrier. “**Carrier**” refers to a diluent, adjuvant, excipient, or vehicle with which the spFv, the heterologous molecule comprising the spFv or the multispecific molecule comprising the spFv of the disclosure is administered. Such vehicles may be liquids, such as water and oils, including those of petroleum, animal, vegetable or synthetic origin, such as peanut oil, soybean oil, mineral oil, sesame oil and the like. For example, 0.4% saline and 0.3% glycine may be used. These solutions are sterile and generally free of particulate matter. They may be sterilized by

conventional, well-known sterilization techniques (*e.g.*, filtration). The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions such as pH adjusting and buffering agents, stabilizing, thickening, lubricating and coloring agents, *etc.* The concentration of the spFv, the heterologous molecule comprising the spFv or the multispecific molecule comprising the spFv of the disclosure in such pharmaceutical formulation may vary, from less than about 0.5%, usually to at least about 1% to as much as 15 or 20% by weight and may be selected primarily based on required dose, fluid volumes, viscosities, *etc.*, according to the mode of administration selected. Suitable vehicles and formulations, inclusive of other human proteins, *e.g.*, human serum albumin, are described, for example, in *e.g.*, Remington: The Science and Practice of Pharmacy, 21st Edition, Troy, D.B. ed., Lipincott Williams and Wilkins, Philadelphia, PA 2006, Part 5, Pharmaceutical Manufacturing pp 691-1092, See especially pp. 958-989.

[00622] The mode of administration of the spFv, the heterologous molecule comprising the spFv or the multispecific molecule comprising the spFv of the disclosure may be any suitable route such as parenteral administration, *e.g.*, intradermal, intramuscular, intraperitoneal, intravenous or subcutaneous, transmucosal (oral, intranasal, intravaginal, rectal) or other means appreciated by the skilled artisan, as well known in the art.

5.8 Process for preparing the spFv of the disclosure

[00623] The disclosure also provides a process for preparing a stabilized scFv, comprising: providing a heavy chain variable region (VH) and a light chain variable region (VL) that form an antigen binding site; providing a linker (L) that comprises or is engineered to comprise a first L Cys; engineering the VH to comprise a VH Cys at a structurally conserved surface exposed VH framework residue position; and forming a disulfide bond between the VH Cys and the first L Cys to prepare the stabilized scFv.

[00624] The disclosure also provides a process for preparing stabilized scFv, comprising: providing a VH and a VL that form an antigen binding site; providing a L that comprises or is engineered to comprise a second L Cys; engineering the VL to comprise a VL Cys at a structurally conserved surface exposed VL framework residue position; and

forming a disulfide bond between the VL Cys and the second L Cys to prepare the stabilized scFv.

[00625] The disclosure also provides a process for preparing a stabilized scFv, comprising: providing a heavy chain variable region (VH) and a light chain variable region (VL) that form an antigen binding site;

providing a linker (L) that comprises or is engineered to comprise a first L Cys and a second L Cys;

engineering the VH to comprise a VH Cys at a structurally conserved surface exposed VH framework residue position;

engineering the VL to comprise a VL Cys at a structurally conserved surface exposed VL framework residue position; and

forming a disulfide bond between the VH Cys and the first L Cys and a disulfide bond between the VL Cys and the second L Cys to prepare the stabilized scFv.

[00626] The disulfide bond is typically formed during expression of the scFv.

[00627] Any known VH/VL pair of scFv that forms an antigen binding domain may be engineered into the stabilized scFvs of the disclosure. Alternatively, antigen binding VH/VL pairs of interest may be identified *de novo* using known methods and the resulting VH/VL pairs may be engineered into spFv format.

[00628] For example, the hybridoma method of Kohler and Milstein may be used to identify VH/VL pairs that bind an antigen of interest and the resulting VH/VL pairs may be engineered as spFvs. Alternatively, transgenic animals, such as mice, rat or chicken carrying human immunoglobulin (Ig) loci in their genome may be used to generate antigen binding fragments, and are described in for example U.S. Patent No. 6,150,584, Int. Patent Publ. No.

WO1999/45962, Int. Patent Publ. Nos. WO2002/066630, WO2002/43478, WO2002/043478 and

WO1990/04036. The endogenous immunoglobulin loci in such animal may be disrupted or deleted, and at least one complete or partial human immunoglobulin locus may be inserted into the genome of the animal using homologous or non-homologous recombination, using transchromosomes, or using minigenes. Companies such as Regeneron

(http://_www_regeneron_com), Harbour Antibodies (http://_www_harbourantibodies_com),

Open Monoclonal Technology, Inc. (OMT) (http://_www_omtinc_net), KyMab (http://_www_kymab_com), Trianni (http://_www_trianni_com) and Ablexis

(http://_www_ablexis_com) may be engaged to provide human antibodies directed against a selected antigen using technologies as described above. Phage display may also be used to generate antigen binding fragments which can be engineered as spFvs.

[00629] In some embodiments, the spFv of the disclosure is humanized. In some
5 embodiments, the spFv of the disclosure is human. In some embodiments, the spFv of the disclosure is non-human..

[00630] In some embodiments, the distance between the VH Cys and the VL Cys is from about 7 Å to about 9 Å in the stabilized scFv.

[00631] In some embodiments, the VH Cys is at H3, H5, H40, H43, H46 or H105, wherein
10 residue numbering is according to Chothia.

[00632] In some embodiments, the VL Cys is at L3, L5, L39, L42, L45, L100 or L102, wherein residue numbering is according to Chothia.

[00633] In some embodiments,
the VH Cys is at H105 and the VL Cys is at L42;
15 the VH Cys is at H43 and the VL Cys is at a L100;
the VH Cys is at H3 and the VL Cys is at L3;
the VH Cys is at H3 and the VL Cys is at L5;
the VH Cys is at H3 and the VL Cys is at L39;
the VH Cys is at H3 and the VL Cys is at L42;
20 the VH Cys is at H3 and the VL Cys is at L45;
the VH Cys is at H3 and the VL Cys is at L100;
the VH Cys is at H3 and the VL Cys is at L102;
the VH Cys is at H5 and the VL Cys is at L3;
the VH Cys is at H5 and the VL Cys is at L5;
25 the VH Cys is at H5 and the VL Cys is at L39;
the VH Cys is at H5 and the VL Cys is at L42;
the VH Cys is at H5 and the VL Cys is at L45;
the VH Cys is at H5 and the VL Cys is at L100;
the VH Cys is at H5 and the VL Cys is at L102;
30 the VH Cys is at H40 and the VL Cys is at L3;
the VH Cys is at H40 and the VL Cys is at L5;

- the VH Cys is at H40 and the VL Cys is at L39;
the VH Cys is at H40 and the VL Cys is at L42;
the VH Cys is at H40 and the VL Cys is at L45;
the VH Cys is at H40 and the VL Cys is at L100;
5 the VH Cys is at H40 and the VL Cys is at L102;
the VH Cys is at H43 and the VL Cys is at L3;
the VH Cys is at H43 and the VL Cys is at L5;
the VH Cys is at H43 and the VL Cys is at L39;
the VH Cys is at H43 and the VL Cys is at L42;
10 the VH Cys is at H43 and the VL Cys is at L45;
the VH Cys is at H43 and the VL Cys is at L102;
the VH Cys is at H46 and the VL Cys is at L3;
the VH Cys is at H46 and the VL Cys is at L5;
the VH Cys is at H46 and the VL Cys is at L39;
15 the VH Cys is at H46 and the VL Cys is at L42;
the VH Cys is at H46 and the VL Cys is at L45;
the VH Cys is at H46 and the VL Cys is at L100;
the VH Cys is at H46 and the VL Cys is at L102;
the VH Cys is at H105 and the VL Cys is at L3;
20 the VH Cys is at H105 and the VL Cys is at L5;
the VH Cys is at H105 and the VL Cys is at L39;
the VH Cys is at H105 and the VL Cys is at L45;
the VH Cys is at H105 and the VL Cys is at L100; or
the VH Cys is at H105 and the VL Cys is at L102, wherein residue numbering is according to
25 Chothia.
- [00634]** In some embodiments, the L comprises a contiguous amino acid sequence derived from an immunoglobulin (Ig) hinge region.
- [00635]** In some embodiments, the Ig hinge region is derived from a human or a non-human Ig hinge region.
- 30 **[00636]** In some embodiments, the Ig hinge region is derived from the human Ig hinge region.

[00637] In some embodiments, the human Ig hinge region is an IgG1, IgG2, IgG3 or IgG4 isotype.

[00638] In some embodiments, the L comprises an amino acid sequence $C(X)_yC$ (**SEQ ID NO: 23**), wherein X is Gly, Ser, Pro, Ala, Arg, Asn, Asp, Glu, Gln, His, Ile, Leu, Lys, Phe, Thr, Trp or Tyr, and y is an integer from 1 to 3

[00639] In some embodiments, the L comprises an amino acid sequence $C(X)_yC$ (**SEQ ID NO: 24**), wherein X is Gly, Ser or Pro, and y is an integer from 1 to 3.

[00640] In some embodiments, the L comprises the amino acid sequence CPC, CGC, CSC, CPPC (**SEQ ID NO: 1**), CGPC (**SEQ ID NO: 28**), CPGC (**SEQ ID NO: 29**), CGGC (**SEQ ID NO: 30**), CSPG (**SEQ ID NO: 31**), CPSC (**SEQ ID NO: 32**), CSSC (**SEQ ID NO: 33**), CGSC (**SEQ ID NO: 34**), CSGC (**SEQ ID NO: 35**), CPPPC (**SEQ ID NO: 36**), CGPPC (**SEQ ID NO: 37**), CPGPC (**SEQ ID NO: 38**), CPPGC (**SEQ ID NO: 39**), CGGPC (**SEQ ID NO: 40**), CPGGC (**SEQ ID NO: 41**), CGGGC (**SEQ ID NO: 42**), CSPPC (**SEQ ID NO: 43**), CPSPC (**SEQ ID NO: 44**), CPPSC (**SEQ ID NO: 45**), CSSPC (**SEQ ID NO: 46**), CPSSC (**SEQ ID NO: 47**), CSSSC (**SEQ ID NO: 48**), CGSPC (**SEQ ID NO: 49**), CPGSC (**SEQ ID NO: 50**), CSGPC (**SEQ ID NO: 51**) or CPSGC (**SEQ ID NO: 52**).

[00641] In some embodiments, the L comprises from about 14 to about 19 amino acids, such as about 14, about 15, about 16, about 17, about 18 or about 19 amino acids.

[00642] In some embodiments, the L comprises the amino acid sequence $(X)_mC(X)_yC(X)_n$ (**SEQ ID NO: 25**); wherein X is Gly, Ser, Pro, Ala, Arg, Asn, Asp, Glu, Gln, His, Ile, leu, Lys, Phe Thr, Trp or Tyr, m is an integer from 6 to 9, y is an integer from 1 to 3 and n is an integer from 4 to 6.

[00643] In some embodiments, the L comprises the amino acid sequence $(X)_mC(X)_yC(X)_n$ (**SEQ ID NO: 26**); wherein X is Gly, Ser, Pro, Ala, Arg, Asn, Asp, Glu, Gln, His, Ile, Leu, Lys, Thr or Tyr, m is an integer from 6 to 9, y is an integer from 1 to 3 and n is an integer from 4 to 6.

[00644] In some embodiments, the L comprises the amino acid sequence $(X)_mC(X)_yC(X)_n$ (**SEQ ID NO: 27**); wherein X is Gly or Pro, m is an integer from 6 to 9, y is an integer from 1 to 3 and n is an integer from 4 to 6.

[00645] In some embodiments, the L comprises the amino acid sequence of **SEQ ID NOs: 2, 3, 4, 5, 6, or 7**.

[00646] In some embodiments, the stabilized spFv of the disclosure is in the VL-L-VH orientation.

[00647] In some embodiments, the stabilized spFv of the disclosure is in the VH-L-VL orientation.

- 5 **[00648]** In some embodiments,
the VH comprises Cys at H105;
the VL comprises Cys at L42;
the L comprises an amino acid sequence of SEQ ID NOs: 2, 3, 4, 5, 6 or 7; and
the scFv is in the VL-L-VH orientation.
- 10 **[00649]** In some embodiments,
the VH comprises Cys at H105;
the VL comprises Cys at L45;
the L comprises an amino acid sequence of SEQ ID NOs: 2, 3, 4, 5, 6 or 7; and
the scFv is in the VL-L-VH orientation.
- 15 **[00650]** In some embodiments,
the VH comprises Cys at H105;
the VL comprises Cys at L39;
the L comprises an amino acid sequence of SEQ ID NOs: 2, 3, 4, 5, 6 or 7; and
the scFv is in the VL-L-VH orientation.
- 20 **[00651]** In some embodiments,
the VH comprises Cys at H5;
the VL comprises Cys at L42;
the L comprises an amino acid sequence of SEQ ID NOs: 2, 3, 4, 5, 6 or 7; and
the scFv is in the VL-L-VH orientation.
- 25 **[00652]** In some embodiments,
the VH comprises Cys at H5;
the VL comprises Cys at L45;
the L comprises an amino acid sequence of SEQ ID NOs: 2, 3, 4, 5, 6 or 7; and
the scFv is in the VL-L-VH orientation.
- 30 **[00653]** In some embodiments,
the VH comprises Cys at H5;

the VL comprises Cys at L39;
the L comprises an amino acid sequence of SEQ ID NOs: 2, 3, 4, 5, 6 or 7; and
the scFv is in the VL-L-VH orientation.

[00654] In some embodiments,

- 5 the VH comprises Cys at H3;
the VL comprises Cys at L42;
the L comprises an amino acid sequence of SEQ ID NOs: 2, 3, 4, 5, 6 or 7; and
the scFv is in the VL-L-VH orientation.

[00655] In some embodiments,

- 10 the VH comprises Cys at H3;
the VL comprises Cys at L45;
the L comprises an amino acid sequence of SEQ ID NOs: 2, 3, 4, 5, 6 or 7; and
the scFv is in the VL-L-VH orientation.

[00656] In some embodiments,

- 15 the VH comprises Cys at H3;
the VL comprises Cys at L39;
the L comprises an amino acid sequence of SEQ ID NOs: 2, 3, 4, 5, 6 or 7; and
the scFv is in the VL-L-VH orientation.

[00657] In some embodiments,

- 20 the VH comprises Cys at H43;
the VL comprises Cys at L100;
the L comprises an amino acid sequence of SEQ ID NOs: 2, 3, 4, 5, 6 or 7; and
the scFv is in the VH-L-VL orientation.

[00658] In some embodiments,

- 25 the VH comprises Cys at H43;
the VL comprises Cys at L102;
the L comprises an amino acid sequence of SEQ ID NOs: 2, 3, 4, 5, 6 or 7; and
the scFv is in the VH-L-VL orientation.

[00659] In some embodiments,

- 30 the VH comprises Cys at H43;
the VL comprises Cys at L5;

the L comprises an amino acid sequence of SEQ ID NOs: 2, 3, 4, 5, 6 or 7; and
the scFv is in the VH-L-VL orientation.

[00660] In some embodiments,

the VH comprises Cys at H43;

5 the VL comprises Cys at L3;

the L comprises an amino acid sequence of SEQ ID NOs: 2, 3, 4, 5, 6 or 7; and

the scFv is in the VH-L-VL orientation.

[00661] In some embodiments,

the VH comprises Cys at H40;

10 the VL comprises Cys at L100;

the L comprises an amino acid sequence of SEQ ID NOs: 2, 3, 4, 5, 6 or 7; and

the scFv is in the VH-L-VL orientation.

[00662] In some embodiments,

the VH comprises Cys at H40;

15 the VL comprises Cys at L102;

the L comprises an amino acid sequence of SEQ ID NOs: 2, 3, 4, 5, 6 or 7; and

the scFv is in the VH-L-VL orientation.

[00663] In some embodiments,

the VH comprises Cys at H40;

20 the VL comprises Cys at L5;

the L comprises an amino acid sequence of SEQ ID NOs: 2, 3, 4, 5, 6 or 7; and

the scFv is in the VH-L-VL orientation.

[00664] In some embodiments,

the VH comprises Cys at H40;

25 the VL comprises Cys at L3;

the L comprises an amino acid sequence of SEQ ID NOs: 2, 3, 4, 5, 6 or 7; and

the scFv is in the VH-L-VL orientation.

[00665] In some embodiments,

the VH comprises Cys at H46;

30 the VL comprises Cys at L100;

the L comprises an amino acid sequence of SEQ ID NOs: 2, 3, 4, 5, 6 or 7; and

the scFv is in the VH-L-VL orientation.

[00666] In some embodiments,

the VH comprises Cys at H46;

the VL comprises Cys at L102;

5 the L comprises an amino acid sequence of SEQ ID NOs: 2, 3, 4, 5, 6 or 7; and

the scFv is in the VH-L-VL orientation.

[00667] In some embodiments,

the VH comprises Cys at H46;

the VL comprises Cys at L5;

10 the L comprises an amino acid sequence of SEQ ID NOs: 2, 3, 4, 5, 6 or 7; and

the scFv is in the VH-L-VL orientation.

[00668] In some embodiments,

the VH comprises Cys at H46;

the VL comprises Cys at L3;

15 the L comprises an amino acid sequence of SEQ ID NOs: 2, 3, 4, 5, 6 or 7; and

the scFv is in the VH-L-VL orientation.

[00669] In some embodiments, the L comprises the amino acid sequence of **SEQ ID NO: 3**.

[00670] In some embodiments, the L comprises the amino acid sequence of **SEQ ID NO: 6**.

[00671] In some embodiments, the L comprises the amino acid sequence of **SEQ ID NO: 7**.

20 **[00672]** In some embodiments, the stabilized spFv of the disclosure binds an antigen with comparable affinity when compared to a parent scFv that is devoid of the disulfide bonds.

[00673] The disclosure also provides a process for preparing a stabilized scFv, comprising providing a polynucleotide encoding a VH, a L and a VL, wherein

the VH comprises Cys at H105 and the VL comprises Cys at L42;

25 the VH comprises Cys at H43 and the VL comprises Cys at a L100;

the VH comprises Cys at H3 and the VL comprises Cys at L3;

the VH comprises Cys at H3 and the VL comprises Cys at L5;

the VH comprises Cys at H3 and the VL comprises Cys at L39;

the VH comprises Cys at H3 and the VL comprises Cys at L42;

30 the VH comprises Cys at H3 and the VL comprises Cys at L45;

the VH comprises Cys at H3 and the VL comprises Cys at L100;

- the VH comprises Cys at H3 and the VL comprises Cys at L102;
the VH comprises Cys at H5 and the VL comprises Cys at L3;
the VH comprises Cys at H5 and the VL comprises Cys at L5;
the VH comprises Cys at H5 and the VL comprises Cys at L39;
5 the VH comprises Cys at H5 and the VL comprises Cys at L42;
the VH comprises Cys at H5 and the VL comprises Cys at L45;
the VH comprises Cys at H5 and the VL comprises Cys at L100;
the VH comprises Cys at H5 and the VL comprises Cys at L102;
the VH comprises Cys at H40 and the VL comprises Cys at L3;
10 the VH comprises Cys at H40 and the VL comprises Cys at L5;
the VH comprises Cys at H40 and the VL comprises Cys at L39;
the VH comprises Cys at H40 and the VL comprises Cys at L42;
the VH comprises Cys at H40 and the VL comprises Cys at L45;
the VH comprises Cys at H40 and the VL comprises Cys at L100;
15 the VH comprises Cys at H40 and the VL comprises Cys at L102;
the VH comprises Cys at H43 and the VL comprises Cys at L3;
the VH comprises Cys at H43 and the VL comprises Cys at L5;
the VH comprises Cys at H43 and the VL comprises Cys at L39;
the VH comprises Cys at H43 and the VL comprises Cys at L42;
20 the VH comprises Cys at H43 and the VL comprises Cys at L45;
the VH comprises Cys at H43 and the VL comprises Cys at L102;
the VH comprises Cys at H46 and the VL comprises Cys at L3;
the VH comprises Cys at H46 and the VL comprises Cys at L5;
the VH comprises Cys at H46 and the VL comprises Cys at L39;
25 the VH comprises Cys at H46 and the VL comprises Cys at L42;
the VH comprises Cys at H46 and the VL comprises Cys at L45;
the VH comprises Cys at H46 and the VL comprises Cys at L100;
the VH comprises Cys at H46 and the VL comprises Cys at L102;
the VH comprises Cys at H105 and the VL comprises Cys at L3;
30 the VH comprises Cys at H105 and the VL comprises Cys at L5;
the VH comprises Cys at H105 and the VL comprises Cys at L39;

the VH comprises Cys at H105 and the VL comprises Cys at L45;
the VH comprises Cys at H105 and the VL comprises Cys at L100; or
the VH comprises Cys at H105 and the VL comprises Cys at L102, wherein residue numbering
is according to Chothia;

- 5 the L comprises an amino acid sequence of SEQ ID NOs: 2, 3, 4, 5, 6 or 7; and
expressing the polynucleotide in a host cell to produce the stabilized scFv.

[00674] In some embodiments, the host cell is a prokaryotic cell.

[00675] In some embodiments, the host cell is an eukaryotic cell.

- [00676] The following examples are provided to further describe some of the embodiments
10 disclosed herein. The examples are intended to illustrate, not to limit, the disclosed
embodiments.

6. EXAMPLES

6.1 Example 1: Design of stabilized scFvs

- [00677] A monoclonal antibody (mAb) recognizes its target antigen through the two variable
15 domains VL and VH. A single chain Fv (scFv) was first designed by Bird *et al.* (1988) *Science*
242:423-426 (1988) as a genetic fusion of VL and VH with a flexible linker in either VL-linker-
VH or VH-linker-VL orientations. The flexible linker is typically three or four repeats of
glycine-serine linker such as (GGGGS)_n; n=1-4 (SEQ ID NO: 2, SEQ ID NO: 53, SEQ ID
NO: 54, SEQ ID NO: 55). A scFv recapitulates the antigen binding specificity and largely the
20 affinity of its parental mAb. These scFv molecules have found wide applications as
detection/diagnostics reagents or as building blocks for making more elaborate molecules such as
bi-, multi-specific therapeutics (Brinkmann and Kontermann (2017) *MAbs* 9: 182-212) or in
CAR-T therapeutics (Gross *et al.*, (1989), *Transplant Proc* 21(1 Pt 1): 127-130; Porter *et al.*,
(2011) *J Cancer* 2: 331-332; Porter *et al.*, (2011) *N Engl J Med* 365: 725-733).

- 25 [00678] One of the challenges of scFv molecules is the low stability and tendencies to
aggregate (reviewed in Worn and Pluckthun (2001) *J Mol Biol* 305: 989-1010; Rothlisberger *et al.*,
(2005) *J Mol Biol* 347: 773-789). A number of strategies have been attempted to improve
their properties (Arnd *et al.*, (2001) *J Mol Biol* 312: 221-228; Monsellier *et al.*, (2006) *J Mol Biol*
362: 580-593; Zhao *et al.*, (2010) *Int J Mol Sci* 12: 1-11; Perchiacca and Tessier (2012) *Annu*
30 *Rev Chem Biomol Eng* 3: 263-286; Asial *et al.*, (2013) *Nat Commun* 4: 2901; Gil and Schrum
(2013) *Adv Biosci Biotechnol* 4: 73-84; Tiller and Tessier (2015) *Annu rev Biomed Eng* 17: 191-

216) These strategies include introducing disulfide bonds between VL/VH domains, improving VL/VH domain stability and/or interface interactions using different experimental methods, using additional dimerization motifs and others. A key difficulty is that most of these strategies are often specific to the VH/VL pair and cannot be readily transferred to other VH/VL pairs.

5 Sometimes, engineering may have negative impact on the VL/VH structure and the scFv property. Recently, Zhang *et al.* introduced a disulfide between position 44 of VH and position 100 of VL of a anti-aflatoxin B₁ scFv (H4) and successfully achieved significant stabilization of the scFv while preserving its binding affinity (Zhao *et al.*, (2010) *Int J Mol Sci* 12: 1-11). However, because of the distance and angle restraints between the chosen two positions, the
10 inter-VL/VH disulfide, if applied to other VL/VH pairs, may restrict/distort the relative orientation between the two domains, which is often required for binding.

[00679] The interface between the heavy and light chains of the Fab fragment comprises VH/VL and CH1/CL interactions. The two independent sets of interactions provide synergistic stabilization effects. In addition, the V/C junction also contributes some stabilization effects. In
15 comparison, in a scFv the VH/VL interface is maintained by the VH/VL interactions only. The linker, being designed to be flexible and non-restrictive except in cases where the length is designed to be so short to promote inter-scFv interactions for dimer and oligomer formation, only loosely couples the two together. It is known that the length and nature of the linker, when long enough, contributes little to the stability of the scFv.

20 6.1.1 “Stapling” design

[00680] The purpose of the work was to design and generate stabilized scFvs by restraining but not negatively impacting the relative movements between the VH and the VL forming the scFv. This was accomplished by stabilizing the scFv by engineering disulfide bonds between the VH and the linker and between the VL and the linker. The restraints (*i.e.*, disulfide bonds), when
25 properly positioned, would then play the role of the synergistic effects afforded by the CH1/CL and V/C interactions discussed above. To this end, two structurally conserved surface exposed framework positions (anchor points) were identified, one each on VH and VL, which were non-overlapping with the typical predicted antigen binding site, and mutated into cysteine (Cys) residues. Two positions were subsequently chosen in the flexible linker for Cys positions.
30 When the distances and locations between the linker Cys residues were designed in a manner that facilitated formation of disulfide bonds between the linker Cys and each anchor point, the

VH and the VL would be tethered more tightly together when compared to tethering in the absence of the disulfide bonds. This scheme is depicted in **FIG. 1** with an exemplary linker containing CPPC sequence (**SEQ ID NO: 1**). The concept of forming disulfide bonds between the flexible linker and anchor points is herein referred to as “stapling”. The resulting “stapled” scFv molecules are herein referred as spFv (“stapled Fv”).

6.1.2 Choice of the anchor points, design of staple sequences and linkers

[00681] For the stapling scheme to be widely applicable, it is important that the anchor points be structurally conserved, exposed on surface of both VL and VH and whose mutation to Cys residue will not impact folding of VL and VH or binding to antigens. The distances and geometry of the anchor points and the N and C termini of the VL and VH domains are also important considerations for proper disulfide formation.

[00682] The anchor points were chosen separately for spFv in the VL-linker-VH and VH-linker-VL orientation. For the VL-linker-VH orientation, Chothia position 42 in the VL and Chothia position 105 in the VH were chosen as anchor points. A graphical illustration of the chosen anchor points for the spFv in the VL-linker-VH orientation is shown in **FIG. 2** within the Fv of a human germline antibody (pdb ID 5I19, GLk1 hereafter). In GLk1, VL Chothia position 42 is lysine (K) and VH Chothia position 105 glutamine (Q). For the VH-linker-VL orientation, Chothia position 100 in the VL and Chothia position 43 in the VH were chosen as anchor points. **FIG. 3** shows the graphical illustration of the chosen anchor points for the spFv in the VH-linker-VL orientation within the Fv of a human germline antibody (pdb ID 5I19, GLk1). In GLk1, VL Chothia position 100 is glutamine (Q) and VH Chothia position 43 is lysine (K). The chosen anchor points were structurally conserved, and the geometry was very similar in antibodies containing either kappa or lambda light chains. The distances between the pairs of the anchor points ranged from approximately 7 Å (for the VL-linker-VH orientation) to approximately 9 Å (for the VH-linker-VL orientation).

[00683] The staple sequences embedded within the linker connecting the VH and the VL were designed to be of similar length with the distances between the anchor points in the spFv. As an initial example of the staple sequence, CPPC (**SEQ ID NO: 1**) was chosen as a possible staple sequence, partly because this sequence occurs natively in human IgG1 hinge as well as in some rodent IgGs. The structures of the hinges of human and mouse IgG molecules demonstrated that the C β (cys1)-C β (cys2) distances in a mouse IgG hinge (**FIG. 4**) and a human IgG (**FIG. 5**)

ranged from about 7 Å to 9 Å. As this range was very similar to the distances between the two anchor points in both VL-linker-VH and VH-linker-VL orientations, the CPPC (**SEQ ID NO: 1**) staple sequence had the potential to provide correct geometry for stapling, *i.e.*, forming proper disulfide bonds efficiently and correctly to the anchor points. In general, the staple sequences were designed to have two Cys residues. For proper stapling, the N-terminal Cys of the staple sequence formed disulfide bond with the spFv N-terminal domain anchor point and the C-terminal Cys of the staple sequence formed a disulfide bond with the spFv C-terminal domain anchor point.

[00684] The linker connecting the VH and the VL was thus designed to comprises the staple sequence and connecting sequences both N-terminal and C-terminal to the staple sequence to extend the linker to provide sufficient linker length to allow intrachain folding of the VH and the VL and to facilitate proper positioning of the staple sequence.

[00685] In the VL-linker-VH design, the distances between the VL anchor point (K42), VH anchor point (Q105), C-terminus of the VL (K107) and the N-terminus of the VH (Q1) are shown in **FIG. 2**. In the VH-linker-VL design, the distances between the VL anchor point (Q100), the VH anchor point (K43), the C-terminus of the VH (S114) and the N-terminus of the VL (D1) are shown in **FIG. 3**. Modeling suggested that these distances can be spanned by linker lengths of about 14-19 residues, in which the staple sequence of 4 residues is flanked by a N-terminal linker extension of about 6-9 residues and a C-terminal linker extension of about 4-6 residues. The designed linker length could thus be expressed as $n+4+m$, in which $n=6-9$ residues and $m=4-6$ residues, and 4 indicates the length of the CPPC (**SEQ ID NO: 1**) staple sequence. The n and m residues could be glycine or serine, or other amino acid residues. These linker lengths are expected to be long and flexible enough to allow stapling but too short to allow scrambling.

6.2 Example 2: Generation and characterization of spFvs

[00686] In order to assess the stapling designs, three human antibodies were chosen to generate scFv and corresponding spFvs: two antibodies with kappa light chains (GLk1 and GLk2) from the synthetic phage antibody libraries (Shi *et al.*, (2010) *J Mol Biol* 397:385-396) and a lambda-containing antibody (CAT2200) obtained from a publication (Gerhardt *et al.* (2009) *J Mol Biol* 394:905-921). For CAT2200, a T28G mutation was introduced in the parental VH to generate a variant (CAT2200a) to reduce some of its interactions with its target, IL-17. In addition, a S42Q

mutation (Chothia) was engineered into the parental CAT2200 VL and paired with the T28G VH to generate CAT2200b. The amino acid sequences of the VL and the VH domains of GLk1, GLk2, CAT2200a and CAT2200b are shown in **FIG. 6** and **FIG. 7**, respectively. The VH domain amino acid sequence is identical between BAT2200a and CAT2200b. GLk1VH is closest to human IGHV2-23*01, GLk2VH to human IGHV5-51, CAT2200VH to human IGHV2-23*01. GLk1VL is closest human IGKV1-39*01, GLk2VL to human IGKV3-20*01, and CAT2200VL to human IGLV6-57*01.

[00687] All scFv and spFv molecules were generated and expressed in both VL-linker-VH and VH-linker-VL orientations. For the scFv constructs, a standard (GGGGS)₄ (**SEQ ID NO: 2**) linker was used. For the spFv, different linker lengths within the **n** and **m** ranges above were used. For GLk1 spFv, 9-4-5 linkers were used for both orientations. For GLk2 spFv, the 9-4-5 and 6-4-6 linker lengths were used for the VL-VH and VH-VL orientations, respectively. For CAT2200a spFv, VL-VH molecules were made with the 8-4-4 and 9-4-4 linkers, respectively, and CAT2200b spFv VH-VL was made with the 9-4-4 linker. **Table 4** shows the generated molecules and their linker sequences. **Table 5** shows the amino acid sequences of the generated molecules.

Table 4.

Molecule name (SEQ ID NO:)	Linker type	Linker amino acid sequence	Linker SEQ ID NO:
GLk1 scFv VL-VH (8)	4x G4S	GGGSGGGSGGGSGGGGS	2
GLk1 spFv VL-VH (9)	9+4+5	GGGSGGSGGCPPCGGSGG	3
GLk1 scFv VH-VL (10)	4x G4S	AGGGSGGGSGGGSGGGGS	2
GLk1 spFv VH-VL (11)	9+4+5	GGGSGGSGGCPPCGGSGG	3
GLk2 scFv VL-VH (12)	4x G4S	GGGSGGGSGGGSGGGGS	2
GLk2 spFv VL-VH (13)	9+4+5	GGGSGGSGGCPPCGGSGG	3
GLk2 scFv VH-VL (14)	4x G4S	GGGSGGGSGGGSGGGGS	2
GLk2 spFv VH-VL (15)	6+4+6	GGGSGGCPPCGGSGG	4
CAT2200a scFv VL-VH (16)	4x G4S	GGGSGGGSGGGSGGGGS	2

Molecule name (SEQ ID NO:)	Linker type	Linker amino acid sequence	Linker SEQ ID NO:
CAT2200a spFv VL-VH (17)	8+4+4	GGSGGSGGCPPCGSGG	5
CAT2200b scFv VL-VH (18)	4x G4S	GGGGSGGGSGGGSGGGGS	2
CAT2200a spFv VL-VH (19)	9+4+4	GGSGGSGGCPPCGSGG	6
CAT2200a scFv VH-VL (20)	4x G4S	GGGGSGGGSGGGSGGGGS	2
CAT2200b spFv VH-VL (21)	9+4+4v2	GGSGGSGGSCPPCGGGG	7

Table 5.

Molecule name	Protein sequence	SEQ ID NO:
GLk1 scFv VL-VH	DIQMTQSPSSLSASVGDRVITTCRASQSISSYLNWYQQKPGK APKLLIYAASSLQSGVPSRFSGSGSGTDFTLTISLQPEDFAT YYCQQSYSTPLTFGQGTKVEIKRGGGSGGGSGGGSGG GGSEVQLLESGLLVQPGGSLRLSCAASGFTFSSYAMSWVR QAPGKGLEWVSAISGSGGSTYYADSVKGRFTISRDNKNTL YLQMNSLRAEDTAVYYCAKYDGIYGELDFWGQGTLLTVSS GHHHHHH	8
GLk1 spFv VL-VH	DIQMTQSPSSLSASVGDRVITTCRASQSISSYLNWYQQKPGC APKLLIYAASSLQSGVPSRFSGSGSGTDFTLTISLQPEDFAT YYCQQSYSTPLTFGQGTKVEIKRGGGSGGSGGCPPCGSGG EVQLLESGLLVQPGGSLRLSCAASGFTFSSYAMSWVRQAP GKGLEWVSAISGSGGSTYYADSVKGRFTISRDNKNTLYLQ MNSLRAEDTAVYYCAKYDGIYGELDFWGCGTLTVSSGHH HHHH	9
GLk1 scFv VH-VL	EVQLLESGLLVQPGGSLRLSCAASGFTFSSYAMSWVRQAP GKGLEWVSAISGSGGSTYYADSVKGRFTISRDNKNTLYLQ MNSLRAEDTAVYYCAKYDGIYGELDFWGQGTLLTVSSAGG GGSGGGSGGGSGGGSGGGSDIQMTQSPSSLSASVGDRVITTC RASQSISSYLNWYQQKPGKAPKLLIYAASSLQSGVPSRFSGS GSGTDFTLTISLQPEDFATYYCQQSYSTPLTFGQGTKVEIKR GHHHHHH	10

Molecule name	Protein sequence	SEQ ID NO:
GLk1 spFv VH-VL	EVQLLES GGGLVQPGGSLRLSCAASGFTFSSYAMSWVRQAP GCGLEWVSAISGSGGSTYYADSVKGRFTISRDN SKNTLYLQ MNSLRAEDTAVYYCAKYDGIYGELDFWGQGT LVTVSSGGG SGGSGGCPPCGSGGDIQMTQSPSSLSASV GDRVITITCRASQ SISSYLNWYQQKPGKAPKLLIYAASSLQSGVPSRFSGSGSGT DFTLTISSLQPEDFATYYCQQSYSTPLTFGCGTKVEIKRGHH HHHH	11
GLk2 scFv VL-VH	EIVLTQSPGTL SLSPGERATLSCRASQSVSSSYLAWYQQKPG QAPRLLIYGASSRATGIPDRFSGSGSGTDFTLTISRLEPEDFA VYYCQQDYGFPWTFGQGTKVEIKGGGSGGGGSGGGGSG GGGSEVQLVQSGAEVKKPGESLKISCKGSGYSFTSYWISWV RQMPGKGLEWMGIIDPSDSDTRYSPSFQGGQVTISADKSISTA YLQWSSLKASDTAMYYCARGDGSTDLDYWGQGT LVTVSS GHHHHHH	12
GLk2 spFv VL-VH	EIVLTQSPGTL SLSPGERATLSCRASQSVSSSYLAWYQQKPG CAPRLLIYGASSRATGIPDRFSGSGSGTDFTLTISRLEPEDFAV YYCQQDYGFPWTFGQGTKVEIKGGGSGGGSGGCPPCGSGG EVQLVQSGAEVKKPGESLKISCKGSGYSFTSYWISWVRQMP GKGLEWMGIIDPSDSDTRYSPSFQGGQVTISADKSISTAYLQW SSLKASDTAMYYCARGDGSTDLDYWGCGT LVTVSSGHHH HHH	13
GLk2 scFv VH-VL	EVQLVQSGAEVKKPGESLKISCKGSGYSFTSYWISWVRQMP GKGLEWMGIIDPSDSDTRYSPSFQGGQVTISADKSISTAYLQW SSLKASDTAMYYCARGDGSTDLDYWGQGT LVTVSSGGGGG GGGGSGGGGSGGGGSEIVLTQSPGTL SLSPGERATLSCRASQ SVSSSYLAWYQQKPGQAPRLLIYGASSRATGIPDRFSGSGSG TDFTLTISRLEPEDFAVYYCQQDYGFPWTFGQGTKVEIKGH HHHHH	14
GLk2 spFv VH-VL	EVQLVQSGAEVKKPGESLKISCKGSGYSFTSYWISWVRQMP GCGLEWMGIIDPSDSDTRYSPSFQGGQVTISADKSISTAYLQW SSLKASDTAMYYCARGDGSTDLDYWGQGT LVTVSSGGGGG	15

Molecule name	Protein sequence	SEQ ID NO:
	GCPPCGGGSGGEIVLTQSPGTLSPGERATLSCRASQSVSSSYLAWYQQKPGQAPRLLIYGASSRATGIPDRFSGSGSGTDFTLTISRLEPEDFAVYYCQQDYGFPWTFGCGTKVEIKGHHHHHH	
CAT2200a scFv VL-VH	NFMLTQPHSVSESPGKTVTISCTRSSGSLANYVQWYQQRPGSSPTIVIFANNQRPSGVPDRFSGSIDSSNSASLTISGLKTED EADYYCQTYDPYSVVFGGGTKLTVLGGGGSGGGSGGGGS GGGGSEVQLLESGGGLVQPGGSLRLSCAASGFGFSSYAMS WVRQAPGKGLEWVSAISGSGGSTYYADSVKGRFTISRDN SKNTLYLQMNSLRAEDTAVYYCARDLIHGVTRN WGQGT LVT VSSGHHHHHH	16
CAT2200a spFv VL-VH	NFMLTQPHSVSESPGKTVTISCTRSSGSLANYVQWYQQRPGCSPTIVIFANNQRPSGVPDRFSGSIDSSNSASLTISGLKTED EADYYCQTYDPYSVVFGGGTKLTVLGGSGSGGCPPCGSG GEVQLLESGGGLVQPGGSLRLSCAASGFGFSSYAMSWVRQ APGKGLEWVSAISGSGGSTYYADSVKGRFTISRDN SKNTLY LQMNSLRAEDTAVYYCARDLIHGVTRN WGCGTLTVSSGH HHHHH	17
CAT2200b scFv VL-VH	NFMLTQPHSVSESPGKTVTISCTRSSGSLANYVQWYQQRPGQSPTIVIFANNQRPSGVPDRFSGSIDSSNSASLTISGLKTED EADYYCQTYDPYSVVFGGGTKLTVLGGGGSGGGSGGGGS GGGGSEVQLLESGGGLVQPGGSLRLSCAASGFGFSSYAMS WVRQAPGKGLEWVSAISGSGGSTYYADSVKGRFTISRDN SKNTLYLQMNSLRAEDTAVYYCARDLIHGVTRN WGQGT LVT VSSGHHHHHH	18
CAT2200a spFv VL-VH	NFMLTQPHSVSESPGKTVTISCTRSSGSLANYVQWYQQRPGCSPTIVIFANNQRPSGVPDRFSGSIDSSNSASLTISGLKTED EADYYCQTYDPYSVVFGGGTKLTVLGGSGSGGCPPCGS GGEVQLLESGGGLVQPGGSLRLSCAASGFGFSSYAMSWVR QAPGKGLEWVSAISGSGGSTYYADSVKGRFTISRDN SKNTLY LQMNSLRAEDTAVYYCARDLIHGVTRN WGCGTLTVSSG HHHHHH	19

Molecule name	Protein sequence	SEQ ID NO:
CAT2200a scFv VH-VL	EVQLLES GGGLVQPGGSLRLSCAASGFGFSSYAMSWVRQAP GKGLEWVSAISGSGGSTYYADSVKGRFTISRDN SKNTLYLQ MNSLRAEDTAVYYCARDLIHGVTNRNWGQGTLVTVSSGGG GSGGGSGGGGSGGGGSNFMLTQPHSVSESPGKTVTISCTR SSGSLANYVQWYQQRPGSSPTIVIFANNQRPSGVPDRFSGS IDSSSNSASLTISGLKTEDEADYYCQTYDPYSVVFVGGGTKLT VLGHHHHHHH	20
CAT2200b spFv VH-VL	EVQLLES GGGLVQPGGSLRLSCAASGFGFSSYAMSWVRQAP GCGLEWVSAISGSGGSTYYADSVKGRFTISRDN SKNTLYLQ MNSLRAEDTAVYYCARDLIHGVTNRNWGQGTLVTVSSGGGS GGGSGCPPCGGGGNFMLTQPHSVSESPGKTVTISCTRSSGSL ANYVQWYQQRPGQSPTIVIFANNQRPSGVPDRFSGSIDSS NSASLTISGLKTEDEADYYCQTYDPYSVVFVGGGCKLTVLGH HHHHH	21

[00688] All scFv and spFv molecules except CAT2200a scFv VL-VH were cloned into a CMV promoter driven mammalian expression vector. These constructs were transfected into Expi293 cells using manufacturer protocols and cells were cultured for 5 days. Each Protein was purified from the clarified supernatant on 1 ml His-TRAP HP columns (GE Healthcare) via an AKTAPRESS system (GE Healthcare). The column was prepared with a gradient of 0-100% Elution Buffer (Wash Buffer: 50 mM Tris, pH 7.5, 500 mM NaCl, 20 mM Imidazole; Elution Buffer: 50 mM Tris, pH 7.5, 500 mM NaCl, 500 mM Imidazole) to remove loosely bound nickel and then re-equilibration in DPBS. The cleared supernatant was first adjusted to 50 mM Tris, pH 7.5 and 20 mM imidazole and then loaded over 1 mL HisTRAP HP column at 4°C 0.8 mL/min. The column was then washed with PBS until stable baseline was obtained. Then the column was further washed with 20 CV of Wash Buffer, eluted with Elution buffer into a single injection loop and desalted in 1X DPBS over 26/10 HiPrep Desalting Column and fractions collected. Fractions containing the purified protein were then pooled and concentrated. The Glk2 scFv and spFv proteins were dialyzed into DPBS for thermal stability measurements (DSC and NanoDSF) and 25 mM Tris, pH 7.5 and 100 mM NaCl for other studies. The other scFv and spFv proteins were dialyzed in 25 mM MES, pH 6.0 and 100 mM NaCl.

[00689] CAT2200a scFv VL-VH was purchased from a vendor. Concentration was 0.77 mg/mL in DPBS, pH 7.2. A mutant of IL-17 (12-132 with K70Q A132Q C106S mutations, IL-17 hereafter for simplicity (**SEQ ID NO: 22**) was purchased from Accelagen (CA). The protein was refolded from *E. coli* inclusion body following their proprietary refolding protocol and provided at 1.50 mg/mL in 20 mM NaCl, 20 mM MES, pH 6.0.

SEQ ID NO: 22 (IL-17A mutant)

MNSEDKNFPRTVMVNLNIHNRNTNTNPKRSSDYNNRSTSPWNLHRNEDPERYPSPVIWE
AQCRHLGCINADGNVDYHMNSVPIQQEILVLRREPPHSPNSFRLEKILVSVGCTCVTPIV
HHVQ

6.2.1 Thermal stability of the generated scFv and spFv molecules

[00690] The thermal stability of the scFv and spFv molecules was investigated by differential thermal calorimetry (DSC). The scFv and spFv proteins were dialyzed overnight against 1x DPBS (Gibco) for GLk1 and CAT2200a/CAT2200b or MES (25 mM MES, pH 6.0, 100 mM NaCl) for GLk2. Dialysis buffer was then 0.22 micron filtered and used as the reference solution and for buffer-buffer blanks in the DSC experiment. Proteins were diluted to ~ 0.5 mg/mL in the filtered buffer and 400 μ L of each protein or buffer sample was loaded into a 96-deepwell plate (MicroLiter Analytical Supplies, 07-2100) and kept at 4°C in the autosampler drawer over the course of the experiment. A MicroCal Capillary DSC with Autosampler (Malvern) was used to perform the DSC experiments. DSC scans were performed from 25-95 °C at a 60°C/h scan rate with no sample rescans. No feedback was selected and the filtering period was set at 15 s. After each sample, cells were cleaned with a 10% Contrad-70 solution and a buffer-buffer blank was run. Data analysis was performed using Origin 7.0 with the MicroCal VP-Capillary DSC Automated Analysis add-on (Malvern). The baseline range and type were manually chosen and then subtracted. The previous buffer blank was subtracted from the sample curve followed by concentration-dependent normalization. The thermal melting profiles were analyzed using both 2-state and non-2-state transitions. Two-state fits (one transition) agreed poorly with the experimental curves. Thus, with two transitions (T_{m1} and T_{m2}) were calculated by manually performing non-2-state fits. The T_m data are reported in **Table 6**. The DSC profiles of all scFv and spFv proteins exhibited a skewness that could only be fitted with non-2-state transitions. Thus, for each scFv or spFv, two transitions (T_{m1} and T_{m2}) were reported (**Table 6**). Most likely, these two transitions correspond to the melting T_m of the VL and VH domains,

respectively. In general, upon comparison, the differences between scFv and spFv for either Tm1 or Tm2, there is a roughly 10°C increase by stapling, regardless of the Tm of the starting scFv. There is only one exception, *i.e.*, the case of GLk2 scFv and spFv (VH-VL orientation) difference, at ~7 °C. This is likely due to the shorter 6+4+6 linker which may have caused slight strain in the stapling geometry. The fact that $\Delta Tm1$ (VL) and $\Delta Tm2$ (VH) were nearly identical suggests that stapling lead to stabilization of the domains themselves in addition to strengthening the VL/VH interactions. Alternatively, stronger VH/VL interactions transmits the stabilization effects into stabilization of the VL/VH domains. In summary, stapling as described in this work significantly increases the stability of scFv.

10 **Table 6.**

Molecule name (SEQ ID NO:)	Linker type	DSC Stability							
		Tm1 (°C)	SD(Tm1) (°C)	Tm2 (°C)	SD(Tm2) (°C)		$\Delta Tm1$ (°C)	$\Delta Tm2$ (°C)	$\Delta(Tm1-Tm2)$
GLk1 scFv VL-VH (8)	4x G4S	68.9	0.2	72.1	0.0		9.7	9.0	3.3
GLk1 spFv VL-VH (9)	9+4+5	78.5	0.2	81.1	0.0				2.6
GLk1 scFv VH-VL (10)	4x G4S	67.7	0.3	70.9	0.0		10.0	9.2	3.3
GLk1 spFv VH-VL (11)	9+4+5	77.7	0.2	80.1	0.1				2.4
GLk2 scFv VL-VH (12)	4x G4S	56.6	0.2	58.6	0.1		10.9	10.5	2.0
GLk2 spFv VL-VH (13)	9+4+5	67.6	0.3	69.2	0.1				1.6
GLk2 scFv VH-VL (14)	4x G4S	56.4	0.1	58.3	0.1		7.3	7.1	1.9
GLk2 spFv VH-VL (15)	6+4+6	63.7	0.3	65.4	0.1				1.7
CAT2200a scFv VL-VH (16)	4x G4S								
CAT2200a spFv VL-VH (17)	8+4+4								
CAT2200b scFv VL-VH (18)	4x G4S	54.7	0.3	57.6	0.0		12.0	11.7	2.9
CAT2200a spFv VL-VH (19)	9+4+4	66.7	0.3	69.3	0.0				2.6
CAT2200a scFv VH-VL (20)	4x G4S	52.6	0.3	56.4	0.0		12.9	11.4	3.8
CAT2200b spFv VH-VL (21)	9+4+4	65.5	0.3	67.9	0.0				2.4
$\Delta Tm1$ (°C): Difference in Tm1 (spFv) and Tm1 (scFv)									
$\Delta Tm2$ (°C): Difference in Tm2 (spFv) and Tm2 (scFv)									
$\Delta(Tm1-Tm2)$: Difference of Tm2 and Tm1 of an scFv or spFv									

[00691] CAT2200 spFvs were tested for their binding to IL-17. The binding was comparable when compared to the CAT2200 scFvs.

6.3 Example 3: Verification of correct stapling by crystallization of generated scFv and spFv molecules

5 [00692] Proteins were concentrated in their respective buffers: GLk1 spFv VL-VH to 8.67 mg/ml in 25 mM MES, pH 6.0, 100 mM NaCl; GLk1 spFv VH-VL to 5 mg/ml in 25 mM MES, pH 6.0, 100 mM NaCl; GLk2 spFv VH-VL to 8.66 mg/ml in 25 mM Tris, pH7.5, 100 mM NaCl; cat2200b spFv VH-VL to 25 mM MES, pH 6.0, 100 mM NaCl. Crystallization was set up for each protein in sitting drop format in Corning 3550 crystal trays using a Mosquito robot. Each well contains 100 nl of protein and 100 nl of reservoir solution and incubated against 70 μ l of reservoir at 20 °C. The reservoir solutions are IH1 and IH2 custom conditions as well as PEG Ion Screen HT (Hampton Research). Some initial conditions were refined by varying reservoir components in optimization attempts. Diffraction quality crystals were obtained for some of scFv and spFv proteins. **Table 7** shows the summary of the conditions used. Crystals were
10 soaked for a few seconds in the mother liquor supplemented with 20% glycerol and flash frozen in liquid N₂. X-ray data were collected at IMCA-CAT Beamline 17ID at Argonne National Lab.

Table 7.

Molecule name	Protein	crystallization Condition	Disulfides?	Resolution (Å)
GLk1 spFv VL-VH	8.67 mg/mL, 25 mM MES, pH 6.0, 100 mM NaCl	0.1 M Na Acet 4.5 pH, 18 %w/v PEG 3350, 1 M LiCl	yes	1.65
Glk1 spFv VH-VL	5 mg/mL, 25 mM MES, pH 6.0, 100 mM NaCl	0.1 M MES 6.5 pH, 5 %v/v PEG 400, 0.75 M (NH ₄) ₂ SO ₄	yes	2.10
GLk2 spFv VH-VL	8.66 mg/mL , 25 mM Tris, pH 7.5, 100 mM NaCl	0.1 M Na Acet 4.5 pH, 0.75 M (NH ₄) ₂ SO ₄	yes	1.50
CAT2200b spFv VH-VL	4.91 mg/mL, 25 mM MES, pH 6.0 100 mM NaCl	0.1 M Na Acet 4.5 pH, 1.5 M (NH ₄) ₂ SO ₄	yes	2.40

Molecule name	Protein	crystallization Condition	Disulfides?	Resolution (Å)
CAT2200a scFv VL-VH/IL-17 complex	2.69 mg/mL, 20 mM HEPES, pH 7.5, 50 mM NaCl	0.1 M Tris, pH 8.5, 18% PEG3K, 0.2M LiSO4	non-stapled	2.30
CAT2200a spFv VL-VH/IL-17 complex	6.0 mg/ml, 20 mM HEPES, pH 7.5, 50 mM NaCl	15.5 % PEG 3350, 0.4 M NaH2PO4	yes	2.00

6.3.1 Crystallization of CAT2200a scFv VL-VH and CAT2200a spFv VL-VH in complex with IL-17

[00693] The IL-17/ CAT2200a scFv VL-VH complex was generated by mixing 333 µL of IL17 (**SEQ ID NO: 22**) (1.5 mg/ml) with 1.74 ml of Cat2200a scFv (0.69 mg/mL) and incubating for 3 hours at 4°C. The mixture was concentrated with 10 kDa cutoff Amicon Ultra concentrator to about 400 µL and loaded onto a Superdex75 column equilibrated in 250 mM NaCl, 20 mM HEPES, pH 7.5. The fractions corresponding to the complex were pooled and concentrated to a volume of 150 µL. The sample was diluted and concentrated 4 times: addition of 350 µL 50 mM NaCl, 20 mM HEPES, pH 7.5 and concentration to just under 150 µL. The volume was brought to ~105 µL and concentration determined to be 2.69 mg/mL. Crystallization was set up in a sitting drop format using a Mosquito crystallization robot with 150 nL protein + 150 nL reservoir in Corning3550 plates against 80 µL reservoir, which is a set of buffer and precipitant conditions pre-formulated in-house. The plates were incubated at 20°C. One of conditions (Na Acetate, pH 4.5, 25% PEG 3K, 0.2M Am Acetate) produced very small crystals. These were harvested and turned into crystallization seeds using Hampton Seed Bead in 100 µL 27% PEG 3350, 200 mM ammonium acetate, 100 mM sodium acetate, pH 4.5 in a Hampton Seed Bead tube.

[00694] Diffraction quality crystals were obtained by the same procedure except with the addition of the seeds above: 150 nL protein + 100 nL reservoir + 50 µL seeds. Crystals grew from 0.1 M Tris 8.5, 18% PEG3K, 0.2M LiSO4 and were transferred to a synthetic mother liquor (0.1 M Tris, pH 8.5, 10% PEG 3350, 0.2 M LiSO4 and 20% glycerol) and flash frozen in liquid

nitrogen. X-ray diffraction data were collected at IMCA-CAT ID17 at Argonne National Laboratory.

[00695] The IL-17/ CAT2200a spFv VL-VH complex were generated by mixing 167 μ l of IL-17 (250 μ g) with 154 μ l MSCW274 (467 μ g in 250 mM NaCl, 20 mM MES, pH 6.5) and incubating at 4°C overnight. The mixture was concentrated in a 10kDa MWCO Amicon Ultra 0.5 mL concentrator to ~100 μ L, then repeatedly diluted and concentrated 5 times: concentrate to ~150 μ L and added 350 μ L 50 mM NaCl, 20 mM HEPES, pH 7.5. The final volume was 100 μ L and the concentration of the complex was determined to be 6.0 mg/ml. Crystallization was set up similarly as for scFv/IL-17 complex in sitting drops using the Mosquito robot. The sitting drop are composed of 150 nL protein + 120 nL reservoir + 30 nL seeds (scFv/IL-17 above). The reservoir solution were a set of conditions varying PEG 3350 concentration and salts. The crystallization plates were incubated at 20°C. Small crystals were obtained from 15.5 % PEG 3350, 0.4 M NaH₂PO₄. Crystals were transferred into 16% PEG 3350, 0.2 M NaH₂PO₄, 20% Glycerol, and flash frozen LN₂. X-ray diffraction data were collected at IMCA-CAT ID17 at Argonne National Laboratory.

[00696] All X-ray diffraction data were processed with XDS (Kabsch *et al.* (2010) *Acta Crystallogr D Biol Crystallogr* 66(Pt. 2):125-132; Monsellier and Bedouelle (2006) *J Mol Biol* 362:580-593) and CCP4 (Collaborative Computational Project, N. (1994) *Acta Crystallogr D Biol Crystallogr* 53:240-255). All crystal structures were solved by molecular replacement (MR) using Phaser (Read (2001) *Acta Crystallogr D Biol Crystallogr* 57(Pt 10):1373-1382) with homology models generated in MOE (Montreal, Canada) except for scFv CAT2200a scFv VL-VH/IL-17 complex, for which the structure of pdb id 2vxs (Gerhardt *et al.* (2009) *J Mol Biol* 394:905-921) was used as search models. The structural models were refined in PHENIX (Adams *et al.* (2004) *J Synchrotron Radiat* 11(Pt 1):53-55) and manually adjusted in Coot (Emsley *et al.* (2010) *Acta Crystallogr D Biol Crystallogr* 66(Pt 4):486-501). Molecular graphics figures were generated in PyMol (www.schrodinger.com).

6.3.2 The structures

[00697] The structures of the unbound scFv and spFv molecules are shown in FIG. 8, FIG. 9, FIG. 10 and FIG. 11. FIG. 8 shows the structure of GLk1 spFv VL-VH. FIG. 9 shows the structure of GLk1 spFv VH-VL. FIG. 10 shows the structure of GLk2 spFv VH-VL. FIG. 11 shows the structure of CAT2200b spFv VH-VL. The structures were consistent with the typical

Fv structures with both VL and VH domains packing against each other. In general, most of the linker residues were ordered and resolved in the electron density maps. The disulfide bonds between the staple and the anchor points were generally well ordered in both VL-VH orientations. In addition to the unbound scFv and spFv structures, we also attempted to reveal any structural impact on antigen binding. CAT2200 scFv and spFv variant molecules were crystallized in complex with its cognate target, IL-17. For the scFv and spFv of CAT2200 variants crystallized, the structures were nearly identical with and without a bound target (FIG. 12, FIG. 13, FIG. 14). FIG. 12 shows the comparison of the unbound CAT2200b spFv VH-VL compared with CAT2200a scFv VL-VH bound to IL-17. FIG. 13 shows the comparison of the front views of the structures of unbound CAT2200b spFv VH-VL compared with CAT2200a spFv VL-VH bound to IL-17. FIG. 14 shows the comparison of the back views of the structures of unbound CAT2200b spFv VH-VL compared with CAT2200a spFv VL-VH bound to IL-17. The structures were also identical regardless of orientation or presence or absence of the staple. The rmsd for all matching Ca atoms between pairs of structures were very small: 0.41 Å between unbound spFv-VH-VL and antigen-bound scFv-VL-VH (FIG. 12), 0.46 Å between unbound spFv-VH-VL and spFv-VL-VH (FIG. 13 and FIG. 14, respectively), and 0.37 Å between bound scFv and bound spFv. The structural evidence shows that the stapling works as designed. Also, stapling does not impact the domain structures of VL and VH or relative VL/VH packing.

6.4 Example 4: Design of additional anchor points

[00698] The approach described in Example 1 was used to identify any additional anchor points for stapling. The following anchor points were identified:

[00699] For VL-linker-VH orientation: VL Chothia position 42, 45 and 39 and VH Chothia positions 105, 5 and 3. In **FIG. 6**, the VL residues on GLk1 VL are K42, K45, K39 and the VH residues on GLk1 are Q105, L5 and Q3. The staple forms between any of the positions indicated.

[00700] For VH-linker-VL orientation: VH Chothia positions 43, 40 and 46, VL Chothia positions 102, 5 and 3, the staple forms between any of the positions.

[00701] The spFvs having the anchor points described in this Example are cloned, expressed and tested for the formation of the staple and their thermostability using assays described herein and in Example 2.

6.5 Example 5: Half-stapling

[00702] Constructs are generated and expressed containing one staple, either between the VH and the linker or between the VL and the linker. The generated constructs are expressed, purified and analyzed using methods described herein.

6.6 Example 6: Multispecific constructs comprising spFvs

[00703] Exemplary multispecific binding molecules incorporating the spFv structures provided herein were constructed and tested in this example. Specifically, bispecific antibodies and control molecules, derived from the target binding sequences shown in **Table 8**, were transiently expressed in CHO suspension cultures in serum-free/animal component-free media, and purified by protein A affinity chromatography, followed by preparative size exclusion chromatography (SEC) on a SUPERDEX 200 10/300 GL column (GE Healthcare) using a ÄKTA PURE instrument (GE Healthcare). Heavy chains contained knob-into-hole (KiH) mutations to promote heterodimerization (Ridgway *et al.*, *Protein Eng.* 9(7):617-21 (1996); Atwell *et al.*, *J. Mol. Biol.* 270(1):26-35 (1997); Merchant *et al.*, *Nat. Biotechnol.* 16(7):677-81 (1998)). Antibodies contained the IgG1sigma Fc comprising a set of seven Fc mutations - L234A, L235A, G237A, P238S, H268A, A330S, and P331S - when compared to the wild type IgG1 to reduce Fc receptor interactions (Tam *et al.*, *Antibodies* (2017)).

[00704] Bispecific antibodies were generated with IgG1sigma mutations and with KiH mutations.

Table 8: Target binding sequences used for the constructs of Example 6.

Antibody / Ligand designation	Target / receptor	VH or Chain 1	VL or Chain 2
LTBRmAb1 (BHA10)	human LTBR	WO2004002431/SEQ ID NO: 69	WO2004002431/SEQ ID NO: 70
EDBmAb1* (L19)	human extra-domain B (EDB) of fibronectin	WO9745544/SEQ ID NO: 71	WO9745544/SEQ ID NO: 72
B21M	RSV	Vafa O <i>et al.</i> (2014) Methods 65(1)/SEQ ID NO: 73	Vafa O <i>et al.</i> (2014) Methods 65(1)/SEQ ID NO: 74
MSLNmAb1	human mesothelin	EP2322560/SEQ ID NO: 75	EP2322560/SEQ ID NO: 76

*: EDBmAb1(WO9745544) used here is an anti-ED-B antibody that has been tested in the clinic, other antibodies binding to ED-B or to adjacent domains have been described previously (Carnemolla *et al. Int. J. Cancer* 68:397-405 (1996))

[00705] The sequences in **Table 8** are as follows:

5

SEQ ID NO: 69 (VH BHA10)

QVQLVQSGAEVKKPGSSVKV SCKASGYTFTTYLHWVRQAPGQGLEWMGWIYPGNV
HAQYNEKFKGRVTITADKSTSTAYMELSSLRSED TAVYYCARSWEGFPYWGQGTTVT
SS

10

SEQ ID NO: 70 (VL BHA10)

DIQMTQSPSSLSASVGDRVTITCKASQNVGINVAWYQQKPGKAPKSLISSASYRYS
GVPS
RFSGSGSGTDFTLTISSLQPEDFATYFCQQYDTPFTFGQGTKVEIK

15

SEQ ID NO: 71 (VH L19)

EVQLLES GGGLVQPGGSLRLSCAASGFTFSSFSMSWVRQAPGKGLEWVSSISGSSGTTY
YADSVKGRFTISRDN SKNTLYLQMNSLRAEDTAVYYCAKPFYFDYWGQGLTVTVSS

SEQ ID NO: 72 (VL L19)

20

EIVLTQSPGTLSPGERATLSCRASQSVSSSFLAWYQQKPGQAPRLLIYYASSRATGIPD
RFSGSGSGTDFTLTISRLEPEDFAVYYCQQTGRIPTFGQGTKVEIK

SEQ ID NO: 73 (VH B21M)

25

QITLKESGPTLVKPTQTLTLCTFSGFSLS TSGMGVSWIRQPPGKALEWLAHIYWDDDKR
YNPSLKSRLTITKDT SKNQVVLMTNMDPVDTATYYCARLYGFTYGFAYWGQGLTV
VSS

SEQ ID NO: 74 (VL B21M)

30

DIVMTQSPDSLAVSLGERATINCRASQSV DYNISYMHYQQKPGQPPKLLIYAASNPE
SGVPDRFSGSGSGTDFTLTISSLQAEDVAVYYCQQIIEDPWTFGQGTKVEIK

SEQ ID NO: 75 (VH MSLNmAb1)

QVQLQQSGPELEKPGASVKISCKASGYSFTGYTMNWVKQSHGKSLEWIGLITPYNGASS
 YNQKFRGKATLTVDKSSSTAYMDLLSLTSEDSAVYFCARGGYDGRGFDYWGSGTPVT
 VSS

5

SEQ ID NO: 76 (VL MSLNmAb1)

DIELTQSPAIMSASPGEKVTMTCSASSSVSYMHWYQQKSGTSPKRWIYDTSKLASGVPG
 RFSGSGSGNSYSLTISSVEAEDDATYYCQQWSKHPLTFGSGTKVEIK

- 10 **[00706]** Protein concentration was determined by absorbance measurement at 280 nm
 (OD280) and purification yield determined. Analytical SEC was performed using a Bio SEC-5
 column (Agilent, 5 μ m particle size, 300Å) on a Thermo VANQUISH HPLC system. 10 μ l
 purified protein was loaded on the column and elution was recorded by OD280.
- [00707]** **Table 9** shows an overview of structural properties of the bispecific antibodies and
 15 control molecules described in this example. The molecules in boldface are exemplary molecules
 according to the invention, while the others are controls for different aspects. **Table 10** shows
 structural properties of another comparative bispecific antibody, targeting LTBR and mesothelin
 (a tumor associated antigen not present in the extracellular matrix), as discussed in Example 9.

Table 9: Overview of the structural properties of the EDB/LTBR bispecific antibodies and control molecules

Name	Format	Binding sites		Fc-domain		scFv features		Comments
		LTBRmAb1	EDBmAb1	B2IM (isotype control mAb)	Protein A mut.*	Stapled linker	Disulfide stabilized	
COVA1480	2:1	1 (scFv)	2 (mAb)	-	yes	yes	-	scFv fused to N-term of EDBmAb1 HC
COVA1481	2:1	1 (scFv)	2 (mAb)	-	yes	yes	-	scFv fused to N-term of EDBmAb1 HC
COVA1482	2:1	1 (scFv)	2 (mAb)	-	yes	yes	-	scFv fused to C-term of EDBmAb1 HC
COVA1483	2:1	1 (scFv)	2 (mAb)	-	yes	yes	-	scFv fused to C-term of EDBmAb1 HC
COVA1484	2:1	1 (scFv)	-	2 (mAb)	yes	yes	-	LTBR/null control to COVA1480
COVA1485	2:1	1 (scFv)	-	2 (mAb)	yes	yes	-	LTBR/null control to COVA1481
COVA1486	2:1	1 (scFv)	-	2 (mAb)	yes	yes	-	LTBR/null control to COVA1482 and to COVA14146
COVA1487	2:1	1 (scFv)	-	2 (mAb)	yes	yes	-	LTBR/null control to COVA1483
COVA14107	2:1	1 (scFv)	2 (mAb)	-	yes	yes	No	scFv (C-Term fusion to EDBmAb1 HC) contains mutations for reduced affinity to LTBR (VL3: Y36F_S49Y_F87)
COVA14108	2:1	1 (scFv)	2 (mAb)	-	yes	yes	No	scFv (C-Term fusion to EDBmAb1 HC) contains mutations for reduced affinity to LTBR (VH_CDR1_Y33A)
COVA14133	2:1	1 (scFv)	2 (mAb)	-	no	yes	No	scFv fused to C-term of EDBmAb1 HC
COVA14136	2:1	1 (scFv)	-	2 (mAb)	no	yes	No	LTBR/null control to COVA14133

*: mutations in the Fc portion to abrogate binding to protein A and facilitate purification of heterodimers, described in WO2010151792.

Table 10: Overview of the structural properties of the MSLN/LTBR bispecific antibody

Name	Format	Binding sites		Fc-domain	scFv features			Comments
		LTBRmAb1	MSLNmAb1	Protein A mut.	Stapled linker	Disulfide stabilized	orientation	
COVA14146	2:1	1 (scFv)	2(mAb)	yes	yes	no	VH-VL	HC C-terminal fusion of scFv derived from LTBRmAb1 to MSLNmAb1. Isotype control molecule for this construct is COVA1486

[00708] The asymmetric antibodies, with 2:1 stoichiometry (all IgG1sigma, all with KiH mutations), listed above were generated as follows:

- i. COVA1484 was generated by co-expression of the anti-RSV B21M antibody heavy chain carrying a N-terminal stapled scFv BHA10 (VH-VL orientation SEQ ID NO: 77) fusion (SEQ ID NO: 78, comprising SEQ ID NO: 79) with the heavy chain (HC; SEQ ID NO: 80) and light chain (LC; SEQ ID NO: 81) of the anti-RSV B21M antibody (**FIG. 15A**).

SEQ ID NO: 77 [stapled scFv BHA10 (VH-VL)]

QVQLVQSGAEVKKPGSSVKVSCKASGYTFTTYYLHWVRQAPGCGLEWMGWIY
PGNVHAQYNEKFKGRVTITADKSTSTAYMELSSLRSEDTAVYYCARSWEGFPY
WGQGTTVTVSSGGSGGGSGCPPCGGGGDIQMTQSPSSLSASVGDRVTITCKAS
QNVGINVAWYQQKPGKAPKSLISSASYRYSQVPSRFSGSGSGTDFTLTISSLQPED
FATYFCQQYDTPFTFGCGTKVEIK

SEQ ID NO: 78 (HC B21M N-term stapled BHA10 (VH-VL), IgG1s, knob, with pA mutations)

QVQLVQSGAEVKKPGSSVKVSCKASGYTFTTYYLHWVRQAPGCGLEWMGWIY
PGNVHAQYNEKFKGRVTITADKSTSTAYMELSSLRSEDTAVYYCARSWEGFPY
WGQGTTVTVSSGGSGGGSGCPPCGGGGDIQMTQSPSSLSASVGDRVTITCKAS

QNVGINVAWYQQKPGKAPKSLISSASYRYSRVPSRFSGSGSGTDFTLTISSLQPED
FATYFCQQYDTPFTFGCGTKVEIKGGGSGGGGSGGGGSGGGGSGGGGSGITL
KESGPTLVKPTQTLTLTCTFSGFSLSTSGMGVSWIRQPPGKALEWLAHIYWDDD
KRYNPSLKSRLTITKDTSKNQVVLMTNMDPVDTATYYCARLYGFTYGFAYWG
QGTLLTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGAL
TSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPK
SCDKTHTCPPCPAPEAAGASSVFLFPPKPKDTLMISRTPEVTCVVDVSAEDPEV
KFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSN
KALPSSIEKTISKAKGQPREPQVYTLPPCREEMTKNQVSLWCLVKGFYPSDIAVE
WESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALH
NRFTQKSLSLSPGK

SEQ ID NO: 79 (HC B21M (RSV) IgG1s knob with pA mutations)

QITLKESGPTLVKPTQTLTLTCTFSGFSLSTSGMGVSWIRQPPGKALEWLAHIYW
DDDKRYNPSLKSRLTITKDTSKNQVVLMTNMDPVDTATYYCARLYGFTYGFAY
YWGQGTLLTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWN
SGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKK
VEPKSCDKTHTCPPCPAPEAAGASSVFLFPPKPKDTLMISRTPEVTCVVDVSAE
DPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKC
KVSNAKALPSSIEKTISKAKGQPREPQVYTLPPCREEMTKNQVSLWCLVKGFYPSD
IAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHE
ALHNRFQKSLSLSPGK

SEQ ID NO: 80 (HC B21M (RSV) IgG1s hole)

QITLKESGPTLVKPTQTLTLTCTFSGFSLSTSGMGVSWIRQPPGKALEWLAHIYW
DDDKRYNPSLKSRLTITKDTSKNQVVLMTNMDPVDTATYYCARLYGFTYGFAY
YWGQGTLLTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWN
SGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKK
VEPKSCDKTHTCPPCPAPEAAGASSVFLFPPKPKDTLMISRTPEVTCVVDVSAE
DPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKC
KVSNAKALPSSIEKTISKAKGQPREPQVCTLPSSREEMTKNQVSLSCAVKGFYPSDI
AVEWESNGQPENNYKTTPPVLDSDGSFFLVSKLTVDKSRWQQGNVFSCSVMHE
ALHNHYTQKSLSLSPGK

SEQ ID NO: 81 [LC B21M (RSV)]

DIVMTQSPDSLAVSLGERATINCRASQSV DYN G I S Y M H W Y Q Q K P G Q P P K L L I Y A
 ASNPESGVPDRFSGSGSGTDFTLTISSLQAEDVAVYYCQQIIEDPWTFGQGTKVEI
 KRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQ
 ESVTEQDSKDSTYLSSTLTLSKADYEEKHKVYACEVTHQGLSSPVTKSFNRGEC

- ii. COVA1485 was generated by co-expression of the anti-RSV B21M antibody heavy chain carrying a N-terminal stapled scFv BHA10 (VL-VH orientation SEQ ID NO: 82) fusion (SEQ ID NO: 83, comprising SEQ ID NO: 79) with the heavy chain (HC; SEQ ID NO: 80) and light chain (LC; SEQ ID NO: 81) of the anti-RSV B21M antibody (**FIG. 15B**).

SEQ ID NO: 82 [stapled scFv BHA10 (VL-VH)]

DIQMTQSPSSLASVGDRVITITCKASQNVGINVAWYQQKPGCAPKSLISSASYRY
 SGVPSRFSGSGSGTDFTLTISSLQPEDFATYFCQQYDTYPFTFGQGTKVEIKGGSG
 GSGGCPPCGSGGQVQLVQSGAEVKKPGSSVKV SCKASGYTFTTYLHWVRQAP
 GQGLEWMGWIYPGNVHAQYNEKFKGRVTITADKSTSTAYMELSSLRSED TAVY
 YCARSWEGFPYWGCGTTVTVSS

SEQ ID NO: 83 (HCB21M N-term stapled BHA10 (VL-VH), IgG1s, knob, with pA mutations)

DIQMTQSPSSLASVGDRVITITCKASQNVGINVAWYQQKPGCAPKSLISSASYRYS
 GVPSRFSGSGSGTDFTLTISSLQPEDFATYFCQQYDTYPFTFGQGTKVEIKGGSGGS
 GGCPPCGSGGQVQLVQSGAEVKKPGSSVKV SCKASGYTFTTYLHWVRQAPGQ
 GLEWMGWIYPGNVHAQYNEKFKGRVTITADKSTSTAYMELSSLRSED TAVYYC
 ARSWEGFPYWGCGTTVTVSSGGGGSGGGSGGGSGGGSGGGGSQITLKESGP
 TLVKPTQTLTLCTFSGFSLSTSGMGVSWIRQPPGKALEWLAHIYWDDDKRYNPS
 LKSRLTITKDTSKNQVVL TMTNMDPVD TATYYCARLYGFTYGFAYWGQGLVT
 VSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTF
 PAVLQSSGLYSLSSVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHT
 CPPCPAPEAAGASSVFLFPPKPKDTLMISRTPEVTCVVVDVSAEDPEVKFNWYVD
 GVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPSSIEK
 TISKAKGQPREPQVYTLPPCREEMTKNQVSLWCLVKGFYPSDIAVEWESNGQPEN

NYKTTTPVLDSGDSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNRFTQKSLSLSPGK

SEQ ID NO: 79 (HC B21M (RSV) IgG1s knob with pA mutations)

QITLKESGPTLVKPTQTLTLTCTFSGFSLSTSGMGVSWIRQPPGKALEWLAHIYW
 DDDKRYNPSLKSRLTITKDTSKNQVVLMTNMDPVDTATYYCARLYGFTYGFA
 YWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWN
 SGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKK
 VEPKSCDKTHTCPPCPAPEAAGASSVFLFPPKPKDTLMISRTPEVTCVVDVSAE
 DPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKC
 KVSNAKALPSSIEKTISKAKGQPREPQVYTLPPCREEMTKNQVSLWCLVKGFYPSD
 IAVEWESNGQPENNYKTTTPVLDSGDSFFLYSKLTVDKSRWQQGNVFSCSVMHE
 ALHNRFTQKSLSLSPGK

SEQ ID NO: 80 (HC B21M (RSV) IgG1s hole)

QITLKESGPTLVKPTQTLTLTCTFSGFSLSTSGMGVSWIRQPPGKALEWLAHIYW
 DDDKRYNPSLKSRLTITKDTSKNQVVLMTNMDPVDTATYYCARLYGFTYGFA
 YWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWN
 SGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKK
 VEPKSCDKTHTCPPCPAPEAAGASSVFLFPPKPKDTLMISRTPEVTCVVDVSAE
 DPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKC
 KVSNAKALPSSIEKTISKAKGQPREPQVCTLPSSREEMTKNQVSLSCAVKGFYPSDI
 AVEWESNGQPENNYKTTTPVLDSGDSFFLVSKLTVDKSRWQQGNVFSCSVMHE
 ALHNHYTQKSLSLSPGK

SEQ ID NO: 81 [LC B21M (RSV)]

DIVMTQSPDSLAVSLGERATINCRASQSVDYNGISYMHYQQKPGQPPKLLIYA
 ASNPESGVPDRFSGSGSGTDFTLTISSLQAEDVAVYYCQQIIEDPWTFGQGTKVEI
 KRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQ
 ESVTEQDSKDSTYLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC

- iii. COVA1486 was generated by co-expression of the anti-RSV B21M antibody heavy chain carrying a C-terminal stapled scFv BHA10 (VH-VL orientation SEQ ID NO: 77) fusion (SEQ ID NO: 84, comprising SEQ ID NO: 79) with the heavy chain (HC; SEQ ID

NO: 80) and light chain (LC; SEQ ID NO: 81) of the anti-RSV B21M antibody (**FIG. 15C**).

SEQ ID NO: 77 [stapled scFv BHA10 (VH-VL)]

QVQLVQSGAEVKKPGSSVKVSCKASGYTFTTYLHWVRQAPGCGLEWMGWIY
PGNVHAQYNEKFKGRVTITADKSTSTAYMELSSLRSEDTAVYYCARSWEGFPY
WGQGTTVTVSSGGGSGGGSGCPPCGGGGDIQMTQSPSSLSASVGDRVTITCKAS
QNVGINVAWYQQKPGKAPKSLISSASYRYSYSGVPSRFSGSGSGTDFTLTISLQPED
FATYFCQQYDTPFTFGCGTKVEIK

SEQ ID NO: 84 (HC B21M C-term stapled BHA (VH-VL), IgG1s, knob, with pA mutations)

QITLKESGPTLVKPTQTLTLCTFSGFSLSTSGMGVSWIRQPPGKALEWLAHIYW
DDDKRYNPSLKSRLTITKDTSKNQVVLMTNMDPVDATYYCARLYGFTYGFA
YWGQGTTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWN
SGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKK
VEPKSCDKTHTCPPCPAPEAAGASSVFLFPPKPKDTLMISRTPEVTCVVVDVSAE
DPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKC
KVSNAKALPSSIEKTISKAKGQPREPQVYTLPPCREEMTKNQVSLWCLVKGFYPSD
IAVEWESNGQPENNYKTTTPVLDSGDSFFLYSKLTVDKSRWQQGNVFSCSVMHE
ALHNRFTQKSLSLSPGKGGGGSGGGGSGGGGSQVQLVQSGAEVKKPGSSVKVS
CKASGYTFTTYLHWVRQAPGCGLEWMGWIYPGNVHAQYNEKFKGRVTITAD
KSTSTAYMELSSLRSEDTAVYYCARSWEGFPYWGQGTTVTVSSGGGSGGGSGC
PPCGGGGDIQMTQSPSSLSASVGDRVTITCKASQNVGINVAWYQQKPGKAPKSLI
SSASYRYSYSGVPSRFSGSGSGTDFTLTISLQPEDFATYFCQQYDTPFTFGCGTKV
EIK

SEQ ID NO: 79 (HC B21M (RSV) IgG1s knob with pA mutations)

QITLKESGPTLVKPTQTLTLCTFSGFSLSTSGMGVSWIRQPPGKALEWLAHIYW
DDDKRYNPSLKSRLTITKDTSKNQVVLMTNMDPVDATYYCARLYGFTYGFA
YWGQGTTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWN
SGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKK
VEPKSCDKTHTCPPCPAPEAAGASSVFLFPPKPKDTLMISRTPEVTCVVVDVSAE

DPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKC
 KVSNAKALPSSIEKTISKAKGQPREPQVYTLPPCREEMTKNQVSLWCLVKGFYPSD
 IAVEWESNGQPENNYKTTTPVLDSGDSFFLYSKLTVDKSRWQQGNVFSCSVMHE
 ALHNRFTQKSLSLSPGK

SEQ ID NO: 80 (HC B21M (RSV) IgG1s hole)

QITLKESGPTLVKPTQTLTLCTFSGFSLSTSGMGVSWIRQPPGKALEWLAHIYW
 DDDKRYNPSLKSRLTITKDTSKNQVVLMTNMDPVDATYTCARLYGFTYGFA
 YWGQGTLLTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWN
 SGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKK
 VEPKSCDKTHTCPPCPAPEAAGASSVFLFPPKPKDTLMISRTPEVTCVVDVSAE
 DPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKC
 KVSNAKALPSSIEKTISKAKGQPREPQVCTLPSPREEMTKNQVSLSCAVKGFYPSDI
 AVEWESNGQPENNYKTTTPVLDSGDSFFLYSKLTVDKSRWQQGNVFSCSVMHE
 ALHNHYTQKSLSLSPGK

SEQ ID NO: 81 [LC B21M (RSV)]

DIVMTQSPDSLAVSLGERATINCRASQSVSDYNGISYMHYQQKPGQPPKLLIYA
 ASNPESGVPDRFSGSGSGTDFTLTISSLQAEDVAVYYCQQIIEDPWTFGQGTKVEI
 KRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPRKAKVQWKVDNALQSGNSQ
 ESVTEQDSKDSSTSLTSLKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC

- iv. COVA1487 was generated by co-expression of the anti-RSV B21M antibody heavy chain carrying a C-terminal stapled scFv BHA10 (VL-VH orientation SEQ ID NO: 82) fusion (SEQ ID NO: 85, comprising SEQ ID NO: 79) with the heavy chain (HC; SEQ ID NO: 80) and light chain (LC; SEQ ID NO: 81) of the anti-RSV B21M antibody (**FIG. 15D**).

SEQ ID NO: 82 [stapled scFv BHA10 (VL-VH)]

DIQMTQSPSSLSASVGDRVTITCKASQNVGINVAWYQQKPGCAPKSLISSASYRY
 SGVPSRFSGSGSGTDFTLTISSLQPEDFATYFCQQYDTPFTFGQGTKVEIKGGSG
 GSGGCPGCGSGGQVQLVQSGAEVKKPGSSVKVSCKASGYTFTTYLHWVRQAP
 GQGLEWMGWIYPGNVHAQYNEKFKGRVTITADKSTSTAYMELSSLRSEDTAVY
 YCARSWEGFPYWGCGTTVTVSS

SEQ ID NO: 85 (HC B21M C-term stapled BHA (VL-VH), IgG1s, knob, with pA mutations)

QITLKESGPTLVKPTQTLTLCTFSGFSLSTSGMGVSWIRQPPGKALEWLAHIYWD
DDKRYNPSLKSRLTITKDTSKNQVVLMTNMDPVDATYYCARLYGFTYGFAY
WGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSG
ALTSQGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEP
KSCDKTHTCPPCPAPEAAGASSVFLFPPKPKDTLMISRTPEVTCVVDVSAEDPEV
KFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSN
KALPSSIEKTISKAKGQPREPQVYTLPPCREEMTKNQVSLWCLVKGFYPSDIAVE
WESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFCFSVMHEALH
NRFTQKSLSLSPGKGGGGSGGGSGGGGSDIQMTQSPSSLSASVGDRVTITCKAS
QNVGINVAWYQQKPGCAPKSLISSASYRYSQVPSRFSGSGSGTDFTLTISSLQPED
FATYFCQQYDTPFTFGQGTKVEIKGGSGSGGCPPCGSGGQVQLVQSGAEVKK
PGSSVKVSCKASGYTFSTYYLHWVRQAPGQGLEWMGWIYPGNVHAQYNEKFK
GRVTITADKSTSTAYMELSSLRSEDTAVYYCARSWEGFPYWGCGTTVTVSS

SEQ ID NO: 79 (HC B21M (RSV) IgG1s knob with pA mutations)

QITLKESGPTLVKPTQTLTLCTFSGFSLSTSGMGVSWIRQPPGKALEWLAHIYW
DDDKRYNPSLKSRLTITKDTSKNQVVLMTNMDPVDATYYCARLYGFTYGFAY
YWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWN
SGALTSQGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKK
VEPKSCDKTHTCPPCPAPEAAGASSVFLFPPKPKDTLMISRTPEVTCVVDVSAE
DPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKC
KVSNAKALPSSIEKTISKAKGQPREPQVYTLPPCREEMTKNQVSLWCLVKGFYPSD
IAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFCFSVMHE
ALHNRFQKSLSLSPGK

SEQ ID NO: 80 (HC B21M (RSV) IgG1s hole)

QITLKESGPTLVKPTQTLTLCTFSGFSLSTSGMGVSWIRQPPGKALEWLAHIYW
DDDKRYNPSLKSRLTITKDTSKNQVVLMTNMDPVDATYYCARLYGFTYGFAY
YWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWN
SGALTSQGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKK
VEPKSCDKTHTCPPCPAPEAAGASSVFLFPPKPKDTLMISRTPEVTCVVDVSAE

DPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKC
KVSNAKALPSSIEKTISKAKGQPREPQVCTLPPSREEMTKNQVSLSCAVKGFYPSDI
AVEWESNGQPENNYKTTTPVLDSGDSFFLVSKLTVDKSRWQQGNVFSCSVMHE
ALHNHYTQKSLSLSPGK

SEQ ID NO: 81 [LC B21M (RSV)]

DIVMTQSPDSLAVSLGERATINCRASQSVDYNGISYMHYQQKPGQPPKLLIYA
ASNPESGVPDRFSGSGSGTDFTLTISSLQAEDVAVYYCQQIIEDPWTFGQGTKVEI
KRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQ
ESVTEQDSKDSSTYLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC

- v. COVA1480 was generated by co-expression of an anti-EDB antibody EDBmAb1 heavy chain carrying a N-terminal stapled scFv BHA10 (VH-VL orientation SEQ ID NO: 77) fusion (SEQ ID NO: 86, comprising SEQ ID NO: 87) with the heavy chain (HC; SEQ ID NO: 88) and light chain (LC; SEQ ID NO: 89) of an anti-EDB antibody EDBmAb1 (FIG. 15E).

SEQ ID NO: 77 [stapled scFv BHA10 (VH-VL)]

QVQLVQSGAEVKKPGSSVKVSCKASGYTFSTYYLHWVRQAPGCGLEWMGWIY
PGNVHAQYNEKFKGRVTITADKSTSTAYMELSSLRSEDTAVYYCARSWEGFPY
WGQGTTTVTVSSGGGSGGGSGCPCGGGGDIQMTQSPSSLSASVGDRVTITCKAS
QNVGINVAWYQQKPGKAPKSLISSASYRYSQVPSRFSGSGSGTDFTLTISLQPED
FATYFCQQYDTPFTFGCGTKVEIK

SEQ ID NO: 86 (HC L19 N-term stapled BHA10 (VH-VL), IgG1s, knob, with pA mutations)

QVQLVQSGAEVKKPGSSVKVSCKASGYTFSTYYLHWVRQAPGCGLEWMGWIY
PGNVHAQYNEKFKGRVTITADKSTSTAYMELSSLRSEDTAVYYCARSWEGFPY
WGQGTTTVTVSSGGGSGGGSGCPCGGGGDIQMTQSPSSLSASVGDRVTITCKAS
QNVGINVAWYQQKPGKAPKSLISSASYRYSQVPSRFSGSGSGTDFTLTISLQPED
FATYFCQQYDTPFTFGCGTKVEIKGGGGSGGGGSGGGGSGGGGSGGGGSEVQ
LLESGLGLVQPGGSLRLSCAASGFTFSSFSMSWVRQAPGKGLEWVSSISGSSGTT
YYADSVKGRFTISRDNKNTLYLQMNSLRAEDTAVYYCAKPFYFDYWGQGT
LTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGV

HTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDK
THTCPPCPAPEAAGASSVFLFPPKPKDTLMISRTPEVTCVVVDVSAEDPEVKFNW
YVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALP
SSIEKTISKAKGQPREPQVYTLPPCREEMTKNQVSLWCLVKGFYPSDIAVEWESN
GQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNRFTQ
KSLSLSPGK

SEQ ID NO: 87 (HC L19 IgG1s knob with pA mutations)

VQLLESGGGLVQPGGSLRLSCAASGFTFSFSMSWVRQAPGKGLEWVSSISGSSG
TTYADSVKGRFTISRDN SKNTLYLQMNSLRAEDTAVYYCAKPFYFDYWGQG
TLTVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTS
GVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSC
DKTHTCPPCPAPEAAGASSVFLFPPKPKDTLMISRTPEVTCVVVDVSAEDPEVKF
NWKYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNK
ALPSSIEKTISKAKGQPREPQVYTLPPCREEMTKNQVSLWCLVKGFYPSDIAVEW
ESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNR
FTQKSLSLSPGK

SEQ ID NO: 88 (HC L19 IgG1s hole)

EVQLLESGGGLVQPGGSLRLSCAASGFTFSFSMSWVRQAPGKGLEWVSSISGSS
GTTYADSVKGRFTISRDN SKNTLYLQMNSLRAEDTAVYYCAKPFYFDYWGQ
GTLTVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALT
SGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKS
CDKTHTCPPCPAPEAAGASSVFLFPPKPKDTLMISRTPEVTCVVVDVSAEDPEVK
FNWKYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNK
ALPSSIEKTISKAKGQPREPQVCTLPSSREEMTKNQVSLSCAVKGFYPSDIAVEWE
SNGQPENNYKTTPPVLDSDGSFFLVSKLTVDKSRWQQGNVFSCSVMHEALHNH
YTQKSLSLSPGK

SEQ ID NO: 89 (LC L19)

EIVLTQSPGTLSPGERATLSCRASQSVSSSFLAWYQQKPGQAPRLLIYYASSRA
TGIPDRFSGSGSGTDFTLTISRLEPEDFAVYYCQQTGRIPPTFGQGTKVEIKRTVAA

PSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQ
DSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC

- vi. COVA1481 was generated by co-expression of an anti-EDB antibody EDBmAb1 heavy chain carrying a N-terminal stapled scFv BHA10 (VL-VH orientation SEQ ID NO: 82) fusion (SEQ ID NO: 90, comprising SEQ ID NO: 87) with the heavy chain (HC; SEQ ID NO: 88) and light chain (LC; SEQ ID NO: 89) of an anti-EDB antibody EDBmAb1 (FIG. 15F).

SEQ ID NO: 82 [stapled scFv BHA10 (VL-VH)]

DIQMTQSPSSLSASVGDRVTITCKASQNVGINVAWYQQKPGCAPKSLISSASYRY
SGVPSRFSGSGSGTDFTLTISLQPEDFATYFCQQYDTPFTFGQGKVEIKGGSG
GSGGCPGCGSGGQVQLVQSGAEVKKPGSSVKVSCKASGYTFTTYLHWVRQAP
GQGLEWMGWIYPGNVHAQYNEKFKGRVTITADKSTSTAYMELSSLRSEDNAVY
YCARSWEGFPYWGCGTTVTVSS

SEQ ID NO: 90 (HC L19 N-term stapled BHA10 (VL-VH), IgG1s, knob, with pA mutations)

DIQMTQSPSSLSASVGDRVTITCKASQNVGINVAWYQQKPGCAPKSLISSASYRY
SGVPSRFSGSGSGTDFTLTISLQPEDFATYFCQQYDTPFTFGQGKVEIKGGSG
GSGGCPGCGSGGQVQLVQSGAEVKKPGSSVKVSCKASGYTFTTYLHWVRQAP
GQGLEWMGWIYPGNVHAQYNEKFKGRVTITADKSTSTAYMELSSLRSEDNAVY
YCARSWEGFPYWGCGTTVTVSSGGGGSGGGSGGGSGGGSGGGSGGGGSEVQLL
ESGGGLVQPGGSLRLSCAASGFTFSFSMSWVRQAPGKGLEWVSSISGSSGTTY
ADSVKGRFTISRDNKNTLYLQMNSLRAEDTAVYYCAKPFYFDYWGQGLVT
VSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHT
FPAVLQSSGLYSLSSVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTH
TCPPCPAPEAAGASSVFLFPPKPKDTLMISRTPEVTCVVVDVSAEDPEVKFNWYV
DGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPSSI
EKTISKAKGQPREPQVYTLPPCREEMTKNQVSLWCLVKGFYPSDIAVEWESNGQ
PENNYKTTTPVLDSDGSFFLYSKLTVDKSRWQQGNVFSQSVMHREALHNRFTQKS
LSLSPGK

SEQ ID NO: 87 (HC L19 IgG1s knob with pA mutations)

VQLLESGGGLVQPGGSLRLSCAASGFTFSFSMSWVRQAPGKGLEWVSSISGSSG
 TTTYADSVKGRFTISRDN SKNTLYLQMNSLRAEDTAVYYCAKPFYFDYWGQG
 TLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTS
 GVHTFPAVLQSSGLYSLSSVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSC
 DKTHTCPPCPAPEAAGASSVFLFPPKPKDTLMISRTPEVTCVVDVSAEDPEVKF
 NWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNK
 ALPSSIEKTISKAKGQPREPQVYTLPPCREEMTKNQVSLWCLVKGFYPSDIAVEW
 ESNQGPENNYKTTTPVLDS DGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNR
 FTQKSLSLSPGK

SEQ ID NO: 88 (HC L19 IgG1s hole)

EVQLLESGGGLVQPGGSLRLSCAASGFTFSFSMSWVRQAPGKGLEWVSSISGSS
 GTTYADSVKGRFTISRDN SKNTLYLQMNSLRAEDTAVYYCAKPFYFDYWGQ
 GTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALT
 SGVHTFPAVLQSSGLYSLSSVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKS
 CDKTHTCPPCPAPEAAGASSVFLFPPKPKDTLMISRTPEVTCVVDVSAEDPEVK
 FNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNK
 ALPSSIEKTISKAKGQPREPQVCTLPSSREEMTKNQVSLSCAVKGFYPSDIAVEWE
 SNGQGPENNYKTTTPVLDS DGSFFLVSKLTVDKSRWQQGNVFSCSVMHEALHNH
 YTQKSLSLSPGK

SEQ ID NO: 89 (LC L19)

EIVLTQSPGTLSPGERATLSCRASQSVSSSFLAWYQQKPGQAPRLLIYYASSRA
 TGIPDRFSGSGSGTDFTLTISRLEPEDFAVYYCQQTGRIPPTFGQGTKVEIKRTVAA
 PSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQ
 DSKDSTYSLSSLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC

- vii. COVA1482 was generated by co-expression of an anti-EDB antibody EDBmAb1 heavy chain carrying a C-terminal stapled scFv BHA10 (VH-VL orientation SEQ ID NO: 77) fusion (SEQ ID NO: 91, comprising SEQ ID NO: 87) with the heavy chain (HC; SEQ ID

NO: 88) and light chain (LC; SEQ ID NO: 89) of an anti-EDB antibody EDBmAb1 (FIG. 15G).

SEQ ID NO: 77 [stapled scFv BHA10 (VH-VL)]

QVQLVQSGAEVKKPGSSVKVSCKASGYTFTTYLHWVRQAPGCGLEWMGWIY
PGNVHAQYNEKFKGRVTITADKSTSTAYMELSSLRSED AVYYCARSWEGFPY
WGQGTTTVTVSSGGGSGGGSGCPPCGGGGDIQMTQSPSSLSASVGDRVTITCKAS
QNVGINVAWYQQKPGKAPKSLISSASYRYSYGVPSRFSGSGSGTDFTLTISLQPED
FATYFCQQYDTYPFTFGCGTKVEIK

SEQ ID NO: 91 (HC L19 C-term stapled BHA10 (VH-VL), IgG1s, knob, with pA mutations)

EVQLLESGGGLVQPGGSLRLSCAASGFTFSSFSMSWVRQAPGKGLEWVSSISGSS
GTTYADSVKGRFTISRDN SKNTLYLQMNSLRAEDTAVYYCAKPFYFDYWGGQ
GTLTVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALT
SGVHTFPAVLQSSGLYSLSSVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKS
CDKTHTCPPCPAPEAAGASSVFLFPPKPKDTLMISRTPEVTCVVVDVSAEDPEVK
FNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNK
ALPSSIEKTISKAKGQPREPQVYTLPPCREEMTKNQVSLWCLVKGFYPSDIAVEW
ESNGQPENNYKTTTPVLDSDGSFFLYSKLTVDKSRWQQGNVFSVMSVMHEALHNR
FTQKSLSLSPGKGGGGSGGGGSGGGGSQVQLVQSGAEVKKPGSSVKVSCKASG
YTFTTYLHWVRQAPGCGLEWMGWIYPGNVHAQYNEKFKGRVTITADKSTSTA
YMELSSLRSED AVYYCARSWEGFPYWGQGTTTVTVSSGGGSGGGSGCPPCGGG
GDIQMTQSPSSLSASVGDRVTITCKASQNVGINVAWYQQKPGKAPKSLISSASYR
YSYGVPSRFSGSGSGTDFTLTISLQPEDFATYFCQQYDTYPFTFGCGTKVEIK

SEQ ID NO: 87 (HC L19 IgG1s knob with pA mutations)

VQLLESGGGLVQPGGSLRLSCAASGFTFSSFSMSWVRQAPGKGLEWVSSISGSSG
TTYADSVKGRFTISRDN SKNTLYLQMNSLRAEDTAVYYCAKPFYFDYWGGQ
TLTVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTS
GVHTFPAVLQSSGLYSLSSVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSC
DKTHTCPPCPAPEAAGASSVFLFPPKPKDTLMISRTPEVTCVVVDVSAEDPEVKF
NWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNK

ALPSSIEKTISKAKGQPREPQVYTLPPCREEMTKNQVSLWCLVKGFYPSDIAVEW
ESNGQPENNYKTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFCFSVMHEALHNR
FTQKSLSLSPGK

SEQ ID NO: 88 (HC L19 IgG1s hole)

EVQLLESGGGLVQPGGSLRLSCAASGFTFSSFSMSWVRQAPGKGLEWVSSISGSS
GTTYADSVKGRFTISRDNKNTLYLQMNSLRAEDTAVYYCAKPFYFDYWGQ
GTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALT
SGVHTFPAVLQSSGLYSLSSVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKS
CDKTHTCPPCPAPEAAGASSVFLFPPKPKDTLMISRTPEVTCVVVDVSAEDPEVK
FNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNK
ALPSSIEKTISKAKGQPREPQVCTLPSSREEMTKNQVSLSCAVKGFYPSDIAVEWE
SNGQPENNYKTPPVLDSDGSFFLVSKLTVDKSRWQQGNVFCFSVMHEALHNR
YTQKSLSLSPGK

SEQ ID NO: 89 (LC L19)

EIVLTQSPGTLSPGERATLSCRASQSVSSSFLAWYQQKPGQAPRLLIYYASSRA
TGIPDRFSGSGSGTDFTLTISRLEPEDFAVYYCQQTGRIPPTFGQGTKVEIKRTVAA
PSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQ
DSKDYSTYLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC

- viii. COVA1483 was generated by co-expression of an anti-EDB antibody EDBmAb1 heavy chain carrying a C-terminal stapled scFv BHA10 (VL-VH orientation SEQ ID NO: 82) fusion (SEQ ID NO: 92, comprising SEQ ID NO: 87) with the heavy chain (HC; SEQ ID NO: 88) and light chain (LC; SEQ ID NO: 89) of an anti-EDB antibody EDBmAb1 (FIG. 15H).

SEQ ID NO: 82 [stapled scFv BHA10 (VL-VH)]

DIQMTQSPSSLSASVGDRVTITCKASQNVGINVAWYQQKPGCAPKSLISSASYRY
SGVPSRFSGSGSGTDFTLTISLQPEDFATYFCQQYDTPFTFGQGTKVEIKGGSG
GSGGCPGCGSGGQVQLVQSGAEVKKPGSSVKVSKKASGYTFTTYLHWVRQAP
GQGLEWMGWIYPGNVHAQYNEKFKGRVTITADKSTSTAYMELSSLRSEDATVY
YCARSWEGFPYWGCGTTVTVSS

SEQ ID NO: 92 (HC L19 C-term stapled BHA10 (VL-VH), IgG1s, knob, with pA mutations)

EVQLLESGGGLVQPGGSLRLSCAASGFTFSSFSMSWVRQAPGKGLEWVSSISGSS
GTTYADSVKGRFTISRDN SKNTLYLQMNSLRAEDTAVYYCAKPFYFDYWGQ
GTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALT
SGVHTFPAVLQSSGLYSLSSVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKS
CDKTHTCPPCPAPEAAGASSVFLFPPKPKDTLMISRTPEVTCVVVDVSAEDPEVK
FNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNK
ALPSSIEKTISKAKGQPREPQVYTLPPCREEMTKNQVSLWCLVKGFYPSDIAVEW
ESNGQPENNYKTTTPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNR
FTQKSLSLSPGKGGGGSGGGGSGGGGSDIQMTQSPSSLSASVGDRVTITCKASQN
VGINVAWYQQKPGCAPKSLISSASYRYSGVPSRFSGSGSGTDFTLTISLQPEDFA
TYFCQQYDTPFTFGQGTKVEIKGGSGSGGCPPCGSGGQVQLVQSGAEVKKPG
SSVKVSCKASGYTFTTYLHWVRQAPGQGLEWMGWIYPGNVHAQYNEKFKGR
VTITADKSTSTAYMELSSLRSEDTAVYYCARSWEGFPYWGCGTTVTVSS

SEQ ID NO: 87 (HC L19 IgG1s knob with pA mutations)

VQLLESGGGLVQPGGSLRLSCAASGFTFSSFSMSWVRQAPGKGLEWVSSISGSSG
TTYADSVKGRFTISRDN SKNTLYLQMNSLRAEDTAVYYCAKPFYFDYWGGQ
TLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTS
GVHTFPAVLQSSGLYSLSSVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSC
DKTHTCPPCPAPEAAGASSVFLFPPKPKDTLMISRTPEVTCVVVDVSAEDPEVKF
NWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNK
ALPSSIEKTISKAKGQPREPQVYTLPPCREEMTKNQVSLWCLVKGFYPSDIAVEW
ESNGQPENNYKTTTPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNR
FTQKSLSLSPGK

SEQ ID NO: 88 (HC L19 IgG1s hole)

EVQLLESGGGLVQPGGSLRLSCAASGFTFSSFSMSWVRQAPGKGLEWVSSISGSS
GTTYADSVKGRFTISRDN SKNTLYLQMNSLRAEDTAVYYCAKPFYFDYWGQ
GTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALT
SGVHTFPAVLQSSGLYSLSSVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKS
CDKTHTCPPCPAPEAAGASSVFLFPPKPKDTLMISRTPEVTCVVVDVSAEDPEVK

FNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNK
ALPSSIEKTISKAKGQPREPQVCTLPSSREEMTKNQVSLSCAVKGFYPSDIAVEWE
SNGQPENNYKTTTPVLDSGDSFLLVSKLTVDKSRWQQGNVFCFSVMHEALHNH
YTQKSLSLSPGK

SEQ ID NO: 89 (LC L19)

EIVLTQSPGTLSPGERATLSCRASQSVSSSFLAWYQQKPGQAPRLLIYYASSRA
TGIPDRFSGSGSGTDFTLTISRLEPEDFAVYYCQQTGRIPPTFGQGTKVEIKRTVAA
PSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQ
DSKDYSTYLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC

- ix. COVA14107 was generated by co-expression of an anti-EDB antibody EDBmAb1 heavy chain carrying a C-terminal stapled scFv BHA10 (VH-VL orientation, VL3 Y36F_S49Y_F87Y SEQ ID NO: 93) fusion (SEQ ID NO: 94, comprising SEQ ID NO: 87) with the heavy chain (HC; SEQ ID NO: 88) and light chain (LC; SEQ ID NO: 89) of an anti-EDB antibody EDBmAb1 (**FIG. 15I**).

SEQ ID NO:93 [stapled scFv (VL3_Y36F_S49Y_F87Y) BHA10 (VH-VL)]

QVQLVQSGAEVKKPGSSVKVSCKASGYTFTTYLHWVRQAPGCGLEWMGWIY
PGNVHAQYNEKFKGRVTITADKSTSTAYMELSSLRSEDFAVYYCARSWEGFPY
WGQGTTTVTVSSGGSGGGSGCPCGGGGDIQMTQSPSSLSASVGRVTITCKAS
QNVGINVAWFQQKPGKAPKSLIYSASYRYSQVPSRFSGSGSGTDFTLTISLQPED
FATYYCQQYDTPFTFGCGTKVEIK

**SEQ ID NO:94 (HC L19 C-term stapled (VL3_Y36F_S49Y_F87Y) BHA (VH-VL),
IgG1s, knob, with pA mutations)**

EVQLLESGLLVQPGGSLRLSCAASGFTFSSFSMSWVRQAPGKGLEWVSSISGSS
GTTYADSVKGRFTISRDNKNTLYLQMNSLRAEDTAVYYCAKPFYFDYWGQ
GTLTVVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPTVSWNSGALT
SGVHTFPAVLQSSGLYSLSSVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKS
CDKTHTCPPCPAPEAAGASSVFLFPPKPKDTLMISRTPEVTCVVVDVSAEDPEVK
FNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNK
ALPSSIEKTISKAKGQPREPQVYTLPPCREEMTKNQVSLWCLVKGFYPSDIAVEW
ESNGQPENNYKTTTPVLDSGDSFLLVSKLTVDKSRWQQGNVFCFSVMHEALHNR

FTQKSLSLSPGKGGGSGGGSGGGGSQVQLVQSGAEVKKPGSSVKVSCKASG
YTFTTYLHWVRQAPGCGLEWMGWIYPGNVHAQYNEKFKGRVTITADKSTSTA
YMESSLRSEDNAVYYCARSWEGFPYWGQTTVTVSSGGGSGGGSGCPPCGGG
GDIQMTQSPSSLSASVGDRVTITCKASQNVGINVAWFQQKPGKAPKSLIYSASYR
YSGVPSRFSGSGSGTDFTLTISLQPEDFATYYCQQYDTYPFTFGCGTKVEIK

SEQ ID NO: 87 (HC L19 IgG1s knob with pA mutations)

VQLLESGGGLVQPGGSLRLSCAASGFTSSFSMSWVRQAPGKGLEWVSSISGSSG
TTYADSVKGRFTISRDN SKNTLYLQMNSLRAEDNAVYYCAKPFYFDYWGGQ
TLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTS
GVHTFPAVLQSSGLYSLSSVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSC
DKTHTCPPCPAPEAAGASSVFLFPPKPKDTLMISRTPEVTCVVVDVSAEDPEVKF
NWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNK
ALPSSIEKTISKAKGQPREPQVYTLPPCREEMTKNQVSLWCLVKGFYPSDIAVEW
ESNGQPENNYKTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNR
FTQKSLSLSPGK

SEQ ID NO: 88 (HC L19 IgG1s hole)

EVQLLESGGGLVQPGGSLRLSCAASGFTSSFSMSWVRQAPGKGLEWVSSISGSS
GTTYADSVKGRFTISRDN SKNTLYLQMNSLRAEDNAVYYCAKPFYFDYWGGQ
GTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALT
SGVHTFPAVLQSSGLYSLSSVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKS
CDKTHTCPPCPAPEAAGASSVFLFPPKPKDTLMISRTPEVTCVVVDVSAEDPEVK
FNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNK
ALPSSIEKTISKAKGQPREPQVCTLPSSREEMTKNQVSLSCAVKGFYPSDIAVEWE
SNGQPENNYKTPPVLDSDGSFFLVSKLTVDKSRWQQGNVFSCSVMHEALHNH
YTQKSLSLSPGK

SEQ ID NO: 89 (LC L19)

EIVLTQSPGTLSPGERATLSCRASQSVSSSFLAWYQQKPGQAPRLLIYYASSRA
TGIPDRFSGSGSGTDFTLTISRLEPEDFAVYYCQQTGRIPPTFGQGTKVEIKRTVAA
PSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQ
DSKDSTYLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC

- x. COVA14108 was generated by co-expression of an anti-EDB antibody EDBmAb1 heavy chain carrying a C-terminal stapled scFv BHA10 (VH-VL orientation, VH_CDR1_Y33A SEQ ID NO: 95) fusion (SEQ ID NO: 96, comprising SEQ ID NO: 87) with the heavy chain (HC; SEQ ID NO: 88) and light chain (LC; SEQ ID NO: 89) of an anti-EDB antibody EDBmAb1 (**FIG. 15J**).

SEQ ID NO: 95 [stapled scFv (VH_CDR1_Y33A) BHA10 (VH-VL)]

QVQLVQSGAEVKKPGSSVKVSCKASGYTFTTYALHWVRQAPGCGLEWMGWIY
PGNVHAQYNEKFKGRVTITADKSTSTAYMELSSLRSEDNAVYYCARSWEGFPY
WGQGTITVTVSSGGGSGGGSGCPPCGGGGDIQMTQSPSSLSASVGDRVTITCKAS
QNVGINVAWYQQKPGKAPKSLISSASYRYSRVPSRFSGSGSGTDFTLTISLQPED
FATYFCQQYDTPFTFGCGTKVEIK

SEQ ID NO: 96 (HC L19 C-term stapled (VH_CDR1_Y33A) BHA10 (VH-VL), IgG1s, knob, with pA mutations)

EVQLLESGGGLVQPGGSLRLSCAASGFTFSSFSMSWVRQAPGKGLEWVSSISGSS
GTTYADSVKGRFTISRDN SKNTLYLQMNSLRAEDNAVYYCAKPFYFDYWGQ
GTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALT
SGVHTFPAVLQSSGLYSLSSVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKS
CDKTHTCPPCPAPEAAGASSVFLFPPKPKDTLMISRTPEVTCVVVDVSAEDPEVK
FNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNK
ALPSSIEKTISKAKGQPREPQVYTLPPCREEMTKNQVSLWCLVKGFYPSDIAVEW
ESNGQPENNYKTTTPVLDSGDSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNR
FTQKSLSLSPGKGGGSGGGGSGGGGSGVQLVQSGAEVKKPGSSVKVSCKASG
YTFTTYALHWVRQAPGCGLEWMGWIYPGNVHAQYNEKFKGRVTITADKSTSTA
YMELSSLRSEDNAVYYCARSWEGFPYWGQGTITVTVSSGGGSGGGSGCPPCGGG
GDIQMTQSPSSLSASVGDRVTITCKASQNVGINVAWYQQKPGKAPKSLISSASYR
YSGVPSRFSGSGSGTDFTLTISLQPEDFATYFCQQYDTPFTFGCGTKVEIK

SEQ ID NO: 87 (HC L19 IgG1s knob with pA mutations)

VQLLESGGGLVQPGGSLRLSCAASGFTFSSFSMSWVRQAPGKGLEWVSSISGSSG
TTYADSVKGRFTISRDN SKNTLYLQMNSLRAEDNAVYYCAKPFYFDYWGQG
TLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTS

GVHTFPAVLQSSGLYSLSSVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSC
DKTHTCPPCPAPEAAGASSVFLFPPKPKDTLMISRTPEVTCVVVDVSAEDPEVKF
NWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNK
ALPSSIEKTISKAKGQPREPQVYTLPPCREEMTKNQVSLWCLVKGFYPSDIAVEW
ESNGQPENNYKTTTPVLDSGDSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNR
FTQKSLSLSPGK

SEQ ID NO: 88 (HC L19 IgG1s hole)

EVQLLESGLLVQPGGSLRLSCAASGFTFSSFSMSWVRQAPGKGLEWVSSISGSS
GTTYADSVKGRFTISRDN SKNTLYLQMNSLRAEDTAVYYCAKPFYFDYWGQ
GTLTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALT
SGVHTFPAVLQSSGLYSLSSVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKS
CDKTHTCPPCPAPEAAGASSVFLFPPKPKDTLMISRTPEVTCVVVDVSAEDPEVK
FNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNK
ALPSSIEKTISKAKGQPREPQVCTLPSSREEMTKNQVSLSCAVKGFYPSDIAVEWE
SNGQPENNYKTTTPVLDSGDSFFLVSKLTVDKSRWQQGNVFSCSVMHEALHNR
YTQKSLSLSPGK

SEQ ID NO: 89 (LC L19)

EIVLTQSPGTLSLSPGERATLSCRASQSVSSSFLAWYQQKPGQAPRLLIYYASSRA
TGIPDRFSGSGSGTDFTLISRLEPEDFAVYYCQQTGRIPPTFGQGTKVEIKRTVAA
PSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQ
DSKDSTYSLSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC

- xi. COVA14133 was generated by co-expression of an anti-EDB antibody EDBmAb1 heavy chain carrying a C-terminal stapled scFv BHA10 (VH-VL orientation SEQ ID NO: 77) fusion (SEQ ID NO: 97, comprising SEQ ID NO: 98) with the heavy chain (HC; SEQ ID NO: 88) and light chain (LC; SEQ ID NO: 89) of an anti-EDB antibody EDBmAb1 (FIG. 15K).

SEQ ID NO: 77 [stapled scFv BHA10 (VH-VL)]

QVQLVQSGAEVKKPGSSVKVSCKASGYTFSTYYLHWVRQAPGCGLEWMGWIY
PGNVHAQYNEKFKGRVTITADKSTSTAYMELSSLRSED TAVYYCARSWEGFPY
WGQGTTTVTVSSGGSGGGSGCPPCGGGGDIQMTQSPSSLSASVGDRVTITCKAS

QNVGINVAWYQQKPGKAPKSLISSASYRYSRVPSRFSGSGSGTDFTLTISSLQPED
FATYFCQQYDTPFTFGCGTKVEIK

SEQ ID NO: 97 (HC L19 C-term stapled BHA10 (VH-VL), IgG1s, knob, no pA
mutations)

EVQLLESGGGLVQPGGSLRLSCAASGFTFSFSMSWVRQAPGKGLEWVSSISGSS
GTTYADSVKGRFTISRDN SKNTLYLQMNSLRAEDTAVYYCAKPFYFDYWGQ
GTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALT
SGVHTFPAVLQSSGLYSLSSVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKS
CDKTHTCPPCPAPEAAGASSVFLFPPKPKDTLMISRTPEVTCVVVDVSAEDPEVK
FNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNK
ALPSSIEKTISKAKGQPREPQVYTLPPCREEMTKNQVSLWCLVKGFYPSDIAVEW
ESNGQPENNYKTTTPVLDS DGSFFLYSKLTVDKSRWQQGNV FSCSVMHEALHN
HYTQKSLSLSPGKGGGGSGGGGSGGGGSQVQLVQSGAEVKKPGSSVKV SCKAS
GYTFTTYYLHWVRQAPGCGLEWMGWIYPGNVHAQYNEKFKGRVTITADKSTST
AYMELSSLRSEDTAVYYCARSWEGFPYWGQGTTVTVSSGGGSGGGSGCPPCGG
GGDIQMTQSPSSLSASVGDRVTITCKASQNVGINVAWYQQKPGKAPKSLISSASY
RYSRVPSRFSGSGSGTDFTLTISSLQPEDFATYFCQQYDTPFTFGCGTKVEIK

SEQ ID NO: 98 (HC L19 IgG1s knob)

EVQLLESGGGLVQPGGSLRLSCAASGFTFSFSMSWVRQAPGKGLEWVSSISGSS
GTTYADSVKGRFTISRDN SKNTLYLQMNSLRAEDTAVYYCAKPFYFDYWGQ
GTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALT
SGVHTFPAVLQSSGLYSLSSVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKS
CDKTHTCPPCPAPEAAGASSVFLFPPKPKDTLMISRTPEVTCVVVDVSAEDPEVK
FNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNK
ALPSSIEKTISKAKGQPREPQVYTLPPCREEMTKNQVSLWCLVKGFYPSDIAVEW
ESNGQPENNYKTTTPVLDS DGSFFLYSKLTVDKSRWQQGNV FSCSVMHEALHN
HYTQKSLSLSPGK

SEQ ID NO: 88 (HC L19 IgG1s hole)

EVQLLESGGGLVQPGGSLRLSCAASGFTFSFSMSWVRQAPGKGLEWVSSISGSS
GTTYADSVKGRFTISRDN SKNTLYLQMNSLRAEDTAVYYCAKPFYFDYWGQ

GTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALT
 SGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKS
 CDKTHTCPPCPAPEAAGASSVFLFPPKPKDTLMISRTPEVTCVVVDVSAEDPEVK
 FNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNK
 ALPSSIEKTISKAKGQPREPQVCTLPSSREEMTKNQVSLSCAVKGFYPSDIAVEWE
 SNGQPENNYKTTPPVLDSDGSFFLVSKLTVDKSRWQQGNVFCFSVMHEALHNH
 YTQKSLSLSPGK

SEQ ID NO: 89 (LC L19)

EIVLTQSPGTLSPGERATLSCRASQSVSSSFLAWYQQKPGQAPRLLIYYASSRA
 TGIPDRFSGSGSGTDFLTISRLEPEDFAVYYCQQTGRIPPTFGQGTKVEIKRTVAA
 PSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQ
 DSKDSTYLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC

- xii. COVA14136 was generated by co-expression of the anti-RSV B21M antibody heavy chain carrying a C-terminal stapled scFv BHA10 (VH-VL orientation SEQ ID NO: 77) fusion (SEQ ID NO: 99, comprising SEQ ID NO: 100) with the heavy chain (HC; SEQ ID NO: 80) and light chain (LC; SEQ ID NO: 81) of an anti-EDB antibody EDBmAb1 (**FIG. 15L**).

SEQ ID NO: 77 [stapled scFv BHA10 (VH-VL)]

QVQLVQSGAEVKKPGSSVKVSCKASGYTFTTYLHWVRQAPGCGLEWMGWIY
 PGNVHAQYNEKFKGRVTITADKSTSTAYMELSSLRSEDTAVYYCARSWEGFPY
 WGQGTTVTVSSGGSGGGSGCPPCGGGGDIQMTQSPSSLSASVGDRTITCKAS
 QNVGINVAWYQQKPGKAPKSLISSASYRYSRVPSRFSGSGSGTDFLTISLQPED
 FATYFCQQYDTPFTFGCGTKVEIK

SEQ ID NO: 99 (HC B21M C-term stapled BHA10 (VH-VL), IgG1s, knob, no pA mutations)

QITLKESGPTLVKPTQTLTLCTFSGFSLSTSGMGVSWIRQPPGKALEWLAHIYW
 DDDKRYNPSLKSRLTITKDTSKNQVVLMTNMDPVDTATYYCARLYGFTYGFA
 YWGQGTTLTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWN
 SGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKK
 VEPKSCDKTHTCPPCPAPEAAGASSVFLFPPKPKDTLMISRTPEVTCVVVDVSAE

DPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKC
KVSNAKALPSSIEKTISKAKGQPREPQVYTLPPCREEMTKNQVSLWCLVKGFYPSD
IAVEWESNGQPENNYKTTTPVLDSGDSFLLYSKLTVDKSRWQQGNVFCFSVMHE
ALHNHYTQKSLSLSPGKGGGGSGGGSGGGGSQVQLVQSGAEVKKPGSSVKVS
CKASGYTFTTYLHWVRQAPGCGLEWMGWIYPGNVHAQYNEKFKGRVTITAD
KSTSTAYMELSSLRSEDTAVYYCARSWEGFPYWGQGTTVTVSSGGGSGGGSGC
PPCGGGGDIQMTQSPSSLSASVGDRTITCKASQNVGINVAWYQQKPKAPKSLI
SSASYRYSQVPSRFSGSGSGTDFTLTISSLQPEDFATYFCQQYDTPFTFGCGTKV
EIK

SEQ ID NO: 100 (HC B21M (RSV) IgG1s knob)

QITLKESGPTLVKPTQTLTLCTFSGFSLSTSGMGVSWIRQPPGKALEWLAHIYW
DDDKRYNPSLKSRLTITKDTSKNQVVLMTNMDPVDTATYYCARLYGFTYGFA
YWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWN
SGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKK
VEPKSCDKTHTCPPCPAPEAAGASSVFLFPPKPKDTLMISRTPEVTCVVDVSAE
DPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKC
KVSNAKALPSSIEKTISKAKGQPREPQVYTLPPCREEMTKNQVSLWCLVKGFYPSD
IAVEWESNGQPENNYKTTTPVLDSGDSFLLYSKLTVDKSRWQQGNVFCFSVMHE
ALHNHYTQKSLSLSPGK

SEQ ID NO: 80 (HC B21M (RSV) IgG1s hole)

QITLKESGPTLVKPTQTLTLCTFSGFSLSTSGMGVSWIRQPPGKALEWLAHIYW
DDDKRYNPSLKSRLTITKDTSKNQVVLMTNMDPVDTATYYCARLYGFTYGFA
YWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWN
SGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKK
VEPKSCDKTHTCPPCPAPEAAGASSVFLFPPKPKDTLMISRTPEVTCVVDVSAE
DPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKC
KVSNAKALPSSIEKTISKAKGQPREPQVCTLPPSREEMTKNQVSLSCAVKGFYPSDI
AVEWESNGQPENNYKTTTPVLDSGDSFLLYSKLTVDKSRWQQGNVFCFSVMHE
ALHNHYTQKSLSLSPGK

SEQ ID NO: 81 [LC B21M (RSV)]

DIVMTQSPDSLAVSLGERATINCRASQSVDYNGISYMHWYQQKPGQPPKLLIYA
 ASNPESGVPDRFSGSGSGTDFTLTISSLQAEDVAVYYCQQIIEDPWTFGQGTKVEI
 KRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQ
 ESVTEQDSKDSTYLSSTLTLSKADYEEKHKVYACEVTHQGLSSPVTKSFNRGEC

- xiii. COVA14146 was generated by co-expression of an anti-mesothelin antibody MSLNmAb1 heavy chain carrying a C-terminal stapled scFv BHA10 (VH-VL orientation SEQ ID NO: 77) fusion (SEQ ID NO: 101, comprising SEQ ID NO: 102) with the heavy chain (HC; SEQ ID NO: 103) and light chain (LC; SEQ ID NO: 104) of an anti-mesothelin antibody MSLNmAb1 (**FIG. 15M**).

SEQ ID NO: 77 [stapled scFv BHA10 (VH-VL)]

QVQLVQSGAEVKKPGSSVKVSCKASGYTFTTYLHWVRQAPGCGLEWMGWIIY
 PGNVHAQYNEKFKGRVTITADKSTSTAYMELSSLRSEDVAVYYCARSWEGFPY
 WGQGTTTVTVSSGGSGGGSGCPPCGGGGDIQMTQSPSSLSASVGDRVTITCKAS
 QNVGINVAWYQQKPGKAPKSLISSASYRYSRVPSRFSGSGSGTDFTLTISSLQPED
 FATYFCQQYDTYPFTFGCGTKVEIK

SEQ ID NO: 101 (MSLNmAb1 HC C-term stapled BHA10 (VH-VL), IgG1s, knob, with pA mutations)

QVQLQQSGPELEKPGASVKISCKASGYSTGYTMNWVKQSHGKSLEWIGLITPY
 NGASSYNQKFRGKATLTVDKSSSTAYMDLLSLTSEDSAVYFCARGGYDGRGFD
 YWGSCTPVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNS
 GALTSGVHTFPAVLQSSGLYSLSSVTVPSSSLGTQTYICNVNHKPSNTKVDKKV
 EPKSCDKTHTCPPCPAPEAAGASSVFLFPPKPKDTLMISRTPEVTCVVVDVSAEDP
 EVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKV
 SNKALPSSIEKTISKAKGQPREPQVYTLPPCREEMTKNQVSLWCLVKGFYPSDIA
 VEWESNGQPENNYKTTTPVLDSGDSFFLYSKLTVDKSRWQQGNVFSVSMHEA
 LHNRFQKSLSLSPGKGGGSGGGSGGGGSQVQLVQSGAEVKKPGSSVKVSC
 KASGYTFTTYLHWVRQAPGCGLEWMGWIIYPGNVHAQYNEKFKGRVTITADK
 STSTAYMELSSLRSEDVAVYYCARSWEGFPYWGQGTTTVTVSSGGSGGGSGCPP
 CGGGGDIQMTQSPSSLSASVGDRVTITCKASQNVGINVAWYQQKPGKAPKSLISS

ASYRYSGVPSRFSGSGSGTDFTLTISSLQPEDFATYFCQQYDTPFTFGCGTKVEIK

SEQ ID NO: 102 (MSLNmAb1 HC, IgG1s, knob, with pA mutations)

QVQLQQSGPELEKPGASVKISCKASGYSTGYTMNWVKQSHGKSLEWIGLITPY
NGASSYNQKFRGKATLTVDKSSSTAYMDLLSLTSEDSAVYFCARGGYDGRGFD
YWGSGTPVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNS
GALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKV
EPKSCDKTHTCPPCPAPEAAGASSVFLFPPKPKDTLMISRTPEVTCVVVDVSAEDP
EVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKV
SNKALPSSIEKTISKAKGQPREPQVYTLPPCREEMTKNQVSLWCLVKGFYPSDIA
VEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEA
LHNRFQKSLSLSPGK

SEQ ID NO: 103 (HC MSLNmAb1 IgG1s hole)

QVQLQQSGPELEKPGASVKISCKASGYSTGYTMNWVKQSHGKSLEWIGLITPY
NGASSYNQKFRGKATLTVDKSSSTAYMDLLSLTSEDSAVYFCARGGYDGRGFD
YWGSGTPVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNS
GALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKV
EPKSCDKTHTCPPCPAPEAAGASSVFLFPPKPKDTLMISRTPEVTCVVVDVSAEDP
EVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKV
SNKALPSSIEKTISKAKGQPREPQVCTLPSSREEMTKNQVSLSCAVKGFYPSDIAV
EWESNGQPENNYKTTPPVLDSDGSFFLVSKLTVDKSRWQQGNVFSCSVMHEAL
HNHYTQKSLSLSPGK

SEQ ID NO: 104 (LC MSLNmAb1)

DIELTQSPAIMSASPGEKVTMTCSASSSVSYMHYQQKSGTSPKRWIYDTSKLAS
GVPGRFSGSGSGNSYSLTISVVEAEDDATYYCQQWSKHPLTFGSGTKVEIKRTVA
APSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTE
QDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC

[00709] All constructs described above could be expressed and purified with high yield and purity (see **Table 11** below), indicating that the bispecific constructs incorporating the spFv provided herein have good biophysical properties.

Table 11: Yield and purities of selected EBD/LTBR bispecifics

Bispecific name	Description	Yield [mg/L]	Purity [% monomer]
COVA1482	2:1 heterodimer consisting of EDBmAb1 HC fused to stapled scFv BHA10 (VH-VL) (contains mutation in the Fc that abrogates binding to Protein A to facilitate purification of heterodimer) paired with EDBmAb1 HC and EDBmAb1 LC	53	100
COVA14133	2:1 heterodimer consisting of EDBmAb1 HC fused to stapled scFv BHA10 (VH-VL) paired with EDBmAb1 HC and EDBmAb1 LC	64.3	100

6.7 Example 7: EDB dependent *in vitro* LTBR activation – NF-κB Luciferase reporter assay

[00710] To show that the EBD/LTBR bispecifics are able to activate LTBR in an EDB-dependent way, the activity of the compounds was tested in an A549 cell NF-κB luciferase reporter assay in the presence or absence of EDB containing fibronectin (EDB+ fibronectin). NF-κB signaling plays a pivotal role in regulating cell development and immune homeostasis. Activation of NF-κB through tumor necrosis factor receptors (TNFR) or the TNFR superfamily members (*e.g.*, LTBR) occurs upon engagement with their respective ligands. The A549 lung epithelial cell line naturally expresses LTBR and the NF-κB luciferase reporter construct is stably integrated into the genome of the A549 lung epithelial cell line. Following activation by stimulants, endogenous NF-κB transcription factors bind to the DNA response elements to induce transcription of the luciferase gene.

[00711] To demonstrate EDB-dependent activation of LTBR, high binding 96-well μClear flat bottom plates (Greiner; Monroe, NC) were coated overnight with 150 ng/well human recombinant EDB+ fibronectin domains 7-B-8-9 (EDB+; SEQ ID NO: 105) or 150 ng/well human recombinant fibronectin domains 7-8-9 (EDB-; SEQ ID NO: 106), which sequences are listed below.

SEQ ID NO: 105 (fibronectin domains 7B89)

PLSPPTNLHLEANPDTGVLTVSWEKSTTPDITGYRITTTPTNGQQGNSLEEVVHAD
QSSCTFDNLSPGLEYNVSVYTVKDDKESVPISDTIPEVPQLTDL SFVDITDSSIGLR
WTPLNSSTIIGYRITVVAAGEGIPIFEDFVDSSVGYYTVTGLEPGIDYDISVITLING

GESAPTTLTQQTAVPPPTDLRFTNIGPDTMRVTWAPPPSIDLTNFLVRYSPVKNEE
 DVAELISPSDNAVVLTNLLPGTEYVVS SVSSVYEQHESTPLRGRQKTGLDSPTGID
 FSDITANSFTVHWIAPRATITGYRIRHHPEHFSGRPREDRVPHSRNSITLTNLTPGT
 EYVVSIVALNGREESPLLIGQQSTHHHHHH

SEQ ID NO: 106 (fibronectin domains 789)

PLSPPTNLHLEANPDTGVLTVSWERSTTPDITGYRITTTPTNGQQGNSLEEVVHAD
 QSSCTFDNLSPGLEYNVSVYTVKDDKESVPISDTIIPAVPPPTDLRFTNIGPDTMRV
 TWAPPPSIDLTNFLVRYSPVKNEEDVAELISPSDNAVVLTNLLPGTEYVVS SVSSV
 YEQHESTPLRGRQKTGLDSPTGIDFSDITANSFTVHWIAPRATITGYRIRHHPEHFS
 GRPREDRVPHSRNSITLTNLTPGTEYVVSIVALNGREESPLLIGQQSTHHHHHH

[00712] After overnight incubation, the coated plates were washed with PBS and blocked for 2 hours at 37°C with assay medium (DMEM + 10% heat inactivated FBS). A 1:5 dilution series of the compounds to be tested was prepared in assay medium as 2-fold concentration stocks (final concentrations tested ranged from 200 nM to 2.6 pM). 50 µl of diluted compounds were added to the pre-blocked plate after the blocking solution was removed by aspiration. 50 µl of a A549 cell suspension (concentration of cell suspension = 0.4 Mio cells /ml assay medium) were added to each well (20,000 cells/well). A549 cells were previously detached from cell culture flask by using Accutase/EDTA and were then transferred in assay medium. Cells were incubated with the compounds for 18-20 hours at 37°C/5% CO₂.

[00713] After incubation for 18 hours, the BIO-GLO Luciferase Assay System (Promega; Madison, WI) was used to detect luciferase activity. Luminescence was measured using a TECAN M1000 Pro instrument with an integration time of 500 milliseconds. From the resulting relative light units (RLU), the fold induction of LTBR signaling was calculated as follows: Fold induction = $RLU_{\text{stimulated cells}} / \text{average } RLU_{\text{unstimulated cells}}$ (unstimulated cells were included as control in each plate tested).

[00714] Dose response curves, including standard deviations, were plotted using GRAPHPAD Prism, and non-linear fits were applied (log(agonist) vs. response (variable slope - three parameters)), if applicable. In order to fit the data, the x-values (concentrations of compounds) were transformed using the $X = \text{Log}(x)$ function of GRAPHPAD Prism.

[00715] COVA1482 was compared in the same A549 NF- κ B reporter assay to COVA1456. COVA1482 differs from COVA1456 only in the stabilization method used for the scFv. The scFv in COVA1482, which was also derived from LTBRmAb1, was stabilized using the stapled platform described herein (*i.e.*, stabilized via VH/linker and VL linker disulfide bond), wherein COVA1456 is disulfide stabilized between the VH and the VL (*i.e.*, stabilized via VH/VL disulfide bond). **FIG. 16A** showed that both COVA1482 and COVA1456 potently activated LTBR in an EDB dependent way. The corresponding isotype controls COVA1486 and COVA1462 did not activate LTBR (**FIG. 16A**). These results demonstrate that incorporation of spFv into a multispecific molecule had no negative impact on the activity of the multispecific molecule. The 2:1 bispecific EDB/LTBR antibodies (COVA1482 or COVA1456) showed increased potency in inducing NF- κ B signaling in this reporter assay. The average EC₅₀ calculated for COVA1482 over several assays with the same experimental set up was of ca. 30 pM $\pm$ 10 pM, whereas a control 1:1 heterodimer construct showed an EC₅₀ of ca. 3 nM in the assay (data not shown here), indicating that the 2:1 bispecifics can be 100 times more potent than 1:1 bispecifics. This could be explained by increased clustering of the LTBR binding site achieved with 2 binding sites to the TAA.

[00716] To study the effects of affinity to LTBR on the ability of such bispecifics to TAA-dependently activate LTBR, lower affinity variants (SEQ ID NO: 107, KD $\approx$ 60 nM and SEQ ID NO: 108, KD $\approx$ 600 nM) of the scFv fragment derived from LTBRmAb1 were generated and used to construct 2:1 bispecifics COVA14107 and COVA14108 (see **Table 9**).

SEQ ID NO: 107 [stapled scFv (VL3_Y36F_S49Y_F87Y) BHA10 (VH-VL)]

QVQLVQSGAEVKKPGSSVKVSCKASGYTFTTYLHWVRQAPGCGLEWMGWIY
PGNVHAQYNEKFKGRVTITADKSTSTAYMELSSLRSEDTAVYYCARSWEGFPY
WGQGTTVTVSSGGSGGGSGCPPCGGGGDIQMTQSPSSLSASVGDRVTITCKAS
QNVGINVAWFQKPGKAPKSLIYSASYRYSRVPSRFSGSGSGTDFLTISLQPED
FATYYCQQYDTYPFTFGCGTKVEIK

SEQ ID NO: 108 [stapled scFv (VH_CDR1_Y33A) BHA10 (VH-VL)]

QVQLVQSGAEVKKPGSSVKVSCKASGYTFTTYALHWVRQAPGCGLEWMGWIY
PGNVHAQYNEKFKGRVTITADKSTSTAYMELSSLRSEDTAVYYCARSWEGFPY

WGQGTTVTVSSGGGSGGGSGCPPCGGGGDIQMTQSPSSLSASVGDRVITITCKAS
 QNVGINVAWYQQKPGKAPKSLISSASYRYSQVPSRFSGSGSGTDFTLTISSLQPED
 FATYFCQQYDTYPFTFGCGTKVEIK

[00717] The generated bispecifics were tested in the A549 NF- κ B reporter assay to see the effects of affinity on activation of LTBR. **FIG. 16B** showed that lower affinity to LTBR corresponded to lower ability of the bispecific to activate LTBR in a TAA-dependent manner in this assay. The data also demonstrated that incorporation of spFv into a multispecific molecules had no negative impact on the activity of the molecule.

[00718] As mentioned in Example 6, mutations (WO2010151792) to abrogate binding to protein A (used for purification of antibodies) were introduced in the Fc of some constructs in order to facilitate the purification of the desired heterodimer. COVA14133 was generated without these mutations, and its activity was compared to COVA1482 to show that the mutations in the Fc region did not influence the activity of the bispecific. COVA14133 and COVA1482 and their respective isotype controls COVA14136 and COVA1486 were compared in the A549 NF- κ B reporter assay. **FIG. 16C** showed that COVA14133 activated LTBR in a TAA-dependent manner with a similar efficiency as COVA1482, demonstrating that the mutations in the Fc did not influence the ability of the bispecific to activate LTBR and had no effect on functioning of the spFv.

[00719] In conclusion, COVA14133 was shown to have excellent ability to activate LTBR in a TAA-dependent manner.

6.8 Example 8: EDB dependent *in vitro* LTBR activation – A375/WI38VA subline2RA co-culture cell assay

[00720] The A375/WI38VA subline2RA co-culture assay was performed to verify if activation of LTBR in the presence of EDB+ fibronectin (produced and deposited in the extracellular matrix by WI38VA cells (Zardi, L., *et al.*, *EMBO J*, 6, 2337-42 (1987)) leads to the release of cytokines and chemokines and upregulation of the adhesion molecule ICAM-1 on the A375 cells and that incorporation of spFv has no negative impact on activity. WI38VA subline2RA (ATCC[®] CCL75.1[™]) cells were seeded in a 96 well plate at a density of 5000 cells/well and incubated for 48 hours in their growth medium (MEM w/o Glutamine + 10% heat inactivated FBS + 0.1 mM NEAA + 2 mM L-Gln + 1 mM Sodium pyruvate) at 37°C/5% CO₂. A 1:5 dilution series of the compounds to be tested was prepared in assay medium (DMEM + 10% heat

inactivated FBS) as 2-fold concentration stocks (final concentrations tested ranged from 40 nM to 0.5 pM). Prior to incubation in the co-culture with the WI38VA subline2RA cells, A375 cells (ATCC® CRL-1619™) were labeled with CELLTRACE violet (CTV, Invitrogen; Carlsbad, CA). For labeling, a cell suspension, with a concentration of 10×10^6 cells/ml and 2.5 μ M CTV in 5% FBS in PBS, was incubated for 5 minutes at RT while protected from light. Cells were then washed and resuspended in assay medium at a density of 0.4×10^6 cells/ml. Careful removal of culture medium from the plate containing the 48 hours WI38VA subline2RA culture, was followed by addition of 50 μ l A375 cell suspension (20,000 cells/well; CTV+ or CTV-) in each well. 50 μ l of the serial diluted compounds (final volume per well 100 μ l) were added to the cells and incubated 24 hours at 37°C/5% CO₂.

[00721] After incubation for 24 hours, the supernatants were cleared by centrifugation and stored for measurement of cytokines and chemokines using MSD assays. The cells were further processed for ICAM-1 measurement by flow cytometry.

6.8.1 Detection of ICAM-1 by flow cytometry

[00722] Any media left in the 96-well plate was carefully removed, cells were detached with Accutase, transferred to a DeepWell 96-well plate (triplicates were pooled in 1 well), washed, resuspended in 100 μ l FACS buffer (PBS + 1 % FBS + 0.1 % NaN₃) and transferred to a round bottom 96-well plate. Antibody, *i.e.*, anti-human ICAM-1 PE labeled (clone 1H4, Thermo; Waltham, MA) or isotype control antibody PE labeled (MPC-11, BioLegend; San Diego, CA) and LIVE/DEAD fixable near-IR stain (Invitrogen), single staining or combination staining was diluted as shown in **Table 12**.

Table 12: Dilution scheme of single staining or combo staining in FACS buffer

	Antibody or stain	Final concentration used (μ g/ml)	Dilution factor
Single stainings	ICAM-1	1.25	20
	Isotype control	1.25	160
	LIVE/DEAD	-	400
Combo staining ICAM-1+LIVE/DEAD	ICAM-1	1.25	20
	LIVE/DEAD	-	400

[00723] Cells were centrifuged at 400 x g at 4°C for 4 minutes, the supernatant was discarded, and 50 μ l antibody solutions were prepared as described in **Table 12**. Cells and antibodies were

incubated in the dark at 4°C for 30 minutes. After incubation, 120 µl were added in each well, and the cells were then centrifuged at 400 x g at 4°C for 4 minutes. Cells were washed once with FACS buffer, centrifuged and resuspended in 90 µl FACS buffer. Cells were then fixed by adding 90 µl of a 3.7% Formalin solution in PBS and were incubated for 15 minutes on ice in the dark. After fixation, cells were centrifuged at 400 x g at 4°C for 4 minutes and resuspended in 100 µl FACS buffer. Cells were measured using a MACS Quant instrument at a high flowrate in screen mode, 49 µl/well were acquired. Data were analyzed using the FLOWLOGICS Software (Version 700.2A) and plotted with GRAPHPAD Prism.

6.8.2 Cytokine measurement in the supernatants of treated cells using MSD platform

[00724] Several cytokines that are known to be under the control of NF-κB signaling were measured using the MSD platform and multiplex MSD plates. Listed here are some examples of measured cytokines:

- RANTES: using R-Plex Antibody Set human RANTES (MSD);
- I-TAC, IP-10, MIP-3b: using 3-PLEX cytokine release assay (MSD);
- IL-8, IP-10, MIP-3b: using 3-PLEX cytokine release assay (MSD); and
- IL-12p70, IL-6, TNF-α, MIP-3a, SDF-1a: using 5-PLEX cytokine release assay (MSD)

[00725] The concentration of cytokines in the supernatant of treated cells was measured using the MSD platform following the manufacturer's instructions. Briefly, the protocol involved following steps:

(1) Preparation of the plate involved coating the provided plate with the linker-coupled capture antibodies. Plates were incubated with shaking overnight at 2-8°C. On the following day, plates were washed with PBST (PBS plus 0.05% Tween-20) using a plate washer (Biotek; Winooski, VT);

(2) Preparation of calibrator standard and detection antibody solution;

(3) Supernatants were diluted 1:3 or 1:5 depending on availability of material.

6.8.3 Assay protocol:

- Step 1: The sample or calibrator standard was added to the plate, and the plate was incubated at RT for 1 hour while shaking;
- Step 2: The plates were washed, and the detection antibody was added. The plates were incubated with shaking for 1 hour at RT

- Step 3: The plates were washed and 2x read buffer T was added. The plate was analyzed on an MSD instrument

Data were analyzed using MESOSCALE Software (MSD discovery work bench program v 4.0.12.1) and plotted using GRAPHPAD Prism.

6.8.4 Results - Detection of ICAM-1 by flow cytometry

[00726] It was previously shown that NF- κ B signaling can lead to upregulation of ICAM-1 on the surface of cells (da Silva Antunes, *et al. Front Immunol*, 9:576, (2018)). Therefore, the levels of ICAM-1 expression on the surface of A375 cells after co-culture incubation with EDB/LTBR bispecifics were measured. As an example, **FIG. 17** shows the upregulation of ICAM-1 after incubation with the EDB/LTBR bispecific COVA1482, demonstrating functionality of the LTBR binding spFv. The isotype control molecule COVA1486 did not cause upregulation of ICAM-1. These findings indicated that the ability to cluster the LTBR scFv via binding to EDB was a prerequisite for LTBR activation, and as a consequence, ICAM-1 upregulation.

6.8.5 Results - Cytokine measurement in the supernatants of treated cells

[00727] Several cytokines and chemokines, that are expressed as a result of LTBR activation were measured in the supernatant of the co-cultures that were treated with EDB/LTBR bispecifics and control molecules as described above. **FIGS. 18A-18D** shows 4 representative examples of cytokines (**FIG. 18A**: RANTES, **FIG. 18B**: IL-6, **FIG. 18C**: IL-8 and **FIG. 18D**: MIP-3b) that were upregulated by activation of LTBR with COVA14133. The untargeted LTBRmAb1 derived scFv in COVA14136 did not activate LTBR, and as a consequence, the concentration of cytokines in the supernatant was not increased above background. The background was represented by the level achieved with the B21M antibody or COVA1440 (a 2(mAb) B21M isotype control mAb) (shown as a single concentration in the plots). The results demonstrate that LTBR binding spFv is functional *in vitro*.

[00728] Taken together, ICAM-1 upregulation and cytokine secretion upon LTBR activation confirmed the expected effects on cells that LTBR activation can have.

[00729] In this example, it was demonstrated that the molecules provided herein achieved efficient tumor associated antigen (here EDB-containing fibronectin) dependent activation of LTBR.

6.9 Example 9: Mesothelin-dependent *in vitro* LTBR activation –co-culture cell assay with A549 NF- κ B reporter cells and CHOK1-huMSLN or H226

[00730] In the Examples 7 and 8, it was demonstrated that bispecific antibodies comprising spFv structures provided herein, targeting EDB (a tumor associated antigen in the extracellular matrix) and LTBR, activated LTBR very efficiently in a tumor antigen dependent way. In order to verify if this finding holds true for any tumor antigen despite its location (deposited in the extracellular matrix or on the cell surface of tumor cells), a bispecific 2:1 antibody targeting mesothelin (MSLN), a tumor associated antigen expressed on different types of tumor (Hassan and Ho, *Eur. J. Cancer*, 44:46-53 (2008)) and LTBR was designed and produced as described in Example 6. COVA14146 is a 2:1 MSLN/LTBR bispecific antibody consisting of an anti-mesothelin antibody (MSLNmAb1) fused to a spFv fragment derived from LTBRmAb1. To show if a LTBR bispecific antibody targeting LTBR and a tumor associated antigen present of the cell surface of tumor cells (*e.g.*, mesothelin) was able to efficiently activate LTBR in a tumor-dependent way, a co-culture cell assay was used. The co-culture assays used were the A549 cell NF- κ B luciferase reporter cell assay (described in Example 7) and H226 cells (mesothelioma cell line; ATCC® CRL-5826) known to express mesothelin (Fan *et al. Mol. Canc. Ther.* 1:595–600 (2002)) and LTBR.

6.9.1 Preparation of H226 cells

[00731] 10,000 cells per well (in 75 μ l assay medium: DMEM + 10 % FBS-HI) of a H226 cell (express about 200,000 copies of mesothelin and 10,000 copies of LTBR) suspension were seeded in a 96-well tissue culture plate and were incubated for 6 hours at 37 °C/5 % CO₂ in their growth media (MEM + 2 mM Glutamine + 10% FBS-HI + 10 μ g/ml Puromycin and RPMI-1640 + 10% FBS + 1mM Na-Pyruvate respectively) to allow the cells to attach to the plate.

6.9.2 Preparation of compounds

[00732] The compounds were tested in a concentration range from 100 nM down to 1.3 pM. A 4-fold 1 in 5 serial dilution of the compounds was prepared in assay medium (DMEM + 10 % FBS-HI) and stored at 4°C until use.

6.9.3 Preparation and addition of A549 reporter cells

[00733] A549 reporter cells were detached from the cell culture flask with Accutase/EDTA and transferred in to assay medium (DMEM + 10 % FBS-HI). After adding a total of 20,000

A549 reporter cells per well to the plates containing H226 cells, 50 μ L of the pre-diluted compounds were added to each well and incubated for 20 hrs. at 37 °C/ 5% CO₂.

6.9.4 Measurement of Luminescence in treated co-cultures

[00734] After incubation for 20 hours, the BIO-GLO Luciferase Assay System (Promega; Madison, WI) was used according to manufacturer's instructions to detect luciferase activity. Luminescence was measured using a TECAN M1000 Pro instrument with an integration time of 500 milliseconds. From the resulting relative light units (RLU), the fold induction of LTBR signaling was calculated as follows: Fold induction = $RLU_{\text{stimulated cells}} / \text{average } RLU_{\text{unstimulated cells}}$ (unstimulated cells were included as control in each plate tested).

[00735] Dose response curves, including standard deviations, were plotted using GRAPHPAD Prism, and non-linear fits were applied (log(agonist) vs. response (variable slope - three parameters)), if applicable. In order to fit the data, the x-values (concentrations of compounds) were transformed using the $X = \text{Log}(x)$ function of GRAPHPAD Prism.

6.9.5 Cytokine measurement in the supernatants of treated cells using MSD platform

[00736] Several cytokines known to be under the control of NF- κ B signaling can be measured using the MSD platform and multiplex MSD plates. As an example, the method for the measurement of RANTES using R-Plex Antibody Set human RANTES (MSD) is described herein.

[00737] The concentration of RANTES in the supernatant of treated cells was measured using the MSD platform following the manufacturer's instructions. Briefly, the protocol involved following steps:

- (1) Preparation of the plate involved coating the provided plate with the linker-coupled capture antibodies. Plates were incubated with shaking overnight at 2-8°C. On the following day, plates were washed with PBST (PBS plus 0.05% Tween-20) using a plate washer (Biotek; Winooski, VT);
- (2) Preparation of calibrator standard and detection antibody solution;
- (3) Supernatants were diluted 1:3 or 1:5 depending on availability of material.

6.9.6 Assay protocol:

- Step 1: The sample or calibrator standard was added to the plate, and the plate was incubated at RT for 1 hour while shaking;

- Step 2: The plates were washed, and the detection antibody was added. The plates were incubated with shaking for 1 hour at RT
- Step 3: The plates were washed and 2x read buffer T was added. The plate was analyzed on an MSD instrument

Data were analyzed using MESOSCALE Software (MSD discovery work bench program v 4.0.12.1) and plotted using GRAPHPAD Prism.

6.9.7 Results – Mesothelin-dependent activation of LTBR in A549 reporter cells / H226 co-culture assay

[00738] A co-culture assay with A549 reporter cells and H226 cells was performed to verify if COVA14146 was able to activate LTBR in a more physiological system, where, due to its broad expression (Lukashev, *et al. Cancer Res.*, 66(19):9617-24 (2006), LTBR and mesothelin (and other tumor associated antigens on the cell surface of tumor cells, *e.g.*, EGFR) are expected to be co-expressed on the cell surface of tumor cells. In **FIG. 19A**, it was shown that under these conditions COVA14146 did not activate LTBR efficiently. The concentrations of RANTES secreted in the supernatants of treated cells were measured to confirm the inability of COVA14146 to efficiently activate LTBR. As expected, **FIG. 19B** shows that RANTES was secreted by cells treated with COVA14146 to same extent as from cells that were treated with the isotype control molecule COVA1486, confirming that LTBR cannot be activated under these conditions.

6.10 Example 10: Bispecific antibodies specifically binding to LTBR and other TAAs present in the extracellular matrix

[00739] The examples above demonstrated that bispecific antibodies targeting LTBR and a TAA expressed in the extracellular matrix, in that case EDB of fibronectin, can activate LTBR selectively in tumor-tissue that expresses EDB. This example demonstrated that this is more generally applicable to bispecific antibodies that target LTBR and another TAA present in the extracellular matrix, by providing two more examples of such TAAs, namely domain A2 of tenascin C, and extra domain A of fibronectin.

[00740] Generally following the methods described in the examples above, further bispecific antibodies that bind to LTBR and other TAAs present in the extracellular matrix, namely domain A2 of tenascin C (TnCA2) or extra domain A of fibronectin (EDA), were prepared. The sequence of these TAAs have, for instance, been described in:

- TnCA2: UniProt Accession number P24821.3;
- EDA: UniProt Accession number P02751.

[00741] In addition, antibodies against these TAAs have, for instance, been previously described in:

- TnCA2: WO2011/020783;
- EDA: EP2142567.

[00742] Exemplary sequences of the binding domains against these targets used for the present example (by combining with an LTBR scFv to create a multispecific binding molecule of the invention) are provided as:

- TnCA2: VH SEQ ID NO: 109 and VL SEQ ID NO: 110;
- EDA: VH SEQ ID NO: 111 and VL SEQ ID NO: 112.

[00743] These sequences are as follows:

SEQ ID NO: 109 (VH of Anti-TnCA2 Ab 2B10)

QVQLVQSGAEVKKPGSSVKVSCKASGGTFSSYAIWVRQAPGQGLEWMGGIPIF
GTANYAQKFQGRVTITADKSTSTAYMELSSLRSEDYAVYYCARLYGYAYYGAF
DYWGQGTTVTVSS

SEQ ID NO: 110 (VL of Anti-TnCA2 Ab 2B10)

DIQMTQSPSSLSASVGDRVTITCRASQGIRNDLGWYQQKPGKAPKRLIYAASSLQ
SGVPSRFSGGSGTEFTLTISLQPEDFATYYCLQNGLQPATFGQGTKVEIK

SEQ ID NO: 111 (VH Anti- EDA Ab F8)

EVQLLESGGGLVQPGGSLRLSCAASGFTFSLFTMSWVRQAPGKGLEWVSAISGS
GGSTYYADSVKGRFTISRDN SKNTLYLQMNSLRAEDTAVYYCAKSTHLYLFDY
WGQGTLLVTVSS

SEQ ID NO: 112 (VL Anti-EDA Ab F8)

EIVLTQSPGTLSPGERATLSCRASQSVMPFLAWYQQKPGQAPRLLIYGASSRA
TGIPDRFSGSGSGTDFTLTISRLEPEDFAVYYCQQMRGRPPTFGQGTKVEIK

[00744] Bispecific antibodies that bind to LTBR and these TAAs were prepared and tested for TAA-specific activation of LTBR in a TAA-dependent *in vitro* LTBR activation – NF-κB Luciferase reporter assay following a similar procedure as described in Example 7.

[00745] Asymmetric antibodies, with 2:1 stoichiometry were generated as follows:

[00746] COVA14198 was generated by co-expression of an anti-EDA antibody heavy chain carrying a C-terminal stapled scFv BHA10 (VH-VL orientation SEQ ID NO: 77) fusion (SEQ ID NO: 113, comprising SEQ ID NO: 114) with the heavy chain (HC; SEQ ID NO: 115) and light chain (LC; SEQ ID NO: 116) of an anti-EDA antibody. The sequences are listed below.

SEQ ID NO: 77 [stapled scFv BHA10 (VH-VL)]

QVQLVQSGAEVKKPGSSVKVSCKASGYTFTTYLHWVRQAPGCGLEWMGWIY
PGNVHAQYNEKFKGRVTITADKSTSTAYMELSSLRSEDNAVYYCARSWEGFPY
WGQGTTVTVSSGGGSGGGSGCPPCGGGGDIQMTQSPSSLSASVGDRVTITCKAS
QNVGINVAWYQQKPGKAPKSLISSASYRYSQVPSRFSGSGSGTDFLTITSLQPED
FATYFCQQYDTYPFTFGCGTKVEIK

SEQ ID NO: 113 (F8 HC with BHA10 stapled (VH-VL) scFv C-ter fused, IgG1 sigma, knob mutations)

EVQLLESGLLVQPGGSLRLSCAASGFTFSLFTMSWVRQAPGKGLEWVSAISGS
GGSTYYADSVKGRFTISRDNSTNTLYLQMNSLRAEDNAVYYCAKSTHLYLFDY
WGQGTLLTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNS
GALTSGVHTFPAVLQSSGLYSLSSVTVPSSSLGTQTYICNVNHKPSNTKVDKKV
EPKSCDKTHTCPPCPAPEAAGASSVFLFPPKPKDTLMISRTPEVTCVVVDVSAEDP
EVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKV
SNKALPSSIEKTISKAKGQPREPQVYTLPPCREEMTKNQVSLWCLVKGFYPSDIA
VEWESNGQPENNYKTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEA
LHNHYTQKSLSLSPGKGGGSGGGGSGGGGSQVQLVQSGAEVKKPGSSVKVSC
KASGYTFTTYLHWVRQAPGCGLEWMGWIYPGNVHAQYNEKFKGRVTITADK
STSTAYMELSSLRSEDNAVYYCARSWEGFPYWGQGTTVTVSSGGGSGGGSGCPP
CGGGGDIQMTQSPSSLSASVGDRVTITCKASQNVGINVAWYQQKPGKAPKSLISS

ASYRYSGVPSRFSGSGSGTDFTLTISSLQPEDFATYFCQQYDTPFTFGCGTKVEIK

SEQ ID NO: 114 (F8 HC, IgG1 sigma, knob mutations)

EVQLLESGGGLVQPGGSLRLSCAASGFTFSLFTMSWVRQAPGKGLEWVSAISGS
GGSTYYADSVKGRFTISRDN SKNTLYLQMNSLRAEDTAVYYCAKSTHL YLFDY
WGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNS
GALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKV
EPKSCDKTHTCPPCPAPEAAGASSVFLFPPKPKDTLMISRTPEVTCVVVDVSAEDP
EVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKV
SNKALPSSIEKTISKAKGQPREPQVYTLPPCREEMTKNQVSLWCLVKGFYPSDIA
VEWESNGQPENNYKTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEA
LHNHYTQKSLSLSPGK

SEQ ID NO: 115 (F8 HC, IgG1 sigma and hole mutations)

EVQLLESGGGLVQPGGSLRLSCAASGFTFSLFTMSWVRQAPGKGLEWVSAISGS
GGSTYYADSVKGRFTISRDN SKNTLYLQMNSLRAEDTAVYYCAKSTHL YLFDY
WGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNS
GALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKV
EPKSCDKTHTCPPCPAPEAAGASSVFLFPPKPKDTLMISRTPEVTCVVVDVSAEDP
EVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKV
SNKALPSSIEKTISKAKGQPREPQVCTLPSSREEMTKNQVSLSCAVKGFYPSDIAV
EWESNGQPENNYKTPPVLDSDGSFFLVSKLTVDKSRWQQGNVFSCSVMHEAL
HNHYTQKSLSLSPGK

SEQ ID NO: 116 (F8 LC)

EIVLTQSPGTLSPGERATLSCRASQSVSMPLAWYQQKPGQAPRLLIYGASSRA
TGIPDRFSGSGSGTDFTLTISRLEPEDFAVYYCQQMRGRPPTFGQGTKVEIKRTVA
APSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTE
QDSKDYSTYLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC

[00747] COVA14202 was generated by co-expression of an anti-domain A2 of tenascin C antibody heavy chain carrying a C-terminal stapled scFv BHA10 (VH-VL orientation SEQ ID NO: 77) fusion (SEQ ID NO: 117, comprising SEQ ID NO: 118) with the heavy chain (HC; SEQ ID NO: 119) and light chain (LC; SEQ ID NO: 120) of an anti- domain A2 of tenascin C antibody. The sequences are listed below.

SEQ ID NO: 77 [stapled scFv BHA10 (VH-VL)]

QVQLVQSGAEVKKPGSSVKVSCKASGYTFTTYLHWVRQAPGCGLEWMGWIY
PGNVHAQYNEKFKGRVTITADKSTSTAYMELSSLRSEDNAVYYCARSWEGFPY
WGQGTITVTVSSGGGSGGGSGCPPCGGGGDIQMTQSPSSLSASVGDRVTITCKAS
QNVGINVAWYQQKPGKAPKSLISSASYRYSRVPSRFSGSGSGTDFTLTISLQPED
FATYFCQQYDTPFTFGCGTKVEIK

SEQ ID NO: 117 (2B10 HC with BHA10 stapled (VH-VL) scFv C-ter fused, IgG1 sigma, knob mutations)

QVQLVQSGAEVKKPGSSVKVSCKASGGTFSSYAISWVRQAPGQGLEWMGGIPIF
GTANYAQKFQGRVTITADKSTSTAYMELSSLRSEDNAVYYCARLYGYAYYGAF
DYWGQGTITVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPTVSW
NSGALTSGVHTFPAVLQSSGLYSLSSVTVPSSSLGTQTYICNVNHKPSNTKVDK
KVEPKSCDKTHTCPPCPAPEAAGASSVFLFPPKPKDTLMISRTPEVTCVVDVSA
EDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYK
CKVSNKALPSSIEKTISKAKGQPREPQVYTLPPCREEMTKNQVSLWCLVKGFYPS
DIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSQSVMH
EALHNHYTQKSLSLSPGKGGGGSGGGGSGGGGSQVQLVQSGAEVKKPGSSVKV
SCKASGYTFTTYLHWVRQAPGCGLEWMGWIYPGNVHAQYNEKFKGRVTITA
DKSTSTAYMELSSLRSEDNAVYYCARSWEGFPYWGQGTITVTVSSGGGSGGGSG
CPPCGGGGDIQMTQSPSSLSASVGDRVTITCKASQNVGINVAWYQQKPGKAPKS
LISSASYRYSRVPSRFSGSGSGTDFTLTISLQPEDFATYFCQQYDTPFTFGCGTK
VEIK

SEQ ID NO: 118 (2B10 HC, IgG1 sigma, knob mutations)

QVQLVQSGAEVKKPGSSVKVSCASGGTFSSYAISWVRQAPGQGLEWMGGIPIF
 GTANYAQKFQGRVTITADKSTSTAYMELSSLRSEDVAVYYCARLYGYAYYGAF
 DYWGQGTTTVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSW
 NSGALTSGVHTFPAVLQSSGLYSLSSVTVPSSSLGTQTYICNVNHKPSNTKVDK
 KVEPKSCDKTHTCPPCPAPEAAGASSVFLFPPKPKDTLMISRTPEVTCVVDVSA
 EDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYK
 CKVSNKALPSSIEKTISKAKGQPREPQVYTLPPCREEMTKNQVSLWCLVKGFYPS
 DIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFCFSVMH
 EALHNHYTQKSLSLSPGK

SEQ ID NO: 119 (2B10 HC, IgG1 sigma and hole mutations)

QVQLVQSGAEVKKPGSSVKVSCASGGTFSSYAISWVRQAPGQGLEWMGGIPIF
 GTANYAQKFQGRVTITADKSTSTAYMELSSLRSEDVAVYYCARLYGYAYYGAF
 DYWGQGTTTVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSW
 NSGALTSGVHTFPAVLQSSGLYSLSSVTVPSSSLGTQTYICNVNHKPSNTKVDK
 KVEPKSCDKTHTCPPCPAPEAAGASSVFLFPPKPKDTLMISRTPEVTCVVDVSA
 EDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYK
 CKVSNKALPSSIEKTISKAKGQPREPQVCTLPSSREEMTKNQVSLSCAVKGFYPSD
 IAVEWESNGQPENNYKTTPPVLDSDGSFFLVSKLTVDKSRWQQGNVFCFSVMHE
 ALHNHYTQKSLSLSPGK

SEQ ID NO: 120 (2B10 LC)

DIQMTQSPSSLSASVGDRVTITCRASQGIRNDLGWYQQKPGKAPKRLIYAASSLQ
 SGVPSRFSGGSGTEFTLTISSLQPEDFATYYCLQNGLQPATFGQGTKVEIKRTVA
 APSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTE
 QDSKDYSTYLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC

[00748] In order to demonstrate TAA-dependent activation of LTBR, high binding 96-well μ Clear flat bottom plates (Greiner; Monroe, NC) were coated overnight with:

- 150 ng/well of recombinant domain A2-containing (A2⁺) tenascin C domains A1-A2-A3 (TnCA2⁺; SEQ ID NO: 121) or 150 ng/well human recombinant tenascin domains A1-A3 (*i.e.*, lacking domain A2, indicated as A2⁻) (TnCA2⁻; SEQ ID NO: 122); or

- 150 ng/well of recombinant EDA-containing (EDA⁺) fibronectin domains 11-A-12 (EDA⁺; SEQ ID NO: 123) or 150 ng/well human recombinant fibronectin domains 11-12 (*i.e.*, not containing EDA, indicated as EDA⁻) (EDA⁻; SEQ ID NO: 124).

[00749] The above mentioned sequences are as follows:

SEQ ID NO: 121 (Hu tenascin C domains A1-A2-A3, including (His)₆- Tag for purification)

EQAPELENLTVTEVGWDGLRLNWTAAADQAYEHFIIQVQEANKVEAARNLTVPG
SLRAVDIPGLKAATPYTVSIYGVIGQYRTPVLSAEASTGETPNLGEVVVAEVDGWD
ALKLNWTAPEGAYEYFFIQVQEADTVEAAQNLTVPGLRSTDLPLGLKAATHYTI
TIRGVTQDFSTTPLSVEVLTEVPDGMGNLTVTEVSWDALRLNWTPDGTQFTI
QVQEADQVEEAHNLTVPGLRSMEIPGLRAGTPYTVTLHGVEVRGHSTRPLAVEV
VTHHHHHH

SEQ ID NO: 122 (Hu tenascin C domains A1-A3, including (His)₆- Tag for purification)

EQAPELENLTVTEVGWDGLRLNWTAAADQAYEHFIIQVQEANKVEAARNLTVPG
SLRAVDIPGLKAATPYTVSIYGVIGQYRTPVLSAEEVPDGMGNLTVTEVSWDALR
LNWTPDGTQFTIQVQEADQVEEAHNLTVPGLRSMEIPGLRAGTPYTVTLH
GEVRGHSTRPLAVEVVTHHHHHH

SEQ ID NO: 123 (Hu fibronectin domains 11-A-12, including (His)₆- Tag for purification)

EIDKPSQMQVTDVQDNSISVKWLPSSSPVTGYRVTTTPKNGPGPTKTKTAGPDQT
EMTIEGLQPTVEYVVSVAQNPSEGSQLVQTAVTNIDRPKGLAFTDQVDSIKI
AWESPQGQVSRVRYTYSSPEDGIHELFPAPDGEEDTAELQGLRPGSEYTVSVVAL
HDDMESQPLIGTQSTAIPAPTDLKFTQVTPTSLAQWTPPNVQLTGVRVRVTPKE
KTGPMKEINLAPDSSSVVVSGLMVATKYEVSVAALKDTLTSRPAQGVVTTLEHH
HHHH

SEQ ID NO: 124 (Hu fibronectin domains 11-12, including (His)₆- Tag for purification)
 EIDKPSQM QVTDVQDNSISVKWLPSSSPVTGYRVTTTPKNGPGPTKTKTAGPDQT
 EMTIEGLQPTVEYVVS VYAQNPSGESQPLVQTAVTTIPAPTDLKFTQVTPTSLSAQ
 WTPPNVQLTGYRVRVTPKEKTGPMKEINLAPDSSSVVVSGLMVATKYEVSVYA
 LKDTLTSRPAQGVVTTLEHHHHHH

[00750] The results of these assays were in line with the results shown above for EDB/LTBR bispecifics, *i.e.*, they showed activation of LTBR mainly in the presence of the tumor antigen of the extracellular matrix targeted by the respective bispecific binding molecule (**Table 13**).

Table 13: Maximal fold inductions of NF- κ B signaling in presence or absence of EDA or TnCA2 for COVA14198 and COVA14202, respectively.

Name	Max. fold induction in presence of TAA	Max. fold induction in absence of TAA	Ratio (max fold induction TAA ⁺)/max fold induction TAA ⁻)
COVA14198 (EDA/LTBR)	4.5	1.3	3.5
COVA14202 (TnCA2/LTBR)	4.2	1.2	3.5

[00751] In particular, the TnCA2/LTBR bispecific binding molecule could activate LTBR, and therefore trigger NF- κ B signaling, strongly in the presence of the TnCA2 antigen, and the EDA/LTBR bispecific binding molecule could activate LTBR, and therefore trigger NF- κ B signaling, strongly in the presence of the EDA antigen. In absence of the respective TAA, these bispecifics did not or only minimally activate LTBR.

[00752] This example demonstrated that the approach to activate LTBR via a multispecific binding molecule of provided herein, *e.g.*, binding to both LTBR and a tumor associated antigen associated with the extracellular matrix, works for all three different tumor associated antigens of the extracellular matrix that have been tested so far, and thus is generally applicable for tumor associated antigens present in the extracellular matrix. The example also demonstrated that molecules incorporating spFv are functional.