natural heterodimerization interface without any introduced mutations, hence minimizing the propensity to induce an immune response. In addition, the heterodimerizing Ig domains can be connected by a disulfide linkage, which is formed between cysteine residues, contained within the naturally occurring sequences and are presented in their evolutionary developed and hence optimal conformation. Moreover these domains can be further equipped with the ability to interact with e.g. the FcRn or with neighbouring Ig domains like CH2 or variable domains, which provide further advantageous properties to the heterodimerizing domains. Surprisingly, it was found that terminal half-life of the generated bivalent format could be improved and bioavailability was increased. Furthermore, these heterodimerizing Ig building blocks were used to generate bi- or tri-valent and bispecific scFv-Fc fusion proteins to retarget CD3 expressing T-cells to FAP (fibroblast activation protein) or Her3 expressing tumor cells or to tumor cells that are surrounded by FAP-expressing fibroblasts.

Summary

The underlying principle of the present disclosure is the generation of novel heterodimerizing regions or heterodimerizing protein domains (HRI and HRII) that can be comprised in two or more proteins of a protein complex, wherein the constant immunoglobulin domains are naturally heterodimerizing (1^{st} and 3^{rd} CRI) and which are modified to provide HRI and HRII with additional properties. These properties are transferred from one or more further constant immunoglobulin domains (2^{nd} and 4^{th} CRI) that are different from the 1^{st} and 3^{rd} CRI to the heterodimerizing constant immunoglobulin domains (1^{st} and 3^{rd} CRI) by replacement of amino acids of the heterodimerizing constant immunoglobulin domains (1^{st} and 3^{rd} CRI) with amino acids of the alternative constant immunoglobulin domains (2^{nd} and 4^{th} CRI). These properties are e.g. the interaction with the neonatal Fc receptor (FcRn) or the interaction with N- or C-terminally connected protein domains (e.g. further constant immunoglobulin domains, which are not 1^{st}, 2^{nd}, 3^{rd} or 4^{th} CRI).

In one aspect, the present disclosure provides a protein complex comprising at least two amino acid chains I and II, which are non-covalently bound to each other through a heterodimerization region I (HRI) comprised in amino acid chain I and a heterodimerization region II (HRII) comprised in amino acid chain II, wherein:

(a) HRI comprises seven antiparallel beta strands AI, BI, CI, DI, EI, FI, and GI, six intervening regions bI, cI, dI, eI, fI, and gI, a N-terminal region aI and a C-terminal region hI positioned from N- to C-terminus in the following order:

aI-AI-bI-BI-cI-CI-dI-DI-eI-EI-fI-FI-gI-GI-hI

wherein the HRI is a fusion protein of a first human constant region of an immunoglobulin or immunoglobulin-like protein (1st CRI, acceptor) interspersed with amino acids of a second human constant region of an immunoglobulin or immunoglobulin-like protein (2nd CRI, donor), wherein the 1st CRI comprises seven antiparallel beta strands A1, B1, C1, D1, E1, F1, and G1, six intervening regions b1, c1, d1, e1, f1, and g1, a N-terminal region a1 and a C-terminal region h1 arranged from N- to C-terminus in the following order:

a1-A1-b1-B1-c1-C1-d1-D1-e1-E1-f1-F1-g1-G1-h1

wherein the 2st CRI comprises seven antiparallel beta strands A2, B2, C2, D2, E2, F2, and G2, six intervening regions b2, c2, d2, e2, f2, and g2, a N-terminal region a2 and a C-terminal region h2 positioned from N- to C-terminus in the following order:

a2-A2-b2-B2-c2-C2-d2-D2-e2-E2-f2-F2-g2-G2-h2

wherein HRI has the amino acid sequence of the 1st CRI and wherein at least the following amino acids of the 1st CRI are replaced with the following amino acids of the 2nd CRI:

(i) at least 1 amino acid of a1 is replaced with at least 1 amino acid of a2 (Replacement 1);

(ii) at least 1 amino acid of c1 is replaced with at least 1 amino acid of c2 (Replacement 2); and

(iii) at least 1 amino acid of g1 is replaced with at least 1 amino acid of g2 (Replacement 3); and

(b) the HRII comprises seven antiparallel beta strands AII, BII, CII, DII, EII, FII, and GII, six intervening regions bII, cII, dII, eII, fII, and gII, a N-terminal region aII and a C-terminal region hII positioned from N- to C-terminus in the following order:

aII-AII-bII-BII-cII-CII-dII-DII-eII-EII-fII-FII-gII-GII-hII

wherein the HRII is a fusion protein of a third human constant region of an immunoglobulin or immunoglobulin-like protein (3rd CRI, acceptor) interspersed with amino acids of a fourth human constant region of an immunoglobulin or immunoglobulin-like protein (4th CRI, donor), and wherein the 3rd CRI comprises seven antiparallel beta strands A3, B3, C3, D3, E3, F3, and G3, six intervening regions b3, c3, d3, e3, f3, and g3, a N-terminal region a3 and a C-terminal region h3 positioned from N- to C-terminus in the following order:

a3-A3-b3-B3-c3-C3-d3-D3-e3-E3-f3-F3-g3-G3-h3

wherein the 4th CRI comprises seven antiparallel beta strands A4, B4, C4, D4, E4, F4, and G4, six intervening regions b4, c4, d4, e4, f4, and g4, a N-terminal region a4 and a C-terminal region h4 positioned from N- to C-terminus in the following order:

a4-A4-b4-B4-c4-C4-d4-D4-e4-E4-f4-F4-g4-G4-h4

wherein HRII has the amino acid sequence of the 3st CRI and wherein at least the following amino acids of the 3rd CRI are replaced with the following amino acids of the 4th CRI:

(i) at least 1 amino acid of a3 is replaced with at least 1 amino acid of a4 (Replacement 4);

(ii) at least 1 amino acid of c3 is replaced with at least 1 amino acid of c4 (Replacement 5); and

(iii) at least 1 amino acid of g3 is replaced with at least 1 amino acid of g4 (Replacement 6);

wherein the 1st CRI and the 3rd CRI are different from each other and specifically bind to each other under physiological conditions.

In another aspect, the present disclosure provides the amino acid chain I or II as defined herein, preferably a combination of amino acid chains, wherein chain I and chain II have the amino acid sequence according to SEQ ID NO: 41 and SEQ ID NO: 42, SEQ ID NO: 34 and SEQ ID NO: 35, SEQ ID NO: 43 and SEQ ID NO: 44, SEQ ID NO: 36 and SEQ ID NO: 37 (scFv13.7-Fc1k), SEQ ID NO: 36 and SEQ ID NO: 38 (scFv13.7-CD3-Hinge-Fc1k), SEQ ID NO: 39 and SEQ ID NO: 38 (scFv3-43-CD3-Hinge-Fc1k) or SEQ ID NO: 40 and SEQ ID NO: 38 (scFvhuMCSP-CD3-Hinge-Fc1k).

Thus, in an example the present disclosure provides a protein complex comprising at least two amino acid chains I and II, which are non-covalently bound to each other through a heterodimerization region I (HRI) comprised in amino acid chain I and a heterodimerization region II (HRII) comprised in amino acid chain II and wherein HRI and HRII respectively comprise the heterodimerizing domains of a human constant region of an immunoglobulin or an immunoglobulin-like protein wherein each heterodimerizing domain is modified in that amino acid sequences of a further immunoglobulin or an immunoglobulin-like protein are inserted into at least two preferably at least three regions of the respective heterodimerizing domain that are outside the heterodimerization interphase of the respective HRI and HRII, i.e. which are preferably solvent accessible after heterodimerization of HRI and HRII.

More specifically the protein complex of the disclosure comprises at least two amino acid chains I and II, which are non-covalently bound to each other through a heterodimerization region I (HRI) comprised in amino acid chain I and a heterodimerization region II (HRII) comprised in amino acid chain II, wherein

(a) HRI comprises seven antiparallel beta strands AI, BI, CI, DI, EI, FI, and GI, six intervening regions bI, cI, dI, eI, fI, and gI, a N-terminal region aI and a C-terminal region hI positioned from N- to C-terminus in the following order:

aI-AI-bI-BI-cI-CI-dI-DI-eI-EI-fI-FI-gI-GI-hI,

wherein HRI is a fusion protein of a first human constant region of an immunoglobulin or immunoglobulin-like protein (1st CRI, acceptor) interspersed with amino acids of a second human constant region of an immunoglobulin or immunoglobulin-like protein (2nd CRI, donor),

wherein the 1st CRI comprises seven antiparallel beta strands A1, B1, C1, D1, E1, F1, and G1, six intervening regions b1, c1, d1, e1, f1, and g1, a N-terminal region a1 and a C-terminal region h1 arranged from N- to C-terminus in the following order:

a1-A1-b1-B1-c1-C1-d1-D1-e1-E1-f1-F1-g1-G1-h1,

wherein the 2nd CRI comprises seven antiparallel beta strands A2, B2, C2, D2, E2, F2, and G2, six intervening regions b2, c2, d2, e2, f2, and g2, a N-terminal region a2 and a C-terminal region h2 positioned from N- to C-terminus in the following order:

a2-A2-b2-B2-c2-C2-d2-D2-e2-E2-f2-F2-g2-G2-h2,

wherein HRI has the amino acid sequence of the 1st CRI and wherein at least the following amino acids of the 1st CRI are replaced with the following amino acids of the 2nd CRI:

(i) at least 1 amino acid of a1 is replaced with at least 1 amino acid of a2 (Replacement 1);

(ii) at least 1 amino acid of c1 is replaced with at least 1 amino acid of c2 (Replacement 2); and

(iii) at least 1 amino acid of g1 is replaced with at least 1 amino acid of g2 (Replacement 3); and wherein

(b) HRII comprises seven antiparallel beta strands AII, BII, CII, DII, EII, FII, and GII, six intervening regions bII, cII, dII, eII, fII, and gII, a N-terminal region aII and a C-terminal region hII positioned from N- to C-terminus in the following order:

aII-AII-bII-BII-cII-CII-dII-DII-eII-EII-fII-FII-gII-GII-hII,

wherein the HRII is a fusion protein of a third human constant region of an immunoglobulin or immunoglobulin-like protein (3rd CRI, acceptor) interspersed with amino acids of a fourth human constant region of an immunoglobulin or immunoglobulin-like protein (4th CRI, donor), and

wherein the 3rd CRI comprises seven antiparallel beta strands A3, B3, C3, D3, E3, F3, and G3, six intervening regions b3, c3, d3, e3, f3, and g3, a N-terminal region a3 and a C-terminal region h3 positioned from N- to C-terminus in the following order:

a3-A3-b3-B3-c3-C3-d3-D3-e3-E3-f3-F3-g3-G3-h3,

wherein the 4th CRI comprises seven antiparallel beta strands A4, B4, C4, D4, E4, F4, and G4, six intervening regions b4, c4, d4, e4, f4, and g4, a N-terminal region a4 and a C-terminal region h4 positioned from N- to C-terminus in the following order:

a4-A4-b4-B4-c4-C4-d4-D4-e4-E4-f4-F4-g4-G4-h4,

wherein HRII has the amino acid sequence of the 3st CRI and wherein at least the following amino acids of the 3rd CRI are replaced with the following amino acids of the 4th CRI:

(i) at least 1 amino acid of a3 is replaced with at least 1 amino acid of a4 (Replacement 4);

(ii) at least 1 amino acid of c3 is replaced with at least 1 amino acid of c4 (Replacement 5); and

(iii) at least 1 amino acid of g3 is replaced with at least 1 amino acid of g4 (Replacement 6),

wherein the 1st CRI and the 3rd CRI are different from each other and specifically bind to each other under physiological conditions.

In another aspect the present disclosure provides a nucleic acid encoding the amino acid chain I and/or II as described herein.

In another aspect, the disclosure provides a vector comprising a nucleic acid as described herein.

In yet another aspect, the present disclosure provides a method of determining (defining) the amino acid sequence of HRI of a, amino acid chain I and/or of HRII of an amino acid sequence II comprising the steps of:

(i) selecting a 1st CRI, a 2nd CRI, a 3rd CRI, and a 4th CRI;

(ii) determining (defining) the seven beta strands A, B, C, D, E, F and G of the 1st CRI, 2nd CRI, 3rd CRI, and 4th CRI, intervening sequences b, c, d, e, f, and g of the 1st CRI, 2nd CRI, 3rd CRI, and 4th CRI, N- and C-terminal sequences a and h, respectively of the 1st CRI, 2nd CRI, 3rd CRI, and 4th CRI;

(iii) replacing at least 1 amino acid of a of the 1st CRI with at least 1 amino acid of a of the 2nd (Replacement 1); replacing at least 1 amino acid of c of the 1st CRI with at least 1 amino acids of c of the 2nd CRI (Replacement 2); replacing at least 1 amino acid of g of the 1st CRI with at least 1 amino acid of g of the 2nd CRI (Replacement 3); replacing at least 1 amino acid of a of the 3rd CRI with at least 1 amino acid of a of the 4th CRI (Replacement 4); replacing at least 1 amino acid of c of the 3rd with at least 1 amino acid of c of the 4th

CRI (Replacement 5); and replacing at least 1 amino acid of g of the 3 with at least 1 amino acid g of the 4th CRI (Replacement 6),

wherein the 1st CRI and the 3rd CRI are different from each other and specifically bind to each other under physiological conditions.

In yet another aspect, the disclosure provides a method of producing amino acid chain I comprising the HRI sequence as described herein and/or amino acid chain II with the HRII sequence as described herein comprising the step of introducing a nucleic acid encoding the amino acid chain I and/or amino acid chain II into a host cell and expressing amino acid chain I and/or II.

In an example, the disclosure provides a method of producing amino acid chain I comprising the HRI sequence determined (defined) as described herein and/or amino acid chain II comprising the HRII sequence determined (defined) as described herein.

In yet another aspect, the disclosure provides the protein complex as described herein or of amino acid chain I and/or II as described herein for use as a medicament, preferably for use in the prophylaxis, treatment or diagnosis of a disorder or disease such as but not limited to inflammatory diseases, autoimmune diseases, allergic diseases, proliferative diseases, cancer type diseases, cutaneous conditions, endocrine diseases, eye diseases and disorders, genetic disorders, metabolic diseases, infectious diseases, intestinal diseases, neurological disorders, and mental illness.

In an example, the disclosure provides the protein complex of the first aspect for use as a medicament.

List of Figures

Figure 1. Schematic picture of constant Ig domain sequence. Represented is the cartoon of a sequence of an Ig domain, while arrows indicate the position of beta strands and bars describe loop regions.

Figure 2. Schematic view of the heterodimerizing Ig domains. Examplary set of heterodimerizing region I and II (HRI and HRII) with human constant regions of an immunoglobulin or immunoglobulin-like protein (CRI). Possible 1st and 3rd CRI sequences originate from two different heterodimerizing Ig domains (white part, 1st CRI and 3rd CRI) that e.g. hold the potential to form inter-chain disulfide bonds (black bar). Possible 2nd and 4th CRI sequences of Ig domains that interact e.g. with FcRn or other Ig domains 2nd CRI and 4th CRI) are indicated in black.

Figure 3. Graphic study on a set of Ig domains. Shown is a cartoon of the superimposition of amino acid backbones of a set of Ig domains (see Fig. 4) where ß-strands are displayed in black, while the loop regions are displayed in grey.

Figure 4. Sequence alignment of several Ig domains. Residues that were predicted to form beta strands are highlighted by black font and dark grey background. Residues that were predicted to form beta strands but excluded from beta strands A-G due to structure or sequence alignment are highlighted by black font and bright grey background. Residues that were not predicted to form beta strands, but included to beta strand A-G due to structure or sequence alignment are highlighted by white font and dark grey background.