Hetero-dimeric Immunoglobulins

Related application

This application claims benefit of US provisional application No. 61/467,727 filed on March 25, 2011; all of which are hereby incorporated by reference in their entirety.

The field of the invention

The present invention relates to engineered hetero-dimeric immunoglobulins or fragments thereof and methods of making the same.

Background of the invention

Methods for making hetero-dimeric proteins have been reported. The first approach to construct and produce hetero-dimeric bispecific antibodies was the quadroma technology (Milstein C and Cuello AC, *Nature*, 305(5934):537-40 (1983)) which consists of a somatic fusion of two different hybridoma cell lines expressing murine monoclonal antibodies with the desired specificities of the bispecific antibody. Because of the random pairing of two different immunoglobulin (Ig) heavy and light chains within the resulting hybrid–hybridoma (or quadroma) cell line, up to ten different immunogloblin species are generated of which only one is the functional bispecific antibody (Kufer P *et al.*, Trends Biotechnol, 22(5):238-44 (2004)). The presence of mispaired by-products reduces significantly the production yield and requires sophisticated purification procedures to achieve product homogeneity. The mispairing of Ig heavy chains can be reduced by using several rational design strategies, most of which engineer the antibody heavy chains for hetero-dimerization via the design of man-made complementary hetero-dimeric interfaces between the two subunits of the CH3 domain homo-dimer. The first report of an engineered CH3 hetero-dimeric domain pair was made by Carter et al. describing a "protuberance-into-cavity" approach for generating a hetero-dimeric Fc moiety (U.S. Patent No. 5,807,706; 'knobs-into-holes'; Merchant AM *et al.*, *Nat Biotechnol*, 16(7):677-81 (1998)). Alternative designs have been recently developed and involved either the design of a new CH3 module pair by modifying the core composition of the modules as described in WO2007/110205 or the design of complementary salt bridges between modules as described in WO2007/147901 or WO2009/089004. The disadvantage of the CH3 engineering strategies is that these techniques still result in the production of a significant amount of undesirable homo-dimers. Hence there remains a need for an engineering technique which minimizes the content of homo-dimeric species.

Regardless of the various approaches at hetero-dimerizing Ig heavy chains as described in these patent publications, the major obstacle facing the development of full bispecific antibodies (i.e., two FAB fragments, each having a unique set of variable heavy and light chain domains that creates a unique antigen binding site and one dimeric Fc region) based on any CH3 domain rational engineering or others, is the requirement of having a common light chain to both FABs in order to circumvent the mispairing of their light chains (Carter P, *J Immunol Methods*, 248(1-2):7-15 (2001)). Although, this can be accomplished by using antibodies with identical light chains that bind to different antigens by virtue of their distinct heavy chains, it does requires the isolation of such antibodies, which usually involves the use of display technologies, and here are no current technologies that will enable the direct use of two distinct human monoclonal antibodies with the desired specificities to be reassembled without further CDR or light chain engineering into a full bispecific antibody. Thus there is a need for generating full, correctly assembled, bispecific antibodies that are similar in their overall structure to natural antibodies, i.e., comprising two FAB fragments, each having a unique set of variable heavy and light chain domains that creates a unique antigen binding site and one dimeric Fc region.

Summary of the invention

The present disclosure relates generally to engineer hetero-dimeric immunoglobulins or fragments thereof and methods of making the same.

In one aspect, the present disclosure provides a hetero-dimeric immunoglobulin or hetero-dimeric fragment thereof comprising:

(a) a first engineered immunoglobulin chain comprising an engineered domain with a protein-protein interface, wherein at least one amino acid residue of the protein-protein interface of the parent domain of said first engineered immunoglobulin chain is substituted with an amino acid residue at the equivalent 3D structural position from a protein-protein interface of a donor domain of a first member of the naturally occurring immunoglobulin super-family; and

(b) a second engineered immunoglobulin chain comprising an engineered domain with a protein-protein interface, wherein at least one amino acid residue of the protein-protein interface of the parent domain of said second engineered immunoglobulin chain is substituted with an amino acid residue at the equivalent 3D structural position from a protein-protein interface of a donor domain of a second member of the naturally occurring immunoglobulin super-family,

wherein the donor domain of the first member of the naturally occurring immunoglobulin super-family and the donor domain of the second member of the naturally occurring immunoglobulin super-family form a naturally occurring hetero-dimer or a naturally occurring homo-dimer and,

wherein the parent domains of the first and the second engineered immunoglobulin chain are not a CH2 domain, not a CH3 domain, not a VL domain and not a VH domain and wherein, if the parent domain of the first engineered immunoglobulin chain is an IGLC domain or an IGKC domain and the parent domain of the second engineered immunoglobulin chain is a CH1 domain and the protein-protein interface of the parent domain of the first engineered immunoglobulin chain is substituted with an amino acid residue at position 12 and the protein-protein interface of the parent domain of the second engineered immunoglobulin chain is substituted with an amino acid residue at position 119, the substituted amino acid residue at position 12 and the substituted amino acid residue at position 119 are not a charged pair,

if the parent domain of the first engineered immunoglobulin chain is a CH1 domain and the parent domain of the second engineered immunoglobulin chain is an IGLC domain and the protein-protein interface of the parent domain of the first engineered immunoglobulin chain is substituted with an amino acid residue at position 26 and the protein-protein interface of the parent domain of the second engineered immunoglobulin chain is substituted with an amino acid residue at position 13, the substituted amino acid residue at position 26 and the substituted amino acid residue at position 13 are not a charged pair,

if the parent domain of the first engineered immunoglobulin chain is an IGLC domain and the parent domain of the second engineered immunoglobulin chain is a CH1 domain and the protein-protein interface of the parent domain of the first engineered immunoglobulin chain is substituted with an amino acid residue at position 5 and the protein-protein interface of the parent domain of the second engineered immunoglobulin chain is substituted with an amino acid residue at position 20, the substituted amino acid residue at position 5 and the substituted amino acid residue at position 20 are not a charged pair,

if the parent domain of the first engineered immunoglobulin chain is a CH1 domain and the parent domain of the second engineered immunoglobulin chain is an IGLC domain and the protein-protein interface of the parent domain of the first engineered immunoglobulin chain is substituted with an amino acid residue at position 27 and the protein-protein interface of the parent domain of the second engineered immunoglobulin chain is substituted with an amino

acid residue at position 18, the substituted amino acid residue at position 27 and the substituted amino acid residue at position 18 are not a charged pair,

if the parent domain of the first engineered immunoglobulin chain is an IGLC domain or an IGKC domain and the parent domain of the second engineered immunoglobulin chain is a CH1 domain and the protein-protein interface of the parent domain of the first engineered immunoglobulin chain is substituted with an amino acid residue at position 20 and the protein-protein interface of the parent domain of the second engineered immunoglobulin chain is substituted with an amino acid residue at position 26, the substituted amino acid residue at position 20 and the substituted amino acid residue at position 26 are not a charged pair,

if the parent domain of the first engineered immunoglobulin chain is an IGLC domain and the parent domain of the second engineered immunoglobulin chain is a CH1 domain and the protein-protein interface of the parent domain of the first engineered immunoglobulin chain is substituted with an amino acid residue at position 79 and the protein-protein interface of the parent domain of the second engineered immunoglobulin chain is substituted with an amino acid residue at position 84.3, the substituted amino acid residue at position 79 and the substituted amino acid residue at position 84.3 are not a charged pair,

if the parent domain of the first engineered immunoglobulin chain is an IGLC domain or an IGKC domain and the parent domain of the second engineered immunoglobulin chain is a CH1 domain and the protein-protein interface of the parent domain of the first engineered immunoglobulin chain is substituted with an amino acid residue at position 86 and the protein-protein interface of the parent domain of the second engineered immunoglobulin chain is substituted with an amino acid residue at position 86, the substituted amino acid residues at both positions 86 are not a charged pair,

if the parent domain of the first engineered immunoglobulin chain is an IGKC domain and the amino acid residue which is substituted in the protein-protein interface of the parent domain of the first engineered immunoglobulin chain is at position 11 and the parent domain of the second engineered immunoglobulin chain is a domain which comprises a hinge region, the parent domain of the second engineered immunoglobulin chain is not substituted at position 3 of the hinge region,

wherein the amino acid position of each group member is indicated according to the IMGT® numbering.

In a further aspect, the present disclosure provides a hetero-dimeric immunoglobulin or hetero-dimeric fragment thereof comprising:

(a) a first engineered immunoglobulin chain comprising an engineered domain with a protein-protein interface, wherein at least one amino acid residue of the protein-protein interface of the parent domain of said first engineered immunoglobulin chain is substituted with an amino acid residue at the equivalent 3D structural position from a protein-protein interface of a donor domain of a first member of the naturally occurring immunoglobulin super-family; and

(b) a second engineered immunoglobulin chain comprising an engineered domain with a protein-protein interface, wherein at least one amino acid residue of the protein-protein interface of the parent domain of said second engineered immunoglobulin chain is substituted with an amino acid residue at the equivalent 3D structural position from a protein-protein interface of a donor domain of a second member of the naturally occurring immunoglobulin super-family,

wherein the donor domain of the first member of the naturally occurring immunoglobulin super-family and the donor domain of the second member of the naturally occurring immunoglobulin super-family form a naturally occurring hetero-dimer or a naturally occurring homo-dimer and,

wherein the parent domains of the first and the second engineered immunoglobulin chain are not a CH2 domain, not a CH3 domain, not a VL domain and not a VH domain and, wherein the protein-protein interface of the parent domain of the first engineered immunoglobulin chain comprises an amino acid residue substitution at a position selected from the group consisting of 20, 22, 85.1, 86, and 88, and wherein,

if the parent domain of the first engineered immunoglobulin chain is an IGLC domain and the parent domain of the second engineered immunoglobulin chain is a CH1 domain and the protein-protein interface of the parent domain of the first engineered immunoglobulin chain is substituted with an amino acid residue at position 5 and the protein-protein interface of the parent domain of the second engineered immunoglobulin chain is substituted with an amino acid residue at position 20, the substituted amino acid residue at position 5 and the substituted amino acid residue at position 20 are not a charged pair,

if the parent domain of the first engineered immunoglobulin chain is an IGLC domain or an IGKC domain and the parent domain of the second engineered immunoglobulin chain is a CH1 domain and the protein-protein interface of the parent domain of the first engineered

immunoglobulin chain is substituted with an amino acid residue at position 20 and the protein-protein interface of the parent domain of the second engineered immunoglobulin chain is substituted with an amino acid residue at position 26, the substituted amino acid residue at position 20 and the substituted amino acid residue at position 26 are not a charged pair,

if the parent domain of the first engineered immunoglobulin chain is an IGLC domain or an IGKC domain and the parent domain of the second engineered immunoglobulin chain is a CH1 domain and the protein-protein interface of the parent domain of the first engineered immunoglobulin chain is substituted with an amino acid residue at position 86 and the protein-protein interface of the parent domain of the second engineered immunoglobulin chain is substituted with an amino acid residue at position 86, the substituted amino acid residues at both positions 86 are not a charged pair, wherein the amino acid position of each group member is indicated according to the IMGT® numbering.

In a further aspect, the present disclosure provides a hetero-dimeric immunoglobulin or hetero-dimeric fragment thereof comprising:

(a) a first engineered immunoglobulin chain comprising an engineered domain with a protein-protein interface, wherein at least one amino acid residue of the protein-protein interface of the parent domain of said first engineered immunoglobulin chain is substituted with an amino acid residue at the equivalent 3D structural position from a protein-protein interface of a donor domain of a first member of the naturally occurring immunoglobulin super-family; and

(b) a second engineered immunoglobulin chain comprising an engineered domain with a protein-protein interface, wherein at least one amino acid residue of the protein-protein interface of the parent domain of said second engineered immunoglobulin chain is substituted with an amino acid residue at the equivalent 3D structural position from a protein-protein interface of a donor domain of a second member of the naturally occurring immunoglobulin super-family,

wherein the engineered domain of the first engineered immunoglobulin chain and the engineered domain of the second engineered immunoglobulin chain are not identical and,

wherein the donor domain of the first member of the naturally occurring immunoglobulin super-family and the donor domain of the second member of the naturally occurring immunoglobulin super-family form a naturally occurring hetero-dimer or a naturally occurring homo-dimer and,

wherein the parent domain of the first and/or the second engineered immunoglobulin chain is a CH3 domain, wherein the amino acid residue which is substituted in the protein-protein interface of the parent domain of the first engineered immunoglobulin chain comprises the amino acid residue at position 88, and optionally a further amino acid residue at a position selected from the group consisting of 3, 5, 7, 20, 22, 26, 27, 79, 81, 84, 84.2, 85.1, 86, and 90 and, wherein the amino acid residue which is substituted in the protein-protein interface of the parent domain of the second engineered immunoglobulin chain comprises the amino acid residue at position 85.1 and/or 86 and optionally a further amino acid residue at a position selected from the group consisting of 3, 5, 7, 20, 22, 26, 27, 79, 81, 84, 84.2, 88, and 90 or at a position selected from the group consisting of 3, 5, 7, 20, 22, 26, 27, 79, 81, 84, 84.2, 85.1, 88, and 90 or at a position selected from the group consisting of 3, 5, 7, 20, 22, 26, 27, 79, 81, 84, 84.2, 86, 88, and 90,

wherein the amino acid residue substituted at position 88 in the parent domain of the first engineered immunoglobulin chain is interacting with the amino acid residue substituted at position 85.1 and/or 86 in the parent domain of the second engineered immunoglobulin chain, wherein the amino acid position of each group member is indicated according to the IMGT® numbering.

In a further aspect, the present disclosure provides a hetero-dimeric immunoglobulin or hetero-dimeric fragment thereof comprising:

(a) a first engineered immunoglobulin chain comprising an engineered domain with a protein-protein interface, wherein at least one amino acid residue of the protein-protein interface of the parent domain of said first engineered immunoglobulin chain is substituted with an amino acid residue at the equivalent 3D structural position from a protein-protein interface of a donor domain of a first member of the naturally occurring immunoglobulin super-family; and

(b) a second engineered immunoglobulin chain comprising an engineered domain with a protein-protein interface, wherein at least one amino acid residue of the protein-protein interface of the parent domain of said second engineered immunoglobulin chain is substituted with an amino acid residue at the equivalent 3D structural position from a protein-protein interface of a donor domain of a second member of the naturally occurring immunoglobulin super-family,

wherein the engineered domain of the first engineered immunoglobulin chain and the engineered domain of the second engineered immunoglobulin chain are not identical and,

wherein the donor domain of the first member of the naturally occurring immunoglobulin super-family and the donor domain of the second member of the naturally occurring immunoglobulin super-family form a naturally occurring hetero-dimer or a naturally occurring homo-dimer and,

wherein the parent domain of the first and/or the second engineered immunoglobulin chain is a CH3 domain, wherein the amino acid residue which is substituted in the protein-protein interface of the parent domain of the first engineered immunoglobulin chain comprises the amino acid residue at position 20, and optionally a further amino acid residue at a position selected from the group consisting of 3, 5, 7, 22, 26, 27, 79, 81, 84, 84.2, 85.1, 86, 88 and 90 and, wherein the amino acid residues which are substituted in the protein-protein interface of the parent domain of the second engineered immunoglobulin chain comprise the amino acid residue at position 26 and at a further position selected from the group consisting of 3, 22, 27, 79, 81, 84, 85.1, 86, and 88, wherein the amino acid residue substituted at position 20 in the parent domain of the first engineered immunoglobulin chain is interacting with the amino acid residue substituted at position 26 in the parent domain of the second engineered immunoglobulin chain, wherein the amino acid position of each group member is indicated according to the IMGT® numbering.

In a further aspect, the present disclosure provides a hetero-dimeric immunoglobulin or hetero-dimeric fragment thereof comprising:

(a) a first engineered immunoglobulin chain comprising an engineered domain with a protein-protein interface, wherein at least one amino acid residue of the protein-protein interface of the parent domain of said first engineered immunoglobulin chain is substituted with an amino acid residue at the equivalent 3D structural position from a protein-protein interface of a donor domain of a first member of the naturally occurring immunoglobulin super-family; and

(b) a second engineered immunoglobulin chain comprising an engineered domain with a protein-protein interface, wherein at least one amino acid residue of the protein-protein interface of the parent domain of said second engineered immunoglobulin chain is substituted with an amino acid residue at the equivalent 3D structural position from a protein-protein interface of a donor domain of a second member of the naturally occurring immunoglobulin super-family,

wherein the engineered domain of the first engineered immunoglobulin chain and the engineered domain of the second engineered immunoglobulin chain are not identical and,

wherein the donor domain of the first member of the naturally occurring immunoglobulin super-family and the donor domain of the second member of the naturally occurring immunoglobulin super-family form a naturally occurring hetero-dimer or a naturally occurring homo-dimer and,

wherein the parent domain of the first and/or the second engineered immunoglobulin chain is a CH2 domain, wherein the amino acid residue which is substituted in the protein-protein interface of the parent domain of the first engineered immunoglobulin chain comprises the amino acid residue at position 88, and optionally a further amino acid residue at a position selected from the group consisting of 3, 5, 7, 20, 22, 26, 27, 79, 81, 83, 84, 84.2, 84.3, 85.1, 86 and 90 and, wherein the amino acid residue which is substituted in the protein-protein interface of the parent domain of the second engineered immunoglobulin chain comprises the amino acid residue at position 85.1 and/or 86 and optionally a further amino acid residue at a position selected from the group consisting of 3, 5, 7, 20, 22, 26, 27, 79, 81, 83, 84, 84.2, 84.3 and 90 or at a position selected from the group consisting of 3, 5, 7, 20, 22, 26, 27, 79, 81, 83, 84, 84.2, 84.3, 85.1 and 90 or at a position selected from the group consisting of 3, 5, 7, 20, 22, 26, 27, 79, 81, 83, 84, 84.2, 84.3, 86 and 90, wherein the amino acid residue substituted at position 88 in the parent domain of the first engineered immunoglobulin chain is interacting with the amino acid residue substituted at position 85.1 and/or 86 in the parent domain of the second engineered immunoglobulin chain, wherein the amino acid position of each group member is indicated according to the IMGT® numbering.

In a further aspect, the present disclosure provides a hetero-dimeric immunoglobulin or hetero-dimeric fragment thereof comprising:

(a) a first engineered immunoglobulin chain comprising an engineered domain with a protein-protein interface, wherein at least one amino acid residue of the protein-protein interface of the parent domain of said first engineered immunoglobulin chain is substituted with an amino acid residue at the equivalent 3D structural position from a protein-protein interface of a donor domain of a first member of the naturally occurring immunoglobulin super-family; and

(b) a second engineered immunoglobulin chain comprising an engineered domain with a protein-protein interface, wherein at least one amino acid residue of the protein-protein interface of the parent domain of said second engineered immunoglobulin chain is substituted with an amino acid residue at the equivalent 3D structural position from a protein-protein interface of a donor domain of a second member of the naturally occurring immunoglobulin

super-family,
wherein the engineered domain of the first engineered immunoglobulin chain and the engineered domain of the second engineered immunoglobulin chain are not identical and,
wherein the donor domain of the first member of the naturally occurring immunoglobulin super-family and the donor domain of the second member of the naturally occurring immunoglobulin super-family form a naturally occurring hetero-dimer or a naturally occurring homo-dimer and,
wherein the parent domain of the first and/or the second engineered immunoglobulin chain is a CH2 domain, wherein the amino acid residue which is substituted in the protein-protein interface of the parent domain of the first engineered immunoglobulin chain comprises the amino acid residue at position 20, and optionally a further amino acid residue at a position selected from the group consisting of 3, 5, 7, 22, 26, 27, 79, 81, 83, 84, 84.2, 84.3, 85.1, 86, 88 and 90 and, wherein the amino acid residue which is substituted in the protein-protein interface of the parent domain of the second engineered immunoglobulin chain comprises the amino acid residue at position 26 and optionally a further amino acid residue at a position selected from the group consisting of 3, 5, 7, 20, 22, 27, 79, 81, 83, 84, 84.2, 84.3, 85.1, 86, 88 and 90, wherein the amino acid residue substituted at position 20 in the parent domain of the first engineered immunoglobulin chain is interacting with the amino acid residue substituted at position 26 in the parent domain of the second engineered immunoglobulin chain, wherein the amino acid position of each group member is indicated according to the IMGT® numbering.

In a further aspect, the present disclosure provides a hetero-dimeric immunoglobulin or hetero-dimeric fragment thereof comprising:
(a) a first engineered immunoglobulin chain comprising an engineered domain with a protein-protein interface, wherein at least one amino acid residue of the protein-protein interface of the parent domain of said first engineered immunoglobulin chain is substituted with an amino acid residue at the equivalent 3D structural position from a protein-protein interface of a donor domain of a first member of the naturally occurring immunoglobulin super-family; and
(b) a second engineered immunoglobulin chain comprising an engineered domain with a protein-protein interface, wherein at least one amino acid residue of the protein-protein interface of the parent domain of said second engineered immunoglobulin chain is substituted with an amino acid residue at the equivalent 3D structural position from a protein-protein

interface of a donor domain of a second member of the naturally occurring immunoglobulin super-family,
wherein the engineered domain of the first engineered immunoglobulin chain and the engineered domain of the second engineered immunoglobulin chain are not identical and,
wherein the donor domain of the first member of the naturally occurring immunoglobulin super-family and the donor domain of the second member of the naturally occurring immunoglobulin super-family form a naturally occurring hetero-dimer or a naturally occurring homo-dimer and,
wherein the parent domain of the first and/or the second engineered immunoglobulin chain is a CH4 domain, wherein the protein-protein interface of the parent domain of the first engineered immunoglobulin chain comprises an amino acid residue substitution at a position selected from the group consisting of 20, 22, 85.1, 86, and 88, and/or wherein the protein-protein interface of the parent domain of the second engineered immunoglobulin chain comprises an amino acid residue substitution at a position selected from the group consisting of 22, 26, 85.1, 86, and 88, wherein the amino acid position of each group member is indicated according to the IMGT® numbering.

In another aspect, the present disclosure provides a method to produce a hetero-dimeric immunoglobulin or hetero-dimeric fragment thereof comprising:
(a) substituting at least one amino acid residue of the protein-protein interface of a parent domain of a first parent immunoglobulin chain at the equivalent 3D structural position from a protein-protein interface of a donor domain of a first member of the naturally occurring immunoglobulin super-family to obtain a first engineered immunoglobulin chain comprising an engineered domain,
(b) substituting at least one amino acid residue of the protein-protein interface of the parent domain of a second parent immunoglobulin chain at the equivalent 3D structural position from a protein-protein interface of a donor domain of a second member of the naturally occurring immunoglobulin super-family to obtain a second engineered immunoglobulin chain comprising an engineered domain,
(c) culturing a host cell comprising a nucleic acid encoding said engineered immunoglobulin chains, wherein the culturing is such that the nucleic acid is expressed and the engineered immunoglobulin chains produced; and
(d) recovering the hetero-dimeric immunoglobulin or hetero-dimeric fragment thereof from

the host cell culture.

In another aspect, the present disclosure provides a method to engineer a protein-protein interface of a domain of a multidomain protein comprising:

(a) providing a multidomain protein comprising a domain with a protein-protein interface;

(b) selecting as a donor domain a naturally occurring immunoglobulin super-family member comprising a domain with a protein-protein interface which is different from the domain of (a);

(c) overlaying 3D structures of the domain with the protein-protein interface of (a) and the donor domain with the protein-protein interface of (b);

(d) identifying exposed protein-protein interface residues in the overlayed 3D structures of the domain with the protein-protein interface of (a) and the donor domain with the protein-protein interface of (b);

e) substituting at least one amino acid residue of the identified exposed protein-protein interface amino acid residues of the domain with the protein-protein interface of (a) with amino acid residues at the equivalent 3D structural position from the identified exposed protein-protein interface amino acid residues from the donor domain with the protein-protein interface of (b).

In a further aspect, the present disclosure provides the use of a donor domain of a first and a second member of the naturally occurring immunoglobulin super-family to engineer a hetero-dimeric immunoglobulin or hetero-dimeric fragment thereof comprising a first engineered immunoglobulin chain comprising at least one engineered domain which has a protein-protein interface which is substituted with at least one amino acid residue from the protein-protein interface of the donor domain of the first member of the naturally occurring immunoglobulin super-family and a second engineered immunoglobulin chain comprising at least one engineered domain which has a protein-protein interface which is substituted with at least one amino acid residue from the protein-protein interface of the donor domain of the second member of the naturally occurring immunoglobulin super-family.

Brief description of the figures

FIG. 1: Depicts the amino-acid sequence alignment of engineered CH3 domain BT alpha chain and engineered CH3 domain BT beta chain with human IgG1 CH3 domain. EU numbering is used.

FIG. 2: Schematic diagram of the various pairings of the BT alpha His and VL-BT beta chains. The BT alpha chain has a polyhistidine tag sequence fused to its C-terminus and BT beta chain has a variable domain antibody fused to its N-terminus.

FIG. 3: SDS-PAGE analysis demonstrating production of both the BT alpha His_VL-BT beta hetero-dimer and the BT alpha His_VL-BT beta F405S hetero-dimer (4-12% SDS Tris-glycine polyacrylamide gel). (M) molecular weight marker as indicated. (1) BT alpha His_VL-BT beta transfection output after protein-A purification, non-reducing conditions. (2) BT alpha His_VL-BT beta transfection output after protein-A purification, reducing conditions. (3) Human IgG1-Fc with hinge transfection output after protein-A purification, non-reducing conditions. (4) Human IgG1-Fc with hinge transfection output after protein-A purification, reducing conditions. (5) BT alpha His_VL-BT beta F405S transfection output after protein-A purification, non-reducing conditions. (6) BT alpha His_VL-BT beta F405S transfection output after protein-A purification, reducing conditions. (A) undefined aggregates. (B) BT alpha His_VL-BT beta hetero-dimer. (C) BT alpha His homo-dimer. (D) VL-BT beta F405S homo-dimer. (E) VL-BT beta F405S monomer. (F) BT alpha His monomer. (G) BT alpha His_VL-BT beta F405S hetero-dimer.

FIG. 4A: Scanning densitometry analysis assessing the relative proportion of the BT alpha His_VL-BT beta hetero-dimer after protein-A purification (4-12% SDS Tris-glycine polyacrylamide gel, non-reducing conditions). (A) undefined aggregates. (B) BT alpha His_VL-BT beta hetero-dimer. (C) BT alpha His homo-dimer. **FIG. 4B**: scanning densitometry analysis assessing the relative proportion of BT alpha His_VL-BT beta hetero-dimer to BT alpha His homo-dimer after protein-A purification when omitting the aggregates (4-12% SDS Tris-glycine polyacrylamide gel, non-reducing conditions). (A) undefined aggregates. (B) BT alpha His_VL-BT beta hetero-dimer. (C) BT alpha His homo-dimer.

FIG. 5: SDS-PAGE analysis demonstrating production of the BT alpha His_VL-BT beta F405A hetero-dimer (4-12% SDS Tris-glycine polyacrylamide gel). (M) molecular weight marker as indicated. (1) BT alpha His_VL-BT beta F405A transfection output after protein-A purification, non-reducing conditions. (2) BT alpha His_VL-BT beta F405A transfection output after protein-A purification, reducing conditions. (3) Human IgG1-Fc with hinge transfection output after protein-A purification, non-reducing conditions. (4) Human IgG1-Fc

with hinge transfection output after protein-A purification, reducing conditions. (A) undefined aggregates. (B) VL-BT beta F405A homo-dimer. (C) BT alpha His_VL-BT beta F405A hetero-dimer. (D) BT alpha His homo-dimer. (E) VL-BT beta F405A monomer.

FIG. 6: Western blot confirming the production of the BT alpha His_VL-BT beta F405A hetero-dimer (4-12% SDS Tris-glycine polyacrylamide gel). Western blot was detected with an anti His tag antibody. (M) molecular weight marker as indicated. (1) BT alpha His_VL-BT beta F405A transfection output after protein-A purification, non-reducing conditions. (2) BT alpha His_VL-BT beta F405A transfection output after protein-A purification, reducing conditions. (3) BT alpha His_VL-BT beta F405A transfection output after protein-A purification and Ni^{2+}-sepharose, non-reducing conditions. (4) BT alpha His_VL-BT beta F405A transfection output after protein-A purification and Ni^{2+}-sepharose, reducing conditions. (5) BT alpha His_VL-BT beta F405A transfection output after protein-A purification, Ni^{2+}-sepharose, and protein-L agarose, non-reducing conditions. (6) BT alpha His_VL-BT beta F405A transfection output after protein-A purification, Ni^{2+}-sepharose, and protein-L agarose, reducing conditions.

FIG. 7A: Scanning densitometry analysis assessing the relative proportion of the BT alpha His_VL-BT beta F405A hetero-dimer after protein-A purification (4-12% SDS Tris-glycine polyacrylamide gel, non-reducing conditions). (A) undefined aggregates. (B) VL-BT beta F405A homo-dimer. (C) BT alpha His_VL-BT beta F405A hetero-dimer. (D) BT alpha His homo-dimer. (E) VL-BT beta F405A monomer. **FIG. 7B**: scanning densitometry analysis assessing the relative proportion of the BT alpha His_VL-BT beta F405A hetero-dimer to homo-dimers after protein-A purification when omitting the aggregates (4-12% SDS Tris-glycine polyacrylamide gel, non-reducing conditions). (A) undefined aggregates. (B) VL-BT beta F405A homo-dimer. (C) BT alpha His_VL-BT beta F405A hetero-dimer. (D) BT alpha His homo-dimer. (E) VL-BT beta F405A monomer.

FIG. 7C: DSC thermogram of the hetero-dimeric BT alpha His_BT beta (F405A) HA hetero-dimer.

FIG. 8: SDS-PAGE analysis demonstrating production of the hetero-dimer Fc (BTA)-S364K-T366V-K370T-K392Y-K409W-T411N_VL-Fc (BTB)-F405A-Y407S (4-12% SDS Tris-glycine polyacrylamide gel). (M) molecular weight marker as indicated. (1) BT alpha His_VL-BT beta F405A hetero-dimer from example 2, transfection output after protein-A purification, non-reducing conditions. (2) BT alpha His_VL-BT beta F405A hetero-dimer from example 2, transfection output after protein-A purification, reducing conditions. (3) Fc

(BTA)-S364K-T366V-K370T-K392Y-K409W-T411N_VL-Fc (BTB)-F405A-Y407S, transfection output after protein-A purification, non-reducing conditions. (4) Fc (BTA)-S364K-T366V-K370T-K392Y-K409W-T411N_VL-Fc (BTB)-F405A-Y407S, transfection output after protein-A purification, reducing conditions. (5) "knob-into-hole" Fc, transfection output after protein-A purification, non-reducing conditions. (6) "knob-into-hole" Fc construct with the "hole" fused to a variable domain antibody, transfection output after protein-A purification, reducing conditions. (A) Fc (BTA)-S364K-T366V-K370T-K392Y-K409W-T411N_VL-Fc (BTB)-F405A-Y407S hetero-dimer. (B) VL-Fc (BTB)-F405A-Y407S homo-dimer. (C) Fc (BTA)-S364K-T366V-K370T-K392Y-K409W-T411N homo-dimer.

FIG. 9: Scanning densitometry analysis assessing the relative proportion of the hetero-dimer Fc (BTA)-S364K-T366V-K370T-K392Y-K409W-T411N_VL-Fc (BTB)-F405A-Y407S after protein-A purification (4-12% SDS Tris-glycine polyacrylamide gel, non-reducing conditions). (A) Fc (BTA)-S364K-T366V-K370T-K392Y-K409W-T411N_VL-Fc (BTB)-F405A-Y407S. (B) VL-Fc (BTB)-F405A-Y407S homo-dimer. (C) Fc (BTA)-S364K-T366V-K370T-K392Y-K409W-T411N homo-dimer.

FIG. 10: SDS-PAGE demonstrating production of the BT delta_VL-BT gamma hetero-dimer after ProteinA purification (4-12% SDS Tris-glycine polyacrylamide gel). (M) Molecular weight marker as indicated. (1) BT delta_VL-BT gamma hetero-dimer, transfection output after protein-A purification, reducing conditions. (2) BT delta_VL-BT gamma hetero-dimer, transfection output after protein-A purification, non-reducing conditions. (A) BT delta_VL-BT gamma hetero-dimer. (B) VL-BT gamma homo-dimer. (C) VL-BT gamma monomer.

FIG. 11A: Scanning densitometry analysis assessing the relative proportion of the BT delta_VL-BT gamma hetero-dimer after protein-A purification (4-12% SDS Tris-glycine polyacrylamide gel, non-reducing conditions). **FIG. 11B**: Scanning densitometry analysis assessing the relative proportion of the BT delta_VL-BT gamma hetero-dimer after protein-A purification omitting traces of aggregates and monomer in the relative ratio measurements (4-12% SDS Tris-glycine polyacrylamide gel, non-reducing conditions).

FIG. 12: Schematic diagram of the possible pairings when expressing the monovalent FAB engineered hetero-dimeric VH(anti-hCD19)-CH1 delta-BT beta c-myc_BT alpha His_VL(anti-hCD19)-CK gamma molecule.

FIG. 13: SDS-PAGE demonstrating production of the monovalent FAB engineered hetero-dimeric VH(anti-hCD19)-CH1 delta-BT beta c-myc_BT alpha His_VL(anti-hCD19)-CK gamma molecule (4-12% SDS Tris-glycine polyacrylamide gel). (M) Molecular weight

marker as indicated. (1) the monovalent FAB engineered hetero-dimeric VH(anti-hCD19)-CH1 delta-BT beta c-myc_BT alpha His_VL(anti-hCD19)-CK gamma molecule, transfection output after protein-A purification, non-reducing conditions. (A) the monovalent FAB engineered hetero-dimeric VH(anti-hCD19)-CH1 delta-BT beta c-myc_BT alpha His_VL(anti-hCD19)-CK gamma molecule. (B) VH(anti-hCD19)-CH1 delta-BT beta c-myc_VL(anti-hCD19)-CK gamma homo-dimer molecule. (C) VH(anti-hCD19)-CH1 delta-BT beta c-myc_VL(anti-hCD19)-CK gamma molecule (half-molecule).

FIG. 14: Scanning densitometry analysis assessing the relative proportion of the monovalent FAB engineered hetero-dimeric VH(anti-hCD19)-CH1 delta-BT beta c-myc_BT alpha His_VL(anti-hCD19)-CK gamma molecule after protein-A purification (4-12% SDS Tris-glycine polyacrylamide gel, non-reducing conditions). (1) VH(anti-hCD19)-CH1 delta-BT beta c-myc_VL(anti-hCD19)-CK gamma homo-dimer molecule. (2) the monovalent FAB engineered hetero-dimeric VH(anti-hCD19)-CH1 delta-BT beta c-myc_BT alpha His_VL(anti-hCD19)-CK gamma molecule. (3) VH(anti-hCD19)-CH1 delta-BT beta c-myc_VL(anti-hCD19)-CK gamma molecule (half-molecule).

FIG. 15: DSC thermogram of the monovalent FAB engineered hetero-dimeric VH(anti-hCD19)-CH1 delta-BT beta c-myc_BT alpha His_VL(anti-hCD19)-CK gamma molecule.

FIG. 16: Schematic diagram of the possible pairings when expressing the monovalent FAB engineered hetero-dimeric VH(anti-hCD19)-CH13-BT beta c-myc_BT alpha His_VL(anti-hCD19)-CK3 molecule.

FIG. 17: SDS-PAGE demonstrating production of the monovalent FAB engineered hetero-dimeric VH(anti-hCD19)-CH13-BT beta c-myc_BT alpha His_VL(anti-hCD19)-CK3 molecule (4-12% SDS Tris-glycine polyacrylamide gel). (M) Molecular weight marker as indicated. (1) the monovalent FAB engineered hetero-dimeric VH(anti-hCD19)-CH13-BT beta c-myc_BT alpha His_VL(anti-hCD19)-CK3 molecule, transfection output after protein-A purification, non-reducing conditions. (A) the monovalent FAB engineered hetero-dimeric VH(anti-hCD19)-CH13-BT beta c-myc_BT alpha His_VL(anti-hCD19)-CK3 molecule. (B) VH(anti-hCD19)-CH13-BT beta c-myc_VL(anti-hCD19)-CK3 homo-dimer molecule. (C) VH(anti-hCD19)-CH13-BT beta c-myc_VL(anti-hCD19)-CK3 molecule (half-molecule).

FIG. 18: scanning densitometry analysis assessing the relative proportion of the monovalent FAB engineered hetero-dimeric VH(anti-hCD19)-CH13-BT beta c-myc_BT alpha His_VL(anti-hCD19)-CK3 molecule after protein-A purification (4-12% SDS Tris-glycine polyacrylamide gel, non-reducing conditions). (1) VH(anti-hCD19)-CH13-BT beta c-

myc_VL(anti-hCD19)-CK3 homo-dimer molecule. (2) the monovalent FAB engineered hetero-dimeric VH(anti-hCD19)-CH13-BT beta c-myc_BT alpha His_VL(anti-hCD19)-CK3 molecule. (3) VH(anti-hCD19)-CH13-BT beta c-myc_VL(anti-hCD19)-CK3 molecule (half-molecule).

FIG. 19A: Sequences of human IGHG1, IGHG2, IGHG3 and IGHG4 CH1 domains; the IMGT® and EU numberings are used; shaded position means no substitution. **FIG. 19B**: Sequences of human IGHG1 hinge region; the IMGT® and EU numberings are used; shaded position means no substitution. **FIG. 19C**: Sequences of human IGHG1, IGHG2, IGHG3 and IGHG4 CH2 domains; the IMGT® and EU numberings are used; shaded position means no substitution; (~) indicates a shift in sequence alignment. **FIG. 19D**: Sequences of human IGHG1, IGHG2, IGHG3 and IGHG4 CH3 domains; the IMGT® and EU numberings are used; shaded position means no substitution.

FIG. 20A: Sequences of engineered human IGHG1 CH1 domains having protein-protein interfaces based on the human TRAC (abbreviated ALPHA), TRBC2 (abbreviated BETA), TRDC (abbreviated DELTA) , TRGC1 (abbreviated GAMMA), and IGHG1 CH3 (abbreviated CH3) constant domains; the IMGT® and EU numberings are used; shaded position means no substitution. **FIG. 20B:** Sequences of engineered human IGHG1 CH2 domains having protein-protein interfaces based on the human TRAC (abbreviated ALPHA), TRBC2 (abbreviated BETA), TRDC (abbreviated DELTA) , TRGC1 (abbreviated GAMMA), and IGHG1 CH3 (abbreviated CH3) constant domains; the IMGT® and EU numberings are used; shaded position means no substitution. **FIG. 20C:** Sequences of engineered human IGHG1 CH3 domains having protein-protein interfaces based on the human TRAC (abbreviated ALPHA), TRBC2 (abbreviated BETA), TRDC (abbreviated DELTA) , and TRGC1 (abbreviated GAMMA) constant domains; the IMGT® and EU numberings are used; shaded position means no substitution.

FIG. 21A: Sequences of engineered human IGHE CH4 domains having protein-protein interfaces based on the human TRAC (abbreviated ALPHA), TRBC2 (abbreviated BETA), TRDC (abbreviated DELTA), TRGC1 (abbreviated GAMMA), and IGHG1 CH3 (abbreviated CH3) constant domains; the IMGT® and EU numberings are used; shaded position means no substitution. **FIG. 21B:** Sequences of engineered human IGHM CH4 domains having protein-protein interfaces based on the human TRAC (abbreviated ALPHA), TRBC2 (abbreviated BETA), TRDC (abbreviated DELTA), TRGC1 (abbreviated GAMMA), and IGHG1 CH3

(abbreviated CH3) constant domains; the IMGT® and EU numberings are used; shaded position means no substitution.

FIG. 22: Sequences of engineered human IGKC domains having protein-protein interfaces based on the human TRAC (abbreviated ALPHA), TRBC2 (abbreviated BETA), TRDC (abbreviated DELTA), TRGC1 (abbreviated GAMMA), and IGHG1 CH3 (abbreviated CH3) constant domains; the IMGT® and EU numberings are used; shaded position means no substitution.

FIG. 23A: Sequences of human IGLC1, IGLC2, IGLC3, IGLC6, IGLC7 and engineered variants having protein-protein interfaces based on the human TRAC (abbreviated ALPHA), TRBC2 (abbreviated BETA), TRDC (abbreviated DELTA), TRGC1 (abbreviated GAMMA), and IGHG1 CH3 (abbreviated CH3) constant domains; Kabat residues 108 to 161 are shown - both the IMGT® and Kabat numberings are used; shaded position means no substitution. **FIG. 23B**: Sequences of human IGLC1, IGLC2, IGLC3, IGLC6, IGLC7 and engineered variants having protein-protein interfaces based on the human TRAC (abbreviated ALPHA), TRBC2 (abbreviated BETA), TRDC (abbreviated DELTA), TRGC1 (abbreviated GAMMA), and IGHG1 CH3 (abbreviated CH3) constant domains; Kabat residues 162 to 215 are shown - both the IMGT® and Kabat numberings are used; shaded position means no substitution.

FIG. 24: Schematic diagram of possible modified heavy chain pairings when expressing the Erbitux FAB-BT alpha_Herceptin scFv-BT beta hetero-dimer.

FIG. 25: SDS-PAGE demonstrating production of the Erbitux FAB-BT alpha_Herceptin scFv-BT beta. Samples of protein pools after each purification step are shown (4-12% SDS Tris-glycine polyacrylamide gel, non-reducing conditions). (M) Molecular weight marker as indicated. (1) after protein-A chromatography. (2) after anion IEX. (3) after cation IEX. (4) after size-exclusion chromatography. (5) control: scFv-Fc fusion protein. (6) control: monoclonal antibody.

FIG. 26: Scanning densitometry analysis assessing the relative proportion of the Erbitux FAB-BT alpha_Herceptin scFv-BT beta hetero-dimer after protein-A purification (4-12% SDS Tris-glycine polyacrylamide gel, non-reducing conditions). (A) VH(anti-EGFR) CH1-BT alpha_VL(anti-EGFR)-CK homo-dimer. (B) Erbitux FAB-BT alpha_Herceptin scFv-BT beta hetero-dimer. (C) scFv(anti-HER2)-BT beta homo-dimer. Impurities have been omitted from measurement.

FIG. 27: DSC thermogram of the Erbitux FAB-BT alpha_Herceptin scFv-BT beta hetero-dimer.

FIG. 28A: BIAcore sensorgram showing the binding and dissociation of the Erbitux FAB-BT alpha_Herceptin scFv-BT beta hetero-dimer to/from immobilized human EGFR-Fc.

FIG. 28B: Demonstration of simultaneous binding of the bispecific Erbitux FAB-BT alpha_Herceptin scFv-BT beta hetero-dimer to both antigens using SPR technology. The Erbitux FAB-BT alpha_Herceptin scFv-BT beta hetero-dimer and the human EGFR-Fc were injected sequentially on immobilized human HER2-Fc (solid line). Controls: (dotted line) binding of the Erbitux FAB-BT alpha_Herceptin scFv-BT beta hetero-dimer to the immobilized human HER2-Fc, (dashed line) absence of binding of the human EGFR-Fc to the immobilized human HER2-Fc.

FIG. 29: Schematic diagram of possible modified heavy chain pairings when expressing the anti-hCD19_anti-hVLA2 bispecific antibody. Both heavy chains, VH(anti-hVLA2)-CH1-BT alpha and VH(anti-hCD19)-BT delta-BT beta are C-terminally fused to the His and c-myc tag respectively.

FIG. 30: SDS-PAGE and Western blot analysis of the anti-hCD19_anti-hVLA2 bispecific antibody. Samples were taken after each purification step (4-12% SDS Tris-glycine polyacrylamide gels under non-reducing conditions); both eluate and unbound pools are shown. (M) Molecular weight marker as indicated. (1) eluate pool from protein-A chromatography. (2) unbound pool from protein-L chromatography. (3) eluate pool from protein-L chromatography. (4) unbound pool from Ni^{2+}-NTA chromatography. (5) eluate pool from Ni^{2+}-NTA chromatography. (A) SDS-PAGE. (B) Western blot detected with an anti His tag antibody. (C) Western blot detected with an anti c-myc tag antibody.

FIG. 31: Flow cytometry detection of the anti-hCD19_anti-hVLA2 bispecific antibody binding to $CD19^+$ $VLA2^-$ and $CD19^-$ $VLA2^+$ cells. Stained cells were acquired on a FACS CyAn™ ADP flow cytometer. Histograms display the normalized % of maximal event (Y axis) according to Phycoerythrin (PE) fluorescence intensity (X-axis) for each staining.

A and B: Raji cells were stained with the anti-hVLA2 antibody (grey histogram) as an isotype control or the anti-hCD19_anti-VLA2 bispecific antibody (white histogram). Binding of the primary antibodies was revealed by (A) a mouse anti c-myc tag antibody or (B) a mouse anti His tag antibody and stained with a PE-labelled anti-mouse Ig antibody.

C and D: HT1080 cells were stained with the anti-hCD19 antibody (grey histogram) as an isotype control or the anti-hCD19_anti-VLA2 bispecific antibody (white histogram). Binding of the primary antibodies was revealed by (C) a mouse anti c-myc tag antibody or (D) a mouse anti His tag antibody and stained with a PE-labelled anti-mouse Ig antibody.

FIG. 32: DSC thermogram of the anti-hCD19_anti-hVLA2 bispecific antibody.

FIG. 33: SDS-PAGE analysis demonstrating production of the BT alpha IGHG3_VL-BT beta F405A hetero-dimer (4-12% SDS Tris-glycine polyacrylamide gel). (M) molecular weight marker as indicated. (1) BT alpha IGHG3_VL-BT beta F405A transfection output after protein-A purification, non-reducing conditions. (2) BT alpha IGHG3_VL-BT beta F405A transfection output after protein-A purification, reducing conditions. (A) VL-BT beta F405A_VL-BT beta F405A homo-dimer. (B) BT alpha IGHG3_VL-BT beta F405A hetero-dimer, (C) VL-BT beta F405A chain (half molecule).

FIG. 34: Scanning densitometry analysis assessing the relative proportion of the BT alpha IGHG3_VL-BT beta F405A hetero-dimer after protein-A purification (4-12% SDS Tris-glycine polyacrylamide gel). (A) VL-BT beta F405A_VL-BT beta F405A homo-dimer. (B) BT alpha IGHG3_VL-BT beta F405A hetero-dimer. (C) VL-BT beta F405A chain (half molecule).

FIG. 35: SDS-PAGE analysis demonstrating production of the Fc IGHAG_VL- Fc IGHGA hetero-dimer (4-12% SDS Tris-glycine polyacrylamide gel). (M) molecular weight marker as indicated. (1) Fc IGHAG_VL- Fc IGHGA hetero-dimer transfection output after protein-A purification, non-reducing conditions. (2) Fc IGHAG_VL- Fc IGHGA hetero-dimer transfection output after protein-A purification, reducing conditions. (A) VL- Fc IGHGA_VL- Fc IGHGA homo-dimer. (B) Fc IGHAG_VL- Fc IGHGA hetero-dimer. (C) Fc IGHAG_ Fc IGHAG homo-dimer. (D) VL- Fc IGHGA chain (half molecule). (E) Fc IGHAG chain (half molecule).

FIG. 36: Scanning densitometry analysis assessing the relative proportion of the Fc IGHAG_VL- Fc IGHGA hetero-dimer after protein-A purification (4-12% SDS Tris-glycine polyacrylamide gel). (A) VL- Fc IGHGA_VL- Fc IGHGA homo-dimer. (B) Fc IGHAG_VL- Fc IGHGA hetero-dimer. (C) Fc IGHAG_ Fc IGHAG homo-dimer. (D) VL- Fc IGHGA chain (half molecule). (E) Fc IGHAG chain (half molecule).

FIG. 37: Schematic diagram of engineered CH4 based Fc chains. (A) BT alpha IGHM-4 His chain. (B) VL-BT beta IGHM-4 chain. (C) BT alpha IGHE-4 His chain. (D) VL-BT beta IGHE-4 chain.

FIG. 38: SDS-PAGE analysis demonstrating production of the BT alpha IGHM-4 His_VL-BT beta IGHM-4 hetero-dimer (4-12% SDS Tris-glycine polyacrylamide gel). (M) molecular weight marker as indicated. (1) BT alpha IGHM-4 His_VL-BT beta IGHM-4 hetero-dimer transfection output, non-reducing conditions. (2) BT alpha IGHM-4 His_VL-BT beta IGHM-

4 hetero-dimer transfection output, reducing conditions. (3) BT alpha IGHM-4 His_VL-BT beta IGHM-4 hetero-dimer transfection output after protein-L purification, non reducing conditions. (4) BT alpha IGHM-4 His_VL-BT beta IGHM-4 hetero-dimer transfection output after protein-L purification, reducing conditions. (A) BT alpha IGHM-4 His_VL-BT beta IGHM-4 beta hetero-dimer.

FIG. 39: Scanning densitometry analysis assessing the relative proportion of the BT alpha IGHM-4 His_VL-BT IGHM-4 beta hetero-dimer after protein-L purification (4-12% SDS Tris-glycine polyacrylamide gel).

FIG. 40: SDS-PAGE analysis demonstrating production of the BT alpha IGHE-4 His_VL-BT beta IGHE-4 hetero-dimer (4-12% SDS Tris-glycine polyacrylamide gel). (M) molecular weight marker as indicated. (1) BT alpha IGHE-4 His_VL-BT beta IGHE-4 hetero-dimer transfection output after protein-A purification, non-reducing conditions. (2) BT alpha IGHE-4 His_VL-BT beta IGHE-4 hetero-dimer transfection output after protein-A purification, reducing conditions. (A) BT alpha IGHE-4 His_VL-BT beta IGHE-4 hetero-dimer. (B) BT alpha IGHE-4 His_ BT alpha IGHE-4 His homo-dimer. (C) VL-BT beta IGHE-4 chain (half molecule). (D) BT alpha IGHE-4 His chain (half molecule).

FIG. 41: Scanning densitometry analysis assessing the relative proportion of the BT alpha IGHE-4 His_VL-BT beta IGHE-4 hetero-dimer after protein-A purification (4-12% SDS Tris-glycine polyacrylamide gel).

FIG. 42: IMGT® unique numbering as used herein e.g. in example 1, 2, 3, 4, 7, 8, 9, 10, 11, and 12 to identify 3D equivalent positions for human CH1, CH2, CH3, and CH4 constant domains of IGHA1, IGHA2, IGHD, IGHE, IGHEP1, IGHG1, IGHG2, IGHG3, IGHG4, IGHGP, and IGHM. GenBank accession numbers are indicated.

FIG. 43: IMGT® unique numbering as used herein e.g. in example 5, 6, 7, and 9 to identify 3D equivalent positions for human IGKC constant domain. GenBank accession number is indicated.

FIG. 44: IMGT® unique numbering as used herein e.g. in example 7 to identify 3D equivalent positions for human IGLC1, IGLC2, IGLC3, IGLC6, and IGLC7 constant domains. GenBank accession numbers are indicated.

FIG. 45: IMGT® unique numbering as used herein e.g. in example 1, 2, 3, 4, 5, 7, 8, 9, 10, and 12 to identify 3D equivalent positions for human TRAC, TRBC1, TRBC2, TRDC, TRGC1, TRGC2 (2x), and TRGC2 (3x) constant domains. GenBank accession numbers are indicated.

Detailed description of the invention

The present disclosure relates generally to engineer hetero-dimeric immunoglobulins or fragments thereof and methods of making the same.

The term "antibody" as referred to herein includes full-length antibodies and any antigen binding fragment or single chains thereof. Antibodies and specifically naturally occurring antibodies are glycoproteins which exist as one or more copies of a Y-shaped unit, composed of four polypeptide chains. Each "Y" shape contains two identical copies of a heavy (H) chain, and two identical copies of a light (L) chain, named as such by their relative molecular weights. Each light chain pairs with a heavy chain, and each heavy chain pairs with another heavy chain. Covalent interchain disulfide bonds and non covalent interactions link the chains together. Antibodies and specifically naturally occurring antibodies contain variable regions, which are the two copies of the antigen binding site. Papain, a proteolytic enzyme splits the "Y" shape into three separate molecules, two so called "Fab" fragments (Fab = fragment antigen binding), and one so called "Fc" fragment or "Fc region" (Fc = fragment crystallizable). A Fab fragment consists of the entire light chain and part of the heavy chain. The heavy chain contains one variable domain (VH) and either three or four constant domains (CH1, CH2, CH3, and CH4, depending on the antibody class or isotype). The region between the CH1 and CH2 domains is called the hinge region and permits flexibility between the two Fab arms of the Y-shaped antibody molecule, allowing them to open and close to accommodate binding to two antigenic determinants separated by a fixed distance. The "hinge region" as referred to herein is a sequence region of 6-62 amino acids in length, only present in IgA, IgD, and IgG, which encompasses the cysteine residues that bridge the two heavy chains. The heavy chains of IgA, IgD, and IgG each have four domains, i.e.one variable domain (VH) and three constant domains (CH1-3). IgE and IgM have one variable and four constant domains (CH1-4) on the heavy chain. The constant regions of the antibodies may mediate the binding to host tissues or factors, including various cells of the immune system (e.g., effector cells) and the first component (C1q) of the complement system classical pathway. Each light chain is usually linked to a heavy chain by one covalent disulfide bond. Each light chain contains one variable domain (VL) and one light chain constant domain. The light chain constant domain is a kappa light chain constant domain designated herein as IGKC or is a lambda light chain constant domain designated herein as IGLC. IGKC is used herein equivalently to Cκ or CK and has the same meaning. IGLC is used herein equivalently to Cλ

or CL and has the same meaning. The term "an IGLC domain" as used herein refer to all lambda light chain constant domains e.g. to all lambda light chain constant domains selected from the group consisting of IGLC1, IGLC2, IGLC3, IGLC6, and IGLC7. The VH and VL regions can be further subdivided into regions of hypervariability, termed complementarity determining regions (CDR), interspersed with regions that are more conserved, termed framework regions (FR or FW). Each VH and VL is composed of three CDRs and four FRs, arranged from amino- terminus to carboxy-terminus in the following order: FR1, CDR1, FR2, CDR2, FR3, CDR3, FR4. The variable regions of the heavy and light chains contain a binding domain that interacts with an antigen.

The term "full length antibody" as used herein includes the structure that constitutes the natural biological form of an antibody, including variable and constant regions. For example, in most mammals, including humans and mice, the full length antibody of the IgG class is a tetramer and consists of two identical pairs of two immunoglobulin chains, each pair having one light and one heavy chain, each light chain comprising immunoglobulin domains VL and a light chain constant region, and each heavy chain comprising immunoglobulin domains VH, CH1 (C[γ]1), CH2 (C[γ]2), CH3 (C[γ]3), and CH4 (C[γ]4, depending on the antibody class or isotype). In some mammals, for example in camels and llamas, IgG antibodies may consist of only two heavy chains, each heavy chain comprising a variable domain attached to the Fc region.

Antibodies are grouped into classes, also referred to as isotypes, as determined genetically by the constant region. Human constant light chains are classified as kappa (CK) and lambda (C[λ]) light chains. Heavy chains are classified as mu (μ), delta (δ), gamma (γ), alpha (α), or epsilon (ε), and define the antibody's isotype as IgM, IgD, IgG, IgA, and IgE, respectively. Thus, "isotype" as used herein is meant any of the classes and/or subclasses of immunoglobulins defined by the chemical and antigenic characteristics of their constant regions. The known human immunoglobulin isotypes are IgG1 (IGHG1), IgG2 (IGHG2), IgG3 (IGHG3), IgG4 (IGHG4), IgA1 (IGHA1), IgA2 (IGHA2), IgM (IGHM), IgD (IGHD), and IgE (IGHE). The so-called human immunoglobulin pseudo-gamma IGHGP gene represents an additional human immunoglobulin heavy constant region gene which has been sequenced but does not encode a protein due to an altered switch region (Bensmana M et al., Nucleic Acids Res, 16(7):3108 (1988)). In spite of having an altered switch region, the human

immunoglobulin pseudo-gamma IGHGP gene has open reading frames for all heavy constant domains (CH1-CH3) and hinge. All open reading frames for its heavy constant domains encode protein domains which align well with all human immunoglobulin constant domains with the predicted structural features. This additional pseudo-gamma isotype is referred herein as IgGP or IGHGP. Other pseudo immunoglobulin genes have been reported such as the human immunoglobulin heavy constant domain epsilon P1 and P2 pseudo-genes (IGHEP1 and IGHEP2). The IgG class is the most commonly used for therapeutic purposes. In humans this class comprises subclasses IgG1, IgG2, IgG3, and IgG4. In mice this class comprises subclasses IgG1, IgG2a, IgG2b, IgG2c and IgG3.

The term "naturally occurring immunoglobulin super-family" as used herein refers to members of the immunoglobulin super-family which can be found in nature (Williams AF and Barclay AN, *Annu Rev Immunol*, 6:381-405 (1988)) i.e. which have not been genetically engineered, and includes, but is not limited to Antigen receptors like antibodies; immunoglobulins; T cell receptor chains including the TCR constant domain family; Antigen presenting molecules like Class I MHC, Class II MHC or beta-2 microglobulin; Co-receptors like CD4, CD8, or CD19; Antigen receptor accessory molecules like CD3-γ, -δ and -ε chains, CD79a or CD79b; Co-stimulatory or inhibitory molecules like CD28, CD80 or CD86 (also known as B7.1 and B7.2 molecules); Receptors on Natural killer cells like Killer-cell immunoglobulin-like receptors (KIR); Adhesion molecules like CD2, CD48, the SIGLEC family (e.g. CD22, CD83), the CTX family (e.g. CTX, JAMs, BT-IgSF, CAR, VSIG, ESAM), Intercellular adhesion molecules (ICAMs), Vascular cell adhesion molecules (e.g. VCAM-1), Neural Cell Adhesion Molecule (NCAM); Cytokine and growth factor receptors like Interleukin-1 receptor type I, Interleukin-1 receptor type II precursor (IL-1R-2, IL-1R-beta, CD121b antigen), Platelet-derived growth factor receptor (PDGFR), Interleukin-6 receptor alpha chain precursor (IL-6R-alpha,CD126 antigen), Colony stimulating factor 1 receptor precursor (CSF-1-R, CD115 antigen, macrophage colony stimulating factor I receptor), Mast/stem cell growth factor receptor precursor (SCFR, c-kit, CD117 antigen), Basic fibroblast growth factor receptor 1 precursor (FGFR-1, Tyrosine kinase receptor CEK1); Receptor tyrosine kinases/phosphatases like Tyrosine-protein kinase receptor Tie-1 precursor or Receptor-type tyrosine-protein phosphatase mu precursor; Ig binding receptors like Polymeric immunoglobulin receptor (PIGR), or selected Fc receptors; and others like CD147, Thymocyte differentiation antigen-1 (Thy-1), also known as CD90, CD7, Butyrophilins (Btn),

Sodium channel subunit beta-1 precursor, Titin (a huge intracellular muscle protein also known as Connectin).

The T cell receptor (TCR) constant domain family as used herein include the human TCR constant domain alpha which is referred herein as "human TCR constant domain alpha" or "TRAC" (SEQ ID NO: 1; GenBank database accession number AAO72258.1 (residues 135-225) which is equivalent to the complete sequence of IMGT® reference TRAC, the human TCR constant domain beta which is referred herein as "human TCR constant domain beta" or "TRBC2" (SEQ ID NO: 2; GenBank database accession number AAA61026.1(residues 134-261), which is equivalent to residues 1.8 – 124 of IMGT® reference TRBC2), the human TCR constant domain delta which is referred herein as "human TCR constant domain delta" or "TRDC" (SEQ ID NO: 32; GenBank database accession number AAA61125.1 (residues 135-221) which is equivalent to residues 1.7 - 120 of IMGT® reference TRDC), the human TCR constant domain gamma which is referred herein as "human TCR constant domain gamma" or "TRGC1" (SEQ ID NO: 33; GenBank database accession number AAA61110.1 (residues 145-245) which is equivalent to residues1.1 - 124 of IMGT® reference TRGC1), and the pre T-cell antigen receptor chains (pre-TCR) as disclosed in Pang SS et al., Nature, 467(7317):844-8 (2010). Within the scope of the invention, allotype variants of the human TCR beta and gamma constant domains (IMGT® reference TRBC1, and TRGC2 (2x) or TRGC2 (3x), respectively) are equally included included.
IMGT® references are according to IMGT® (the international ImMunoGeneTics information system® (Lefranc MP *et al., Nucleic Acids Res*, 27(1):209-12 (1999); Ruiz M *et al., Nucleic Acids Res*, 28(1):219-21 (2000); Lefranc MP, *Nucleic Acids Res*, 29(1):207-9 (2001); Lefranc MP, *Nucleic Acids Res*, 31(1):307-10 (2003); Lefranc MP *et al., Dev Comp Immunol*, 29(3):185-203 (2005); Kaas Q *et al., Briefings in Functional Genomics & Proteomics*, 6(4):253-64 (2007)).

Preferred naturally occurring immunoglobulin super-family members which can be used for the present invention as donor domains are selected from the group consisting of the human TCR constant domain alpha, the human TCR constant domain beta, the human TCR constant domain gamma, the human TCR constant domain delta, the human TCR variable domain alpha, the human TCR variable domain beta, the human TCR variable domain gamma, the human TCR variable domain delta, pre T-cell antigen receptor chains and the CH1, CH2,

CH3, CH4, IGKC, IGLC1, IGLC2, IGLC3, IGLC6, and IGLC7 domains. More preferred are donor domains selected from the group consisting of the human TCR constant domain alpha, the human TCR constant domain beta, the human TCR constant domain gamma, the human TCR constant domain delta, and the CH3 domain, in particular donor domains selected from the group consisting of the human TCR constant domain alpha, the human TCR constant domain beta, the human TCR constant domain gamma and the human TCR constant domain delta. Preferably naturally occurring immunoglobulin super-family members referred to in the present invention are human naturally occurring immunoglobulins.

The term "Immunoglobulin fragments" as used herein include, but is not limited to, (i) a domain, (ii) the Fab fragment consisting of VL, VH, CL or CK and CH1 domains, including Fab′ and Fab′-SH, (ii) the Fd fragment consisting of the VH and CH1 domains, (iii) the dAb fragment (Ward ES *et al.*, *Nature*, 341(6242):544-6 (1989)) which consists of a single variable domain (iv) $F(ab')_2$ fragments, a bivalent fragment comprising two linked Fab fragments (v) single chain Fv molecules (scFv), wherein a VH domain and a VL domain are linked by a peptide linker which allows the two domains to associate to form an antigen binding site (Bird RE *et al.*, *Science*, 242(4877):423-6 (1988); Huston JS *et al.*, *Proc Natl Acad Sci U S A*, 85(16):5879-83 (1988)), (vi) "diabodies" or "triabodies", multivalent or multispecific fragments constructed by gene fusion (Holliger P *et al.*, *Proc Natl Acad Sci U S A*, 90(14):6444-8 (1993); Tomlinson I and Holliger P, *Methods Enzymol*, 326:461-79 (2000)), (vii) scFv, diabody or domain antibody fused to an Fc region and (viii) scFv fused to the same or a different antibody.

The term "hetero-dimeric immunoglobulin" or "hetero-dimeric fragment" or "hetero-dimer" as used herein includes an immunoglobulin molecule or part of comprising at least a first and a second polypeptide, like a first and a second domain, wherein the second polypeptide differs in amino acid sequence from the first polypeptide. Preferably, a hetero-dimeric immunoglobulin comprises two polypeptide chains, wherein the first chain has at least one non identical domain to the second chain, and wherein both chains assemble, i.e. interact through their non identical domains. Specifically, a hetero-dimeric immunoglobulin comprises at least two domains, wherein the first domain is non identical to the second domain, and wherein both domains assemble, i.e. interact through their protein-protein interfaces. More preferably the hetero-dimeric immunoglobulin, has binding specificity for at

least two different ligands, antigens or binding sites, i.e. is bispecific. Hetero-dimeric immunoglobulin as used herein includes but is not limited to full length bispecific antibodies, bispecifc Fab, bispecifc $F(ab')_2$,bispecific scFv fused to an Fc region, diabody fused to an Fc region and domain antibody fused to an Fc region. Preferably, a hetero-dimeric immunoglobulin fragment comprises at least two domains, wherein the first domain is non identical to the second domain, and wherein both domains assemble, i.e. interact through their protein-protein interfaces. More preferably, a hetero-dimeric immunoglobulin fragment comprises at least two engineered domains, wherein the first engineered domain is non identical to the second engineered domain i.e. the first engineered domain differs in amino acid sequence from the second engineered domain, and wherein both engineered domains assemble by interaction through their protein-protein interfaces.

"Naturally occurring hetero-dimers" as used herein includes but are not limited to an IGKC domain or an IGLC domain (IGLC1 or IGLC2 or IGLC3 or IGLC6 or IGLC7) which hetero-dimerizes with any heavy chain CH1 domain (IGHA1 CH1, IGHA2 CH1, IGHD CH1, IGHE CH1, IGHG1 CH1, IGHG2 CH1, IGHG3 CH1, IGHG4 CH1, IGHGP CH1, IGHM CH1), e.g. IGKC/IGHA1 CH1, IGLC2/IGHA1 CH1, IGLC3/IGHA1 CH1, IGLC6/IGHA1 CH1, IGLC7/IGHA1 CH1, IGKC/IGHA2 CH1, IGLC2/IGHA2 CH1, IGLC3/IGHA2 CH1, IGLC6/IGHA2 CH1, IGLC7/IGHA2 CH1, IGKC/IGHD CH1, IGLC2/IGHD CH1, IGLC3/IGHD CH1, IGLC6/IGHD CH1, IGLC7/IGHD CH1, IGKC/IGHE CH1, IGLC2/IGHE CH1, IGLC3/IGHE CH1, IGLC6/IGHE CH1, IGLC7/IGHE CH1, IGKC/IGHG1 CH1, IGLC2/IGHG1 CH1, IGLC3/IGHG1 CH1, IGLC6/IGHG1 CH1, IGLC7/IGHG1 CH1, IGKC/IGHG2 CH1, IGLC2/IGHG2 CH1, IGLC3/IGHG2 CH1, IGLC6/IGHG2 CH1, IGLC7/IGHG2 CH1, IGKC/IGHG3 CH1, IGLC2/IGHG3 CH1, IGLC3/IGHG3 CH1, IGLC6/IGHG3 CH1, IGLC7/IGHG3 CH1, IGKC/IGHG4 CH1, IGLC2/IGHG4 CH1, IGLC3/IGHG4 CH1, IGLC6/IGHG4 CH1, IGLC7/IGHG4 CH1, IGKC/IGHGP CH1, IGLC2/IGHGP CH1, IGLC3/IGHGP CH1, IGLC6/IGHGP CH1, IGLC7/IGHGP CH1, IGKC/IGHM CH1, IGLC2/IGHM CH1, IGLC3/IGHM CH1, IGLC6/IGHM CH1, IGLC7/IGHM CH1. Other examples of "naturally occurring hetero-dimers" encompass an antibody variable heavy chain domain which hetero-dimerizes with an antibody variable light chain domain (kappa or lambda), a TCR alpha variable domain which hetero-dimerizes with a TCR beta variable domain, a TCR gamma variable domain which hetero-dimerizes with a TCR delta variable domain, a TCR alpha constant domain which

hetero-dimerizes with a TCR beta constant domain, a TCR gamma constant domain which hetero-dimerizes with a TCR delta constant domain.

The term "homo-dimeric immunoglobulin" or "homo-dimeric fragment" or "homo-dimer" as used herein includes an immunoglobulin molecule or part of comprising at least a first and a second polypeptide, like a first and a second domain, wherein the second polypeptide is identical in amino acid sequence to the first polypeptide. Preferably, a homo-dimeric immunoglobulin comprises two polypeptide chains, wherein the first chain has at least one identical domain to the second chain, and wherein both chains assemble, i.e. interact through their identical domains. Specifically, a homo-dimeric immunoglobulin comprises at least two identical domains and wherein both domains assemble, i.e. interact through their protein-protein interfaces. Preferably, a homo-dimeric immunoglobulin fragment comprises at least two domains, wherein the first domain is identical to the second domain, and wherein both domains assemble, i.e. interact through their protein-protein interfaces. "Naturally occurring homo-dimers" as used herein include but are not limited to e.g. two CH3 domains of the same species, isotype and subclass e.g. human IGHG1 CH3/IGHG1 CH3, human IGHG2 CH3/IGHG2 CH3, human IGHG3 CH3/IGHG3 CH3, human IGHG4 CH3/IGHG4 CH3, human IGHA1 CH3/IGHA1 CH3, human IGHA2 CH3/IGHA2 CH3, human IGHE CH3/IGHE CH3, human IGHEP1 CH3/IGHEP1 CH3, human IGHM CH3/IGHM CH3, human IGHD CH3/IGHD CH3, human IGHGP CH3/IGHGP CH3, two CH2 domains of the same species, isotype and subclass e.g. human IGHG1 CH2/IGHG1 CH2, human IGHG2 CH2/IGHG2 CH2, human IGHG3 CH2/IGHG3 CH2, human IGHG4 CH2/IGHG4 CH2, human IGHA1 CH2/IGHA1 CH2, human IGHA2 CH2/IGHA2 CH2, human IGHE CH2/IGHE CH2, human IGHEP1 CH2/IGHEP1 CH2, human IGHM CH2/IGHM CH2, human IGHD CH2/IGHD CH2, human IGHGP CH2/IGHGP CH2, or two CH4 domains of the same species, isotype and subclass e.g. human IGHE CH4/IGHE CH4, human IGHM CH4/IGHM CH4. Prefered "Naturally occurring homo-dimers" are selected from the group consisting of human IGHG1 CH3/IGHG1 CH3, human IGHG2 CH3/IGHG2 CH3, human IGHG3 CH3/IGHG3 CH3, human IGHG4 CH3/IGHG4 CH3, human IGHA1 CH3/IGHA1 CH3, human IGHA2 CH3/IGHA2 CH3, human IGHE CH3/IGHE CH3, human IGHM CH3/IGHM CH3, human IGHD CH3/IGHD CH3, human IGHGP CH3/IGHGP CH3, human IGHE CH2/IGHE CH2, human IGHM CH2/IGHM CH2, human IGHE CH4/IGHE CH4, and human IGHM CH4/IGHM CH4.

Most immunoglobulin light chains associate into dimers (Novotny J and Haber E, *Proc Natl Acad Sci U S A*, 82(14):4592-6 (1985)). Both kappa and lambda light chains have been reported to homo-dimerize, and several crystal structures of kappa and lambda light chain dimers are available from the Protein Data Bank (PDB) database (Roussel A *et al.*, *Eur J Biochem*, 260(1):192-9 (1999), Huang DB *et al.*, *Proc Natl Acad Sci U S A*, 93(14):7017-21 (1996); www.pdb.org; Bernstein FC *et al.*, *Eur J Biochem*, 80(2):319-24 (1977)).Within the scope of the invention homo-dimers of an IGKC domain or an IGLC domain (IGLC1 or IGLC2 or IGLC3 or IGLC6 or IGLC7) can be considered as parent or donor domains for protein-protein interface engineering. Thus, "naturally occurring homo-dimers" as used herein also include but are not limited to two IGKC domains of the same species, isotype and subclass e.g. human IGKC/IGKC, two IGLC domains of the same species, isotype and subclass e.g. human IGLC1/IGLC1, human IGLC2/IGLC2, human IGLC3/IGLC3, human IGLC6/IGLC6, human IGLC7/IGLC7 and any other pairwise combination of IGLC1, IGLC2, IGLC3, IGLC6, and IGLC7.

The term "formation of a hetero-dimer" or "forming a hetero-dimer" or "capable to form a hetero-dimer" in relation to two domains e.g. in relation to two parent domains or to two donor domains as used herein means that the first domain is not identical to the second domain and wherein both domains assemble, i.e. interact through their protein-protein interface wherein the protein-protein interfaces are normally not identical.The first domain is not identical to the second domain if both domains differ in amino acid sequence by at least one amino acid residue. The protein-protein interfaces are normally not identical if the protein-protein interface of the first parent or donor domain has at least one 3D structural position with a different amino acid residue compared to the protein-protein interface of the second parent or donor domain.

The term "formation of a naturally occurring hetero-dimer" or "forming a naturally occurring hetero-dimer" or "capable to form a naturally occurring hetero-dimer" in relation to two domains e.g. in relation to two naturally occurring parent domains or to two naturally occurring donor domains as used herein means that the first domain is not identical to the second domain and wherein both domains assemble, i.e. interact through their protein-protein interface wherein the protein-protein interfaces are normally not identical. The first domain is not identical to the second domain if both domains differ in amino acid sequence by at least one amino acid residue. The protein-protein interfaces are normally not identical if the

protein-protein interface of the first parent or donor domain has at least one 3D structural position with a different amino acid residue compared to the protein-protein interface of the second parent or donor domain.

The term "formation of a homo-dimer" or "forming a homo-dimer" or "capable to form a homo-dimer" in relation to two domains e.g. in relation to two parent domains or to two donor domains as used herein means that the first domain is identical to the second domain and wherein both domains assemble, i.e. interact through their protein-protein interface, wherein the protein-protein interfaces are normally identical. The first domain is identical to the second domain if their amino acid sequences are identical. The protein-protein interfaces are normally identical if the protein-protein interface of the first parent or donor domain has the identical amino acid at the identical 3D structural position compared to the protein-protein interface of the second parent or donor domain.

The term "formation of a naturally occurring homo-dimer" or "forming a naturally occurring homo-dimer" or "capable to form a naturally occurring homo-dimer" in relation to two domains e.g. in relation to two naturally occurring parent domains or to two naturally occurring donor domains as used herein means that the first domain is identical to the second domain and wherein both domains assemble, i.e. interact through their protein-protein interface, wherein the protein-protein interfaces are normally identical. The first domain is identical to the second domain if their amino acid sequences are identical. The protein-protein interfaces are normally identical if the protein-protein interface of the first parent or donor domain has the identical amino acid at the identical 3D structural position compared to the protein-protein interface of the second parent or donor domain.

The term "domain" as used herein includes any region of a polypeptide that is responsible for selectively assembling with a protein partner (i.e., another protein (or region of) or another domain) and/or can perform a complete biological function or part of like binding a receptor, or a substrate, independently or within a multidomain entity. Usually a domain as referred to herein is not a hinge region and/or does not contain a hinge region. The domain can exist independently of the rest of a protein chain. A domain forms a compact three-dimensional structure and is independently stable and folded. Domains vary in length from between about 25 amino acids up to 500 amino acids in length. Preferably the domains as used herein vary in length from between about 70 amino acids up to about 120 amino acids in length. The shortest

domains such as zinc fingers are stabilized by metal ions or disulfide bridges. Domains often form functional units, such as the calcium-binding EF hand domain of calmodulin. Because they are self-stable, domains can be "swapped" by genetic engineering between one protein and another to make chimeric proteins. Immunoglobulins are made of variable and constant domains belonging to the immunoglobulin superfamily (Williams AF and Barclay AN, *Annu Rev Immunol*, 6:381-405 (1988); Bork P *et al.*, *J Mol Biol*, 242(4):309-20 (1994)). Domains, which are included herein are CH1 and CH3, specifically naturally occurring CH1 and CH3, from IGHA1, IGHA2, IGHD, IGHE, IGHG1, IGHG2, IGHG3, IGHG4, IGHGP and IGHM; CH4, specifically naturally occurring CH4, from IGHE and IGHM; IGKC, IGLC1, IGLC2, IGLC3, IGLC6, and IGLC7, specifically naturally occurring IGKC, IGLC1, IGLC2, IGLC3, IGLC6, and IGLC7. Further domains which are included herein are CH2, specifically naturally occurring CH2, from IGHA1, IGHA2, IGHD, IGHE, IGHG1, IGHG2, IGHG3, IGHG4, IGHGP and IGHM. Preferably unglycosylated CH2 domains are used herein which in their unglycosylated form are homo-dimers as referred herein. The CH2 domains in an unglycosylated Fc fragment approach each other much more closely compared to the CH2 domains in a naturally glycosylated Fc fragment. The crystal structure of the murine unglycosylated IgG1 Fc fragment has shown that a fully unglycosylated Fc fragment can adopt a "closed" structure with the distance between the Pro 332 from the CH2 domain of the first unglycosylated immunoglobulin chain and the Pro 332 from the CH2 domain of the second unglycosylated immunoglobulin chain is only 11.6 Å (Feige MJ *et al.*, *J Mol Biol*, 391(3):599-608 (2009)). Further domains which are included herein are VH and VL domains which do not have an engineered protein-protein interface according to the invention, i.e. which are not specifically engineered to modify its naturally occurring protein-protein interface except for backmutations arising out of the humanization process. The term "naturally occurring domain" as used herein refers to domains which can be found in nature i.e. which have not been genetically engineered.

For all immunoglobulin constant domains included in the present invention, numbering can be according to the IMGT® (IMGT®, the international ImMunoGeneTics information system®; Lefranc MP *et al.*, *Nucleic Acids Res*, 27(1):209-12 (1999); Ruiz M *et al.*, *Nucleic Acids Res*, 28(1):219-21 (2000); Lefranc MP, *Nucleic Acids Res*, 29(1):207-9 (2001); Lefranc MP, *Nucleic Acids Res*, 31(1):307-10 (2003); Lefranc MP *et al.*, *Dev Comp Immunol*, 29(3):185-

203 (2005); Kaas Q *et al.*, *Briefings in Functional Genomics & Proteomics*, 6(4):253-64 (2007)).

For all human CH1, CH2, CH3 immunoglobulin heavy chain constant domains selected from the group consisting of IGHG1, IGHG2, IGHG3, and IGHG4, numbering can be according to the "EU numbering system" (Edelman GM *et al.*, *Proc Natl Acad Sci U S A*, 63(1):78-85 (1969)). The correspondence between the IMGT unique numbering and the EU numbering for all human CH1, CH2, CH3 immunoglobulin heavy chain constant domains selected from the group consisting of IGHG1, IGHG2, IGHG3, and IGHG4 is shown in FIG. 19. A complete correspondence for the human CH1, hinge, CH2, and CH3 constant regions of IGHG1 can be found at the IMGT database (IMGT®, the international ImMunoGeneTics information system®; Lefranc MP *et al.*, *Nucleic Acids Res*, 27(1):209-12 (1999); Ruiz M *et al.*, *Nucleic Acids Res*, 28(1):219-21 (2000); Lefranc MP, *Nucleic Acids Res*, 29(1):207-9 (2001); Lefranc MP, *Nucleic Acids Res*, 31(1):307-10 (2003); Lefranc MP *et al.*, *Dev Comp Immunol*, 29(3):185-203 (2005)); Kaas Q *et al.*, *Briefings in Functional Genomics & Proteomics*, 6(4):253-64 (2007)).

For the human kappa immunoglobulin light chain constant domain (IGKC), numbering can be according to the "EU numbering system" (Edelman GM et al., Proc Natl Acad Sci U S A, 63(1):78-85 (1969)). The correspondence between the IMGT unique numbering and the EU numbering for the human IGKC immunoglobulin light chain constant domain is shown in FIG. 22. A complete correspondence for the human CK domain can be found at IMGT database (IMGT®, the international ImMunoGeneTics information system®; Lefranc MP *et al.*, *Nucleic Acids Res*, 27(1):209-12 (1999); Ruiz M *et al.*, *Nucleic Acids Res*, 28(1):219-21 (2000); Lefranc MP, *Nucleic Acids Res*, 29(1):207-9 (2001); Lefranc MP, *Nucleic Acids Res*, 31(1):307-10 (2003); Lefranc MP *et al.*, *Dev Comp Immunol*, 29(3):185-203 (2005)); Kaas Q *et al.*, *Briefings in Functional Genomics & Proteomics*, 6(4):253-64 (2007)).

For the human lambda immunoglobulin light chain constant domains (IGLC1, IGLC2, IGLC3, IGLC6, and IGLC7), numbering can be according to the "Kabat numbering system" (Kabat EA *et al.*, *Sequences of proteins of immunological interest.* 5th Edition - US Department of Health and Human Services, NIH publication n° 91-3242 (1991)) as described by Dariavach P *et al.*, *Proc Natl Acad Sci U S A*, 84(24):9074-8 (1987) and Frangione B *et al.*, *Proc Natl Acad Sci U S A*, 82(10):3415-9 (1985). The correspondence between the IMGT unique numbering and the Kabat numbering for human immunoglobulin light chain constant domains (IGLC1, IGLC2, IGLC3, IGLC6, and IGLC7) is shown in FIG. 23. A complete

correspondence for human IGLC domains can be found at the IMGT database (IMGT®, the international ImMunoGeneTics information system®; Lefranc MP *et al.*, *Nucleic Acids Res*, 27(1):209-12 (1999); Ruiz M *et al.*, *Nucleic Acids Res*, 28(1):219-21 (2000); Lefranc MP, *Nucleic Acids Res*, 29(1):207-9 (2001); Lefranc MP, *Nucleic Acids Res*, 31(1):307-10 (2003); Lefranc MP *et al.*, *Dev Comp Immunol*, 29(3):185-203 (2005)); Kaas Q *et al.*, *Briefings in Functional Genomics & Proteomics*, 6(4):253-64 (2007)).

The human IGHG1 immunoglobulin heavy chain constant domains referred herein as the following domain boundaries: CH1 [EU numbering: 118-215], Hinge γ1 [EU numbering: 216-230], CH2 [EU numbering: 231-340], and CH3 [EU numbering: 341-447]. The human CK domain referred herein spans residues 108 to 214 (EU numbering). The human IGLC1, IGLC2, IGLC3, IGLC6, and IGLC7domains referred herein span residues 108-215 (Kabat numbering).

A "multidomain entity" or "multidomain protein" as used herein comprises at least two domains. These two domains can be on one immunoglobulin chain (one polypeptide) or can be on two immunoglobulin chains (two polypeptides).

The term "engineered immunoglobulin chain" as used herein includes an immunoglobulin chain comprising at least one engineered domain with a protein-protein interface which differs from the parent domain.
The term "parent immunoglobulin chain" as used herein includes any existing immunoglobulin chain, specifically naturally occurring immunoglobulin chains, which can be used as a parent sequence for designing an engineered immunoglobulin chain. Preferably, the the parent immunoglobulin chains, e.g. the parent immunoglobulin chain of the first engineered immunoglobulin chain and/or the parent immunoglobulin chain of the second engineered immunoglobulin chain and/or the parent immunoglobulin chain of the third engineered immunoglobulin chain are naturally occurring immunoglobulin chains.

The term "parent domain" as used herein includes any existing domain, specifically naturally occurring domains, more specifically naturally occurring domains which form a naturally occurring hetero-dimer or a naturally occurring homo-dimer, which can be used as a parent

sequence for designing an engineered domain. The parent domain of the engineered domain of the first, second, third and fourth engineered immunoglobulin chain is not a zinc finger. The parent domain is usually an immunoglobulin domain, e.g. a domain of an immunoglobulin chain. Preferably, the parent domains, e.g. the parent domain of a first and/or a second engineered immunoglobulin chain and/or the parent domain of a further engineered domain of the first and/or the second engineered immunoglobulin chain and/or the parent domain of an engineered domain of a third engineered immunoglobulin chain are naturally occurring domains.

The term "donor domain" as used herein includes naturally occurring domains, specifically naturally occurring domains which form a naturally occurring hetero-dimer or a naturally occurring homo-dimer, which can be used as a donor sequence for designing an engineered domain, i.e. which can be used as donor sequence for substituting amino acid residues at the equivalent 3D structural position in a parent domain.

The term "engineered domain" as used herein includes a domain engineered from a parent domain and a donor domain.

The term "protein-protein interface" as used herein includes amino acid residues that mediate direct-contact association of a protein domain with amino acid residues of another protein domain thereby defining a 3D interface. These amino acid residues that mediate direct-contact association between two domains include any amino acid from one partner interacting with one or more amino acid from the other partner. The term "interacting" in relation to interacting amino acid residues as used herein includes any amino acid from one partner having at least one heavy atom that is less than 15 Å away from any heavy atom of an amino acid residue in the other partner. The term "heavy atom of an amino acid residue" refers herein to any atom of an amino acid residue which is not a hydrogen atom. Preferably, interacting amino acid residues include any amino acids from one partner having at least one heavy atom that is less than 10 Å away from any heavy atom of an amino acid residue in the other partner. Most preferably, interacting amino acid residues include any amino acids from one partner having at least one heavy atom that is less than 5 Å away from any heavy atom of an amino acid residue in the other partner. The interaction of amino acid residues can be mediated by forces which include van der Waals forces, hydrogen bonds, water-mediated

hydrogen bonds, salt bridges or electrostatic forces, hydrophobic contacts, and disulfide bonds or other forces known to one skilled in the art. Protein-protein interfaces are more hydrophobic and bury twice as much protein surfaces as in protein complexes, and usually assemble at the time they fold (Bahadur RP *et al.*, *Proteins*, 53(3):708-19 (2003)). Analysis of 47 FAB fragments experimentally determined 3D structures showed that up to 50 residues in the constant domain light chain and up to 52 residues in the constant domain heavy chain compose the domain protein-protein interfaces (Potapov V *et al.*, *J Mol Biol*, 342(2):665-79 (2004)). The CH3 protein interface involves 16 residues located on four antiparallel beta-strands that make intermolecular contacts and are buried 1090 Å2 from each surface (Dall'Acqua W *et al.*, *Biochemistry*, 37(26):9266-73 (1998)).

The protein-protein interfaces of the engineered domains are considered to interact by homo-dimerization if the protein-protein interface of the donor domain of e.g. the first member of the naturally occurring immunoglobulin super-family and the protein-protein interface of the donor domain of e.g. the second member of the naturally occurring immunoglobulin super-family interact by homo-dimerization, e.g. if both donor domains form a homo-dimer, and if all amino acid residues of the protein-protein interfaces of the parent domains are substituted with amino acid residues of the donor domain of the first member of the naturally occurring immunoglobulin super-family and the donor domain of the second member of the naturally occurring immunoglobulin super-family. Thus protein-protein interfaces of the engineered domains are considered to interact by homo-dimerization if all amino acid residues of the protein-protein interface of the first engineered domain are identical to the amino acid residues of the protein-protein interface of the second engineered domain at identical 3D positions.

The protein-protein interfaces of the engineered domains are considered to interact by hetero-dimerization if the protein-protein interface of the donor domain of e.g. the first member of the naturally occurring immunoglobulin super-family and the protein-protein interface of the donor domain of e.g. the second member of the naturally occurring immunoglobulin super-family interact by homo-dimerization, e.g. if both donor domains form a homo-dimer, and if not all amino acid residues of the protein-protein interfaces of the parent domains are substituted with amino acid residues of the donor domain of the first member of the naturally occurring immunoglobulin super-family and the donor domain of the second member of the naturally occurring immunoglobulin super-family. More specifically the protein-protein

interfaces of the engineered domains are considered to interact by hetero-dimerization if the protein-protein interface of the donor domain of e.g. the first member of the naturally occurring immunoglobulin super-family and the protein-protein interface of the donor domain of e.g. the second member of the naturally occurring immunoglobulin super-family interact by homo-dimerization, e.g. if both donor domains form a homo-dimer, and if at least one amino acid residue in the first parent domain is substituted with an amino acid residue at the 3D equivalent structural position in the donor domain of the first member of the naturally occurring immunoglobulin super-family and wherein the second parent domain is not substituted with the identical amino acid residue which was substituted in the first parent domain at the identical 3D structural position.

The protein-protein interfaces of the engineered domains are considered to interact by hetero-dimerization if the protein-protein interface of the donor domain of e.g. the first member of the naturally occurring immunoglobulin super-family and the protein-protein interface of the donor domain of e.g. the second member of the naturally occurring immunoglobulin super-family interact by hetero-dimerization e.g. if both donor domains form a hetero-dimer, and if all or not all amino acid residues of the protein-protein interfaces of the parent domains are substituted with amino acid residues of the donor domain of the first member of the naturally occurring immunoglobulin super-family and the donor domain of the second member of the naturally occurring immunoglobulin super-family.
Thus protein-protein interfaces of the engineered domains are considered to interact by hetero-dimerization if not all amino acid residues of the protein-protein interface of the first engineered domain are identical to the amino acid residues of the protein-protein interface of the second engineered domain at identical 3D positions.

The term "equivalent 3D structural position" or "equivalent 3D position" or "3D equivalent structural position" or "3D equivalent position" are used interchangeably herein and include the position of an amino acid residue in the protein-protein interface of a donor domain which when overlaid on the protein-protein interface of a parent domain by superimposing the carbon alpha traces of both domains, occupies a 3D position within less than a distance of 6 Å to the closest residue of the parent domain, wherein the donor domain is different from the parent domain. Superimposition can be performed by comparing the coordinates of the atoms

in the same frame of reference. Beside the carbon alpha traces, backbone atoms (N, C and O) of both domains can be superimposed. The 3D structural positions can be selected as well based on visual inspection (i.e. intuition based design or knowledge based design) or positions can be selected after enumerating energy terms like van der Waals interaction, hydrogen bond interaction etc. (i.e. rational design). 3D structures of the domains as used herein includes experimentally solved 3D structure in crystalline or solution state which can be retrieved the Protein Data Bank (www.pdb.org; Bernstein FC *et al.*, *Eur J Biochem*, 80(2):319-24 (1977); Berman HM *et al.*, *Nucleic Acids Res*, 28(1):235-42 (2000)) or other databases. In addition, 3D structures of the domains which are not available from public databases or have not been solved can be modelled using a number of computational methods such as *Ab initio* prediction methods where only the amino acid sequence of the protein is required or threading and homology modeling methods where a 3D model for the protein domain is build from experimental structures of evolutionary related proteins (Zhang Y, *Curr Opin Struct Biol*, 18(3):342-8 (2008)). Without being bound by theory, alternatively the IMGT® numbering system can be used to identify equivalent 3D structural positions in immunoglobulin domains since this system is based on the comparative analysis of the 3D structure of the immunoglobulin super-family domains.

The term "Non essential to the core integrity of the domain" as used herein includes residues which are non-essential for correct folding and/or the hydrophobic core of the protein domain. Folded proteins usually have a hydrophobic core in which side chain packing stabilizes the folded state, and charged or polar side chains occupy the solvent-exposed surface where they interact with surrounding water, ions or others ligands such as other proteins or protein domains or carbohydrates. Formation of intramolecular hydrogen bonds provides another important contribution to protein core stability. Non essential residues to the core integrity of a protein domain include but are not limited to non-proline residues, non cysteine residues involved in an intramolecular disulfide bond, exposed residues, and residues which are not involved intramolecular hydrogen bonds.

The terms "protein" and "polypeptide" as used herein have the same meaning and are used interchangeably.

The terms “amino acid” or “amino acid residue” as used herein includes natural amino acids as well as non-natural amino acids. Preferably natural amino acids are included.

The term “charged amino acid” as used herein includes the amino acids lysine (positively charged), arginine (positively charged), histidine (positively charged), aspartic acid (negatively charged) and glutamic acid (negatively charged). Normally, charged amino acid as used herein are lysine, arginine, histidine, aspartic acid and glutamic acid. The term “charged pair” or “charged pair of amino acids” or “charged amino acid pair” have the same meaning and are used interchangeably and include any combination of one positively charged amino acid with one negatively charged amino acid. This includes the following pairs of charged amino acids: lysine/aspartic acid, lysine/glutamic acid, arginine/aspartic acid, arginine/glutamic acid, histidine/aspartic acid, and histidine/glutamic acid.

The terms “substitution” or “amino acid substitution” or “amino acid residue substitution” as used herein refers to a substitution of a first amino acid residue in an amino acid sequence with a second amino acid residue, whereas the first amino acid residue is different from the second amino acid residue i.e. the substituted amino acid residue is different from the amino acid which has been substituted.

The term “amino acid residues which are not adjacent” or “not adjacent amino acid residues” as used herein refers to two amino acids within an amino acid sequence which are not immediately adjoining i.e. which have at least one intervening amino acid residue in between them.

The term "Fab" or "Fab region" as used herein includes the polypeptides that comprise the VH, CH1, VL, and light chain constant immunoglobulin domains. Fab may refer to this region in isolation, or this region in the context of a full length antibody or antibody fragment.

The term "Fc" or "Fc region", as used herein includes the polypeptide comprising the constant region of an antibody heavy chain excluding the first constant region immunoglobulin domain. Thus Fc refers to the last two constant region immunoglobulin domains of IgA, IgD, and IgG, and the last three constant region immunoglobulin domains of IgE and IgM, and the flexible hinge N-terminal to these domains. For IgA and IgM, Fc may include the J chain. For

IgG, Fc comprises immunoglobulin domains Cgamma2 and Cgamma3 (C[γ]2 and C[γ]3) and the hinge between Cgammal (C[γ]l) and Cgamma2 (C[γ]2). Although the boundaries of the Fc region may vary, the human IgG heavy chain Fc region is usually defined to comprise residues C226 or P230 to its carboxyl-terminus, wherein the numbering is according to the EU index. Fc may refer to this region in isolation or this region in the context of an Fc polypeptide, for example an antibody.

The term "amino acid modification" herein includes an amino acid substitution, insertion, and/or deletion in a polypeptide sequence. By "amino acid substitution" or "substitution" herein is meant the replacement of an amino acid at a particular position in a parent polypeptide sequence with another amino acid. For example, the substitution R94K refers to a variant polypeptide, in which the arginine at position 94 is replaced with a lysine. For example 94K indicates the substitution of position 94 with a lysine. For the purposes herein, multiple substitutions are typically separated by a slash or a comma. For example, "R94K/L78V" or "R94K, L78V" refers to a double variant comprising the substitutions R94K and L78V. By "amino acid insertion" or "insertion" as used herein is meant the addition of an amino acid at a particular position in a parent polypeptide sequence. For example, insert -94 designates an insertion at position 94. By "amino acid deletion" or "deletion" as used herein is meant the removal of an amino acid at a particular position in a parent polypeptide sequence. For example, R94- designates the deletion of arginine at position 94.

The term "conservative modifications" or "conservative sequence modifications" is intended to refer to amino acid modifications that do not significantly affect or alter the binding characteristics of the antibody containing the amino acid sequence. Such conservative modifications include amino acid substitutions, insertions and deletions. Modifications can be introduced into an antibody of the invention by standard techniques known in the art, such as site-directed mutagenesis and PCR-mediated mutagenesis.
"Conservative amino acid substitutions" as used herein includes amino acid substitutions in which the amino acid residue is replaced with an amino acid residue having a similar side chain. Families of amino acid residues having similar side chains have been defined in the art. These families include amino acids with basic side chains (e.g., lysine, arginine, histidine), acidic side chains (e.g., aspartic acid, glutamic acid), uncharged polar side chains (e.g., glycine, asparagine, glutamine, serine, threonine, tyrosine, cysteine, tryptophan), nonpolar

side chains (e.g., alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine), beta-branched side chains (e.g., threonine, valine, isoleucine) and aromatic side chains (e.g., tyrosine, phenylalanine, tryptophan, histidine).

Hetero-dimeric immunoglobulins

In one aspect, the present disclosure provides a hetero-dimeric immunoglobulin or hetero-dimeric fragment thereof comprising:

(a) a first engineered immunoglobulin chain comprising an engineered domain with a protein-protein interface, wherein at least one amino acid residue of the protein-protein interface of the parent domain of said first engineered immunoglobulin chain is substituted with an amino acid residue at the equivalent 3D structural position from a protein-protein interface of a donor domain of a first member of the naturally occurring immunoglobulin super-family; and

(b) a second engineered immunoglobulin chain comprising an engineered domain with a protein-protein interface, wherein at least one amino acid residue of the protein-protein interface of the parent domain of said second engineered immunoglobulin chain is substituted with an amino acid residue at the equivalent 3D structural position from a protein-protein interface of a donor domain of a second member of the naturally occurring immunoglobulin super-family,

wherein the donor domain of the first member of the naturally occurring immunoglobulin super-family and the donor domain of the second member of the naturally occurring immunoglobulin super-family form a naturally occurring hetero-dimer or a naturally occurring homo-dimer and,

wherein the parent domains of the first and the second engineered immunoglobulin chain are not a CH2 domain, not a CH3 domain, not a VL domain and not a VH domain and wherein,

if the parent domain of the first engineered immunoglobulin chain is an IGLC domain or an IGKC domain and the parent domain of the second engineered immunoglobulin chain is a CH1 domain and the protein-protein interface of the parent domain of the first engineered immunoglobulin chain is substituted with an amino acid residue at position 12 and the protein-protein interface of the parent domain of the second engineered immunoglobulin chain is substituted with an amino acid residue at position 119, the substituted amino acid residue at position 12 and the substituted amino acid residue at position 119 are not a charged pair,

if the parent domain of the first engineered immunoglobulin chain is a CH1 domain and the

parent domain of the second engineered immunoglobulin chain is an IGLC domain and the protein-protein interface of the parent domain of the first engineered immunoglobulin chain is substituted with an amino acid residue at position 26 and the protein-protein interface of the parent domain of the second engineered immunoglobulin chain is substituted with an amino acid residue at position 13, the substituted amino acid residue at position 26 and the substituted amino acid residue at position 13 are not a charged pair,

if the parent domain of the first engineered immunoglobulin chain is an IGLC domain and the parent domain of the second engineered immunoglobulin chain is a CH1 domain and the protein-protein interface of the parent domain of the first engineered immunoglobulin chain is substituted with an amino acid residue at position 5 and the protein-protein interface of the parent domain of the second engineered immunoglobulin chain is substituted with an amino acid residue at position 20, the substituted amino acid residue at position 5 and the substituted amino acid residue at position 20 are not a charged pair,

if the parent domain of the first engineered immunoglobulin chain is a CH1 domain and the parent domain of the second engineered immunoglobulin chain is an IGLC domain and the protein-protein interface of the parent domain of the first engineered immunoglobulin chain is substituted with an amino acid residue at position 27 and the protein-protein interface of the parent domain of the second engineered immunoglobulin chain is substituted with an amino acid residue at position 18, the substituted amino acid residue at position 27 and the substituted amino acid residue at position 18 are not a charged pair,

if the parent domain of the first engineered immunoglobulin chain is an IGLC domain or an IGKC domain and the parent domain of the second engineered immunoglobulin chain is a CH1 domain and the protein-protein interface of the parent domain of the first engineered immunoglobulin chain is substituted with an amino acid residue at position 20 and the protein-protein interface of the parent domain of the second engineered immunoglobulin chain is substituted with an amino acid residue at position 26, the substituted amino acid residue at position 20 and the substituted amino acid residue at position 26 are not a charged pair,

if the parent domain of the first engineered immunoglobulin chain is an IGLC domain and the parent domain of the second engineered immunoglobulin chain is a CH1 domain and the protein-protein interface of the parent domain of the first engineered immunoglobulin chain is substituted with an amino acid residue at position 79 and the protein-protein interface of the parent domain of the second engineered immunoglobulin chain is substituted with an amino

acid residue at position 84.3, the substituted amino acid residue at position 79 and the substituted amino acid residue at position 84.3 are not a charged pair,
if the parent domain of the first engineered immunoglobulin chain is an IGLC domain or an IGKC domain and the parent domain of the second engineered immunoglobulin chain is a CH1 domain and the protein-protein interface of the parent domain of the first engineered immunoglobulin chain is substituted with an amino acid residue at position 86 and the protein-protein interface of the parent domain of the second engineered immunoglobulin chain is substituted with an amino acid residue at position 86, the substituted amino acid residues at both positions 86 are not a charged pair,
if the parent domain of the first engineered immunoglobulin chain is an IGKC domain and the amino acid residue which is substituted in the protein-protein interface of the parent domain of the first engineered immunoglobulin chain is at position 11 and the parent domain of the second engineered immunoglobulin chain is a domain which comprises a hinge region, the parent domain of the second engineered immunoglobulin chain is not substituted at position 3 of the hinge region, wherein the amino acid position of each group member is indicated according to the IMGT® numbering.

Preferably, the present disclosure provides a hetero-dimeric immunoglobulin or hetero-dimeric fragment thereof comprising:
(a) a first engineered immunoglobulin chain comprising an engineered domain with a protein-protein interface, wherein at least one amino acid residue of the protein-protein interface of the parent domain of said first engineered immunoglobulin chain is substituted with an amino acid residue at the equivalent 3D structural position from a protein-protein interface of a donor domain of a first member of the naturally occurring immunoglobulin super-family; and
(b) a second engineered immunoglobulin chain comprising an engineered domain with a protein-protein interface, wherein at least one amino acid residue of the protein-protein interface of the parent domain of said second engineered immunoglobulin chain is substituted with an amino acid residue at the equivalent 3D structural position from a protein-protein interface of a donor domain of a second member of the naturally occurring immunoglobulin super-family,
wherein the donor domain of the first member of the naturally occurring immunoglobulin super-family and the donor domain of the second member of the naturally occurring immunoglobulin super-family form a naturally occurring hetero-dimer or a naturally

occurring homo-dimer and,

wherein the parent domains of the first and the second engineered immunoglobulin chain are not a CH2 domain, not a CH3 domain, not a VL domain and not a VH domain and wherein,

if the parent domain of the first engineered immunoglobulin chain is an IGLC domain or an IGKC domain and the parent domain of the second engineered immunoglobulin chain is a CH1 domain and the protein-protein interface of the parent domain of the first engineered immunoglobulin chain is substituted with an amino acid residue at position 12 and the protein-protein interface of the parent domain of the second engineered immunoglobulin chain is substituted with an amino acid residue at position 119, the substituted amino acid residue at position 12 and the substituted amino acid residue at position 119 are not a charged pair,

if the parent domain of the first engineered immunoglobulin chain is a CH1 domain and the parent domain of the second engineered immunoglobulin chain is an IGLC domain and the protein-protein interface of the parent domain of the first engineered immunoglobulin chain is substituted with an amino acid residue at position 26 and the protein-protein interface of the parent domain of the second engineered immunoglobulin chain is substituted with an amino acid residue at position 13, the substituted amino acid residue at position 26 and the substituted amino acid residue at position 13 are not a charged pair,

if the parent domain of the first engineered immunoglobulin chain is an IGLC domain and the parent domain of the second engineered immunoglobulin chain is a CH1 domain and the protein-protein interface of the parent domain of the first engineered immunoglobulin chain is substituted with an amino acid residue at position 5 and the protein-protein interface of the parent domain of the second engineered immunoglobulin chain is substituted with an amino acid residue at position 20, the substituted amino acid residue at position 5 and the substituted amino acid residue at position 20 are not a charged pair,

if the parent domain of the first engineered immunoglobulin chain is a CH1 domain and the parent domain of the second engineered immunoglobulin chain is an IGLC domain and the protein-protein interface of the parent domain of the first engineered immunoglobulin chain is substituted with an amino acid residue at position 27 and the protein-protein interface of the parent domain of the second engineered immunoglobulin chain is substituted with an amino acid residue at position 18, the substituted amino acid residue at position 27 and the substituted amino acid residue at position 18 are not a charged pair,

if the parent domain of the first engineered immunoglobulin chain is an IGLC domain or an

IGKC domain and the parent domain of the second engineered immunoglobulin chain is a CH1 domain and the protein-protein interface of the parent domain of the first engineered immunoglobulin chain is substituted with an amino acid residue at position 20 and/or the protein-protein interface of the parent domain of the second engineered immunoglobulin chain is substituted with an amino acid residue at position 26, the substituted amino acid residue at position 20 and the unsubstituted or substituted amino acid residue at position 26 are not a charged pair,

if the parent domain of the first engineered immunoglobulin chain is an IGLC domain and the parent domain of the second engineered immunoglobulin chain is a CH1 domain and the protein-protein interface of the parent domain of the first engineered immunoglobulin chain is substituted with an amino acid residue at position 79 and/or the protein-protein interface of the parent domain of the second engineered immunoglobulin chain is substituted with an amino acid residue at position 84.3, the unsubstituted or substituted amino acid residue at position 79 and the substituted amino acid residue at position 84.3 are not a charged pair,

if the parent domain of the first engineered immunoglobulin chain is an IGLC domain or an IGKC domain and the parent domain of the second engineered immunoglobulin chain is a CH1 domain and the protein-protein interface of the parent domain of the first engineered immunoglobulin chain is substituted with an amino acid residue at position 86 and the protein-protein interface of the parent domain of the second engineered immunoglobulin chain is substituted with an amino acid residue at position 86, the substituted amino acid residues at both positions 86 are not a charged pair,

if the parent domain of the first engineered immunoglobulin chain is an IGKC domain and the amino acid residue which is substituted in the protein-protein interface of the parent domain of the first engineered immunoglobulin chain is at position 11 and the parent domain of the second engineered immunoglobulin chain is a domain which comprises a hinge region, the parent domain of the second engineered immunoglobulin chain is not substituted at position 3 of the hinge region, wherein the amino acid position of each group member is indicated according to the IMGT® numbering.

Even more preferably, the present disclosure provides a hetero-dimeric immunoglobulin or hetero-dimeric fragment thereof comprising:

(a) a first engineered immunoglobulin chain comprising an engineered domain with a protein-protein interface, wherein at least one amino acid residue of the protein-protein interface of

the parent domain of said first engineered immunoglobulin chain is substituted with an amino acid residue at the equivalent 3D structural position from a protein-protein interface of a donor domain of a first member of the naturally occurring immunoglobulin super-family; and

(b) a second engineered immunoglobulin chain comprising an engineered domain with a protein-protein interface, wherein at least one amino acid residue of the protein-protein interface of the parent domain of said second engineered immunoglobulin chain is substituted with an amino acid residue at the equivalent 3D structural position from a protein-protein interface of a donor domain of a second member of the naturally occurring immunoglobulin super-family,

wherein the donor domain of the first member of the naturally occurring immunoglobulin super-family and the donor domain of the second member of the naturally occurring immunoglobulin super-family form a naturally occurring hetero-dimer or a naturally occurring homo-dimer and,

wherein the parent domains of the first and the second engineered immunoglobulin chain are not a CH2 domain, not a CH3 domain, not a VL domain and not a VH domain and wherein, if the parent domain of the first engineered immunoglobulin chain is an IGLC domain or an IGKC domain and the parent domain of the second engineered immunoglobulin chain is a CH1 domain and the protein-protein interface of the parent domain of the first engineered immunoglobulin chain is substituted with an amino acid residue at position 12 and the protein-protein interface of the parent domain of the second engineered immunoglobulin chain is substituted with an amino acid residue at position 119, the substituted amino acid residue at position 12 and the substituted amino acid residue at position 119 are not charged amino acids,

if the parent domain of the first engineered immunoglobulin chain is a CH1 domain and the parent domain of the second engineered immunoglobulin chain is an IGLC domain and the protein-protein interface of the parent domain of the first engineered immunoglobulin chain is substituted with an amino acid residue at position 26 and the protein-protein interface of the parent domain of the second engineered immunoglobulin chain is substituted with an amino acid residue at position 13, the substituted amino acid residue at position 26 and the substituted amino acid residue at position 13 are not charged amino acids,

if the parent domain of the first engineered immunoglobulin chain is an IGLC domain and the parent domain of the second engineered immunoglobulin chain is a CH1 domain and the protein-protein interface of the parent domain of the first engineered immunoglobulin chain is

substituted with an amino acid residue at position 5 and the protein-protein interface of the parent domain of the second engineered immunoglobulin chain is substituted with an amino acid residue at position 20, the substituted amino acid residue at position 5 and the substituted amino acid residue at position 20 are not charged amino acids,

if the parent domain of the first engineered immunoglobulin chain is a CH1 domain and the parent domain of the second engineered immunoglobulin chain is an IGLC domain and the protein-protein interface of the parent domain of the first engineered immunoglobulin chain is substituted with an amino acid residue at position 27 and the protein-protein interface of the parent domain of the second engineered immunoglobulin chain is substituted with an amino acid residue at position 18, the substituted amino acid residue at position 27 and the substituted amino acid residue at position 18 are not charged amino acids,

if the parent domain of the first engineered immunoglobulin chain is an IGLC domain or an IGKC domain and the parent domain of the second engineered immunoglobulin chain is a CH1 domain and the protein-protein interface of the parent domain of the first engineered immunoglobulin chain is substituted with an amino acid residue at position 20 and the protein-protein interface of the parent domain of the second engineered immunoglobulin chain is substituted with an amino acid residue at position 26, the substituted amino acid residue at position 20 and the substituted amino acid residue at position 26 are not charged amino acids,

if the parent domain of the first engineered immunoglobulin chain is an IGLC domain and the parent domain of the second engineered immunoglobulin chain is a CH1 domain and the protein-protein interface of the parent domain of the first engineered immunoglobulin chain is substituted with an amino acid residue at position 79 and the protein-protein interface of the parent domain of the second engineered immunoglobulin chain is substituted with an amino acid residue at position 84.3, the substituted amino acid residue at position 79 and the substituted amino acid residue at position 84.3 are not charged amino acids,

if the parent domain of the first engineered immunoglobulin chain is an IGLC domain or an IGKC domain and the parent domain of the second engineered immunoglobulin chain is a CH1 domain and the protein-protein interface of the parent domain of the first engineered immunoglobulin chain is substituted with an amino acid residue at position 86 and the protein-protein interface of the parent domain of the second engineered immunoglobulin chain is substituted with an amino acid residue at position 86, the substituted amino acid residues at both positions 86 are not charged amino acids,

if the parent domain of the first engineered immunoglobulin chain is an IGKC domain and the amino acid residue which is substituted in the protein-protein interface of the parent domain of the first engineered immunoglobulin chain is at position 11 and the parent domain of the second engineered immunoglobulin chain is a domain which comprises a hinge region, the parent domain of the second engineered immunoglobulin chain is not substituted at position 3 of the hinge region, wherein the amino acid position of each group member is indicated according to the IMGT® numbering.

Position 5 of an IGLC domain in the protein-protein interface of the parent domain of the first engineered immunoglobulin chain indicated according to the IMGT® numbering correspond to position 116 according to Kabat numbering.
Position 12 of an IGLC domain in the protein-protein interface of the parent domain of the first engineered immunoglobulin chain indicated according to the IMGT® numbering correspond to position 123 according to Kabat numbering.
Position 13 of an IGLC domain in the protein-protein interface of the parent domain of the first engineered immunoglobulin chain indicated according to the IMGT® numbering correspond to position 124 according to Kabat numbering.
Position 18 of an IGLC domain in the protein-protein interface of the parent domain of the first engineered immunoglobulin chain indicated according to the IMGT® numbering correspond to position 129 according to Kabat numbering.
Position 20 of an IGLC domain in the protein-protein interface of the parent domain of the first engineered immunoglobulin chain indicated according to the IMGT® numbering correspond to position 131 according to Kabat numbering.
Position 79 of an IGLC domain in the protein-protein interface of the parent domain of the first engineered immunoglobulin chain indicated according to the IMGT® numbering correspond to position 160 according to Kabat numbering.
Position 11 of the IGKC domain in the protein-protein interface of the parent domain of the first engineered immunoglobulin chain indicated according to the IMGT® numbering correspond to position 122 according to EU numbering.
Position 12 of the IGKC domain in the protein-protein interface of the parent domain of the first engineered immunoglobulin chain indicated according to the IMGT® numbering correspond to position 123 according to EU numbering.

Position 20 of an IGKC domain in the protein-protein interface of the parent domain of the first engineered immunoglobulin chain indicated according to the IMGT® numbering correspond to position 131 according to EU numbering.

Position 86 of an IGKC domain in the protein-protein interface of the parent domain of the first engineered immunoglobulin chain indicated according to the IMGT® numbering correspond to position 176 according to EU numbering.

Position 20 of a CH1 domain in the protein-protein interface of the parent domain of the second engineered immunoglobulin chain indicated according to the IMGT® numbering correspond to position 141 according to EU numbering.

Position 26 of a CH1 domain in the protein-protein interface of the parent domain of the second engineered immunoglobulin chain indicated according to the IMGT® numbering correspond to position 147 according to EU numbering.

Position 27 of a CH1 domain in the protein-protein interface of the parent domain of the second engineered immunoglobulin chain indicated according to the IMGT® numbering correspond to position 148 according to EU numbering.

Position 84.3 of a CH1 domain in the protein-protein interface of the parent domain of the second engineered immunoglobulin chain indicated according to the IMGT® numbering correspond to position 176 according to EU numbering.

Position 86 of the CH1 domain in the protein-protein interface of the parent domain of the second engineered immunoglobulin chain indicated according to the IMGT® numbering correspond to position 183 according to EU numbering.

Position 119 of a CH1 domain in the protein-protein interface of the parent domain of the second engineered immunoglobulin chain indicated according to the IMGT® numbering correspond to position 213 according to EU numbering.

In a further aspect, the present disclosure provides a hetero-dimeric immunoglobulin or hetero-dimeric fragment thereof comprising:

(a) a first engineered immunoglobulin chain comprising an engineered domain with a protein-protein interface, wherein at least one amino acid residue of the protein-protein interface of the parent domain of said first engineered immunoglobulin chain is substituted with an amino acid residue at the equivalent 3D structural position from a protein-protein interface of a donor domain of a first member of the naturally occurring immunoglobulin super-family; and

(b) a second engineered immunoglobulin chain comprising an engineered domain with a protein-protein interface, wherein at least one amino acid residue of the protein-protein interface of the parent domain of said second engineered immunoglobulin chain is substituted with an amino acid residue at the equivalent 3D structural position from a protein-protein interface of a donor domain of a second member of the naturally occurring immunoglobulin super-family,

wherein the donor domain of the first member of the naturally occurring immunoglobulin super-family and the donor domain of the second member of the naturally occurring immunoglobulin super-family form a naturally occurring hetero-dimer or a naturally occurring homo-dimer and,

wherein the parent domains of the first and the second engineered immunoglobulin chain are not a CH2 domain, not a CH3 domain, not a VL domain and not a VH domain and, wherein the protein-protein interface of the parent domain of the first engineered immunoglobulin chain comprises an amino acid residue substitution at a position selected from the group consisting of 20, 22, 85.1, 86, and 88, and wherein,

if the parent domain of the first engineered immunoglobulin chain is an IGLC domain and the parent domain of the second engineered immunoglobulin chain is a CH1 domain and the protein-protein interface of the parent domain of the first engineered immunoglobulin chain is substituted with an amino acid residue at position 5 and the protein-protein interface of the parent domain of the second engineered immunoglobulin chain is substituted with an amino acid residue at position 20, the substituted amino acid residue at position 5 and the substituted amino acid residue at position 20 are not a charged pair,

if the parent domain of the first engineered immunoglobulin chain is an IGLC domain or an IGKC domain and the parent domain of the second engineered immunoglobulin chain is a CH1 domain and the protein-protein interface of the parent domain of the first engineered immunoglobulin chain is substituted with an amino acid residue at position 20 and the protein-protein interface of the parent domain of the second engineered immunoglobulin chain is substituted with an amino acid residue at position 26, the substituted amino acid residue at position 20 and the substituted amino acid residue at position 26 are not a charged pair,

if the parent domain of the first engineered immunoglobulin chain is an IGLC domain or an IGKC domain and the parent domain of the second engineered immunoglobulin chain is a CH1 domain and the protein-protein interface of the parent domain of the first engineered

immunoglobulin chain is substituted with an amino acid residue at position 86 and the protein-protein interface of the parent domain of the second engineered immunoglobulin chain is substituted with an amino acid residue at position 86, the substituted amino acid residues at both positions 86 are not a charged pair, wherein the amino acid position of each group member is indicated according to the IMGT® numbering.

Preferably the present disclosure provides a hetero-dimeric immunoglobulin or hetero-dimeric fragment thereof comprising:

(a) a first engineered immunoglobulin chain comprising an engineered domain with a protein-protein interface, wherein at least one amino acid residue of the protein-protein interface of the parent domain of said first engineered immunoglobulin chain is substituted with an amino acid residue at the equivalent 3D structural position from a protein-protein interface of a donor domain of a first member of the naturally occurring immunoglobulin super-family; and

(b) a second engineered immunoglobulin chain comprising an engineered domain with a protein-protein interface, wherein at least one amino acid residue of the protein-protein interface of the parent domain of said second engineered immunoglobulin chain is substituted with an amino acid residue at the equivalent 3D structural position from a protein-protein interface of a donor domain of a second member of the naturally occurring immunoglobulin super-family,

wherein the donor domain of the first member of the naturally occurring immunoglobulin super-family and the donor domain of the second member of the naturally occurring immunoglobulin super-family form a naturally occurring hetero-dimer or a naturally occurring homo-dimer and,

wherein the parent domains of the first and the second engineered immunoglobulin chain are not a CH2 domain, not a CH3 domain, not a VL domain and not a VH domain and, wherein the protein-protein interface of the parent domain of the first engineered immunoglobulin chain comprises an amino acid residue substitution at a position selected from the group consisting of 20, 22, 85.1, 86, and 88, and wherein,

if the parent domain of the first engineered immunoglobulin chain is an IGLC domain and the parent domain of the second engineered immunoglobulin chain is a CH1 domain and the protein-protein interface of the parent domain of the first engineered immunoglobulin chain is substituted with an amino acid residue at position 5 and the protein-protein interface of the parent domain of the second engineered immunoglobulin chain is substituted with an amino

acid residue at position 20, the substituted amino acid residue at position 5 and the substituted amino acid residue at position 20 are not a charged pair,
if the parent domain of the first engineered immunoglobulin chain is an IGLC domain or an IGKC domain and the parent domain of the second engineered immunoglobulin chain is a CH1 domain and the protein-protein interface of the parent domain of the first engineered immunoglobulin chain is substituted with an amino acid residue at position 20 and/or the protein-protein interface of the parent domain of the second engineered immunoglobulin chain is substituted with an amino acid residue at position 26, the substituted amino acid residue at position 20 and the unsubstituted or substituted amino acid residue at position 26 are not a charged pair,
if the parent domain of the first engineered immunoglobulin chain is an IGLC domain or an IGKC domain and the parent domain of the second engineered immunoglobulin chain is a CH1 domain and the protein-protein interface of the parent domain of the first engineered immunoglobulin chain is substituted with an amino acid residue at position 86 and the protein-protein interface of the parent domain of the second engineered immunoglobulin chain is substituted with an amino acid residue at position 86, the substituted amino acid residues at both positions 86 are not a charged pair, wherein the amino acid position of each group member is indicated according to the IMGT® numbering.

Even more preferably the present disclosure provides a hetero-dimeric immunoglobulin or hetero-dimeric fragment thereof comprising:
(a) a first engineered immunoglobulin chain comprising an engineered domain with a protein-protein interface, wherein at least one amino acid residue of the protein-protein interface of the parent domain of said first engineered immunoglobulin chain is substituted with an amino acid residue at the equivalent 3D structural position from a protein-protein interface of a donor domain of a first member of the naturally occurring immunoglobulin super-family; and
(b) a second engineered immunoglobulin chain comprising an engineered domain with a protein-protein interface, wherein at least one amino acid residue of the protein-protein interface of the parent domain of said second engineered immunoglobulin chain is substituted with an amino acid residue at the equivalent 3D structural position from a protein-protein interface of a donor domain of a second member of the naturally occurring immunoglobulin super-family,
wherein the donor domain of the first member of the naturally occurring immunoglobulin

super-family and the donor domain of the second member of the naturally occurring immunoglobulin super-family form a naturally occurring hetero-dimer or a naturally occurring homo-dimer and,

wherein the parent domains of the first and the second engineered immunoglobulin chain are not a CH2 domain, not a CH3 domain, not a VL domain and not a VH domain and, wherein the protein-protein interface of the parent domain of the first engineered immunoglobulin chain comprises an amino acid residue substitution at a position selected from the group consisting of 20, 22, 85.1, 86, and 88, and wherein,

if the parent domain of the first engineered immunoglobulin chain is an IGLC domain and the parent domain of the second engineered immunoglobulin chain is a CH1 domain and the protein-protein interface of the parent domain of the first engineered immunoglobulin chain is substituted with an amino acid residue at position 5 and the protein-protein interface of the parent domain of the second engineered immunoglobulin chain is substituted with an amino acid residue at position 20, the substituted amino acid residue at position 5 and the substituted amino acid residue at position 20 are not charged amino acids,

if the parent domain of the first engineered immunoglobulin chain is an IGLC domain or an IGKC domain and the parent domain of the second engineered immunoglobulin chain is a CH1 domain and the protein-protein interface of the parent domain of the first engineered immunoglobulin chain is substituted with an amino acid residue at position 20 and the protein-protein interface of the parent domain of the second engineered immunoglobulin chain is substituted with an amino acid residue at position 26, the substituted amino acid residue at position 20 and the substituted amino acid residue at position 26 are not charged amino acids,

if the parent domain of the first engineered immunoglobulin chain is an IGLC domain or an IGKC domain and the parent domain of the second engineered immunoglobulin chain is a CH1 domain and the protein-protein interface of the parent domain of the first engineered immunoglobulin chain is substituted with an amino acid residue at position 86 and the protein-protein interface of the parent domain of the second engineered immunoglobulin chain is substituted with an amino acid residue at position 86, the substituted amino acid residues at both positions 86 are not charged amino acids, wherein the amino acid position of each group member is indicated according to the IMGT® numbering.

In a further aspect, the present disclosure provides a hetero-dimeric immunoglobulin or

hetero-dimeric fragment thereof comprising:

(a) a first engineered immunoglobulin chain comprising an engineered domain with a protein-protein interface, wherein at least one amino acid residue of the protein-protein interface of the parent domain of said first engineered immunoglobulin chain is substituted with an amino acid residue at the equivalent 3D structural position from a protein-protein interface of a donor domain of a first member of the naturally occurring immunoglobulin super-family; and

(b) a second engineered immunoglobulin chain comprising an engineered domain with a protein-protein interface, wherein at least one amino acid residue of the protein-protein interface of the parent domain of said second engineered immunoglobulin chain is substituted with an amino acid residue at the equivalent 3D structural position from a protein-protein interface of a donor domain of a second member of the naturally occurring immunoglobulin super-family,

wherein the donor domain of the first member of the naturally occurring immunoglobulin super-family and the donor domain of the second member of the naturally occurring immunoglobulin super-family form a naturally occurring hetero-dimer or a naturally occurring homo-dimer and,

wherein the parent domains of the first and the second engineered immunoglobulin chain are not a CH2 domain, not a CH3 domain, not a VL domain and not a VH domain and, wherein the protein-protein interface of the parent domain of the first engineered immunoglobulin chain comprises an amino acid residue substitution at a position selected from the group consisting of 20, 22, 85.1, 86, and 88, and wherein,

if the parent domain of the first engineered immunoglobulin chain is an IGLC domain or an IGKC domain and the parent domain of the second engineered immunoglobulin chain is a CH1 domain and the protein-protein interface of the parent domain of the first engineered immunoglobulin chain is substituted with an amino acid residue at position 20 and the protein-protein interface of the parent domain of the second engineered immunoglobulin chain is substituted with an amino acid residue at position 26, the substituted amino acid residue at position 20 and the substituted amino acid residue at position 26 are not a charged pair,

if the parent domain of the first engineered immunoglobulin chain is an IGLC domain or an IGKC domain and the parent domain of the second engineered immunoglobulin chain is a CH1 domain and the protein-protein interface of the parent domain of the first engineered immunoglobulin chain is substituted with an amino acid residue at position 86 and the

protein-protein interface of the parent domain of the second engineered immunoglobulin chain is substituted with an amino acid residue at position 86, the substituted amino acid residues at both positions 86 are not a charged pair, wherein the amino acid position of each group member is indicated according to the IMGT® numbering.

Preferably the present disclosure provides a hetero-dimeric immunoglobulin or hetero-dimeric fragment thereof comprising:

(a) a first engineered immunoglobulin chain comprising an engineered domain with a protein-protein interface, wherein at least one amino acid residue of the protein-protein interface of the parent domain of said first engineered immunoglobulin chain is substituted with an amino acid residue at the equivalent 3D structural position from a protein-protein interface of a donor domain of a first member of the naturally occurring immunoglobulin super-family; and

(b) a second engineered immunoglobulin chain comprising an engineered domain with a protein-protein interface, wherein at least one amino acid residue of the protein-protein interface of the parent domain of said second engineered immunoglobulin chain is substituted with an amino acid residue at the equivalent 3D structural position from a protein-protein interface of a donor domain of a second member of the naturally occurring immunoglobulin super-family,

wherein the donor domain of the first member of the naturally occurring immunoglobulin super-family and the donor domain of the second member of the naturally occurring immunoglobulin super-family form a naturally occurring hetero-dimer or a naturally occurring homo-dimer and,

wherein the parent domains of the first and the second engineered immunoglobulin chain are not a CH2 domain, not a CH3 domain, not a VL domain and not a VH domain and, wherein the protein-protein interface of the parent domain of the first engineered immunoglobulin chain comprises an amino acid residue substitution at a position selected from the group consisting of 20, 22, 85.1, 86, and 88, and wherein,

if the parent domain of the first engineered immunoglobulin chain is an IGLC domain or an IGKC domain and the parent domain of the second engineered immunoglobulin chain is a CH1 domain and the protein-protein interface of the parent domain of the first engineered immunoglobulin chain is substituted with an amino acid residue at position 20 and/or the protein-protein interface of the parent domain of the second engineered immunoglobulin chain is substituted with an amino acid residue at position 26, the substituted amino acid

residue at position 20 and the unsubstituted or substituted amino acid residue at position 26 are not a charged pair,
if the parent domain of the first engineered immunoglobulin chain is an IGLC domain or an IGKC domain and the parent domain of the second engineered immunoglobulin chain is a CH1 domain and the protein-protein interface of the parent domain of the first engineered immunoglobulin chain is substituted with an amino acid residue at position 86 and the protein-protein interface of the parent domain of the second engineered immunoglobulin chain is substituted with an amino acid residue at position 86, the substituted amino acid residues at both positions 86 are not a charged pair, wherein the amino acid position of each group member is indicated according to the IMGT® numbering.

Even more preferably the present disclosure provides a hetero-dimeric immunoglobulin or hetero-dimeric fragment thereof comprising:
(a) a first engineered immunoglobulin chain comprising an engineered domain with a protein-protein interface, wherein at least one amino acid residue of the protein-protein interface of the parent domain of said first engineered immunoglobulin chain is substituted with an amino acid residue at the equivalent 3D structural position from a protein-protein interface of a donor domain of a first member of the naturally occurring immunoglobulin super-family; and
(b) a second engineered immunoglobulin chain comprising an engineered domain with a protein-protein interface, wherein at least one amino acid residue of the protein-protein interface of the parent domain of said second engineered immunoglobulin chain is substituted with an amino acid residue at the equivalent 3D structural position from a protein-protein interface of a donor domain of a second member of the naturally occurring immunoglobulin super-family,
wherein the donor domain of the first member of the naturally occurring immunoglobulin super-family and the donor domain of the second member of the naturally occurring immunoglobulin super-family form a naturally occurring hetero-dimer or a naturally occurring homo-dimer and,
wherein the parent domains of the first and the second engineered immunoglobulin chain are not a CH2 domain, not a CH3 domain, not a VL domain and not a VH domain and, wherein the protein-protein interface of the parent domain of the first engineered immunoglobulin chain comprises an amino acid residue substitution at a position selected from the group consisting of 20, 22, 85.1, 86, and 88, and wherein,

if the parent domain of the first engineered immunoglobulin chain is an IGLC domain or an IGKC domain and the parent domain of the second engineered immunoglobulin chain is a CH1 domain and the protein-protein interface of the parent domain of the first engineered immunoglobulin chain is substituted with an amino acid residue at position 20 and the protein-protein interface of the parent domain of the second engineered immunoglobulin chain is substituted with an amino acid residue at position 26, the substituted amino acid residue at position 20 and the substituted amino acid residue at position 26 are not charged amino acids,

if the parent domain of the first engineered immunoglobulin chain is an IGLC domain or an IGKC domain and the parent domain of the second engineered immunoglobulin chain is a CH1 domain and the protein-protein interface of the parent domain of the first engineered immunoglobulin chain is substituted with an amino acid residue at position 86 and the protein-protein interface of the parent domain of the second engineered immunoglobulin chain is substituted with an amino acid residue at position 86, the substituted amino acid residues at both positions 86 are not charged amino acids, wherein the amino acid position of each group member is indicated according to the IMGT® numbering.

In some aspects the present disclosure provides a hetero-dimeric immunoglobulin or hetero-dimeric fragment comprising:

(a) a first engineered immunoglobulin chain comprising an engineered domain with a protein-protein interface, wherein at least one amino acid residue of the protein-protein interface of the parent domain of said first engineered immunoglobulin chain is substituted with an amino acid residue at the equivalent 3D structural position from a protein-protein interface of a donor domain of a first member of the naturally occurring immunoglobulin super-family; and

(b) a second engineered immunoglobulin chain comprising an engineered domain with a protein-protein interface, wherein at least one amino acid residue of the protein-protein interface of the parent domain of said second engineered immunoglobulin chain is substituted with an amino acid residue at the equivalent 3D structural position from a protein-protein interface of a donor domain of a second member of the naturally occurring immunoglobulin super-family,

wherein the donor domain of the first member of the naturally occurring immunoglobulin super-family and the donor domain of the second member of the naturally occurring immunoglobulin super-family form a naturally occurring hetero-dimer or a naturally

occurring homo-dimer and,

wherein the parent domains of the first and the second engineered immunoglobulin chain are not a CH2 domain, not a CH3 domain, not a VL domain and not a VH domain and, wherein the protein-protein interface of the parent domain of the first engineered immunoglobulin chain comprises an amino acid residue substitution at a position selected from the group consisting of 20, 22, 85.1, 86, and 88, and wherein,

if the parent domain of the first engineered immunoglobulin chain is an IGLC domain and the parent domain of the second engineered immunoglobulin chain is a CH1 domain and the protein-protein interface of the parent domain of the first engineered immunoglobulin chain is substituted with an amino acid residue at position 5 and the protein-protein interface of the parent domain of the second engineered immunoglobulin chain is substituted with an amino acid residue at position 20, the substituted amino acid residue at position 5 and the substituted amino acid residue at position 20 are not a charged pair,

if the parent domain of the first engineered immunoglobulin chain is an IGLC domain or an IGKC domain and the parent domain of the second engineered immunoglobulin chain is a CH1 domain and the protein-protein interface of the parent domain of the first engineered immunoglobulin chain is substituted with an amino acid residue at position 20 and the protein-protein interface of the parent domain of the second engineered immunoglobulin chain is substituted with an amino acid residue at position 26, the substituted amino acid residue at position 20 and the substituted amino acid residue at position 26 are not a charged pair,

if the parent domain of the first engineered immunoglobulin chain is an IGLC domain or an IGKC domain and the parent domain of the second engineered immunoglobulin chain is a CH1 domain and the protein-protein interface of the parent domain of the first engineered immunoglobulin chain is substituted with an amino acid residue at position 86 and the protein-protein interface of the parent domain of the second engineered immunoglobulin chain is substituted with an amino acid residue at position 86, the substituted amino acid residues at both positions 86 are not a charged pair,

if the parent domain of the first engineered immunoglobulin chain is a CH1 domain and the parent domain of the second engineered immunoglobulin chain is an IGLC domain and the protein-protein interface of the parent domain of the first engineered immunoglobulin chain is substituted with an amino acid residue at position 20 and the protein-protein interface of the parent domain of the second engineered immunoglobulin chain is substituted with an amino

acid residue at position 5, the substituted amino acid residue at position 5 and the substituted amino acid residue at position 20 are not a charged pair, preferably are not charged amino acids, and wherein, if the parent domain of the first engineered immunoglobulin chain is a CH1 domain and the parent domain of the second engineered immunoglobulin chain is an IGLC domain or an IGKC domain and the protein-protein interface of the parent domain of the first engineered immunoglobulin chain is substituted with an amino acid residue at position 86 and the protein-protein interface of the parent domain of the second engineered immunoglobulin chain is substituted with an amino acid residue at position 86, the substituted amino acid residues at both positions 86 are not a charged pair, preferably are not charged amino acids, wherein the amino acid position of each group member is indicated according to the IMGT® numbering.

In some aspects the present disclosure provides a hetero-dimeric immunoglobulin or hetero-dimeric fragment comprising:

(a) a first engineered immunoglobulin chain comprising an engineered domain with a protein-protein interface, wherein at least one amino acid residue of the protein-protein interface of the parent domain of said first engineered immunoglobulin chain is substituted with an amino acid residue at the equivalent 3D structural position from a protein-protein interface of a donor domain of a first member of the naturally occurring immunoglobulin super-family; and

(b) a second engineered immunoglobulin chain comprising an engineered domain with a protein-protein interface, wherein at least one amino acid residue of the protein-protein interface of the parent domain of said second engineered immunoglobulin chain is substituted with an amino acid residue at the equivalent 3D structural position from a protein-protein interface of a donor domain of a second member of the naturally occurring immunoglobulin super-family,

wherein the donor domain of the first member of the naturally occurring immunoglobulin super-family and the donor domain of the second member of the naturally occurring immunoglobulin super-family form a naturally occurring hetero-dimer or a naturally occurring homo-dimer and,

wherein the parent domains of the first and the second engineered immunoglobulin chain are not a CH2 domain, not a CH3 domain, not a VL domain and not a VH domain and, wherein the protein-protein interface of the parent domain of the first engineered immunoglobulin chain comprises an amino acid residue substitution at a position selected from the group

consisting of 20, 22, 85.1, 86, and 88, and wherein,
if the parent domain of the first engineered immunoglobulin chain is an IGLC domain or an IGKC domain and the parent domain of the second engineered immunoglobulin chain is a CH1 domain and the protein-protein interface of the parent domain of the first engineered immunoglobulin chain is substituted with an amino acid residue at position 20 and the protein-protein interface of the parent domain of the second engineered immunoglobulin chain is substituted with an amino acid residue at position 26, the substituted amino acid residue at position 20 and the substituted amino acid residue at position 26 are not a charged pair, preferably are not charged amino acids,
if the parent domain of the first engineered immunoglobulin chain is an IGLC domain or an IGKC domain and the parent domain of the second engineered immunoglobulin chain is a CH1 domain and the protein-protein interface of the parent domain of the first engineered immunoglobulin chain is substituted with an amino acid residue at position 86 and the protein-protein interface of the parent domain of the second engineered immunoglobulin chain is substituted with an amino acid residue at position 86, the substituted amino acid residues at both positions 86 are not a charged pair, preferably are not charged amino acids,
if the parent domain of the first engineered immunoglobulin chain is a CH1 domain and the parent domain of the second engineered immunoglobulin chain is an IGLC domain and the protein-protein interface of the parent domain of the first engineered immunoglobulin chain is substituted with an amino acid residue at position 20 and the protein-protein interface of the parent domain of the second engineered immunoglobulin chain is substituted with an amino acid residue at position 5, the substituted amino acid residue at position 5 and the substituted amino acid residue at position 20 are not a charged pair, preferably are not charged amino acids, and wherein, if the parent domain of the first engineered immunoglobulin chain is a CH1 domain and the parent domain of the second engineered immunoglobulin chain is an IGLC domain or an IGKC domain and the protein-protein interface of the parent domain of the first engineered immunoglobulin chain is substituted with an amino acid residue at position 86 and the protein-protein interface of the parent domain of the second engineered immunoglobulin chain is substituted with an amino acid residue at position 86, the substituted amino acid residues at both positions 86 are not a charged pair, preferably are not charged amino acids, wherein the amino acid position of each group member is indicated according to the IMGT® numbering..

In a further aspect, the present disclosure provides a hetero-dimeric immunoglobulin or hetero-dimeric fragment thereof comprising:

(a) a first engineered immunoglobulin chain comprising an engineered domain with a protein-protein interface, wherein at least one amino acid residue of the protein-protein interface of the parent domain of said first engineered immunoglobulin chain is substituted with an amino acid residue at the equivalent 3D structural position from a protein-protein interface of a donor domain of a first member of the naturally occurring immunoglobulin super-family; and

(b) a second engineered immunoglobulin chain comprising an engineered domain with a protein-protein interface, wherein at least one amino acid residue of the protein-protein interface of the parent domain of said second engineered immunoglobulin chain is substituted with an amino acid residue at the equivalent 3D structural position from a protein-protein interface of a donor domain of a second member of the naturally occurring immunoglobulin super-family,

wherein the engineered domain of the first engineered immunoglobulin chain and the engineered domain of the second engineered immunoglobulin chain are not identical and, wherein the donor domain of the first member of the naturally occurring immunoglobulin super-family and the donor domain of the second member of the naturally occurring immunoglobulin super-family form a naturally occurring hetero-dimer or a naturally occurring homo-dimer and,

wherein the parent domain of the first and/or the second engineered immunoglobulin chain is a CH3 domain, wherein the amino acid residue which is substituted in the protein-protein interface of the parent domain of the first engineered immunoglobulin chain comprises the amino acid residue at position 88, and optionally a further amino acid residue at a position selected from the group consisting of 3, 5, 7, 20, 22, 26, 27, 79, 81, 84, 84.2, 85.1, 86, and 90 and, wherein the amino acid residue which is substituted in the protein-protein interface of the parent domain of the second engineered immunoglobulin chain comprises the amino acid residue at position 85.1 and/or 86 and optionally a further amino acid residue at a position selected from the group consisting of 3, 5, 7, 20, 22, 26, 27, 79, 81, 84, 84.2, 88, and 90 or at a position selected from the group consisting of 3, 5, 7, 20, 22, 26, 27, 79, 81, 84, 84.2, 85.1, 88, and 90 or at a position selected from the group consisting of 3, 5, 7, 20, 22, 26, 27, 79, 81, 84, 84.2, 86, 88, and 90,

wherein the amino acid residue substituted at position 88 in the parent domain of the first

engineered immunoglobulin chain is interacting with the amino acid residue substituted at position 85.1 and/or 86 in the parent domain of the second engineered immunoglobulin chain, wherein the amino acid position of each group member is indicated according to the IMGT® numbering.

In a further aspect, the present disclosure provides a hetero-dimeric immunoglobulin or hetero-dimeric fragment thereof comprising:

(a) a first engineered immunoglobulin chain comprising an engineered domain with a protein-protein interface, wherein at least one amino acid residue of the protein-protein interface of the parent domain of said first engineered immunoglobulin chain is substituted with an amino acid residue at the equivalent 3D structural position from a protein-protein interface of a donor domain of a first member of the naturally occurring immunoglobulin super-family; and

(b) a second engineered immunoglobulin chain comprising an engineered domain with a protein-protein interface, wherein at least one amino acid residue of the protein-protein interface of the parent domain of said second engineered immunoglobulin chain is substituted with an amino acid residue at the equivalent 3D structural position from a protein-protein interface of a donor domain of a second member of the naturally occurring immunoglobulin super-family,

wherein the engineered domain of the first engineered immunoglobulin chain and the engineered domain of the second engineered immunoglobulin chain are not identical and, wherein the donor domain of the first member of the naturally occurring immunoglobulin super-family and the donor domain of the second member of the naturally occurring immunoglobulin super-family form a naturally occurring hetero-dimer or a naturally occurring homo-dimer and,

wherein the parent domain of the first and/or the second engineered immunoglobulin chain is a CH3 domain, wherein the amino acid residue which is substituted in the protein-protein interface of the parent domain of the first engineered immunoglobulin chain comprises the amino acid residue at position 20, and optionally a further amino acid residue at a position selected from the group consisting of 3, 5, 7, 22, 26, 27, 79, 81, 84, 84.2, 85.1, 86, 88 and 90 and, wherein the amino acid residues which are substituted in the protein-protein interface of the parent domain of the second engineered immunoglobulin chain comprise the amino acid residue at position 26 and at a further position selected from the group consisting of 3, 22, 27, 79, 81, 84, 85.1, 86, and 88, wherein the amino acid residue substituted at position 20 in the

parent domain of the first engineered immunoglobulin chain is interacting with the amino acid residue substituted at position 26 in the parent domain of the second engineered immunoglobulin chain, wherein the amino acid position of each group member is indicated according to the IMGT® numbering.

In a further aspect, the present disclosure provides a hetero-dimeric immunoglobulin or hetero-dimeric fragment thereof comprising:

(a) a first engineered immunoglobulin chain comprising an engineered domain with a protein-protein interface, wherein at least one amino acid residue of the protein-protein interface of the parent domain of said first engineered immunoglobulin chain is substituted with an amino acid residue at the equivalent 3D structural position from a protein-protein interface of a donor domain of a first member of the naturally occurring immunoglobulin super-family; and

(b) a second engineered immunoglobulin chain comprising an engineered domain with a protein-protein interface, wherein at least one amino acid residue of the protein-protein interface of the parent domain of said second engineered immunoglobulin chain is substituted with an amino acid residue at the equivalent 3D structural position from a protein-protein interface of a donor domain of a second member of the naturally occurring immunoglobulin super-family,

wherein the engineered domain of the first engineered immunoglobulin chain and the engineered domain of the second engineered immunoglobulin chain are not identical and, wherein the donor domain of the first member of the naturally occurring immunoglobulin super-family and the donor domain of the second member of the naturally occurring immunoglobulin super-family form a naturally occurring hetero-dimer or a naturally occurring homo-dimer and,

wherein the parent domain of the first and/or the second engineered immunoglobulin chain is a CH2 domain, wherein the amino acid residue which is substituted in the protein-protein interface of the parent domain of the first engineered immunoglobulin chain comprises the amino acid residue at position 88, and optionally a further amino acid residue at a position selected from the group consisting of 3, 5, 7, 20, 22, 26, 27, 79, 81, 83, 84, 84.2, 84.3, 85.1, 86 and 90 and, wherein the amino acid residue which is substituted in the protein-protein interface of the parent domain of the second engineered immunoglobulin chain comprises the amino acid residue at position 85.1 and/or 86 and optionally a further amino acid residue at a position selected from the group consisting of 3, 5, 7, 20, 22, 26, 27, 79, 81, 83, 84, 84.2, 84.3

and 90 or at a position selected from the group consisting of 3, 5, 7, 20, 22, 26, 27, 79, 81, 83, 84, 84.2, 84.3, 85.1 and 90 or at a position selected from the group consisting of 3, 5, 7, 20, 22, 26, 27, 79, 81, 83, 84, 84.2, 84.3, 86 and 90, wherein the amino acid residue substituted at position 88 in the parent domain of the first engineered immunoglobulin chain is interacting with the amino acid residue substituted at position 85.1 and/or 86 in the parent domain of the second engineered immunoglobulin chain, wherein the amino acid position of each group member is indicated according to the IMGT® numbering.

In a further aspect, the present disclosure provides a hetero-dimeric immunoglobulin or hetero-dimeric fragment thereof comprising:

(a) a first engineered immunoglobulin chain comprising an engineered domain with a protein-protein interface, wherein at least one amino acid residue of the protein-protein interface of the parent domain of said first engineered immunoglobulin chain is substituted with an amino acid residue at the equivalent 3D structural position from a protein-protein interface of a donor domain of a first member of the naturally occurring immunoglobulin super-family; and

(b) a second engineered immunoglobulin chain comprising an engineered domain with a protein-protein interface, wherein at least one amino acid residue of the protein-protein interface of the parent domain of said second engineered immunoglobulin chain is substituted with an amino acid residue at the equivalent 3D structural position from a protein-protein interface of a donor domain of a second member of the naturally occurring immunoglobulin super-family,

wherein the engineered domain of the first engineered immunoglobulin chain and the engineered domain of the second engineered immunoglobulin chain are not identical and,

wherein the donor domain of the first member of the naturally occurring immunoglobulin super-family and the donor domain of the second member of the naturally occurring immunoglobulin super-family form a naturally occurring hetero-dimer or a naturally occurring homo-dimer and,

wherein the parent domain of the first and/or the second engineered immunoglobulin chain is a CH2 domain, wherein the amino acid residue which is substituted in the protein-protein interface of the parent domain of the first engineered immunoglobulin chain comprises the amino acid residue at position 20, and optionally a further amino acid residue at a position selected from the group consisting of 3, 5, 7, 22, 26, 27, 79, 81, 83, 84, 84.2, 84.3, 85.1, 86, 88 and 90 and, wherein the amino acid residue which is substituted in the protein-protein

interface of the parent domain of the second engineered immunoglobulin chain comprises the amino acid residue at position 26 and optionally a further amino acid residue at a position selected from the group consisting of 3, 5, 7, 20, 22, 27, 79, 81, 83, 84, 84.2, 84.3, 85.1, 86, 88 and 90, wherein the amino acid residue substituted at position 20 in the parent domain of the first engineered immunoglobulin chain is interacting with the amino acid residue substituted at position 26 in the parent domain of the second engineered immunoglobulin chain, wherein the amino acid position of each group member is indicated according to the IMGT® numbering.

In a further aspect, the present disclosure provides a hetero-dimeric immunoglobulin or hetero-dimeric fragment thereof comprising:

(a) a first engineered immunoglobulin chain comprising an engineered domain with a protein-protein interface, wherein at least one amino acid residue of the protein-protein interface of the parent domain of said first engineered immunoglobulin chain is substituted with an amino acid residue at the equivalent 3D structural position from a protein-protein interface of a donor domain of a first member of the naturally occurring immunoglobulin super-family; and

(b) a second engineered immunoglobulin chain comprising an engineered domain with a protein-protein interface, wherein at least one amino acid residue of the protein-protein interface of the parent domain of said second engineered immunoglobulin chain is substituted with an amino acid residue at the equivalent 3D structural position from a protein-protein interface of a donor domain of a second member of the naturally occurring immunoglobulin super-family,

wherein the engineered domain of the first engineered immunoglobulin chain and the engineered domain of the second engineered immunoglobulin chain are not identical and,

wherein the donor domain of the first member of the naturally occurring immunoglobulin super-family and the donor domain of the second member of the naturally occurring immunoglobulin super-family form a naturally occurring hetero-dimer or a naturally occurring homo-dimer and,

wherein the parent domain of the first and/or the second engineered immunoglobulin chain is a CH4 domain, wherein the protein-protein interface of the parent domain of the first engineered immunoglobulin chain comprises an amino acid residue substitution at a position selected from the group consisting of 20, 22, 85.1, 86, and 88, and/or wherein the protein-protein interface of the parent domain of the second engineered immunoglobulin chain

comprises an amino acid residue substitution at a position selected from the group consisting of 22, 26, 85.1, 86, and 88, wherein the amino acid position of each group member is indicated according to the IMGT® numbering.

In some embodiments the donor domain of the first member of the naturally occurring immunoglobulin super-family and the donor domain of the second member of the naturally occurring immunoglobulin super-family form a naturally occurring hetero-dimer or a naturally occurring homo-dimer. In some embodiments the donor domain of the third member of the naturally occurring immunoglobulin super-family and the donor domain of the fourth member of the naturally occurring immunoglobulin super-family form a naturally occurring hetero-dimer or a naturally occurring homo-dimer.

In some embodiments, the first member of the naturally occurring immunoglobulin super-family and its donor domain is identical in amino acid sequence to the second member of the naturally occurring immunoglobulin super-family and its donor domain respectively.

In some embodiments, the first member of the naturally occurring immunoglobulin super-family and its donor domain is different in amino acid sequence from the second member of the naturally occurring immunoglobulin super-family and its donor domain respectively.

In some embodiments the third and the fourth member of the naturally occurring immunoglobulin super-family, in particular the donor domains of the third and the fourth member of the naturally occurring immunoglobulin super-family, are different from the first and the second member of the naturally occurring immunoglobulin super-family, in particular the donor domains of the first and the second member of the naturally occurring immunoglobulin super-family. In some embodiments the first, second, third and fourth member of the naturally occurring immunoglobulin super-family, in particular its donor domains are each different from the other.

In some embodiments the engineered domain of the first engineered immunoglobulin chain and the engineered domain of the second engineered immunoglobulin chain are not identical. In some embodiments the first engineered immunoglobulin chain and its engineered domain are not identical to the second engineered immunoglobulin chain and its engineered domain, i.e. the engineered immunoglobulin chains, in particular its engineered domains are different in amino acid sequence from each other e.g. the first and the second engineered immunoglobulin chains in particular its engineered domains have amino acid sequences

which are different from each other by at least one amino acid or by at least two amino acids or by at least three amino acids or by at least four amino acids or by at least five amino acids or by at least five to ten amino acids or by at least ten to thirty amino acids.

In some embodiments, the first engineered immunoglobulin chain and/or its engineered domain is different in amino acid sequence from the second engineered immunoglobulin chain and/or its engineered domain, wherein the protein-protein interfaces of the engineered domains of the engineered immunoglobulin chains interact by hetero-dimerization.

In some embodiments, the first engineered immunoglobulin chain and/or its engineered domain is different in amino acid sequence from the second engineered immunoglobulin chain and/or its engineered domain, wherein the protein-protein interfaces of the engineered domains of the engineered immunoglobulin chains interact by homo-dimerization.

In some embodiments, at least one substituted amino acid residue in the protein-protein interface of the parent domain of the first engineered immunoglobulin chain is interacting with at least one substituted amino acid residue in the protein-protein interface of the parent domain of the second engineered immunoglobulin chain. Preferably at least two, more preferably at least three, most preferably at least four, in particular at least five substituted amino acid residue in the protein-protein interface of the parent domain of the first engineered immunoglobulin chain are interacting with at least one substituted amino acid residue in the protein-protein interface of the parent domain of the second engineered immunoglobulin chain.

In some embodiments at least one, preferably at least two, more preferably at least three, most preferably at least four, in particular at least five amino acid residue of the protein-protein interface of the engineered domain of the first engineered immunoglobulin chain is different from the protein-protein interface of the engineered domain of the second engineered immunoglobulin chain.

In some embodiments, each of the first and second engineered immunoglobulin chains and its engineered domains are different in amino acid sequence from the first and second member of the naturally occurring immunoglobulin super-family and its donor domains.

In some embodiments, the parent domain of the first engineered immunoglobulin chain is different in amino acid sequence from the first member of the naturally occurring immunoglobulin super-family and its donor domain. In some embodiments, the parent domain of the second engineered immunoglobulin chain is different in amino acid sequence from

second member of the naturally occurring immunoglobulin super-family and its donor domain.

In some embodiments, the protein-protein interface of the parent domain of the first engineered immunoglobulin chain and the protein-protein interface of the parent domain of the second engineered immunoglobulin chain are not substituted with charged amino acids, preferably are not substituted with a charged pair.

In some embodiments, the protein-protein interface of the parent domain of the first engineered immunoglobulin chain is not substituted with 26A and/or 26E and/or 86T and/or the protein-protein interface of the parent domain of the second engineered immunoglobulin chain is not substituted with 20T and/or 26K and/or 85.1A and/or 88T, wherein the amino acid position is indicated according to the IMGT® numbering.

In some embodiments, the protein-protein interface of the engineered domain of the first engineered immunoglobulin chain is interacting with the protein-protein interface of the engineered domain of the second engineered immunoglobulin chain by homo-dimerization, wherein the engineered domains of the first engineered immunoglobulin chain and the second engineered immunoglobulin chain are not identical in amino acid sequence.

In some embodiments, the parent domains of the engineered domains of the engineered immunoglobulin chains are not substituted in the loop region of the domain.

In some embodiments, at least one 3D structural position of the protein-protein interface of the engineered domain of the first engineered immunoglobulin chain is different in amino acid residue compared to the identical 3D structural position of the protein-protein interface of the engineered domain of the second engineered immunoglobulin chain.

In some embodiments, the further engineered domain of the first engineered immunoglobulin chain and/or the second engineered immunoglobulin chain are not identical to the third engineered immunoglobulin chain and its engineered domain, i.e. the engineered immunoglobulin chains, in particular its engineered domains are different in amino acid sequence from each other e.g. the first engineered immunoglobulin chain and/or the second engineered immunoglobulin chain and/or the third engineered immunoglobulin chain, in particular the further engineered domain of the first engineered immunoglobulin chain and/or the second engineered immunoglobulin chain and th eengineered domain of the third engineered immunoglobulin chain have amino acid sequences which are different from each other by at least one amino acid or by at least two amino acids or by at least three amino acids or by at least four amino acids or by at least five amino acids or by at least five to ten amino

acids or by at least ten to thirty amino acids.

In some embodiments, the further engineered domain of the first engineered immunoglobulin chain and/or the second engineered immunoglobulin chain is different in amino acid sequence from the engineered domain of the third engineered immunoglobulin chain, wherein the protein-protein interfaces of the engineered domains of the engineered immunoglobulin chains interact by hetero-dimerization.

In some embodiments, the further engineered domain of the first engineered immunoglobulin chain and/or the second engineered immunoglobulin chain is different in amino acid sequence from the engineered domain of the third engineered immunoglobulin chain, wherein the protein-protein interfaces of the engineered domains of the engineered immunoglobulin chains interact by homo-dimerization.

In some embodiments, the third member of the naturally occurring immunoglobulin super-family and its donor domain is different in amino acid sequence from the fourth member of the naturally occurring immunoglobulin super-family and its donor domain respectively.

In some embodiments, the third member of the naturally occurring immunoglobulin super-family and its donor domain is identical in amino acid sequence to the fourth member of the naturally occurring immunoglobulin super-family and its donor domain respectively.

In some embodiments, each of the further engineered domain of the first engineered immunoglobulin chain and/or the second engineered immunoglobulin chain and the engineered domain of the third engineered immunoglobulin chain are different in amino acid sequence from the third and fourth member of the naturally occurring immunoglobulin super-family and its donor domains.

In some embodiments, the parent domain of the further engineered domain of the first engineered immunoglobulin chain and/or the second engineered immunoglobulin chain is different in amino acid sequence from the third member of the naturally occurring immunoglobulin super-family and its donor domain.

In some embodiments, the parent domain of the engineered domain of the third engineered immunoglobulin chain is different in amino acid sequence from the fourth member of the naturally occurring immunoglobulin super-family and its donor domain.

In some embodiments the first and second engineered immunoglobulin chains have parent domains which are identical. In some embodiments the first and second engineered immunoglobulin chains have parent domains which are not identical. In some embodiments

the first and second engineered immunoglobulin chains have parent domains which have amino acid sequences which are different from each other by one amino acid or by two amino acids or by three amino acids or by four amino acids or by five amino acids or by five to ten amino acids or by ten to thirty amino acids, preferably by at least one amino acid or by at least two amino acids or by at least three amino acids or by at least four amino acids or by at least five amino acids or by at least five to ten amino acids or by at least ten to thirty amino acids. In some embodiments the parent domain of the further engineered domain of the first and/or second engineered immunoglobulin chains and the parent domain of the engineered domain of the third engineered immunoglobulin chain are identical. In some embodiments the parent domain of the further engineered domain of the first and/or second engineered immunoglobulin chains and the parent domain of the engineered domain of the third engineered immunoglobulin chain are not identical. In some embodiments the parent domain of the further engineered domain of the first and/or second engineered immunoglobulin chains and the parent domain of the engineered domain of the third engineered immunoglobulin chain are different from each other by one amino acid or by two amino acids or by three amino acids or by four amino acids or by five amino acids or by five to ten amino acids or by ten to thirty amino acids, preferably by at least one amino acid or by at least two amino acids or by at least three amino acids or by at least four amino acids or by at least five amino acids or by at least five to ten amino acids or by at least ten to thirty amino acids.

In some embodiments the present disclosure provides a hetero-dimeric immunoglobulin or fragment thereof comprising a parent domain of the first and/or second engineered immunoglobulin chain, in particular a parent domain of the first and second engineered immunoglobulin chain which is selected from the group consisting of CH1, CH2, CH3, CH4, IGKC, IGLC1, IGLC2, IGLC3, IGLC6, and IGLC7. CH1, CH3 can be from IGHA1, IGHA2, IGHD, IGHE, IGHG1, IGHG2, IGHG3, IGHG4, IGHGP and IGHM. CH4 can be from IGHE and IGHM. CH2 can be from IGHA1, IGHA2, IGHD, IGHE, IGHG1, IGHG2, IGHG3, IGHG4, IGHGP and IGHM.

In some embodiments the parent domain of the first and/or the second engineered immunoglobulin chain, in particular the parent domain of the first and second engineered immunoglobulin chain, is a domain selected from the group consisting of CH1 domain, CH4 domain, IGKC domain, IGLC1 domain, IGLC2 domain, IGLC3 domain, IGLC6 domain, and IGLC7 domain, preferably from the group consisting of CH1 domain, IGKC domain, IGLC1

domain, IGLC2 domain, IGLC3 domain, IGLC6 domain, and IGLC7 domain.

Normally the parent domain of the first and/or second engineered immunoglobulin chain is a naturally occurring domain. Usually the parent domain of the first and/or second engineered immunoglobulin chain is from human e.g a naturally occurring human domain. Preferably the parent domain of the first and/or second engineered immunoglobulin chain is from human and from the same isotype, species and subclass. In some embodiments, the preferred parent domain of the first and/or second engineered immunoglobulin chain, in particular the parent domain of the first and second engineered immunoglobulin chain, is a CH3 domain, more preferably a human CH3 domain, in particular a human CH3 domain from IGHG1. Equally preferred, the parent domains of the first and second engineered immunoglobulin chain are from a different isotype. In some embodiments the parent domain of the first engineered immunoglobulin chain is a CH3 domain from IGHG1, preferably a human CH3 domain from IGHG1 and the parent domain of the second engineered immunoglobulin chain is a CH3 domain from IGHG3 preferably a human CH3 domain from IGHG3.

In some embodiments, the parent domain of the first and/or second engineered immunoglobulin chain, in particular the parent domain of the first and second engineered immunoglobulin chain, is a CH4 domain, more preferably a human CH4 domain, in particular a human CH4 domain from IGHM or a human CH4 domain from IGHE.

Equally, the preferred the parent domain of the first engineered immunoglobulin chain is a CH1 domain and the parent domain of the second engineered immunoglobulin chain is a light chain constant domain such as an IGLC domain or an IGKC domain. Equally, the preferred parent domain of the first engineered immunoglobulin chain is a light chain constant domain such as an IGLC domain or an IGKC domain and the preferred parent domain of the second engineered immunoglobulin chain is a CH1 domain. More preferred is a human CH1 domain selected from the group consisting of IGHG1, IGHG2, IGHG3, and IGHG4, in particular a human CH1 domain from IGHG1. Most preferably the parent domain of the first engineered immunoglobulin chain is an IGLC domain selected from the group consisting of IGLC1, IGLC2, IGLC3, IGLC6, and IGLC7 or is an IGKC domain and the parent domain of the second engineered immunoglobulin chain is a CH1 domain or the parent domain of the first engineered immunoglobulin chain is a CH1 domain and the parent domain of the second engineered immunoglobulin chain is an IGLC domain selected from the group consisting of

IGLC1, IGLC2, IGLC3, IGLC6, and IGLC7 or is an IGKC domain.

In some embodiments the parent domain of the further engineered domain of the first engineered immunoglobulin chain and/or the second engineered immunoglobulin chain and the engineered domain of the third engineered immunoglobulin chain are selected from the group consisting of CH1, CH2, CH3, CH4, IGKC, IGLC1, IGLC2, IGLC3, IGLC6 and IGLC7. Normally the parent domain of the further engineered domain of the first engineered immunoglobulin chain and/or the second engineered immunoglobulin chain and the parent domain of the engineered domain of the third engineered immunoglobulin chain is a naturally occurring domain. Usually the parent domain of the further engineered domain of the first engineered immunoglobulin chain and/or the second engineered immunoglobulin chain and the engineered domain of the third engineered immunoglobulin chain is from human. Preferably the parent domain of the further engineered domain of the first engineered immunoglobulin chain and/or the second engineered immunoglobulin chain and the engineered domain of the third engineered immunoglobulin chain is from human and from the same isotyp, species and subclass. The preferred parent domain of the further engineered domain of the first engineered immunoglobulin chain and/or the second engineered immunoglobulin chain and the engineered domain of the third engineered immunoglobulin chain is a CH3 domain, more preferably a human CH3 domain, in particular a human CH3 domain from IGHG1. Equally preferred, the parent domains of the further engineered domain of the first engineered immunoglobulin chain and/or the second engineered immunoglobulin chain and/or the engineered domain of the third engineered immunoglobulin chain are from a different isotype. In some embodiments the parent domain of the further engineered domain of the first engineered immunoglobulin chain and/or the second engineered immunoglobulin chain is a CH3 domain from IGHG1, preferably a human CH3 domain from IGHG1, and the parent domain of the the engineered domain of the third engineered immunoglobulin chain is a CH3 domain from IGHG3 preferably a human CH3 domain from IGHG3.

In some embodiments, the parent domains of the further engineered domain of the first engineered immunoglobulin chain and/or the second engineered immunoglobulin chain and/or the engineered domain of the third engineered immunoglobulin chain is a CH4 domain, more preferably a human CH4 domain, in particular a human CH4 domain from IGHM or a human CH4 domain from IGHE. Equally preferred the parent domain of the further engineered

domain of the first engineered immunoglobulin chain and/or the the second engineered immunoglobulin is a CH1 domain and the parent domain of the engineered domain of the third engineered immunoglobulin chain is a light chain constant domain such as an IGLC domain or an IGKC domain. Equally, the preferred parent domain of the further engineered domain of the first engineered immunoglobulin chain and/or the second engineered immunoglobulin is a light chain constant domain such as an IGLC domain or an IGKC domain and the preferred parent domain of the engineered domain of the third engineered immunoglobulin chain is a CH1domain. More preferred is a human CH1 domain selected from the group consisting of IGHG1, IGHG2, IGHG3, and IGHG4, in particular a human CH1 domain from IGHG1.

Parent domains from the same species, isotype and subclass, e.g. human CH3 domain from IGHG1 as parent domain of e.g. the first and second engineered immunoglobulin chain, form a homo-dimer. In some embodiments the parent domains of the first and the second engineered immunoglobulin chain form a homo-dimer, specifically a naturally occurring homo-dimer.

In some embodiments the parent domains of the first and the second engineered immunoglobulin chain form a hetero-dimer specifically a naturally occurring hetero-dimer, e.g. a CH1 domain and an IGKC domain or a CH1 domain and an IGLC domain.

In some embodiments the parent domains of the first and the second engineered immunoglobulin chain form a hetero-dimer e.g. a CH3 domain from IGHG1 and a CH3 domain from IGHG3.

In some embodiments the parent domains of the further engineered domain of the first and/or second engineered immunoglobulin chain and the parent domain of the engineered domain of the third engineered immunoglobulin chain form a homo-dimer, specifically a naturally occurring homo-dimer.

In some embodiments the parent domains of the further engineered domain of the first and/or second engineered immunoglobulin chain and the parent domain of the engineered domain of the third engineered immunoglobulin chain form a hetero-dimer, specifically a naturally occurring hetero-dimer, e.g. a CH1 domain and an IGKC domain or a CH1 domain and an IGLC domain.

In some embodiments the parent domains of the further engineered domain of the first and/or second engineered immunoglobulin chain and the parent domain of the engineered domain of

the third engineered immunoglobulin chain form a hetero-dimer e.g. a CH3 domain from IGHG1 and a CH3 domain from IGHG3.

In some embodiments the parent domains of the first and the second engineered immunoglobulin chain are not a CH2 domain.

In some embodiments the parent domains of the first and the second engineered immunoglobulin chain are not a CH3 domain.

In some embodiments the parent domains of the first and the second engineered immunoglobulin chains are not a VH domain and not a VL domain.

In some embodiments the parent domains of the first and the second engineered immunoglobulin chain are not from IgG2.

In some embodiments the preferred parent domain of the first engineered immunoglobulin chain is a CH1 domain and the preferred parent domain of the second engineered immunoglobulin chain is an IGKC domain or, the preferred parent domain of the first engineered immunoglobulin chain is an IGKC domain and the preferred parent domain of the second engineered immunoglobulin chain is a CH1 domain. Even more preferably, the preferred parent domain of the first engineered immunoglobulin chain is a human CH1 domain and the preferred parent domain of the second engineered immunoglobulin chain is a human IGKC domain or, the preferred parent domain of the first engineered immunoglobulin chain is a human IGKC domain and the preferred parent domain of the second engineered immunoglobulin chain is a human CH1 domain, more preferably a human CH1 domain selected from the group consisting of IGHG1, IGHG2, IGHG3, and IGHG4, in particular a human CH1 domain from IGHG1.

In some embodiments the preferred parent domain of the first engineered immunoglobulin chain is a CH1 domain and the preferred parent domain of the second engineered immunoglobulin chain is an IGLC domain or, the preferred parent domain of the first engineered immunoglobulin chain is an IGLC domain and the preferred parent domain of the second engineered immunoglobulin chain is a CH1 domain. Even more preferably, the preferred parent domain of the first engineered immunoglobulin chain is a human CH1 domain and the preferred the parent domain of the second engineered immunoglobulin chain is a human IGLC domain or, the preferred parent domain of the first engineered immunoglobulin chain is a human IGLC domain and the preferred parent domain of the second engineered immunoglobulin chain is a human CH1 domain, more preferably a human

CH1 domain selected from the group consisting of IGHG1, IGHG2, IGHG3, and IGHG4, in particular a human CH1 domain from IGHG1.

In some embodiments the preferred parent domain of the further engineered domain of the first and/or second engineered immunoglobulin chain is a CH1 domain and the preferred parent domain of the engineered domain of the third engineered immunoglobulin chain is an IGKC domain or, the preferred parent domain of the further engineered domain of the first and/or second engineered immunoglobulin chain is an IGKC domain and the preferred parent domain of the engineered domain of the third engineered immunoglobulin chain. Even more preferably, the preferred parent domain of the further engineered domain of the first and/or second engineered immunoglobulin chain is a human CH1 domain and the preferred parent domain of the engineered domain of the third engineered immunoglobulin chain is a human IGKC domain or, the preferred parent domain of the further engineered domain of the first and/or second engineered immunoglobulin chain is a human IGKC domain and the preferred parent domain of the engineered domain of the third engineered immunoglobulin chain is a human CH1 domain, more preferably a human CH1 domain selected from the group consisting of IGHG1, IGHG2, IGHG3, and IGHG4, in particular a human CH1 domain from IGHG1.

In some embodiments the preferred parent domain of the further engineered domain of the first and/or second engineered immunoglobulin chain is a CH1 domain and the preferred parent domain of the engineered domain of the third engineered immunoglobulin chain is an IGLC domain or, the preferred parent domain of the further engineered domain of the first and/or second engineered immunoglobulin chain is an IGLC domain and the preferred parent domain of the engineered domain of the third engineered immunoglobulin chain. Even more preferably, the preferred parent domain of the further engineered domain of the first and/or second engineered immunoglobulin chain is a human CH1 domain and the preferred parent domain of the engineered domain of the third engineered immunoglobulin chain is a human IGLC domain or, the preferred parent domain of the further engineered domain of the first and/or second engineered immunoglobulin chain is a human IGLC domain and the preferred parent domain of the engineered domain of the third engineered immunoglobulin chain is a human CH1 domain.,More preferred is a human CH1 domain selected from the group consisting of IGHG1, IGHG2, IGHG3, and IGHG4, in particular a human CH1 domain from IGHG1.

In some embodiments the parent domain of the first and/or second engineered immunoglobulin chain, in particular the parent domain of the first and second engineered immunoglobulin chain, is a CH3 domain, preferably a CH3 domain of the same species, isotype and subclass, and the parent domain of the further engineered domain of the first engineered immunoglobulin chain and/or the second engineered immunoglobulin is a light chain constant domain such as an IGLC domain or an IGKC domain and the parent domain of the engineered domain of the third engineered immunoglobulin chain is a CH1 domain.
In some embodiments the parent domain of the first engineered immunoglobulin chain is a light chain constant domain such as an IGLC domain or an IGKC domain and the parent domain of the engineered domain of the second engineered immunoglobulin chain is a CH1 domain, and the parent domain of the further engineered domain of the first engineered immunoglobulin chain and/or the second engineered immunoglobulin and the parent domain of the engineered domain of the third engineered immunoglobulin chain is a CH3 domain, preferably a CH3 domain of the same species, isotype and subclass.

In some embodiments e.g if the hetero-dimeric immunoglobulin is a $F(ab')_2$, the parent domain of the engineered domain of the first engineered immunoglobulin chain is a CH1 domain and the parent domain of the engineered domain of the second engineered immunoglobulin chain is a light chain constant domain such as an IGLC domain or an IGKC domain and the parent domain of the further engineered domain of the first engineered immunoglobulin chain is a CH1 domain, and the parent domain of the further engineered domain of the second engineered immunoglobulin chain is a light chain constant domain such as an IGLC domain or an IGKC domain, wherein the first engineered immunoglobulin chain comprises a hinge region between the parent domain of the engineered domain and the parent domain of the further engineered domain.

In some embodiments the present disclosure provides a hetero-dimeric immunoglobulin or fragment thereof, wherein the amino acid residues from the protein-protein interface of the donor domain of the first and second member of the naturally occurring immunoglobulin super-family are amino acids non essential to the core integrity of the domain, preferably wherein the amino acid residues from the protein-protein interface of the donor domain of the first and second member of the naturally occurring immunoglobulin super-family used for

substitution of the protein-protein interface of the parent domain of the first and/or second engineered immunoglobulin chain are amino acids non essential to the core integrity of the domain.

In some embodiments, wherein the protein-protein interface of the parent domain of the first engineered immunoglobulin chain comprises an amino acid residue substitution at a position selected from the group consisting of 20, 22, 85.1, 86, and 88, the protein-protein interface of the parent domain of the second engineered immunoglobulin chain comprises an amino acid residue substitution at a position selected from the group consisting of 22, 26, 85.1, 86, and 88, preferably the protein-protein interface of the parent domain of the first engineered immunoglobulin chain comprises an amino acid residue substitution at a position selected from the group consisting of 22, 85.1, 86, and 88, and the protein-protein interface of the parent domain of the second engineered immunoglobulin chain comprises an amino acid residue substitution at a position selected from the group consisting of 22, 85.1, 86, and 88 or comprises an amino acid residue substitution at a position selected from the group consisting of 85.1 and 86, more preferably the protein-protein interface of the parent domain of the first engineered immunoglobulin chain comprises an amino acid residue substitution at a position selected from the group consisting of 22, 85.1 and 88, and the protein-protein interface of the parent domain of the second engineered immunoglobulin chain comprises an amino acid residue substitution at a position selected from the group consisting of 22, 85.1, 86, and 88 or comprises an amino acid residue substitution at a position selected from the group consisting of 85.1 and 86, in particular the protein-protein interface of the parent domain of the first engineered immunoglobulin chain comprises an amino acid residue substitution at position 88, and the protein-protein interface of the parent domain of the second engineered immunoglobulin chain comprises an amino acid residue substitution at a position selected from the group consisting of 22, 85.1, 86, and 88 or comprises an amino acid residue substitution at a position selected from the group consisting of 85.1 and 86, wherein the amino acid position of each group member is indicated according to the IMGT® numbering.

Thus in some embodiments, the present disclosure provides a hetero-dimeric immunoglobulin or hetero-dimeric fragment thereof comprising:

(a) a first engineered immunoglobulin chain comprising an engineered domain with a protein-protein interface, wherein at least one amino acid residue of the protein-protein interface of

the parent domain of said first engineered immunoglobulin chain is substituted with an amino acid residue at the equivalent 3D structural position from a protein-protein interface of a donor domain of a first member of the naturally occurring immunoglobulin super-family; and (b) a second engineered immunoglobulin chain comprising an engineered domain with a protein-protein interface, wherein at least one amino acid residue of the protein-protein interface of the parent domain of said second engineered immunoglobulin chain is substituted with an amino acid residue at the equivalent 3D structural position from a protein-protein interface of a donor domain of a second member of the naturally occurring immunoglobulin super-family,

wherein the donor domain of the first member of the naturally occurring immunoglobulin super-family and the donor domain of the second member of the naturally occurring immunoglobulin super-family form a naturally occurring hetero-dimer or a naturally occurring homo-dimer and,

wherein the parent domains of the first and the second engineered immunoglobulin chain are not a CH2 domain, not a CH3 domain, not a VL domain and not a VH domain and, wherein the protein-protein interface of the parent domain of the first engineered immunoglobulin chain comprises an amino acid residue substitution at a position selected from the group consisting of 22, 85.1, 86, 88 and/or, wherein the protein-protein interface of the parent domain of the second engineered immunoglobulin chain comprises an amino acid residue substitution at a position selected from the group consisting of 22, 85.1, 86, 88, wherein if the parent domain of the first engineered immunoglobulin chain is an IGLC domain or an IGKC domain and the parent domain of the second engineered immunoglobulin chain is a CH1 domain and the protein-protein interface of the parent domain of the first engineered immunoglobulin chain is substituted with an amino acid residue at position 86 and the protein-protein interface of the parent domain of the second engineered immunoglobulin chain is substituted with an amino acid residue at position 86, the substituted amino acid residues at both positions 86 are not a charged pair, preferably not charged amino acids, wherein the amino acid position of each group member is indicated according to the IMGT® numbering.

In some embodiments, wherein the protein-protein interface of the parent domain of the first engineered immunoglobulin chain comprises an amino acid residue substitution at a position selected from the group consisting of 20, 22, 85.1, 86, and 88, the protein-protein interface of the parent domain of the first engineered immunoglobulin chain comprises an amino acid

residue substitution at position 20 selected from the group consisting of 20A, 20E, 20K, 20N, 20Q, 20S, 20T, 20V and 20W and/or conservative amino substitutions thereof, preferably selected from the group consisting of 20A, 20N, 20Q, 20S, 20T, 20V and 20W and/or conservative amino substitutions thereof, wherein the amino acid position of each group member is indicated according to the IMGT® numbering.

In some embodiments, wherein the protein-protein interface of the parent domain of the first engineered immunoglobulin chain comprises an amino acid residue substitution at a position selected from the group consisting of 20, 22, 85.1, 86, and 88, the protein-protein interface of the parent domain of the first engineered immunoglobulin chain comprises an amino acid residue substitution at position 22 selected from the group consisting of 22A, 22G, 22I, 22L, 22T and 22V and/or conservative amino substitutions thereof, wherein the amino acid position of each group member is indicated according to the IMGT® numbering.

In some embodiments, wherein the protein-protein interface of the parent domain of the first engineered immunoglobulin chain comprises an amino acid residue substitution at a position selected from the group consisting of 20, 22, 85.1, 86, and 88, the protein-protein interface of the parent domain of the first engineered immunoglobulin chain comprises an amino acid residue substitution at position 85.1 selected from the group consisting of 85.1A, 85.1C, 85.1F, 85.1H, 85.1K, 85.1M, 85.1N, 85.1R, 85.1S, 85.1T, and 85.1W and/or conservative amino substitutions thereof, wherein the amino acid position of each group member is indicated according to the IMGT® numbering.

In some embodiments, wherein the protein-protein interface of the parent domain of the first engineered immunoglobulin chain comprises an amino acid residue substitution at a position selected from the group consisting of 20, 22, 85.1, 86, and 88, the protein-protein interface of the parent domain of the first engineered immunoglobulin chain comprises an amino acid residue substitution at position 86 selected from the group consisting of 86F, 86H, 86I, 86T, 86Q, 86S, 86V, 86W, 86Y and/or conservative amino substitutions thereof, wherein the amino acid position of each group member is indicated according to the IMGT® numbering.

In some embodiments, wherein the protein-protein interface of the parent domain of the first engineered immunoglobulin chain comprises an amino acid residue substitution at a position selected from the group consisting of 20, 22, 85.1, 86, and 88, the protein-protein interface of the parent domain of the first engineered immunoglobulin chain comprises an amino acid residue substitution at position 88 selected from the group consisting of 88E, 88I, 88K, 88L, 88Q, 88R, 88T, 88V, 88W, 88Y and/or conservative amino substitutions thereof, wherein the

amino acid position of each group member is indicated according to the IMGT® numbering.

In some embodiments, wherein the protein-protein interface of the parent domain of the first engineered immunoglobulin chain comprises an amino acid residue substitution at a position selected from the group consisting of 20, 22, 85.1, 86, and 88, and the protein-protein interface of the parent domain of the second engineered immunoglobulin chain comprises an amino acid residue substitution at a position selected from the group consisting of 22, 26, 85.1, 86, and 88, the protein-protein interface of the parent domain of the second engineered immunoglobulin chain comprises an amino acid residue substitution at position 22 selected from the group consisting of 22A, 22G, 22I, 22L, 22T and 22V and/or conservative amino substitutions thereof, wherein the amino acid position of each group member is indicated according to the IMGT® numbering.

In some embodiments, wherein the protein-protein interface of the parent domain of the first engineered immunoglobulin chain comprises an amino acid residue substitution at a position selected from the group consisting of 20, 22, 85.1, 86, and 88, and the protein-protein interface of the parent domain of the second engineered immunoglobulin chain comprises an amino acid residue substitution at a position selected from the group consisting of 22, 26, 85.1, 86, and 88, the protein-protein interface of the parent domain of the second engineered immunoglobulin chain comprises an amino acid residue substitution at position 26 selected from the group consisting of 26K, 26Q, 26R, 26S, 26T, and 26V and/or conservative amino substitutions thereof, wherein the amino acid position of each group member is indicated according to the IMGT® numbering.

In some embodiments, wherein the protein-protein interface of the parent domain of the first engineered immunoglobulin chain comprises an amino acid residue substitution at a position selected from the group consisting of 20, 22, 85.1, 86, and 88, and the protein-protein interface of the parent domain of the second engineered immunoglobulin chain comprises an amino acid residue substitution at a position selected from the group consisting of 22, 26, 85.1, 86, and 88, the protein-protein interface of the parent domain of the second engineered immunoglobulin chain comprises an amino acid residue substitution at position 85.1 selected from the group consisting of 85.1A, 85.1C, 85.1F, 85.1H, 85.1K, 85.1M, 85.1N, 85.1R ,85.1S, 85.1T, and 85.1W and/or conservative amino substitutions thereof, wherein the amino acid position of each group member is indicated according to the IMGT® numbering.

In some embodiments, wherein the protein-protein interface of the parent domain of the first

engineered immunoglobulin chain comprises an amino acid residue substitution at a position selected from the group consisting of 20, 22, 85.1, 86, and 88, and the protein-protein interface of the parent domain of the second engineered immunoglobulin chain comprises an amino acid residue substitution at a position selected from the group consisting of 22, 26, 85.1, 86, and 88, the protein-protein interface of the parent domain of the second engineered immunoglobulin chain comprises an amino acid residue substitution at position 86 selected from the group consisting of 86F, 86H, 86I, 86T, 86Q, 86S, 86V, 86W, 86Y and/or conservative amino substitutions thereof, wherein the amino acid position of each group member is indicated according to the IMGT® numbering.

In some embodiments, wherein the protein-protein interface of the parent domain of the first engineered immunoglobulin chain comprises an amino acid residue substitution at a position selected from the group consisting of 20, 22, 85.1, 86, and 88, and the protein-protein interface of the parent domain of the second engineered immunoglobulin chain comprises an amino acid residue substitution at a position selected from the group consisting of 22, 26, 85.1, 86, and 88, the protein-protein interface of the parent domain of the second engineered immunoglobulin chain comprises an amino acid residue substitution at position 88 selected from the group consisting of 88E, 88I, 88K, 88L, 88Q, 88R, 88T, 88V, 88W, 88Y and/or conservative amino substitutions thereof, wherein the amino acid position of each group member is indicated according to the IMGT® numbering.

In some embodiments, if the substitution in the parent domain of the first engineered immunoglobulin chain is at position 88, the substituted amino acid residue in the parent domain of the second engineered immunoglobulin chain which interacts with the substituted amino acid residue at position 88 in the parent domain of the first engineered immunoglobulin chain is at position 85.1 and/or 86, or if the substitution in the parent domain of the second engineered immunoglobulin chain is at position 88, the substituted amino acid residue in the parent domain of the first engineered immunoglobulin chain which interacts with the substituted amino acid residue at position 88 in the parent domain of the second engineered immunoglobulin chain is at position 85.1 and/or 86, wherein the amino acid position of each group member is indicated according to the IMGT® numbering.

In some embodiments, the substituted amino acid residue in the parent domain of the first engineered immunoglobulin chain comprises an amino acid residue at position 88 and the substituted amino acid residue in the parent domain of the second engineered immunoglobulin

chain comprises an amino acid residue at position 85.1 and/or 86, preferably at position 85.1 and 86, wherein the amino acid position is indicated according to the IMGT® numbering. Usually the substituted amino acid residue in the parent domain of the first engineered immunoglobulin chain at position 88 and the substituted amino acid residue in the parent domain of the second engineered immunoglobulin chain at position 85.1 and/or 86 interact with each other, wherein the amino acid position is indicated according to the IMGT® numbering.

In some embodiments, if the substitution in the parent domain of the first engineered immunoglobulin chain is at position 85.1, the substituted amino acid residue in the parent domain of the second engineered immunoglobulin chain which interacts with the substituted amino acid residue at position 85.1 in the parent domain of the first engineered immunoglobulin chain is at position 86, or if the substitution in the parent domain of the second engineered immunoglobulin chain is at position 85.1, the substituted amino acid residue in the parent domain of the first engineered immunoglobulin chain which interacts with the substituted amino acid residue at position 85.1 in the parent domain of the second engineered immunoglobulin chain is at position 86, wherein the amino acid position of each group member is indicated according to the IMGT® numbering.

In some embodiments, the substituted amino acid residue in the parent domain of the first engineered immunoglobulin chain comprises an amino acid residue at position 85.1 and the substituted amino acid residue in the parent domain of the second engineered immunoglobulin chain comprises an amino acid residue at position 86, wherein the amino acid position is indicated according to the IMGT® numbering. Usually the substituted amino acid residue in the parent domain of the first engineered immunoglobulin chain at position 85.1 and the substituted amino acid residue in the parent domain of the second engineered immunoglobulin chain at position 86 interact with each other, wherein the amino acid position is indicated according to the IMGT® numbering.

In some embodiments, if the substitution in the parent domain of the first engineered immunoglobulin chain is at position 22, the substituted amino acid residue in the parent domain of the second engineered immunoglobulin chain which interacts with the substituted amino acid residue at position 22 in the parent domain of the first engineered immunoglobulin chain is at position 22 and/or 86, or if the substitution in the parent domain of the second

engineered immunoglobulin chain is at position 22, the substituted amino acid residue in the parent domain of the first engineered immunoglobulin chain which interacts with the substituted amino acid residue at position 22 in the parent domain of the second engineered immunoglobulin chain is at position 22 and/or 86, wherein the amino acid position of each group member is indicated according to the IMGT® numbering.

In some embodiments, the substituted amino acid residue in the parent domain of the first engineered immunoglobulin chain comprises an amino acid residue at position 22 and the substituted amino acid residue in the parent domain of the second engineered immunoglobulin chain comprises an amino acid residue at position 22 and/or 86, preferably at position 22 and 86, wherein the amino acid position is indicated according to the IMGT® numbering.Usually the substituted amino acid residue in the parent domain of the first engineered immunoglobulin chain at position 22 and the substituted amino acid residue in the parent domain of the second engineered immunoglobulin chain at position 22 and/or 86 interact with each other, wherein the amino acid position is indicated according to the IMGT® numbering.

In some embodiments, if the substitution in the parent domain of the first engineered immunoglobulin chain is at position 20, the substituted amino acid residue in the parent domain of the second engineered immunoglobulin chain which interacts with the substituted amino acid residue at position 20 in the parent domain of the first engineered immunoglobulin chain is at position 26, or if the substitution in the parent domain of the second engineered immunoglobulin chain is at position 20, the substituted amino acid residue in the parent domain of the first engineered immunoglobulin chain which interacts with the substituted amino acid residue at position 20 in the parent domain of the second engineered immunoglobulin chain is at position 26, wherein the amino acid position of each group member is indicated according to the IMGT® numbering.

In some embodiments, the substituted amino acid residue in the parent domain of the first engineered immunoglobulin chain comprises an amino acid residue at position 20 and the substituted amino acid residue in the parent domain of the second engineered immunoglobulin chain comprises an amino acid residue at position 26, wherein the amino acid position is indicated according to the IMGT® numbering. Usually the substituted amino acid residue in the parent domain of the first engineered immunoglobulin chain at position 20 and the substituted amino acid residue in the parent domain of the second engineered immunoglobulin chain at position 26 interact with each other, wherein the amino acid position is indicated

according to the IMGT® numbering.

In some embodiments, the donor domains of the first and second member of the naturally occurring immunoglobulin super-family are a human IGHA1 CH2 or a human IGHA2 CH2, wherein the amino acid substitutions in the protein-protein interface of the engineered domain of the first immunoglobulin chain comprise 88V and conservative amino acid substitutions thereof, and the amino acid substitutions in the protein-protein interface of the engineered domain of the second immunoglobulin chain comprise 86S and conservative amino acid substitutions thereof, and/or 85.1S and conservative amino acid substitutions thereof, wherein the amino acid position is indicated according to the IMGT® numbering.

In some embodiments, the donor domains of the first and second member of the naturally occurring immunoglobulin super-family are a human IGHA1 CH2 or a human IGHA2 CH2, wherein the amino acid substitutions in the protein-protein interface of the engineered domain of the first immunoglobulin chain comprise 86S and conservative amino acid substitutions thereof, and the amino acid substitutions in the protein-protein interface of the engineered domain of the second immunoglobulin chain comprise 85.1S and conservative amino acid substitutions thereof, wherein the amino acid position is indicated according to the IMGT® numbering.

In some embodiments, the donor domains of the first and second member of the naturally occurring immunoglobulin super-family are a human IGHA1 CH2 or a human IGHA2 CH2, wherein the amino acid substitutions in the protein-protein interface of the engineered domain of the first immunoglobulin chain comprise 86S and conservative amino acid substitutions thereof, and the amino acid substitutions in the protein-protein interface of the engineered domain of the second immunoglobulin chain comprise 22T and conservative amino acid substitutions thereof, wherein the amino acid position is indicated according to the IMGT® numbering.

In some embodiments, the donor domains of the first and second member of the naturally occurring immunoglobulin super-family are a human IGHD CH2, wherein the amino acid substitutions in the protein-protein interface of the engineered domain of the first immunoglobulin chain comprise 88R and conservative amino acid substitutions thereof, and the amino acid substitutions in the protein-protein interface of the engineered domain of the second immunoglobulin chain comprise 86H and conservative amino acid substitutions

thereof, and/or 85.1S and conservative amino acid substitutions thereof, wherein the amino acid position is indicated according to the IMGT® numbering.

In some embodiments, the donor domains of the first and second member of the naturally occurring immunoglobulin super-family are a human IGHD CH2, wherein the amino acid substitutions in the protein-protein interface of the engineered domain of the first immunoglobulin chain comprise 86H and conservative amino acid substitutions thereof, and the amino acid substitutions in the protein-protein interface of the engineered domain of the second immunoglobulin chain comprise 85.1S and conservative amino acid substitutions thereof, wherein the amino acid position is indicated according to the IMGT® numbering.

In some embodiments, the donor domains of the first and second member of the naturally occurring immunoglobulin super-family are a human IGHD CH2, wherein the amino acid substitutions in the protein-protein interface of the engineered domain of the first immunoglobulin chain comprise 86H and conservative amino acid substitutions thereof, and the amino acid substitutions in the protein-protein interface of the engineered domain of the second immunoglobulin chain comprise 22T and conservative amino acid substitutions thereof, wherein the amino acid position is indicated according to the IMGT® numbering.

In some embodiments, the donor domains of the first and second member of the naturally occurring immunoglobulin super-family are a human IGHE CH2, wherein the amino acid substitutions in the protein-protein interface of the engineered domain of the first immunoglobulin chain comprise 88E and conservative amino acid substitutions thereof, and the amino acid substitutions in the protein-protein interface of the engineered domain of the second immunoglobulin chain comprise 86Q and conservative amino acid substitutions thereof, and/or 85.1S and conservative amino acid substitutions thereof, wherein the amino acid position is indicated according to the IMGT® numbering.

In some embodiments, the donor domains of the first and second member of the naturally occurring immunoglobulin super-family are a human IGHE CH2, wherein the amino acid substitutions in the protein-protein interface of the engineered domain of the first immunoglobulin chain comprise 86Q and conservative amino acid substitutions thereof, and the amino acid substitutions in the protein-protein interface of the engineered domain of the second immunoglobulin chain comprise 85.1S and conservative amino acid substitutions thereof, wherein the amino acid position is indicated according to the IMGT® numbering.

In some embodiments, the donor domains of the first and second member of the naturally occurring immunoglobulin super-family are a human IGHE CH2, wherein the amino acid

substitutions in the protein-protein interface of the engineered domain of the first immunoglobulin chain comprise 86Q and conservative amino acid substitutions thereof, and the amino acid substitutions in the protein-protein interface of the engineered domain of the second immunoglobulin chain comprise 22L and conservative amino acid substitutions thereof, wherein the amino acid position is indicated according to the IMGT® numbering.

In some embodiments, the donor domains of the first and second member of the naturally occurring immunoglobulin super-family are a human IGHG1 CH2 or a human IGHG2 CH2 or a human IGHG3 CH2 or a human IGHG4 CH2, wherein the amino acid substitutions in the protein-protein interface of the engineered domain of the first immunoglobulin chain comprise 88V and conservative amino acid substitutions thereof, and the amino acid substitutions in the protein-protein interface of the engineered domain of the second immunoglobulin chain comprise 86V and conservative amino acid substitutions thereof, and/or 85.1R and conservative amino acid substitutions thereof, wherein the amino acid position is indicated according to the IMGT® numbering.

In some embodiments, the donor domains of the first and second member of the naturally occurring immunoglobulin super-family are a human IGHG1 CH2 or a human IGHG2 CH2 or a human IGHG3 CH2 or a human IGHG4 CH2, wherein the amino acid substitutions in the protein-protein interface of the engineered domain of the first immunoglobulin chain comprise 86V and conservative amino acid substitutions thereof, and the amino acid substitutions in the protein-protein interface of the engineered domain of the second immunoglobulin chain comprise 85.1R and conservative amino acid substitutions thereof, wherein the amino acid position is indicated according to the IMGT® numbering.

In some embodiments, the donor domains of the first and second member of the naturally occurring immunoglobulin super-family are a human IGHG1 CH2 or a human IGHG2 CH2 or a human IGHG3 CH2 or a human IGHG4 CH2, wherein the amino acid substitutions in the protein-protein interface of the engineered domain of the first immunoglobulin chain comprise 86V and conservative amino acid substitutions thereof, and the amino acid substitutions in the protein-protein interface of the engineered domain of the second immunoglobulin chain comprise 22T and conservative amino acid substitutions thereof, wherein the amino acid position is indicated according to the IMGT® numbering.

In some embodiments, the donor domains of the first and second member of the naturally occurring immunoglobulin super-family are a human IGHP CH2, wherein the amino acid substitutions in the protein-protein interface of the engineered domain of the first

immunoglobulin chain comprise 88V and conservative amino acid substitutions thereof, and the amino acid substitutions in the protein-protein interface of the engineered domain of the second immunoglobulin chain comprise 86V and conservative amino acid substitutions thereof, and/or 85.1H and conservative amino acid substitutions thereof, wherein the amino acid position is indicated according to the IMGT® numbering.

In some embodiments, the donor domains of the first and second member of the naturally occurring immunoglobulin super-family are a human IGHP CH2, wherein the amino acid substitutions in the protein-protein interface of the engineered domain of the first immunoglobulin chain comprise 86V and conservative amino acid substitutions thereof, and the amino acid substitutions in the protein-protein interface of the engineered domain of the second immunoglobulin chain comprise 85.1H and conservative amino acid substitutions thereof, wherein the amino acid position is indicated according to the IMGT® numbering.In some embodiments, the donor domains of the first and second member of the naturally occurring immunoglobulin super-family are a human IGHP CH2, wherein the amino acid substitutions in the protein-protein interface of the engineered domain of the first immunoglobulin chain comprise 86V and conservative amino acid substitutions thereof, and the amino acid substitutions in the protein-protein interface of the engineered domain of the second immunoglobulin chain comprise 22T and conservative amino acid substitutions thereof, wherein the amino acid position is indicated according to the IMGT® numbering.

In some embodiments, the donor domains of the first and second member of the naturally occurring immunoglobulin super-family are a human IGHM CH2, wherein the amino acid substitutions in the protein-protein interface of the engineered domain of the first immunoglobulin chain comprise 88T and conservative amino acid substitutions thereof, and the amino acid substitutions in the protein-protein interface of the engineered domain of the second immunoglobulin chain comprise 86T and conservative amino acid substitutions thereof, and/or 85.1K and conservative amino acid substitutions thereof, wherein the amino acid position is indicated according to the IMGT® numbering.

In some embodiments, the donor domains of the first and second member of the naturally occurring immunoglobulin super-family are a human IGHM CH2, wherein the amino acid substitutions in the protein-protein interface of the engineered domain of the first immunoglobulin chain comprise 86T and conservative amino acid substitutions thereof, and the amino acid substitutions in the protein-protein interface of the engineered domain of the

second immunoglobulin chain comprise 85.1K and conservative amino acid substitutions thereof, wherein the amino acid position is indicated according to the IMGT® numbering. In some embodiments, the donor domains of the first and second member of the naturally occurring immunoglobulin super-family are a human IGHM CH2, wherein the amino acid substitutions in the protein-protein interface of the engineered domain of the first immunoglobulin chain comprise 86T and conservative amino acid substitutions thereof, and the amino acid substitutions in the protein-protein interface of the engineered domain of the second immunoglobulin chain comprise 22I and conservative amino acid substitutions thereof, wherein the amino acid position is indicated according to the IMGT® numbering. In some embodiments, the donor domains of the first and second member of the naturally occurring immunoglobulin super-family are a human IGHA1 CH3 or a human IGHA2 CH3, wherein the amino acid substitutions in the protein-protein interface of the engineered domain of the first immunoglobulin chain comprise 88I and conservative amino acid substitutions thereof, and the amino acid substitutions in the protein-protein interface of the engineered domain of the second immunoglobulin chain comprise 86T and conservative amino acid substitutions thereof, and/or 85.1A and conservative amino acid substitutions thereof, wherein the amino acid position is indicated according to the IMGT® numbering.In some embodiments, the donor domains of the first and second member of the naturally occurring immunoglobulin super-family are a human IGHA1 CH3 or a human IGHA2 CH3, wherein the amino acid substitutions in the protein-protein interface of the engineered domain of the first immunoglobulin chain comprise 86T and conservative amino acid substitutions thereof, and the amino acid substitutions in the protein-protein interface of the engineered domain of the second immunoglobulin chain comprise 85.1A and conservative amino acid substitutions thereof, wherein the amino acid position is indicated according to the IMGT® numbering. In some embodiments, the donor domains of the first and second member of the naturally occurring immunoglobulin super-family are a human IGHA1 CH3 or a human IGHA2 CH3, wherein the amino acid substitutions in the protein-protein interface of the engineered domain of the first immunoglobulin chain comprise 86T and conservative amino acid substitutions thereof, and the amino acid substitutions in the protein-protein interface of the engineered domain of the second immunoglobulin chain comprise 22T and conservative amino acid substitutions thereof, wherein the amino acid position is indicated according to the IMGT® numbering.

In some embodiments, the donor domains of the first and second member of the naturally occurring immunoglobulin super-family are a human IGHD CH3, wherein the amino acid substitutions in the protein-protein interface of the engineered domain of the first immunoglobulin chain comprise 88V and conservative amino acid substitutions thereof, and the amino acid substitutions in the protein-protein interface of the engineered domain of the second immunoglobulin chain comprise 86W and conservative amino acid substitutions thereof, and/or 85.1W and conservative amino acid substitutions thereof, wherein the amino acid position is indicated according to the IMGT® numbering.

In some embodiments, the donor domains of the first and second member of the naturally occurring immunoglobulin super-family are a human IGHD CH3, wherein the amino acid substitutions in the protein-protein interface of the engineered domain of the first immunoglobulin chain comprise 86W and conservative amino acid substitutions thereof, and the amino acid substitutions in the protein-protein interface of the engineered domain of the second immunoglobulin chain comprise 85.1W and conservative amino acid substitutions thereof, wherein the amino acid position is indicated according to the IMGT® numbering.

In some embodiments, the donor domains of the first and second member of the naturally occurring immunoglobulin super-family are a human IGHD CH3, wherein the amino acid substitutions in the protein-protein interface of the engineered domain of the first immunoglobulin chain comprise 86W and conservative amino acid substitutions thereof, and the amino acid substitutions in the protein-protein interface of the engineered domain of the second immunoglobulin chain comprise 22L and conservative amino acid substitutions thereof, wherein the amino acid position is indicated according to the IMGT® numbering.In some embodiments, the donor domains of the first and second member of the naturally occurring immunoglobulin super-family are a human IGHE CH3, wherein the amino acid substitutions in the protein-protein interface of the engineered domain of the first immunoglobulin chain comprise 88T and conservative amino acid substitutions thereof, and the amino acid substitutions in the protein-protein interface of the engineered domain of the second immunoglobulin chain comprise 86T and conservative amino acid substitutions thereof, and/or 85.1T and conservative amino acid substitutions thereof, wherein the amino acid position is indicated according to the IMGT® numbering.

In some embodiments, the donor domains of the first and second member of the naturally occurring immunoglobulin super-family are a human IGHE CH3, wherein the amino acid substitutions in the protein-protein interface of the engineered domain of the first

immunoglobulin chain comprise 86T and conservative amino acid substitutions thereof, and the amino acid substitutions in the protein-protein interface of the engineered domain of the second immunoglobulin chain comprise 85.1T and conservative amino acid substitutions thereof, wherein the amino acid position is indicated according to the IMGT® numbering.

In some embodiments, the donor domains of the first and second member of the naturally occurring immunoglobulin super-family are a human IGHE CH3, wherein the amino acid substitutions in the protein-protein interface of the engineered domain of the first immunoglobulin chain comprise 86T and conservative amino acid substitutions thereof, and the amino acid substitutions in the protein-protein interface of the engineered domain of the second immunoglobulin chain comprise 22T and conservative amino acid substitutions thereof, wherein the amino acid position is indicated according to the IMGT® numbering.

In some embodiments, the donor domains of the first and second member of the naturally occurring immunoglobulin super-family are a human IGHG1 CH3 or a human IGHG2 CH3 or a human IGHG3 CH3, wherein the amino acid substitutions in the protein-protein interface of the engineered domain of the first immunoglobulin chain comprise 88K and conservative amino acid substitutions thereof, and the amino acid substitutions in the protein-protein interface of the engineered domain of the second immunoglobulin chain comprise 86Y and conservative amino acid substitutions thereof, and/or 85.1F and conservative amino acid substitutions thereof, wherein the amino acid position of each group member is indicated according to the IMGT® numbering.

In some embodiments, the donor domains of the first and second member of the naturally occurring immunoglobulin super-family are a human IGHG1 CH3 or a human IGHG2 CH3 or a human IGHG3 CH3, wherein the amino acid substitutions in the protein-protein interface of the engineered domain of the first immunoglobulin chain comprise 86Y and conservative amino acid substitutions thereof, and the amino acid substitutions in the protein-protein interface of the engineered domain of the second immunoglobulin chain comprise 85.1F and conservative amino acid substitutions thereof, wherein the amino acid position is indicated according to the IMGT® numbering.

In some embodiments, the donor domains of the first and second member of the naturally occurring immunoglobulin super-family are a human IGHG1 CH3 or a human IGHG2 CH3 or a human IGHG3 CH3, wherein the amino acid substitutions in the protein-protein interface of the engineered domain of the first immunoglobulin chain comprise 86Y and conservative amino acid substitutions thereof, and the amino acid substitutions in the protein-protein

interface of the engineered domain of the second immunoglobulin chain comprise 22T and conservative amino acid substitutions thereof, wherein the amino acid position is indicated according to the IMGT® numbering.

In some embodiments, the donor domains of the first and second member of the naturally occurring immunoglobulin super-family are a human IGHG4 CH3, wherein the amino acid substitutions in the protein-protein interface of the engineered domain of the first immunoglobulin chain comprise 88R and conservative amino acid substitutions thereof, and the amino acid substitutions in the protein-protein interface of the engineered domain of the second immunoglobulin chain comprise 86Y and conservative amino acid substitutions thereof, and/or 85.1F and conservative amino acid substitutions thereof, wherein the amino acid position is indicated according to the IMGT® numbering.

In some embodiments, the donor domains of the first and second member of the naturally occurring immunoglobulin super-family are a human IGHG4 CH3, wherein the amino acid substitutions in the protein-protein interface of the engineered domain of the first immunoglobulin chain comprise 86Y and conservative amino acid substitutions thereof, and the amino acid substitutions in the protein-protein interface of the engineered domain of the second immunoglobulin chain comprise 85.1F and conservative amino acid substitutions thereof, wherein the amino acid position is indicated according to the IMGT® numbering.

In some embodiments, the donor domains of the first and second member of the naturally occurring immunoglobulin super-family are a human IGHG4 CH3, wherein the amino acid substitutions in the protein-protein interface of the engineered domain of the first immunoglobulin chain comprise 86Y and conservative amino acid substitutions thereof, and the amino acid substitutions in the protein-protein interface of the engineered domain of the second immunoglobulin chain comprise 22T and conservative amino acid substitutions thereof, wherein the amino acid position is indicated according to the IMGT® numbering.In some embodiments, the donor domains of the first and second member of the naturally occurring immunoglobulin super-family are a human IGHP CH3, wherein the amino acid substitutions in the protein-protein interface of the engineered domain of the first immunoglobulin chain comprise 88K and conservative amino acid substitutions thereof, and the amino acid substitutions in the protein-protein interface of the engineered domain of the second immunoglobulin chain comprise 86Y and conservative amino acid substitutions thereof, and/or 85.1F and conservative amino acid substitutions thereof, wherein the amino acid position is indicated according to the IMGT® numbering.

In some embodiments, the donor domains of the first and second member of the naturally occurring immunoglobulin super-family are a human IGHP CH3, wherein the amino acid substitutions in the protein-protein interface of the engineered domain of the first immunoglobulin chain comprise 86Y and conservative amino acid substitutions thereof, and the amino acid substitutions in the protein-protein interface of the engineered domain of the second immunoglobulin chain comprise 85.1F and conservative amino acid substitutions thereof, wherein the amino acid position is indicated according to the IMGT® numbering.

In some embodiments, the donor domains of the first and second member of the naturally occurring immunoglobulin super-family are a human IGHP CH3, wherein the amino acid substitutions in the protein-protein interface of the engineered domain of the first immunoglobulin chain comprise 86Y and conservative amino acid substitutions thereof, and the amino acid substitutions in the protein-protein interface of the engineered domain of the second immunoglobulin chain comprise 22T and conservative amino acid substitutions thereof, wherein the amino acid position is indicated according to the IMGT® numbering.

In some embodiments, the donor domains of the first and second member of the naturally occurring immunoglobulin super-family are a human IGHM CH3, wherein the amino acid substitutions in the protein-protein interface of the engineered domain of the first immunoglobulin chain comprise 88E and conservative amino acid substitutions thereof, and the amino acid substitutions in the protein-protein interface of the engineered domain of the second immunoglobulin chain comprise 86V and conservative amino acid substitutions thereof, and/or 85.1S and conservative amino acid substitutions thereof, wherein the amino acid position is indicated according to the IMGT® numbering.

In some embodiments, the donor domains of the first and second member of the naturally occurring immunoglobulin super-family are a human IGHM CH3, wherein the amino acid substitutions in the protein-protein interface of the engineered domain of the first immunoglobulin chain comprise 86V and conservative amino acid substitutions thereof, and the amino acid substitutions in the protein-protein interface of the engineered domain of the second immunoglobulin chain comprise 85.1S and conservative amino acid substitutions thereof, wherein the amino acid position is indicated according to the IMGT® numbering.In some embodiments, the donor domains of the first and second member of the naturally occurring immunoglobulin super-family are a human IGHM CH3, wherein the amino acid substitutions in the protein-protein interface of the engineered domain of the first immunoglobulin chain comprise 86V and conservative amino acid substitutions thereof, and

the amino acid substitutions in the protein-protein interface of the engineered domain of the second immunoglobulin chain comprise 22T and conservative amino acid substitutions thereof, wherein the amino acid position is indicated according to the IMGT® numbering.

In some embodiments, the donor domains of the first and second member of the naturally occurring immunoglobulin super-family are a human IGHE CH4, wherein the amino acid substitutions in the protein-protein interface of the engineered domain of the first immunoglobulin chain comprise 88R and conservative amino acid substitutions thereof, and the amino acid substitutions in the protein-protein interface of the engineered domain of the second immunoglobulin chain comprise 86F and conservative amino acid substitutions thereof, and/or 85.1F and conservative amino acid substitutions thereof, wherein the amino acid position is indicated according to the IMGT® numbering.

In some embodiments, the donor domains of the first and second member of the naturally occurring immunoglobulin super-family are a human IGHE CH4, wherein the amino acid substitutions in the protein-protein interface of the engineered domain of the first immunoglobulin chain comprise 86F and conservative amino acid substitutions thereof, and the amino acid substitutions in the protein-protein interface of the engineered domain of the second immunoglobulin chain comprise 85.1F and conservative amino acid substitutions thereof, wherein the amino acid position is indicated according to the IMGT® numbering.In some embodiments, the donor domains of the first and second member of the naturally occurring immunoglobulin super-family are a human IGHE CH4, wherein the amino acid substitutions in the protein-protein interface of the engineered domain of the first immunoglobulin chain comprise 86F and conservative amino acid substitutions thereof, and the amino acid substitutions in the protein-protein interface of the engineered domain of the second immunoglobulin chain comprise 22A and conservative amino acid substitutions thereof, wherein the amino acid position is indicated according to the IMGT® numbering.

In some embodiments, the donor domains of the first and second member of the naturally occurring immunoglobulin super-family are a human IGHM CH4, wherein the amino acid substitutions in the protein-protein interface of the engineered domain of the first immunoglobulin chain comprise 88I and conservative amino acid substitutions thereof, and the amino acid substitutions in the protein-protein interface of the engineered domain of the second immunoglobulin chain comprise 86H and conservative amino acid substitutions thereof, and/or 85.1F and conservative amino acid substitutions thereof, wherein the amino acid position is indicated according to the IMGT® numbering.

In some embodiments, the donor domains of the first and second member of the naturally occurring immunoglobulin super-family are a human IGHM CH4, wherein the amino acid substitutions in the protein-protein interface of the engineered domain of the first immunoglobulin chain comprise 86H and conservative amino acid substitutions thereof, and the amino acid substitutions in the protein-protein interface of the engineered domain of the second immunoglobulin chain comprise 85.1F and conservative amino acid substitutions thereof, wherein the amino acid position is indicated according to the IMGT® numbering.

In some embodiments, the donor domains of the first and second member of the naturally occurring immunoglobulin super-family are a human IGHM CH4, wherein the amino acid substitutions in the protein-protein interface of the engineered domain of the first immunoglobulin chain comprise 86H and conservative amino acid substitutions thereof, and the amino acid substitutions in the protein-protein interface of the engineered domain of the second immunoglobulin chain comprise 22T and conservative amino acid substitutions thereof, wherein the amino acid position is indicated according to the IMGT® numbering.

In some embodiments, the donor domains of the first and second member of the naturally occurring immunoglobulin super-family are a human IGKC, wherein the amino acid substitutions in the protein-protein interface of the engineered domain of the first immunoglobulin chain comprise 88T and conservative amino acid substitutions thereof, and the amino acid substitutions in the protein-protein interface of the engineered domain of the second immunoglobulin chain comprise 86S and conservative amino acid substitutions thereof, and/or 85.1S and conservative amino acid substitutions thereof, wherein the amino acid position is indicated according to the IMGT® numbering.

In some embodiments, the donor domains of the first and second member of the naturally occurring immunoglobulin super-family are a human IGKC, wherein the amino acid substitutions in the protein-protein interface of the engineered domain of the first immunoglobulin chain comprise 86S and conservative amino acid substitutions thereof, and the amino acid substitutions in the protein-protein interface of the engineered domain of the second immunoglobulin chain comprise 85.1S and conservative amino acid substitutions thereof, wherein the amino acid position is indicated according to the IMGT® numbering.

In some embodiments, the donor domains of the first and second member of the naturally occurring immunoglobulin super-family are a human IGKC, wherein the amino acid substitutions in the protein-protein interface of the engineered domain of the first immunoglobulin chain comprise 86S and conservative amino acid substitutions thereof, and

the amino acid substitutions in the protein-protein interface of the engineered domain of the second immunoglobulin chain comprise 22V and conservative amino acid substitutions thereof, wherein the amino acid position is indicated according to the IMGT® numbering.

In some embodiments, the donor domains of the first and second member of the naturally occurring immunoglobulin super-family are a human IGLC1 or a human IGLC2 or a human IGLC3 or a human IGLC6 or a human IGLC7, wherein the amino acid substitutions in the protein-protein interface of the engineered domain of the first immunoglobulin chain comprise 88Y and conservative amino acid substitutions thereof, and the amino acid substitutions in the protein-protein interface of the engineered domain of the second immunoglobulin chain comprise 86S and conservative amino acid substitutions thereof, and/or 85.1A and conservative amino acid substitutions thereof, wherein the amino acid position of each group member is indicated according to the IMGT® numbering.

In some embodiments, the donor domains of the first and second member of the naturally occurring immunoglobulin super-family are a human IGLC1 or a human IGLC2 or a human IGLC3 or a human IGLC6 or a human IGLC7, wherein the amino acid substitutions in the protein-protein interface of the engineered domain of the first immunoglobulin chain comprise 86S and conservative amino acid substitutions thereof, and the amino acid substitutions in the protein-protein interface of the engineered domain of the second immunoglobulin chain comprise 85.1A and conservative amino acid substitutions thereof, wherein the amino acid position is indicated according to the IMGT® numbering.

In some embodiments, the donor domains of the first and second member of the naturally occurring immunoglobulin super-family are a human IGLC1 or a human IGLC2 or a human IGLC3 or a human IGLC6 or a human IGLC7, wherein the amino acid substitutions in the protein-protein interface of the engineered domain of the first immunoglobulin chain comprise 86S and conservative amino acid substitutions thereof, and the amino acid substitutions in the protein-protein interface of the engineered domain of the second immunoglobulin chain comprise 22V and conservative amino acid substitutions thereof, wherein the amino acid position is indicated according to the IMGT® numbering.

In some embodiments, the donor domain of the first member of the naturally occurring immunoglobulin super-family is a human IGHA1 CH1 or human IGHA2 CH1, and the donor domain of the second member of the naturally occurring immunoglobulin super-family is a human IGKC, wherein the amino acid substitutions in the protein-protein interface of the engineered domain of the first immunoglobulin chain comprise 88Q and conservative amino

acid substitutions thereof, and the amino acid substitutions in the protein-protein interface of the engineered domain of the second immunoglobulin chain comprise 86S and conservative amino acid substitutions thereof, and/or 85.1S and conservative amino acid substitutions thereof, wherein the amino acid position is indicated according to the IMGT® numbering.

In some embodiments, the donor domain of the first member of the naturally occurring immunoglobulin super-family is a human IGHA1 CH1 or human IGHA2 CH1, and the donor domain of the second member of the naturally occurring immunoglobulin super-family is a human IGKC, wherein the amino acid substitutions in the protein-protein interface of the engineered domain of the first immunoglobulin chain comprise 86S and conservative amino acid substitutions thereof, and the amino acid substitutions in the protein-protein interface of the engineered domain of the second immunoglobulin chain comprise 85.1S and conservative amino acid substitutions thereof, wherein the amino acid position is indicated according to the IMGT® numbering.

In some embodiments, the donor domain of the first member of the naturally occurring immunoglobulin super-family is a human IGHA1 CH1 or human IGHA2 CH1, and the donor domain of the second member of the naturally occurring immunoglobulin super-family is a human IGKC, wherein the amino acid substitutions in the protein-protein interface of the engineered domain of the first immunoglobulin chain comprise 86S and conservative amino acid substitutions thereof, and the amino acid substitutions in the protein-protein interface of the engineered domain of the second immunoglobulin chain comprise 22V and conservative amino acid substitutions thereof, wherein the amino acid position is indicated according to the IMGT® numbering.

In some embodiments, the donor domain of the first member of the naturally occurring immunoglobulin super-family is a human IGHD CH1, and the donor domain of the second member of the naturally occurring immunoglobulin super-family is a human IGKC, wherein the amino acid substitutions in the protein-protein interface of the engineered domain of the first immunoglobulin chain comprise 88Q and conservative amino acid substitutions thereof, and the amino acid substitutions in the protein-protein interface of the engineered domain of the second immunoglobulin chain comprise 86S and conservative amino acid substitutions thereof, and/or 85.1S and conservative amino acid substitutions thereof, wherein the amino acid position is indicated according to the IMGT® numbering.

In some embodiments, the donor domain of the first member of the naturally occurring immunoglobulin super-family is a human IGHD CH1, and the donor domain of the second

member of the naturally occurring immunoglobulin super-family is a human IGKC, wherein the amino acid substitutions in the protein-protein interface of the engineered domain of the first immunoglobulin chain comprise 86S and conservative amino acid substitutions thereof, and the amino acid substitutions in the protein-protein interface of the engineered domain of the second immunoglobulin chain comprise 85.1S and conservative amino acid substitutions thereof, wherein the amino acid position is indicated according to the IMGT® numbering.In some embodiments, the donor domain of the first member of the naturally occurring immunoglobulin super-family is a human IGHD CH1, and the donor domain of the second member of the naturally occurring immunoglobulin super-family is a human IGKC, wherein the amino acid substitutions in the protein-protein interface of the engineered domain of the first immunoglobulin chain comprise 86S and conservative amino acid substitutions thereof, and the amino acid substitutions in the protein-protein interface of the engineered domain of the second immunoglobulin chain comprise 22V and conservative amino acid substitutions thereof, wherein the amino acid position is indicated according to the IMGT® numbering.

In some embodiments, the donor domain of the first member of the naturally occurring immunoglobulin super-family is a human IGHE CH1, and the donor domain of the second member of the naturally occurring immunoglobulin super-family is a human IGKC, wherein the amino acid substitutions in the protein-protein interface of the engineered domain of the first immunoglobulin chain comprise 88L and conservative amino acid substitutions thereof, and the amino acid substitutions in the protein-protein interface of the engineered domain of the second immunoglobulin chain comprise 86S and conservative amino acid substitutions thereof, and/or 85.1S and conservative amino acid substitutions thereof, wherein the amino acid position is indicated according to the IMGT® numbering.

In some embodiments, the donor domain of the first member of the naturally occurring immunoglobulin super-family is a human IGHE CH1, and the donor domain of the second member of the naturally occurring immunoglobulin super-family is a human IGKC, wherein the amino acid substitutions in the protein-protein interface of the engineered domain of the first immunoglobulin chain comprise 86I and conservative amino acid substitutions thereof, and the amino acid substitutions in the protein-protein interface of the engineered domain of the second immunoglobulin chain comprise 85.1S and conservative amino acid substitutions thereof, wherein the amino acid position is indicated according to the IMGT® numbering.

In some embodiments, the donor domain of the first member of the naturally occurring immunoglobulin super-family is a human IGHE CH1, and the donor domain of the second

member of the naturally occurring immunoglobulin super-family is a human IGKC, wherein the amino acid substitutions in the protein-protein interface of the engineered domain of the first immunoglobulin chain comprise 86I and conservative amino acid substitutions thereof, and the amino acid substitutions in the protein-protein interface of the engineered domain of the second immunoglobulin chain comprise 22V and conservative amino acid substitutions thereof, wherein the amino acid position is indicated according to the IMGT® numbering.

In some embodiments, the donor domain of the first member of the naturally occurring immunoglobulin super-family is a human IGHG1 CH1 or a human IGHG2 CH1 or a human IGHG3 CH1 or a human IGHG4 CH1 or a human IGHGP CH1, and the donor domain of the second member of the naturally occurring immunoglobulin super-family is a human IGKC, wherein the amino acid substitutions in the protein-protein interface of the engineered domain of the first immunoglobulin chain comprise 88V and conservative amino acid substitutions thereof, and the amino acid substitutions in the protein-protein interface of the engineered domain of the second immunoglobulin chain comprise 86S and conservative amino acid substitutions thereof, and/or 85.1S and conservative amino acid substitutions thereof, wherein the amino acid position is indicated according to the IMGT® numbering.

In some embodiments, the donor domain of the first member of the naturally occurring immunoglobulin super-family is a human IGHG1 CH1 or a human IGHG2 CH1 or a human IGHG3 CH1 or a human IGHG4 CH1 or a human IGHGP CH1, and the donor domain of the second member of the naturally occurring immunoglobulin super-family is a human IGKC, wherein the amino acid substitutions in the protein-protein interface of the engineered domain of the first immunoglobulin chain comprise 86S and conservative amino acid substitutions thereof, and the amino acid substitutions in the protein-protein interface of the engineered domain of the second immunoglobulin chain comprise 85.1S and conservative amino acid substitutions thereof, wherein the amino acid position is indicated according to the IMGT® numbering.

In some embodiments, the donor domain of the first member of the naturally occurring immunoglobulin super-family is a human IGHG1 CH1 or a human IGHG2 CH1 or a human IGHG3 CH1 or a human IGHG4 CH1 or a human IGHGP CH1, and the donor domain of the second member of the naturally occurring immunoglobulin super-family is a human IGKC, wherein the amino acid substitutions in the protein-protein interface of the engineered domain of the first immunoglobulin chain comprise 86S and conservative amino acid substitutions thereof, and the amino acid substitutions in the protein-protein interface of the engineered

domain of the second immunoglobulin chain comprise 22V and conservative amino acid substitutions thereof, wherein the amino acid position is indicated according to the IMGT® numbering.

In some embodiments, the donor domain of the first member of the naturally occurring immunoglobulin super-family is a human IGHM CH1, and the donor domain of the second member of the naturally occurring immunoglobulin super-family is a human IGKC, wherein the amino acid substitutions in the protein-protein interface of the engineered domain of the first immunoglobulin chain comprise 88Q and conservative amino acid substitutions thereof, and the amino acid substitutions in the protein-protein interface of the engineered domain of the second immunoglobulin chain comprise 86S and conservative amino acid substitutions thereof, and/or 85.1S and conservative amino acid substitutions thereof, wherein the amino acid position is indicated according to the IMGT® numbering.

In some embodiments, the donor domain of the first member of the naturally occurring immunoglobulin super-family is a human IGHM CH1, and the donor domain of the second member of the naturally occurring immunoglobulin super-family is a human IGKC, wherein the amino acid substitutions in the protein-protein interface of the engineered domain of the first immunoglobulin chain comprise 86T and conservative amino acid substitutions thereof, and the amino acid substitutions in the protein-protein interface of the engineered domain of the second immunoglobulin chain comprise 85.1S and conservative amino acid substitutions thereof, wherein the amino acid position is indicated according to the IMGT® numbering.

In some embodiments, the donor domain of the first member of the naturally occurring immunoglobulin super-family is a human IGHM CH1, and the donor domain of the second member of the naturally occurring immunoglobulin super-family is a human IGKC, wherein the amino acid substitutions in the protein-protein interface of the engineered domain of the first immunoglobulin chain comprise 86T and conservative amino acid substitutions thereof, and the amino acid substitutions in the protein-protein interface of the engineered domain of the second immunoglobulin chain comprise 22V and conservative amino acid substitutions thereof, wherein the amino acid position is indicated according to the IMGT® numbering.

In some embodiments, the donor domain of the first member of the naturally occurring immunoglobulin super-family is a human IGHA1 CH1 or a human IGHA2 CH1, and the donor domain of the second member of the naturally occurring immunoglobulin super-family is a human IGLC1 or a human IGLC2 or a human IGLC3 or a human IGLC6 or a human IGLC7, wherein the amino acid substitutions in the protein-protein interface of the engineered

domain of the first immunoglobulin chain comprise 88Q and conservative amino acid substitutions thereof, and the amino acid substitutions in the protein-protein interface of the engineered domain of the second immunoglobulin chain comprise 86S and conservative amino acid substitutions thereof, and/or 85.1A and conservative amino acid substitutions thereof, wherein the amino acid position is indicated according to the IMGT® numbering.

In some embodiments, the donor domain of the first member of the naturally occurring immunoglobulin super-family is a human IGHA1 CH1 or a human IGHA2 CH1, and the donor domain of the second member of the naturally occurring immunoglobulin super-family is a human IGLC1 or a human IGLC2 or a human IGLC3 or a human IGLC6 or a human IGLC7, wherein the amino acid substitutions in the protein-protein interface of the engineered domain of the first immunoglobulin chain comprise 86S and conservative amino acid substitutions thereof, and the amino acid substitutions in the protein-protein interface of the engineered domain of the second immunoglobulin chain comprise 85.1A and conservative amino acid substitutions thereof, wherein the amino acid position is indicated according to the IMGT® numbering.

In some embodiments, the donor domain of the first member of the naturally occurring immunoglobulin super-family is a human IGHA1 CH1 or a human IGHA2 CH1, and the donor domain of the second member of the naturally occurring immunoglobulin super-family is a human IGLC1 or a human IGLC2 or a human IGLC3 or a human IGLC6 or a human IGLC7, wherein the amino acid substitutions in the protein-protein interface of the engineered domain of the first immunoglobulin chain comprise 86S and conservative amino acid substitutions thereof, and the amino acid substitutions in the protein-protein interface of the engineered domain of the second immunoglobulin chain comprise 22V and conservative amino acid substitutions thereof, wherein the amino acid position is indicated according to the IMGT® numbering.

In some embodiments, the donor domain of the first member of the naturally occurring immunoglobulin super-family is a human IGHD CH1, and the donor domain of the second member of the naturally occurring immunoglobulin super-family is a human IGLC1 or a human IGLC2 or a human IGLC3 or a human IGLC6 or a human IGLC7, wherein the amino acid substitutions in the protein-protein interface of the engineered domain of the first immunoglobulin chain comprise 88Q and conservative amino acid substitutions thereof, and the amino acid substitutions in the protein-protein interface of the engineered domain of the second immunoglobulin chain comprise 86S and conservative amino acid substitutions

thereof, and/or 85.1A and conservative amino acid substitutions thereof, wherein the amino acid position is indicated according to the IMGT® numbering.

In some embodiments, the donor domain of the first member of the naturally occurring immunoglobulin super-family is a human IGHD CH1, and the donor domain of the second member of the naturally occurring immunoglobulin super-family is a human IGLC1 or a human IGLC2 or a human IGLC3 or a human IGLC6 or a human IGLC7, wherein the amino acid substitutions in the protein-protein interface of the engineered domain of the first immunoglobulin chain comprise 86S and conservative amino acid substitutions thereof, and the amino acid substitutions in the protein-protein interface of the engineered domain of the second immunoglobulin chain comprise 85.1A and conservative amino acid substitutions thereof, wherein the amino acid position is indicated according to the IMGT® numbering.

In some embodiments, the donor domain of the first member of the naturally occurring immunoglobulin super-family is a human IGHD CH1, and the donor domain of the second member of the naturally occurring immunoglobulin super-family is a human IGLC1 or a human IGLC2 or a human IGLC3 or a human IGLC6 or a human IGLC7, wherein the amino acid substitutions in the protein-protein interface of the engineered domain of the first immunoglobulin chain comprise 86S and conservative amino acid substitutions thereof, and the amino acid substitutions in the protein-protein interface of the engineered domain of the second immunoglobulin chain comprise 22V and conservative amino acid substitutions thereof, wherein the amino acid position is indicated according to the IMGT® numbering.

In some embodiments, the donor domain of the first member of the naturally occurring immunoglobulin super-family is a human IGHE CH1, and the donor domain of the second member of the naturally occurring immunoglobulin super-family is a human IGLC1 or a human IGLC2 or a human IGLC3 or a human IGLC6 or a human IGLC7, wherein the amino acid substitutions in the protein-protein interface of the engineered domain of the first immunoglobulin chain comprise 88L and conservative amino acid substitutions thereof, and the amino acid substitutions in the protein-protein interface of the engineered domain of the second immunoglobulin chain comprise 86S and conservative amino acid substitutions thereof, and/or 85.1A and conservative amino acid substitutions thereof, wherein the amino acid position is indicated according to the IMGT® numbering.

In some embodiments, the donor domain of the first member of the naturally occurring immunoglobulin super-family is a human IGHE CH1, and the donor domain of the second member of the naturally occurring immunoglobulin super-family is a human IGLC1 or a

human IGLC2 or a human IGLC3 or a human IGLC6 or a human IGLC7, wherein the amino acid substitutions in the protein-protein interface of the engineered domain of the first immunoglobulin chain comprise 86I and conservative amino acid substitutions thereof, and the amino acid substitutions in the protein-protein interface of the engineered domain of the second immunoglobulin chain comprise 85.1A and conservative amino acid substitutions thereof, wherein the amino acid position is indicated according to the IMGT® numbering.In some embodiments, the donor domain of the first member of the naturally occurring immunoglobulin super-family is a human IGHE CH1, and the donor domain of the second member of the naturally occurring immunoglobulin super-family is a human IGLC1 or a human IGLC2 or a human IGLC3 or a human IGLC6 or a human IGLC7, wherein the amino acid substitutions in the protein-protein interface of the engineered domain of the first immunoglobulin chain comprise 86I and conservative amino acid substitutions thereof, and the amino acid substitutions in the protein-protein interface of the engineered domain of the second immunoglobulin chain comprise 22V and conservative amino acid substitutions thereof, wherein the amino acid position is indicated according to the IMGT® numbering.

In some embodiments, the donor domain of the first member of the naturally occurring immunoglobulin super-family is a human IGHG1 CH1 or a human IGHG2 CH1 or a human IGHG3 CH1 or a human IGHG4 CH1 or a human IGHGP CH1, and the donor domain of the second member of the naturally occurring immunoglobulin super-family is a human IGLC1 or a human IGLC2 or a human IGLC3 or a human IGLC6 or a human IGLC7, wherein the amino acid substitutions in the protein-protein interface of the engineered domain of the first immunoglobulin chain comprise 88V and conservative amino acid substitutions thereof, and the amino acid substitutions in the protein-protein interface of the engineered domain of the second immunoglobulin chain comprise 86S and conservative amino acid substitutions thereof, and/or 85.1A and conservative amino acid substitutions thereof, wherein the amino acid position is indicated according to the IMGT® numbering.

In some embodiments, the donor domain of the first member of the naturally occurring immunoglobulin super-family is a human IGHG1 CH1 or a human IGHG2 CH1 or a human IGHG3 CH1 or a human IGHG4 CH1 or a human IGHGP CH1, and the donor domain of the second member of the naturally occurring immunoglobulin super-family is a human IGLC1 or a human IGLC2 or a human IGLC3 or a human IGLC6 or a human IGLC7, wherein the amino acid substitutions in the protein-protein interface of the engineered domain of the first immunoglobulin chain comprise 86S and conservative amino acid substitutions thereof, and

the amino acid substitutions in the protein-protein interface of the engineered domain of the second immunoglobulin chain comprise 85.1A and conservative amino acid substitutions thereof, wherein the amino acid position is indicated according to the IMGT® numbering.In some embodiments, the donor domain of the first member of the naturally occurring immunoglobulin super-family is a human IGHG1 CH1 or a human IGHG2 CH1 or a human IGHG3 CH1 or a human IGHG4 CH1 or a human IGHGP CH1, and the donor domain of the second member of the naturally occurring immunoglobulin super-family is a human IGLC1 or a human IGLC2 or a human IGLC3 or a human IGLC6 or a human IGLC7, wherein the amino acid substitutions in the protein-protein interface of the engineered domain of the first immunoglobulin chain comprise 86S and conservative amino acid substitutions thereof, and the amino acid substitutions in the protein-protein interface of the engineered domain of the second immunoglobulin chain comprise 22V and conservative amino acid substitutions thereof, wherein the amino acid position is indicated according to the IMGT® numbering.

In some embodiments, the donor domain of the first member of the naturally occurring immunoglobulin super-family is a human IGHM CH1, and the donor domain of the second member of the naturally occurring immunoglobulin super-family is a human IGLC1 or a human IGLC2 or a human IGLC3 or a human IGLC6 or a human IGLC7, wherein the amino acid substitutions in the protein-protein interface of the engineered domain of the first immunoglobulin chain comprise 88Q and conservative amino acid substitutions thereof, and the amino acid substitutions in the protein-protein interface of the engineered domain of the second immunoglobulin chain comprise 86S and conservative amino acid substitutions thereof, and/or 85.1A and conservative amino acid substitutions thereof, wherein the amino acid position is indicated according to the IMGT® numbering.In some embodiments, the donor domain of the first member of the naturally occurring immunoglobulin super-family is a human IGHM CH1, and the donor domain of the second member of the naturally occurring immunoglobulin super-family is a human IGLC1 or a human IGLC2 or a human IGLC3 or a human IGLC6 or a human IGLC7, wherein the amino acid substitutions in the protein-protein interface of the engineered domain of the first immunoglobulin chain comprise 86T and conservative amino acid substitutions thereof, and the amino acid substitutions in the protein-protein interface of the engineered domain of the second immunoglobulin chain comprise 85.1A and conservative amino acid substitutions thereof, wherein the amino acid position is indicated according to the IMGT® numbering.

In some embodiments, the donor domain of the first member of the naturally occurring immunoglobulin super-family is a human IGHM CH1, and the donor domain of the second member of the naturally occurring immunoglobulin super-family is a human IGLC1 or a human IGLC2 or a human IGLC3 or a human IGLC6 or a human IGLC7, wherein the amino acid substitutions in the protein-protein interface of the engineered domain of the first immunoglobulin chain comprise 86T and conservative amino acid substitutions thereof, and the amino acid substitutions in the protein-protein interface of the engineered domain of the second immunoglobulin chain comprise 22V and conservative amino acid substitutions thereof, wherein the amino acid position is indicated according to the IMGT® numbering.

In some embodiments, the donor domain of the first member of the naturally occurring immunoglobulin super-family is a human IGKC, and the donor domain of the second member of the naturally occurring immunoglobulin super-family is a human IGHA1 CH1 or a human IGHA2 CH1, wherein the amino acid substitutions in the protein-protein interface of the engineered domain of the first immunoglobulin chain comprise 88T and conservative amino acid substitutions thereof, and the amino acid substitutions in the protein-protein interface of the engineered domain of the second immunoglobulin chain comprise 86S and conservative amino acid substitutions thereof, and/or 85.1T and conservative amino acid substitutions thereof, wherein the amino acid position is indicated according to the IMGT® numbering.

In some embodiments, the donor domain of the first member of the naturally occurring immunoglobulin super-family is a human IGKC, and the donor domain of the second member of the naturally occurring immunoglobulin super-family is a human IGHA1 CH1 or a human IGHA2 CH1, wherein the amino acid substitutions in the protein-protein interface of the engineered domain of the first immunoglobulin chain comprise 86S and conservative amino acid substitutions thereof, and the amino acid substitutions in the protein-protein interface of the engineered domain of the second immunoglobulin chain comprise 85.1T and conservative amino acid substitutions thereof, wherein the amino acid position is indicated according to the IMGT® numbering.

In some embodiments, the donor domain of the first member of the naturally occurring immunoglobulin super-family is a human IGKC, and the donor domain of the second member of the naturally occurring immunoglobulin super-family is a human IGHA1 CH1 or a human IGHA2 CH1, wherein the amino acid substitutions in the protein-protein interface of the engineered domain of the first immunoglobulin chain comprise 86S and conservative amino acid substitutions thereof, and the amino acid substitutions in the protein-protein interface of

the engineered domain of the second immunoglobulin chain comprise 22A and conservative amino acid substitutions thereof, wherein the amino acid position is indicated according to the IMGT® numbering.

In some embodiments, the donor domain of the first member of the naturally occurring immunoglobulin super-family is a human IGKC, and the donor domain of the second member of the naturally occurring immunoglobulin super-family is a human IGHD CH1, wherein the amino acid substitutions in the protein-protein interface of the engineered domain of the first immunoglobulin chain comprise 88T and conservative amino acid substitutions thereof, and the amino acid substitutions in the protein-protein interface of the engineered domain of the second immunoglobulin chain comprise 86S and conservative amino acid substitutions thereof, and/or 85.1M and conservative amino acid substitutions thereof, wherein the amino acid position is indicated according to the IMGT® numbering.

In some embodiments, the donor domain of the first member of the naturally occurring immunoglobulin super-family is a human IGKC, and the donor domain of the second member of the naturally occurring immunoglobulin super-family is a human IGHD CH1, wherein the amino acid substitutions in the protein-protein interface of the engineered domain of the first immunoglobulin chain comprise 86S and conservative amino acid substitutions thereof, and the amino acid substitutions in the protein-protein interface of the engineered domain of the second immunoglobulin chain comprise 85.1M and conservative amino acid substitutions thereof, wherein the amino acid position is indicated according to the IMGT® numbering.

In some embodiments, the donor domain of the first member of the naturally occurring immunoglobulin super-family is a human IGKC, and the donor domain of the second member of the naturally occurring immunoglobulin super-family is a human IGHD CH1, wherein the amino acid substitutions in the protein-protein interface of the engineered domain of the first immunoglobulin chain comprise 86S and conservative amino acid substitutions thereof, and the amino acid substitutions in the protein-protein interface of the engineered domain of the second immunoglobulin chain comprise 22A and conservative amino acid substitutions thereof, wherein the amino acid position is indicated according to the IMGT® numbering.

In some embodiments, the donor domain of the first member of the naturally occurring immunoglobulin super-family is a human IGKC, and the donor domain of the second member of the naturally occurring immunoglobulin super-family is a human IGHE CH1, wherein the amino acid substitutions in the protein-protein interface of the engineered domain of the first immunoglobulin chain comprise 88T and conservative amino acid substitutions thereof, and

the amino acid substitutions in the protein-protein interface of the engineered domain of the second immunoglobulin chain comprise 86I and conservative amino acid substitutions thereof, and/or 85.1A and conservative amino acid substitutions thereof, wherein the amino acid position is indicated according to the IMGT® numbering.

In some embodiments, the donor domain of the first member of the naturally occurring immunoglobulin super-family is a human IGKC, and the donor domain of the second member of the naturally occurring immunoglobulin super-family is a human IGHE CH1, wherein the amino acid substitutions in the protein-protein interface of the engineered domain of the first immunoglobulin chain comprise 86S and conservative amino acid substitutions thereof, and the amino acid substitutions in the protein-protein interface of the engineered domain of the second immunoglobulin chain comprise 85.1A and conservative amino acid substitutions thereof, wherein the amino acid position is indicated according to the IMGT® numbering.

In some embodiments, the donor domain of the first member of the naturally occurring immunoglobulin super-family is a human IGKC, and the donor domain of the second member of the naturally occurring immunoglobulin super-family is a human IGHE CH1, wherein the amino acid substitutions in the protein-protein interface of the engineered domain of the first immunoglobulin chain comprise 86S and conservative amino acid substitutions thereof, and the amino acid substitutions in the protein-protein interface of the engineered domain of the second immunoglobulin chain comprise 22G and conservative amino acid substitutions thereof, wherein the amino acid position is indicated according to the IMGT® numbering.

In some embodiments, the donor domain of the first member of the naturally occurring immunoglobulin super-family is a human IGKC, and the donor domain of the second member of the naturally occurring immunoglobulin super-family is a human IGHG1 CH1 or a human IGHG2 CH1 or a human IGHG3 CH1 or a human IGHG4 CH1 or a human IGHGP CH1, wherein the amino acid substitutions in the protein-protein interface of the engineered domain of the first immunoglobulin chain comprise 88T and conservative amino acid substitutions thereof, and the amino acid substitutions in the protein-protein interface of the engineered domain of the second immunoglobulin chain comprise 86S and conservative amino acid substitutions thereof, and/or 85.1S and conservative amino acid substitutions thereof, wherein the amino acid position is indicated according to the IMGT® numbering.

In some embodiments, the donor domain of the first member of the naturally occurring immunoglobulin super-family is a human IGKC, and the donor domain of the second member of the naturally occurring immunoglobulin super-family is a human IGHG1 CH1 or a human

IGHG2 CH1 or a human IGHG3 CH1 or a human IGHG4 CH1 or a human IGHGP CH1, wherein the amino acid substitutions in the protein-protein interface of the engineered domain of the first immunoglobulin chain comprise 86S and conservative amino acid substitutions thereof, and the amino acid substitutions in the protein-protein interface of the engineered domain of the second immunoglobulin chain comprise 85.1S and conservative amino acid substitutions thereof, wherein the amino acid position is indicated according to the IMGT® numbering.

In some embodiments, the donor domain of the first member of the naturally occurring immunoglobulin super-family is a human IGKC, and the donor domain of the second member of the naturally occurring immunoglobulin super-family is a human IGHG1 CH1 or a human IGHG2 CH1 or a human IGHG3 CH1 or a human IGHG4 CH1 or a human IGHGP CH1, wherein the amino acid substitutions in the protein-protein interface of the engineered domain of the first immunoglobulin chain comprise 86S and conservative amino acid substitutions thereof, and the amino acid substitutions in the protein-protein interface of the engineered domain of the second immunoglobulin chain comprise 22G and conservative amino acid substitutions thereof, wherein the amino acid position is indicated according to the IMGT® numbering.

In some embodiments, the donor domain of the first member of the naturally occurring immunoglobulin super-family is a human IGKC, and the donor domain of the second member of the naturally occurring immunoglobulin super-family is a human IGHM CH1, wherein the amino acid substitutions in the protein-protein interface of the engineered domain of the first immunoglobulin chain comprise 88T and conservative amino acid substitutions thereof, and the amino acid substitutions in the protein-protein interface of the engineered domain of the second immunoglobulin chain comprise 86T and conservative amino acid substitutions thereof, and/or 85.1A and conservative amino acid substitutions thereof, wherein the amino acid position is indicated according to the IMGT® numbering.

In some embodiments, the donor domain of the first member of the naturally occurring immunoglobulin super-family is a human IGKC, and the donor domain of the second member of the naturally occurring immunoglobulin super-family is a human IGHM CH1, wherein the amino acid substitutions in the protein-protein interface of the engineered domain of the first immunoglobulin chain comprise 86S and conservative amino acid substitutions thereof, and the amino acid substitutions in the protein-protein interface of the engineered domain of the

second immunoglobulin chain comprise 85.1A and conservative amino acid substitutions thereof, wherein the amino acid position is indicated according to the IMGT® numbering.

In some embodiments, the donor domain of the first member of the naturally occurring immunoglobulin super-family is a human IGKC, and the donor domain of the second member of the naturally occurring immunoglobulin super-family is a human IGHM CH1, wherein the amino acid substitutions in the protein-protein interface of the engineered domain of the first immunoglobulin chain comprise 86S and conservative amino acid substitutions thereof, and the amino acid substitutions in the protein-protein interface of the engineered domain of the second immunoglobulin chain comprise 22G and conservative amino acid substitutions thereof, wherein the amino acid position is indicated according to the IMGT® numbering.

In some embodiments, the donor domain of the first member of the naturally occurring immunoglobulin super-family is a human IGLC1 or a human IGLC2 or a human IGLC3 or a human IGLC6 or a human IGLC7, and the donor domain of the second member of the naturally occurring immunoglobulin super-family is a human IGHA1 CH1 or a human IGHA2 CH1, wherein the amino acid substitutions in the protein-protein interface of the engineered domain of the first immunoglobulin chain comprise 88Y and conservative amino acid substitutions thereof, and the amino acid substitutions in the protein-protein interface of the engineered domain of the second immunoglobulin chain comprise 86S and conservative amino acid substitutions thereof, and/or 85.1T and conservative amino acid substitutions thereof, wherein the amino acid position is indicated according to the IMGT® numbering.

In some embodiments, the donor domain of the first member of the naturally occurring immunoglobulin super-family is a human IGLC1 or a human IGLC2 or a human IGLC3 or a human IGLC6 or a human IGLC7, and the donor domain of the second member of the naturally occurring immunoglobulin super-family is a human IGHA1 CH1 or a human IGHA2 CH1, wherein the amino acid substitutions in the protein-protein interface of the engineered domain of the first immunoglobulin chain comprise 86S and conservative amino acid substitutions thereof, and the amino acid substitutions in the protein-protein interface of the engineered domain of the second immunoglobulin chain comprise 85.1T and conservative amino acid substitutions thereof, wherein the amino acid position is indicated according to the IMGT® numbering.

In some embodiments, the donor domain of the first member of the naturally occurring immunoglobulin super-family is a human IGLC1 or a human IGLC2 or a human IGLC3 or a human IGLC6 or a human IGLC7, and the donor domain of the second member of the

naturally occurring immunoglobulin super-family is a human IGHA1 CH1 or a human IGHA2 CH1, wherein the amino acid substitutions in the protein-protein interface of the engineered domain of the first immunoglobulin chain comprise 86S and conservative amino acid substitutions thereof, and the amino acid substitutions in the protein-protein interface of the engineered domain of the second immunoglobulin chain comprise 22A and conservative amino acid substitutions thereof, wherein the amino acid position is indicated according to the IMGT® numbering.

In some embodiments, the donor domain of the first member of the naturally occurring immunoglobulin super-family is a human IGLC1 or a human IGLC2 or a human IGLC3 or a human IGLC6 or a human IGLC7, and the donor domain of the second member of the naturally occurring immunoglobulin super-family is a human IGHD CH1, wherein the amino acid substitutions in the protein-protein interface of the engineered domain of the first immunoglobulin chain comprise 88Y and conservative amino acid substitutions thereof, and the amino acid substitutions in the protein-protein interface of the engineered domain of the second immunoglobulin chain comprise 86S and conservative amino acid substitutions thereof, and/or 85.1M and conservative amino acid substitutions thereof, wherein the amino acid position is indicated according to the IMGT® numbering.

In some embodiments, the donor domain of the first member of the naturally occurring immunoglobulin super-family is a human IGLC1 or a human IGLC2 or a human IGLC3 or a human IGLC6 or a human IGLC7, and the donor domain of the second member of the naturally occurring immunoglobulin super-family is a human IGHD CH1, wherein the amino acid substitutions in the protein-protein interface of the engineered domain of the first immunoglobulin chain comprise 86S and conservative amino acid substitutions thereof, and the amino acid substitutions in the protein-protein interface of the engineered domain of the second immunoglobulin chain comprise 85.1M and conservative amino acid substitutions thereof, wherein the amino acid position is indicated according to the IMGT® numbering.In some embodiments, the donor domain of the first member of the naturally occurring immunoglobulin super-family is a human IGLC1 or a human IGLC2 or a human IGLC3 or a human IGLC6 or a human IGLC7, and the donor domain of the second member of the naturally occurring immunoglobulin super-family is a human IGHD CH1, wherein the amino acid substitutions in the protein-protein interface of the engineered domain of the first immunoglobulin chain comprise 86S and conservative amino acid substitutions thereof, and the amino acid substitutions in the protein-protein interface of the engineered domain of the

second immunoglobulin chain comprise 22A and conservative amino acid substitutions thereof, wherein the amino acid position is indicated according to the IMGT® numbering.

In some embodiments, the donor domain of the first member of the naturally occurring immunoglobulin super-family is a human IGLC1 or a human IGLC2 or a human IGLC3 or a human IGLC6 or a human IGLC7, and the donor domain of the second member of the naturally occurring immunoglobulin super-family is a human IGHE CH1, wherein the amino acid substitutions in the protein-protein interface of the engineered domain of the first immunoglobulin chain comprise 88Y and conservative amino acid substitutions thereof, and the amino acid substitutions in the protein-protein interface of the engineered domain of the second immunoglobulin chain comprise 86I and conservative amino acid substitutions thereof, and/or 85.1A and conservative amino acid substitutions thereof, wherein the amino acid position is indicated according to the IMGT® numbering.

In some embodiments, the donor domain of the first member of the naturally occurring immunoglobulin super-family is a human IGLC1 or a human IGLC2 or a human IGLC3 or a human IGLC6 or a human IGLC7, and the donor domain of the second member of the naturally occurring immunoglobulin super-family is a human IGHE CH1, wherein the amino acid substitutions in the protein-protein interface of the engineered domain of the first immunoglobulin chain comprise 86S and conservative amino acid substitutions thereof, and the amino acid substitutions in the protein-protein interface of the engineered domain of the second immunoglobulin chain comprise 85.1A and conservative amino acid substitutions thereof, wherein the amino acid position is indicated according to the IMGT® numbering.

In some embodiments, the donor domain of the first member of the naturally occurring immunoglobulin super-family is a human IGLC1 or a human IGLC2 or a human IGLC3 or a human IGLC6 or a human IGLC7, and the donor domain of the second member of the naturally occurring immunoglobulin super-family is a human IGHE CH1, wherein the amino acid substitutions in the protein-protein interface of the engineered domain of the first immunoglobulin chain comprise 86S and conservative amino acid substitutions thereof, and the amino acid substitutions in the protein-protein interface of the engineered domain of the second immunoglobulin chain comprise 22G and conservative amino acid substitutions thereof, wherein the amino acid position is indicated according to the IMGT® numbering.

In some embodiments, the donor domain of the first member of the naturally occurring immunoglobulin super-family is a human IGLC1 or a human IGLC2 or a human IGLC3 or a human IGLC6 or a human IGLC7, and the donor domain of the second member of the

naturally occurring immunoglobulin super-family is a human IGHG1 CH1 or a human IGHG2 CH1 or a human IGHG3 CH1 or a human IGHG4 CH1 or a human IGHGP CH1, wherein the amino acid substitutions in the protein-protein interface of the engineered domain of the first immunoglobulin chain comprise 88Y and conservative amino acid substitutions thereof, and the amino acid substitutions in the protein-protein interface of the engineered domain of the second immunoglobulin chain comprise 86S and conservative amino acid substitutions thereof, and/or 85.1S and conservative amino acid substitutions thereof, wherein the amino acid position is indicated according to the IMGT® numbering.

In some embodiments, the donor domain of the first member of the naturally occurring immunoglobulin super-family is a human IGLC1 or a human IGLC2 or a human IGLC3 or a human IGLC6 or a human IGLC7, and the donor domain of the second member of the naturally occurring immunoglobulin super-family is a human IGHG1 CH1 or a human IGHG2 CH1 or a human IGHG3 CH1 or a human IGHG4 CH1 or a human IGHGP CH1, wherein the amino acid substitutions in the protein-protein interface of the engineered domain of the first immunoglobulin chain comprise 86S and conservative amino acid substitutions thereof, and the amino acid substitutions in the protein-protein interface of the engineered domain of the second immunoglobulin chain comprise 85.1S and conservative amino acid substitutions thereof, wherein the amino acid position is indicated according to the IMGT® numbering.

In some embodiments, the donor domain of the first member of the naturally occurring immunoglobulin super-family is a human IGLC1 or a human IGLC2 or a human IGLC3 or a human IGLC6 or a human IGLC7, and the donor domain of the second member of the naturally occurring immunoglobulin super-family is a human IGHG1 CH1 or a human IGHG2 CH1 or a human IGHG3 CH1 or a human IGHG4 CH1 or a human IGHGP CH1, wherein the amino acid substitutions in the protein-protein interface of the engineered domain of the first immunoglobulin chain comprise 86S and conservative amino acid substitutions thereof, and the amino acid substitutions in the protein-protein interface of the engineered domain of the second immunoglobulin chain comprise 22G and conservative amino acid substitutions thereof, wherein the amino acid position is indicated according to the IMGT® numbering.

In some embodiments, the donor domain of the first member of the naturally occurring immunoglobulin super-family is a human IGLC1 or a human IGLC2 or a human IGLC3 or a human IGLC6 or a human IGLC7, and the donor domain of the second member of the

naturally occurring immunoglobulin super-family is a human IGHM CH1, wherein the amino acid substitutions in the protein-protein interface of the engineered domain of the first immunoglobulin chain comprise 88Y and conservative amino acid substitutions thereof, and the amino acid substitutions in the protein-protein interface of the engineered domain of the second immunoglobulin chain comprise 86T and conservative amino acid substitutions thereof, and/or 85.1A and conservative amino acid substitutions thereof, wherein the amino acid position is indicated according to the IMGT® numbering.

In some embodiments, the donor domain of the first member of the naturally occurring immunoglobulin super-family is a human IGLC1 or a human IGLC2 or a human IGLC3 or a human IGLC6 or a human IGLC7, and the donor domain of the second member of the naturally occurring immunoglobulin super-family is a human IGHM CH1, wherein the amino acid substitutions in the protein-protein interface of the engineered domain of the first immunoglobulin chain comprise 86S and conservative amino acid substitutions thereof, and the amino acid substitutions in the protein-protein interface of the engineered domain of the second immunoglobulin chain comprise 85.1A and conservative amino acid substitutions thereof, wherein the amino acid position is indicated according to the IMGT® numbering.

In some embodiments, the donor domain of the first member of the naturally occurring immunoglobulin super-family is a human IGLC1 or a human IGLC2 or a human IGLC3 or a human IGLC6 or a human IGLC7, and the donor domain of the second member of the naturally occurring immunoglobulin super-family is a human IGHM CH1, wherein the amino acid substitutions in the protein-protein interface of the engineered domain of the first immunoglobulin chain comprise 86S and conservative amino acid substitutions thereof, and the amino acid substitutions in the protein-protein interface of the engineered domain of the second immunoglobulin chain comprise 22G and conservative amino acid substitutions thereof, wherein the amino acid position is indicated according to the IMGT® numbering.

In some embodiments, the donor domain of the first member of the naturally occurring immunoglobulin super-family is a human TRAC, and the donor domain of the second member of the naturally occurring immunoglobulin super-family is a human TRBC1 or a human TRBC2, wherein the amino acid substitutions in the protein-protein interface of the engineered domain of the first immunoglobulin chain comprise 88W and conservative amino acid substitutions thereof, and the amino acid substitutions in the protein-protein interface of the engineered domain of the second immunoglobulin chain comprise 86S and conservative amino acid substitutions thereof, and/or 85.1C or 85.1A or 85.1C and conservative amino acid

substitutions thereof, wherein the amino acid position is indicated according to the IMGT® numbering.

In some embodiments, the donor domain of the first member of the naturally occurring immunoglobulin super-family is a human TRAC, and the donor domain of the second member of the naturally occurring immunoglobulin super-family is a human TRBC1 or a human TRBC2, wherein the amino acid substitutions in the protein-protein interface of the engineered domain of the first immunoglobulin chain comprise 86V and conservative amino acid substitutions thereof, and the amino acid substitutions in the protein-protein interface of the engineered domain of the second immunoglobulin chain comprise 85.1C or 85.1A or 85.1C and conservative amino acid substitutions thereof, wherein the amino acid position is indicated according to the IMGT® numbering.

In some embodiments, the donor domain of the first member of the naturally occurring immunoglobulin super-family is a human TRAC, and the donor domain of the second member of the naturally occurring immunoglobulin super-family is a human TRBC1 or a human TRBC2, wherein the amino acid substitutions in the protein-protein interface of the engineered domain of the first immunoglobulin chain comprise 86V and conservative amino acid substitutions thereof, and the amino acid substitutions in the protein-protein interface of the engineered domain of the second immunoglobulin chain comprise 22V and conservative amino acid substitutions thereof, wherein the amino acid position is indicated according to the IMGT® numbering.

In some embodiments, the donor domain of the first member of the naturally occurring immunoglobulin super-family is a human TRBC1 or a human TRBC2, and the donor domain of the second member of the naturally occurring immunoglobulin super-family is a human TRAC, wherein the amino acid substitutions in the protein-protein interface of the engineered domain of the first immunoglobulin chain comprise 88R and conservative amino acid substitutions thereof, and the amino acid substitutions in the protein-protein interface of the engineered domain of the second immunoglobulin chain comprise 86V and conservative amino acid substitutions thereof, and/or 85.1S and conservative amino acid substitutions thereof, wherein the amino acid position is indicated according to the IMGT® numbering.

In some embodiments, the donor domain of the first member of the naturally occurring immunoglobulin super-family is a human TRBC1 or a human TRBC2, and the donor domain of the second member of the naturally occurring immunoglobulin super-family is a human TRAC, wherein the amino acid substitutions in the protein-protein interface of the engineered

domain of the first immunoglobulin chain comprise 86S and conservative amino acid substitutions thereof, and the amino acid substitutions in the protein-protein interface of the engineered domain of the second immunoglobulin chain comprise 85.1S and conservative amino acid substitutions thereof, wherein the amino acid position is indicated according to the IMGT® numbering.

In some embodiments, the donor domain of the first member of the naturally occurring immunoglobulin super-family is a human TRBC1 or a human TRBC2, and the donor domain of the second member of the naturally occurring immunoglobulin super-family is a human TRAC, wherein the amino acid substitutions in the protein-protein interface of the engineered domain of the first immunoglobulin chain comprise 86S and conservative amino acid substitutions thereof, and the amino acid substitutions in the protein-protein interface of the engineered domain of the second immunoglobulin chain comprise 22V and conservative amino acid substitutions thereof, wherein the amino acid position is indicated according to the IMGT® numbering.In some embodiments, the donor domain of the first member of the naturally occurring immunoglobulin super-family is a human TRDC, and the donor domain of the second member of the naturally occurring immunoglobulin super-family is a human TRGC1 or a human TRGC2 (2x) or a human TRGC2 (3x), wherein the amino acid substitutions in the protein-protein interface of the engineered domain of the first immunoglobulin chain comprise 88L and conservative amino acid substitutions thereof, and the amino acid substitutions in the protein-protein interface of the engineered domain of the second immunoglobulin chain comprise 86F and conservative amino acid substitutions thereof, and/or 85.1M and conservative amino acid substitutions thereof, wherein the amino acid position is indicated according to the IMGT® numbering.

In some embodiments, the donor domain of the first member of the naturally occurring immunoglobulin super-family is a human TRDC, and the donor domain of the second member of the naturally occurring immunoglobulin super-family is a human TRGC1 or a human TRGC2 (2x) or a human TRGC2 (3x), wherein the amino acid substitutions in the protein-protein interface of the engineered domain of the first immunoglobulin chain comprise 86V and conservative amino acid substitutions thereof, and the amino acid substitutions in the protein-protein interface of the engineered domain of the second immunoglobulin chain comprise 85.1M and conservative amino acid substitutions thereof, wherein the amino acid position is indicated according to the IMGT® numbering.In some embodiments, the donor domain of the first member of the naturally occurring

immunoglobulin super-family is a human TRDC, and the donor domain of the second member of the naturally occurring immunoglobulin super-family is a human TRGC1 or a human TRGC2 (2x) or a human TRGC2 (3x), wherein the amino acid substitutions in the protein-protein interface of the engineered domain of the first immunoglobulin chain comprise 86V and conservative amino acid substitutions thereof, and the amino acid substitutions in the protein-protein interface of the engineered domain of the second immunoglobulin chain comprise 22L and conservative amino acid substitutions thereof, wherein the amino acid position is indicated according to the IMGT® numbering.

In some embodiments, the donor domain of the first member of the naturally occurring immunoglobulin super-family is a human TRGC1 or a human TRGC2 (2x) or a human TRGC2 (3x), and the donor domain of the second member of the naturally occurring immunoglobulin super-family is a human TRDC, wherein the amino acid substitutions in the protein-protein interface of the engineered domain of the first immunoglobulin chain comprise 88W and conservative amino acid substitutions thereof, and the amino acid substitutions in the protein-protein interface of the engineered domain of the second immunoglobulin chain comprise 86V and conservative amino acid substitutions thereof, and/or 85.1N and conservative amino acid substitutions thereof, wherein the amino acid position is indicated according to the IMGT® numbering.In some embodiments, the donor domain of the first member of the naturally occurring immunoglobulin super-family is a human TRGC1 or a human TRGC2 (2x) or a human TRGC2 (3x), and the donor domain of the second member of the naturally occurring immunoglobulin super-family is a human TRDC, wherein the amino acid substitutions in the protein-protein interface of the engineered domain of the first immunoglobulin chain comprise 86F and conservative amino acid substitutions thereof, and the amino acid substitutions in the protein-protein interface of the engineered domain of the second immunoglobulin chain comprise 85.1N and conservative amino acid substitutions thereof, wherein the amino acid position is indicated according to the IMGT® numbering.

In some embodiments, the donor domain of the first member of the naturally occurring immunoglobulin super-family is a human TRGC1 or a human TRGC2 (2x) or a human TRGC2 (3x), and the donor domain of the second member of the naturally occurring immunoglobulin super-family is a human TRDC, wherein the amino acid substitutions in the protein-protein interface of the engineered domain of the first immunoglobulin chain comprise 86F and conservative amino acid substitutions thereof, and the amino acid

substitutions in the protein-protein interface of the engineered domain of the second immunoglobulin chain comprise 22A and conservative amino acid substitutions thereof, wherein the amino acid position is indicated according to the IMGT® numbering.

In some embodiments, wherein the protein-protein interface of the parent domain of the first engineered immunoglobulin chain comprises an amino acid residue substitution at a position selected from the group consisting of 20, 22, 85.1, 86, and 88, and/or the protein-protein interface of the parent domain of the second engineered immunoglobulin chain comprises an amino acid residue substitution at a position selected from the group consisting of 22, 26, 85.1, 86, and 88, the protein-protein interface of the parent domain of the first engineered immunoglobulin chain comprises an amino acid residue substitution selected from the group consisting of 20V, 20T, 20A, 20N, 20Q, 20E, 20K, 20W, 20S, 22A, 22G, 22T, 22L, 22I, 22V, 85.1T, 85.1M, 85.1A, 85.1S, 85.1R, 85.1H, 85.1K, 85.1W, 85.1F, 85.1C, 85.1N, 86S, 86I, 86T, 86H, 86Q, 86V, 86W, 86Y, 86F, 88Q, 88L, 88V, 88R, 88E, 88T, 88I, 88K, 88Y, and 88W and/or wherein the protein-protein interface of the parent domain of the second engineered immunoglobulin chain comprises an amino acid residue substitution selected from the group consisting of 22A, 22G, 22T, 22L, 22I, 22V, 26Q, 26T, 26K, 26V, 26S, 26R, 26N, 26E, 85.1T, 85.1M, 85.1A, 85.1S, 85.1R, 85.1H, 85.1K, 85.1W, 85.1F, 85.1C, 85.1N, 86S, 86I, 86T, 86H, 86Q, 86V, 86W, 86Y, 86F, 88Q, 88L, 88V, 88R, 88E, 88I, 88T, 88K, 88Y, and 88W, wherein the amino acid position of each group member is indicated according to the IMGT® numbering.

In some embodiments, wherein the protein-protein interface of the parent domain of the first engineered immunoglobulin chain comprises an amino acid residue substitution at a position selected from the group consisting of 20, 22, 85.1, 86, and 88, and/or the protein-protein interface of the parent domain of the second engineered immunoglobulin chain comprises an amino acid residue substitution at a position selected from the group consisting of 22, 26, 85.1, 86, and 88, the protein-protein interface of the parent domain of the first engineered immunoglobulin chain comprises an amino acid residue substitution selected from the group consisting of 20K, 20N, 20T, 20S, 22A, 22L, 22V, 22T, 85.1S, 85.1A, 85.1N, 85.1M, 85.1F, 86S, 86F, 86Y, 86V, 88W, 88R, 88L, and 88K and/or conservative amino acid substitutions thereof and/or the protein-protein interface of the parent domain of the second engineered immunoglobulin chain comprises an amino acid residue substitution selected from the group consisting of 22A, 22L, 22V, 22T, 26E, 26T, 26K, 85.1S, 85.1A, 85.1N, 85.1M, 85.1F, 86S,

86F, 86Y, 86V, 88W, 88R, 88L, and 88K and/or conservative amino acid substitutions thereof, wherein the amino acid position of each group member is indicated according to the IMGT® numbering.

In some embodiments, wherein the protein-protein interface of the parent domain of the first engineered immunoglobulin chain comprises an amino acid residue substitution at a position selected from the group consisting of 20, 22, 85.1, 86, and 88, and/or the protein-protein interface of the parent domain of the second engineered immunoglobulin chain comprises an amino acid residue substitution at a position selected from the group consisting of 22, 26, 85.1, 86, and 88, the protein-protein interface of the parent domain of the first engineered immunoglobulin chain comprises an amino acid residue substitution selected from the group consisting of 20K, 20N, 20T, 20S, 22A, 22L, 22V, 22T, 85.1S, 85.1A, 85.1N, 85.1M, 85.1F, 86S, 86T, 86F, 86Y, 86V, 88W, 88R, 88L, 88I, and 88K and/or conservative amino acid substitutions thereof and/or the protein-protein interface of the parent domain of the second engineered immunoglobulin chain comprises an amino acid residue substitution selected from the group consisting of 22A, 22L, 22V, 22T, 26E, 26T, 26K, 26R, 85.1S, 85.1A, 85.1N, 85.1M, 85.1F, 86S, 86T, 86F, 86Y, 86V, 88W, 88R, 88L, 88I and 88K and/or conservative amino acid substitutions thereof, wherein the amino acid position of each group member is indicated according to the IMGT® numbering.

In some embodiments, wherein the protein-protein interface of the parent domain of the first engineered immunoglobulin chain comprises an amino acid residue substitution at a position selected from the group consisting of 20, 22, 85.1, 86, and 88, the protein-protein interface of the parent domain of the first engineered immunoglobulin chain comprises a further amino acid residue substitution at a position selected from the group consisting of 3, 5, 7, 11, 12, 13, 18, 26, 27, 79, 81, 83, 84, 84.2 and 90, wherein the amino acid position of each group member is indicated according to the IMGT® numbering.

In some embodiments, wherein the protein-protein interface of the parent domain of the first engineered immunoglobulin chain comprises an amino acid residue substitution at a position selected from the group consisting of 20, 22, 85.1, 86, and 88, and the protein-protein interface of the parent domain of the second engineered immunoglobulin chain comprises an amino acid residue substitution at a position selected from the group consisting of 22, 26, 85.1, 86, and 88, the protein-protein interface of the parent domain of the second engineered

immunoglobulin chain comprises a further amino acid residue substitution at a position selected from the group consisting of 3, 5, 7, 11, 12, 13, 18, 20, 27, 79, 81, 83, 84, 84.2 and 90, wherein the amino acid position of each group member is indicated according to the IMGT® numbering.

Preferably the protein-protein interface of the parent domain of the first engineered immunoglobulin chain comprises an amino acid residue substitution at a position selected from the group consisting of 20, 22, 85.1, 86, 88 and a further amino acid residue substitution at a position selected from the group consisting of 26, 79, and 90, and the protein-protein interface of the parent domain of the second engineered immunoglobulin chain comprises an amino acid residue substitution at a position selected from the group consisting of 22, 26, 85.1, 86, 88 and a further amino acid residue substitution at a position selected from the group consisting of 3, 5, 7, 20, 81, 84, 84.2, and 90, wherein the amino acid position of each group member is indicated according to the IMGT® numbering.

Preferably the protein-protein interface of the parent domain of the first engineered immunoglobulin chain comprises an amino acid residue substitution at a position selected from the group consisting of 20, 22, 85.1, 86, 88 and a further amino acid residue substitution at a position selected from the group consisting of 3, 5, 26, 27, 81, 83, and 84, and the protein-protein interface of the parent domain of the second engineered immunoglobulin chain comprises an amino acid residue substitution at a position selected from the group consisting of 22, 85.1, 86, 88 and a further amino acid residue substitution at a position selected from the group consisting of 7, 20, 27, 79, 81, 84.2, and 90, wherein the amino acid position of each group member is indicated according to the IMGT® numbering.

Preferably the protein-protein interface of the parent domain of the first engineered immunoglobulin chain comprises an amino acid residue substitution at a position selected from the group consisting of 20, 22, 85.1, 86, 88 and a further amino acid residue substitution at a position selected from the group consisting of 3, 5, 7, 11, 12, 13, 18, 26, 79, 81, 83, and 84.2 , and the protein-protein interface of the parent domain of the second engineered immunoglobulin chain comprises an amino acid residue substitution at a position selected from the group consisting of 22, 26, 85.1, 86, 88 and a further amino acid residue substitution at a position selected from the group consisting of 3, 5, 7, 11, 12, 13, 18, 20, 79, 81, 83, and 84.2, wherein the amino acid position of each group member is indicated according to the IMGT® numbering.

In some embodiments, wherein the protein-protein interface of the parent domain of the first engineered immunoglobulin chain comprises an amino acid residue substitution at a position selected from the group consisting of 20, 22, 85.1, 86, and 88, and/or the protein-protein interface of the parent domain of the second engineered immunoglobulin chain comprises an amino acid residue substitution at a position selected from the group consisting of 22, 26, 85.1, 86, and 88, the parent domain of the first and/or the second engineered immunoglobulin chain is a domain selected from the group consisting of CH1 domain, CH4 domain, IGKC domain, IGLC1 domain, IGLC2 domain, IGLC3 domain, IGLC6 domain, and IGLC7 domain, wherein the amino acid position of each group member is indicated according to the IMGT® numbering.

Preferably, the parent domain of the first engineered immunoglobulin chain is a CH1 domain and the parent domain of the second engineered immunoglobulin chain is selected from the group consisting of IGKC domain, IGLC1 domain, IGLC2 domain, IGLC3 domain, IGLC6 domain, and IGLC7 domain. Equally preferably the parent domain of the first and the second engineered immunoglobulin chain is a CH4 domain, in particular a CH4 domain of the same isotype, species and subclass.

Preferably, the parent domain of the first engineered immunoglobulin chain is a CH1 domain and the parent domain of the second engineered immunoglobulin chain is an IGKC domain or, the parent domain of the first engineered immunoglobulin chain is an IGKC domain and the parent domain of the second engineered immunoglobulin chain is a CH1 domain.

Preferably,the parent domain of the first engineered immunoglobulin chain is a CH1 domain and the parent domain of the second engineered immunoglobulin chain is an IGLC domain selected from the group consisting of IGLC1, IGLC2, IGLC3, IGLC6, and IGLC7 or, the parent domain of the first engineered immunoglobulin chain is an IGLC domain selected from the group consisting of IGLC1, IGLC2, IGLC3, IGLC6, and IGLC7 and the parent domain of the second engineered immunoglobulin chain is a CH1 domain.

In some embodiments, wherein the protein-protein interface of the parent domain of the first engineered immunoglobulin chain comprises an amino acid residue substitution at a position selected from the group consisting of 20, 22, 85.1, 86, and 88, and/or the protein-protein interface of the parent domain of the second engineered immunoglobulin chain comprises an amino acid residue substitution at a position selected from the group consisting of 22, 26, 85.1, 86, and 88, the parent domain of the first and/or the second engineered immunoglobulin

chain is a domain selected from the group consisting of CH1 domain, CH4 domain, IGKC domain, IGLC1 domain, IGLC2 domain, IGLC3 domain, IGLC6 domain, and IGLC7 domain, wherein the amino acid position of each group member is indicated according to the IMGT® numbering.

In some embodiments, wherein the parent domains of the first and the second engineered immunoglobulin chain are not a CH2 domain, not a CH3 domain, not a VL domain and not a VH domain and wherein,

if the parent domain of the first engineered immunoglobulin chain is an IGLC domain or an IGKC domain and the parent domain of the second engineered immunoglobulin chain is a CH1 domain and the protein-protein interface of the parent domain of the first engineered immunoglobulin chain is substituted with an amino acid residue at position 12 and the protein-protein interface of the parent domain of the second engineered immunoglobulin chain is substituted with an amino acid residue at position 119, the substituted amino acid residue at position 12 and the substituted amino acid residue at position 119 are not a charged pair,

if the parent domain of the first engineered immunoglobulin chain is a CH1 domain and the parent domain of the second engineered immunoglobulin chain is an IGLC domain and the protein-protein interface of the parent domain of the first engineered immunoglobulin chain is substituted with an amino acid residue at position 26 and the protein-protein interface of the parent domain of the second engineered immunoglobulin chain is substituted with an amino acid residue at position 13, the substituted amino acid residue at position 26 and the substituted amino acid residue at position 13 are not a charged pair,

if the parent domain of the first engineered immunoglobulin chain is an IGLC domain and the parent domain of the second engineered immunoglobulin chain is a CH1 domain and the protein-protein interface of the parent domain of the first engineered immunoglobulin chain is substituted with an amino acid residue at position 5 and the protein-protein interface of the parent domain of the second engineered immunoglobulin chain is substituted with an amino acid residue at position 20, the substituted amino acid residue at position 5 and the substituted amino acid residue at position 20 are not a charged pair,

if the parent domain of the first engineered immunoglobulin chain is a CH1 domain and the parent domain of the second engineered immunoglobulin chain is an IGLC domain and the protein-protein interface of the parent domain of the first engineered immunoglobulin chain is

substituted with an amino acid residue at position 27 and the protein-protein interface of the parent domain of the second engineered immunoglobulin chain is substituted with an amino acid residue at position 18, the substituted amino acid residue at position 27 and the substituted amino acid residue at position 18 are not a charged pair,

if the parent domain of the first engineered immunoglobulin chain is an IGLC domain or an IGKC domain and the parent domain of the second engineered immunoglobulin chain is a CH1 domain and the protein-protein interface of the parent domain of the first engineered immunoglobulin chain is substituted with an amino acid residue at position 20 and the protein-protein interface of the parent domain of the second engineered immunoglobulin chain is substituted with an amino acid residue at position 26, the substituted amino acid residue at position 20 and the substituted amino acid residue at position 26 are not a charged pair,

if the parent domain of the first engineered immunoglobulin chain is an IGLC domain and the parent domain of the second engineered immunoglobulin chain is a CH1 domain and the protein-protein interface of the parent domain of the first engineered immunoglobulin chain is substituted with an amino acid residue at position 79 and the protein-protein interface of the parent domain of the second engineered immunoglobulin chain is substituted with an amino acid residue at position 84.3, the substituted amino acid residue at position 79 and the substituted amino acid residue at position 84.3 are not a charged pair,

if the parent domain of the first engineered immunoglobulin chain is an IGLC domain or an IGKC domain and the parent domain of the second engineered immunoglobulin chain is a CH1 domain and the protein-protein interface of the parent domain of the first engineered immunoglobulin chain is substituted with an amino acid residue at position 86 and the protein-protein interface of the parent domain of the second engineered immunoglobulin chain is substituted with an amino acid residue at position 86, the substituted amino acid residues at both positions 86 are not a charged pair,

if the parent domain of the first engineered immunoglobulin chain is an IGKC domain and the amino acid residue which is substituted in the protein-protein interface of the parent domain of the first engineered immunoglobulin chain is at position 11 and the parent domain of the second engineered immunoglobulin chain is a domain which comprises a hinge region, the parent domain of the second engineered immunoglobulin chain is not substituted at position 3 of the hinge region,

the parent domain of the first and/or the second engineered immunoglobulin chain is a domain

selected from the group consisting of CH1 domain, CH4 domain, IGKC domain, IGLC1 domain, IGLC2 domain, IGLC3 domain, IGLC6 domain, and IGLC7 domain and/or the amino acid residue which is substituted in the protein-protein interface of the parent domain of the first and/or the second engineered immunoglobulin chain comprises an amino acid residue at a position selected from the group consisting of 3, 5, 7, 11, 12, 13, 18, 20, 22, 26, 27, 79, 81, 83, 84, 84.2, 85.1, 86, 88 and 90, wherein the amino acid position of each group member is indicated according to the IMGT® numbering.

Preferably the protein-protein interface of the parent domain of the first engineered immunoglobulin chain comprises an amino acid residue substitution at a position selected from the group consisting of 20, 22, 26, 79, 85.1, 86, 88 and 90 and the protein-protein interface of the parent domain of the second engineered immunoglobulin chain comprises an amino acid residue substitution at a position selected from the group consisting of 3, 5, 7, 20, 22, 26, 81, 84, 84.2, 85.1, 86, 88 and 90, wherein the amino acid position of each group member is indicated according to the IMGT® numbering.

Preferably the protein-protein interface of the parent domain of the first engineered immunoglobulin chain comprises an amino acid residue substitution at a position selected from the group consisting of 3, 5, 20, 22, 26, 27, 81, 83, 84, 85.1, 86, and 88 , and the protein-protein interface of the parent domain of the second engineered immunoglobulin chain comprises an amino acid residue substitution at a position selected from the group consisting of 7, 20, 22, 27, 79, 81, 84.2, 85.1, 86, 88, and 90, wherein the amino acid position of each group member is indicated according to the IMGT® numbering.

Preferably the protein-protein interface of the parent domain of the first engineered immunoglobulin chain comprises an amino acid residue substitution at a position selected from the group consisting of 3, 5, 7, 11, 12, 13, 18, 20, 22, 26, 79, 81, 83, 84.2, 85.1, 86, and 88 , and the protein-protein interface of the parent domain of the second engineered immunoglobulin chain comprises an amino acid residue substitution at a position selected from the group consisting of 3, 5, 7, 11, 12, 13, 18, 20, 22, 26, 79, 81, 83, 84.2, 85.1, 86, and 88, wherein the amino acid position of each group member is indicated according to the IMGT® numbering.

In some embodiments the first and the second member of the naturally occurring immunoglobulin super-family are selected from the TCR constant domain family. In some

embodiments the third and the fourth member of the naturally occurring immunoglobulin super-family are selected from the TCR constant domain family.

Preferably the donor domain of first member of the naturally occurring immunoglobulin super-family is the TCR constant domain alpha (SEQ ID NO: 1) and the donor domain of the second member of the naturally occurring immunoglobulin super-family is the TCR constant domain beta (SEQ ID NO: 2). Equally the donor domain of the first member of the naturally occurring immunoglobulin super-family can be the TCR constant domain beta (SEQ ID NO: 2) and the donor domain of the second member of the naturally occurring immunoglobulin super-family can be the TCR constant domain alpha (SEQ ID NO: 1).

Preferably the donor domain of the third member of the naturally occurring immunoglobulin super-family is the TCR constant domain alpha (SEQ ID NO: 1) and the donor domain of the fourth member of the naturally occurring immunoglobulin super-family is the TCR constant domain beta (SEQ ID NO: 2). Equally the donor domain of the third member of the naturally occurring immunoglobulin super-family can be the TCR constant domain beta (SEQ ID NO: 2) and the donor domain of the fouth member of the naturally occurring immunoglobulin super-family can be the TCR constant domain alpha (SEQ ID NO: 1).

In a further embodiment the donor domain of the first member of the naturally occurring immunoglobulin super-family is the TCR constant domain alpha (SEQ ID NO: 1) and the donor domain of the second member of the naturally occurring immunoglobulin super-family is the TCR constant domain beta (SEQ ID NO: 2), wherein the cysteine (C) at amino acid position 75 in SEQ ID NO: 2 is substituted with alanine (A) or serine (S), preferably with alanine (A).

In a further embodiment the donor domain of the third member of the naturally occurring immunoglobulin super-family is the TCR constant domain alpha (SEQ ID NO: 1) and the donor domain of the fourth member of the naturally occurring immunoglobulin super-family is the TCR constant domain beta (SEQ ID NO: 2), wherein the cysteine (C) at amino acid position 75 in SEQ ID NO: 2 is substituted with alanine (A) or serine (S), preferably with alanine (A).

In a further embodiment the donor domain of the third member of the naturally occurring immunoglobulin super-family is the TCR constant domain gamma (SEQ ID NO: 33) and the donor domain of the fourth member of the naturally occurring immunoglobulin super-family is the TCR constant domain delta (SEQ ID NO: 32) or wherein the donor domain of the third

member of the naturally occurring immunoglobulin super-family is the TCR constant domain delta (SEQ ID NO: 32) and the donor domain of the fourth member of the naturally occurring immunoglobulin super-family is the TCR constant domain gamma (SEQ ID NO: 33).

In a further embodiment the donor domain of the first and/or second member of the naturally occurring immunoglobulin super-family is an IgA1 CH3 domain, preferably an IGHA1 CH3 domain, more preferably the human IGHA1 CH3 domain (SEQ ID NO: 96). In a further embodiment the donor domain of the first and/or second member of the naturally occurring immunoglobulin super-family is IgA2 CH3 domain, preferably an IGHA2 CH3 domain, more preferably the human IGHA2 CH3 domain (SEQ ID NO: 97).
In a further embodiment the donor domain of the third and/or fourth member of the naturally occurring immunoglobulin super-family is an IgA1 CH3 domain, preferably an IGHA1 CH3 domain, more preferably the human IGHA1 CH3 domain (SEQ ID NO: 96). In a further embodiment the donor domain of the third and/or fourth member of the naturally occurring immunoglobulin super-family is an IgA2 CH3 domain, preferably an IGHA2 CH3 domain, more preferably the human IGHA2 CH3 domain (SEQ ID NO: 97).

The IGHA1 CH3 domain or the IGHA2 CH3 domain as donor domains are particularily useful if the parent domains of the first engineered immunoglobulin chain and/or the second engineered immunoglobulin chain or the further engineered domain of the first engineered immunoglobulin chain and/or the second engineered immunoglobulin chain and the engineered domain of the third engineered immunoglobulin chain are CH3 domains from an isotype different from IGHA1 or IGHA2, e.g. IGHG1 or IGHG2 or IGHG3 or IGHG4 CH3 domains or preferably IGHG1 CH3, more preferably human IGHG1 CH3.

In a further embodiment at least one amino acid residue of the protein-protein interface of the parent domain of said first engineered immunoglobulin chain is substituted with an amino acid residue at the equivalent 3D structural position from a protein-protein interface of a donor domain of a first member of the naturally occurring immunoglobulin super-family and additionally at least one amino acid residue of the protein-protein interface of the parent domain of said first engineered immunoglobulin chain is substituted with an amino acid residue at the equivalent 3D structural position from a protein-protein interface of a donor domain of a third member of the naturally occurring immunoglobulin super-family and

wherein at least one amino acid residue of the protein-protein interface of the parent domain of said second engineered immunoglobulin chain is substituted with an amino acid residue at the equivalent 3D structural position from a protein-protein interface of a donor domain of a second member of the naturally occurring immunoglobulin super-family and additionally at least one amino acid residue of the protein-protein interface of the parent domain of said second engineered immunoglobulin chain is substituted with an amino acid residue at the equivalent 3D structural position from a protein-protein interface of a donor domain of a fourth member of the naturally occurring immunoglobulin super-family.

In case the parent domains are from IgG1, the donor domain of the first member of the naturally occurring immunoglobulin super-family can be selected from e.g. a CH3 domain of IgA, IgM or IgE, the donor domain of the third member of the naturally occurring immunoglobulin super-family can be selected from e.g. a CH4 domain of IgM or IgE or from a CH3 domain of IgG2, IgG3 or IgG4, the donor domain of the second member of the naturally occurring immunoglobulin super-family can be selected from e.g. a CH3 domain of IgA, IgM or IgE and the donor domain of the fourth member of the naturally occurring immunoglobulin super-family can be selected from e.g. a CH4 domain of IgM or IgE or from a CH3 domain of IgG2, IgG3 or IgG4. Usually the donor domain of the first member of the naturally occurring immunoglobulin super-family and the the donor domain of the second member of the naturally occurring immunoglobulin super-family which are selected are the same and the donor domain of the third member of the naturally occurring immunoglobulin super-family and the the donor domain of the fourth member of the naturally occurring immunoglobulin super-family are the same.

Provided that the parent domain of the first engineered immunoglobulin chain and the parent domain of the second engineered immunoglobulin chain form a hetero-dimer, specifically a naturally occurring hetero-dimer, or are spefically a CH1 domain and an IGKC domain, the donor domain of the first member of the naturally occurring immunoglobulin super-family and the donor domain of the second member of the naturally occurring immunoglobulin can be domains which form a homo-dimer, usually domains of the same isotype, species and subclass, preferably a CH3 domain, more preferred a human CH3 domain, even more preferred an IgG1 CH3 domain, in particular a human IgG1 CH3 domain. Equally preferred is an IgA1 CH3 domain or an IgA2 CH3 domain, preferably the human IGHA1 CH3 domain or the human IGHA2 CH3 domain.

Provided that the parent domain of the first engineered immunoglobulin chain and the parent

domain of the second engineered immunoglobulin chain form a hetero-dimer, specifically a naturally occurring hetero-dimer, or are spefically a CH1 domain and an IGKC domain, the donor domain of the first member of the naturally occurring immunoglobulin super-family and the donor domain of the second member of the naturally occurring immunoglobulin can be domains which form a hetero-dimer, preferably domains from the TCR constant domain family, more preferably TCR constant domain alpha (SEQ ID NO: 1) and TCR constant domain beta (SEQ ID NO: 2) or TCR constant domain gamma (SEQ ID NO: 33) and the TCR constant domain delta (SEQ ID NO: 32).

Provided that the parent domain of the first engineered immunoglobulin chain and the parent domain of the second engineered immunoglobulin chain form a homo-dimer, specifically a naturally occurring homo-dimer, or are spefically domains of the same isotype, species and subclass, the donor domain of the first member of the naturally occurring immunoglobulin super-family and the donor domain of the second member of the naturally occurring immunoglobulin are domains which form a hetero-dimer, preferably domains from the TCR constant domain family, more preferably TCR constant domain alpha (SEQ ID NO: 1) and TCR constant domain beta (SEQ ID NO: 2) or TCR constant domain gamma (SEQ ID NO: 33) and the TCR constant domain delta (SEQ ID NO: 32).

Provided that the parent domain of the further engineered domain of the first and/or second engineered immunoglobulin chain and the parent domain of the engineered domain of the third engineered immunoglobulin chain form a hetero-dimer, specifically a naturally occurring hetero-dimer, or are spefically a CH1 domain and a IGKC domain, the donor domain of the third member of the naturally occurring immunoglobulin super-family and the donor domain of the fourth member of the naturally occurring immunoglobulin are preferably domains which form a homo-dimer, usually domains of the same isotype, species and subclass, preferably a CH3 domain, more preferred a human CH3 domain, even more preferred an IgG1 CH3 domain, in particular a human IgG1 CH3 domain.

Provided that the parent domain of the further engineered domain of the first and/or second engineered immunoglobulin chain and the parent domain of the engineered domain of the third engineered immunoglobulin chain form a homo-dimer, specifically a naturally occurring homo-dimer, or are spefically domains of the same isotype, species and subclass, the donor domain of the third member of the naturally occurring immunoglobulin super-family and the donor domain of the fourth member of the naturally occurring immunoglobulin are preferably

domains which form a hetero-dimer, preferably domains from the TCR constant domain family, more preferably TCR constant domain alpha (SEQ ID NO: 1) and TCR constant domain beta (SEQ ID NO: 2) or TCR constant domain gamma (SEQ ID NO: 33) and the TCR constant domain delta (SEQ ID NO: 32).

Provided that the parent domain of the further engineered domain of the first and/or second engineered immunoglobulin chain and the parent domain of the engineered domain of the third engineered immunoglobulin chain form a hetero-dimer, specifically a naturally occurring hetero-dimer, the donor domain of the third member of the naturally occurring immunoglobulin super-family and the donor domain of the fourth member of the naturally occurring immunoglobulin are domains which form a hetero-dimer, preferably domains from the TCR constant domain family, more preferably TCR constant domain alpha (SEQ ID NO: 1) and TCR constant domain beta (SEQ ID NO: 2) or TCR constant domain gamma (SEQ ID NO: 33) and the TCR constant domain delta (SEQ ID NO: 32).

In some embodiments the donor domain of the first member of the naturally occurring immunoglobulin super-family is the TCR constant domain alpha (SEQ ID NO: 1), the donor domain of the second member of the naturally occurring immunoglobulin super-family is the TCR constant domain beta (SEQ ID NO: 2), the donor domain of the third member of the naturally occurring immunoglobulin super-family is the TCR constant domain delta (SEQ ID NO: 32) and the donor domain of the fourth member of the naturally occurring immunoglobulin super-family is the TCR constant domain gamma (SEQ ID NO: 33).

In some embodiments the the donor domain of first member of the naturally occurring immunoglobulin super-family is the TCR constant domain delta (SEQ ID NO: 32), the donor domain of the second member of the naturally occurring immunoglobulin super-family is the TCR constant domain gamma (SEQ ID NO: 33), the donor domain of the third member of the naturally occurring immunoglobulin super-family is the TCR constant domain alpha (SEQ ID NO: 1) and the donor domain of the fourth member of the naturally occurring immunoglobulin super-family is the TCR constant domain beta (SEQ ID NO: 2).

In some embodiments the donor domain of the first member of the naturally occurring immunoglobulin super-family is the TCR constant domain alpha (SEQ ID NO: 1) or the TCR constant domain delta (SEQ ID NO: 32), the donor domain of the second member of the naturally occurring immunoglobulin super-family is the TCR constant domain beta (SEQ ID NO: 2) or the TCR constant domain gamma (SEQ ID NO: 33), the donor domain of the third

and the fourth member of the naturally occurring immunoglobulin super-family is the IgG1 CH3 domain (SEQ ID NO: 47), with the proviso that if the donor domain of the first member of the naturally occurring immunoglobulin super-family is the TCR constant domain alpha (SEQ ID NO: 1), the donor domain of the second member of the naturally occurring immunoglobulin super-family is the TCR constant domain beta (SEQ ID NO: 2) and if the donor domain of the first member of the naturally occurring immunoglobulin super-family is the TCR constant domain delta (SEQ ID NO: 32), the donor domain of the second member of the naturally occurring immunoglobulin super-family is the TCR constant domain gamma (SEQ ID NO: 33).

In some embodiments the donor domain of the first member of the naturally occurring immunoglobulin super-family is the TCR constant domain alpha (SEQ ID NO: 1) or the TCR constant domain delta (SEQ ID NO: 32), the donor domain of the second member of the naturally occurring immunoglobulin super-family is the TCR constant domain beta (SEQ ID NO: 2) or the TCR constant domain gamma (SEQ ID NO: 33), the donor domain of the third and the fourth member of the naturally occurring immunoglobulin super-family is the human IGHA1 CH3 domain (SEQ ID NO: 96) or the human IGHA2 CH3 domain (SEQ ID NO: 97), with the proviso that if the first member of the naturally occurring immunoglobulin super-family is the TCR constant domain alpha (SEQ ID NO: 1), the donor domain of the second member of the naturally occurring immunoglobulin super-family is the TCR constant domain beta (SEQ ID NO: 2) and if the donor domain of the first member of the naturally occurring immunoglobulin super-family is the TCR constant domain delta (SEQ ID NO: 32), the donor domain of the second member of the naturally occurring immunoglobulin super-family is the TCR constant domain gamma (SEQ ID NO: 33).

In some embodiments the protein-protein interface of the engineered domain of the first engineered immunoglobulin chain is interacting with the protein-protein interface of the engineered domain of the second engineered immunoglobulin chain by hetero-dimerization.

In some embodiments the protein-protein interface of the engineered domain of the first engineered immunoglobulin chain is interacting with the protein-protein interface of the engineered domain of the second engineered immunoglobulin chain by homo-dimerization.

In some embodiments the parent domain of the first engineered immunoglobulin chain is an IGKC domain and the amino acid residue which is substituted in the protein-protein interface of the parent domain of the engineered domain of the first engineered immunoglobulin chain comprises an amino acid residue at a position selected from the group consisting of 114, 116, 118, 123, 124, 131, 133, 137, 138, 160, 162, 164, 165, 167, 174, 176, 178, and 180, wherein the amino acid position of each group member is indicated according to the EU numbering.

In some embodiments the parent domain of the first engineered immunoglobulin chain is an IGLC domain selected from the group consisting of IGLC1, IGLC2, IGLC3, IGLC6, and IGLC7 and the amino acid residue which is substituted in the protein-protein interface of the parent domain of the first engineered immunoglobulin chain comprises an amino acid residue at a position selected from the group consisting of 114, 116, 118, 122, 131, 133, 137, 138, 160, 162, 165, 167, 174, 176, 178, and 180, wherein the amino acid position of each group member is indicated according to the Kabat numbering.

In some embodiments the parent domain of the first engineered immunoglobulin chain is an IGKC domain and the amino acid residue which is substituted in the protein-protein interface of the parent domain of the first engineered immunoglobulin chain comprises an amino acid residue selected from the group consisting of S131V, S131T, S131A, S131N, S131Q, S131E, S131K, S131W, V133A, V133G, V133T, V133L, V133I, N137Q, N137T, N137K, N137V, N137S, N137R, N137E, S174T, S174M, S174A, S174R, S174H, S174K, S174W, S174F, S174C, S174N, S176I, S176T, S176H, S176Q, S176V, S176W, S176Y, S176F, T178Q, T178L, T178V, T178R, T178E, T178I, T178K, T178Y, and T178W, wherein the amino acid position of each group member is indicated according to the EU numbering.

In some embodiments the parent domain of the first engineered immunoglobulin chain is an IGKC domain and the amino acid residue which is substituted in the protein-protein interface of the parent domain of the first engineered immunoglobulin chain comprises an amino acid residue selected from the group consisting of S114E, S114K, S114Q, F116A, F116T, F116Y, F118M, F118L, E123D, Q124E, S131K, S131T, S131N, V133L, V133A, V133T, N137T, N137E, N137K, N138K, N138E, Q160Y, Q160F, Q160K, S162A, S162G, S162D, S162T, T164V, T164M, E165L, D167E, D167S, S174A, S174M, S174N, S174F, S176F, S176V, S176Y, T178W, T178R, T178L, T178K, T180N, T180R, and T180K or is selected from the group consisting of S114E, S114K, S114Q, F116A, F116T, F116Y, F118M, F118L, E123D, Q124E, S131K, S131T, S131N, V133L, V133A, V133T, N137T, N137E, N137K, N138K, N138E, Q160Y, Q160F, Q160K, S162A, S162G, S162D, S162T, T164V, T164M, E165L,

D167E, D167S, S174A, S174M, S174N, S174F, S176F, S176V, S176Y, T178W, T178R, T178L, T178K, T180N, T180R, and T180K and conservative amino acid substitutions thereof, wherein the amino acid position of each group member is indicated according to the EU numbering.

In some embodiments the parent domain of the first engineered immunoglobulin chain is an IGLC domain selected from the group consisting of IGLC1, IGLC2, IGLC3, IGLC6, and IGLC7 and the amino acid residue which is substituted in the protein-protein interface of the parent domain of the first engineered immunoglobulin chain comprises an amino acid residue selected from the group consisting of 131V, 131A, 131N, 131Q, 131E, 131K, 131W, 131S, 133A, 133G, 133T, 133L, 133I, 137Q, 137T, 137K, 137V, 137R, 137N, 137E, 174T, 174M, 174S, 174R, 174H, 174K, 174W, 174F, 174C, 174N, 176I, 176T, 176H, 176Q, 176W, 176Y, 176V, 176F, 178Q, 178L, 178V, 178R, 178E, 178T, 178I, 178K, 178E, and 178W, wherein the amino acid position of each group member is indicated according to the Kabat numbering.

In some embodiments the parent domain of the first engineered immunoglobulin chain is an IGLC domain selected from the group consisting of IGLC1, IGLC2, IGLC3, IGLC6, and IGLC7 and the amino acid residue which is substituted in the protein-protein interface of the parent domain of the first engineered immunoglobulin chain comprises an amino acid residue selected from the group consisting of 114E, 114K, 114Q, 116A, 116Y, 118M, 118L, 122D, 131K, 131N, 133A, 133L, 133T, 137T, 137E, 137K, 138E, 138K, 160Y, 160F, 160K, 162D, 162A, 162G, 165L, 165M, 167E, 167S, 174N, 174M, 174F, 174S, 176V, 176F, 176Y, 178W, 178R, 178L, 178K, 180N, 180R, and 180K or selected from the group consisting of 114E, 114K, 114Q, 116A, 116Y, 118M, 118L, 122D, 131K, 131N, 133A, 133L, 133T, 137T, 137E, 137K, 138E, 138K, 160Y, 160F, 160K, 162D, 162A, 162G, 165L, 165M, 167E, 167S, 174N, 174M, 174F, 174S, 176V, 176F, 176Y, 178W, 178R, 178L, 178K, 180N, 180R, and 180K and conservative amino acid substitutions thereof, wherein the amino acid position of each group member is indicated according to the Kabat numbering.

In some embodiments the parent domain of the second engineered immunoglobulin chain is a CH1 domain and the amino acid residue which is substituted in the protein-protein interface of the parent domain of the second engineered immunoglobulin chain comprises an amino acid residue at a position selected from the group consisting of 124, 126, 128, 133, 134, 139, 141, 143, 147, 148, 168, 170, 173, 175, 181, 183, 185, and 187, wherein the amino acid position of each group member is indicated according to the EU numbering.

In some embodiments the parent domain of the second engineered immunoglobulin chain is a

CH1 domain and and the amino acid residue which is substituted in the protein-protein interface of the parent domain of the second engineered immunoglobulin chain comprises an amino acid residue selected from the group consisting of 141V, 141T, 141N, 141Q, 141E, 141K, 141W, 141S, 143A, 143T, 143L, 143I, 143V, 147Q, 147T, 147V, 147S, 147R, 147N, 147E, 181T, 181M, 181A, 181R, 181H, 181K, 181W, 181F, 181C, 181N, 183I, 183H, 183Q, 183V, 183T, 183W, 183Y, 183F, 185Q, 185L, 185R, 185E, 185I, 185T, 185K, 185Y, and 185W, wherein the amino acid position of each group member is indicated according to the EU numbering.

In some embodiments the parent domain of the second engineered immunoglobulin chain is a CH1 domain and the amino acid residue which is substituted in the protein-protein interface of the parent domain of the second engineered immunoglobulin chain comprises an amino acid residueselected from the group consisting of S124E, S124K, S124Q, F126A, F126T, F126Y, L128F, L128M, K133D, S134E, T139Q, A141K, A141T, A141N, A141S, G143V, G143A, G143L, G143T, K147T, K147E, D148E, D148K, H168Y, H168F, H168K, F170D, F170A, F170G, F170T, V173L, V173M, Q175E, Q175S, Q175D, S181A, S181N, S181M, S181F, S183V, S183F, S183Y, V185W, V185R, V185L, V185K, T187N, T187K and T187R or is selected from the group consisting of S124E, S124K, S124Q, F126A, F126T, F126Y, L128F, L128M, K133D, S134E, T139Q, A141K, A141T, A141N, A141S, G143V, G143A, G143L, G143T, K147T, K147E, D148E, D148K, H168Y, H168F, H168K, F170D, F170A, F170G, F170T, V173L, V173M, Q175E, Q175S, Q175D, S181A, S181N, S181M, S181F, S183V, S183F, S183Y, V185W, V185R, V185L, V185K, T187N, T187K and T187R and conservative amino acid substitutions thereof, wherein the amino acid position of each group member is indicated according to the EU numbering.

Positions 114, 116, 118, 122, 131, 133, 137, 138, 160, 162, 165, 167, 174, 176, 178, and 180 in the protein-protein interface of an IGLC domain as parent domain according to Kabat numbering correspond in the same order to positions 3, 5, 7, 11, 20, 22, 26, 27, 79, 81, 84, 84.2, 85.1, 86, 88, and 90 indicated according to the IMGT® numbering.

Positions 114, 116, 118, 123, 124, 131, 133, 137, 138, 160, 162, 164, 165, 167, 174, 176, 178, and 180 in the protein-protein interface of the IGKC domain as parent domain according to EU numbering correspond in the same order to positions 3, 5, 7, 12, 13, 20, 22, 26, 27, 79, 81,

83, 84, 84.2, 85.1, 86, 88, and 90 indicated according to the IMGT® numbering.

Positions 124, 126, 128, 133, 134, 139, 141, 143, 147, 148, 168, 170, 173, 175, 181, 183, 185, and 187 in the protein-protein interface of the CH1 domain as parent domain according to EU numbering correspond in the same order to positions 3, 5, 7, 12, 13, 18, 20, 22, 26, 27, 79, 81, 84, 84.2, 85.1, 86, 88, and 90 indicated according to the IMGT® numbering.

Positions 124, 126, 128, 133, 134, 139, 141, 143, 147, 148, 168, 170, 173, 175, 181, 183, 185, and 187 in the protein-protein interface of the CH4 domain as parent domain according to EU numbering correspond in the same order to positions 3, 5, 7, 12, 13, 18, 20, 22, 26, 27, 79, 81, 84, 84.2, 85.1, 86, 88, and 90 indicated according to the IMGT® numbering.

Thus equivalently in some embodiments the present disclosure provides a hetero-dimeric immunoglobulin or fragment thereof, wherein the parent domain of the engineered domain of the first engineered immunoglobulin chain is an IGKC domain and the amino acid residue which is substituted in the protein-protein interface of the parent domain of the engineered domain of the first engineered immunoglobulin chain comprises an amino acid residue at a position selected from the group consisting of 3, 5, 7, 12, 13, 20, 22, 26, 27, 79, 81, 83, 84, 84.2, 85.1, 86, 88, and 90, wherein the amino acid position of each group member is indicated according to the IMGT® numbering.

Thus equivalently in some embodiments the present disclosure provides a hetero-dimeric immunoglobulin or fragment thereof, wherein the parent domain of the first engineered immunoglobulin chain is an IGLC domain selected from the group consisting of IGLC1, IGLC2, IGLC3, IGLC6, and IGLC7 and the amino acid residue which is substituted in the protein-protein interface of the parent domain of the first engineered immunoglobulin chain comprises an amino acid residue at a position selected from the group consisting of 3, 5, 7, 11, 20, 22, 26, 27, 79, 81, 84, 84.2, 85.1, 86, 88, and 90, wherein the amino acid position of each group member is indicated according to the IMGT® numbering.

Thus equivalently in some embodiments the present disclosure provides a hetero-dimeric immunoglobulin or fragment thereof, wherein the parent domain of the engineered domain of the second engineered immunoglobulin chain is a CH1 domain and the amino acid residue which is substituted in the protein-protein interface of the parent domain of the engineered domain of the second engineered immunoglobulin chain comprises an amino acid residue at a

position selected from the group consisting of 3, 5, 7, 12, 13, 18, 20, 22, 26, 27, 79, 81, 84, 84.2, 85.1, 86, 88, and 90, wherein the amino acid position of each group member is indicated according to the IMGT® numbering.

Thus equivalently in some embodiments the present disclosure provides a hetero-dimeric immunoglobulin or fragment thereof, wherein the parent domain of the engineered domain of the first and/or second engineered immunoglobulin chain is a CH4 domain and the amino acid residue which is substituted in the protein-protein interface of the parent domain of the engineered domain of the first and/or second engineered immunoglobulin chain comprises an amino acid residue at a position selected from the group consisting of 3, 5, 7, 12, 13, 18, 20, 22, 26, 27, 79, 81, 84, 84.2, 85.1, 86, 88, and 90, wherein the amino acid position of each group member is indicated according to the IMGT® numbering.

In some embodiments the parent domain of the first engineered immunoglobulin chain is an IGKC domain and the amino acid residue which is substituted in the protein-protein interface of the parent domain of the first engineered immunoglobulin chain comprises an amino acid residue at a position selected from the group consisting of 131, 137, 160, 176, 178, and 180, and/or the parent domain of the second engineered immunoglobulin chain is a CH1 domain and the amino acid residue which is substituted in the protein-protein interface of the parent domain of the second engineered immunoglobulin chain comprises an amino acid residue at a position selected from the group consisting of 124, 126, 128, 141, 143, 147, 170, 173, 175, 181, 185, and 187, wherein the amino acid position of each group member is indicated according to the EU numbering.

In some embodiments the parent domain of the first engineered immunoglobulin chain is an IGKC domain and the amino acid residue which is substituted in the protein-protein interface of the parent domain of the first engineered immunoglobulin chain comprises the amino acid residue at postion 178 or comprises T178W or comprises T178W and conservative amino acid substitutions thereof, and optionally a further amino acid residue selected from the group consisting of 131, 137, 160, 176, and 180, and/or the parent domain of the second engineered immunoglobulin chain is a CH1 domain and the amino acid residue which is substituted in the protein-protein interface of the parent domain of the second engineered immunoglobulin chain comprises an amino acid residue at postion 181 or comprises S181A or comprises S181A and conservative amino acid substitutions thereof and optionally a further amino acid

residue selected from the group consisting of 124, 126, 128, 141, 143, 147, 170, 173, 175, 185, and 187, wherein the amino acid position of each group member is indicated according to the EU numbering.

In some embodiments the parent domain of the first engineered immunoglobulin chain is an IGKC domain and the amino acid residue which is substituted in the protein-protein interface of the parent domain of the first engineered immunoglobulin chain comprises an amino acid residue selected from the group consisting of S131K, N137T, Q160Y, S176V, T178W, and T180N or comprises an amino acid residue selected from the group consisting of S131K, N137T, Q160Y, S176V, T178W, and T180N and conservative amino acid substitutions thereof, and/or the parent domain of the second engineered immunoglobulin chain is a CH1 domain and the amino acid residue which is substituted in the protein-protein interface of the parent domain of the second engineered immunoglobulin chain comprises an amino acid residue selected from the group consisting of S124E, F126A, L128F, A141T, G143V, K147T, F170D, V173L, Q175E, S181A, V185R or comprises an amino acid residue selected from the group consisting of S124E, F126A, L128F, A141T, G143V, K147T, F170D, V173L, Q175E, S181A, V185R and T187R and conservative amino acid substitutions thereof, wherein the amino acid position of each group member is indicated according to the EU numbering.

In some embodiments the parent domain of the first engineered immunoglobulin chain is an IGKC domain and the amino acid residue which is substituted in the protein-protein interface of the parent domain of the first engineered immunoglobulin chain comprises an amino acid residue at a position selected from the group consisting of 114, 116, 131, 137, 162, 165, 167, 174, 178, and 180, and/or the parent domain of the second engineered immunoglobulin chain is a CH1 domain and the amino acid residue which is substituted in the protein-protein interface of the parent domain of the second engineered immunoglobulin chain comprises an amino acid residue at a position selected from the group consisting of 141, 143, 147, 168, 183, 185 and 187, wherein the amino acid position of each group member is indicated according to the EU numbering.

In some embodiments the parent domain of the first engineered immunoglobulin chain is an IGKC domain and the amino acid residue which is substituted in the protein-protein interface

of the parent domain of the first engineered immunoglobulin chain comprises an amino acid residue at postion 174 or comprises S174A or comprises S174A and conservative amino acid substitutions thereof and optionally a further amino acid residue selected from the group consisting of 114, 116, 131, 137, 162, 165, 167, 178, and 180, and/or the parent domain of the second engineered immunoglobulin chain is a CH1 domain and the amino acid residue which is substituted in the protein-protein interface of the parent domain of the second engineered immunoglobulin chain comprises an amino acid residue at postion 185 or comprises V185W or comprises V185W and conservative amino acid substitutions thereof and optionally a further amino acid residue selected from the group consisting of 141, 143, 147, 168, 183, and 187, wherein the amino acid position of each group member is indicated according to the EU numbering.

In some embodiments the parent domain of the first engineered immunoglobulin chain is an IGKC domain and the amino acid residue which is substituted in the protein-protein interface of the parent domain of the first engineered immunoglobulin chain comprises an amino acid residue selected from the group consisting of S114E, F116A, S131T, N137T, S162D, E165L, D167E, S174A, T178R, and T180R or comprises an amino acid residue selected from the group consisting of S114E, F116A, S131T, N137T, S162D, E165L, D167E, S174A, T178R, and T180R and conservative amino acid substitutions thereof, and/or the parent domain of the second engineered immunoglobulin chain is a CH1 domain and the amino acid residue which is substituted in the protein-protein interface of the parent domain of the second engineered immunoglobulin chain comprises an amino acid residue selected from the group consisting of A141K, G143V, K147T, H168Y, S183V, V185W, and T187N or comprises an amino acid residue selected from the group consisting of A141K, G143V, K147T, H168Y, S183V, V185W, and T187N and conservative amino acid substitutions thereof, wherein the amino acid position of each group member is indicated according to the EU numbering.

In some embodiments the parent domain of the first engineered immunoglobulin chain is an IGKC domain and the amino acid residue which is substituted in the protein-protein interface of the parent domain of the first engineered immunoglobulin chain comprises an amino acid residue at a position selected from the group consisting of 114, 116, 131, 133, 137, 138, 162, 164, 174, 176, 178 and 180, and/or the parent domain of the second engineered immunoglobulin chain is a CH1 domain and the amino acid residue which is substituted in the protein-protein interface of the parent domain of the second engineered immunoglobulin

chain comprises an amino acid residue at a position selected from the group consisting of 128 , 141, 143, 148, 168, 170, 175, 181, 183, 185, and 187, wherein the amino acid position of each group member is indicated according to the EU numbering.

In some embodiments the parent domain of the first engineered immunoglobulin chain is an IGKC domain and the amino acid residue which is substituted in the protein-protein interface of the parent domain of the first engineered immunoglobulin chain comprises the amino acid residue at postion 178 or comprises T178W or comprises T178W and conservative amino acid substitutions thereof and optionally a further amino acid residue selected from the group consisting of 114,116, 131, 133, 137, 138, 162, 164, 174, and 176, and/or the parent domain of the second engineered immunoglobulin chain is a CH1 domain and the amino acid residue which is substituted in the protein-protein interface of the parent domain of the second engineered immunoglobulin chain comprises an amino acid residue at postion 183 or comprises S183V or comprises S183V and conservative amino acid substitutions thereof and optionally a further amino acid residue selected from the group consisting of 128, 141, 143, 148, 168, 170, 175, 181, 185, and 187, wherein the amino acid position of each group member is indicated according to the EU numbering.

In some embodiments the parent domain of the first engineered immunoglobulin chain is an IGKC domain and the amino acid residue which is substituted in the protein-protein interface of the parent domain of the first engineered immunoglobulin chain comprises an amino acid residue selected from the group consisting of S114K, F116T, S131T, V133L, N137E, N138K, S162G, T164M, S174M, S176F, and T178W or comprises an amino acid residue selected from the group consisting of S114K, F116T, S131T, V133L, N137E, N138K, S162G, T164M, S174M, S176F, and T178W and conservative amino acid substitutions thereof, and/or the parent domain of the second engineered immunoglobulin chain is a CH1 domain and the amino acid residue which is substituted in the protein-protein interface of the parent domain of the second engineered immunoglobulin chain comprises an amino acid residue selected from the group consisting of L128M , A141N, G143A, D148E, H168F, F170A, Q175S, S181N, S183V, V185L, and T187K or comprises an amino acid residue selected from the group consisting of L128M , A141N, G143A, D148E, H168F, F170A, Q175S, S181N, S183V, V185L, and T187K and conservative amino acid substitutions thereof, wherein the amino acid position of each group member is indicated according to the EU numbering.

In some embodiments the parent domain of the first engineered immunoglobulin chain is an

IGKC domain and the amino acid residue which is substituted in the protein-protein interface of the parent domain of the first engineered immunoglobulin chain comprises an amino acid residue at a position selected from the group consisting of 118, 131, 133, 138, 160, 162, 167, 174, 176, 178, and 180 and/or the parent domain of the second engineered immunoglobulin chain is a CH1 domain and the amino acid residue which is substituted in the protein-protein interface of the parent domain of the second engineered immunoglobulin chain comprises an amino acid residue at a position selected from the group consisting of 124, 126, 141, 143, 147, 148, 170, 173, 181, 183 and 185, wherein the amino acid position of each group member is indicated according to the EU numbering.

In some embodiments the parent domain of the first engineered immunoglobulin chain is an IGKC domain and the amino acid residue which is substituted in the protein-protein interface of the parent domain of the engineered domain of the first engineered immunoglobulin chain comprises the amino acid residue at postion 176 or comprises S176V or comprises S176V and conservative amino acid substitutions thereof and optionally a further amino acid residue selected from the group consisting of 118, 131, 133, 138, 160, 162, 167, 174, 178, 180, and/or the parent domain of the second engineered immunoglobulin chain is a CH1 domain and the amino acid residue which is substituted in the protein-protein interface of the parent domain of the second engineered immunoglobulin chain comprises the amino acid residue at postion 185 or comprises V185W or comprises V185W and conservative amino acid substitutions thereof and optionally a further amino acid residue selected from the group consisting of 124, 126, 141, 143, 147, 148, 170, 173, 181, and 183, wherein the amino acid position of each group member is indicated according to the EU numbering.

In some embodiments the parent domain of the first engineered immunoglobulin chain is an IGKC domain and the amino acid residue which is substituted in the protein-protein interface of the parent domain of the first engineered immunoglobulin chain comprises an amino acid residue selected from the group consisting of F118M, S131N, V133A, N138E, Q160F, S162A, D167S, S174N, S176V, T178L, and T180K or comprises an amino acid residue selected from the group consisting of F118M, S131N, V133A, N138E, Q160F, S162A, D167S, S174N, S176V, T178L, and T180K and conservative amino acid substitutions thereof and/or the parent domain of the second engineered immunoglobulin chain is a CH1 domain and the amino acid residue which is substituted in the protein-protein interface of the parent domain of the second engineered immunoglobulin chain comprises an amino acid residue selected from the group consisting of S124K, F126T, A141T, G143L, K147E, D148K,

F170G, V173M, S181M, S183F, and V185W or comprises an amino acid residue selected from the group consisting of S124K, F126T, A141T, G143L, K147E, D148K, F170G, V173M, S181M, S183F, and V185W and conservative amino acid substitutions thereof, wherein the amino acid position of each group member is indicated according to the EU numbering.

In some embodiments the parent domain of the first engineered immunoglobulin chain is an IGKC domain and the amino acid residue which is substituted in the protein-protein interface of the parent domain of the first engineered immunoglobulin chain comprises an amino acid residue at a position selected from the group consisting of 114, 116, 118, 123, 124, 133, 137, 160, 162, 164, 174, 176, and 178, and/or the parent domain of the second engineered immunoglobulin chain is a CH1 domain and the amino acid residue which is substituted in the protein-protein interface of the parent domain of the second engineered immunoglobulin chain comprises an amino acid residue at a position selected from the group consisting of 124, 126, 133, 134, 139, 141, 143, 168, 170, 175, 181, 183, and 185, wherein the amino acid position of each group member is indicated according to the EU numbering.

In some embodiments the parent domain of the first engineered immunoglobulin chain is an IGKC domain and the amino acid residue which is substituted in the protein-protein interface of the parent domain of the first engineered immunoglobulin chain comprises the amino acid residue at postion 176 or comprises S176Y or comprises S176Y and conservative amino acid substitutions thereof and optionally a further amino acid residue selected from the group consisting of 114, 116, 118, 123, 124, 133, 137, 160, 162, 164, 174, and 178, and/or the parent domain of the second engineered immunoglobulin chain is a CH1 domain and the amino acid residue which is substituted in the protein-protein interface of the parent domain of the second engineered immunoglobulin chain comprises the amino acid residue at postion 183 or comprises S183Y or comprises S183Y and conservative amino acid substitutions thereof and optionally a further amino acid residue selected from the group consisting of 124, 126, 133, 134, 139, 141, 143, 168, 170, 175, 181, and 185, wherein the amino acid position of each group member is indicated according to the EU numbering.

In some embodiments the parent domain of the first engineered immunoglobulin chain is an IGKC domain and the amino acid residue which is substituted in the protein-protein interface of the parent domain of the first engineered immunoglobulin chain comprises an amino acid residue selected from the group consisting of S114Q, F116Y, F118L, E123D, Q124E, V133T,

N137K, Q160K, S162T, T164V, S174F, S176Y, and T178K or comprises an amino acid residue selected from the group consisting of S114Q, F116Y, F118L, E123D, Q124E, V133T, N137K, Q160K, S162T, T164V, S174F, S176Y, and T178K and conservative amino acid substitutions thereof, and/or the parent domain of the second engineered immunoglobulin chain is a CH1 domain and the amino acid residue which is substituted in the protein-protein interface of the parent domain of the second engineered immunoglobulin chain comprises an amino acid residue selected from the group consisting of S124Q, F126Y, K133D, S134E, T139Q, A141S, G143T, H168K, F170T, Q175D, S181F, S183Y, and V185K or comprises an amino acid residue selected from the group consisting of S124Q, F126Y, K133D, S134E, T139Q, A141S, G143T, H168K, F170T, Q175D, S181F, S183Y, and V185K and conservative amino acid substitutions thereof, wherein the amino acid position of each group member is indicated according to the EU numbering.

In some embodiments the parent domain of the first engineered immunoglobulin chain is a CH1 domain and the parent domain of the second engineered immunoglobulin chain is a IGKC domain, wherein the amino acid residue which are substituted in the protein-protein interface of the parent domain of the first engineered immunoglobulin chain comprises V185W and S183V or comprises V185W and S183V and conservative amino acid substitutions thereof , and/or wherein the amino acid residues which are substituted in the protein-protein interface of the parent domain of the second engineered immunoglobulin chain comprises S174A or S174A and conservative amino acid substitutions thereof and/or a further amino acid selected from the group consisting of S114E, F116A, F118F, S131T, N137T, S162D, E165L, D167E, T178R, T180R or selected from the group consisting of S114E, F116A, F118F, S131T, N137T, S162D, E165L, D167E, T178R, T180R and conservative amino acid substitutions thereof, wherein the amino acid position is indicated according to the EU numbering.

In some embodiments the parent domain of the first engineered immunoglobulin chain is an IGKC domain and the parent domain of the second engineered immunoglobulin chain is a CH1 domain, wherein the amino acid residues which are substituted in the protein-protein interface of the parent domain of the first engineered immunoglobulin chain comprises S176F and/or T178W or comprises S176F and/or T178W and conservative amino acid substitutions thereof, and/or wherein the amino acid residues which are substituted in the protein-protein

interface of the parent domain of the second engineered immunoglobulin chain comprises H168F and/or F170A or comprises H168F and/or F170A and conservative amino acid substitutions thereof, wherein the amino acid position is indicated according to the EU numbering.

In some embodiments the parent domain of the first engineered immunoglobulin chain is an IGKC domain and the parent domain of the second engineered immunoglobulin chain is a CH1 domain, wherein the amino acid residues which are substituted in the protein-protein interface of the parent domain of the first engineered immunoglobulin chain comprises T178W or comprises T178W and conservative amino acid substitutions thereof, and/or wherein the amino acid residues which are substituted in the protein-protein interface of the parent domain of the second engineered immunoglobulin chain comprises L128M or comprises L128M and conservative amino acid substitutions thereof, wherein the amino acid position is indicated according to the EU numbering.

In some embodiments the parent domain of the first engineered immunoglobulin chain is an IGKC domain and the parent domain of the second engineered immunoglobulin chain is a CH1 domain, wherein the amino acid residues which are substituted in the protein-protein interface of the parent domain of the first engineered immunoglobulin chain comprises T178W or comprises T178W and conservative amino acid substitutions thereof, and/or wherein the amino acid residues which are substituted in the protein-protein interface of the parent domain of the second engineered immunoglobulin chain comprises F170A or comprises F170A and conservative amino acid substitutions thereof, wherein the amino acid position is indicated according to the EU numbering.

In some embodiments the parent domain of the first engineered immunoglobulin chain is a CH1 domain and the parent domain of the second engineered immunoglobulin chain is an IGKC domain, wherein the amino acid residues which are substituted in the protein-protein interface of the parent domain of the first engineered immunoglobulin chain comprises S183Y or S183Y and conservative amino acid substitutions thereof and a further amino acid selected from the group consisting of S124Q, F126Y, K133D, S134E, T139Q, A141S, G143T, H168K, F170T, Q175D, S181F, and V185K or selected from the group consisting of S124Q, F126Y, K133D, S134E, T139Q, A141S, G143T, H168K, F170T, Q175D, S181F, and V185K

and conservative amino acid substitutions thereof, and/or wherein the amino acid residues which are substituted in the protein-protein interface of the parent domain of the second engineered immunoglobulin chain comprises S174F and/or S176Y or comprises S174F and/or S176Y and conservative amino acid substitutions thereof, wherein the amino acid position is indicated according to the EU numbering.

In some embodiments the parent domain of the first engineered immunoglobulin chain is a CH1 domain and the parent domain of the second engineered immunoglobulin chain is an IGKC domain, wherein the amino acid residues which are substituted in the protein-protein interface of the parent domain of the first engineered immunoglobulin chain comprises S183Y or S183Y and conservative amino acid substitutions thereof and a further amino acid selected from the group consisting of S124Q, F126Y, K133D, S134E, T139Q, A141S, G143T, H168K, F170T, Q175D, S181F, and V185K or selected from the group consisting of S124Q, F126Y, K133D, S134E, T139Q, A141S, G143T, H168K, F170T, Q175D, S181F, and V185K and conservative amino acid substitutions thereof, and/or wherein the amino acid residues which are substituted in the protein-protein interface of the parent domain of the second engineered immunoglobulin chain comprises S162T or S162T and conservative amino acid substitutions thereof and a further amino acid selected from the group consisting of S114Q, F116Y, F118L, E123D, Q124E, V133T, N137K, Q160K, T164V, S174F, S176Y, and T178K or selected from the group consisting of S114Q, F116Y, F118L, E123D, Q124E, V133T, N137K, Q160K, T164V, S174F, S176Y, and T178K and conservative amino acid substitutions thereof, wherein the amino acid position is indicated according to the EU numbering.

In some embodiments the parent domain of the engineered domain of the third engineered immunoglobulin chain is an IGKC domain and the amino acid residue which is substituted in the protein-protein interface of the parent domain of the engineered domain of the third engineered immunoglobulin chain comprises an amino acid residue at a position selected from the group consisting of 114, 116, 118, 123, 124, 131, 133, 137, 138, 160, 162, 164, 165, 167, 174, 176, 178, and 180, wherein the amino acid position of each group member is indicated according to the EU numbering.

In some embodiments the parent domain of the engineered domain of the third engineered immunoglobulin chain is an IGKC domain and the amino acid residue which is substituted in the protein-protein interface of the parent domain of the engineered domain of the third engineered immunoglobulin chain comprises an amino acid residue selected from the group consisting of S114E, S114K, S114Q, F116A, F116T, F116Y, F118M, F118L, E123D, Q124E, S131K, S131T, S131N, V133L, V133A, V133T, N137T, N137E, N137K, N138K, N138E, Q160Y, Q160F, Q160K, S162A, S162G, S162D, S162T, T164V, T164M, E165L, D167E, D167S, S174A, S174M, S174N, S174F, S176V, S176F, S176Y, T178W, T178R, T178L, T178K, T180N, T180R, and T180K or comprises an amino acid residue selected from the group consisting of S114E, S114K, S114Q, F116A, F116T, F116Y, F118M, F118L, E123D, Q124E, S131K, S131T, S131N, V133L, V133A, V133T, N137T, N137E, N137K, N138K, N138E, Q160Y, Q160F, Q160K, S162A, S162G, S162D, S162T, T164V, T164M, E165L, D167E, D167S, S174A, S174M, S174N, S174F, S176V, S176F, S176Y, T178W, T178R, T178L, T178K, T180N, T180R, and T180K and conservative amino acid substitutions thereof, wherein the amino acid position of each group member is indicated according to the EU numbering.

In some embodiments the parent domain of the further engineered domain of the first and/or second engineered immunoglobulin chain is a CH1 domain and the amino acid residue which is substituted in the protein-protein interface of the parent domain of the further engineered domain of the first and/or second engineered immunoglobulin chain comprises an amino acid residue at a position selected from the group consisting of 124, 126, 128, 133, 134, 139, 141, 143, 147, 148, 168, 170, 173, 175, 181, 183, 185, and 187, wherein the amino acid position of each group member is indicated according to the EU numbering.

In some embodiments the parent domain of the further engineered domain of the first and/or second engineered immunoglobulin chain is a CH1 domain and the amino acid residue which is substituted in the protein-protein interface of the parent domain of the further engineered domain of the first and/or second engineered immunoglobulin chain comprises an amino acid residue selected from the group consisting of S124E, S124K, S124Q, F126A, F126T, F126Y, L128F, L128M, K133D, S134E, T139Q, A141K, A141T, A141N, A141S, G143V, G143A, G143L, G143T, K147T, K147E, D148E, D148K, H168Y, H168F, H168K, F170D, F170A, F170G, F170T, V173L, V173M, Q175E, Q175S, Q175D, S181A, S181N, S181M, S181F,

S183V, S183F, S183Y, V185W, V185R, V185L, V185K, T187N, T187K and T187R or comprises an amino acid residue selected from the group consisting of S124E, S124K, S124Q, F126A, F126T, F126Y, L128F, L128M, K133D, S134E, T139Q, A141K, A141T, A141N, A141S, G143V, G143A, G143L, G143T, K147T, K147E, D148E, D148K, H168Y, H168F, H168K, F170D, F170A, F170G, F170T, V173L, V173M, Q175E, Q175S, Q175D, S181A, S181N, S181M, S181F, S183V, S183F, S183Y, V185W, V185R, V185L, V185K, T187N, T187K and T187R and conservative amino acid substitutions thereof, wherein the amino acid position of each group member is indicated according to the EU numbering.

In some embodiments the parent domain of the engineered domain of the third engineered immunoglobulin chainis an IGKC domain and the amino acid residue which is substituted in the protein-protein interface of the parent domain of the engineered domain of the third engineered immunoglobulin chain comprises an amino acid residue at a position selected from the group consisting of 131, 137, 160, 176, 178, and 180, and/or the parent domain of the further engineered domain of the first and/or second engineered immunoglobulin chain is a CH1 domain and the amino acid residue which is substituted in the protein-protein interface of the parent domain of the further engineered domain of the first and/or second engineered immunoglobulin chain comprises an amino acid residue at a position selected from the group consisting of 124, 126, 128, 141, 143, 147, 170, 173, 175, 181, 185, and 187, wherein the amino acid position of each group member is indicated according to the EU numbering.

In some embodiments the parent domain of the engineered domain of the third engineered immunoglobulin chain is an IGKC domain and the amino acid residue which is substituted in the protein-protein interface of the parent domain of the engineered domain of the third engineered immunoglobulin chain comprises an amino acid residue at postion 178 or comprises T178W or comprises T178W and conservative amino acid substitutions thereof and optionally a further amino acid residue selected from the group consisting of 131, 137, 160, 176, 180, and/or the parent domain of the further engineered domain of the first and/or second engineered immunoglobulin chain is a CH1 domain and the amino acid residue which is substituted in the protein-protein interface of the parent domain of the further engineered domain of the first and/or second engineered immunoglobulin chain comprises an amino acid residue at postion 181or comprises S181A or comprises S181A and conservative amino acid substitutions thereof and optionally a further amino acid residue selected from the group

consisting of 124, 126, 128, 141, 143, 147, 170, 173, 175, 185, and 187, wherein the amino acid position of each group member is indicated according to the EU numbering.

In some embodiments the parent domain of the engineered domain of the third engineered immunoglobulin chain is an IGKC domain and the amino acid residue which is substituted in the protein-protein interface of the parent domain of the engineered domain of the third engineered immunoglobulin chain comprises an amino acid residue selected from the group consisting of S131K, N137T, Q160Y, S176V, T178W, T180N or comprises an amino acid residue selected from the group consisting of S131K, N137T, Q160Y, S176V, T178W, T180N and conservative amino acid substitutions thereof, and/or the parent domain of the further engineered domain of the first and/or second engineered immunoglobulin chain is a CH1 domain and the amino acid residue which is substituted in the protein-protein interface of the parent domain of the further engineered domain of the first and/or second engineered immunoglobulin chain comprises an amino acid residue selected from the group consisting of S124E, F126A, L128F, A141T, G143V, K147T, F170D, V173L, Q175E, S181A, V185R, and T187R or are selected from the group consisting of S124E, F126A, L128F, A141T, G143V, K147T, F170D, V173L, Q175E, S181A, V185R, and T187R and conservative amino acid substitutions thereof, wherein the amino acid position of each group member is indicated according to the EU numbering.

In some embodiments the parent domain of the engineered domain of the third engineered immunoglobulin chain is an IGKC domain and the amino acid residue which is substituted in the protein-protein interface of the parent domain of the engineered domain of the third engineered immunoglobulin chain comprises an amino acid residue at a position selected from the group consisting of 114, 116, 131, 137, 162, 165, 167, 174, 176, 178, 180, and/or the parent domain of the further engineered domain of the first and/or second engineered immunoglobulin chain is a CH1 domain and the amino acid residue which is substituted in the protein-protein interface of the parent domain of the further engineered domain of the first and/or second engineered immunoglobulin chain comprises an amino acid residue at a position selected from the group consisting of 141, 143, 147, 168, 183, 185 and 187, wherein the amino acid position of each group member is indicated according to the EU numbering.

In some embodiments the parent domain of the engineered domain of the third engineered

immunoglobulin chain is an IGKC domain and the amino acid residue which is substituted in the protein-protein interface of the parent domain of the engineered domain of the third engineered immunoglobulin chain comprises the amino acid residue at postion 174 or comprises 174A or comprises 174A and conservative amino acid substitutions thereof and optionally a further amino acid residue selected from the group consisting of 114, 116, 131, 137, 162, 165, 167, 178, and 180, and/or the parent domain of the further engineered domain of the first and/or second engineered immunoglobulin chain is a CH1 domain and the amino acid residue which is substituted in the protein-protein interface of the parent domain of the further engineered domain of the first and/or second engineered immunoglobulin chain comprises an amino acid residue at postion 185 or comprises V185W or comprises V185W and conservative amino acid substitutions thereof and optionally a further amino acid residue selected from the group consisting of 141, 143, 147, 168, 183, and 187, wherein the amino acid position of each group member is indicated according to the EU numbering.

In some embodiments the parent domain of the engineered domain of the third engineered immunoglobulin chain is an IGKC domain and the amino acid residue which is substituted in the protein-protein interface of the parent domain of the engineered domain of the third engineered immunoglobulin chain comprises an amino acid residue selected from the group consisting of S114E, F116A, S131T, N137T, S162D, E165L, D167E, S174A, T178R, T180R or comprises an amino acid residue selected from the group consisting of S114E, F116A, S131T, N137T, S162D, E165L, D167E, S174A, T178R, T180R and conservative amino acid substitutions thereof, and/or the parent domain of the further engineered domain of the first and/or second engineered immunoglobulin chain is a CH1 domain and the amino acid residue which is substituted in the protein-protein interface of the parent domain of the further engineered domain of the first and/or second engineered immunoglobulin chain comprises an amino acid residue selected from the group consisting of A141K, G143V, K147T, H168Y, S183V, V185W, and T187N or comprises an amino acid residue selected from the group consisting of A141K, G143V, K147T, H168Y, S183V, V185W, and T187N and conservative amino acid substitutions thereof, wherein the amino acid position of each group member is indicated according to the EU numbering.

In some embodiments the parent domain of the engineered domain of the third engineered immunoglobulin chain is an IGKC domain and the amino acid residue which is substituted in

the protein-protein interface of the parent domain of the engineered domain of the third engineered immunoglobulin chain comprises an amino acid residue at a position selected from the group consisting of 114, 116, 131, 133, 137, 138, 162, 164, 174, 176, and 178, and/or the parent domain of the further engineered domain of the first and/or second engineered immunoglobulin chain is a CH1 domain and the amino acid residue which is substituted in the protein-protein interface of the parent domain of the further engineered domain of the first and/or second engineered immunoglobulin chain comprises an amino acid residue at a position selected from the group consisting of 128 , 141, 143, 148, 168, 170, 175, 181, 183, 185, and 187, wherein the amino acid position of each group member is indicated according to the EU numbering.

In some embodiments the parent domain of the engineered domain of the third engineered immunoglobulin chain is an IGKC domain and the amino acid residue which is substituted in the protein-protein interface of the parent domain of the engineered domain of the third engineered immunoglobulin chain comprises the amino acid residue at postion 178 or comprises T178W or comprises T178W and conservative amino acid substitutions thereof and optionally a further amino acid residue selected from the group consisting of 114,116, 131, 133, 137, 138, 162, 164, 174, and 176, and/or the parent domain of the further engineered domain of the first and/or second engineered immunoglobulin chain is a CH1 domain and the amino acid residue which is substituted in the protein-protein interface of the parent domain of the further engineered domain of the first and/or second engineered immunoglobulin chain comprises an amino acid residue at postion 183 or comprises S183V or comprises S183V and conservative amino acid substitutions thereof and optionally a further amino acid residue selected from the group consisting of 128 , 141, 143, 148, 168, 170, 175, 181, 185, and 187, wherein the amino acid position of each group member is indicated according to the EU numbering.

In some embodiments the parent domain of the engineered domain of the third engineered immunoglobulin chain is an IGKC domain and the amino acid residue which is substituted in the protein-protein interface of the parent domain of the engineered domain of the third engineered immunoglobulin chain comprises an amino acid residue selected from the group consisting of S114K, F116T, S131T, V133L, N137E, N138K, S162G, T164M, S174M, S176F, and T178W or comprises an amino acid residue selected from the group consisting of

S114K, F116T, S131T, V133L, N137E, N138K, S162G, T164M, S174M, S176F, and T178W and conservative amino acid substitutions thereof, and/or the parent domain of the further engineered domain of the first and/or second engineered immunoglobulin chain is a CH1 domain and the amino acid residue which is substituted in the protein-protein interface of the parent domain of the further engineered domain of the first and/or second engineered immunoglobulin chain comprises an amino acid residue selected from the group consisting of L128M , A141N, G143A, D148E, H168F, F170A, Q175S, S181N, S183V, V185L, and T187K or comprises an amino acid residue selected from the group consisting of L128M , A141N, G143A, D148E, H168F, F170A, Q175S, S181N, S183V, V185L, and T187K and conservative amino acid substitutions thereof, wherein the amino acid position of each group member is indicated according to the EU numbering.

In some embodiments the parent domain of the engineered domain of the third engineered immunoglobulin chain is an IGKC domain and the amino acid residue which is substituted in the protein-protein interface of the parent domain of the engineered domain of the third engineered immunoglobulin chain comprises an amino acid residue at a position selected from the group consisting of 118, 131, 133, 138, 160, 162, 167, 174, 176, 178, and 180 and/or the parent domain of the further engineered domain of the first and/or second engineered immunoglobulin chain is a CH1 domain and the amino acid residue which is substituted in the protein-protein interface of the parent domain of the further engineered domain of the first and/or second engineered immunoglobulin chain comprises an amino acid residue at a position selected from the group consisting of 124, 126, 141, 143, 147, 148, 170, 173, 181, 183 and 185, wherein the amino acid position of each group member is indicated according to the EU numbering.

In some embodiments the parent domain of the engineered domain of the third engineered immunoglobulin chain is an IGKC domain and the amino acid residue which is substituted in the protein-protein interface of the parent domain of the engineered domain of the third engineered immunoglobulin chain comprises the amino acid residue at postion 176 or comprises S176V or comprises S176V and conservative amino acid substitutions thereof and optionally a further amino acid residue selected from the group consisting of 118, 131, 133, 138, 160, 162, 167, 174, 178, 180, and/or the parent domain of the further engineered domain of the first and/or second engineered immunoglobulin chain is a CH1 domain and the amino acid residue which is substituted in the protein-protein interface of the parent domain of the

further engineered domain of the first and/or second engineered immunoglobulin chain comprises an amino acid residue at postion 185 or comprises V185W or comprises V185W and conservative amino acid substitutions thereof and optionally a further amino acid residue selected from the group consisting of 124, 126, 141, 143, 147, 148, 170, 173, 181, and 183, wherein the amino acid position of each group member is indicated according to the EU numbering.

In some embodiments the parent domain of the engineered domain of the third engineered immunoglobulin chain is an IGKC domain and the amino acid residue which is substituted in the protein-protein interface of the parent domain of the engineered domain of the third engineered immunoglobulin chain comprises an amino acid residue selected from the group consisting of F118M, S131N, V133A, N138E, Q160F, S162A, D167S, S174N, S176V, and T178L or comprises an amino acid residue selected from the group consisting of F118M, S131N, V133A, N138E, Q160F, S162A, D167S, S174N, S176V, and T178L and conservative amino acid substitutions thereof, and T180K and/or the parent domain of the further engineered domain of the first and/or second engineered immunoglobulin chain is a CH1 domain and the amino acid residue which is substituted in the protein-protein interface of the parent domain of the further engineered domain of the first and/or second engineered immunoglobulin chain comprises an amino acid residue selected from the group consisting of S124K, F126T, A141T, G143L, K147E, D148K, F170G, V173M, S181M, S183F, and V185W or comprises an amino acid residue selected from the group consisting of S124K, F126T, A141T, G143L, K147E, D148K, F170G, V173M, S181M, S183F, and V185W and conservative amino acid substitutions thereof, wherein the amino acid position of each group member is indicated according to the EU numbering.

In some embodiments the parent domain of the engineered domain of the third engineered immunoglobulin chain is an IGKC domain and the amino acid residue which is substituted in the protein-protein interface of the parent domain of the engineered domain of the third engineered immunoglobulin chain comprises an amino acid residue at a position selected from the group consisting of 114, 116, 118, 123, 124, 133, 137, 160, 162, 164, 174, 176, and 178, and/or the parent domain of the further engineered domain of the first and/or second engineered immunoglobulin chain is a CH1 domain and the amino acid residue which is substituted in the protein-protein interface of the parent domain of the further engineered

domain of the first and/or second engineered immunoglobulin chain comprises an amino acid residue at a position selected from the group consisting of 124, 126, 133, 134, 139, 141, 143, 168, 170, 175, 181, 183, and 185, wherein the amino acid position of each group member is indicated according to the EU numbering.

In some embodiments the parent domain of the engineered domain of the third engineered immunoglobulin chain is an IGKC domain and the amino acid residue which is substituted in the protein-protein interface of the parent domain of the engineered domain of the third engineered immunoglobulin chain comprises the amino acid residue at postion 176 or comprises S176Y or comprises S176Y and conservative amino acid substitutions thereof and optionally a further amino acid residue selected from the group consisting of 114, 116, 118, 123, 124, 133, 137, 160, 162, 164, 174, and 178, and/or the parent domain of the further engineered domain of the first and/or second engineered immunoglobulin chain is a CH1 domain and the amino acid residue which is substituted in the protein-protein interface of the parent domain of the further engineered domain of the first and/or second engineered immunoglobulin chain comprises the amino acid residue at postion 183 or comprises S183Y or comprises S183Y and conservative amino acid substitutions thereof and optionally a further amino acid residue selected from the group consisting of 124, 126, 133, 134, 139, 141, 143, 168, 170, 175, 181, and 185, wherein the amino acid position of each group member is indicated according to the EU numbering.

In some embodiments the parent domain of the engineered domain of the third engineered immunoglobulin chain is an IGKC domain and the amino acid residue which is substituted in the protein-protein interface of the parent domain of the engineered domain of the third engineered immunoglobulin chain comprises an amino acid residue selected from the group consisting of S114Q, F116Y, F118L, E123D, Q124E, V133T, N137K, Q160K, S162T, T164V, S174F, S176Y, and T178K or are selected from the group consisting of S114Q, F116Y, F118L, E123D, Q124E, V133T, N137K, Q160K, S162T, T164V, S174F, S176Y, and T178K and conservative amino acid substitutions thereof, and/or the parent domain of the further engineered domain of the first and/or second engineered immunoglobulin chain is a CH1 domain and the amino acid residue which is substituted in the protein-protein interface of the parent domain of the further engineered domain of the first and/or second engineered immunoglobulin chain comprises an amino acid residue selected from the group consisting of

S124Q, F126Y, K133D, S134E, T139Q, A141S, G143T, H168K, F170T, Q175D, S181F, S183Y, and V185K or are selected from the group consisting of S124Q, F126Y, K133D, S134E, T139Q, A141S, G143T, H168K, F170T, Q175D, S181F, S183Y, and V185K and conservative amino acid substitutions thereof, wherein the amino acid position of each group member is indicated according to the EU numbering.

In some embodiments the parent domain of the further engineered domain of the first and/or second engineered immunoglobulin chain is a CH1 domain and the parent domain of the engineered domain of the third engineered immunoglobulin chain is a IGKC domain, wherein the amino acid residues which are substituted in the protein-protein interface of the parent domain of the further engineered domain of the first and/or second engineered immunoglobulin chain comprises V185W and S183V or comprises V185W and S183V and conservative amino acid substitutions thereof, and/or the amino acid residues which are substituted in the protein-protein interface of the parent domain of the engineered domain of the third engineered immunoglobulin chain comprises S174A or S174A and conservative amino acid substitutions thereof and/or a further amino acid selected from the group consisting of S114E, F116A, S131T, N137T, S162D, E165L, D167E, T178R, T180R or are selected from the group consisting of S114E, F116A, S131T, N137T, S162D, E165L, D167E, T178R, T180R and conservative amino acid substitutions thereof, wherein the amino acid position is indicated according to the EU numbering.

In some embodiments the parent domain of the engineered domain of the third engineered immunoglobulin chain is an IGKC domain and the parent domain of the further engineered domain of the first and/or second engineered immunoglobulin chain is a CH1 domain, wherein the amino acid residues which are substituted in the protein-protein interface of the parent domain of the engineered domain of the third engineered immunoglobulin chain comprises S176F and/or T178W or comprises S176F and/or T178W and conservative amino acid substitutions thereof, and/or wherein the amino acid residues which are substituted in the protein-protein interface of the parent domain of the further engineered domain of the first and/or second engineered immunoglobulin chain comprises H168F and/or F170A or comprises H168F and/or F170A and conservative amino acid substitutions thereof, wherein the amino acid position is indicated according to the EU numbering.

In some embodiments the parent domain of the engineered domain of the third engineered immunoglobulin chain is an IGKC domain and the parent domain of the further engineered domain of the first and/or second engineered immunoglobulin chain is a CH1 domain, wherein the amino acid residue which is substituted in the protein-protein interface of the parent domain of the engineered domain of the third engineered immunoglobulin chain comprises T178W or comprises T178W and conservative amino acid substitutions thereof , and/or wherein the amino acid residue which is substituted in the protein-protein interface of the parent domain of the further engineered domain of the first and/or second engineered immunoglobulin chain comprises L128M or comprises L128M and conservative amino acid substitutions thereof, wherein the amino acid position is indicated according to the EU numbering.

In some embodiments the parent domain of the engineered domain of the third engineered immunoglobulin chain is an IGKC domain and the parent domain of the further engineered domain of the first and/or second engineered immunoglobulin chain is a CH1 domain, wherein the amino acid residues which are substituted in the protein-protein interface of the parent domain of the engineered domain of the third engineered immunoglobulin chain comprises T178W or comprises T178W and conservative amino acid substitutions thereof, and/or wherein the amino acid residues which are substituted in the protein-protein interface of the parent domain of the further engineered domain of the first and/or second engineered immunoglobulin chain comprises F170A or comprises F170A and conservative amino acid substitutions thereof, wherein the amino acid position is indicated according to the EU numbering.

In some embodiments the parent domain of the further engineered domain of the first and/or second engineered immunoglobulin chain is a CH1 domain and the parent domain of the engineered domain of the third engineered immunoglobulin chain is an IGKC domain, wherein the amino acid residues which are substituted in the protein-protein interface of the parent domain of the further engineered domain of the first and/or second engineered immunoglobulin chain comprises S183Y or comprises S183Y and conservative amino acid substitutions thereof and a further amino acid selected from the group consisting of S124Q, F126Y, K133D, S134E, T139Q, A141S, G143T, H168K, F170T, Q175D, S181F, and V185K or are selected from the group consisting of S124Q, F126Y, K133D, S134E, T139Q, A141S,

G143T, H168K, F170T, Q175D, S181F, and V185K and conservative amino acid substitutions thereof, and/or wherein the amino acid residues which are substituted in the protein-protein interface of the parent domain of the engineered domain of the third engineered immunoglobulin chain comprises S174F and/or S176Y or comprises S174F and/or S176Y and conservative amino acid substitutions thereof, wherein the amino acid position is indicated according to the EU numbering.

In some embodiments the parent domain of the further engineered domain of the first and/or second engineered immunoglobulin chain is a CH1 domain and the parent domain of the engineered domain of the third engineered immunoglobulin chain is an IGKC domain, wherein the amino acid residues which are substituted in the protein-protein interface of the parent domain of the further engineered domain of the first and/or second engineered immunoglobulin chain comprises S183Y or comprises S183Y and conservative amino acid substitutions thereof and a further amino acid selected from the group consisting of S124Q, F126Y, K133D, S134E, T139Q, A141S, G143T, H168K, F170T, Q175D, S181F, and V185K or are selected from the group consisting of S124Q, F126Y, K133D, S134E, T139Q, A141S, G143T, H168K, F170T, Q175D, S181F, and V185K and conservative amino acid substitutions thereof, and/or wherein the amino acid residues which are substituted in the protein-protein interface of the parent domain of the engineered domain of the third engineered immunoglobulin chain comprises S162T or comprises S162T and conservative amino acid substitutions thereof and a further amino acid selected from the group consisting of S114Q, F116Y, F118L, E123D, Q124E, V133T, N137K, Q160K, T164V, S174F, S176Y, and T178K or are selected from the group consisting of S114Q, F116Y, F118L, E123D, Q124E, V133T, N137K, Q160K, T164V, S174F, S176Y, and T178K and conservative amino acid substitutions thereof, wherein the amino acid position is indicated according to the EU numbering.

In some embodiments the present disclosure provides a hetero-dimeric immunoglobulin or fragment thereof, wherein the parent domain of the first and/or the second engineered immunoglobulin chain is a CH3 domain, wherein the amino acid residue which is substituted in the protein-protein interface of the parent domain of the first engineered immunoglobulin chain comprises the amino acid residue at position 88, and optionally a further amino acid residue at a position selected from the group consisting of 3, 5, 7, 20, 22, 26, 27, 79, 81, 84,

84.2, 85.1, 86, and 90 and, wherein the amino acid residue which is substituted in the protein-protein interface of the parent domain of the second engineered immunoglobulin chain comprises the amino acid residue at position 85.1 and/or 86 and optionally a further amino acid residue at a position selected from the group consisting of 3, 5, 7, 20, 22, 26, 27, 79, 81, 84, 84.2, 88, and 90 or at a position selected from the group consisting of 3, 5, 7, 20, 22, 26, 27, 79, 81, 84, 84.2, 85.1, 88, and 90 or at a position selected from the group consisting of 3, 5, 7, 20, 22, 26, 27, 79, 81, 84, 84.2, 86, 88, and 90,

wherein the amino acid residue substituted at position 88 in the parent domain of the first engineered immunoglobulin chain is interacting with the amino acid residue substituted at position 85.1 and/or 86 in the parent domain of the second engineered immunoglobulin chain, wherein the amino acid position of each group member is indicated according to the IMGT® numbering.

Positions 3, 5, 7, 20, 22, 26, 27, 79, 81, 84, 84.2, 85.1, 86, 88 and 90 in the protein-protein interface of the CH3 domain as parent domain indicated according to IMGT® numbering correspond in the same order to positions 347, 349, 351, 364, 366, 370, 371, 392, 394, 397, 399, 405, 407, 409 and 411 indicated according to the EU numbering,

In some embodiments the parent domain of the first and the second engineered immunoglobulin chain is a CH3 domain.

In some embodiments the parent domains of the first and the second engineered immunoglobulin chain are CH3 domains of different isotypes.

In some embodiments the parent domain of the first engineered immunoglobulin chain is an IgG1 CH3 domain, preferably a human CH3 domain from IGHG1 and the parent domain of the second engineered immunoglobulin chain is an IgG3 CH3 domain, preferably a human CH3 domain from IGHG3 or wherein the parent domain of the first engineered immunoglobulin chain is an IgG3 CH3 domain, preferably a human CH3 domain from IGHG3 and the parent domain of the second engineered immunoglobulin chain is an IgG1 CH3 domain preferably a human CH3 domain from IGHG1.

In some embodiments the amino acid residue which is substituted in the protein-protein interface of the parent domain of the second engineered immunoglobulin chain comprises the amino acid residue at position 85.1 and 86, wherein the amino acid position is indicated according to the IMGT® numbering.

In some embodiments the amino acid residue which is substituted in the protein-protein interface of the parent domain of the first engineered immunoglobulin chain at position 88 is

not a charged amino acid, wherein the amino acid position is indicated according to the IMGT® numbering.

In some embodiments the amino acid residue which is substituted in the protein-protein interface of the parent domain of the first engineered immunoglobulin chain at position 88 is not 88I, wherein the amino acid position is indicated according to the IMGT® numbering.

In some embodiments the amino acid residue which is substituted in the protein-protein interface of the parent domain of the first engineered immunoglobulin chain at position 85.1 and/or 86 is not a charged amino acid, wherein the amino acid position is indicated according to the IMGT® numbering.

In some embodiments the amino acid residue which is substituted in the protein-protein interface of the parent domain of the first engineered immunoglobulin chain at position 88 and the amino acid residue which is substituted in the protein-protein interface of the parent domain of the first engineered immunoglobulin chain at position 85.1 and/or 86 are not a charged pair.

In some embodiments the amino acid residue which is substituted in the protein-protein interface of the parent domain of the first engineered immunoglobulin chain at position 88 is 88W or 88W and conservative amino acid substitutions thereof, wherein the amino acid position is indicated according to the IMGT® numbering

In some embodiments the CH3 domain is a domain selected from the group consisting of human IgG1 (IGHG1), human IgG2 (IGHG2), human IgG3 (IGHG3) and human IgGP (IGHGP) and the amino acid residue which is substituted in the protein-protein interface of the parent domain of the first engineered immunoglobulin chain is K88W or K88W and conservative amino acid substitutions thereof and optionally a further amino acid residue selected from the group consisting of 3, 5, 7, 20, 22, 26, 27, 79, 81, 84, 84.2, 85.1, 86, and 90, wherein the amino acid position is indicated according to the IMGT® numbering.

In some embodiments the CH3 domain is a human IgA1 (IGHA1) or a IgA2 (IGHA2) CH3 domain and the amino acid residue which is substituted in the protein-protein interface of the parent domain of the first engineered immunoglobulin chain is I88W or I88W and conservative amino acid substitutions thereof and optionally a further amino acid residue selected from the group consisting of 3, 5, 7, 20, 22, 26, 27, 79, 81, 84, 84.2, 85.1, 86, and 90, wherein the amino acid position is indicated according to the IMGT® numbering.

In some embodiments the CH3 domain is a human IgD (IGHD) CH3 domain and the amino acid residue which is substituted in the protein-protein interface of the parent domain of the

first engineered immunoglobulin chain is V88W or V88W and conservative amino acid substitutions thereof and optionally a further amino acid residue selected from the group consisting of 3, 5, 7, 20, 22, 26, 27, 79, 81, 84, 84.2, 85.1, 86, and 90, wherein the amino acid position is indicated according to the IMGT® numbering.

In some embodiments the CH3 domain is a human IgE CH3 (IGHE) domain and the amino acid residue which is substituted in the protein-protein interface of the parent domain of the first engineered immunoglobulin chain is T88W or T88W and conservative amino acid substitutions thereof and optionally a further amino acid residue selected from the group consisting of 3, 5, 7, 20, 22, 26, 27, 79, 81, 84, 84.2, 85.1, 86, and 90, wherein the amino acid position is indicated according to the IMGT® numbering.

In some embodiments the CH3 domain is a human IgG4 (IGHG4) CH3 domain and the amino acid residue which is substituted in the protein-protein interface of the parent domain of the first engineered immunoglobulin chain is R88W or R88W and conservative amino acid substitutions thereof and optionally a further amino acid residue selected from the group consisting of 3, 5, 7, 20, 22, 26, 27, 79, 81, 84, 84.2, 85.1, 86, and 90, wherein the amino acid position is indicated according to the IMGT® numbering.

In some embodiments the CH3 domain is a human IgM (IGHM) CH3 domain and the amino acid residue which is substituted in the protein-protein interface of the parent domain of the first engineered immunoglobulin chain is E88W or E88W and conservative amino acid substitutions thereof and optionally a further amino acid residue selected from the group consisting of 3, 5, 7, 20, 22, 26, 27, 79, 81, 84, 84.2, 85.1, 86, and 90, wherein the amino acid position is indicated according to the IMGT® numbering.

In some embodiments the amino acid residue which is substituted in the protein-protein interface of the parent domain of the first engineered immunoglobulin chain at position 88 is selected from the group consisting of 88Q, 88L, 88V, 88R, 88E, 88I, 88T, 88Y, 88K and 88W and/or the further amino acid residue substituted in the protein-protein interface of the parent domain of the first engineered immunoglobulin chain is selected from the group consisting of 20V, 20T, 20A, 20N, 20Q, 20E, 20S, 20K, 20W, 22A, 22G, 22T, 22L, 22I, 22V, 26R, 26Q, 26T, 26K, 26V, 26S, 26N, 26E, 85.1T, 85.1M, 85.1A, 85.1S, 85.1R, 85.1H, 85.1K, 85.1F, 85.1C, 85.1N, 85.1W, 86S, 86I, 86T, 86H, 86Q, 86V, 86W, 86Y and 86F, wherein the amino acid position of each group member is indicated according to the IMGT® numbering.

In some embodiments the amino acid residue which is substituted in the protein-protein interface of the parent domain of the first engineered immunoglobulin chain at position 88 is selected from the group consisting of 88Q, 88L, 88V, 88R, 88E, 88I, 88T, 88Y, and 88W, wherein the amino acid position is indicated according to the IMGT® numbering.

In some embodiments the further amino acid residue substituted in the protein-protein interface of the parent domain of the first engineered immunoglobulin chain is selected from the group consisting of 20V, 20T, 20A, 20N, 20Q, 20E, 20S, 22A, 22G, 22T, 22L, 22I, 22V, 26Q, 26T, 26K, 26V, 26S, 26N, 26E, 85.1T, 85.1M, 85.1A, 85.1S, 85.1R, 85.1H, 85.1K, 85.1F, 85.1C, 85.1N, 86S, 86I, 86T, 86H, 86Q, 86V, and 86F, wherein the amino acid position of each group member is indicated according to the IMGT® numbering.

In some embodiments the further amino acid residue substituted in the protein-protein interface of the parent domain of the first engineered immunoglobulin chain is selected from the group consisting of 20V, 20T, 20A, 20N, 20Q, 20E, 20S, 20K, 22A, 22G, 22T, 22L, 22I, 22V, 26Q, 26T, 26K, 26V, 26S, 26N, 26E, 85.1T, 85.1M, 85.1A, 85.1S, 85.1R, 85.1H, 85.1K, 85.1F, 85.1C, 85.1N, 86S, 86I, 86T, 86H, 86Q, 86V, and 86F, wherein the amino acid position of each group member is indicated according to the IMGT® numbering.

In some embodiments the amino acid residue which is substituted in the protein-protein interface of the parent domain of the first engineered immunoglobulin chain at position 88 is selected from the group consisting of 88Q, 88L, 88V, 88R, 88E, 88T, 88Y, and 88W, wherein the amino acid position is indicated according to the IMGT® numbering.

In some embodiments the further amino acid residue substituted in the protein-protein interface of the parent domain of the first engineered immunoglobulin chain is selected from the group consisting of 20V, 20A, 20N, 20Q, 20E, 20S, 22A, 22G, 22T, 22L, 22I, 22V, 26Q, 26T, 26K, 26V, 26S, 26N, 26E, 85.1T, 85.1M, 85.1S, 85.1R, 85.1H, 85.1K, 85.1F, 85.1C, 85.1N, 86S, 86I, 86H, 86Q, 86V, and 86F, wherein the amino acid position of each group member is indicated according to the IMGT® numbering.

In some embodiments the further amino acid residue substituted in the protein-protein interface of the parent domain of the first engineered immunoglobulin chain is selected from the group consisting of 3E, 3K, 5A, 5T, 7F, 7M, 20K, 20N, 20T, 22A, 22L, 22V, 26E, 26T, 27E, 27K, 79F, 79Y, 81A, 81G, 81D, 84L, 84M, 84.2E, 84.2S, 85.1A, 85.1C, 85.1M, 85.1N, 85.1S, 86F, 86S, 86V, 90K, 90N, and 90R or is selected from the group consisting of 3E, 3K, 5A, 5T, 7F, 7M, 20K, 20N, 20T, 22A, 22L, 22V, 26E, 26T, 27E, 27K, 79F, 79Y, 81A, 81G,

81D, 84L, 84M, 84.2E, 84.2S, 85.1A, 85.1C, 85.1M, 85.1N, 85.1S, 86F, 86S, 86V, 90K, 90N, and 90R and conservative amino acid substitutions thereof, wherein the amino acid position of each group member is indicated according to the IMGT® numbering.

In some embodiments relating to a CH3 domain as parent domain of the first and/or the second engineered immunoglobulin chain the amino acid residue which is substituted in the protein-protein interface of the parent domain of the first engineered immunoglobulin chain is 88I and conservative amino acid substitutions thereof and the optional further amino acid residue which is substituted in the protein-protein interface of the parent domain of the first engineered immunoglobulin chain is 81W and conservative amino acid substitutions thereof, wherein the amino acid position is indicated according to the IMGT® numbering.

In some embodiments relating to a CH3 domain as parent domain of the first and/or the second engineered immunoglobulin chain the amino acid residue which is substituted in the protein-protein interface of the parent domain of the first engineered immunoglobulin chain is 88I and conservative amino acid substitutions thereof and the optional further amino acid residue which is substituted in the protein-protein interface of the parent domain of the first engineered immunoglobulin chain is 81W and conservative amino acid substitutions and/or the optional further amino acid residue or an additional further amino acid residue which is substituted in the protein-protein interface of the parent domain of the second engineered immunoglobulin chain is 86T and conservative amino acid substitutions thereof and/or 84R and conservative amino acid substitutions thereof, wherein the amino acid position is indicated according to the IMGT® numbering.

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In some embodiments the amino acid residue which is substituted in the protein-protein interface of the parent domain of the first engineered immunoglobulin chain comprises the amino acid residues at position 88 and at position 20, and optionally a further amino acid residue at a position selected from the group consisting of 3, 5, 7, 22, 26, 27, 79, 81, 84, 84.2, 85.1, 86, and 90, wherein the amino acid position of each group member is indicated according to the IMGT® numbering.

In some embodiments the amino acid residue which is substituted in the protein-protein interface of the parent domain of the second engineered immunoglobulin chain comprises the amino acid residues at position 85.1 and/or 86 and at position 26 and optionally an amino acid residue at a further position, wherein the amino acid position is indicated according to the IMGT® numbering.

In some embodiments the amino acid residue which is substituted at position 85.1 and/or 86 in the protein-protein interface of the parent domain of the second engineered immunoglobulin chain is selected from the group consisting of 85.1A, 85.1S, 85.1C and 85.1N or is selected from the group consisting of 85.1A, 85.1S, 85.1C and 85.1N and conservative amino acid substitutions thereof and/or is selected from the group consisting of 86S and 86V or is selected from the group consisting of 86S and 86V and conservative amino acid substitutions thereof, wherein the amino acid position is indicated according to the IMGT® numbering.

In some embodiments the further amino acid residue which is substituted in the protein-protein interface of the parent domain of the second engineered immunoglobulin chain is at a position selected from the group consisting of 3, 5, 7, 20, 22, 26, 81, 84, 84.2, 88, and 90 or at a position selected from the group consisting of 3, 5, 7, 20, 22, 26, 81, 84, 84.2, 85.1, 88, and 90 or at a position selected from the group consisting of 3, 5, 7, 20, 22, 26, 81, 84, 84.2, 86, 88, and 90, wherein the amino acid position of each group member is indicated according to the IMGT® numbering.

In some embodiments the further amino acid residue which is substituted in the protein-protein interface of the parent domain of the first engineered immunoglobulin chain is at a position selected from the group consisting of 20, 22, 26, 79, 85.1, 86, and 90, and/or the further amino acid residue which is substituted in the protein-protein interface of the parent domain of the second engineered immunoglobulin chain is at position 84 or is 84L or is 84L and conservative amino acid substitutions thereof, and optionally a further amino acid at a position selected from the group consisting of 3, 5, 7, 20, 22, 26, 81, 84.2, 88, and 90 or at a position selected from the group consisting of 3, 5, 7, 20, 22, 26, 81, 84.2, 85.1, 88, and 90 or at a position selected from the group consisting of 3, 5, 7, 20, 22, 26, 81, 84.2, 86, 88, and 90, wherein the amino acid position of each group member is indicated according to the IMGT® numbering.

In some embodiments the further amino acid residue substituted in the protein-protein interface of the parent domain of the first engineered immunoglobulin chain is selected from the group consisting of 20K, 22V, 26T, 79Y, 85.1S, 86V, and 90N or is selected from the

group consisting of 20K, 22V, 26T, 79Y, 85.1S, 86V, and 90N and conservative amino acid substitutions thereof and/or the amino acid residue which is substituted in the protein-protein interface of the parent domain of the second engineered immunoglobulin chain comprises the amino acid residues at position 85.1 and 86 or comprises 85.1C and conservative amino acid substitutions thereof and 86S and conservative amino acid substitutions thereof, and the further amino acid residue which is substituted in the protein-protein interface of the parent domain of the second engineered immunoglobulin chain is selected from the group consisting of 3, 5, 7, 20, 22, 26, 81, 84, 84.2, 88, and 90 , preferably from the group consisting of 3E, 5A, 7F, 20T, 22V, 26T, 81D, 84L, 84.2E, 88R and 90R or from the group consisting of 3E, 5A, 7F, 20T, 22V, 26T, 81D, 84L, 84.2E, 88R and 90R and conservative amino acid substitutions thereof, wherein the amino acid position of each group member is indicated according to the IMGT® numbering.

In some embodiments the amino acid residue substitution 85.1C is replaced by amino acid residue substitution 85.1A or 85.1S wherein the amino acid position of each group member is indicated according to the IMGT® numbering.

In some embodiments the further amino acid residue substituted in the protein-protein interface of the parent domain of the first engineered immunoglobulin chain is at a position selected from the group consisting of 3, 5, 20, 22, 26, 27, 81, 84, 85.1, and 86, and/or the further amino acid residue which is substituted in the protein-protein interface of the parent domain of the second engineered immunoglobulin chain is at a position selected from the group consisting of 7, 20, 22, 27, 79, 81, 84.2, 88, and 90 or at a position selected from the group consisting of 7, 20, 22, 27, 79, 81, 84.2, 85.1, 88, and 90 or at a position selected from the group consisting of 7, 20, 22, 27, 79, 81, 84.2, 86, 88, and 90, wherein the amino acid position of each group member is indicated according to the IMGT® numbering.

In some embodiments the further amino acid residue substituted in the protein-protein interface of the parent domain of the first engineered immunoglobulin chain is selected from the group consisting of 3K, 5T, 20T, 22L, 26E, 27K, 81G, 84M, 85.1M, 86F or is selected from the group consisting of 3K, 5T, 20T, 22L, 26E, 27K, 81G, 84M, 85.1M, 86F and conservative amino acid substitutions thereof, and/or

the further amino acid residue which is substituted in the protein-protein interface of the parent domain of the second engineered immunoglobulin chain is at position 79 or is 79F or is 79F and conservative amino acid substitutions thereof, and optionally a further amino acid

residue at a position selected from the group consisting of 7, 20, 22, 27, 81, 84.2, 88, and 90 or at a position selected from the group consisting of 7, 20, 22, 27, 81, 84.2, 85.1, 88, and 90 or at a position selected from the group consisting of 7, 20, 22, 27, 81, 84.2, 86, 88, and 90, wherein the amino acid position of each group member is indicated according to the IMGT® numbering.

In some embodiments the further amino acid residue which is substituted in the protein-protein interface of the parent domain of the first engineered immunoglobulin chain is selected from the group consisting of 3K, 5T, 20T, 22L, 26E, 27K, 81G, 84M, 85.1M, 86F or is selected from the group consisting of 3K, 5T, 20T, 22L, 26E, 27K, 81G, 84M, 85.1M, 86F and conservative amino acid substitutions thereof, and/or the amino acid residue which is substituted in the protein-protein interface of the parent domain of the second engineered immunoglobulin chain comprises the amino acid residues at position 85.1 and 86 or comprises 85.1N and conservative amino acid substitutions thereof and 86V and conservative amino acid substitutions thereof, and the further amino acid residue which is substituted in the protein-protein interface of the parent domain of the second engineered immunoglobulin chain is selected from the group consisting of 7M, 20N, 22A, 27E, 79F, 81A, 84.2S, 88L, and 90K or is selected from the group consisting of 7M, 20N, 22A, 27E, 79F, 81A, 84.2S, 85.1N, 86V, 88L, and 90K and conservative amino acid substitutions thereof, wherein the amino acid position of each group member is indicated according to the IMGT® numbering.

In some embodiments the present disclosure provides a hetero-dimeric immunoglobulin or fragment thereof, wherein the parent domain of the first and/or the second engineered immunoglobulin chain is a CH3 domain, wherein the amino acid residue which is substituted in the protein-protein interface of the parent domain of the first engineered immunoglobulin chain comprises the amino acid residue at position 20, and optionally a further amino acid residue at a position selected from the group consisting of 3, 5, 7, 22, 26, 27, 79, 81, 84, 84.2, 85.1, 86, 88 and 90 and, wherein the amino acid residues which are substituted in the protein-protein interface of the parent domain of the second engineered immunoglobulin chain comprise the amino acid residue at position 26 and at a further position selected from the group consisting of 3, 22, 27, 79, 81, 84, 85.1, 86, and 88, wherein the amino acid residue substituted at position 20 in the parent domain of the first engineered immunoglobulin chain is interacting with the amino acid residue substituted at position 26 in the parent domain of the

second engineered immunoglobulin chain, wherein the amino acid position of each group member is indicated according to the IMGT® numbering.

In some embodiments the amino acid residue which is substituted in the protein-protein interface of the parent domain of the first engineered immunoglobulin chain comprises the amino acid residue at position 20, and optionally a further amino acid residue at a position selected from the group consisting of 3, 5, 7, 22, 26, 27, 79, 81, 84, 84.2, 85.1, 86, 88 and 90 and, the amino acid residues which are substituted in the protein-protein interface of the parent domain of the second engineered immunoglobulin chain comprises the amino acid residues at positions 26 and 86 and optionally at a further position selected from the group consisting of 3, 5, 22, 27, 79, 81, 84, 85.1, 88 and 90, wherein the amino acid position of each group member is indicated according to the IMGT® numbering.

In some embodiments the amino acid residues which are substituted in the protein-protein interface of the parent domain of the first engineered immunoglobulin chain comprise the amino acid residues at positions 20 and 22, and optionally a further amino acid residue at a position selected from the group consisting of 3, 5, 7, 26, 27, 79, 81, 84, 84.2, 85.1, 86, 88 and 90 and, the amino acid residues which are substituted in the protein-protein interface of the parent domain of the second engineered immunoglobulin chain comprise the amino acid residues at positions 26 and at a further position selected from the group consisting of 3, 5, 22, 27, 79, 81, 84, 85.1, 86, 88 and 90, wherein the amino acid position of each group member is indicated according to the IMGT® numbering.

In some embodiments the amino acid residues which are substituted in the protein-protein interface of the parent domain of the first engineered immunoglobulin chain comprise the amino acid residues at positions 20 and 22, and optionally a further amino acid residue at a position selected from the group consisting of 3, 5, 7, 26, 27, 79, 81, 84, 84.2, 85.1, 86, 88 and 90 and, the amino acid residues which are substituted in the protein-protein interface of the parent domain of the second engineered immunoglobulin chain comprise the amino acid residues at positions 26 and 86 and optionally at a further position selected from the group consisting of 3, 5, 22, 27, 79, 81, 84, 85.1, 88 and 90, wherein the amino acid position of each group member is indicated according to the IMGT® numbering.

In some embodiments the amino acid residue which is substituted at position 20 in the protein-protein interface of the parent domain of the first engineered immunoglobulin chain is

selected from the group consisting of 20N and 20T, and conservative amino acid substitutions thereof, wherein the amino acid position is indicated according to the IMGT® numbering.

In some embodiments the amino acid residue which is substituted at position 26 in the protein-protein interface of the parent domain of the second engineered immunoglobulin chain is selected from the group consisting of 26T and 26E and conservative amino acid substitutions thereof, wherein the amino acid position is indicated according to the IMGT® numbering.

In some embodiments the amino acid residue which is substituted at position 22 in the protein-protein interface of the parent domain of the first engineered immunoglobulin chain is selected from the group consisting of 22A and 22V, and conservative amino acid substitutions thereof, wherein the amino acid position is indicated according to the IMGT® numbering.

In some embodiments the amino acid residue which is substituted at position 86 in the protein-protein interface of the parent domain of the second engineered immunoglobulin chain is selected from the group consisting of 86V and 86F and conservative amino acid substitutions thereof, wherein the amino acid position is indicated according to the IMGT® numbering.

In some embodiments the amino acid residue which is substituted at position 20 in the protein-protein interface of the parent domain of the first engineered immunoglobulin chain is selected from the group consisting of 20V, 20T, 20A, 20N, 20Q, 20K, 20S, 20W and 20E and/or wherein the further amino acid residue substituted in the protein-protein interface of the parent domain of the first engineered immunoglobulin chain is selected from the group consisting of 22A, 22G, 22L, 22I, 22V, 22T, 26K, 26R, 26Q, 26T, 26V, 26S, 26N, 26E, 85.1W, 85.1F, 85.1T, 85.1M, 85.1A, 85.1S, 85.1R, 85.1H, 85.1K, 85.1C, 85.1N, 86W, 86Y, 86S, 86I, 86H, 86Q, 86V, 86T, 86F, 88Q, 88L, 88V, 88R, 88E, 88T, 88I, 88Y, 88K and 88W, wherein the amino acid position of each group member is indicated according to the IMGT® numbering.

In some embodiments the amino acid residue which is substituted at position 20 in the protein-protein interface of the parent domain of the first engineered immunoglobulin chain is selected from the group consisting of 20V, 20T, 20A, 20N, 20Q, and 20E and/or wherein the further amino acid residue substituted in the protein-protein interface of the parent domain of

the first engineered immunoglobulin chain is selected from the group consisting of 22A, 22G, 22L, 22I, 22V, 26Q, 26T, 26V, 26S, 26N, 26E, 85.1T, 85.1M, 85.1A, 85.1S, 85.1R, 85.1H, 85.1K, 85.1C, 85.1N, 86S, 86I, 86T, 86H, 86Q, 86V, 86T, 86F, 88Q, 88L, 88V, 88R, 88E, 88T, 88I, 88Y, and 88W, wherein the amino acid position of each group member is indicated according to the IMGT® numbering.

In some embodiments the amino acid residue which is substituted at position 20 in the protein-protein interface of the parent domain of the first engineered immunoglobulin chain is selected from the group consisting of 20V, 20T, 20A, 20N, 20Q, 20K, 20S and 20E and/or wherein the further amino acid residue substituted in the protein-protein interface of the parent domain of the first engineered immunoglobulin chain is selected from the group consisting of 22A, 22G, 22L, 22I, 22V, 26K, 26Q, 26T, 26V, 26S, 26N, 26E, 85.1T, 85.1M, 85.1A, 85.1S, 85.1R, 85.1H, 85.1K, 85.1C, 85.1N, 86S, 86I, 86H, 86Q, 86V, 86T, 86F, 88Q, 88L, 88V, 88R, 88E, 88T, 88I, 88Y, and 88W, wherein the amino acid position of each group member is indicated according to the IMGT® numbering.

In some embodiments the amino acid residues which are substituted at position 20 and position 22 in the protein-protein interface of the parent domain of the first engineered immunoglobulin chain are selected from the group consisting of 20V, 20T, 20A, 20N, 20Q, 20K, 20S, 20W and 20E and 22A, 22G, 22L, 22I, 22V, 22T and/or wherein the further amino acid residue substituted in the protein-protein interface of the parent domain of the first engineered immunoglobulin chain is selected from the group consisting of 26K, 26R, 26Q, 26T, 26V, 26S, 26N, 26E, 85.1W, 85.1F, 85.1T, 85.1M, 85.1A, 85.1S, 85.1R, 85.1H, 85.1K, 85.1C, 85.1N, 86W, 86Y, 86S, 86I, 86H, 86Q, 86V, 86T, 86F, 88Q, 88L, 88V, 88R, 88E, 88T, 88I, 88Y, 88K and 88W, wherein the amino acid position of each group member is indicated according to the IMGT® numbering.

In some embodiments the further amino acid residue substituted in the protein-protein interface of the parent domain of the first engineered immunoglobulin chain is selected from the group consisting of 3E, 3K, 5A, 5T, 7F, 7M, 22A, 22L, 22V, 26E, 26T, 27E, 27K, 79F, 79Y, 81A, 81G, 81D, 84L, 84M, 84.2E, 84.2S, 85.1A, 85.1C, 85.1M, 85.1N, 85.1S, 86F, 86S, 86V, 88L, 88R, 88W, 90K, 90N, and 90R and conservative amino acid substitutions thereof, wherein the amino acid position of each group member is indicated according to the IMGT® numbering.

In some embodiments the further amino acid residue substituted in the protein-protein interface of the parent domain of the first engineered immunoglobulin chain is selected from the group consisting of 3E, 3K, 5A, 5T, 7F, 7M, 26E, 26T, 27E, 27K, 79F, 79Y, 81A, 81G, 81D, 84L, 84M, 84.2E, 84.2S, 85.1A, 85.1C, 85.1M, 85.1N, 85.1S, 86F, 86S, 86V, 88L, 88R, 88W, 90K, 90N, and 90R and conservative amino acid substitutions thereof, wherein the amino acid position of each group member is indicated according to the IMGT® numbering.

In some embodiments the amino acid residue which is substituted at position 26 in the protein-protein interface of the parent domain of the second engineered immunoglobulin chain is selected from the group consisting of 26K, 26R, 26Q, 26T, 26V, 26S, 26N, 26E and/or wherein the further amino acid residue substituted in the protein-protein interface of the parent domain of the first engineered immunoglobulin chain is selected from the group consisting of 22A, 22G, 22L, 22I, 22V, 22T, 85.1W, 85.1F, 85.1T, 85.1M, 85.1A, 85.1S, 85.1R, 85.1H, 85.1K, 85.1C, 85.1N, 86W, 86Y, 86S, 86I, 86H, 86Q, 86V, 86T, 86F, 88Q, 88L, 88V, 88R, 88E, 88T, 88I, 88Y, 88K and 88W wherein the amino acid position of each group member is indicated according to the IMGT® numbering.

In some embodiments the amino acid residues which are substituted at position 26 and position 86 in the protein-protein interface of the parent domain of the second engineered immunoglobulin chain is selected from the group consisting of 26K, 26R, 26Q, 26T, 26V, 26S, 26N, 26E and 86W, 86Y, 86S, 86I, 86H, 86Q, 86V, 86T, 86F and/or wherein the further amino acid residue substituted in the protein-protein interface of the parent domain of the first engineered immunoglobulin chain is selected from the group consisting of 22A, 22G, 22L, 22I, 22V, 22T, 85.1W, 85.1F, 85.1T, 85.1M, 85.1A, 85.1S, 85.1R, 85.1H, 85.1K, 85.1C, 85.1N, 88Q, 88L, 88V, 88R, 88E, 88T, 88I, 88Y, 88K and 88W, wherein the amino acid position of each group member is indicated according to the IMGT® numbering.

In some embodiments the further amino acid residue substituted in the protein-protein interface of the parent domain of the second engineered immunoglobulin chain is selected from the group consisting of 3E, 3K, 22A, 22L, 22V, 27E, 27K, 79F, 79Y, 81A, 81G, 81D, 84L, 84M, 85.1A, 85.1C, 85.1M, 85.1N, 85.1S, 86F, 86S, 86V, 88L, 88R, and 88W, and conservative amino acid substitutions thereof, wherein the amino acid position of each group member is indicated according to the IMGT® numbering.

In some embodiments the present disclosure provides a hetero-dimeric immunoglobulin or

fragment thereof, wherein the parent domain of the first and/or the second engineered immunoglobulin chain is a CH2 domain, wherein the amino acid residue which is substituted in the protein-protein interface of the parent domain of the first engineered immunoglobulin chain comprises the amino acid residue at position 88, and optionally a further amino acid residue at a position selected from the group consisting of 3, 5, 7, 20, 22, 26, 27, 79, 81, 83, 84, 84.2, 84.3, 85.1, 86 and 90 and, wherein the amino acid residue which is substituted in the protein-protein interface of the parent domain of the second engineered immunoglobulin chain comprises the amino acid residue at position 85.1 and/or 86 and optionally a further amino acid residue at a position selected from the group consisting of 3, 5, 7, 20, 22, 26, 27, 79, 81, 83, 84, 84.2, 84.3 and 90 or at a position selected from the group consisting of 3, 5, 7, 20, 22, 26, 27, 79, 81, 83, 84, 84.2, 84.3, 85.1 and 90 or at a position selected from the group consisting of 3, 5, 7, 20, 22, 26, 27, 79, 81, 83, 84, 84.2, 84.3, 86 and 90, wherein the amino acid residue substituted at position 88 in the parent domain of the first engineered immunoglobulin chain is interacting with the amino acid residue substituted at position 85.1 and/or 86 in the parent domain of the second engineered immunoglobulin chain, wherein the amino acid position of each group member is indicated according to the IMGT® numbering. . Positions 3, 5, 7, 20, 22, 26, 27, 79, 81, 83, 84, 84.2, 84.3, 85.1, 86 and 90 in the protein-protein interface of the CH2 domain as parent domain indicated according to IMGT® numbering correspond in the same order to positions 239, 241, 243, 258, 260, 264, 265, 288, 290, 292, 293, 295, 296, 301, 303, 305, 307 indicated according to the EU numbering.

In some embodiments the parent domain of the first and the second engineered immunoglobulin chain is a CH2 domain.

In some embodiments the parent domains of the first and the second engineered immunoglobulin chain are CH2 domains of different isotypes.

In some embodiments the parent domain of the first engineered immunoglobulin chain is an IgG1 CH2 domain, preferably a human CH2 domain from IGHG1 and the parent domain of the second engineered immunoglobulin chain is an IgG3 CH2 domain, preferably a human CH2 domain from IGHG3 or wherein the parent domain of the first engineered immunoglobulin chain is an IgG3 CH2 domain, preferably a human CH2 domain from IGHG3 and the parent domain of the second engineered immunoglobulin chain is an IgG1 CH2 domain preferably a human CH2 domain from IGHG1.

In some embodiments the amino acid residue which is substituted in the protein-protein

interface of the parent domain of the first engineered immunoglobulin chain at position 88 is 88W or 88W and conservative amino acid substitutions thereof, wherein the amino acid position is indicated according to the IMGT® numbering.

In some embodiments the amino acid residue which is substituted in the protein-protein interface of the parent domain of the second engineered immunoglobulin chain at position 85.1 and/or 86 is selected from the group consisting of 85.1A and 85.1N or is selected from the group consisting of 85.1A and 85.1N and conservative amino acid substitutions thereof, and/or is 86S or 86S and conservative amino acid substitutions thereof, wherein the amino acid position is indicated according to the IMGT® numbering.

In some embodiments the amino acid residue which is substituted in the protein-protein interface of the parent domain of the first engineered immunoglobulin chain at position 88 is selected from the group consisting of 88Q, 88L, 88V, 88R, 88I, 88T, 88K, 88E, 88Y, and 88W and/or wherein the further amino acid residue substituted in the protein-protein interface of the parent domain of the first engineered immunoglobulin chain is selected from the group consisting of 20V, 20T, 20A, 20N, 20K, 20W, 20S, 20E, 20Q, 22A, 22G, 22T, 22L, 22V, 26Q, 26T, 26K, 26V, 26S, 26R, 26N, 26E, 85.1R, 85.1H, 85.1K, 85.1T, 85.1M, 85.1A, 85.1S, 85.1W, 85.1F, 85.1C, 85.1N, 86S, 86I, 86H, 86T, 86W, 86Y, 86V, 86Q and 86F, wherein the amino acid position of each group member is indicated according to the IMGT® numbering.

In some embodiments the amino acid residue which is substituted in the protein-protein interface of the parent domain of the first engineered immunoglobulin chain at position 88 is selected from the group consisting of 88Q, 88L, 88V, 88R, 88I, 88T, 88K, 88E, 88Y, and 88W and/or wherein the further amino acid residue substituted in the protein-protein interface of the parent domain of the first engineered immunoglobulin chain is selected from the group consisting of 20V, 20T, 20A, 20N, 20K, 20W, 20S, 22A, 22G, 22T, 22L, 22V, 26Q, 26T, 26K, 26V, 26S, 26R, 26N, 26E, 85.1T, 85.1M, 85.1A, 85.1S, 85.1W, 85.1F, 85.1C, 85.1N, 86S, 86I, 86H, 86T, 86W, 86Y, 86V, and 86F, wherein the amino acid position of each group member is indicated according to the IMGT® numbering.

In some embodiments the further amino acid residue substituted in the protein-protein interface of the parent domain of the first engineered immunoglobulin chain is selected from the group consisting of 3E, 3K, 5A, 5T, 5Y, 7M, 7L, 20K, 20N, 20T, 20S, 22A, 22L, 22V, 26E, 26T, 27E, 27K, 79F, 79Y, 81A, 81G, 81D, 83M, 83T, 84L, 84.2E, 84.2S, 84.3D, 85.1S, 85.1A, 85.1N, 85.1M, 85.1F, 86S, 86F, 86Y, 90K, 90N, and 90R or is selected from the group consisting of 3E, 3K, 5A, 5T, 5Y, 7M, 7L, 20K, 20N, 20T, 20S, 22A, 22L, 22V, 26E, 26T,

27E, 27K, 79F, 79Y, 81A, 81G, 81D, 83M, 83T, 84L, 84.2E, 84.2S, 84.3D, 85.1S, 85.1A, 85.1N, 85.1M, 85.1F, 86S, 86F, 86Y, 90K, 90N, and 90R and conservative amino acid substitutions thereof, wherein the amino acid position of each group member is indicated according to the IMGT® numbering.

In some embodiments the amino acid residue which is substituted in the protein-protein interface of the parent domain of the first engineered immunoglobulin chain comprises the amino acid residues at position 88 and at position 20, and optionally a further amino acid residue at a position selected from the group consisting of 3, 5, 7, 22, 26, 27, 79, 81, 83, 84, 84.2, 84.3, 85.1, 86 and 90, wherein the amino acid position is indicated according to the IMGT® numbering.

In some embodiments the amino acid residue which is substituted in the protein-protein interface of the parent domain of the second engineered immunoglobulin chain comprises the amino acid residues at position 85.1 and/or 86 and at position 26 and optionally an amino acid residue at a further position, wherein the amino acid position is indicated according to the IMGT® numbering.

In some embodiments the further amino acid residue which is substituted in the protein-protein interface of the parent domain of the first engineered immunoglobulin chain is at a position selected from the group consisting of 20, 22, 26, 79, 85.1, and 90, and/or the further amino acid residue which is substituted in the protein-protein interface of the parent domain of the second engineered immunoglobulin chain is at position 84 or is 84L or is 84L and conservative amino acid substitutions thereof, and optionally a further amino acid at a position selected from the group consisting of 3, 5, 7, 20, 22, 26, 81, 84.2, 88, and 90 or at a position selected from the group consisting of 3, 5, 7, 20, 22, 26, 81, 84.2, 85.1, 88, and 90 or at a position selected from the group consisting of 3, 5, 7, 20, 22, 26, 81, 84.2, 86, 88, and 90, wherein the amino acid position of each group member is indicated according to the IMGT® numbering.

In some embodiments the further amino acid residue substituted in the protein-protein interface of the parent domain of the first engineered immunoglobulin chain is selected from the group consisting of 20K, 22V, 26T, 79Y, 85.1S, and 90N or is selected from the group consisting of 20K, 22V, 26T, 79Y, 85.1S, and 90N and conservative amino acid substitutions thereof and/or the amino acid residue which is substituted in the protein-protein interface of the parent domain of the second engineered immunoglobulin chain at position 85.1 or is 85.1C or is 85.1C and conservative amino acid substitutions thereof, and wherein the amino

acid residue which is substituted in the protein-protein interface of the parent domain of the second engineered immunoglobulin chain at position 86 or is 86S or is 86S and conservative amino acid substitutions thereof, and wherein the further amino acid residue which is substituted in the protein-protein interface of the parent domain of the second engineered immunoglobulin chain is selected from the group consisting of 3, 5, 20, 22, 26, 81, 84, 84.2, 88, and 90 , preferably from the group consisting of 3E, 5A, 20T, 22V, 26T, 81D, 84L, 84.2E, 88R and 90R or from the group consisting of 3E, 5A, 20T, 22V, 26T, 81D, 84L, 84.2E, 88R and 90R and conservative amino acid substitutions thereof, wherein the amino acid position of each group member is indicated according to the IMGT® numbering.

In some embodiments the amino acid residue substitution 85.1C is replaced by amino acid residue substitution 85.1A or 85.1S wherein the amino acid position of each group member is indicated according to the IMGT® numbering.

In some embodiments the further amino acid residue substituted in the protein-protein interface of the parent domain of the first engineered immunoglobulin chain is at a position selected from the group consisting of 3, 5, 20, 22, 26, 27, 81, 83, 85.1, and 86, and/or the further amino acid residue which is substituted in the protein-protein interface of the parent domain of the second engineered immunoglobulin chain is at a position selected from the group consisting of 7, 20, 22, 27, 79, 81, 84.2, 88, and 90 or at a position selected from the group consisting of 7, 20, 22, 27, 79, 81, 84.2, 85.1, 88, and 90 or at a position selected from the group consisting of 7, 20, 22, 27, 79, 81, 84.2, 86, 88, and 90, wherein the amino acid position of each group member is indicated according to the IMGT® numbering.

In some embodiments the further amino acid residue substituted in the protein-protein interface of the parent domain of the first engineered immunoglobulin chain is selected from the group consisting of 3K, 5T, 20T, 22L, 26E, 27K, 81G, 83M, 85.1M, and 86F or is selected from the group consisting of 3K, 5T, 20T, 22L, 26E, 27K, 81G, 83M, 85.1M, and 86F and conservative amino acid substitutions thereof, and/or

the further amino acid residues which are substituted in the protein-protein interface of the parent domain of the second engineered immunoglobulin chain is at position 79 or is 79F or is 79F and conservative amino acid substitutions thereof, and optionally a further amino acid residue at a position selected from the group consisting of 7, 20, 22, 27, 81, 84.2, 88, and 90 or at a position selected from the group consisting of 7, 20, 22, 27, 81, 84.2, 85.1, 88, and 90, or at a position selected from the group consisting of 7, 20, 22, 27, 81, 84.2, 86, 88, and 90,

wherein the amino acid position of each group member is indicated according to the IMGT® numbering.

In some embodiments the further amino acid residue substituted in the protein-protein interface of the parent domain of the first engineered immunoglobulin chain is selected from the group consisting of 3K, 5T, 20T, 22L, 26E, 27K, 81G, 83M, 85.1M, and 86F or is selected from the group consisting of 3K, 5T, 20T, 22L, 26E, 27K, 81G, 83M, 85.1M, and 86F and conservative amino acid substitutions thereof, and/or the amino acid residue which is substituted in the protein-protein interface of the parent domain of the second engineered immunoglobulin chain at position 85.1 is 85.1N or is 85.1N and conservative amino acid substitutions thereof, and wherein the amino acid residue which is substituted in the protein-protein interface of the parent domain of the second engineered immunoglobulin chain at position 22 is 22A or is 22A and conservative amino acid substitutions thereof, and/or wherein the further amino acid residue which is substituted in the protein-protein interface of the parent domain of the second engineered immunoglobulin chain is selected from the group consisting of 7M, 20N, 27E, 79F, 81A, 84.2S, 88L, and 90K or is selected from the group consisting of 7M, 20N, 27E, 79F, 81A, 84.2S, 88L, and 90K and conservative amino acid substitutions thereof, wherein the amino acid position of each group member is indicated according to the IMGT® numbering.

In some embodiments the further amino acid residue substituted in the protein-protein interface of the parent domain of the first engineered immunoglobulin chain is at a position selected from the group consisting of 5, 7, 20, 83, 84.3, 85.1, and 86 and/or the further amino acid residue which is substituted in the protein-protein interface of the parent domain of the second engineered immunoglobulin chain is at a position selected from the group consisting of 5, 7, 20, 83, 84.3, and 88 or at a position selected from the group consisting of 5, 7, 20, 83, 84.3, 85.1, and 88, or at a position selected from the group consisting of 5, 7, 20, 83, 84.3, 86, and 88 wherein the amino acid position of each group member is indicated according to the IMGT® numbering.

In some embodiments the further amino acid residue substituted in the protein-protein interface of the parent domain of the first engineered immunoglobulin chain is selected from the group consisting of 5Y, 7L, 20S, 83T, 84.3D, 85.1F, and 86Y or is selected from the group consisting of 5Y, 7L, 20S, 83T, 84.3D, 85.1F, and 86Y and conservative amino acid substitutions thereof, and/or the further amino acid residues which are substituted in the protein-protein interface of the parent domain of the second engineered immunoglobulin

chain is at position 20 or is 20S or is 20S and conservative amino acid substitutions thereof, and optionally a further amino acid residue at a position selected from the group consisting of 5, 7, 83, 84.3, and 88 or at a position selected from the group consisting of 5, 7, 83, 84.3, 85.1 and 88 or at a position selected from the group consisting of 5, 7, 83, 84.3, 86 and 88, wherein the amino acid position of each group member is indicated according to the IMGT® numbering.

In some embodiments the further amino acid residue substituted in the protein-protein interface of the parent domain of the first engineered immunoglobulin chain is selected from the group consisting of 5Y, 7L, 20S, 83T, 84.3D, 85.1F and 86Y or is selected from the group consisting of 5Y, 7L, 20S, 83T, 84.3D, 85.1F and 86Y and conservative amino acid substitutions thereof, and/or the amino acid residue which is substituted in the protein-protein interface of the parent domain of the second engineered immunoglobulin chain is at position 85.1 or is 85.1F or is 85.1F and conservative amino acid substitutions thereof, and wherein the further amino acid residue which is substituted in the protein-protein interface of the parent domain of the second engineered immunoglobulin chain is at position 20 or is 20S or is 20S and conservative amino acid substitutions thereof, and/or wherein a further amino acid residue which is substituted in the protein-protein interface of the parent domain of the second engineered immunoglobulin chain is selected from the group consisting of 5Y, 7L, 83T, 84.3D, 86Y, and 88K or is selected from the group consisting of 5Y, 7L, 83T, 84.3D, 86Y and 88K and conservative amino acid substitutions thereof, wherein the amino acid position of each group member is indicated according to the IMGT® numbering.

In some embodiments the present disclosure provides a hetero-dimeric immunoglobulin or fragment thereof, wherein the parent domain of the first and/or the second engineered immunoglobulin chain is a CH2 domain, wherein the amino acid residue which is substituted in the protein-protein interface of the parent domain of the first engineered immunoglobulin chain comprises the amino acid residue at position 20, and optionally a further amino acid residue at a position selected from the group consisting of 3, 5, 7, 22, 26, 27, 79, 81, 83, 84, 84.2, 84.3, 85.1, 86, 88 and 90 and, wherein the amino acid residue which is substituted in the protein-protein interface of the parent domain of the second engineered immunoglobulin chain comprises the amino acid residue at position 26 and optionally a further amino acid residue at a position selected from the group consisting of 3, 5, 7, 20, 22, 27, 79, 81, 83, 84, 84.2, 84.3, 85.1, 86, 88 and 90, wherein the amino acid residue substituted at position 20 in

the parent domain of the first engineered immunoglobulin chain is interacting with the amino acid residue substituted at position 26 in the parent domain of the second engineered immunoglobulin chain, wherein the amino acid position of each group member is indicated according to the IMGT® numbering.

In some embodiments the present disclosure provides a hetero-dimeric immunoglobulin or fragment thereof, wherein the parent domain of the first and/or the second engineered immunoglobulin chain is a CH2 domain, wherein the amino acid residue which is substituted in the protein-protein interface of the parent domain of the first engineered immunoglobulin chain comprises the amino acid residue at position 20, and optionally a further amino acid residue at a position selected from the group consisting of 3, 5, 7, 22, 26, 27, 79, 81, 84, 84.2, 85.1, 86, 88 and 90 and, wherein the amino acid residues which are substituted in the protein-protein interface of the parent domain of the second engineered immunoglobulin chain are at position 26 and at a further position selected from the group consisting of 3, 22, 27, 79, 81, 84, 85.1, 86, and 88, wherein the amino acid residue substituted at position 20 in the parent domain of the first engineered immunoglobulin chain is interacting with the amino acid residue substituted at position 26 in the parent domain of the second engineered immunoglobulin chain, wherein the amino acid position of each group member is indicated according to the IMGT® numbering.

In some embodiments the amino acid residue which is substituted in the protein-protein interface of the parent domain of the first engineered immunoglobulin chain comprises the amino acid residue at position 20, and optionally a further amino acid residue at a position selected from the group consisting of 3, 5, 7, 22, 26, 27, 79, 81, 84, 84.2, 85.1, 86, 88 and 90 and, the amino acid residues which are substituted in the protein-protein interface of the parent domain of the second engineered immunoglobulin chain comprises the amino acid residues at positions 26 and 86 and optionally at a further position selected from the group consisting of 3, 5, 22, 27, 79, 81, 84, 85.1, 88 and 90, wherein the amino acid position of each group member is indicated according to the IMGT® numbering.

In some embodiments the amino acid residues which are substituted in the protein-protein interface of the parent domain of the first engineered immunoglobulin chain comprise the amino acid residues at positions 20 and 22, and optionally a further amino acid residue at a position selected from the group consisting of 3, 5, 7, 26, 27, 79, 81, 84, 84.2, 85.1, 86, 88 and 90 and, the amino acid residues which are substituted in the protein-protein interface of the parent domain of the second engineered immunoglobulin chain comprise the amino acid

residues at positions 26 and at a further position selected from the group consisting of 3, 5, 22, 27, 79, 81, 84, 85.1, 86, 88 and 90, wherein the amino acid position of each group member is indicated according to the IMGT® numbering.

In some embodiments the amino acid residues which are substituted in the protein-protein interface of the parent domain of the first engineered immunoglobulin chain comprise the amino acid residues at positions 20 and 22, and optionally a further amino acid residue at a position selected from the group consisting of 3, 5, 7, 26, 27, 79, 81, 84, 84.2, 85.1, 86, 88 and 90 and, the amino acid residues which are substituted in the protein-protein interface of the parent domain of the second engineered immunoglobulin chain comprise the amino acid residues at positions 26 and 86 and optionally at a further position selected from the group consisting of 3, 5, 22, 27, 79, 81, 84, 85.1, 88 and 90, wherein the amino acid position of each group member is indicated according to the IMGT® numbering.

In some embodiments the amino acid residue which is substituted at position 20 in the protein-protein interface of the parent domain of the first engineered immunoglobulin chain is selected from the group consisting of 20N and 20T, and conservative amino acid substitutions thereof, wherein the amino acid position is indicated according to the IMGT® numbering.

In some embodiments the amino acid residue which is substituted at position 26 in the protein-protein interface of the parent domain of the second engineered immunoglobulin chain is selected from the group consisting of 26T and 26E and conservative amino acid substitutions thereof, wherein the amino acid position is indicated according to the IMGT® numbering.

In some embodiments the amino acid residue which is substituted at position 22 in the protein-protein interface of the parent domain of the first engineered immunoglobulin chain is selected from the group consisting of 22A and 22V, and conservative amino acid substitutions thereof, wherein the amino acid position is indicated according to the IMGT® numbering.

In some embodiments the amino acid residue which is substituted at position 86 in the protein-protein interface of the parent domain of the second engineered immunoglobulin chain is selected from the group consisting of 86V and 86F and conservative amino acid substitutions thereof, wherein the amino acid position is indicated according to the IMGT® numbering.

In some embodiments the amino acid residue which is substituted in the protein-protein interface of the parent domain of the first engineered immunoglobulin chain at position 20 is

selected from the group consisting of 20V, 20T, 20A, 20N, 20K, 20Q, 20E, 20W and 20S and/or wherein the further amino acid residue substituted in the protein-protein interface of the parent domain of the first engineered immunoglobulin chain is selected from the group consisting of 22A, 22G, 22T, 22L, 22V, 22I, 26Q, 26T, 26K, 26V, 26S, 26R, 26N, 26E, 85.1R, 85.1H, 85.1K, 85.1T, 85.1M, 85.1A, 85.1S, 85.1W, 85.1F, 85.1C, 85.1N, 86Q, 86S, 86I, 86H, 86T, 86W, 86Y, 86V, 86F, 88Q, 88L, 88V, 88R, 88E, 88I, 88T, 88K, 88Y, and 88W, wherein the amino acid position of each group member is indicated according to the IMGT® numbering.

In some embodiments the amino acid residue which is substituted in the protein-protein interface of the parent domain of the first engineered immunoglobulin chain at position 20 is selected from the group consisting of 20V, 20T, 20A, 20N, 20K, 20W and 20S and/or wherein the further amino acid residue substituted in the protein-protein interface of the parent domain of the first engineered immunoglobulin chain is selected from the group consisting of 22A, 22G, 22T, 22L, 22V, 26Q, 26T, 26K, 26V, 26S, 26R, 26N, 26E, 85.1T, 85.1M, 85.1A, 85.1S, 85.1W, 85.1F, 85.1C, 85.1N, 86S, 86I, 86H, 86T, 86W, 86Y, 86V, 86F, 88Q, 88L, 88V, 88R, 88E, 88I, 88T, 88K, 88Y, and 88W, wherein the amino acid position of each group member is indicated according to the IMGT® numbering.

In some embodiments the further amino acid residue substituted in the protein-protein interface of the parent domain of the first engineered immunoglobulin chain is selected from the group consisting of 3E, 3K, 5A, 5T, 5Y, 7M, 7L, 22A, 22L, 22V, 26E, 26T, 27E, 27K, 79F, 79Y, 81A, 81G, 81D, 83M, 83T, 84L, 84.2E, 84.2S, 84.3D, 85.1S, 85.1A, 85.1N, 85.1M, 85.1F, 86S, 86F, 86Y, 88K, 88L, 88R, 88W, 90K, 90N, and 90R or selected from the group consisting of 3E, 3K, 5A, 5T, 5Y, 7M, 7L, 22A, 22L, 22V, 26E, 26T, 27E, 27K, 79F, 79Y, 81A, 81G, 81D, 83M, 83T, 84L, 84.2E, 84.2S, 84.3D, 85.1S, 85.1A, 85.1N, 85.1M, 85.1F, 86S, 86F, 86Y, 88K, 88L, 88R, 88W, 90K, 90N, and 90R and conservative amino acid substitutions thereof, wherein the amino acid position of each group member is indicated according to the IMGT® numbering.

In some embodiments the present disclosure provides a hetero-dimeric immunoglobulin or hetero-dimeric fragment thereof comprising:

(a) a first engineered immunoglobulin chain comprising an engineered domain with a protein-protein interface, wherein at least one amino acid residue of the protein-protein interface of the parent domain of said first engineered immunoglobulin chain is substituted with an amino

acid residue at the equivalent 3D structural position from a protein-protein interface of a donor domain of a first member of the naturally occurring immunoglobulin super-family; and (b) a second engineered immunoglobulin chain comprising an engineered domain with a protein-protein interface, wherein at least one amino acid residue of the protein-protein interface of the parent domain of said second engineered immunoglobulin chain is substituted with an amino acid residue at the equivalent 3D structural position from a protein-protein interface of a donor domain of a second member of the naturally occurring immunoglobulin super-family,

wherein the engineered domain of the first engineered immunoglobulin chain and the engineered domain of the second engineered immunoglobulin chain are not identical and, wherein the donor domain of the first member of the naturally occurring immunoglobulin super-family and the donor domain of the second member of the naturally occurring immunoglobulin super-family form a naturally occurring hetero-dimer or a naturally occurring homo-dimer and,

wherein the parent domain of the first and/or the second engineered immunoglobulin chain is a CH4 domain, wherein the protein-protein interface of the parent domain of the first engineered immunoglobulin chain comprises an amino acid residue substitution at a position selected from the group consisting of 20, 22, 85.1, 86, and 88, and/or wherein the protein-protein interface of the parent domain of the second engineered immunoglobulin chain comprises an amino acid residue substitution at a position selected from the group consisting of 22, 26, 85.1, 86, and 88, wherein the amino acid position of each group member is indicated according to the IMGT® numbering. In some embodiments the parent domain of the first and the second engineered immunoglobulin chain is a naturally occurring CH4 domain.

In some embodiments the amino acid residue which is substituted in the protein-protein interface of the parent domain of the first engineered immunoglobulin chain at position 88 is 88W and conservative amino acid substitutions thereof, wherein the amino acid position is indicated according to the IMGT® numbering.

In some embodiments preferably at least two, more preferably at least three, most preferably at least four, in particular at least five amino acid residues, more particular at least six, even more particular at least seven, most particular all amino acid residues of the protein-protein interface of the parent domain of the first and/or the second engineered immunoglobulin chain

are substituted. Equally preferred two, more preferably three, most preferably four, in particular five amino acid residues, more particular six, even more particular seven amino acid residues of the protein-protein interface of the parent domain of the first and/or the second engineered immunoglobulin chain are substituted. Equally preferred less than two, more preferably less than three, most preferably less than four, in particular less than five amino acid residues, more particular less than six, even more particular less than seven amino acid residues of the protein-protein interface of the parent domain of the first and/or the second engineered immunoglobulin chain are substituted.

In some embodiments at least one, preferably at least two, more preferably at least three, most preferably at least four, in particular at least five amino acid residues, more particular at least six, even more particular at least seven, most particular all amino acid residues of the protein-protein interface of the engineered domain of the first engineered immunoglobulin chain is different from the protein-protein interface of the engineered domain of the second engineered immunoglobulin chain

In some embodiments the amino acid residues of the protein-protein interface of the parent domain of the first and/or second engineered immunoglobulin chain which are substituted are not adjacent.

In some embodiments the present disclosure provides a hetero-dimeric immunoglobulin or fragment thereof, wherein, at least two, more preferably at least three, most preferably at least four, in particular at least five amino acid residues of the protein-protein interface of the parent domain of the first and/or second engineered immunoglobulin chain which are substituted are not adjacent.

In some embodiments the amino acid sequence of the engineered domains of the first engineered immunoglobulin chain and/or the second engineered immunoglobulin chain do not contain an insertion of one or more amino acid residues compared to the amino acid sequence of the parent domains of the first engineered immunoglobulin chain and/or the second engineered immunoglobulin chain.

In some embodiments the amino acid sequence of the further engineered domain of the first engineered immunoglobulin chain and/or the second engineered immunoglobulin chain do not contain an insertion of one or more amino acid residues compared to the amino acid sequence of the parent domain of the further engineered domain of the first engineered immunoglobulin chain and/or the second engineered immunoglobulin.

In some embodiments the amino acid sequence of the engineered domain of the third engineered immunoglobulin chain do not contain an insertion of one or more amino acid residues compared to the amino acid sequence of the parent domain of the third engineered immunoglobulin chain.

In some embodiments the first engineered immunoglobulin chain and/or the second engineered immunoglobulin chain comprise a further engineered domain with a protein-protein interface, wherein at least one amino acid residue of the protein-protein interface of the parent domain of said further engineered domain of said first engineered immunoglobulin chain and/or said second engineered immunoglobulin chain is substituted with an amino acid residue at the equivalent 3D structural position from a protein-protein interface of a donor domain of a third member of the naturally occurring immunoglobulin super-family, and wherein the protein-protein interface of the further engineered domain of the first engineered immunoglobulin chain and/or the second engineered immunoglobulin chain is interacting with the protein-protein interface of an engineered domain of a third engineered immunoglobulin chain by hetero-dimerization or by homo-dimerization,

wherein at least one amino acid residue of the protein-protein interface of the parent domain of said engineered domain of said third engineered immunoglobulin chain is substituted with an amino acid residue at the equivalent 3D structural position from a protein-protein interface of a donor domain of a fourth member of the naturally occurring immunoglobulin super-family, and

wherein the donor domain of the third member of the naturally occurring immunoglobulin super-family and the donor domain of the fourth member of the naturally occurring immunoglobulin super-family form a naturally occurring hetero-dimer or a naturally occurring homo-dimer and,

wherein the protein-protein interface of the further engineered domain of the first engineered immunoglobulin chain and/or the second engineered immunoglobulin chain and of the engineered domain of the third engineered immunoglobulin chain is different from the protein-protein interface of the engineered domain of the first engineered immunoglobulin chain and of the engineered domain of the second engineered immunoglobulin chain.

Preferably, the further engineered domain of the first engineered immunoglobulin chain and/or the second engineered immunoglobulin chain and the engineered domain of the third engineered immunoglobulin chain are not identical.

Preferably at least two, more preferably at least three, most preferably at least four, in particular at least five amino acid residues, more particular at least six, even more particular at least seven, most particular all amino acid residues of the protein-protein interface of the parent domain of the further engineered domain of the first and/or second engineered immunoglobulin chain and/or the parent domain of the engineered domain of the third engineered immunoglobulin chain are substituted. Equally preferred two, more preferably three, most preferably four, in particular five amino acid residues, more particular six, even more particular seven amino acid residues of the protein-protein interface of the parent domain of the further engineered domain of the first and/or second engineered immunoglobulin chain and/or the parent domain of the engineered domain of the third engineered immunoglobulin chain are substituted. Equally preferred less than two, more preferably less than three, most preferably less than four, in particular less than five amino acid residues, more particular less than six, even more particular less than seven amino acid residues of the protein-protein interface of the parent domain of the further engineered domain of the first and/or second engineered immunoglobulin chain and/or the parent domain of the engineered domain of the third engineered immunoglobulin chain are substituted.
In some embodiments at least one, preferably at least two, more preferably at least three, most preferably at least four, in particular at least five amino acid residues, more particular at least six, even more particular at least seven, most particular all amino acid residues of the protein-protein interface of the parent domain of the further engineered domain of the first and/or second engineered immunoglobulin chain is different from the protein-protein interface of the engineered domain of the third engineered immunoglobulin chain,

In some embodiments the present disclosure provides a hetero-dimeric immunoglobulin or fragment thereof, wherein the amino acid residues of the protein-protein interface of the parent domain of the further engineered domain of the first engineered immunoglobulin chain and the parent domain of the engineered domain of the third engineered immunoglobulin chain which are substituted are not adjacent. In some embodiments the present disclosure provides a hetero-dimeric immunoglobulin or fragment thereof, wherein, at least two, more preferably at least three, most preferably at least four, in particular at least five amino acid residues of the protein-protein interface of the parent domain of the further engineered domain of the first engineered immunoglobulin chain and the parent domain of the engineered domain

of the third engineered immunoglobulin chain which are substituted are not adjacent.

In some embodiments the third engineered immunoglobulin chain comprises a further engineered domain with a protein-protein interface, wherein at least one amino acid residue of the protein-protein interface of the parent domain of said further engineered domain of said third engineered immunoglobulin chain is substituted with an amino acid residue at the equivalent 3D structural position from a protein-protein interface of a donor domain of a fifth member of the naturally occurring immunoglobulin super-family, and

wherein the protein-protein interface of the further engineered domain of the third engineered immunoglobulin chain is interacting with the protein-protein interface of an engineered domain of a fourth engineered immunoglobulin chain by hetero-dimerization or by homo-dimerization,

wherein at least one amino acid residue of the protein-protein interface of the parent domain of said engineered domain of said third engineered immunoglobulin chain is substituted with an amino acid residue at the equivalent 3D structural position from a protein-protein interface of a donor domain of a sixth member of the naturally occurring immunoglobulin super-family, and

wherein the donor domain of the fifth member of the naturally occurring immunoglobulin super-family and the donor domain of the sixth member of the naturally occurring immunoglobulin super-family form a naturally occurring hetero-dimer or a naturally occurring homo-dimer and,

wherein the protein-protein interface of the further engineered domain of the third engineered immunoglobulin chain and of the engineered domain of the fourth engineered immunoglobulin chain is different from the protein-protein interface of the engineered domain of the first engineered immunoglobulin chain and of the engineered domain of the second engineered immunoglobulin chain and from the protein-protein interface of the further engineered domain of the first engineered immunoglobulin chain and/or the second engineered immunoglobulin chain and from the protein-protein interface of the engineered domain of a third engineered immunoglobulin chain.

The parent domains of the further engineered domain of the third engineered immunoglobulin chain and/or of the engineered domain of the fourth engineered immunoglobulin chain is selected from consisting of CH1, CH2, CH3, CH4, IGKC, IGLC1, IGLC2, IGLC3, IGLC6, and IGLC7, wherein the amino acid residues of the protein-protein

interface of the further engineered domain of the third engineered immunoglobulin chain and/or of the engineered domain of the fourth engineered immunoglobulin chain which are substituted are e.g. selected from the group of the amino acid residues of the protein-protein interface for the parent domains of the first and/or second engineered immunoglobulin chains as described supra.

The donor domain of the fifth and/or a sixth member of the naturally occurring immunoglobulin super-family are e.g. selected from for the group of donor domain of the first and/or second member of the naturally occurring immunoglobulin super-family as described supra.

In some embodiments the first engineered immunoglobulin chain comprises an engineered domain with a protein-protein interface comprising the amino acid sequence of SEQ ID NO: 13 and/or the second engineered immunoglobulin chain comprises an engineered domain with a protein-protein interface comprising the amino acid sequence selected from the group consisting of SEQ ID NO: 14, SEQ ID NO: 15 and SEQ ID NO: 16, preferably SEQ ID NO: 14 or SEQ ID NO: 15.

In some embodiments the first engineered immunoglobulin chain comprises an engineered domain with a protein-protein interface comprising the amino acid sequence of SEQ ID NO: 13 and/or the second engineered immunoglobulin chain comprises an engineered domain with a protein-protein interface comprising the amino acid sequence of SEQ ID NO: 14.

Thus in a further aspect the present invention provides a hetero-dimeric immunoglobulin or fragment thereof comprising a first engineered immunoglobulin chain comprising an engineered domain with a protein-protein interface comprising the amino acid sequence of SEQ ID NO: 13 and a second engineered immunoglobulin chain comprising an engineered domain with a protein-protein interface.

In some embodiments the engineered domain with a protein-protein interface of the first engineered immunoglobulin chain comprises an amino acid sequence which is at least 80 %, preferably at least 90%, more preferably at least 95%, most preferred at least 96%, in particular at least 97%, more particular at least 98%, most particular at least 99% identical to the amino acid sequence of SEQ ID NO: 13, with the proviso that the amino acid sequence which is at least 80 % identical to the amino acid sequence of SEQ ID NO: 13 contains at least two, preferably at least three, more preferably at least four, most preferred at least five,

in particular at least six, more particular at least seven, most particular all of the amino acid residues selected from the group consisting of K24, V26, T30, Y52, S65, V67, W69, and N71, wherein the amino acid position of each group member is indicated according to the numbering of the amino acid residues of SEQ ID NO: 13 in the sequence listing.

In a further aspect the present invention provides a hetero-dimeric immunoglobulin or fragment thereof comprising a first engineered immunoglobulin chain comprising an engineered domain with a protein-protein interface and a second engineered immunoglobulin chain comprising an engineered domain with a protein-protein interface comprising the amino acid sequence of SEQ ID NO: 14. In some embodiments the engineered domain with a protein-protein interface of the second engineered immunoglobulin chain comprises an amino acid sequence which is at least 80 %, preferably at least 90%, more preferably at least 95%, most preferred at least 96%, in particular at least 97%, more particular at least 98%, most particular at least 99% identical to the amino acid sequence of SEQ ID NO: 14, with the proviso that the amino acid sequence which is at least 80 % identical to the amino acid sequence of SEQ ID NO: 14 contains at least one, usually two, preferably at least three, more preferably at least four, most preferred at least five, in particular at least six, more particular at least seven, most particular all of the amino acid residues selected from the group consisting of E7, A9, F11, T24, V26, T30, D54, L57, E59, A65, S67, R69, and R71, wherein the amino acid position of each group member is indicated according to the numbering of the amino acid residues of SEQ ID NO: 14 in the sequence listing.

In a further aspect the present invention provides a hetero-dimeric immunoglobulin or fragment thereof comprising a first engineered immunoglobulin chain comprising an engineered domain with a protein-protein interface and a second engineered immunoglobulin chain comprising an engineered domain with a protein-protein interface comprising the amino acid sequence of SEQ ID NO: 15.

In some embodiments the engineered domain with a protein-protein interface of the second engineered immunoglobulin chain comprises an amino acid sequence which is at least 80 %, preferably at least 90%, more preferably at least 95%, most preferred at least 96%, in particular at least 97%, more particular at least 98%, most particular at least 99% identical to the amino acid sequence of SEQ ID NO: 15, with the proviso that the amino acid sequence which is at least 80 % identical to the amino acid sequence of SEQ ID NO: 15 contains at least one, usually two, preferably at least three, more preferably at least four, most preferred at least five, in particular at least six, more particular at least seven, most particular all of the

amino acid residues selected from the group consisting of E7, A9, F11, T24, V26, T30, D54, L57, E59, C65, S67, R69, and R71, wherein the amino acid position of each group member is indicated according to the numbering of the amino acid residues of SEQ ID NO: 15 in the sequence listing.

In a further aspect the present invention provides a hetero-dimeric immunoglobulin or fragment thereof comprising a first engineered immunoglobulin chain comprising an engineered domain with a protein-protein interface and a second engineered immunoglobulin chain comprising an engineered domain with a protein-protein interface comprising the amino acid sequence of SEQ ID NO: 16, wherein the protein-protein interface of the engineered domain of the first engineered immunoglobulin chain is interacting with the protein-protein interface of the engineered domain of the second engineered immunoglobulin chain by hetero-dimerization.

In some embodiments the engineered domain with a protein-protein interface of the second engineered immunoglobulin chain comprises an amino acid sequence which is at least 80 %, preferably at least 90%, more preferably at least 95%, most preferred at least 96%, in particular at least 97%, more particular at least 98%, most particular at least 99% identical to the amino acid sequence of SEQ ID NO: 16, with the proviso that the amino acid sequence which is at least 80 % identical to the amino acid sequence of SEQ ID NO: 16 contains at least one, usually two, preferably at least three, more preferably at least four, most preferred at least five, in particular at least six, more particular at least seven, most particular all of the amino acid residues selected from the group consisting of E7, A9, F11, T24, V26, T30, D54, L57, E59, S65, S67, R69, and R71, wherein the amino acid position of each group member is indicated according to the numbering of the amino acid residues of SEQ ID NO: 16 in the sequence listing.

In a further aspect the present invention provides a hetero-dimeric immunoglobulin or fragment thereof comprising a first engineered immunoglobulin chain comprising an engineered domain with a protein-protein interface, wherein the first engineered immunoglobulin chain comprises the amino acid sequence of SEQ ID NO: 3 and a second engineered immunoglobulin chain comprising an engineered domain with a protein-protein interface, wherein the protein-protein interface of the engineered domain of the first engineered immunoglobulin chain is interacting with the protein-protein interface of the engineered domain of the second engineered immunoglobulin chain by hetero-dimerization.

In some embodiments the first engineered immunoglobulin chain comprises an amino acid sequence which is at least 80 %, preferably at least 90%, more preferably at least 95%, most preferred at least 96%, in particular at least 97%, more particular at least 98%, most particular at least 99% identical to the amino acid sequence of SEQ ID NO: 3, with the proviso that the amino acid sequence which is at least 80 % identical to the amino acid sequence of SEQ ID NO: 3 contains at least one, usually two, preferably at least three, more preferably at least four, most preferred at least five, in particular at least six, more particular at least seven, most particular all of the amino acid residues selected from the group consisting of S144K, T146V, K150T, K172Y, F185S, Y187V, K189W, and T191N, wherein the amino acid position of each group member is indicated according to the numbering of the amino acid residues of SEQ ID NO: 3 in the sequence listing.

In a further aspect the present invention provides a hetero-dimeric immunoglobulin or fragment thereof comprising a first engineered immunoglobulin chain comprising an engineered domain with a protein-protein interface and a second engineered immunoglobulin chain comprising an engineered domain with a protein-protein interface, wherein the second engineered immunoglobulin chain comprises the amino acid sequence of SEQ ID NO: 4, wherein the protein-protein interface of the engineered domain of the first engineered immunoglobulin chain is interacting with the protein-protein interface of the engineered domain of the second engineered immunoglobulin chain by hetero-dimerization.

In some embodiments the second engineered immunoglobulin chain comprises an amino acid sequence which is at least 80 %, preferably at least 90%, more preferably at least 95%, most preferred at least 96%, in particular at least 97%, more particular at least 98%, most particular at least 99% identical to the amino acid sequence of SEQ ID NO: 4, with the proviso that the amino acid sequence which is at least 80 % identical to the amino acid sequence of SEQ ID NO: 4 contains at least one, usually two, preferably at least three, more preferably at least four, most preferred at least five, in particular at least six, more particular at least seven or eight or nine or ten, or eleven or twelve, most particular all of the amino acid residues selected from the group consisting of Q127E, Y129A, L131F, S144T, T146V, K150T, T174D, V177L, D179E, F185C, Y187S, K189R, and T191R, wherein the amino acid position of each group member is indicated according to the numbering of the amino acid residues of SEQ ID NO: 4 in the sequence listing.

In some embodiments the first engineered immunoglobulin chain comprises an engineered domain with a protein-protein interface comprising the amino acid sequence of SEQ ID NO:

35 and/or the second engineered immunoglobulin chain comprises an engineered domain with a protein-protein interface comprising the amino acid sequence of SEQ ID NO: 34.

In a further aspect the present invention provides a hetero-dimeric immunoglobulin or fragment thereof comprising a first engineered immunoglobulin chain comprising an engineered domain with a protein-protein interface comprising the amino acid sequence of SEQ ID NO: 35and a second engineered immunoglobulin chain comprising an engineered domain with a protein-protein interface.

In some embodiments the engineered domain with a protein-protein interface of the first engineered immunoglobulin chain comprises an amino acid sequence which is at least 80 %, preferably at least 90%, more preferably at least 95%, most preferred at least 96%, in particular at least 97%, more particular at least 98%, most particular at least 99% identical to the amino acid sequence of SEQ ID NO: 35, with the proviso that the amino acid sequence which is at least 80 % identical to the amino acid sequence of SEQ ID NO: 35 contains at least one, usually two, preferably at least three, more preferably at least four, most preferred at least five, in particular at least six, more particular at least seven, most particular all of the amino acid residues selected from the group consisting of K127, T129, T144, L146, E150, K151, G174, M177, M185, F187, and W189, wherein the amino acid position of each group member is indicated according to the numbering of the amino acid residues of SEQ ID NO: 35 in the sequence listing.

In a further aspect the present invention provides a hetero-dimeric immunoglobulin or fragment thereof comprising a first engineered immunoglobulin chain comprising an engineered domain with a protein-protein interface and a second engineered immunoglobulin chain comprising an engineered domain with a protein-protein interface comprising the amino acid sequence of SEQ ID NO: 34.

In some embodiments the engineered domain with a protein-protein interface of the second engineered immunoglobulin chain comprises an amino acid sequence which is at least 80 %, preferably at least 90%, more preferably at least 95%, most preferred at least 96%, in particular at least 97%, more particular at least 98%, most particular at least 99% identical to the amino acid sequence of SEQ ID NO: 34, with the proviso that the amino acid sequence which is at least 80 % identical to the amino acid sequence of SEQ ID NO: 34 contains at least one, usually two, preferably at least three, more preferably at least four, most preferred at least five, in particular at least six, more particular at least seven, most particular all of the amino acid residues selected from the group consisting of M131, N144, A146, E151, F172,

A174, S179, N185, V187, L189, and K191, wherein the amino acid position of each group member is indicated according to the numbering of the amino acid residues of SEQ ID NO: 34 in the sequence listing.

In some embodiments the first engineered immunoglobulin chain comprises an engineered domain with a protein-protein interface comprising the amino acid sequence of SEQ ID NO: 40 and/or the second engineered immunoglobulin chain comprises an engineered domain with a protein-protein interface comprising the amino acid sequence of SEQ ID NO: 39.

In a further aspect the present invention provides a hetero-dimeric immunoglobulin or fragment thereof comprising a first engineered immunoglobulin chain comprising an engineered domain with a protein-protein interface comprising the amino acid sequence of SEQ ID NO: 40 and a second engineered immunoglobulin chain comprising an engineered domain with a protein-protein interface.

In some embodiments the engineered domain with a protein-protein interface of the first engineered immunoglobulin chain comprises an amino acid sequence which is at least 80 %, preferably at least 90%, more preferably at least 95%, most preferred at least 96%, in particular at least 97%, more particular at least 98%, most particular at least 99% identical to the amino acid sequence of SEQ ID NO: 40, with the proviso that the amino acid sequence which is at least 80 % identical to the amino acid sequence of SEQ ID NO: 40 contains at least one, usually two, preferably at least three, more preferably at least four, most preferred at least five, in particular at least six, more particular at least seven, most particular all of the amino acid residues selected from the group consisting K7, T9, T24, L26, E30, K31, G55, M57, M67, F69, and W71, wherein the amino acid position of each group member is indicated according to the numbering of the amino acid residues of SEQ ID NO: 40 in the sequence listing.

In a further aspect the present invention provides a hetero-dimeric immunoglobulin or fragment thereof comprising a first engineered immunoglobulin chain comprising an engineered domain with a protein-protein interface and a second engineered immunoglobulin chain comprising an engineered domain with a protein-protein interface comprising the amino acid sequence of SEQ ID NO: 39.

In some embodiments the engineered domain with a protein-protein interface of the second engineered immunoglobulin chain comprises an amino acid sequence which is at least 80 %, preferably at least 90%, more preferably at least 95%, most preferred at least 96%, in particular at least 97%, more particular at least 98%, most particular at least 99% identical to

the amino acid sequence of SEQ ID NO: 39, with the proviso that the amino acid sequence which is at least 80 % identical to the amino acid sequence of SEQ ID NO: 39 contains at least one, usually two, preferably at least three, more preferably at least four, most preferred at least five, in particular at least six, more particular at least seven, most particular all of the amino acid residues selected from the group consisting of M11, N24, A26, E31, F51, A53, S58, N64, V66, L68, and K70, wherein the amino acid position of each group member is indicated according to the numbering of the amino acid residues of SEQ ID NO: 39 in the sequence listing.

In some embodiments the first engineered immunoglobulin chain comprises an engineered domain with a protein-protein interface comprising the amino acid sequence of SEQ ID NO: 48 and/or the second engineered immunoglobulin chain comprises an engineered domain with a protein-protein interface comprising the amino acid sequence of SEQ ID NO: 49.

In a further aspect the present invention provides a hetero-dimeric immunoglobulin or fragment thereof comprising a first engineered immunoglobulin chain comprising an engineered domain with a protein-protein interface comprising the amino acid sequence of SEQ ID NO: 48 and a second engineered immunoglobulin chain comprising an engineered domain with a protein-protein interface.

In some embodiments the engineered domain with a protein-protein interface of the first engineered immunoglobulin chain comprises an amino acid sequence which is at least 80 %, preferably at least 90%, more preferably at least 95%, most preferred at least 96%, in particular at least 97%, more particular at least 98%, most particular at least 99% identical to the amino acid sequence of SEQ ID NO: 48, with the proviso that the amino acid sequence which is at least 80 % identical to the amino acid sequence of SEQ ID NO: 48 contains at least one, usually two, preferably at least three, more preferably at least four, most preferred at least five, in particular at least six, more particular at least seven, most particular all of the amino acid residues selected from the group consisting of Q7, Y9, D16, E17, Q22, S24, T26, K51, T53, D58, F64, Y66, and K68, wherein the amino acid position of each group member is indicated according to the numbering of the amino acid residues of SEQ ID NO: 48 in the sequence listing.

In a further aspect the present invention provides a hetero-dimeric immunoglobulin or fragment thereof comprising a first engineered immunoglobulin chain comprising an engineered domain with a protein-protein interface and a second engineered immunoglobulin

chain comprising an engineered domain with a protein-protein interface comprising the amino acid sequence of SEQ ID NO: 49.

In some embodiments the engineered domain with a protein-protein interface of the second engineered immunoglobulin chain comprises an amino acid sequence which is at least 80 %, preferably at least 90%, more preferably at least 95%, most preferred at least 96%, in particular at least 97%, more particular at least 98%, most particular at least 99% identical to the amino acid sequence of SEQ ID NO: 49, with the proviso that the amino acid sequence which is at least 80 % identical to the amino acid sequence of SEQ ID NO: 49 contains at least one, usually two, preferably at least three, more preferably at least four, most preferred at least five, in particular at least six, more particular at least seven, most particular all of the amino acid residues selected from the group consisting of Q7, Y9, L11, D16, E17, T26, K30, K53, T55, V57, F67, Y69, and K71, wherein the amino acid position of each group member is indicated according to the numbering of the amino acid residues of SEQ ID NO: 49 in the sequence listing.

In some embodiments the first engineered immunoglobulin chain comprises an engineered domain with a protein-protein interface comprising the amino acid sequence of SEQ ID NO: 76 and/or the second engineered immunoglobulin chain comprises an engineered domain with a protein-protein interface comprising the amino acid sequence of SEQ ID NO: 14.

In some embodiments the engineered domain with a protein-protein interface of the first engineered immunoglobulin chain comprises an amino acid sequence which is at least 80 %, preferably at least 90%, more preferably at least 95%, most preferred at least 96%, in particular at least 97%, more particular at least 98%, most particular at least 99% identical to the amino acid sequence of SEQ ID NO: 76, with the proviso that the amino acid sequence which is at least 80 % identical to the amino acid sequence of SEQ ID NO: 76 contains at least one, usually two, preferably at least three, more preferably at least four, most preferred at least five, in particular at least six, more particular at least seven, most particular all of the amino acid residues selected from the group consisting of K24, V26, T30, Y52, S65, V67, W69, and N71,wherein the amino acid position of each group member is indicated according to the numbering of the amino acid residues of SEQ ID NO: 76 in the sequence listing.

In some embodiments the engineered domain with a protein-protein interface of the second engineered immunoglobulin chain comprises an amino acid sequence which is at least 80 %, preferably at least 90%, more preferably at least 95%, most preferred at least 96%, in particular at least 97%, more particular at least 98%, most particular at least 99% identical to

the amino acid sequence of SEQ ID NO: 14, with the proviso that the amino acid sequence which is at least 80 % identical to the amino acid sequence of SEQ ID NO: 14 contains at least one, usually two, preferably at least three, more preferably at least four, most preferred at least five, in particular at least six, more particular at least seven, most particular all of the amino acid residues selected from the group consisting of E7, A9, F11, T24, V26, T30, D54, L57, E59, A65, S67, R69, and R71, wherein the amino acid position of each group member is indicated according to the numbering of the amino acid residues of SEQ ID NO: 14 in the sequence listing.

In some embodiments the first engineered immunoglobulin chain comprises an engineered domain with a protein-protein interface comprising the amino acid sequence of SEQ ID NO: 78 and/or the second engineered immunoglobulin chain comprises an engineered domain with a protein-protein interface comprising the amino acid sequence of SEQ ID NO: 79. In a further aspect the present invention provides a hetero-dimeric immunoglobulin or fragment thereof comprising a first engineered immunoglobulin chain comprising an engineered domain with a protein-protein interface comprising the amino acid sequence of SEQ ID NO: 78 and a second engineered immunoglobulin chain comprising an engineered domain with a protein-protein interface.

In some embodiments the engineered domain with a protein-protein interface of the first engineered immunoglobulin chain comprises an amino acid sequence which is at least 80 %, preferably at least 90%, more preferably at least 95%, most preferred at least 96%, in particular at least 97%, more particular at least 98%, most particular at least 99% identical to the amino acid sequence of SEQ ID NO: 78, with the proviso that the amino acid sequence which is at least 80 % identical to the amino acid sequence of SEQ ID NO: 78 contains at least one, usually two, preferably at least three, more preferably at least four, most preferred at least five, in particular at least six, more particular at least seven, most particular all of the amino acid residues selected from the group consisting of E7, H9, R30, R57, E59, A65, and T67, wherein the amino acid position of each group member is indicated according to the numbering of the amino acid residues of SEQ ID NO: 78 in the sequence listing.

In a further aspect the present invention provides a hetero-dimeric immunoglobulin or fragment thereof comprising a first engineered immunoglobulin chain comprising an engineered domain with a protein-protein interface and a second engineered immunoglobulin

chain comprising an engineered domain with a protein-protein interface comprising the amino acid sequence of SEQ ID NO: 79.

In some embodiments the engineered domain with a protein-protein interface of the second engineered immunoglobulin chain comprises an amino acid sequence which is at least 80 %, preferably at least 90%, more preferably at least 95%, most preferred at least 96%, in particular at least 97%, more particular at least 98%, most particular at least 99% identical to the amino acid sequence of SEQ ID NO: 79, with the proviso that the amino acid sequence which is at least 80 % identical to the amino acid sequence of SEQ ID NO: 79 contains at least one, usually two, preferably at least three, more preferably at least four, most preferred at least five, in particular at least six, more particular at least seven, most particular all of the amino acid residues selected from the group consisting of T24, L52, W54, I69, and R71, wherein the amino acid position of each group member is indicated according to the numbering of the amino acid residues of SEQ ID NO: 79 in the sequence listing.

In some embodiments the first engineered immunoglobulin chain comprises an engineered domain with a protein-protein interface comprising the amino acid sequence of SEQ ID NO: 84 and/or the second engineered immunoglobulin chain comprises an engineered domain with a protein-protein interface comprising the amino acid sequence of SEQ ID NO: 85.

In a further aspect the present invention provides a hetero-dimeric immunoglobulin or fragment thereof comprising a first engineered immunoglobulin chain comprising an engineered domain with a protein-protein interface comprising the amino acid sequence of SEQ ID NO: 84 and a second engineered immunoglobulin chain comprising an engineered domain with a protein-protein interface.

In some embodiments the engineered domain with a protein-protein interface of the first engineered immunoglobulin chain comprises an amino acid sequence which is at least 80 %, preferably at least 90%, more preferably at least 95%, most preferred at least 96%, in particular at least 97%, more particular at least 98%, most particular at least 99% identical to the amino acid sequence of SEQ ID NO: 84, with the proviso that the amino acid sequence which is at least 80 % identical to the amino acid sequence of SEQ ID NO: 84 contains at least one, usually two, preferably at least three, more preferably at least four, most preferred at least five, in particular at least six, more particular at least seven, most particular all of the amino acid residues selected from the group consisting of K26, V28, Y56, S71, V73, W75,

and N77, wherein the amino acid position of each group member is indicated according to the numbering of the amino acid residues of SEQ ID NO: 84 in the sequence listing.

In a further aspect the present invention provides a hetero-dimeric immunoglobulin or fragment thereof comprising a first engineered immunoglobulin chain comprising an engineered domain with a protein-protein interface and a second engineered immunoglobulin chain comprising an engineered domain with a protein-protein interface comprising the amino acid sequence of SEQ ID NO: 85.

In some embodiments the engineered domain with a protein-protein interface of the second engineered immunoglobulin chain comprises an amino acid sequence which is at least 80 %, preferably at least 90%, more preferably at least 95%, most preferred at least 96%, in particular at least 97%, more particular at least 98%, most particular at least 99% identical to the amino acid sequence of SEQ ID NO: 85, with the proviso that the amino acid sequence which is at least 80 % identical to the amino acid sequence of SEQ ID NO: 85 contains at least one, usually two, preferably at least three, more preferably at least four, most preferred at least five, in particular at least six, more particular at least seven, most particular all of the amino acid residues selected from the group consisting of E8, A10, F12, V28, D58, L61, A71, S73, R75, and R77, wherein the amino acid position of each group member is indicated according to the numbering of the amino acid residues of SEQ ID NO: 85 in the sequence listing.

In some embodiments the present disclosure provides a hetero-dimeric immunoglobulin or fragment thereof wherein the first engineered immunoglobulin chain comprises the amino acid sequence selected from the group consisting of SEQ ID NOs: 3, 4, 6, 7, 13-29, 34, 35, 36, 39, 40, 41, 42, 48, 49, 50, 51, 59, 61, and 68 and/or the second engineered immunoglobulin chain comprises the amino acid sequence selected from the group consisting of SEQ ID NOs: 3, 4, 6, 7, 13-29, 34, 35, 36, 39,, 40, 41, 42, 48, 49, 50, 51, 59, 61, and 68.

In some embodiments the present disclosure provides a hetero-dimeric immunoglobulin or fragment thereof wherein the first engineered immunoglobulin chain comprises the amino acid sequence selected from the group consisting of SEQ ID NOs: 3, 4, 6, 7, 13-29, 34, 35, 36, 39, 40, 41, 42, 48, 49, 50, 51, 59, 61, 68, 73, 78 and 84 and/or the second engineered immunoglobulin chain comprises the amino acid sequence selected from the group consisting of SEQ ID NOs: 3, 4, 6, 7, 13-29, 34, 35, 36, 39, 40, 41, 42, 48, 49, 50, 51, 59, 61, 68, 74, 79 and 85.

In some embodiments the present disclosure provides a hetero-dimeric immunoglobulin or fragment thereof wherein the first engineered immunoglobulin chain comprises the amino acid sequence selected from the group consisting of SEQ ID NOs: 3, 7, 13, 59, and 68 and/or the second engineered immunoglobulin chain comprises the amino acid sequence selected from the group consisting of SEQ ID NOs: 4, 6, 14, 15, 16, 17, 41, 50, and 61.

In some embodiments the present disclosure provides a hetero-dimeric immunoglobulin or fragment thereof wherein the first engineered immunoglobulin chain comprises the amino acid sequence selected from the group consisting of SEQ ID NOs: 3, 7, 13, 59, 68, 73 and 84 and/or the second engineered immunoglobulin chain comprises the amino acid sequence selected from the group consisting of SEQ ID NOs: 4, 6, 14, 15, 16, 17, 41, 50, 61, 74 and 85.

In some embodiments the present disclosure provides a hetero-dimeric immunoglobulin or fragment thereof wherein the first engineered immunoglobulin chain comprises the amino acid sequence selected from the group consisting of SEQ ID NOs: 34, 39, and 41 and/or the second engineered immunoglobulin chain comprises the amino acid sequence selected from the group consisting of SEQ ID NOs: 35, 36, 40, and 42 .

In some embodiments the present disclosure provides a hetero-dimeric immunoglobulin or fragment thereof wherein the first engineered immunoglobulin chain comprises the amino acid sequence selected from the group consisting of SEQ ID NOs: 48, 49, 50, and 51 and/or the second engineered immunoglobulin chain comprises the amino acid sequence selected from the group consisting of SEQ ID NOs: 48, 49, 50, and 51. Preferably the first engineered immunoglobulin chain comprises the amino acid sequence selected from the group consisting of SEQ ID NO: 48 and the second engineered immunoglobulin chain comprises the amino acid sequence of SEQ ID NO: 49 or of SEQ ID NO: 51. Equally preferred the first engineered immunoglobulin chain comprises the amino acid sequence selected from the group consisting of SEQ ID NO: 50 and the second engineered immunoglobulin chain comprises the amino acid sequence of SEQ ID NO: 49 or of SEQ ID NO: 51.

In some embodiments the present disclosure provides a hetero-dimeric immunoglobulin or fragment thereof wherein the first engineered immunoglobulin chain comprises the amino acid sequence of SEQ ID NO: 78 and/or the second engineered immunoglobulin chain comprises the amino acid sequence of SEQ ID NO: 79.

In some embodiments the present disclosure provides a hetero-dimeric immunoglobulin or fragment thereof wherein the first and/or second engineered immunoglobulin chain comprises the amino acid sequence selected from the group consisting o SEQ ID NOs: 3, 7, 13, 59, and

68 and the third engineered immunoglobulin chain comprises the amino acid sequence selected from the group consisting of SEQ ID NOs: 4, 6, 14, 15, 16, 17, 41, 50, and 61.

In some embodiments the present disclosure provides a hetero-dimeric immunoglobulin or fragment thereof wherein the first and/or second engineered immunoglobulin chain comprises the amino acid sequence selected from the group consisting of SEQ ID NOs: 34, 39 and 41 and/or the third engineered immunoglobulin chain comprises the amino acid sequence selected from the group consisting of SEQ ID NOs 35, 36, 40, and 42.

In some embodiments the present disclosure provides a hetero-dimeric immunoglobulin or fragment thereof wherein the first and/or second engineered immunoglobulin chain comprises the amino acid sequence selected from the group consisting of SEQ ID NOs: 3, 4, 6, 7, 13-29, 34, 35, 36, 39, 40, 41, 42, 48, 49, 50, 51, 59, 61, 68, 73, 78 and 84 and/or the third engineered immunoglobulin chain comprises the amino acid sequence selected from the group consisting of SEQ ID NOs 3, 4, 6, 7, 13-29, 34, 35, 36, 39,, 40, 41, 42, 48, 49, 50, 51, 59, 61, 68, 74, 79 and 85.

In some embodiments the present disclosure provides a hetero-dimeric immunoglobulin or fragment thereof wherein the first and/or second engineered immunoglobulin chain comprises the amino acid sequence of SEQ ID NO: 78 and/or the third engineered immunoglobulin chain comprises the amino acid sequence of SEQ ID NO 79.

In some embodiments the present disclosure provides a hetero-dimeric immunoglobulin or fragment thereof wherein the first and/or second engineered immunoglobulin chain comprises the amino acid sequence selected from the group consisting of SEQ ID NOs: 3, 7, 13, 59, 68 and/or the third engineered immunoglobulin chain comprises the amino acid sequence selected from the group consisting of SEQ ID NOs: 3, 7, 13, 59, 68.

In some embodiments the present disclosure provides a hetero-dimeric immunoglobulin or fragment thereof wherein the first engineered immunoglobulin chain comprises the amino acid sequence of SEQ ID NO: 72, and the second engineered immunoglobulin chain comprises the amino acid sequence of SEQ ID NO: 71 and the third engineered immunoglobulin chain which interacts with the protein-protein interface of the engineered domain of the second engineered immunoglobulin chain comprises the amino acid sequence of SEQ ID NO: 51.

In some embodiments the present disclosure provides a hetero-dimeric immunoglobulin or fragment thereof which comprises a first engineered immunoglobulin chain comprising the amino acid sequence of SEQ ID NO: 72, a second engineered immunoglobulin chain comprising the amino acid sequence of SEQ ID NO: 70, a third engineered immunoglobulin chain with an engineered domain which interacts with the protein-protein interface of the engineered domain of the first engineered immunoglobulin chain comprising the amino acid sequence of SEQ ID NO: 69 and a third engineered immunoglobulin chain with an engineered domain which interacts with the protein-protein interface of the engineered domain of the second engineered immunoglobulin chain comprising the amino acid sequence of SEQ ID NO: 42.

In some embodiments the present disclosure provides a hetero-dimeric immunoglobulin or fragment thereof, wherein the first and second engineered immunoglobulin chains comprise an Fc region. In some embodiments the present disclosure provides a hetero-dimeric immunoglobulin or fragment thereof, wherein the first and/or the second and the third engineered immunoglobulin chains comprise an Fc region. Usually the Fc region is selected from the group consisting of IgA, IgE, IgD, IgG, and IgM Fc region.

In some embodiments the present disclosure provides a hetero-dimeric immunoglobulin or fragment thereof, wherein the first and second engineered immunoglobulin chains comprise an Fc region from the same species, preferably from the same species and isotype, more preferably from the same species, isotype and subclass.

In some embodiments the present disclosure provides a hetero-dimeric immunoglobulin or fragment thereof, wherein the first and/or the second and the third engineered immunoglobulin chains comprise an Fc region from the same species, preferably from the same species and isotype, more preferably from the same species, isotype and subclass.

In some embodiments the present disclosure provides a hetero-dimeric immunoglobulin or fragment thereof, wherein the Fc regions of the first and second engineered immunoglobulin chains are are not identical. In some embodiments the present disclosure provides a hetero-dimeric immunoglobulin or fragment thereof, wherein the Fc regions of the first and second engineered immunoglobulin chains have amino acid sequences which are different from each other by one amino acid or by two amino acids or by three amino acids or by four amino acids or by five amino acids or by five to ten amino acids or by ten to thirty amino acids, preferably by at least two amino acids or by at least three amino acids or by at least four amino acids or

by at least five amino acids or by at least five to ten amino acids or by at least ten to thirty amino acids.

In some embodiments the present disclosure provides a hetero-dimeric immunoglobulin or fragment thereof which is bispecific.

In some embodiments the present disclosure provides a hetero-dimeric immunoglobulin or fragment thereof, wherein the hetero-dimeric immunoglobulin or fragment thereof is a full-length antibody.

In some embodiments the present disclosure provides a hetero-dimeric immunoglobulin or fragment thereof, wherein the hetero-dimeric immunoglobulin or fragment thereof is a full-length antibody which is bispecific.

In some embodiments the present disclosure provides a hetero-dimeric immunoglobulin or fragment thereof, wherein the bispecific hetero-dimeric immunoglobulin or fragment thereof or the bispecific antibody binds to antigens selected from the group consisting of HER2, EGFR, CD19 and VLA-2. Preferably the bispecific hetero-dimeric immunoglobulin or fragment thereof or the bispecific antibody is specific to HER2 and EGFR. Equally preferably the bispecific hetero-dimeric immunoglobulin or fragment thereof or the bispecific antibody is specific to CD19 and VLA-2.

In some embodiments the present disclosure provides a hetero-dimeric immunoglobulin or fragment thereof which is a Fab, with a first engineered immunoglobulin chain and a second engineered immunoglobulin chain.

In some embodiments the present disclosure provides a hetero-dimeric immunoglobulin or fragment thereof is a $F(ab')_2$ fragment, wherein the first engineered immunoglobulin chain comprises an engineered domain and a further engineered domain, whereas the protein interface of the engineered domain of the first engineered immunoglobulin chain interacts with an engineered domain of a third immunoglobulin chain and the protein interface of the further engineered domain interacts with an engineered domain of a third immunoglobulin chain, wherein the protein interfaces of these engineered domain of these two third immunoglobulin chains are the same or are different form each other.

In some embodiments the present disclosure provides a hetero-dimeric immunoglobulin or fragment thereof, wherein wherein at least one additional polypeptide is fused to the first and/or second engineered immunoglobulin chain. Preferably the one additional polypeptide is

fused to the first engineered immunoglobulin chain. Suitably the additional polypeptide is selected from the group consiting of Fab, scFv, diabody, domain antibody, pharmacologically active peptide or protein, receptor extracellular domain, CDR grafted polypeptide and therapeutic engineered protein scaffold. Fusion is usually achieved by genetic engineering as known to the skilled person and may involve amino acid sequence linkers which do not form part of the amino acid sequences to be fused.

In some embodiments the present disclosure provides a multidomain protein comprising at least a first and a second nonidentical engineered domain, each of the first and the second engineered domain containing a protein-protein interface, wherein at least one amino acid residue of the protein-protein interface of the first parent domain are substituted with amino acid residues at the equivalent 3D structural position from a protein-protein interface of a donor domain of a first member of the naturally occurring immunoglobulin super-family and at least one amino acid residue of the protein-protein interface of the second parent domain are substituted with amino acid residues at the equivalent 3D structural position from a protein-protein interface of a donor domain of a second member of the naturally occurring immunoglobulin super-family,
wherein the donor domain of the first member of the naturally occurring immunoglobulin super-family and the donor domain of the second member of the naturally occurring immunoglobulin super-family form a hetero-dimer or a homo-dimer.
The multidomain protein comprises preferably a Fc region. Usually the Fc region is selected from the group consisting of IgA, IgE, IgD, IgG, and IgM Fc region.

In some embodiments the present disclosure provides a hetero-dimeric immunoglobulin or fragment thereof or methods of making hetero-dimeric immunoglobulins or fragments thereof as described below wherein the amino acid residue of the protein-protein interface of the parent domain of the parent immunoglobulin chain which is substituted at the equivalent 3D structural position from the protein-protein interface of a donor domain is an amino acid residue in the protein-protein interface of a donor domain which when overlaid on the protein-protein interface of the parent domain by superimposing the carbon alpha traces of both domains, occupies a 3D position within less than a distance of 6 Å to the closest residue of the

parent domain, wherein the donor domain is different from the parent domain.

Methods of making hetero-dimeric immunoglobulins or fragments thereof

In a further aspect the present invention provides a method to produce a hetero-dimeric immunoglobulin or hetero-dimeric fragment thereof comprising:

(a) substituting at least one amino acid residue of the protein-protein interface of a parent domain of a first parent immunoglobulin chain at the equivalent 3D structural position from a protein-protein interface of a donor domain of a first member of the naturally occurring immunoglobulin super-family to obtain a first engineered immunoglobulin chain comprising an engineered domain,

(b) substituting at least one amino acid residue of the protein-protein interface of the parent domain of a second parent immunoglobulin chain at the equivalent 3D structural position from a protein-protein interface of a donor domain of a second member of the naturally occurring immunoglobulin super-family to obtain a second engineered immunoglobulin chain comprising an engineered domain,

(c) culturing a host cell comprising a nucleic acid encoding said engineered immunoglobulin chains, wherein the culturing is such that the nucleic acid is expressed and the engineered immunoglobulin chains produced; and

(d) recovering the hetero-dimeric immunoglobulin or hetero-dimeric fragment thereof from the host cell culture.

In some embodiments the present disclosure provides a method wherein the engineered domain of the first engineered immunoglobulin chain and the engineered domain of the second engineered immunoglobulin chain are not identical.

In some embodiments the present disclosure provides a method wherein the protein-protein interface of the engineered domain of the first engineered immunoglobulin chain is interacting with the protein-protein interface of the engineered domain of the second engineered immunoglobulin chain by hetero-dimerization or by homo-dimerization.

In some embodiments the present disclosure provides a method comprising a parent domain of the first and/or second parent immunoglobulin chain which is selected from the group consisting of CH1, CH2, CH3, CH4, IGKC, IGLC1, IGLC2, IGLC3, IGLC6, and IGLC7. CH1, CH3 can be from IGHA1, IGHA2, IGHD, IGHE, IGHG1, IGHG2, IGHG3, IGHG4,

IGHGP and IGHM; CH4 can be from IGHE and IGHM. CH2 can be from IGHA1, IGHA2, IGHD, IGHE, IGHG1, IGHG2, IGHG3, IGHG4, IGHGP and IGHM. In some embodiments the parent domain of the first and/or the second engineered immunoglobulin chain is a domain selected from the group consisting of CH1 domain, CH4 domain, IGKC domain, IGLC1 domain, IGLC2 domain, IGLC3 domain, IGLC6 domain, and IGLC7 domain, preferably from the group consisting of CH1 domain, IGKC domain, IGLC1 domain, IGLC2 domain, IGLC3 domain, IGLC6 domain, and IGLC7 domain.

Usually the parent domain of the first and/or second engineered immunoglobulin chain is from human. Preferably the parent domain of the first and/or second engineered immunoglobulin chain is from human and from the same isotype, species and subclass. The preferred parent domain of the first and/or second engineered immunoglobulin chain, in particular the parent domain of the first and second engineered immunoglobulin chain, is a CH3 domain, more preferably a human CH3 domain, in particular a human CH3 domain from IGHG1.

Equally preferred the parent domain of the first engineered immunoglobulin chain is a CH1 domain and the parent domain of the second engineered immunoglobulin chain is a light chain constant domain such as an IGLC domain or an IGKC domain. Equally, the preferred parent domain of the first engineered immunoglobulin chain is a light chain constant domain such as an IGLC domain or an IGKC domain and the preferred parent domain of the second engineered immunoglobulin chain is a CH1 domain more preferably a human CH1 domain selected from the group consisting of IGHG1, IGHG2, IGHG3, and IGHG4, in particular a human CH1 domain from IGHG1.

In some embodiments the present disclosure provides a method, wherein the parent domains of the first and the second engineered immunoglobulin chain form a naturally occurring homo-dimer. In some embodiments the present disclosure provides a method, wherein the parent domains of the first and the second engineered immunoglobulin chain form a naturally occurring hetero-dimer.

In some embodiments the present disclosure provides a method, wherein the donor domain of the first member of the naturally occurring immunoglobulin super-family and the donor domain of the second member of the naturally occurring immunoglobulin super-family form a naturally occurring hetero-dimer or a naturally occurring homo-dimer and/or wherein the protein-protein interface of the engineered domain of the first engineered immunoglobulin

chain is interacting with the protein-protein interface of the engineered domain of the second engineered immunoglobulin chain by hetero-dimerization or by homo- dimerization. Preferably the donor domain of the first member of the naturally occurring immunoglobulin super-family and the donor domain of the second member of the naturally occurring immunoglobulin super-family form a naturally occurring hetero-dimer.

In some embodiments the present disclosure provides a method, wherein the amino acid residues from the protein-protein interface of the donor domain of the first and second member of the naturally occurring immunoglobulin super-family are amino acids non essential to the core integrity of the domain.

In some embodiments the present disclosure provides a method, wherein the first and the second member of the naturally occurring immunoglobulin super-family are selected from the TCR constant domain family. Preferably the donor domain of the first member of the naturally occurring immunoglobulin super-family is the TCR constant domain alpha (SEQ ID NO: 1) and the donor domain of the second member of the naturally occurring immunoglobulin super-family is the TCR constant domain beta (SEQ ID NO: 2). Equally the donor domain of the first member of the naturally occurring immunoglobulin super-family can be the TCR constant domain beta (SEQ ID NO: 2) and the donor domain of the second member of the naturally occurring immunoglobulin super-family can be the TCR constant domain alpha (SEQ ID NO: 1). In a further embodiment the present disclosure provides a method, wherein the donor domain of the first member of the naturally occurring immunoglobulin super-family is the TCR constant domain alpha (SEQ ID NO: 1) and the donor domain of the second member of the naturally occurring immunoglobulin super-family is the TCR constant domain beta (SEQ ID NO: 2), wherein the cysteine (C) at amino acid position 75 in SEQ ID NO: 2 is substituted with alanine (A) or serine (S), preferably with alanine (A).

In a further embodiment the present disclosure provides a method, wherein the donor domain of the first member of the naturally occurring immunoglobulin super-family is the TCR constant domain gamma (SEQ ID NO: 33) and the donor domain of the second member of the naturally occurring immunoglobulin super-family is the TCR constant domain delta (SEQ ID NO: 32) or wherein the donor domain of the first member of the naturally occurring immunoglobulin super-family is the TCR constant domain delta (SEQ ID NO: 32) and the donor domain of the second member of the naturally occurring immunoglobulin super-family

is the TCR constant domain gamma (SEQ ID NO: 33).

In some embodiments the present disclosure provides a method, wherein the donor domain of the first member of the naturally occurring immunoglobulin super-family is the TCR constant domain alpha (SEQ ID NO: 1), the donor domain of the second member of the naturally occurring immunoglobulin super-family is the TCR constant domain beta (SEQ ID NO: 2), the donor domain of the third member of the naturally occurring immunoglobulin super-family is the TCR constant domain delta (SEQ ID NO: 32) and the donor domain of the fourth member of the naturally occurring immunoglobulin super-family is the TCR constant domain gamma (SEQ ID NO: 33).

In some embodiments the present disclosure provides a method, wherein the donor domain of the first member of the naturally occurring immunoglobulin super-family is the TCR constant domain delta (SEQ ID NO: 32), the donor domain of the second member of the naturally occurring immunoglobulin super-family is the TCR constant domain gamma (SEQ ID NO: 33), the donor domain of the third member of the naturally occurring immunoglobulin super-family is the TCR constant domain alpha (SEQ ID NO: 1) and the donor domain of the fourth member of the naturally occurring immunoglobulin super-family is the TCR constant domain beta (SEQ ID NO: 2).

In some embodiments the present disclosure provides a method, wherein the donor domain of the first member of the naturally occurring immunoglobulin super-family is the TCR constant domain alpha (SEQ ID NO: 1) or the TCR constant domain delta (SEQ ID NO: 32), the donor domain of the second member of the naturally occurring immunoglobulin super-family is the TCR constant domain beta (SEQ ID NO: 2) or the TCR constant domain gamma (SEQ ID NO: 33), the donor domain of the third and the fourth member of the naturally occurring immunoglobulin super-family is the IgG1 CH3 domain (SEQ ID NO: 47), with the proviso that if the donor domain of the first member of the naturally occurring immunoglobulin super-family is the TCR constant domain alpha (SEQ ID NO: 1), the donor domain of the second member of the naturally occurring immunoglobulin super-family is the TCR constant domain beta (SEQ ID NO: 2) and if the donor domain of the first member of the naturally occurring immunoglobulin super-family is the TCR constant domain delta (SEQ ID NO: 32), the donor domain of the second member of the naturally occurring immunoglobulin super-family is the

TCR constant domain gamma (SEQ ID NO: 33).

In a further embodiment the present disclosure provides a method, wherein the first engineered immunoglobulin chain and/or the second engineered immunoglobulin chain comprise a further engineered domain with a protein-protein interface, wherein at least one amino acid residue of the protein-protein interface of the parent domain of said further engineered domain of said first engineered immunoglobulin chain and/or said second engineered immunoglobulin chain is substituted with an amino acid residue at the equivalent 3D structural position from a protein-protein interface of a donor domain of a third member of the naturally occurring immunoglobulin super-family, and
wherein the protein-protein interface of the further engineered domain of the first engineered immunoglobulin chain and/or the second engineered immunoglobulin chain is interacting with the protein-protein interface of an engineered domain of a third engineered immunoglobulin chain by hetero-dimerization or by homo-dimerization,
wherein at least one amino acid residue of the protein-protein interface of the parent domain of said engineered domain of said third engineered immunoglobulin chain is substituted with an amino acid residue at the equivalent 3D structural position from a protein-protein interface of a donor domain of a fourth member of the naturally occurring immunoglobulin super-family and,
wherein the protein-protein interface of the further engineered domain of the first engineered immunoglobulin chain and/or the second engineered immunoglobulin chain and of the engineered domain of the third engineered immunoglobulin chain is different from the protein-protein interface of the engineered domain of the first engineered immunoglobulin chain and of the engineered domain of the second engineered immunoglobulin chain.

In some embodiments the present disclosure provides a method, wherein the further engineered domain of the first engineered immunoglobulin chain and/or the second engineered immunoglobulin chain and the engineered domain of the third engineered immunoglobulin chain are not identical.

In some embodiments the parent domain of the first and/or second engineered immunoglobulin chain, in particular the parent domain of the first and second engineered immunoglobulin chain is a CH3 domain, preferably a CH3 domain of the same species, isotype and subclass, and the parent domain of the further engineered domain of the first

engineered immunoglobulin chain and/or the second engineered immunoglobulin is a CH1 domain and the parent domain of the engineered domain of the third engineered immunoglobulin chain is a a light chain constant domain such as an IGLC domain or an IGKC domain.

In some embodiments the parent domain of the first engineered immunoglobulin chain is a light chain constant domain such as an IGLC domain or an IGKC domain and the parent domain of the engineered domain of the second engineered immunoglobulin chain is a CH1 domain and the parent domain of the further engineered domain of the first engineered immunoglobulin chain and/or the second engineered immunoglobulin and the parent domain of the engineered domain of the third engineered immunoglobulin chain is a CH3 domain, preferably a CH3 domain of the same species, isotype and subclass.

In some embodiments the present disclosure provides a method, wherein the amino acid residues of the protein-protein interface of the parent domain of the first and/or second parent immunoglobulin chain which are substituted are not adjacent.

In some embodiments the present disclosure provides a method, wherein the amino acid residues of the protein-protein interface of the parent domain of the further engineered domain of the first engineered immunoglobulin chain and/or the second engineered immunoglobulin and/or the parent domain of the engineered domain of the third engineered immunoglobulin chain which are substituted are not adjacent.

In some embodiments the present disclosure provides a method, wherein, preferably at least two, more preferably at least three, most preferably at least four, in particular at least five amino acid residues of the protein-protein interface of the parent domain of the first and/or second engineered immunoglobulin chain which are substituted are not adjacent.

In some embodiments the present disclosure provides a method, wherein, preferably at least two, more preferably at least three, most preferably at least four, in particular at least five amino acid residues of the protein-protein interface of the parent domain of the further engineered domain of the first engineered immunoglobulin chain and/or the second engineered immunoglobulin and/or the parent domain of the engineered domain of the third engineered immunoglobulin chain which are substituted are not adjacent.

In some embodiments the present disclosure provides a method, wherein preferably at least two, more preferably at least three, most preferably at least four, in particular at least five more particular at least six, most particular at least seven amino acid residues of the protein-protein interface of the parent domain of the first and/or second parent immunoglobulin chain

are substituted.

In some embodiments the present disclosure provides a method, wherein preferably at least two, more preferably at least three, most preferably at least four, in particular at least five more particular at least six, most particular at least seven amino acid residues of the protein-protein interface of the parent domain of the further engineered domain of the first engineered immunoglobulin chain and/or the second engineered immunoglobulin and/or the parent domain of the engineered domain of the third engineered immunoglobulin chain are substituted.

In some embodiments the present disclosure provides a method, wherein the amino acid sequence of the engineered domains of the first engineered immunoglobulin chain and/or the second engineered immunoglobulin chain do not contain an insertion of one or more amino acid residues compared to the amino acid sequence of the parent domains of the first engineered immunoglobulin chain and/or the second engineered immunoglobulin chain.

In some embodiments the present disclosure provides a method, wherein at least one 3D structural position of the protein-protein interface of the engineered domain of the first engineered immunoglobulin chain is different in amino acid residue compared to the identical 3D structural position of the protein-protein interface of the engineered domain of the second engineered immunoglobulin chain.

In some embodiments the present disclosure provides a method, wherein the first and second engineered immunoglobulin chains have parent domains from the same species, preferably from the same species and isotype, more preferably from the same species, isotype and subclass. In some embodiments the present disclosure provides a method, wherein the first and second engineered immunoglobulin chains have identical parent domains, i.e. parent domains which have the identical amino acid sequence.

In some embodiments the present disclosure provides a method, wherein the first and second engineered immunoglobulin chains have parent domains from IgG1, preferably human IgG1.

In some embodiments the present disclosure provides a method, wherein the first and second engineered immunoglobulin chains have parent domains which are not identical. In some embodiments the present disclosure provides a method, wherein the first and second engineered immunoglobulin chains have parent domains which have amino acid sequences which are different from each other by one amino acid or by two amino acids or by three

amino acids or by four amino acids or by five amino acids or by five to ten amino acids or by ten to thirty amino acids

In some embodiments the present disclosure provides a method, wherein the first and second engineered immunoglobulin chains comprise an Fc region. In some embodiments the present disclosure provides a method, wherein the first and/or the second and the third engineered immunoglobulin chains comprise an Fc region. Usually the Fc region is selected from the group consisting of IgA, IgE, IgD, IgG, and IgM Fc region.

In some embodiments the present disclosure provides a method, wherein the first and second engineered immunoglobulin chains comprise an Fc region from the same species, preferably from the same species and isotype, more preferably from the same species, isotype and subclass.

In some embodiments the present disclosure provides a method, wherein the first and/or the second and the third engineered immunoglobulin chains comprise an Fc region from the same species, preferably from the same species and isotype, more preferably from the same species, isotype and subclass.

In some embodiments the present disclosure provides a method, wherein the Fc regions of the first and second engineered immunoglobulin chains are are not identical. In some embodiments the present disclosure provides a method, wherein the Fc regions of the first and second engineered immunoglobulin chains have amino acid sequences which are different from each other by one amino acid or by two amino acids or by three amino acids or by four amino acids or by five amino acids or by five to ten amino acids or by ten to thirty amino acids

In some embodiments the present disclosure provides a method, wherein the hetero-dimeric immunoglobulin or fragment thereof is a full-length antibody.

In some embodiments the present disclosure provides a method, wherein the hetero-dimeric immunoglobulin or fragment thereof is a full-length antibody which is bispecific.

In some embodiments the present disclosure provides a bispecific hetero-dimeric immunoglobulin or fragment thereof or a bispecific full-length antibody which binds to antigens selected from the group consisting of HER2, EGFR, CD19 and VLA-2. Preferably the bispecific hetero-dimeric immunoglobulin or fragment thereof or the bispecific antibody is specific to HER2 and EGFR. Equally preferably the bispecific hetero-dimeric

immunoglobulin or fragment thereof or the bispecific antibody is specific to CD19 and VLA-2.

In some embodiments the present disclosure provides a method, wherein wherein at least one additional polypeptide is fused to the first and/or second engineered immunoglobulin chain. Preferably the additional domain is fused to the first engineered immunoglobulin chain. Optionally the additional domain can be fused to the first and/or second parent immunoglobulin chain before engineering the parent domain. Suitably the additional domain is selected from the group consiting of FAB, scFv, diabody, domain antibody, pharmacologically active peptide or protein, receptor extracellular domain, CDR grafted polypeptide and therapeutic engineered protein scaffold.

In a further aspect the present invention provides a method to engineer a protein-protein interface of a domain of a multidomain protein comprising:

(a) providing a multidomain protein comprising a domain with a protein-protein interface;

(b) selecting as a donor domain a naturally occurring immunoglobulin super-family member comprising a domain with a protein-protein interface which is different from the domain of (a);

(c) overlaying 3D structures of the domain with the protein-protein interface of (a) and the donor domain with the protein-protein interface of (b);

(d) identifying exposed protein-protein interface residues in the overlayed 3D structures of the domain with the protein-protein interface of (a) and the donor domain with the protein-protein interface of (b);

e) substituting at least one amino acid residue of the identified exposed protein-protein interface amino acid residues of the domain with the protein-protein interface of (a) with amino acid residues at the equivalent 3D structural position from the identified exposed protein-protein interface amino acid residues from the donor domain with the protein-protein interface of (b).

In some embodiments the present disclosure provides a method, wherein the naturally occurring immunoglobulin super-family member is a naturally occurring homo-dimer.

In some embodiments the present disclosure provides a method, wherein the naturally occurring immunoglobulin super-family member is a naturally occurring hetero-dimer.

Usually at least two, preferably at least three, more preferably at least four, most preferred at least five, in particular at least six, more particular at least seven amino acid residues are

substituted. Preferably the domain with a protein-protein interface of the multidomain protein of (a) is capable to form a homo-dimer, preferably a naturally occurring homo-dimer, with another domain,. Preferably the domain with a protein-protein interface of the multidomain protein of (a) is capable to form a hetero-dimer, preferably a naturally occurring hetero-dimer, with another domain. Preferably, the exposed interface residues identified in the immunoglobulin chain and in the selected immunoglobulin super-family members are non essential to the domain core integrity. Preferably, the naturally occurring immunoglobulin super-family member is selected from the TCR constant domain family. More preferably, the donor domain is the TCR constant domain alpha (SEQ ID NO: 1) or the TCR constant domain beta (SEQ ID NO: 2), whereas in an alternative embodiment the cysteine (C) at amino acid position 75 in SEQ ID NO: 2 is substituted with alanine (A) or serine (S). As well preferred as donor domain is a CH3 domain, more preferably a human CH3 domain, in particular a human CH3 domain from IGHG1.

In a further embodiment the present disclosure provides a method, wherein the donor domain is the TCR constant domain gamma (SEQ ID NO: 33) or the TCR constant domain delta (SEQ ID NO: 32).

In some embodiments the present disclosure provides a method, wherein the amino acid residues of the protein-protein interface of the parent domain of the first and/or second parent immunoglobulin chain which are substituted are not adjacent.

Preferably, the identified exposed protein-protein interface amino acid residues of the donor domain with the protein-protein interface of (b) which are used for substituting at least one amino acid residue of the identified exposed protein-protein interface amino acid residues of the domain with the protein-protein interface of (a) are selected from the group consisting of K20, V22, T26, Y43, S61, V63, W65 and N67 wherein the amino acid position of each group member corresponds to the amino acid position in SEQ ID NO: 1. Alternatively preferred, the identified exposed protein-protein interface amino acid residues of the donor domain with the protein-protein interface of (b) which are used for substituting at least one amino acid residue of the identified exposed protein-protein interface amino acid residues of the domain with the protein-protein interface of (a) are selected from the group consisting of E10, A12, F14, T28, V30, T34, D59, L63, E65, C75, S77, R79 and R81 wherein the amino acid position of each group member corresponds to the amino acid position in SEQ ID NO: 2.

Preferably, the identified exposed protein-protein interface amino acid residues of the donor domain with the protein-protein interface of (b) which are used for substituting at least one

amino acid residue of the identified exposed protein-protein interface amino acid residues of the domain with the protein-protein interface of (a) are selected from the group consisting of K4,T6, T22, L24, E28, K29, G53, M56, M63, F65, W67, wherein the amino acid position of each group member corresponds to the amino acid position in SEQ ID NO: 33 Alternatively preferred, the identified exposed protein-protein interface amino acid residues of the donor domain with the protein-protein interface of (b) which are used for substituting at least one amino acid residue of the identified exposed protein-protein interface amino acid residues of the domain with the protein-protein interface of (a) are selected from the group consisting of M13, N18, A20, E25, F44, A47, S51, N57, V59, L61, K63, wherein the amino acid position of each group member corresponds to the amino acid position in SEQ ID NO: 32.
Preferably, the identified exposed protein-protein interface amino acid residues of the donor domain with the protein-protein interface of (b) which are used for substituting at least one amino acid residue of the identified exposed protein-protein interface amino acid residues of the domain with the protein-protein interface of (a) are selected from the group consisting of Q127, Y129, L131, D136, E137, Q142, S144, T146, K150, K172, T174, V177, D179, F185, Y187, K189, wherein the amino acid position of each group member corresponds to the amino acid position in SEQ ID NO: 12.

In a further aspect the present invention provides the use of a donor domain of a first and a second member of the naturally occurring immunoglobulin super-family to engineer a hetero-dimeric immunoglobulin or hetero-dimeric fragment thereof comprising a first engineered immunoglobulin chain comprising at least one engineered domain which has a protein-protein interface which is substituted with at least one amino acid residue from the protein-protein interface of the donor domain of the first member of the naturally occurring immunoglobulin super-family and a second engineered immunoglobulin chain comprising at least one engineered domain which has a protein-protein interface which is substituted with at least one amino acid residue from the protein-protein interface of the donor domain of the second member of the naturally occurring immunoglobulin super-family.
Usually, the donor domain of the first member of the naturally occurring immunoglobulin super-family and the donor domain of the second member of the naturally occurring immunoglobulin super-family form a naturally occurring hetero-dimer or a naturally occurring homo-dimer, preferably a naturally occurring hetero-dimer. Preferably the first and the second member of the naturally occurring immunoglobulin super-family are selected from

T cell receptor chains.

More preferably the donor domain of the first member of the naturally occurring immunoglobulin super-family is the TCR constant domain alpha (SEQ ID NO: 1) and the donor domain of the second member of the naturally occurring immunoglobulin super-family is the TCR constant domain beta (SEQ ID NO: 2).

Equally more preferred, the donor domain of the first member of the naturally occurring immunoglobulin super-family is the TCR constant domain delta (SEQ ID NO: 32) and the donor domain of the second member of the naturally occurring immunoglobulin super-family is the TCR constant domain gamma (SEQ ID NO: 33). Equally more preferred, the donor domain of the first and the second member of the naturally occurring immunoglobulin super-family is a CH3 domain.

Examples

Example 1: construction of an immunoglobulin Fc hetero-dimer having a CH3-CH3 protein-protein interface based on the human α/β T cell receptor constant domains.

This example demonstrates that two human IGHG1 Fc chains (each chain consisting of human hinge (γ1), CH2 and CH3 constant domains; EU residues 221-447) having mutations in the protein-protein interface of their CH3 domains (EU residues 341-447) carefully selected from a subset of the protein-protein interface residues from the human T-cell receptor (TCR) constant domain alpha (GenBank database accession number AAO72258.1 [residues 135-225]; SEQ ID NO: 1; IMGT® reference TRAC [complete sequence], the international ImMunoGeneTics information system®; Lefranc MP *et al.*, *Nucleic Acids Res*, 27(1):209-12 (1999); Ruiz M *et al.*, *Nucleic Acids Res*, 28(1):219-21 (2000); Lefranc MP, *Nucleic Acids Res*, 29(1):207-9 (2001); Lefranc MP, *Nucleic Acids Res*, 31(1):307-10 (2003); Lefranc MP *et al.*, *Dev Comp Immunol*, 29(3):185-203 (2005); Kaas Q *et al.*, *Briefings in Functional Genomics & Proteomics*, 6(4):253-64 (2007)) for one chain and the human TCR constant domain beta (GenBank database accession number AAA61026.1 [residues 134-261], SEQ ID NO: 2; IMGT® reference TRBC2 [IMGT® residues 1.8-124]) for the second chain, assemble into a hetero-dimeric Fc molecule with at least 76 % efficacy. TRBC2 is one of two naturally occurring allotypes for the human TCR constant domain beta. Both TRBC1 and TRBC2 can be equivalently used for the purpose to mutate the CH3 domain of IGHG1 Fc chains since there is no difference in the amino acid sequence of their protein-protein interfaces.

Mutations were derived from the analysis of an overlay of the crystal structure of the LC13 TCR molecule (Kjer-Nielsen L *et al.*, *Structure*, 10(11):1521-32 (2002)) with the crystal structure of the Fc fragment from human IGHG1 (Krapp S *et al.*, *J Mol Biol*, 325(5):979-89 (2003)). Both TCR and IGHG1 Fc 3D structures were retrieved from the Protein Data Bank (PDB codes 1KGC and 1H3Y for TRBC2 (SEQ ID NO: 2) and human IGHG1, respectively; www.pdb.org; Bernstein FC *et al.*, *Eur J Biochem*, 80(2):319-24 (1977)), overlaid with the Coot software (Emsley P and Cowtan K, *Acta Crystallogr D Biol Crystallogr*, 60(Pt 12 Pt 1):2126-32 (2004)) and further visualized with the Discovery-Studio software from Accelrys (Cambridge, UK). Examination of the protein-protein interface of the

overlaid 3D structures of the TCR hetero-dimeric constant domains and the CH3 homo-dimer was used as a starting point for rational design. Several parameters were considered; these included but were not limited to: preservation of the prolines residues, and preservation of amino acid positions involved in the integrity of the domain cores as well as some of the electrostatic contacts from the IGHG1 CH3 homo-dimer; abrogating specific hydrophobic contacts found in the IGHG1 CH3 homo-dimer, and replacing those with selected hydrophobic contacts found in the TCR constant domain hetero-dimer. This analysis led to the design of two subsets of amino acid substitutions, one subset originating from equivalent 3D positions between the protein-protein interface of one subunit of the CH3 homo-dimer and the protein-protein interface of the TCR constant domain alpha, and a second subset originating from equivalent 3D positions between the protein-protein interface of the second subunit of the CH3 homo-dimer and the protein-protein interface of the TCR constant domain beta; each subset creating two new and unique CH3 engineered domain sequences which can hetero-dimerize.

The engineered human IGHG1 Fc chain thereof having mutations derived from the protein-protein interface of the human TCR constant domain alpha in the protein-protein interface of its CH3 domain is designated "BT alpha chain" (FIG. 1), while the engineered human IGHG1 Fc chain thereof having mutations derived from the protein-protein interface of the human TCR constant domain beta in the protein-protein interface of its CH3 domain is designated "BT beta chain" (FIG. 1). More specifically, the BT alpha chain consists of an immunoglobulin Fc chain from human IGHG1 having an engineered CH3 domain with the following substitutions: S364K, T366V, K370T, K392Y, F405S, Y407V, K409W, and T411N (SEQ ID NO: 3) (EU numbering); and accordingly, the "BT beta chain" consists of an immunoglobulin Fc chain from human IGHG1 having an engineered CH3 domain with the following substitutions: Q347E, Y349A, L351F, S364T, T366V, K370T, T394D, V397L, D399E, F405C, Y407S, K409R, and T411R (SEQ ID NO: 4) (EU numbering).

The formation of the hetero-dimer Fc can be assessed by SDS-PAGE analysis of samples under non- reducing and reducing conditions. Because the BT alpha chain and the BT beta chain have similar molecular weights, the BT beta chain was fused to a variable light-chain Kappa domain antibody (abbreviated VL, a subfamily KappaI-O12 member derived from the VBASE2 humIGKV115 sequence with the IGKJ1*01 junction, SEQ ID NO: 5;

Retter I *et al.*, *Nucleic Acids Res*, 33(Database issue):D671-4 (2005)), to generate a difference in SDS-PAGE mobility and consequently facilitate the identification of hetero-dimer formation (SEQ ID NO: 6) (FIG.2). Furthermore, to enable confirmation of the presence of the BT alpha chain in the hetero-dimer, a series of six histidine residues was included at its C-terminus (SEQ ID NO: 7). This hetero-dimeric construct is abbreviated BT alpha His_VL-BT beta.

To create the BT alpha His and the VL-BT beta chain cDNA coding sequences, a cDNA coding the engineered CH3 domain of the BT alpha chain (SEQ ID NO: 8) and the engineered CH3 domain of the BT beta chain were synthesized by GENEART AG (Regensburg, Germany). The BT beta chain was originally synthesized with the F405A mutation, which was later converted to F405C (SEQ ID NO: 9) by standard mutagenesis. Using PCR assembly techniques, each chain had their respective engineered CH3 domain cDNA coding sequence fused downstream of a synthetic cDNA encoding for the human IGHG1 hinge (DKTHTCPPCP) and IGHG1 CH2 constant domain (separately synthesized by GENEART AG). The polyhistidine sequence located at the C-terminus of the BT alpha His chain was included in the anti-sense oligonucleotide during PCR amplification; while the variable Kappa domain antibody located at the N-terminus of the VL-BT beta chain was engineered by fusing the domain cDNA (separately synthesized by GENEART AG) upstream of the BT beta chain cDNA coding sequence using PCR assembly techniques. Finally, the BT alpha His chain and VL-BT beta chain coding DNA sequences (SEQ ID NOs: 10, and 11, respectively) were ligated in independent vectors which are based on a modified pREP4 (Invitrogen, CA, USA) vector carrying CMV promoter and Bovine Growth Hormone poly-adenylation signal. In both chain expression-vectors, secretion was driven by the murine VJ2C leader peptide.

For transient expression of the BT alpha His_VL-BT beta hetero-dimer, equal quantities of each engineered chains vectors were co-transfected into suspension-adapted HEK-EBNA cells (ATCC-CRL-10852) using Polyethyleneimine (PEI). Typically, 100 ml of cells in suspension at a density of 0.8-1.2 million cells per ml is transfected with a DNA-PEI mixture containing 50 µg of expression vector encoding the BT alpha His chain and 50 µg expression vector encoding the VL-BT beta chain. When recombinant expression vectors encoding each engineered chain genes are introduced into the host cells, the hetero-dimeric

construct is produced by further culturing the cells for a period of 4 to 5 days to allow for secretion into the culture medium (EX-CELL 293, HEK293-serum-free medium (Sigma, Buchs, Switzerland), supplemented with 0.1% pluronic acid, 4mM glutamine, and 0.25 µg/ml geneticin). The hetero-dimeric construct was then purified from cell-free supernatant using recombinant Streamline rProtein A media (GE Healthcare Europe GmbH, Glattbrugg, Switzerland), and used for further analysis.

Transient transfection production yields were above 20 mg/l. The results of a typical SDS-PAGE analysis are shown in FIG.3. Post protein-A purification the BT alpha His_VL-BT beta hetero-dimer is the main species produced with little homo-dimeric species present (lane 1). Of particular interest is the comparison with the SDS-PAGE mobility of a standard homo-dimeric human IGHG1 Fc (GenBank database accession number AAC82527.1 residues 103-329; SEQ ID NO: 12 (lane 3)), under reducing conditions, the BT alpha His_VL-BT beta hetero-dimer breaks down into the two expected molecular weight bands for the BT alpha His chain and the VL-BT beta chain (lane 2; 25.5 kDa, and 37.2 kDa, respectively; an additional 3 kDa needs to be added to each of these molecular weights to account for chain N-glycosylation at Asn 297) whereas the standard homo-dimeric human IGHG1 Fc (lane 4) collapses into one unique molecular weight band having a similar mobility to the BT alpha His chain. Accordingly, under non-reducing condition, comparison with a standard homo-dimeric human IGHG1 Fc allows identification of traces of BT alpha His homo-dimer present at a very low abundance. Finally aggregates can be also detected at a very low abundance.

It is possible to assess the proportion of hetero-dimer to homo-dimer in the protein-A purified preparation by scanning densitometry analysis of the non-reduced SDS-polyacrylamide (4-12%) gel bands. Relative ratios of the different species mentioned above were quantified using a FluorChem SP imaging system (Witec AG, Littau, Switzerland) following the protocol provided by the manufacturer. The BT alpha chain His_VL-BT beta chain hetero-dimer represents overall at least 76.1 % of the protein-A purified material (FIG.4A); when omitting traces of aggregates in the relative ratio measurements, the BT alpha chain His_VL-BT beta chain hetero-dimer represents overall at least 83.8 % of the protein-A purified material (FIG.4B).

Example 2: construction of immunoglobulin Fc hetero-dimer variants having a CH3-CH3 protein-protein interface based on the human α/β T cell receptor constant domains.

T-cell receptor domains are very prone to aggregation and misassociation during folding, and this is often compounded by incorrect disulfide bond formation (Pecorari F *et al.*, *J Mol Biol*, 285(4):1831-43 (1999)). Mutation of the unpaired cysteine in the TCR constant domain beta has been shown to facilitate the production of TCR molecules in E. coli (Willcox BE *et al.*, *Protein Sci*, 8(11):2418-23 (1999)). In addition, this unpaired cysteine has been mutated in several TCR molecule crystal structures. Mutations of C405A and C405S in the BT beta chain coding DNA sequences were derived from the analysis of an overlay of the crystal structure of the Fc fragment from human IGHG1 (Krapp S *et al.*, *J Mol Biol*, 325(5):979-89 (2003)) with the crystal structure of the CD1d–α-GalCer–specific TCR molecule 5E (PDB code 2CDF, Gadola SD *et al.*, *J Exp Med*, 203(3):699-710 (2006)) and the crystal structure of the T cell receptor (TCR) HA1.7 (PDB code 1J8H, Hennecke J and Wiley DC, *J Exp Med*, 195(5):571-81 (2002)). Since the engineered CH3 domain of the BT beta chain was originally synthesized with the F405A mutation, this construct was directly used to build a new VL-BT beta chain identical to the VL-BT beta chain described in example 1 except for the introduction of the F405A mutation. The VL-BT beta chain having the F405A mutation was then modified to create a second VL-BT beta chain having the F405S mutation using standard mutagenesis techniques. These two new chains identical to the VL-BT beta chain described in example 1 expect for their F405A and F405S mutations respectively, are referred herein as "VL-BT beta F405A chain" or "VL-BT beta F405A" and "VL-BT beta F405S chain" or "VL-BT beta F405S".

Transient expression of the BT alpha His_VL-BT beta F405S hetero-dimer and BT alpha His_VL-BT beta F405A hetero-dimer were performed as described in example 1. Production yields for the BT alpha His_VL-BT beta F405A hetero-dimer were similar to the BT alpha His_VL-BT beta hetero-dimer (22 mg/l each). For the BT alpha His_VL-BT beta F405S hetero-dimer production yields of 37 mg/l were obtained. To assess the quality of the BT alpha His_VL-BT beta F405S hetero-dimer formation over the BT alpha His_VL-BT beta hetero-dimer described in example 1, both BT hetero-dimers were run on the same SDS-PAGE gel (FIG.3, lane 1 (BT alpha His_VL-BT beta) and 5 (BT alpha His_VL-BT beta F405S)). Post protein-A purification the BT alpha His_VL-BT beta F405S hetero-dimer is the

main species produced, however when compared to the BT alpha His_VL-BT beta hetero-dimer described in example 1, significantly higher amounts of monomeric species as well as VL-BT beta F405S homo-dimer species were observed, indicating a higher hetero-dimeric assembly efficacy of the naturally occurring F405C substitution over the mutated F405S substitution. Similarly to the BT alpha His_VL-BT beta hetero-dimer, under reducing conditions, the BT alpha His_VL-BT beta F405S hetero-dimer breaks down into the two expected molecular weight bands for the BT alpha His chain and the VL-BT beta F405S chain (lane 6).

The results of a typical SDS-PAGE analysis for the BT alpha His_VL-BT beta F405A hetero-dimer are shown in FIG.5. Post protein-A purification the BT alpha His_VL-BT beta F405A hetero-dimer is the main species produced with little homo-dimeric species present (lane 1). Similarly to the BT alpha His_VL-BT beta hetero-dimer, under reducing conditions, the BT alpha His_VL-BT beta F405A hetero-dimer breaks down into the two expected molecular weight bands for the BT alpha His chain and the VL-BT beta F405A chain (lane 2). Accordingly, under non-reducing condition, comparison with a standard homo-dimeric human IGHG1 Fc (lane 3: non-reducing conditions, lane 4: reducing conditions) allows identification of traces of BT alpha His homo-dimer present at a very low abundance. Finally traces of VL-BT beta F405A homo-dimer and momomer as well as aggregates can be also detected at a very low abundance.

To further demonstrate the identity of BT alpha His_VL-BT beta F405A hetero-dimer, the protein-A purified material was loaded on Ni^{2+} affinity sepharose (GE Healthcare Europe GmbH, Glattbrugg, Switzerland), a chromatographic step which is specific for species containing the BT alpha His chain due to its polyhistidine sequence. After a wash step, bound and eluted fractions from the Ni^{2+} affinity step were pooled and loaded onto protein-L affinity agarose (GenScript USA Inc., Piscataway, NJ, USA), a superantigen affinity resin which specifically select for species having the variable light-chain Kappa domain antibody, i.e. the VL-BT beta F405A chain. Outputs of the above purification steps were then assessed by Western blotting with an antibody against the polyhistidine sequence labeled with horse-radish peroxidase, thereby detecting the BT alpha His chain. The blot was stained using Sigma Fast 3,3′-Diaminobenzidine precipitating substrate (Sigma, Buchs, Switzerland). FIG.6 shows that the BT alpha His chain is consistently detected before, and after Ni^{2+}

sepharose but also and more importantly after protein-L agarose chromatography, demonstrating the identity of the hetero-dimer.

It is possible to assess the proportion of hetero-dimer to homo-dimer in the protein-A purified preparation by scanning densitometry analysis of the non-reduced SDS-polyacrylamide (4-12%) gel bands. Relative ratios of the different species mentioned above were quantified using a FluorChem SP imaging system (Witec AG, Littau, Switzerland) following the protocol provided by the manufacturer. The BT alpha chain His_VL-BT beta chain hetero-dimer represents overall at least 80.7 % of the protein-A purified material (FIG.7A); when omitting traces of aggregates and VL-BT beta F405A chain monomer in the relative ratio measurements, the BT alpha chain His_VL-BT beta F405A chain hetero-dimer represents overall at least 86.9 % of the protein-A purified material (FIG.7B).

Protein stability is a crucial issue for the development of recombinant antibody therapeutics. A poor stability can affect the ability of an antibody to fold when expressed in various cell types (Garber E and Demarest SJ, *Biochem Biophys Res Commun*, 355(3):751-7 (2007)), and lead to a fraction of the clinical material to be misfolded, non functional and/or prone to aggregate, and thus potentially immunogenic (Chirino AJ *et al.*, *Drug Discov Today*, 9(2):82-90 (2004)). To assess the intrinsic stability of the hetero-dimeric Fc fragment without any interference from an additional protein domain, a BT beta chain without variable kappa domain (this chain was engineered similarly to the other BT beta chains but had a C-terminal HA tag (YPYDVPDYA) and no VL domain, abbreviated BT beta (F405A) HA chain; SEQ ID NO: 17) was co-expressed with a BT alpha His chain. The resulting hetero-dimeric molecule: BT alpha His_BT beta (F405A) HA was purified as above and analyzed using differential scanning calorimetry (DSC). The calorimetric measurements were carried out on a VP-DSC differential scanning microcalorimeter (GE Healthcare Europe GmbH, Glattbrugg, Switzerland). The cell volume was 0.128 ml, the heating rate was 1 °C/min, and the excess pressure was kept at 64 p.s.i. The protein was used at a concentration of 2 mg/ml in PBS (pH 7.4). The molar heat capacity of the protein was estimated by comparison with duplicate samples containing identical buffer from which the protein had been omitted. The partial molar heat capacities and melting curves were analyzed using standard procedures. Thermograms were baseline corrected and concentration normalized before being further analyzed using a Non-Two State model in the Origin (v7.0) software (OriginLab Corporation,

Northampton, MA, USA; provided by GE Healthcare Europe GmbH, Glattbrugg, Switzerland).

The expected melting profiles for the human IgG subclasses are known (Garber E and Demarest SJ, *Biochem Biophys Res Commun*, 355(3):751-7 (2007)), and all profiles have been shown to contain three unfolding transitions corresponding to the independent unfolding of the CH2, CH3, and FAB domains. Of the four human IgG subclasses, IGHG1 has the most stable CH3 domain (~85 °C); while other subclasses CH3 domains are less stable, although none are known to melt below 70 °C. Similarly, all subclasses are known to have a melting temperature of ~70 °C for the CH2 domain (Garber E and Demarest SJ, *Biochem Biophys Res Commun*, 355(3):751-7 (2007)). Overall no melting transition is expected to be lower than 70 °C for a stable antibody Fc fragment (hinge-CH2-CH3). FIG. 7C shows a thermogram of the BT alpha His_BT beta (F405A) HA hetero-dimer exhibiting a single sharp transition at 70 °C, accounting for the melting transitions of both the CH2 domain and the engineered CH3 domain. It is important to note that no sharp decrease after the heat absorption peak was recorded, indicating that no precipitation or aggregate formation occurred after thermal unfolding as expected for a stable antibody Fc fragment (Liu H *et al.*, *Immunol Lett*, 106(2):144-53 (2006)). Furthermore, the hetero-dimeric Fc fragment has stability similar to the naturally occurring Fc fragments since the melting transitions of its domains are similar to the ones observed for the human IGHG4 Fc fragment where both the CH2 domain and the CH3 domain melt at approximately 70 °C (Garber E and Demarest SJ, *Biochem Biophys Res Commun*, 355(3):751-7 (2007)). In conclusion, this thermal unfolding study shows that the hetero-dimeric Fc fragment with engineered protein-protein interfaces based on the TCR alpha and beta constant domains is stable and suitable for building further therapeutic hetero-dimeric immunoglobulins.

Example 3: construction of immunoglobulin Fc hetero-dimer variants having a CH3-CH3 protein-protein interface based on the human α/β T cell receptor constant domains with minimal substitutions.

To identify pairs of BT alpha and beta chains that will form hetero-dimers over the formation of possible homo-dimers with a reduced number of amino acid substitutions originating from the key protein-protein interface residues from the human T-cell receptor (TCR) constant domain alpha (GenBank database accession number AAO72258.1, residues 135-225; SEQ ID NO: 1; IMGT® reference TRAC [complete sequence], the international ImMunoGeneTics information system®; Lefranc MP *et al.*, *Nucleic Acids Res*, 27(1):209-12 (1999); Ruiz M *et al.*, *Nucleic Acids Res*, 28(1):219-21 (2000); Lefranc MP, *Nucleic Acids Res*, 29(1):207-9 (2001); Lefranc MP, *Nucleic Acids Res*, 31(1):307-10 (2003); Lefranc MP *et al.*, *Dev Comp Immunol*, 29(3):185-203 (2005); Kaas Q *et al.*, *Briefings in Functional Genomics & Proteomics*, 6(4):253-64 (2007)) for one chain and the human TCR constant domain beta (GenBank database accession number AAA61026.1 [residues 134-261], SEQ ID NO: 2; IMGT® reference TRBC2 [IMGT® residues 1.8-124]) for the second chain, two or more of the substitutions described in example 1 and 2 were gradually introduced in each chain.

There are multiple subsets of amino acid substitutions originating from the key protein-protein interface residues from the human TCR constant domain alpha and beta that can be chosen to alternate between the TCR constant domain sequence and the human IGHG1 Fc sequence in the BT alpha and beta chains, respectively. The choice of residues for each considered subset is based on structural considerations, for example, protein-protein surface interaction calculations. All substitution subsets were engineered by mutating the human IGHG1 Fc DNA coding sequence (SEQ ID NO: 12) using standard molecular biology techniques (example 1). The resulting variants were then ligated independently into the modified pREP4 vector mentioned previously and co-transfected into HEK293-EBNA cells as described in example 1. Protein production was also according to example 1. Each subset of substitutions was assessed for its rate of hetero-dimerization using a combination of SDS-PAGE analysis of samples under non-reducing and reducing conditions, and scanning densitometry (as stated in example 1).

Because the engineered chains have close molecular weights, similarly to example 1, the BT beta chain variants were fused to a variable light-chain kappa domain antibody (abbreviated VL, a subfamily Kappa I - O12 member derived from the VBASE2 humIGKV115 sequence with the IGKJ1*01 junction, SEQ ID NO: 5; Retter I. et al, 2005, Nucleic Acids Res., 33, Database issue D671-D674) to generate a difference in SDS-PAGE mobility and consequently facilitate the identification of hetero-dimer formation. To differentiate these chains from example 1, and since these BT chains have identical amino acid sequence to the human IGHG1 Fc sequence except for the specific TCR alpha and beta constant domain based substitutions which have been introduced by site-directed mutagenesis, the BT alpha and VL-BT beta chains are herein abbreviated Fc (BTA) chain and VL-Fc (BTB) chain, respectively. For each chain, the specific substitutions which have been introduced into the human IGHG1 Fc sequence are indicated using the EU numbering. The result of these expression experiments, in terms of yield and hetero-dimerization rate are summarized in Table 1. Two to six substitutions were made in each chain.

The results of a typical SDS-PAGE analysis are shown in FIG. 8. Post protein-A purification the hetero-dimer is the main species produced with little homo-dimeric species present (lane1). Under reducing conditions the hetero-dimer breaks down into the two expected molecular weight bands for the engineered Fc (BTA) chain and the other engineered VL-Fc (BTB) chain (lane 2; ~25 kDa and ~37 kDa, respectively). Transient transfection production yields were up to 50 mg/l while the best hetero-dimerization rate was 90%.

The best combination of substitution subsets was: Fc (BTA)-S364K-T366V-K370T-K392Y-K409W-T411N_VL-Fc (BTB)-F405A-Y407S (SEQ ID NOs: 23 and 24) with a 90% hetero-dimerization rate (FIG. 9) and a transient transfection yield of 30 mg/l. The minimal number of substitutions tested in each chain was two with a 64% hetero-dimerization rate and a transient transfection yield of 35 mg/l. To compare the hetero-dimerization technology described herein with existing methods, a "knob-into-hole" Fc variant (Merchant AM *et al.*, *Nat Biotechnol*, 16(7):677-81 (1998)) with the portion encompassing the "hole" fused to a variable light-chain kappa domain antibody (VL, SEQ ID NO: 5) was created (abbreviated Fc-T366W_VL-Fc-T366S-L368A-Y407V; EU numbering; SEQ ID NOs: 30 and 31). In side by side expression experiments, the "knob-into-hole" based molecule had only a 66% hetero-dimerization rate (data not shown).

Table1: Production yields of minimal BT chains. Hetero-dimer formation percentage is indicated in brackets and represents the percentage of hetero-dimer in the protein-A purified material when omitting impurities in the relative ratio measurements.

VL-Fc (BTB) chain / Fc (BTA) chain	F405A Y407S SEQ ID NO: 24	F405A K409R SEQ ID NO: 25	Y407S K409R SEQ ID NO: 26	K409R T411R SEQ ID NO: 27	F405A Y407S K409R SEQ ID NO: 28	F405A Y407S K409R T411R SEQ ID NO: 29
Y407V K409W SEQ ID NO: 18	35 mg/l (64)	11 mg/l (32)	39 mg/l (30)	41 mg/l (36)	45 mg/l (47)	28 mg/l (36)
K392Y K409W SEQ ID NO: 19	35 mg/l (52)	46 mg/l (46)	30 mg/l (46)	18 mg/l (14)	35 mg/l (50)	29 mg/l (30)
T366V K409W SEQ ID NO: 20	51 mg/l (57)	50 mg/l (46)	27 mg/l (49)	26 mg/l (26)	26 mg/l (49)	26 mg/l (34)
Y407V K409W T411N SEQ ID NO: 21	18 mg/l (50)	20 mg/l (25)	5 mg/l (25)	25 mg/l (not quantifiable)	4 mg/l (52)	21 mg/l (53)
F405S Y407V K409W T411N SEQ ID NO: 22	8 mg/l (7)	7 mg/l (4)	8 mg/l (5)	12 mg/l (not quantifiable)	4 mg/l (15)	29 mg/l (9)
S364K T366V K370T K392Y K409W T411N SEQ ID NO: 23	30 mg/l (90)	5 mg/l (60)	7 mg/l (74)	9 mg/l (32)	10 mg/l (72)	42 mg/l (88)

Example 4: construction of an immunoglobulin Fc hetero-dimer having a CH3-CH3 protein-protein interface based on the human δ/γ T cell receptor constant domains.

This example demonstrates that two human IGHG1 Fc chains (each chain consisting of human hinge (γ1), CH2 and CH3 constant domains; EU residues 221-447) having mutations in the protein-protein interface of their CH3 domains (EU residues 341-447) carefully selected from a subset of the protein-protein interface residues from the human T-cell receptor (TCR) constant domain delta (GenBank database accession number AAA61125.1 [residues 135-221]; SEQ ID NO: 32; IMGT® reference TRDC [IMGT® residues 1.7-120], the international ImMunoGeneTics information system®; Lefranc MP *et al.*, *Nucleic Acids Res*, 27(1):209-12 (1999); Ruiz M *et al.*, *Nucleic Acids Res*, 28(1):219-21 (2000); Lefranc MP, *Nucleic Acids Res*, 29(1):207-9 (2001); Lefranc MP, *Nucleic Acids Res*, 31(1):307-10 (2003); Lefranc MP *et al.*, *Dev Comp Immunol*, 29(3):185-203 (2005); Kaas Q *et al.*, *Briefings in Functional Genomics & Proteomics*, 6(4):253-64 (2007)) for one chain and the human TCR constant domain gamma (GenBank database accession number AAA61110.1 [residues 145-245], SEQ ID NO: 33; IMGT® reference TRGC1 [IMGT® residues 1.1-124]) for the second chain, assemble into a hetero-dimeric Fc molecule with at least 52% efficacy. TRGC1 and TRDC are naturally occurring isotypes. TRGC1 is one of two naturally occurring allotypes for the human TCR constant domain gamma. Both TRGC1 and TRGC2 (termed 2x or 3x) can be equivalently used for the purpose to mutate the CH3 domain of IGHG1 Fc chains since there is no difference in the amino acid sequence of their protein-protein interfaces.

Mutations were derived from the analysis of an overlay of the crystal structure of the the G115 γδTCR molecule (Allison TJ *et al.*, *Nature*, 411(6839):820-4 (2001)) with the crystal structure of the Fc fragment from human IGHG1 (Krapp S *et al.*, *J Mol Biol*, 325(5):979-89 (2003)). Both TCR and IGHG1 Fc 3D structures were retrieved from the Protein Data Bank (PDB codes 1HXM and 1H3Y for human TCR and human IGHG1, respectively; www.pdb.org; Bernstein FC *et al.*, *Eur J Biochem*, 80(2):319-24 (1977)), overlaid with the Coot software (Emsley P and Cowtan K, *Acta Crystallogr D Biol Crystallogr*, 60(Pt 12 Pt 1):2126-32 (2004)) and further visualized with the Discovery-Studio software from Accelrys (Cambridge, UK). Examination of the protein-protein interfaces of the overlaid 3D structures of the TCR hetero-dimeric constant domains and the CH3 homo-dimer was used as a starting point for rational design. Several parameters were considered;

these included but were not limited to: the preservation of the prolines residues and the preservation of amino acid positions involved in the integrity of the domain cores as well as some of the electrostatic contacts from the IGHG1 CH3 homo-dimer; abrogating specific hydrophobic contacts found in the IGHG1 CH3 homo-dimer, and replacing those with selected hydrophobic contacts found in the TCR constant domain hetero-dimer. This analysis led to the design of two subsets of amino acid substitutions, one subset originating from equivalent 3D positions between the protein-protein interface of one subunit of the CH3 homo-dimer and the protein-protein interface of the TCR constant domain delta, and a second subset originating from equivalent 3D positions between the protein-protein interface of the second subunit of the CH3 homo-dimer and the protein-protein interface of the TCR constant domain gamma; each subset creating two new and unique CH3 engineered domain sequences which can hetero-dimerize.

The engineered human IGHG1 Fc chain thereof having mutations derived from the protein-protein interface of the human TCR constant domain delta in the protein-protein interface of its CH3 domain is designated "BT delta chain", while the engineered human IGHG1 Fc chain thereof having mutations derived from the protein-protein interface of the human TCR constant domain gamma in the protein-protein interface of its CH3 domain is designated "BT gamma chain". More specifically, the BT delta chain consists of an immunoglobulin Fc chain from human IGHG1 having an engineered CH3 domain with the following substitutions: L351M, S364N, T366A, G371E, K392F, T394A, D399S, F405N, Y407V, K409L, and T411K (SEQ ID NO: 34) (EU numbering); and accordingly, the "BT gamma chain" consists of an immunoglobulin Fc chain from human IGHG1 having an engineered CH3 domains with the following substitutions: Q347K, Y349T, S364T, T366L, K370E, G371K, T394G, V397M, F405M, Y407F, K409W (SEQ ID NO: 35) (EU numbering).

The formation of the Fc hetero-dimer can be assessed by SDS-PAGE analysis of samples under non-reducing and reducing conditions. Because the BT delta chain and the BT gamma chain have similar molecular weights, the BT gamma chain was fused to a variable light-chain kappa domain antibody (abbreviated VL, a subfamily Kappa I - O12 member derived from the VBASE2 humIGKV115 sequence with the IGKJ1*01 junction, SEQ ID NO: 5; Retter I *et al.*, *Nucleic Acids Res*, 33(Database issue):D671-4 (2005)) (VL-BT gamma, SEQ

ID NO: 36), to generate a difference in SDS-PAGE mobility and consequently facilitate the identification of hetero-dimer formation. The resulting hetero-dimeric construct is abbreviated BT delta_VL-BT gamma.

To create the BT delta and the VL-BT gamma chain cDNA coding sequences, a cDNA coding the engineered CH3 domain of the BT delta chain (SEQ ID NO: 37) and the engineered CH3 domain of the BT gamma chain (SEQ ID NO: 38) were synthesized by GENEART AG (Regensburg, Germany). Using PCR assembly techniques, each chain had their respective engineered CH3 domain cDNA coding sequence fused downstream of a synthetic cDNA coding for the human hinge IGHG1 (DKTHTCPPCP) and CH2 constant domain (separately synthesized by GENEART AG). The variable kappa domain antibody located at the N-terminus of the VL-BT gamma chain was engineered by fusing the domain cDNA (separately synthesized by GENEART AG) upstream of the BT gamma chain cDNA coding sequence using PCR assembly techniques.

The resulting variants were then ligated independently into the modified pREP4 vector mentioned previously and co-transfected into HEK293-EBNA cells as described in example 1. Protein production was also according to example 1. Transient transfection yields were up to 60 mg/l. The results of a typical SDS-PAGE analysis are shown in FIG. 10.

To assess the proportion of hetero-dimer to homo-dimer in the protein-A purified material, the relative ratios of the different species were quantified by scanning densitometry analysis of the non-reduced SDS-polyacrylamide (4-12%) gel bands according to the procedure described in example 1. The BT delta_VL-BT gamma hetero-dimer represents at least 52.1% of the protein-A purified material (FIG. 11A); when omitting traces of aggregates and monomer in the relative ratio measurements, the BT delta_VL-BT gamma hetero-dimer represents at least 57.5% of the protein-A purified material (FIG. 11B).

Example 5: construction of a monovalent immunoglobulin with a hetero-dimeric Fc having a CH3-CH3 protein-protein interface based on the human α/β T cell receptor constant domains, and a FAB fragment having a CH1-CK protein-protein interface based on the hetero-dimeric δ/γ T cell receptor constant domains.

This example demonstrates that at least three human immunoglobulin chains: one chain consisting of a heavy chain variable domain fused to the human heavy chain constant domains CH1 (γ1), hinge (γ1), CH2 (γ1) and engineered CH3 domain from the BT alpha or BT beta chain, one BT alpha or one BT beta chain, and one chain consisting of a light chain variable domain fused to the human Cκ light chain constant domain wherein the protein-protein interface of the CH1 (γ1) (IGHG1, EU residues 118-215) and Cκ (IGKC, EU residues 108-214) domains have been carefully substituted at selected positions with a subset of the protein-protein interface residues from the naturally occurring human TCR constant domain delta (GenBank database accession number AAA61125.1 [residues 135-221]; SEQ ID NO: 32; IMGT® reference TRDC [IMGT® residues 1.7-120], the international ImMunoGeneTics information system®; Lefranc MP *et al.*, *Nucleic Acids Res*, 27(1):209-12 (1999); Ruiz M *et al.*, *Nucleic Acids Res*, 28(1):219-21 (2000); Lefranc MP, *Nucleic Acids Res*, 29(1):207-9 (2001); Lefranc MP, *Nucleic Acids Res*, 31(1):307-10 (2003); Lefranc MP *et al.*, *Dev Comp Immunol*, 29(3):185-203 (2005); Kaas Q *et al.*, *Briefings in Functional Genomics & Proteomics*, 6(4):253-64 (2007)), and the naturally occurring human TCR constant domain gamma (GenBank database accession number AAA61110.1 [residues 145-245]; SEQ ID NO: 33; IMGT® reference TRGC1 [IMGT® residues 1.1-124]) respectively, assemble into a FAB engineered hetero-dimeric immunoglobulin molecule with at least 50 % efficacy.

Mutations were derived from the analysis of an overlay of the crystal structure of the TCR molecule with the crystal structure of a human FAB (γ1) fragment. Both TCR and human FAB (γ1) fragment 3D structures were retrieved from the Protein Data Bank (PDB code 1HXM, and 1VGE, respectively; Allison TJ *et al.*, *Nature*, 411(6839):820-4 (2001); Chacko S *et al.*, *J Biol Chem*, 271(21):12191-8 (1996)), overlaid with the Coot software (Emsley P and Cowtan K, *Acta Crystallogr D Biol Crystallogr*, 60(Pt 12 Pt 1):2126-32 (2004)) and further visualized with the Discovery-Studio software from Accelrys (Cambridge, UK). Examination of the protein-protein interfaces of the overlaid 3D structures of the δ/γ TCR hetero-dimeric constant domains and the CH1 (γ1) - Cκ hetero-dimer was used as a

starting point for rational design. Several parameters were considered; these included but were not limited to: the preservation of the proline residues and the preservation of amino acid positions involved in the integrity of the domain cores as well as some of the electrostatic contacts between the CH1 (γ1), and the Cκ domains; abrogating specific hydrophobic contacts found in the CH1 (γ1) - Cκ hetero-dimer, and replacing those with selected hydrophobic contacts found in the TCR constant domain gamma-delta hetero-dimer. This analysis led to the design of two subsets of amino acid substitutions, one subset originating from equivalent 3D positions between the protein-protein interface of the CH1 (γ1) domain and the protein-protein interface of the TCR constant domain delta, and a second subset originating from equivalent 3D positions between the protein-protein interface of the Cκ domain and the protein-protein interface of the TCR constant domain gamma; thereby creating two engineered, novel and unique domains which can still hetero-dimerize but differently from the naturally occurring CH1 (γ1) - Cκ hetero-dimeric domain pair.

An engineered heavy chain of a FAB fragment comprising a variable heavy chain domain, and a human CH1 (γ1) domain having mutations derived from the protein-protein interface of the human TCR constant domain delta in its protein-protein interface is designated "VH-CH1 delta" (FIG. 12), while an engineered kappa light chain comprising a variable light chain domain, and a human Cκ domain having mutations derived from the protein-protein interface of the human TCR constant domain gamma in its protein-protein interface is designated "VL-CK gamma" (FIG. 12). More specifically, a VH-CH1 delta consists of an engineered heavy chain FAB fragment having an engineered CH1 (γ1) domain with the following substitutions: L128M , A141N, G143A, D148E, H168F, F170A, Q175S, S181N, S183V, V185L, T187K (SEQ ID NO: 39) (EU numbering); and accordingly, a VL-CK gamma consists of an engineered kappa light chain having an engineered Cκ domain with the following substitutions: S114K, F116T, S131T, V133L, N137E, N138K, S162G, T164M, S174M, S176F, T178W (SEQ ID NO: 40) (EU numbering).

In this example, the variable heavy chain and light chain domains from a humanized anti human CD19 antibody disclosed in the PCT Publication NO: WO 2010/095031 (abbreviated VH(anti-hCD19), and VL(anti-hCD19)) were used as inputs to build the VH-CH1 delta and the VL-CK gamma chains, respectively. The combination of a VH-CH1 delta chain fused upstream of a previously described BT beta chain (examples 1-3) (note that in this

example, the chain had a C-terminal c-myc tag (EQKLISEEDLN), abbreviated BT beta c-myc chain) results in a newly engineered immunoglobulin heavy chain which once co-expressed with the newly engineered VL-CK gamma light chain described above, and a BT alpha His chain described in examples 1-3, creates a new type of hetero-dimeric monovalent (i.e. having one specificity for one antigen) immunoglobulin molecule having a novel FAB fragment wherein the CH1 (γ1) and Cκ constant domains have novel protein-protein interfaces based on the TCR delta and TCR gamma constant domain protein-protein interfaces, respectively, and a Fc fragment with hetero-dimeric engineered CH3 domains based on the TCR alpha and beta constant domains (examples 1- 3). This novel immunoglobulin molecule is abbreviated VH(anti-hCD19)-CH1 delta-BT beta c-myc_BT alpha His_VL(anti-hCD19)-CK gamma (SEQ ID NOs: 41, 7, and 42).

To create the VH(anti-hCD19)-CH1 delta-BT beta c-myc chain and the VL(anti-hCD19)-CK gamma chain cDNA coding sequences (SEQ ID NOs: 43 and 44, respectively), a unique cDNA encompassing both the CH1 delta domain sequence (SEQ ID NO: 45) and the CK gamma domain sequence (SEQ ID NO: 46) was synthesized by GENEART AG (Regensburg, Germany). Using PCR, each chain was amplified individually, and the respective engineered constant domain cDNA coding sequences were fused downstream of a synthetic cDNA coding for their respective variable domains (separately synthesized by GENEART AG). The resulting VH(anti-hCD19)-CH1 delta cDNA fragment was then further fused upstream of a BT beta F405A chain having a C-terminal c-myc tag to generate the final VH(anti-hCD19)-CH1 delta-BT beta c-myc chain. Subsequently, the two chains were then ligated independently in the modified pREP4 vector described in example 1. The BT alpha His chain originated from example 1 (SEQ ID NO: 10).

For transient expression of the VH(anti-hCD19)-CH1 delta-BT beta c-myc_BT alpha His_VL(anti-hCD19)-CK gamma molecule, equal quantities of three engineered chain vectors were co-transfected into suspension-adapted HEK293-EBNA cells as described previously (example 1). The FAB engineered hetero-dimeric construct was then purified from the cell-free supernatant using protein-A affinity chromatography and used for further analysis (procedure according to example 1). The correct assembly of the FAB engineered hetero-dimer can be assessed by SDS-PAGE analysis of samples under non-reducing conditions,

since the correctly assembled molecule is expected to have a different SDS-PAGE mobility from the possible homo-dimeric species.

Transient transfection yields were above 16 mg/l. The results of a typical SDS-PAGE analysis are shown in FIG. 13. Post protein-A purification the VH(anti-hCD19)-CH1 delta-BT beta c-myc_BT alpha His_VL(anti-hCD19)-CK gamma molecule (lane1) is the main species produced (A) with some homo-dimeric species (B) and some half-molecules (C) present.

It is possible to assess the proportion of hetero-dimer to homo-dimer in the protein-A purified preparation by scanning densitometry analysis of the non-reduced SDS-polyacrylamide (4-12%) gel bands (procedure according to example 1). FIG. 14 shows that the VH(anti-hCD19)-CH1 delta-BT beta c-myc_ BT alpha His_VL(anti-hCD19)-CK gamma molecule represents at least 50 % of the protein-A purified material.

The thermal stability of the VH(anti-hCD19)-CH1 delta-BT beta c-myc_BT alpha His_VL(anti-hCD19)-CK gamma molecule was assessed using calorimetric measurements (DSC) (see example 2 for methods). Monoclonal antibodies have melting profiles which are characteristic of their isotypes, and which usually combine transitions for the FAB fragment, the CH2 and the CH3 domains (see example 2); within an antibody melting profile, the specific transition for the FAB fragment can easily be identified (Garber E and Demarest SJ, *Biochem Biophys Res Commun*, 355(3):751-7 (2007)). FIG. 15 shows an example of a thermal unfolding experiment for the VH(anti-hCD19)-CH1 delta-BT beta c-myc_BT alpha His_VL(anti-hCD19)-CK gamma molecule: two overlapping sharp transitions at 70.7 °C and ~75 °C are observed, accounting for the melting transitions of the engineered FAB fragment, the CH2 domains, and the engineered CH3 domains. Since the BT alpha His_BT beta (F405A) HA hetero-dimeric Fc displayed a single sharp melting transition at 70 °C in previous measurements (example 2), it is possible to conclude that the melting transition of the engineered FAB fragment of the VH(anti-hCD19)-CH1 delta-BT beta c-myc_BT alpha His_VL(anti-hCD19)-CK gamma molecule has a mid-point of ~75 °C. It is important to note that no sharp decrease after the heat absorption peak was recorded, indicating that no precipitation or aggregate formation occurred after thermal unfolding (Liu H *et al.*, *Immunol Lett*, 106(2):144-53 (2006)). In conclusion, this thermal denaturation study shows that the novel FAB fragment with engineered protein-protein interfaces based on the TCR delta and

gamma constant domains is stable and suitable for building further therapeutic hetero-dimeric immunoglobulins; more precisely, immunoglobulin molecules comprising two novel hetero-dimeric pairs of constant domains, one pair having engineered protein-protein interfaces based on the human T-cell receptor constant domain alpha and beta, and the other pair having engineered protein-protein interfaces based on the human T-cell receptor constant domain delta and gamma.

Example 6: construction of a monovalent immunoglobulin with a hetero-dimeric Fc having a CH3-CH3 protein-protein interface based on the human α/β T cell receptor constant domains, and a FAB fragment having a CH1-CK protein-protein interface based on the homo-dimeric IGHG1 CH3 constant domains.

This example demonstrates that at least three human immunoglobulin chains: one chain consisting of a heavy chain variable domain fused to the human heavy chain constant domains CH1 (γ1), hinge (γ1), CH2 (γ1) and engineered CH3 domain from the BT alpha or BT beta chain, one BT alpha or one BT beta chain, and one chain consisting of a light chain variable domain fused to the human Cκ light chain constant domain wherein the protein-protein interface of the CH1 (γ1) (IGHG1, EU residues 118-215) and Cκ (IGKC, EU residues 108-214) domains have been carefully substituted at selected positions with a subset of the protein-protein interface residues from the human CH3 (γ1) constant domain (IGHG1, EU residues 341-447; SEQ ID NO: 47), assemble into a FAB engineered hetero-dimeric immunoglobulin molecule with at least 51.9 % efficacy.

Mutations were derived from the analysis of an overlay of the crystal structure of a Fc (γ1) fragment with the crystal structure of a human FAB (γ1) fragment. Both Fc (γ1) fragment and human FAB (γ1) fragment 3D structures were retrieved from the Protein Data Bank (PDB code 1H3U, and 1VGE, respectively; Krapp S *et al.*, *J Mol Biol*, 325(5):979-89 (2003), Chacko S *et al.*, *J Biol Chem*, 271(21):12191-8 (1996)), overlaid with the Coot software (Emsley P and Cowtan K, *Acta Crystallogr D Biol Crystallogr*, 60(Pt 12 Pt 1):2126-32 (2004)) and further visualized with the Discovery-Studio software from Accelrys (Cambridge, UK). Examination of the protein-protein interfaces of the overlaid 3D structures of the homo-dimeric CH3 (γ1) constant domain pair and the CH1 (γ1) - Cκ hetero-dimer was used as a starting point for rational design. Several parameters were considered; these

included but were not limited to: the preservation of the proline residues and the preservation of amino acid positions involved in the integrity of the domain cores as well as some of the electrostatic contacts from the CH1 (γ1) - Cκ hetero-dimer, abrogating specific hydrophobic contacts found in the CH1 (γ1) - Cκ hetero-dimer, and replacing those with selected hydrophobic contacts found in the homo-dimeric CH3 constant domain pair. This analysis led to the design of two subsets of amino acid substitutions, one subset originating from equivalent 3D positions between the protein-protein interface of the CH1 (γ1) constant domain and the protein-protein interface of the human CH3 (γ1) constant domain, and a second subset originating from equivalent 3D positions between the protein-protein interface of the Cκ domain and the protein-protein interface of the human CH3 (γ1) constant domain; thereby creating two engineered, novel and unique domains which can still hetero-dimerize but differently from the naturally occurring CH1 (γ1) - Cκ hetero-dimeric domain pair.

An engineered heavy chain of a FAB fragment comprising a variable heavy chain domain, and a human CH1 (γ1) domain having mutations derived from the protein-protein interface of the CH3 (γ1) constant domain in its protein-protein interface is designated "VH-CH13" (FIG. 16), while an engineered kappa light chain comprising a variable light chain domain, and an engineered human Cκ constant domain having mutations derived from the protein-protein interface of the CH3 (γ1) constant domain in its protein-protein interface is designated "VL-CK3" (FIG. 16). More specifically, a VH-CH13 chain consists of an engineered heavy chain FAB fragment having an engineered CH1 (γ1) domain with the following substitutions: S124Q, F126Y, K133D, S134E, T139Q, A141S, G143T, H168K, F170T, Q175D, S181F, S183Y, V185K (SEQ ID NO: 48) (EU numbering); and accordingly, a VL-CK3 domain consists of an engineered light chain FAB fragment having an engineered Cκ domain with the following substitutions: S114Q, F116Y, F118L, E123D, Q124E, V133T, N137K, Q160K, S162T, T164V, S174F, S176Y, T178K (SEQ ID NO: 49) (EU numbering).

In this example, the variable heavy chain and light chain domains from a humanized anti human CD19 antibody disclosed in the PCT Publication NO: WO 2010/095031 (abbreviated VH(anti-hCD19), and VL(anti-hCD19)) were used as inputs to build the VH-CH13 and the VL-CK3 chains, respectively. The combination of a VH-CH13 chain fused upstream of a previously described BT beta chain (note that in this example, the chain had a

C-terminal c-myc tag (EQKLISEEDLN), abbreviated BT beta c-myc chain), results in a newly engineered immunoglobulin heavy chain which once co-expressed with the newly engineered VL-CK3 light chain described above, and a BT alpha His chain described in examples 1-3, creates a new type of hetero-dimeric monovalent (i.e. having one specificity for one antigen) immunoglobulin molecule having a novel FAB fragment wherein the CH1 (γ1) and Cκ constant domains have novel protein-protein interfaces based on the CH3 (γ1) constant domain homo-dimeric protein-protein interface, and a Fc fragment with hetero-dimeric engineered CH3 domains based on the TCR alpha and beta constant domains (see examples 1- 3). This novel immunoglobulin molecule is abbreviated VH(anti-hCD19)-CH13-BT beta c-myc_BT alpha His_VL(anti-hCD19)-CK3 (SEQ ID NOs: 50, 7, and 51).

To create the VH(anti-hCD19)-CH13-BT beta c-myc chain and the VL(anti-hCD19)-CK3 chain cDNA coding sequences (SEQ ID NOs: 52 and 53, respectively), a unique cDNA encompassing both the CH13 domain sequence (SEQ ID NO: 54) and the CK3 domain sequence (SEQ ID NO: 55) was synthesized by GENEART AG (Regensburg, Germany). Using PCR, each chain was amplified individually, and the respective engineered constant domain cDNA coding sequences were fused downstream of a synthetic cDNA coding for their respective variable domains (separately synthesized by GENEART AG). The resulting VH(anti-hCD19)-CH13 DNA fragment was further fused upstream of a BT beta F405A chain having a C-terminal c-myc tag to generate the final VH(anti-hCD19)-CH13-BT beta c-myc chain. Subsequently, the two chains were ligated independently in the modified pREP4 vector described in example 1. The BT alpha His chain originated from example 1 (SEQ ID NO: 10).

For transient expression of the VH(anti-hCD19)-CH13-BT beta c-myc_BT alpha His_VL(anti-hCD19)-CK3 molecule, equal quantities of the three engineered chain vectors were co-transfected into suspension-adapted HEK293-EBNA cells as described previously (example 1). The FAB engineered hetero-dimeric construct was then purified from the cell-free supernatant using protein-A affinity chromatography and used for further analysis (procedure according to example 1). The correct assembly of the FAB engineered hetero-dimer can be assessed by SDS-PAGE analysis of samples under non-reducing conditions, since the correctly assembled molecule is expected to have a different SDS-PAGE mobility from the possible homo-dimeric species.

Transient transfection yields were 50 mg/l. The results of a typical SDS-PAGE analysis are shown in FIG. 17. Post protein-A purification the VH(anti-hCD19)-CH13-BT beta c-myc_BT alpha His_VL(anti-hCD19)-CK3 molecule (lane1) is the main species produced (A) with some homo-dimeric species (B) and some half-molecule (C) present.

It is possible to assess the proportion of hetero-dimer to homo-dimer in the protein-A purified preparation by scanning densitometry analysis of the non-reduced SDS-polyacrylamide (4-12%) gel bands (procedure according to example 1). FIG. 18 shows that the VH(anti-hCD19)-CH13-BT beta c-myc_BT alpha His_VL(anti-hCD19)-CK3 molecule represents at least 51.9 % of the protein-A purified material.

In conclusion, this example shows that the novel FAB fragment with engineered protein-protein interfaces based on the protein-protein interface of the homo-dimeric CH3 (γ1) constant domain is suitable for building further therapeutic hetero-dimeric immunoglobulins; more precisely, immunoglobulin molecules comprising two novel hetero-dimeric pairs of constant domains, one pair having engineered protein-protein interfaces based on the human T-cell receptor constant domain alpha and beta, and the other pair having engineered protein-protein interfaces based on the homo-dimeric CH3 (γ1) constant domain.

Example 7: immunoglobulin domains with engineered protein-protein interfaces derived from naturally occurring homo-dimeric or hetero-dimeric immunoglobulin super-family members.

This present example provides various set of mutations in human immunoglobulin domains to create novel protein-protein interfaces in acceptor domains and allow for one engineered domain to interact by hetero-dimerization or homo-dimerization with another engineered domain.

The mutations can be derived from naturally occurring dimeric immunoglobulin super-family members for example from the naturally occuring homo-dimeric human IGHG1 CH3 domain or from a naturally occurring hetero-dimers such as the human TCRα (IMGT® reference TRAC, the international ImMunoGeneTics information system®; Lefranc MP *et al.*, *Nucleic Acids Res*, 27(1):209-12 (1999); Ruiz M *et al.*, *Nucleic Acids Res*, 28(1):219-21 (2000); Lefranc MP, *Nucleic Acids Res*, 29(1):207-9 (2001); Lefranc MP, *Nucleic Acids Res*, 31(1):307-10 (2003); Lefranc MP *et al.*, *Dev Comp Immunol*, 29(3):185-203 (2005); Kaas Q *et al.*, *Briefings in Functional Genomics & Proteomics*, 6(4):253-64 (2007)), TCRβ (IMGT® reference TRBC1 and TRBC2), TCRγ (IMGT® reference TRGC1, TRGC2(2x), and TRGC2(3x)), and TCRδ (IMGT® reference TRDC) constant domains and introduced into the human heavy chain immunoglobulin constant domains of the γ1, γ2, γ3, γ4, α1, α2, ε, and μ isotypes (IMGT® reference IGHG1, IGHG2, IGHG3, IGHG4, IGHA1, IGHA2, IGHE, IGHM, respectively) or into the human immunoglobulin light chain constant domain κ (IMGT® reference IGKC) or into the human immunoglobulin light chain constant domains λ1, λ2, λ3, λ6, and λ7 (IMGT® reference IGLC1, IGLC2, IGLC3, IGLC6, and IGLC7, respectively).

In this example, amino acid substitutions were derived from the analysis of overlays of 3D structures of TCR domains (PDB codes: 1KGC or 1J8H (TCRα-TCRβ) and 1HXM (TCRδ-TCRγ)) with 3D structures of human IgG domains which were either retrieved form public databases (see Table 2) or modelled depending on availability (CH1 domains of α1, α2, ε; CH2 domains of γ2, γ3, α2, μ; CH3 domains of γ2, γ3, α2, μ and CH4 domain of μ). In addition, amino acid substitutions were derived from the analysis of overlays of 3D structures of human IgG domains in a pair wise fashion; for example, the human IGHG1 CH3 homo-

dimeric pair of domains was overlaid onto the human IGHG1 CH1-Cκ hetero-dimer. Models were calculated using the structure homology-modelling server SWISS-MODEL (Arnold K *et al.*, *Bioinformatics*, 22(2):195-201 (2006); http://swissmodel.expasy.org) in the automated mode with the following inputs: the protein domain amino acid sequence retrieved from the IMGT® database with specific amino acid substitutions depending on the project requirements (the international ImMunoGeneTics information system®; Lefranc MP *et al.*, *Nucleic Acids Res*, 27(1):209-12 (1999); Ruiz M *et al.*, *Nucleic Acids Res*, 28(1):219-21 (2000); Lefranc MP, *Nucleic Acids Res*, 29(1):207-9 (2001); Lefranc MP, *Nucleic Acids Res*, 31(1):307-10 (2003); Lefranc MP *et al.*, *Dev Comp Immunol*, 29(3):185-203 (2005); Kaas Q *et al.*, *Briefings in Functional Genomics & Proteomics*, 6(4):253-64 (2007)), and a 3D structure template consisting of an experimentally solved 3D structure from a related protein domain automatically identified by the SWISS-MODEL software. All experimentally solved 3D structures were retrieved from the RCSB Protein Data Bank (www.pdb.org; Berman HM *et al.*, *Nucleic Acids Res*, 28(1):235-42 (2000)). Overlays were performed using the Coot software (Emsley P and Cowtan K, *Acta Crystallogr D Biol Crystallogr*, 60(Pt 12 Pt 1):2126-32 (2004)). Visualization for analysis was done in the Discovery-Studio software from Accelrys (Cambridge, UK). The selection of each subset of mutations followed similar criteria to the ones described in example 1.

Examples of homo-dimeric or hetero-dimeric immunoglobulin domains to be used as donor domains or parent domain are selected from the group consisting of human IGHA1 CH1, IGHA1 CH2, IGHA1 CH3, IGHA2 CH1, IGHA2 CH2, IGHA2 CH3, IGHD CH1, IGHD CH2, IGHD CH3, IGHG1 CH1, IGHG1 CH2, IGHG1 CH3, IGHG2 CH1, IGHG2 CH2, IGHG2 CH3, IGHG3 CH1, IGHG3 CH2, IGHG3 CH3, IGHG4 CH1, IGHG4 CH2, IGHG4 CH3, IGHGP CH1, IGHGP CH2, IGHGP CH3, IGHE CH1, IGHE CH2, IGHE CH3, IGHE CH4, IGHM CH1, IGHM CH2, IGHM CH3, IGHM CH4, IGKC, IGLC1, IGLC2, IGLC3, IGLC6, IGLC7, TRAC, TRBC1, TRBC2, TRDC, TRGC1, TRGC2 (2x), and TRGC2 (3x).

Table 2: PDB codes used for the overlay analysis of human IgG molecules.

Domain / Isotype	CH1	CH2-CH3	CH4	Cκ	Cλ
γ1	1VGE	1H3U		1VGE	7FAB
γ2	3KYM				
γ3	1Q1J				
γ4	3NAA	1ADQ			
μ	1QLR				
α1		1OW0			
ε		2WQR	2WQR		

Details of the amino acid variations for the CH1-CH3 domains between the human IGHG1, IGHG2, IGHG3, and IGHG4 isotypes can be found in FIG. 19.

Complete lists of the substitutions derived from the protein-protein interface of the human TCR constant domains (TRAC, TRBC2, TRDC, and TRGC1) or the human IGHG1 CH3 domain that can be introduced in the human IGHG1-G4 CH1, IGHG1-G4 CH2, IGHG1-G4 CH3, IHGE CH4, IGHM CH4, IGKC, and IGLC (IGLC1, IGLC2, IGLC3, IGLC6, and IGLC7) protein-protein interfaces are provided in FIG. 20-23 which corresponds to IMGT® numbering as referenced in the IMGT® database (http://imgt.cines.fr/, the international ImMunoGeneTics information system®; Lefranc MP *et al.*, *Nucleic Acids Res*, 27(1):209-12 (1999); Ruiz M *et al.*, *Nucleic Acids Res*, 28(1):219-21 (2000); Lefranc MP, *Nucleic Acids Res*, 29(1):207-9 (2001); Lefranc MP, *Nucleic Acids Res*, 31(1):307-10 (2003); Lefranc MP *et al.*, *Dev Comp Immunol*, 29(3):185-203 (2005); Kaas Q *et al.*, *Briefings in Functional Genomics & Proteomics*, 6(4):253-64 (2007)).

A complete overview of the preferred positions considered for substitution with key amino acid residues derived from the protein-protein interface of the TCR constant domains (α, β, δ, γ) or the CH3 domain of IGHG1 is also provided below. Since the IMGT® numbering is based on the comparative analysis of the 3D structures of the immunoglobulin

super-family domains, it defines the 3D equivalent positions of the substitutions between donor and acceptor (parent) domains.

7.1 Examples of molecular modelling.

7.1.1 Modelling hetero-dimeric domain pairs based on the human TCRα-TCRβ constant domain protein-protein interface.

a. TCRα based substitutions.

The amino acid sequence positions from the protein-protein interface of the human TCRα constant domain (donor domain) which are used to substitute the 3D equivalent amino acid sequence positions from the protein-protein interface of the parent immunoglobulin domain are listed below:

- The donor positions from human TCRα are as follows: 20, 22, 26, 79, 85.1, 86, 88, and 90 (having the following amino acids: K20, V22, T26, Y79, S85.1, V86, W88, and N90), wherein the amino acid position of each group member is indicated according to the IMGT® numbering (the international ImMunoGeneTics information system®; Lefranc MP *et al.*, *Nucleic Acids Res*, 27(1):209-12 (1999); Ruiz M *et al.*, *Nucleic Acids Res*, 28(1):219-21 (2000); Lefranc MP, *Nucleic Acids Res*, 29(1):207-9 (2001); Lefranc MP, *Nucleic Acids Res*, 31(1):307-10 (2003); Lefranc MP *et al.*, *Dev Comp Immunol*, 29(3):185-203 (2005); Kaas Q *et al.*, *Briefings in Functional Genomics & Proteomics*, 6(4):253-64 (2007)).

- The acceptor positions in all human immunoglobulin domains are as follows: 20, 22, 26, 79, 85.1, 86, 88, and 90; except for position 79 in α1: CH2 (78), α2: CH2 (78), ε: CH2 (81) and ε: CH3 (80), wherein the amino acid position of each group member is indicated according to the IMGT® numbering.

b. TCRβ based substitutions.

The amino acid sequence positions from the protein-protein interface of the human TCRβ constant domain (donor domain) which are used to substitute the 3D equivalent amino acid sequence positions from the protein-protein interface of the parent immunoglobulin domain are listed below:

- The donor positions from the human TCR beta are as follows: 3, 5, 7, 20, 22, 26, 81, 84.1, 84.3, 85.1, 86, 88, and 90 (having the following amino acids: E3, A5, F7, T20, V22, T26, D81, L84.1, E84.3, C85.1 or A85.1 or S85.1, S86, R88, and R90), wherein the amino acid position of each group member is indicated according to the IMGT® numbering.

- The acceptor positions in all human immunoglobulin domains are as follows: 3, 5, 7, 20, 22, 26, 81, 84, 84.2, 85.1, 86, 88, and 90; except for position 81 in α1: CH2 (80), α2: CH2 (80), ε: CH2 (83); except for position 84 in α1: CH2 (83), α2: CH2 (83); except for position 84.2 in α1: CH2 (84), α2: CH2 (84), wherein the amino acid position of each group member is indicated according to the IMGT® numbering.

c. Modelling the human TCRα-TCRβ constant domain protein-protein interface on the human IGHG1 CH3 homo-dimer.

To model a novel hetero-dimer based on the human IGHG1 CH3 homo-dimer with protein-protein interfaces derived from the human TCRα/β constant domains, two models of a human IGHG1 CH3 monomer substituted in its protein-protein interface with selected amino acids were calculated (see above for methods). One monomer was substituted at selected equivalent 3D positions in its protein-protein interface with amino acids from the protein-protein interface of the human TCRα constant domain, and a second monomer was substituted at selected equivalent 3D positions in its protein-protein interface with amino acids from the protein-protein interface of the human TCRβ constant domain. The Cα traces of the resulting models for the two

engineered protein domains were further overlaid on the experimentally solved parental CH3 homo-dimer 3D structure (PDB code 1H3U) for visual inspection. In addition, a computational analysis of the protein-protein interface of the engineered domains was performed using the online PIC web server (http://crick.mbu.iisc.ernet.in/~PIC/, Tina KG *et al.*, *Nucleic Acids Res*, 35(Web Server issue):W473-6 (2007)). Tables 3 and 4 list the interacting residues in the protein-protein interface of the engineered hetero-dimer identified from computational analysis, and direct measurements; the amino acid position of each group member is indicated according to the IMGT® numbering. From the modelling of a large number of immunoglobulin domain pairs engineered in their protein-protein interface with substitutions derived from naturally occurring homo-dimeric and hetero-dimeric protein-protein interface residues, the residues 20, 22, 26, 85.1, 86 and 88, in particular the residues 22, 85.1, 86 and 88 (numbering according to IMGT®) were repeatedly found to provide the most important contacts between the protein-protein interfaces of the engineered domains. More generally, by visual inspection and calculation of protein-protein interactions (using the online PIC web server (http://crick.mbu.iisc.ernet.in/~PIC/, Tina KG *et al.*, *Nucleic Acids Res*, 35(Web Server issue):W473-6 (2007) or other protein-protein interaction programs known in the art), the residues 20, 22, 26, 85.1, 86 and 88, in particular the residues 22, 85.1, 86 and 88 were found to mediate the most important interactions for the hetero-dimerization or homo-dimerization of all engineered domains.

Without being bound by theory, the analysis of the newly engineered domain protein-protein interfaces identified four important subsets of interacting residues: residue 88 in the first engineered immunoglobulin (BT alpha) interacts with residue 85.1 and 86 in the second engineered immunoglobulin (BT beta), residue 85.1 in the first engineered immunoglobulin (BT alpha) interacts with residue 86 in the second engineered immunoglobulin (BT beta), residue 22 in the first engineered immunoglobulin (BT alpha) interacts with residue 22 and residue 86 in the second engineered immunoglobulin (BT beta), and residue 20 in the first engineered immunoglobulin (BT alpha) interacts

with residue 26 in the second engineered immunoglobulin (BT beta). Conversely, residue 88 in the second engineered immunoglobulin (BT beta) interacts with residue 85.1 and 86 in the first engineered immunoglobulin (BT alpha), residue 85.1 in the second engineered immunoglobulin (BT beta) interacts with residue 86 in the first engineered immunoglobulin (BT alpha), residue 22 in the second engineered immunoglobulin (BT beta) interacts with residue 22 and residue 86 in the first engineered immunoglobulin (BT alpha), and residue 20 in the second engineered immunoglobulin (BT beta) interacts with residue 26 in the first engineered immunoglobulin (BT alpha).

Table 3: Interactions between positions from the protein-protein interface of the TCRα-CH3 monomer to the protein-protein interface of TCRβ-CH3 monomer in the newly engineered CH3 hetero-dimer. The IMGT® numbering is used.

TCRα-CH3 (BT alpha)	TCRβ-CH3 (BT beta)
20	3, 5, 7, 26, 84.2, 85.1, 86
22	5, 7, 22, 86, 88
26	20, 88, 90
79	26, 81, 84, 85.1, 86
85.1	20, 81, 86, 88, 90
86	22, 81, 84, 86, 88
88	26, 81, 84, 84.2, 85.1, 86
90	26,84.2,85.1

Table 4: Interactions between positions from the protein-protein interface of the TCRβ-CH3 monomer to the protein-protein interface of TCRα-CH3 monomer in the newly engineered CH3 hetero-dimer. The IMGT® numbering is used.

TCRβ-CH3 (BT beta)	TCRα-CH3 (BT alpha)
3	20
5	20,22
7	20,22
20	26,85.1,86
22	22,86
26	20,79,88,90
81	79,85.1,86,88
84	79,86,88
84.2	20,79,88,90
85.1	20,79,86,88,90
86	20,22,79,85.1,86
88	22,26,85.1,86
90	26, 85.1

7.1.2 Modelling hetero-dimeric domain pairs based on the human TCRδ-TCRγ constant domain protein-protein interface.

a. TCRδ based substitutions.

The amino acid sequence positions from the protein-protein interface of the human TCRδ constant domain (donor domain) which are used to substitute the 3D equivalent amino acid sequence positions from the protein-protein interface of the parent immunoglobulin domain are listed below:

- The donor positions from the human TCR delta are as follows: 7, 20, 22, 27, 79, 82, 84.2, 85.1, 86, 88, and 90 (having the following amino acids: M7, N20, A22, E27, F79, A82, S84.2, N85.1, V86, L88, and K90), wherein the amino acid position of each group member is indicated according to the IMGT® numbering.

- The acceptor positions in all human immunoglobulin domains are as follows: 7, 20, 22, 27, 79, 81, 84.2, 85.1, 86, 88, 90; except for position 79 in α1: CH2 (78), α2: CH2 (78), ε: CH2 (81) and ε: CH3 (80); except for position 81 in α1: CH2 (80), α2: CH2 (80), ε: CH2 (83) and ε: CH3 (82); except for position 84.2 in α1: CH2 (84), α2: CH2 (84), ε: CH2 (84.4), wherein the amino acid position of each group member is indicated according to the IMGT® numbering.

b. TCRγ based substitutions.

The amino acid sequence positions from the protein-protein interface of the human TCRγ constant domain (donor domain) which are used to substitute the 3D equivalent amino acid sequence positions from the protein-protein interface of the parent immunoglobulin domain are listed below:

- The donor positions from the human TCR gamma are as follows: 3, 5, 20, 22, 26, 27, 81, 84, 85.1, 86, and 88 (having the following amino acids: K3, T5, T20, L22, E26, K27, G81, M84, M85.1, F86, and W88), wherein the amino acid position of each group member is indicated according to the IMGT® numbering.

- The acceptor positions in all human immunoglobulin domains are as follows: 3, 5, 20, 22, 26, 27, 81, 84, 85.1, 86, 88; except for position 81 in α1: CH2 (80), α2: CH2 (80), ε: CH2 (83); except for position 84 in γ1, γ2, γ3, γ3: CH2 (for all 83), α1: CH2 (83), α2: CH2 (83), ε: CH2 (84.1), μ: CH2 (83) and μ: CH3 (83), wherein the amino acid position of each group member is indicated according to the IMGT® numbering.

c. Modelling the human TCRδ-TCRγ constant domain protein-protein interface on the human IGHG1 CH3 homo-dimer.

To model a novel hetero-dimer based on the human IGHG1 CH3 homo-dimer with protein-protein interfaces derived from the human TCRδ/γ constant domains, two models of a human IGHG1 CH3 monomer substituted in its protein-protein interface with selected amino acids were calculated (see above for methods). One monomer was substituted at selected equivalent 3D positions in its protein-protein interface with amino acids from the protein-protein interface of the human TCRδ constant domain, and a second monomer was substituted at selected equivalent 3D positions in its protein-protein interface with amino acids from the protein-protein interface of the human TCRγ constant domain. The Cα traces of the resulting models for the two engineered protein domains were further overlaid on the experimentally solved parental CH3 homo-dimer 3D structure (PDB code 1H3U) for visual inspection. In addition, a computational analysis of the protein-protein interface of the engineered domains was performed using the online PIC web server (http://crick.mbu.iisc.ernet.in/~PIC/, Tina KG *et al.*, *Nucleic Acids Res*, 35(Web Server issue):W473-6 (2007)). Tables 5 and 6 list the interacting residues in the protein-protein interface of the engineered hetero-dimer identified from computational analysis, and direct measurements; the amino acid position of each group member is indicated according to the IMGT® numbering. From the modelling of a large number of immunoglobulin domain pairs engineered in their protein-protein interface with substitutions derived from naturally occurring homo-dimeric and hetero-dimeric protein-protein interface residues, the residues 20, 22, 26, 85.1, 86 and 88, in particular the residues 22, 85.1, 86 and 88 (numbering according to IMGT®) were repeatedly found to provide the most important contacts between the protein-protein interfaces of the engineered domains. More generally, by visual inspection and calculation of protein-protein interactions (using the online PIC web server (http://crick.mbu.iisc.ernet.in/~PIC/, Tina KG *et al.*, *Nucleic Acids Res*, 35(Web Server issue):W473-6 (2007) or other protein-protein interaction

programs known in the art), the residues 20, 22, 26, 85.1, 86 and 88, in particular the residues 22, 85.1, 86 and 88 were found to mediate the most important interactions for the hetero-dimerization or homo-dimerization of all engineered domains.

Without being bound by theory, the analysis of the newly engineered domain protein-protein interfaces identified four important subsets of interacting residues: residue 88 in the first engineered immunoglobulin (BT delta) interacts with residue 85.1 and 86 in the second engineered immunoglobulin (BT gamma), residue 85.1 in the first engineered immunoglobulin (BT delta) interacts with residue 86 in the second engineered immunoglobulin (BT gamma), residue 22 in the first engineered immunoglobulin (BT delta) interacts with residue 22 and residue 86 in the second engineered immunoglobulin (BT gamma), and residue 20 in the first engineered immunoglobulin (BT delta) interacts with residue 26 in the second engineered immunoglobulin (BT gamma). Conversely, residue 88 in the second engineered immunoglobulin (BT gamma) interacts with residue 85.1 and 86 in the first engineered immunoglobulin (BT delta), residue 85.1 in the second engineered immunoglobulin (BT gamma) interacts with residue 86 in the first engineered immunoglobulin (BT delta), residue 22 in the second engineered immunoglobulin (BT gamma) interacts with residue 22 and residue 86 in the first engineered immunoglobulin (BT delta), and residue 20 in the second engineered immunoglobulin (BT gamma) interacts with residue 26 in the first engineered immunoglobulin (BT delta).

Table 5: Interactions between positions from the protein-protein interface of the TCRδ-CH3 monomer to the protein-protein interface of TCRγ-CH3 monomer in the newly engineered CH3 hetero-dimer. The IMGT® numbering is used.

TCRδ-CH3 (BT delta)	TCRγ-CH3 (BT gamma)
7	20, 22
20	3, 5, 26, 27, 85.1, 86
22	5, 22, 26, 86
26	20, 88
27	13
79	26, 81, 84, 85.1, 86
81	81, 84, 85.1, 86
84.2	20, 81, 88
85.1	20, 81, 86, 88
86	20, 22, 84, 86, 88
88	26, 81, 84, 85.1, 86
90	26, 27, 85.1, 90

Table 6: Interactions between positions from the protein-protein interface of the TCRγ-CH3 monomer to the protein-protein interface of TCRδ-CH3 monomer in the newly engineered CH3 hetero-dimer. The IMGT® numbering is used.

TCRγ-CH3 (BT gamma)	TCRδ-CH3 (BT delta)
3	20
5	20, 22
20	7, 26, 84.2, 85.1, 86
22	7, 22, 79, 86
26	20, 79, 88, 90
27	20, 90
81	81, 84.2, 85.1, 88
84	79, 81, 86, 88
85.1	20, 79, 81, 86, 88, 90
86	20, 22, 79, 81, 85.1, 86, 88
88	84.2, 85.1, 86

7.1.3 Modelling hetero-dimeric domain pairs based on the human IGHG1 CH3 constant domain pair protein-protein interface.

The amino acid sequence positions from the protein-protein interface of the human IGHG1 CH3 constant domain (donor domain) which are used to substitute the 3D equivalent amino acid sequence positions from the protein-protein interface of the parent immunoglobulin domain are listed below:

a. A first set of donor positions from the human CH3 of IGHG1 comprises the following positions: 3, 5, 12, 13, 18, 20, 22, 79, 81, 84.2, 85.1, 86, and 88 (having the following amino acids: Q3, Y5, D12, E13, Q18, S20, T22, K79, T81, D84.2, F85.1, Y86, and K88), wherein the amino acid position of each group member is indicated according to the IMGT® numbering.

The acceptor positions for the first set in all human immunoglobulin domains except for all human immunoglobulin light chain domains are as follows: 3, 5, 12, 13, 18, 20, 22, 79, 81, 84.2, 85.1, 86, and 88, except for position 81 in γ1, γ2, γ3, γ3: CH2 (for all 83), except for position 84.2 in γ1, γ2, γ3, γ3: CH2 (for all 84.3), wherein the amino acid position of each group member is indicated according to the IMGT® numbering.

b. A second set of donor positions from the human CH3 of IGHG1 comprises the following positions: 3, 5, 7, 12, 13, 22, 26, 79, 81, 84, 85.1, 86, and 88 (having the following amino acids: Q3, Y5, L7, D12, E13, T22, K26, K79, T81, V84, F85.1, Y86, and K88), wherein the amino acid position of each group member is indicated according to the IMGT® numbering.

The acceptor positions for the second set in all human immunoglobulin light chain domains are as follows: 3, 5, 7, 12, 13, 22, 26, 79, 81, 83, 85.1, 86, and 88, except for a human IGLC domain where the second set in is as follows: 3, 5, 7, 11, 12, 22, 26, 79, 81, 83, 85.1, 86, and 88, wherein the amino acid

position of each group member is indicated according to the IMGT® numbering.

c. Modelling the human IGHG1 CH3 homo-dimer protein-protein interface on the human CH1-CK hetero-dimer protein-protein interface.

A model for the human IGHG1 CH1 domain substituted in its protein-protein interface with amino acids at equivalent 3D positions derived from the protein-protein interface of a human IGHG1 CH3 constant domain (CH13), and a model for the human Cκ domain substituted in its protein-protein interface with amino acids at equivalent 3D positions derived from the protein-protein interface of a human IGHG1 CH3 constant domain (CK3) were calculated (see above for methods). The Cα traces of the resulting models for the two engineered protein domains were further overlaid on the experimentally solved parental human IGHG1 CH1-Cκ hetero-dimer 3D structure (PDB code 1VGE) for visual inspection. In addition, a computational analysis of the protein-protein interface of the engineered domains was performed using the online PIC web server (http://crick.mbu.iisc.ernet.in/~PIC/, Tina KG *et al.*, *Nucleic Acids Res*, 35(Web Server issue):W473-6 (2007)). Tables 7 and 8 list the interacting residues in the protein-protein interface of the engineered hetero-dimer identified from computational analysis, and direct measurements; the amino acid position of each group member is indicated according to the IMGT® numbering. From the modelling of a large number of immunoglobulin domain pairs engineered in their protein-protein interface with substitutions derived from naturally occurring homo-dimeric and hetero-dimeric protein-protein interface residues, the residues 20, 22, 26, 85.1, 86 and 88, in particular the residues 22, 85.1, 86 and 88 (numbering according to IMGT®) were repeatedly found to provide the most important contacts between the protein-protein interfaces of the engineered domains. More generally, by visual inspection and calculation of protein-protein interactions (using the online PIC web server (http://crick.mbu.iisc.ernet.in/~PIC/, Tina KG *et al.*, *Nucleic Acids Res*, 35(Web Server issue):W473-6 (2007) or other protein-protein interaction

programs known in the art), the residues 20, 22, 26, 85.1, 86 and 88, in particular the residues 22, 85.1, 86 and 88 were found to mediate the most important interactions for the hetero-dimerization or homo-dimerization of all engineered domains.

Without being bound by theory, the analysis of the newly engineered domain protein-protein interfaces identified four important subsets of interacting residues: residue 88 in the first engineered immunoglobulin (CH13) interacts with residue 85.1 and 86 in the second engineered immunoglobulin (CK3), residue 85.1 in the first engineered immunoglobulin (CH13) interacts with residue 86 in the second engineered immunoglobulin (CK3), residue 22 in the first engineered immunoglobulin (CH13) interacts with residue 22 and residue 86 in the second engineered immunoglobulin (CK3), and residue 20 in the first engineered immunoglobulin (CH13) interacts with residue 26 in the second engineered immunoglobulin (CK3). Conversely, residue 88 in the second engineered immunoglobulin (CK3) interacts with residue 85.1 and 86 in the first engineered immunoglobulin (CH13), residue 85.1 in the second engineered immunoglobulin (CK3) interacts with residue 86 in the first engineered immunoglobulin (CH13), residue 22 in the second engineered immunoglobulin (CK3) interacts with residue 22 and residue 86 in the first engineered immunoglobulin (CH13), and residue 20 in the second engineered immunoglobulin (CK3) interacts with residue 26 in the first engineered immunoglobulin (CH13).

Table 7: Interactions between positions from the protein-protein interface of the CH13 monomer to the protein-protein interface of CK3 monomer in the newly engineered CH1-CK hetero-dimer. The IMGT® numbering is used.

CH13	CK3
3	13
5	12, 13
12	79, 88
13	3, 5, 7
18	3, 5, 26
20	3, 5, 7, 26, 85.1, 86
22	7, 22, 86
26	20
79	26, 81, 83, 85.1, 86
81	79, 81, 83, 85.1, 86
84.2	79, 88
85.1	79, 81, 86, 88
86	79, 81, 83, 85.1, 86
88	26, 81, 83, 85.1, 86

Table 8: Interactions between positions from the protein-protein interface of the CK3 monomer to the protein-protein interface of CH13 monomer in the newly engineered CH1-CK hetero-dimer. The IMGT® numbering is used.

CK3	CH13
3	13, 18, 20
5	13, 18, 20
7	13, 20, 22
12	5
13	3, 5
20	26
22	22, 86
26	20, 79, 88
79	12, 81, 84.2, 85.1, 86
81	79, 81, 85.1, 86, 88
83	79, 81, 86, 88
85.1	20, 79, 81, 86, 88
86	20, 22, 79, 81, 86, 88
88	12, 84.2, 85.1, 86

7.2 Summary of donor positions derived from human TCR constant domains (TRAC, TRBC2, TRDC, TRGC1) and IGHG1 CH3.

A summary of the donor positions derived from the human TRAC (TCRα, abbreviated ALPHA), TRBC2 (TCRβ, abbreviated BETA), TRDC (TCRδ, abbreviated DELTA), TRGC1 (TCRγ, abbreviated GAMMA) and IGHG1 CH3 (abbreviated CH3) is found in Table 9. The amino acid position of each group member is indicated according to the IMGT® numbering. Amino acid positions for CH3 (set i) are donor positions for substituting all human immunoglobulin domains except for light chain domains. Amino acid positions for CH3 (set ii) are donor positions for substituting all human immunoglobulin light chain domains.

Table 9: Donor positions derived from the human TRAC, TRBC2, TRDC, TRGC1 and IGHG1 CH3. The IMGT® numbering is used.

IMGT®	3	5	7	12	13	18	20	22	26	27	79
ALPHA							K	V	T		Y
BETA	E	A	F				T	V	T		
DELTA			M				N	A		E	F
GAMMA	K	T					T	L	E	K	
CH3 set i	Q	Y		D	E	Q	S	T			K
CH3 set ii	Q	Y	L	D	E			T	K		K

IMGT®	81	82	84	84.1	84.2	84.3	85.1	86	88	90
ALPHA							S	V	W	N
BETA	D			L		E	C*	S	R	R
DELTA		A			S		N	V	L	K
GAMMA	G		M				M	F	W	
CH3set i	T				D		F	Y	K	
CH3set ii	T		V				F	Y	K	

(* or C or S)

7.3 Basis for additional substitutions.

In a more general approach, the protein-protein interface residues from naturally occurring dimeric protein domains of the immunoglobulin super-family selected from the group consisting of 3, 5, 7, 12, 13, 18, 20, 22, 26, 27, 79, 81, 82, 84, 84.1, 84.2, 84.3, 85.1, 86, 88, and 90, can be used as donor residues to create new dimers, wherein the amino acid position of each group member is indicated according to the IMGT® numbering.

Table 10 shows the amino acid residues of selected positions 20, 22, 26, 85.1, 86, and 88 from the hetero-dimeric donor domain CH1, and homo-dimeric domains CH2, CH3 and CH4 originating from different isotypes which can be used to substitute amino acids at the same positions in parent domains. Table 11 shows the amino acid residues of selected positions 20, 22, 26, 85.1, 86, and 88 from various hetero-dimeric human immunoglobulin superfamily members which can be used to substitute amino acids at the same positions in parent domains.

Table 10: Amino acid residues of selected positions 20, 22, 26, 85.1, 86, and 88 of the hetero-dimeric donor domain CH1, and homo-dimeric CH2, CH3 and CH4 domains. The IMGT® numbering is used.

	20	22	26	85.1	86	88
CH1						
IGHA1	V	A	Q	T	S	Q
IGHA2	V	A	Q	T	S	Q
IGHD	V	A	T	M	S	Q
IGHE	T	G	T	A	I	L
IGHG1	A	G	K	S	S	V
IGHG2	A	G	K	S	S	V
IGHG3	A	G	K	S	S	V
IGHG4	A	G	K	S	S	V
IGHGP	A	G	K	S	S	V
IGHM	A	G	Q	A	T	Q
CH2						
IGHA1	N	T	T	S	S	V
IGHA2	N	T	T	S	S	V
IGHD	T	T	V	S	H	R
IGHE	Q	L	S	S	Q	E
IGHG1	E	T	V	R	V	V
IGHG2	E	T	V	R	V	V
IGHG3	E	T	V	R	V	V
IGHG4	E	T	V	R	V	V
IGHGP	E	T	V	H	V	V
IGHM	K	I	T	K	T	T
CH3						
IGHA1	T	T	R	A	T	I
IGHA2	T	T	R	A	T	I
IGHD	W	L	S	W	W	V
IGHE	T	T	V	T	T	T
IGHG1	S	T	K	F	Y	K
IGHG2	S	T	K	F	Y	K
IGHG3	S	T	K	F	Y	K
IGHG4	S	T	K	F	Y	R
IGHGP	T	T	K	F	Y	K
IGHM	K	T	T	S	V	E
CH4						
IGHE	T	A	Q	F	F	R
IGHM	T	T	T	F	H	I

Table 11: Amino acid residues of selected positions 20, 22, 26, 85.1, 86, and 88 of various hetero-dimeric human immunoglobulin superfamily members. The IMGT® numbering is used.

	20	22	26	85.1	86	88
Human IGKC	S	V	N	S	S	T
Human IGLC1-IGLC7	T	V	S	A	S	Y
Human TRAC	K	V	T	S	V	W
Human TRBC1, TRBC2	T	V	T	C	S	R
Human TRDC	N	A	K	N	V	L
Human TRGC1, TRGC2 (2x, 3x)	T	L	E	M	F	W

7.4 Correspondence between the IMGT® unique numbering, the EU numbering, and the Kabat numbering.

The correspondence between the IMGT® unique numbering and the EU numbering for most of the human heavy chain constant domains cited above is found in Table 12. The correspondence between the IMGT® unique numbering and the EU numbering for the human kappa constant light chain domain, and the correspondence between the IMGT® unique numbering and the Kabat numbering for the human lambda constant light chain domains are both found in Table 13.

A complete correspondence for the human IGHG1 CH1-CH3, IGKC, and IGLC domains is found in Figures 19A, 19B, 19C, 19D, 22, 23A, and 23B and alternatively at the IMGT® database (the international ImMunoGeneTics information system®; Lefranc MP *et al.*, *Nucleic Acids Res*, 27(1):209-12 (1999); Ruiz M *et al.*, *Nucleic Acids Res*, 28(1):219-21 (2000); Lefranc MP, *Nucleic Acids Res*, 29(1):207-9 (2001); Lefranc MP, *Nucleic Acids Res*, 31(1):307-10 (2003); Lefranc MP *et al.*, *Dev Comp Immunol*, 29(3):185-203 (2005); Kaas Q *et al.*, *Briefings in Functional Genomics & Proteomics*, 6(4):253-64 (2007)), more specifically from the pages found at:

http://imgt.cines.fr/textes/IMGTScientificChart/Numbering/Hu_IGHGnber.html;
http://imgt.cines.fr/textes/IMGTScientificChart/Numbering/Hu_IGKCnber.html;
http://imgt.cines.fr/textes/IMGTScientificChart/Numbering/Hu_IGLCnber.html.

Table 12: Correspondence between the IMGT® unique numbering and the EU numbering for the CH1, CH2, and CH3 domains of human IGHG1, IGHG2, IGHG3, and IGHG4.

IMGT®	3	5	7	12	13	18	20	22	26	27	79
CH1 EU	124	126	128	133	134	139	141	143	147	148	168
CH2 EU	239	241	243	248	249	256	258	260	264	265	288
CH3 EU	347	349	351	356	357	362	364	366	370	371	392

IMGT®	81	83	84	84.2	85.1	86	88	90
CH1 EU	170	172	173	175	181	183	185	187
CH2 EU	290	292	293	295	301	303	305	307
CH3 EU	394	396	397	399	405	407	409	411

Table 13: Correspondence between the IMGT® unique numbering and the EU numbering for human IGKC, and correspondence between the IMGT® unique numbering and the Kabat numbering for human IGLC (IGLC1, IGLC2, IGLC3, IGLC6, and IGLC7 domains).

IMGT®	3	5	7	12	13	18	20	22	26	27	79
IGKC EU	114	116	118	123	124	129	131	133	137	138	160
IGLC Kabat	114	116	118	123	124	129	131	133	137	138	160

IMGT®	81	83	84	84.2	85.1	86	88	90
IGKC EU	162	164	165	167	174	176	178	180
IGLC Kabat	162	164	165	167	174	176	178	180

Example 8: construction of a bispecific hetero-dimeric immunoglobulin having a CH3-CH3 protein-protein interface based on the human α/β T cell receptor constant domains.

The HER family of receptor tyrosine kinases are important mediators of cell growth, differentiation, and survival (Earp HS *et al.*, *Breast Cancer Res Treat*, 35(1):115-32 (1995)); the receptor family includes four distinct members: epidermal growth factor receptor EGFR (ErbB1, or HER1), HER2 (ErbB2 or $p185^{neu}$), HER3 (ErbB3) and HER4 (ErbB4 or tyro2) which are generally found in various combinations in cells (Rowinsky EK, *Annu Rev Med*, 55:433-57 (2004); Hynes NE and Lane HA, *Nat Rev Cancer*, 5(5):341-54 (2005)). Receptor dimerization is an essential requirement for ErbB function and for the signaling activity of these receptors (Baselga J and Swain SM, *Nat Rev Cancer*, 9(7):463-75 (2009)); more specifically, receptor hetero-dimerization is thought to increase the diversity of cellular responses to various HER ligands. Therapeutics that target a single receptor from the HER family are presently in use in treating diseases such as breast cancer, non-small cell lung cancer, colorectal cancer, head and neck cancer and pancreatic cancer (Engelman JA and Janne PA, *Clin Cancer Res*, 14(10):2895-9 (2008); Baselga J and Swain SM, *Nat Rev Cancer*, 9(7):463-75 (2009)). Although these therapies had some success, issues related to native and induced resistance and toxicity are currently limiting their efficacies (Robert C *et al.*, *Lancet Oncol*, 6(7):491-500 (2005); Jones KL and Buzdar AU, *Lancet Oncol*, 10(12):1179-87 (2009)). Therapeutics that could target multiple receptors from the HER family may offer improved clinical efficacy and lower toxicity.

To produce a bispecific molecule that could specifically bind to at least two HER receptors (FIG. 24), the antigen binding sites derived from the recombinant humanized anti-HER2 antibody 4D5 (rhuMAbHER2, huMAB4D5-8, Trastuzumab or Herceptin®; U.S. Patent No.5,821,337), and the recombinant chimeric anti-EGFR antibody C225 (IMC-C225, Cetuximab or Erbitux®; PCT Publication NO: WO 96/40210) were selected as inputs for gene synthesis at GENEART AG (Regensburg, Germany). For both antibodies, the heavy chain variable sequence (VH) and the light chain variable sequence (VL) were synthesized and reformatted into a scFv fragment (VH and VL domains were fused with a 15 amino acid GlySer linker: $(Gly_4Ser)_3$) and a chimeric FAB fragment (i.e., a murine VH - human CH1 (γ1) chain assembled with a murine VL - human CK chain) for the anti-HER2 antibody 4D5 and the anti-EGFR antibody C225, respectively. All DNA manipulations followed standard

molecular biology techniques and primarily involved PCR amplification, and PCR fragment assembly methods. To create the anti-EGFR portion of the bispecific molecule, the anti-EGFR antibody C225 murine VH - human CH1 (γ1) chain was fused upstream of a BT alpha chain described in example 1 (abbreviated VH(anti-EGFR)-CH1-BT alpha chain, SEQ ID NO: 56), while the anti-EGFR antibody C225 murine VL was fused upstream of a human Kappa constant domain (abbreviated VL(anti-EGFR)-CK chain, SEQ ID NO: 57). To create the anti-HER2 portion of the bispecific molecule, the anti-HER2 scFv was fused upstream of a BT beta F405A chain described in example 2 with a short GlySer amino acid linker (GGGS) between the two sequences (abbreviated scFv(anti-HER2)-BT beta chain, SEQ ID NO: 58). All three resulting chains were further ligated independently into the modified pREP4 vector mentioned in example 1 and co-transfected into HEK293-EBNA cells as described in example 1. Protein production was also according to the procedure described in example 1.

The expected bispecific molecule consisting of one VH(anti-EGFR)-CH1-BT alpha chain, one VL(anti-EGFR)-CK chain, and one scFv(anti-HER2)-BT beta chain (SEQ ID NOs: 59, 60, and 61), is designated "Erbitux FAB-BT alpha_Herceptin scFv-BT beta".

To isolate the Erbitux FAB-BT alpha_Herceptin scFv-BT beta hetero-dimer from homo-dimers, ion-exchange chromatography (IEX) steps were performed post protein-A purification (procedure according to example 1). First, an anion-IEX step was performed: the protein-A eluate was buffer exchanged (into 50 mM Tris-HCl pH 9.0) and loaded on a 4 ml SourceQ 30 packed column operated on an ÄKTA Purifier (both from GE Healthcare Europe GmbH, Glattbrugg, Switzerland). The Erbitux FAB-BT alpha_Herceptin scFv-BT beta was then eluted with a shallow pH gradient ranging from pH 9.0 to pH 5.0 (final buffer condition was 50 mM sodium acetate pH 5.0). Following SDS-PAGE analysis, the fractions containing the majority of the Erbitux FAB-BT alpha_Herceptin scFv-BT beta hetero-dimer were pooled and rebuffered to 50 mM sodium acetate pH 5.0. In a second step, cation IEX was performed by loading the rebuffered fractions from the anion IEX step on a 1 ml MonoS column (GE Healthcare Europe GmbH, Glattbrugg, Switzerland) and running a shallow gradient from 50 mM sodium acetate pH 5.0 to 50 mM Tris-HCl pH 9.0. Fractions containing the Erbitux FAB-BT alpha_Herceptin scFv-BT beta hetero-dimer were selected by SDS-PAGE analysis. Finally the purified the Erbitux FAB-BT alpha_Herceptin scFv-BT beta hetero-dimer was polished on a size-exclusion chromatography column (Superdex 200 HR16/60, GE Healthcare

Europe GmbH, Glattbrugg, Switzerland) to remove traces of aggregates and buffer exchange the final bispecific molecule into PBS. FIG. 25 shows a polyacrylamide SDS gel profile after each purification step as well as two control molecules: a scFv-Fc fusion protein and a monoclonal antibody. The production yields from transient transfections were up to 30 mg/l with a hetero-dimerization rate of 49.5% as judged by scanning densitometry analysis of non-reduced SDS-polyacrylamide (4-12%) gel bands (see example 1 for methods) (FIG. 26).

To assess the stability of Erbitux FAB-BT alpha_Herceptin scFv-BT beta hetero-dimer, the purified material was analysed using differential scanning calorimetry (DSC). The calorimetric measurements were carried out on a VP-DSC differential scanning microcalorimeter (GE Healthcare Europe GmbH, Glattbrugg, Switzerland) as described in example 2. The cell volume was 0.128 ml, the heating rate was 1 °C/min, and the excess pressure was kept at 64 p.s.i. The protein was used at a concentration of 0.95 mg/ml in PBS (pH 7.4). FIG. 27 shows a thermogram of the Erbitux FAB-BT alpha_Herceptin scFv-BT beta hetero-dimer exhibiting two thermal transitions at 69.5 and 75.9 °C. It is important to note that no sharp decrease after the heat absorption peak was recorded, indicating that no precipitation or aggregate formation occurred after thermal unfolding (Liu H *et al.*, *Immunol Lett*, 106(2):144-53 (2006)). Hence, the melting transitions observed for the Erbitux FAB-BT alpha_Herceptin scFv-BT beta hetero-dimer are similar to the ones observed for a human IGHG4 Fc fragment CH2 and CH3 domains, and similar to the melting transitions generally observed for stable human FAB fragments (Garber E and Demarest SJ, *Biochem Biophys Res Commun*, 355(3):751-7 (2007)). In conclusion, this thermal unfolding study shows that the Erbitux FAB-BT alpha_Herceptin scFv-BT beta hetero-dimer with engineered protein-protein interface based on the TCR alpha and beta constant domains has similar thermo-stability to naturally occurring immunoglobulins.

The ability of the Erbitux FAB-BT alpha_Herceptin scFv-BT beta hetero-dimer to bind simultaneously to both of its cognate antigens (human EGFR and human HER2) was tested by surface plasmon resonance (SPR) on a BIAcore 2000 instrument (GE Healthcare Europe GmbH, Glattbrugg, Switzerland). In the present example, the recombinant extracellular domains from the human EGFR and HER2 receptors were individually fused upstream of a human Fc (γ1) fragment (abbreviated EGFR-Fc and HER2-Fc, SEQ ID NO: 62

and 63, respectively). Briefly, the extracellular domains of the human EGFR and HER2 were amplified by PCR from the imaGenes clone NO: EX-A0275-MO2 and Ex-BOO17-M10 (imaGenes GmbH, campus Berlin-Buch, Berlin, Germany), respectively. PCR products were then fused upstream of a human Fc (γ1) portion using PCR assembly methods (SEQ ID NO: 64 and 65, respectively) and independently cloned into a mammalian cell expression vector based on the pcDNA3.1 vector DNA from Invitrogen (Invitrogen AG, Basel, Switzerland). Finally each recombinant vector was transfected into HEK293 cells; after 4-5 days post transfection, supernatants were harvested and recombinant proteins were purified to homogeneity by protein-A affinity chromatography. SPR experiments were performed at 25°C in 1 x HBS-EP buffer (GE Healthcare Europe GmbH, Glattbrugg, Switzerland) at a flow rate of 30 µl/min. The purified Fc-fused antigens were immobilized on a research-grade CM5 chip (GE Healthcare Europe GmbH, Glattbrugg, Switzerland) via amine coupling using a standard protocol provided by the manufacturer. EFGR-Fc and HER2-Fc, which were immobilized on different channels of the chip led to a signal of 410 and 880 response units (RU), respectively. A channel lacking immobilized antigens was used as reference channel. Responses from this channel were subtracted from measurements. For the binding and co-binding experiments, the Erbitux FAB-BT alpha_Herceptin scFv-BT beta hetero-dimer and the EGFR-Fc were diluted in 1 x HBS- EP buffer (Healthcare Europe GmbH, Glattbrugg, Switzerland) to a final concentration of 25 and 100 nM, respectively. For all analytes the injections were limited to two minutes. FIG. 28A shows the binding and dissociation between the Erbitux FAB-BT alpha_Herceptin scFv-BT beta hetero-dimer and the immobilized EGFR-Fc. Co-binding of the Erbitux FAB-BT alpha_Herceptin scFv-BT beta and the EGFR-Fc as analytes to the immobilized HER2-Fc is shown in FIG. 28B. In this binding experiment, the Erbitux FAB-BT alpha_Herceptin scFv-BT beta hetero-dimer was first injected on the channel with immobilized HER2-Fc, showing the binding of Erbitux FAB-BT alpha_Herceptin scFv-BT beta hetero-dimer to the antigen. Three minutes after this first injection, EGFR-Fc was injected, resulting in a co-binding event with the Erbitux FAB-BT alpha_Herceptin scFv-BT beta hetero-dimer; to verify that the observed response was a true

co-binding event and not an interaction between both antigens, the EGFR-Fc was injected on the immobilized HER2-Fc and no significant response was recorded (FIG. 28B).

Example 9: construction of a bispecific hetero-dimeric immunoglobulin having a CH3-CH3 protein-protein interface based on the human α/β T cell receptor constant domains, and a FAB fragment having a CH1-CK protein-protein interface based on the hetero-dimeric δ/γ T cell receptor constant domains.

Example 5 provides a novel set of modified CH1 (γ1) and Cκ constant domains based on the protein-protein interfaces of the human TCR constant domains delta and gamma. This new pair of FAB constant domains offers the opportunity to create human bispecific antibodies without having to compromise in using a common light chain.

This novel type of bispecific antibody will be based on two distinct heavy chains having different variable domains, and two distinct light chains having different variable domains. The correct assembly of the four distinct chains of the bispecific antibody is based on two principles: (1) to force the two different antibody heavy chains to hetero-dimerize, the novel pair of engineered hetero-dimeric CH3 domains described in example 1-3 will be used, (2) to circumvent any potential light chain mispairing, the novel set of CH1 (γ1) and Cκ constant domains described in example 5 will be introduced in one of the two FAB arms of the bispecific antibody. Hence, the bispecific antibody will encompass at least three different protein-protein interfaces in three different portions: the first FAB arm having the naturally occurring hetero-dimeric pair of CH1 (γ1) and Cκ constant domains, the second FAB arm having the novel hetero-dimeric pair of CH1 (γ1) and Cκ constant domains with protein-protein interfaces originating from the hetero-dimeric pair of TCR delta and TCR gamma constant domains, and one Fc fragment having the novel hetero-dimeric pair of engineered CH3 (γ1) constant domains with protein-protein interfaces originating from the hetero-dimeric TCR alpha and TCR beta constant domains (FIG. 29).

To produce a full and correctly assembled bispecific antibody that could specifically bind to at least two antigens, the variable heavy chain and variable light chain domains from a humanized anti human CD19 antibody (abbreviated VH(anti-hCD19) and VL(anti-hCD19)

respectively , disclosed in the PCT Publication NO: WO 2010/095031), and the variable heavy chain and variable light chain domains from a humanized anti alpha 2 subunit of the human VLA-2 receptor (VLA2) antibody (abbreviated VH(anti-hVLA2) and VL(anti-hVLA2) respectively, disclosed in the PCT Publication NO: WO 2007/056858), were selected as inputs for expression and functional studies. The coding cDNAs for VH(anti-hVLA2) and VL(anti-hVLA2) domains were available from studies described in the PCT Publication NO: WO 2007/056858.

To create the four distinct chains of the bispecific antibody, two new immunoglobulin chains were created using PCR techniques: a first chain consisting of the variable heavy chain VH(anti-hVLA2) fused upstream of a human CH1(γ1) - BT alpha His chain (example 1 and 2) (abbreviated VH(anti-hVLA2)-CH1-BT alpha His, SEQ ID NO: 66), and a second chain consisting of the variable light chain VL(hVLA2) fused upstream of the naturally occurring human Cκ constant domain (abbreviated VL(anti-hVLA2)-CK, SEQ ID NO: 67). Following PCR assembly, both chain cDNAs were ligated independently into the modified pREP4 vector described example 1. The final two immunoglobulin chains needed to produce the complete bispecific antibody originated from example 5: a third chain consisting of the VH(anti-hCD19)-CH1 delta - BT beta c-myc chain (SEQ ID NO: 43), and a fourth chain consisting of the VL(anti-hCD19)-CK gamma chain (SEQ ID NO: 44). The resulting bispecific antibody having all the four distinct chains is abbreviated anti-hCD19_anti-hVLA2 bispecific antibody (SEQ ID NOs: 68, 69, 41, and 42).

All four vectors carrying the recombinant immunoglobulin chains were co-transfected into HEK293-EBNA cells and protein production was performed according to the procedure described in example 1. Small quantities of antibody species having a single specificity towards either the human VLA2 antigen (abbreviated anti-hVLA2 antibody) or the human CD19 antigen (abbreviated hCD19 antibody) were obtained by co-transfection of chain "one" with chain "two", and chain "three" with chain "four", respectively. These antibodies were later used as controls in FACS experiments (see below).

The production yields of transient transfections were about18 mg/l post protein A purification (procedure according to example 1). To isolate fully assembled bispecific molecules from traces of homo-dimeric species, two affinity chromatographic steps were

sequentially implemented. The first step was based on protein L affinity chromatography (Protein L resin from GenScript, Piscataway, NJ USA; used according to the manufacturer's protocol), and made use of the specificity of protein-L towards the light chain variable domains originating from Kappa subgroup κ1 and κ3, but not κ2 (Nilson BH *et al.*, *J Biol Chem*, 267(4):2234-9 (1992)). Since the variable VL(anti-hVLA2) light chain domain belongs to the κ2 subclass, and the variable VL(anti-hCD19) light chain domain belongs to the κ1 subclass, this step allowed for the removal of any homo-dimeric antibody species lacking the variable VL(anti-hCD19) domain (see above, chain "four"). For the second step, the protein pool after protein L purification was buffer-exchanged into 50 mM Tris-HCl pH 9.0, 200 mM NaCl and loaded on a 1 ml HisTrap Ni^{2+}-NTA affinity chromatography column operated on an ÄKTA Purifier (both from GE Healthcare Europe GmbH, Glattbrugg, Switzerland) at a flow rate of 0.6 ml/min. This step allowed for the removal of any homo-dimeric antibody species lacking the BT alpha chain (see above, chain "one").

To confirm the presence of the anti-hCD19_anti-hVLA2 bispecific antibody, a Western blot analysis was performed on sample aliquots isolated at different steps of the purification process. For detection, a horse radish peroxidase conjugated anti His tag (Sigma, Buchs, Switzerland) and anti c-myc tag (Roche Diagnostics (Schweiz) AG, Rotkreuz, Switzerland) antibodies were used. For staining, the peroxidase substrate SIGMA FAST 3,3'-Diaminobenzidine (Sigma, Buchs, Switzerland) was used. Comparison of two Western blots, one detected with the anti His tag antibody and one detected with the anti c-myc antibody confirmed the presence of the anti-hCD19_anti-hVLA2 bispecific antibody after the two purification steps (FIG. 30, lane 5 in panels B and C).

To confirm the dual specificity of the anti-hCD19_anti-hVLA2 bispecific antibody, two independent FACS binding experiments were designed. In both experiments, the bispecific antibody was tested for binding to one of its two cognate antigens displayed at the cell-surface of target cells while detecting with the affinity tag present on the chain having the second specificity. Since the anti-hCD19_anti-hVLA2 bispecific antibody specificities are towards both the human CD19 cell-surface protein and the alpha 2 subunit of the human VLA-2 receptor, the Raji cell line (a human Burkitt's lymphoma cell line, DSMZ clone NO: ACC319), and the HT1080 cell-line (human fibrosarcoma cell line, ATCC clone NO: CCL-

121) were selected as target cells, respectively. Raji cells are strictly $CD19^+$ and $VLA2^-$, while HT1080 cells are $VLA2^+$ and $CD19^-$ (data not shown), thereby preventing any false positives. Both cell lines were harvested from standard culture conditions and counted (viability > 90% according to trypan blue exclusion). Cells were washed once in staining buffer: a PBS solution supplemented with 2.5% FBS from PAA (PAA Laboratories, Pasching, Austria; catalogue NO: A15-101) and 10% versene from GIBCO-Invitrogen AG (Basel, Switzerland, catalogue NO: 15040) prior to the staining procedure. Staining was performed on 2 x 10^5 cells per condition, in V-bottom 96-well plates, on ice. Each incubation step was done in 50 µl of staining buffer supplemented with 10 µg/ml of anti-hCD19_anti-hVLA2 bispecific antibody and further incubated for 20 min. Cells were then washed twice with cold staining buffer and incubated 20 min with either a mouse anti His tag antibody (Sigma, Buchs, Switzerland; catalogue NO: A7058-1VL), or a mouse anti c-myc tag antibody (Roche Diagnostics (Schweiz) AG, Rotkreuz, Switzerland; catalogue NO: 11667149001), both at a concentration of 10 µg/ml in staining buffer. A control sample with cells kept only in staining buffer was also made for both cell lines. For detection, cells were washed twice with staining buffer and incubated 20 min with a Phycoerythrin-conjugated mouse-anti-Human Ig Fc fragment specific antibody (eBioscience, San Diego, USA; distributor: eBioscience CBI Medicals PV, Baar, Switzerland; catalogue NO: 12-4998-82) used at a 1:200 dilution in staining buffer. Finally, cells were washed once with staining buffer and resuspended in 300 µl of cold staining buffer and promptly analyzed by flow cytometry (CyAn™ ADP, Beckman Coulter International S.A., Nyon, Switzerland). Data were processed using the Flowjo® software (Tree Star, Inc., Ashland, OR, USA); histograms were plotted on live cells based on Forward and Side Scatter parameter gating. FIG. 31A shows the binding of the anti-hCD19_anti-hVLA2 bispecific antibody to Raji cells detected with a mouse-anti c-myc tag antibody, in this experiment the anti-hVLA2 antibody lacking the c-myc tag is used as a negative control. FIG. 31B shows the binding of the anti-hCD19_anti-hVLA2 bispecific antibody to Raji cells detected with a mouse-anti His tag antibody, in this experiment the anti-hVLA2 antibody is also used as a negative control, since it lacks the hCD19 specificity in spite of having the polyhistidine tag sequence. FIG. 31C shows the binding of the anti-hCD19_anti-hVLA2 bispecific antibody to HT1080 cells detected with a mouse-anti c-myc tag antibody, in this experiment the anti-hCD19 antibody is used as a negative control since it lacks the hVLA2 specificity in spite of having the c-myc tag sequence. FIG. 31D shows the binding of the anti-hCD19_anti-hVLA2 bispecific antibody to HT1080 cells detected with a mouse-anti His tag antibody, in this

experiment the anti-hCD19 antibody lacking the polyhistidine tag sequence is used as a negative control. When taken together these four independent binding experiments show that when engaging target cells with one FAB arm, the anti-hCD19_anti-hVLA2 bispecific antibody is always detectable via the tag located on the opposite Fc subunit (carrying the other FAB arm, see FIG. 31B and 31C). This demonstrates the identity of the anti-hCD19_anti-hVLA2 as being a full bispecific antibody consisting of four different chains correctly assembled into two distinct FAB fragments each having a unique specificity brought together by a hetero-dimeric Fc region.

The thermal stability of the anti-hCD19_anti-hVLA2 bispecific antibody was assessed using calorimetric measurements (DSC) (see example 2 for methods; the protein was used at a concentration of 0.57 mg/ml in PBS). FIG. 32 shows a thermogram of the anti-hCD19_anti-hVLA2 bispecific antibody exhibiting three sharp transitions at 70.9, 76.6 and 82.8 °C, none of which are below 70°C thereby implying a stable immunoglobulin molecule (example 2). It is important to note that no sharp decrease after the heat absorption peak was recorded, indicating that no precipitation or aggregate formation occurred after thermal unfolding (Liu H *et al.*, *Immunol Lett*, 106(2):144-53 (2006)). Since the BT alpha His_BT beta hetero-dimeric Fc fragment from example 2 displayed a single sharp melting transition at 70 °C, it is possible to conclude that the melting transitions for both FAB arms have a mid-point superior to 75 °C as expected for a properly assembled and stable FAB fragments (Garber E and Demarest SJ, *Biochem Biophys Res Commun*, 355(3):751-7 (2007)). In conclusion, this thermal unfolding study shows that the anti-hCD19_anti-hVLA2 bispecific antibody is stable.

This example also shows that when combined, two or more novel hetero-dimeric pairs of constant domains having engineered protein-protein interfaces derived form the human T-cell receptor constant domains can be used as building blocks to create novel bispecific antibodies which are stable and suitable for therapeutic use in humans.

Example 10: construction of immunoglobulin Fc hetero-dimer variants with mixed gamma immunoglobulin isotype backgrounds.

This example demonstrates that two human CH3 domains, one domain derived from IGHG1 and the second domain derived from IGHG3 having mutations in the protein-protein interface of their CH3 domains carefully selected from the T-cell receptor (TCR) constant domain alpha and beta (as described in example1), assemble into a hetero-dimeric Fc molecule.

Mutations were derived from the analysis of an overlay of the crystal structure of the human LC13 TCR molecule (PDB code 1KGC; Bernstein FC *et al.*, *Eur J Biochem*, 80(2):319-24 (1977)) with the crystal structure of the Fc fragment from human IGHG1 (PDB code 1H3Y) as described in example 1. Since human IGHG1 and IGHG3 CH3 domains only differ at position 384, 392, 397, 422, 435, and 436 (EU numbering), their protein-protein interfaces are identical excepting for the residue at position 392, and allow for the design of a mixed isotype hetero-dimeric immunoglobulin based on the 3D equivalent positions described in example 1.

The hetero-dimeric immunoglobulin described in this example consists of the assembly of two Fc chain variants: one chain from human IGHG3 origin having mutations in the protein-protein interface of its CH3 domain carefully selected from the TCR constant domain alpha protein-protein interface (referred as BT alpha IGHG3 chain) (SEQ ID NO: 73), and one chain from human IGHG1 origin having mutations in the protein-protein interface of its CH3 domain carefully selected from the TCR constant domain beta protein-protein interface (referred as BT beta F405A chain) (SEQ ID NO: 74). To generate a difference in SDS-PAGE mobility, the latter Fc chain variant was fused to a variable light-chain kappa domain antibody (abbreviated VL) resulting in an engineered immunoglobulin chain referred as VL-BT beta F405A chain (SEQ ID NO: 75). The engineered CH3 domain from IGHG3 origin described herein is abbreviated CH3-BT alpha IGHG3 domain, and has SEQ ID NO: 76. The VL-BT beta F405A chain encompasses the CH3-BT beta F405A domain described in example 2 (SEQ ID NO: 14).The hetero-dimeric immunoglobulin consisting of the assembly of the two aforementioned chains is abbreviated BT alpha IGHG3_VL-BT beta F405A hetero-dimer.

cDNA encoding the VL-BT beta F405A chain was built as described in example 2. cDNA encoding the BT alpha IGHG3 chain encompassed the CH3-BT alpha IGHG3 domain coding sequence (SEQ ID NO: 77) which was originally synthesized by GENEART AG (Regensburg, Germany). Human IGHG1 hinge and CH2 domain coding sequences were subsequently added upstream to this synthesized fragment using standard PCR assembly methods. Each chain encoding PCR product was digested, purified, and ligated independently into the modified pREP4 vector mentioned previously (example 1). The two resulting sequence-verified recombinant vectors were then co-transfected into HEK293-EBNA cells as described in example 1. Protein production and purification were also according to example 1. For the BT alpha IGHG3_VL-BT beta F405A hetero-dimer, a production yield of 10 mg/l was obtained.

To assess the proportion of hetero-dimer to homo-dimer in the protein-A purified material, the relative ratios of the different species were quantified by scanning densitometry analysis of the non-reduced SDS-polyacrylamide (4-12%) gel bands according to the procedure described in example 1. Post protein-A purification the BT alpha IGHG3_VL-BT beta F405A hetero-dimer is the main species produced, followed by the VL-BT beta F405A _VL-BT beta F405A homo-dimer, and the VL-BT beta F405A chain (half molecule) (FIG. 33; lane 1).Under reducing conditions, the BT alpha IGHG3_VL-BT beta F405A hetero-dimer breaks down into the two expected molecular weight bands for the BT alpha IGHG3 and the VL-BT beta F405A chains (half molecules) (FIG. 33; lane 2). FIG. 34 shows that BT alpha IGHG3_VL-BT beta F405A hetero-dimer represents at least 91 % of the protein-A purified material.

Example 11: construction of immunoglobulin Fc hetero-dimer variants with mixed immunoglobulin class backgrounds.

This example demonstrates that two human Fc chains, derived from IGHG1 having mutations in the protein-protein interface of their CH3 domains carefully selected from the protein-protein interface of the CH3 domains from IGHA1 or IGHA2, assemble into a hetero-dimeric Fc molecule, thereby demonstrating that protein-protein interfaces from two different pairs of homo-dimeric immunoglobulin domains can be combined to create a novel hetero-dimeric protein-protein interface pair which upon grafting onto a naturally occurring pair of homo-dimeric immunoglobulin domains induce domain hetero-dimerization. Note that the scope of the method described herein is not limited to IGHA1, but has broad application using all type of homo-dimeric immunoglobulin domains. IGHA1 and IGHA2 have identical CH3 domain amino acid sequences, and IGHA1 CH3 amino acid sequence (SEQ ID NO: 96) used herein is fully interchangeable with IGHA2 CH3 amino acid sequence (SEQ ID NO: 97).

Mutations were derived from the analysis of the CH3 domain protein-protein interface interactions of the crystal structure of human IGHG1 and IGHA1 Fc fragments (Krapp S *et al.*, *J Mol Biol*, 325(5):979-89 (2003) and Herr AB *et al.*, *Nature*, 423(6940):614-20 (2003), respectively). The IGHG1 and IGHA1 Fc 3D structures were retrieved from the Protein Data Bank (PDB code 1H3Y and 1OW0 respectively; www.pdb.org; Bernstein FC *et al.*, *Eur J Biochem*, 80(2):319-24 (1977)) and further analyzed as described in example 1.

The hetero-dimeric immunoglobulin described in this example consists of the assembly of two Fc chain variants: one human IGHG1 Fc chain thereof having mutations in the protein-protein interface of its CH3 domain derived from a selected group of 3D equivalent positions found in the protein-protein interface of the naturally occurring human IGHA1 CH3 constant domain pair (designated "Fc IGHAG" chain), and a second engineered human IGHG1 Fc chain thereof having mutations in the protein-protein interface of its CH3 domain derived from a selected group of 3D equivalent positions found in the protein-protein interface of the naturally occurring human IGHA1 CH3 constant domain pair (designated "Fc IGHGA" chain), wherein the selected group of 3D equivalent positions found in the protein-

protein interface of the naturally occurring human IGHA1 CH3 constant domain pair is different in each engineered CH3 domain. More specifically, the "Fc IGHAG" chain consists of an immunoglobulin Fc chain from human IGHG1 having a CH3 domain with the following substitutions (abbreviated CH3 IGHAG domain; SEQ ID NO: 78): Q347E, Y349H, K370R, V397R, D399E, F405A, and Y407T (EU numbering); and conversely, the "Fc IGHGA" consists of an immunoglobulin Fc chain from human IGHG1 having a CH3 domain with the following substitutions (abbreviated CH3 IGHGA domain; SEQ ID NO: 79): S364T, K392L, T394W, K409I, and T411R (EU numbering). To generate a difference in SDS-PAGE mobility, one Fc chain variant was fused to a variable light-chain kappa domain antibody (abbreviated VL). The different PCR steps gave two final chains: the Fc IGHAG chain (SEQ ID NO: 80), and the VL-Fc IGHGA chain (SEQ ID NO: 81). The hetero-dimeric immunoglobulin construct described herein resulting from the assembly of these two chains is designated Fc IGHAG_VL-Fc IGHGA hetero-dimer.

cDNA coding sequences for the CH3 IGHGA and CH3 IGHAG domains (SEQ ID NO: 82 and 83, respectively) were synthesized by GENEART AG (Regensburg, Germany), and used to create their respective engineered chains by PCR assembly methods. Each chain encoding PCR product was digested, purified, and ligated independently into the modified pREP4 vector mentioned previously (example 1). The two resulting sequence-verified recombinant vectors were then co-transfected into HEK293-EBNA cells as described in example 1. Protein production and purification were also according to example 1. For the Fc IGHAG_VL-Fc IGHGA hetero-dimer, a production yield of 12 mg/l was obtained.

To assess the proportion of hetero-dimer to homo-dimer in the protein-A purified material, the relative ratios of the different species were quantified by scanning densitometry analysis of the non-reduced SDS-polyacrylamide (4-12%) gel bands according to the procedure described in example 1. Post protein-A purification, the Fc IGHAG_VL-Fc IGHGA hetero-dimer is the main species produced followed by the VL-Fc IGHGA_VL-Fc IGHGA homo-dimer and single chains (Fc IGHAG chain and VL-Fc IGHGA chains, i.e. half molecules) (FIG. 35, lane 1). Under reducing conditions, the Fc IGHAG_VL-Fc IGHGA hetero-dimer breaks down into the two expected molecular weight bands for the Fc IGHAG and the VL-Fc IGHGA chains (FIG. 35, lane 2). FIG. 36 shows that the Fc IGHAG_VL-Fc IGHGA hetero-dimer represents at least 54% of the protein-A purified material.

Example 12: construction of immunoglobulin Fc hetero-dimer variants with different immunoglobulin class backgrounds

12.1 Construction of a hetero-dimer variant with a chimeric IGHG1-IGHM immunoglobulin class background

This example demonstrates that two chains, each consisting of a human IGHG1 hinge, a human IGHG1 CH2 domain and an engineered human IGHM CH4 domain, wherein one chain has mutations in the protein-protein interface of its IGHM CH4 domain carefully selected from 3D equivalent positions of the protein-protein interface of the T-cell receptor (TCR) constant domain alpha and the second chain has mutations in the protein-protein interface of its IGHM CH4 domain carefully selected from 3D equivalent positions of the protein-protein interface of the TCR constant domain beta, assemble into a hetero-dimeric immunoglobulin; the 3D equivalent positions are identical to the ones described in example 1.

Specifically, the hetero-dimeric immunoglobulin described in this example consists of the assembly of two Fc chain variants: one engineered chain comprising an engineered IGHM CH4 domain wherein its protein-protein interface is substituted with a selected group of 3D equivalent positions found in the protein-protein interface of the naturally occurring human TCR constant domain alpha (designated "BT alpha IGHM-4" chain), and a second engineered chain comprising an engineered IGHM CH4 domain wherein its protein-protein interface is substituted with a selected group of 3D equivalent positions found in the protein-protein interface of the naturally occurring human TCR constant domain beta (designated "BT beta IGHM-4" chain). More specifically, the BT alpha IGHM-4 chain encompasses a CH4 domain from human IGHM with the following substitutions (abbreviated IGHM CH4 alpha domain; SEQ ID NO: 84): T20K, T22V, V79Y, F85.1S, H86V, I88W, and T90N (IMGT numbering); and conversely, the BT beta IGHM-4 chain encompasses of a CH4 domain from human IGHM with the following substitutions (abbreviated IGHM CH4 beta domain; SEQ ID NO: 85): D3E, Y5A, L7F, T22V, S81D, M84L, F85.1A, H86S, I88R, and T90R (IMGT numbering).

cDNA coding sequences for the IGHM CH4 alpha domain and IGHM CH4 beta domain (SEQ ID NO: 86 and 87, respectively) were synthesized by GENEART AG

(Regensburg, Germany), and used to create their respective engineered chains by PCR assembly methods. A short stretch of six histidine residues was appended at the C-terminus of the BT alpha IGHM-4 chain (abbreviated His) by including a polyhistidine coding sequence in the PCR anti-sense oligonucleotide. To generate a difference in SDS-PAGE mobility, one Fc chain variant was fused to a variable light-chain kappa domain antibody (abbreviated VL). The different PCR steps gave two final chains: the BT alpha IGHM-4 His chain (SEQ ID NO: 88), and the VL-BT beta IGHM-4 chain (SEQ ID NO: 89) (FIG. 37 labelled A and FIG. 37 labelled B). The hetero-dimeric immunoglobulin construct described herein resulting from the assembly of these two chains is designated BT alpha IGHM-4 His_VL-BT IGHM-4 beta hetero-dimer.

Each chain encoding PCR product was digested, purified, and ligated independently into the modified pREP4 vector mentioned previously. The two resulting sequence-verified recombinant vectors were subsequently co-transfected into HEK293-EBNA cells as described in example 1. Protein expression was also according to example 1. Purification was performed using protein-L affinity agarose (GenScript USA Inc., Piscataway, NJ, USA). The proportion of hetero-dimer to homo-dimer in the cell-culture supernatants and protein-L purified preparations were assessed by scanning densitometry analysis of the non-reduced SDS-polyacrylamide (4-12%) gel bands (procedure according to example 1).

Post protein-L purification, the BT alpha IGHM-4 His_VL-BT IGHM-4 beta hetero-dimer is isolated without any contaminants (FIG. 38, lane 3, labelled as A), representing 100% of the protein-L eluted material as measured by scanning densitometry analysis of the non-reduced SDS-PAGE band (FIG. 39). Under reducing conditions, the BT alpha IGHM-4 His_VL-BT beta IGHM-4 hetero-dimer band breaks down into the two expected molecular weight bands for the BT alpha IGHM-4 His and VL-BT beta IGHM-4 chains (half molecules) (FIG. 38, lane 4).

12.2 Construction of a hetero-dimer with a chimeric IGHG1-IGHE immunoglobulin class background

This example demonstrates that two chains, each consisting of a human IGHG1 hinge, a human IGHG1 CH2 domain and an engineered human IGHE CH4 domain, wherein one chain has mutations in the protein-protein interface of its IGHE CH4 domain carefully selected from 3D equivalent positions of the protein-protein interface of the T-cell receptor (TCR) constant domain alpha and the second chain has mutations in the protein-protein interface of its IGHE CH4 domain carefully selected from 3D equivalent positions of the protein-protein interface of the TCR constant domain beta, assemble into a hetero-dimeric immunoglobulin; the 3D equivalent positions are identical to the ones described in example 1.

Specifically, the hetero-dimeric immunoglobulin described in this example consists of the assembly of two Fc chain variants: one engineered chain comprising an engineered IGHE CH4 domain wherein its protein-protein interface is substituted with a selected group of 3D equivalent positions found in the protein-protein interface of the naturally occurring human TCR constant domain alpha (designated "BT alpha IGHE-4" chain), and a second engineered chain comprising an engineered IGHE CH4 domain wherein its protein-protein interface is substituted with a selected group of 3D equivalent positions found in the protein-protein interface of the naturally occurring human TCR constant domain beta (designated "BT beta IGHE-4" chain). More specifically, the BT alpha IGHE-4 chain encompasses a CH4 domain from human IGHE with the following substitutions (abbreviated IGHE CH4 alpha domain; SEQ ID NO: 90): T20K, A22V, Q26T, S79Y, F85.1S, F86V, R88W, and E90N (IMGT numbering); and conversely, the BT beta IGHGE4 chain encompasses a CH4 domain from human IGHE with the following substitutions (abbreviated IGHE CH4 beta domain; SEQ ID NO: 91): Y5A, A22V, Q26T, T81D, R84L, T84.2E, F85.1A, F86S, and E90R (IMGT numbering).

Similarly to example 12.1, cDNA coding sequences for the IGHE CH4 alpha and IGHE CH4 beta domains (SEQ ID NO: 92 and 93, respectively) were synthesized by GENEART AG (Regensburg, Germany), and used to create their respective engineered chains by PCR assembly methods. A short stretch of six histidine residues was appended at the C-terminus of the BT alpha IGHE-4 chain (abbreviated His) by including a polyhistidine coding

sequence in the PCR anti-sense oligonucleotide. To generate a difference in SDS-PAGE mobility, one Fc chain variant was fused to a variable light-chain kappa domain antibody (abbreviated VL). The different PCR steps gave two final chains: the BT alpha IGHE-4 His chain (SEQ ID NO: 94), and the VL-BT IGHE-4 beta chain (SEQ ID NO: 95) (FIG. 37 labelled C and FIG. 37 labelled D). The hetero-dimeric immunoglobulin construct described herein resulting from the assembly of these two chains is designated BT alpha IGHE-4 His_VL-BT IGHE-4 beta hetero-dimer.

Each chain encoding PCR product was digested, purified, and ligated independently into the modified pREP4 vector mentioned previously. The two resulting sequence-verified recombinant vectors were subsequently co-transfected into HEK293-EBNA cells as described in example 1. Protein production and purification were also according to example 1. The proportion of hetero-dimer to homo-dimer in the protein-A purified preparations were assessed by scanning densitometry analysis of the non-reduced SDS-polyacrylamide (4-12%) gel bands (procedure according to example 1).

Post protein-A purification, the hetero-dimer is isolated (FIG. 40, lane 1, labelled as A) with a hetero-dimerization rate of 10 % (FIG. 41, peak 2), and bands corresponding to the BT alpha IGHE-4 His homo-dimer (FIG. 40, lane 1, labelled as B), the VL-BT beta IGHE-4 chain (half molecule) (FIG. 40, lane 1, labelled as C), and the BT alpha IGHE-4 His chain (half molecule) (FIG. 40, lane 1, labelled as D) are observed. Under reducing conditions, the BT alpha IGHE-4 His_VL-BT beta IGHE-4 hetero-dimer breaks down into the two expected molecular weight bands for the BT alpha IGHE-4 His and VL-BT beta IGHE-4 chains (half molecules) (FIG. 40, lane 2).

CLAIMS

1. A hetero-dimeric immunoglobulin or hetero-dimeric fragment thereof comprising:
(a) a first engineered immunoglobulin chain comprising an engineered domain with a protein-protein interface, wherein at least one amino acid residue of the protein-protein interface of the parent domain of said first engineered immunoglobulin chain is substituted with an amino acid residue at the equivalent 3D structural position from a protein-protein interface of a donor domain of a first member of the naturally occurring immunoglobulin super-family; and
(b) a second engineered immunoglobulin chain comprising an engineered domain with a protein-protein interface, wherein at least one amino acid residue of the protein-protein interface of the parent domain of said second engineered immunoglobulin chain is substituted with an amino acid residue at the equivalent 3D structural position from a protein-protein interface of a donor domain of a second member of the naturally occurring immunoglobulin super-family,
wherein the engineered domain of the first engineered immunoglobulin chain and the engineered domain of the second engineered immunoglobulin chain are not identical and,
wherein the donor domain of the first member of the naturally occurring immunoglobulin super-family and the donor domain of the second member of the naturally occurring immunoglobulin super-family form a naturally occurring hetero-dimer or a naturally occurring homo-dimer and,
wherein the parent domain of the first and/or the second engineered immunoglobulin chain is a CH3 domain, wherein the amino acid residue which is substituted in the protein-protein interface of the parent domain of the first engineered immunoglobulin chain comprises the amino acid residue at position 88, and optionally a further amino acid residue at a position selected from the group consisting of 3, 5, 7, 20, 22, 26, 27, 79, 81, 84, 84.2, 85.1, 86, and 90 and, wherein the amino acid residue which is substituted in the protein-protein interface of the parent domain of the second engineered immunoglobulin chain comprises the amino acid residue at position 85.1 and/or 86 and optionally a further amino acid residue at a position selected from the group consisting of 3, 5, 7, 20, 22, 26, 27, 79, 81, 84, 84.2, 88, and 90 or at a position selected from the group consisting of 3, 5, 7, 20, 22, 26, 27, 79, 81, 84, 84.2, 85.1, 88, and 90 or at a position selected from the group consisting of 3, 5, 7, 20, 22, 26, 27, 79, 81, 84, 84.2, 86, 88, and 90,
wherein the amino acid residue substituted at position 88 in the parent domain of the first engineered immunoglobulin chain is interacting with the amino acid residue substituted at position 85.1 and/or 86 in the parent domain of the second engineered immunoglobulin chain,
wherein the amino acid position of each group member is indicated according to the IMGT® numbering.

2. The hetero-dimeric immunoglobulin or hetero-dimeric fragment thereof of claim 1, wherein the parent domain of the first and/or the second engineered immunoglobulin chain is a naturally occurring CH3 domain.

3. The hetero-dimeric immunoglobulin or hetero-dimeric fragment thereof of claim 1, wherein:
a) the amino acid residue which is substituted in the protein-protein interface of the parent domain of the first engineered immunoglobulin chain at position 88 is 88W and conservative amino acid substitutions thereof, wherein the amino acid position is indicated according to the IMGT® numbering;
b) the amino acid residue which is substituted at position 85 and/or 86 in the protein-protein interface of the parent domain of the second engineered immunoglobulin chain is selected from the group consisting of 85.1A, 85.1S, 85.1C and 85.1N and conservative amino acid substitutions thereof and/or is selected from the group consisting of 86S and 86V and conservative amino acid substitutions thereof, wherein the amino acid position is indicated according to the IMGT® numbering;
c) the amino acid residue which is substituted in the protein-protein interface of the parent domain of the first engineered immunoglobulin chain at position 88 is selected from the group consisting of 88Q, 88L, 88V, 88R, 88E, 88I, 88T, 88Y, 88K and 88W and/or wherein the further amino acid residue substituted in the protein-protein interface of the parent domain of the first engineered immunoglobulin chain is selected from the group consisting of 20V, 20T, 20A, 20N, 20Q, 20E, 20S, 20K, 20W, 22A, 22G, 22T, 22L, 22I, 22V, 26R, 26Q, 26T, 26K, 26V, 26S, 26N, 26E, 85.1T, 85.1M, 85.1A, 85.1S, 85.1R, 85.1H, 85.1K, 85.1F, 85.1C, 85.1N, 85.1W, 86S, 86I, 86T, 86H, 86Q, 86V, 86W, 86Y and 86F, wherein the amino acid position of each group member is indicated according to the IMGT® numbering;
d) the amino acid residue which is substituted in the protein-protein interface of the parent domain of the first engineered immunoglobulin chain at position 88 is selected from the group consisting of 88Q, 88L, 88V, 88R, 88E, 88I, 88T, 88Y, and 88W and/or wherein the further amino acid residue substituted in the protein-protein interface of the parent domain of the first engineered immunoglobulin chain is selected from the group consisting of 20V, 20T, 20A, 20N, 20Q, 20E, 20S, 20K, 22A, 22G, 22T, 22L, 22I, 22V, 26Q, 26T, 26K, 26V, 26S, 26N, 26E, 85.1T, 85.1M, 85.1A, 85.1S, 85.1R, 85.1H, 85.1K, 85.1F, 85.1C, 85.1N, 86S, 86I, 86T, 86H, 86Q, 86V, and 86F, wherein the amino acid position of each group member is indicated according to the IMGT® numbering;
e) the amino acid residue which is substituted in the protein-protein interface of the parent domain of the first engineered immunoglobulin chain comprises the amino acid residues at position 88 and at position 20, and optionally a further amino acid residue at a position

selected from the group consisting of 3, 5, 7, 22, 26, 27, 79, 81, 84, 84.2, 85.1, 86, and 90, wherein the amino acid position of each group member is indicated according to the IMGT® numbering;

f) the amino acid residue which is substituted in the protein-protein interface of the parent domain of the second engineered immunoglobulin chain comprises the amino acid residues at position 85.1 and/or 86 and at position 26 and optionally an amino acid residue at a further position, wherein the amino acid position is indicated according to the IMGT® numbering;

g) the amino acid residue which is substituted in the protein-protein interface of the parent domain of the second engineered immunoglobulin chain comprises the amino acid residues at position 85.1 and 86 or comprises 85.1C and conservative amino acid substitutions thereof and 86S and conservative amino acid substitutions thereof, and wherein the further amino acid residue which is substituted in the protein-protein interface of the parent domain of the second engineered immunoglobulin chain is selected from the group consisting of 3E, 5A, 7F, 20T, 22V, 26T, 81D, 84L, 84.2E, 88R and 90R and conservative amino acid substitutions thereof, wherein the amino acid position of each group member is indicated according to the IMGT® numbering;

h) the amino acid residue which is substituted in the protein-protein interface of the parent domain of the second engineered immunoglobulin chain comprises the amino acid residues at position 85.1 and 86 or comprises 85.1N and conservative amino acid substitutions thereof and 86V and conservative amino acid substitutions thereof, and wherein the further amino acid residue which is substituted in the protein-protein interface of the parent domain of the second engineered immunoglobulin chain is selected from the group consisting of 7M, 20N, 22A, 27E, 79F, 81A, 84.2S, 85.1N, 86V, 88L, and 90K and conservative amino acid substitutions thereof, wherein the amino acid position of each group member is indicated according to the IMGT® numbering; or

i) the amino acid residue which is substituted in the protein-protein interface of the parent domain of the first engineered immunoglobulin chain is 88I and conservative amino acid substitutions therof and the optional further amino acid residue which is substituted in the protein-protein interface of the parent domain of the first engineered immunoglobulin chain is 81W and conservative amino acid substitutions thereof, wherein the amino acid position of each group member is indicated according to the IMGT® numbering.

4. The hetero-dimeric immunoglobulin or hetero-dimeric fragment thereof of claim 1, wherein:

a) the further amino acid residue which is substituted in the protein-protein interface of the parent domain of the first engineered immunoglobulin chain is selected from the group consisting of 3E, 3K, 5A, 5T, 7F, 7M, 20K, 20N, 20T, 22A, 22L, 22V, 26E, 26T, 27E, 27K, 79F, 79Y, 81A, 81G, 81D, 84L, 84M, 84.2E, 84.2S, 85.1A, 85.1C, 85.1M, 85.1N, 85.1S, 86F,

86S, 86V, 90K, 90N, and 90R and conservative amino acid substitutions thereof, wherein the amino acid position of each group member is indicated according to the IMGT® numbering;
b) wherein the further amino acid residue which is substituted in the protein-protein interface of the parent domain of the first engineered immunoglobulin chain is at a position selected from the group consisting of 20, 22, 26, 79, 85.1, 86, and 90, wherein the amino acid position of each group member is indicated according to the IMGT® numbering;
c) the further amino acid residue which is substituted in the protein-protein interface of the parent domain of the first engineered immunoglobulin chain is selected from the group consisting of 20K, 22V, 26T, 79Y, 85.1S, 86V, and 90N and conservative amino acid substitutions thereof, wherein the amino acid position of each group member is indicated according to the IMGT® numbering;
d) the further amino acid residue which is substituted in the protein-protein interface of the parent domain of the second engineered immunoglobulin chain is at position 84 or is 84L and conservative amino acid substitutions thereof, and optionally a further amino acid at a position selected from the group consisting of 3, 5, 7, 20, 22, 26, 81, 84.2, 88, and 90 or at a position selected from the group consisting of 3, 5, 7, 20, 22, 26, 81, 84.2, 85.1, 88, and 90 or at a position selected from the group consisting of 3, 5, 7, 20, 22, 26, 81, 84.2, 86, 88, and 90, wherein the amino acid position of each group member is indicated according to the IMGT® numbering;
e) the further amino acid residue which is substituted in the protein-protein interface of the parent domain of the second engineered immunoglobulin chain is at a position selected from the group consisting of 3, 5, 7, 20, 22, 26, 81, 84, 84.2, 88, and 90 or at a position selected from the group consisting of 3, 5, 7, 20, 22, 26, 81, 84, 84.2, 85.1, 88, and 90 or at a position selected from the group consisting of 3, 5, 7, 20, 22, 26, 81, 84, 84.2, 86, 88, and 90, wherein the amino acid position of each group member is indicated according to the IMGT® numbering;
f) wherein the further amino acid residue which is substituted in the protein-protein interface of the parent domain of the first engineered immunoglobulin chain is at a position selected from the group consisting of 3, 5, 20, 22, 26, 27, 81, 84, 85.1, and 86, wherein the amino acid position of each group member is indicated according to the IMGT® numbering;
g) the further amino acid residue which is substituted in the protein-protein interface of the parent domain of the first engineered immunoglobulin chain is selected from the group consisting of 3K, 5T, 20T, 22L, 26E, 27K, 81G, 84M, 85.1M, 86F and conservative amino acid substitutions thereof, wherein the amino acid position of each group member is indicated according to the IMGT® numbering;
h) the further amino acid residue which is substituted in the protein-protein interface of the parent domain of the second engineered immunoglobulin chain is at a position selected from

the group consisting of 7, 20, 22, 27, 79, 81, 84.2, 88, and 90 or at a position selected from the group consisting of 7, 20, 22, 27, 79, 81, 84.2, 85.1, 88, and 90 or at a position selected from the group consisting of 7, 20, 22, 27, 79, 81, 84.2, 86, 88, and 90, wherein the amino acid position of each group member is indicated according to the IMGT® numbering; or

i) the further amino acid residue which is substituted in the protein-protein interface of the parent domain of the second engineered immunoglobulin chain is at position 79 or is 79F and conservative amino acid substitutions thereof, and optionally a further amino acid residue at a position selected from the group consisting of 7, 20, 22, 27, 81, 84.2, 88, and 90 or at a position selected from the group consisting of 7, 20, 22, 27, 81, 84.2, 85.1, 88, and 90 or at a position selected from the group consisting of 7, 20, 22, 27, 81, 84.2, 86, 88, and 90, wherein the amino acid position of each group member is indicated according to the IMGT® numbering.

5. The hetero-dimeric immunoglobulin or hetero-dimeric fragment thereof of claim 4, wherein the amino acid residue substitution 85.1C is replaced by amino acid residue substitution 85.1A or 85.1S, wherein the amino acid position of each group member is indicated according to the IMGT® numbering.

6. The hetero-dimeric immunoglobulin or hetero-dimeric fragment thereof of claim 1, wherein the first engineered immunoglobulin chain and/or the second engineered immunoglobulin chain comprise a further engineered domain with a protein-protein interface, wherein at least one amino acid residue of the protein-protein interface of the parent domain of said further engineered domain of said first engineered immunoglobulin chain and/or said second engineered immunoglobulin chain is substituted with an amino acid residue at the equivalent 3D structural position from a protein-protein interface of a donor domain of a third member of the naturally occurring immunoglobulin super-family, and

wherein the protein-protein interface of the further engineered domain of the first engineered immunoglobulin chain and/or the second engineered immunoglobulin chain is interacting with the protein-protein interface of an engineered domain of a third engineered immunoglobulin chain by hetero-dimerization or by homo-dimerization,

wherein at least one amino acid residue of the protein-protein interface of the parent domain of said engineered domain of said third engineered immunoglobulin chain is substituted with an amino acid residue at the equivalent 3D structural position from a protein-protein interface of a donor domain of a fourth member of the naturally occurring immunoglobulin super-family, and

wherein the donor domain of the third member of the naturally occurring immunoglobulin super-family and the donor domain of the fourth member of the naturally occurring

immunoglobulin super-family form a naturally occurring hetero-dimer or a naturally occurring homo-dimer and,
wherein the protein-protein interface of the further engineered domain of the first engineered immunoglobulin chain and/or the second engineered immunoglobulin chain and of the engineered domain of the third engineered immunoglobulin chain is different from the protein-protein interface of the engineered domain of the first engineered immunoglobulin chain and of the engineered domain of the second engineered immunoglobulin chain.

7. The hetero-dimeric immunoglobulin or hetero-dimeric fragment thereof of any one of claims 1 to 6, wherein the first and second engineered immunoglobulin chains comprise an Fc region.

8. The hetero-dimeric immunoglobulin or hetero-dimeric fragment thereof of claim 6, wherein at least two amino acid residues of the protein-protein interface of the parent domain of the further engineered domain of the first engineered immunoglobulin chain and/or the second engineered immunoglobulin chain and/or of the parent domain of the engineered domain of the third engineered immunoglobulin chain are substituted.

9. The hetero-dimeric immunoglobulin or hetero-dimeric fragment thereof of claim 6, wherein the hetero-dimeric immunoglobulin or hetero-dimeric fragment thereof or the full-length antibody is bispecific.

10. The hetero-dimeric immunoglobulin or hetero-dimeric fragment thereof of claim 9, wherein the bispecific hetero-dimeric immunoglobulin or hetero-dimeric fragment thereof or the bispecific full-length antibody binds to antigens selected from the group consisting of HER2, EGFR, CD19, and VLA-2.

11. The hetero-dimeric immunoglobulin or hetero-dimeric fragment thereof of any one of claims 1 to 10, wherein at least one additional polypeptide is fused to the first and/or second engineered immunoglobulin chain.

12. The hetero-dimeric immunoglobulin or hetero-dimeric fragment thereof of claim 11, wherein said additional polypeptide is selected from the group consisting of Fab, scFv, diabody, domain antibody, pharmacologically active peptide or protein, receptor extracellular domain, CDR grafted polypeptide, and therapeutic engineered protein scaffold.

13. The hetero-dimeric immunoglobulin or hetero-dimeric fragment thereof of any one of claims 1 to 12, wherein the amino acid residues of the protein-protein interface of the parent domain of

the first and/or second engineered immunoglobulin chain which are substituted are not adjacent.

14. The hetero-dimeric immunoglobulin or hetero-dimeric fragment thereof of any one of claims 1 to 13, wherein at least two amino acid residues of the protein-protein interface of the parent domain of the first and/or second engineered immunoglobulin chain are substituted.

15. The hetero-dimeric immunoglobulin or hetero-dimeric fragment thereof of any one of claims 1 to 14. wherein the parent domain of the first engineered immunoglobulin chain is an IgG1 CH3 domain and the parent domain of the second engineered immunoglobulin chain is an IgG3 CH3 domain or wherein the parent domain of the first engineered immunoglobulin chain is an IgG3 CH3 domain and the parent domain of the second engineered immunoglobulin chain is an IgG1 CH3 domain.

16. The hetero-dimeric immunoglobulin or hetero-dimeric fragment thereof of any one of claims 1 to 15, wherein the amino acid sequence of the engineered domains of the first engineered immunoglobulin chain and/or the second engineered immunoglobulin chain do not contain an insertion of one or more amino acid residues compared to the amino acid sequence of the parent domains of the first engineered immunoglobulin chain and/or the second engineered immunoglobulin chain.

17. A method of producing a hetero-dimeric immunoglobulin or hetero-dimeric fragment thereof of any one of claims 1 to 16, the method comprising:
(a) substituting at least one amino acid residue of the protein-protein interface of a parent domain of a first parent immunoglobulin chain at the equivalent 3D structural position from a protein-protein interface of a donor domain of a first member of the naturally occurring immunoglobulin super-family to obtain a first engineered immunoglobulin chain comprising an engineered domain;
(b) substituting at least one amino acid residue of the protein-protein interface of the parent domain of a second parent immunoglobulin chain at the equivalent 3D structural position from a protein-protein interface of a donor domain of a second member of the naturally occurring immunoglobulin super-family to obtain a second engineered immunoglobulin chain comprising an engineered domain;
(c) culturing a host cell comprising a nucleic acid encoding said engineered immunoglobulin chains, wherein the culturing is such that the nucleic acid is expressed and the engineered immunoglobulin chains produced; and

(d) recovering the hetero-dimeric immunoglobulin or hetero-dimeric fragment thereof from the host cell culture.

18. The method of claim 17, wherein the amino acid residue of the protein-protein interface of the parent domain of the parent immunoglobulin chain which is substituted at the equivalent 3D structural position from the protein-protein interface of a donor domain is an amino acid residue in the protein-protein interface of a donor domain which when overlaid on the protein-protein interface of the parent domain by superimposing the carbon alpha traces of both domains, occupies a 3D position within less than a distance of 6 Å to the closest residue of the parent domain, wherein the donor domain is different from the parent domain.

19. The method of claim 17, wherein:
a) the first and the second member of a naturally occurring immunoglobulin super-family are selected from the TCR constant domain family;
b) the donor domain of the first member of the naturally occurring immunoglobulin super-family is the TCR constant domain alpha (SEQ ID NO: 1) and the donor domain of the second member of the naturally occurring immunoglobulin super-family is the TCR constant domain beta (SEQ ID NO: 2);
c) the donor domain of the first member of the naturally occurring immunoglobulin super-family is the TCR constant domain alpha (SEQ ID NO: 1) and the donor domain of the second member of the naturally occurring immunoglobulin super-family is the TCR constant domain beta (SEQ ID NO: 2), wherein the cysteine (C) at amino acid position 75 in SEQ ID NO: 2 is substituted with alanine (A) or serine (S); or
d) the donor domain of the first member of the naturally occurring immunoglobulin super-family is the TCR constant domain gamma (SEQ ID NO: 33) and second member of the naturally occurring immunoglobulin super-family is the TCR constant domain delta (SEQ ID NO: 32).

20. The method of claim 17, wherein the first engineered immunoglobulin chain and/or the second engineered immunoglobulin chain comprise a further engineered domain with a protein-protein interface, wherein at least one amino acid residue of the protein-protein interface of the parent domain of said further engineered domain of said first engineered immunoglobulin chain and/or said second engineered immunoglobulin chain is substituted with an amino acid residue at the equivalent 3D structural position from a protein-protein interface of a donor domain of a third member of the naturally occurring immunoglobulin super-family, and wherein the protein-protein interface of the further engineered domain of the first engineered immunoglobulin chain and/or the second engineered immunoglobulin chain is interacting with

the protein-protein interface of an engineered domain of a third engineered immunoglobulin chain by hetero-dimerization or by homo-dimerization, wherein at least one amino acid residue of the protein-protein interface of the parent domain of said engineered domain of said third engineered immunoglobulin chain is substituted with an amino acid residue at the equivalent 3D structural position from a protein-protein interface of a donor domain of a fourth member of the naturally occurring immunoglobulin super-family and,
wherein the protein-protein interface of the further engineered domain of the first engineered immunoglobulin chain and/or the second engineered immunoglobulin chain and of the engineered domain of the third engineered immunoglobulin chain is different from the protein-protein interface of the engineered domain of the first engineered immunoglobulin chain and of the engineered domain of the second engineered immunoglobulin chain.