MODIFIED Cκ AND CH1 DOMAINS

BACKGROUND

[0001] A bispecific monoclonal antibody (BsMAb, BsAb) is an artificial protein that can simultaneously bind to two different types of antigen or two different epitopes of the same antigen. BsAbs can be manufactured in several structural formats, and current applications have been explored for cancer immunotherapy and drug delivery.

[0002] There are many formats of BsAb. An IgG-like BsAb retains the traditional monoclonal antibody (mAb) structure of two Fab arms and one Fc region, except the two Fab sites bind different antigens. The most common types are called trifunctional antibodies, as they have three unique binding sites on the antibody: the two Fab regions, and the Fc region. Each heavy and light chain pair is from a unique mAb. The Fc region made from the two heavy chains forms the third binding site. These BsAbs are often manufactured with the quadroma, or the hybrid hybridoma, method.

[0003] However, the quadroma method relies on random chance to form usable BsAbs, and can be inefficient. Another method for manufacturing IgG-like BsAbs is called "knobs into holes," and relies on introducing a mutation for a large amino acid in the heavy chain from one mAb, and a mutation for a small amino acid in the other mAb's heavy chain. This allows the target heavy chains (and their corresponding light chains) to fit together better, and makes BsAb production more reliable.

[0004] While this knob-into-holes approach solves the heavy chain homodimerazation problem, it did not address the issues regarding mispairing between the light chain and heavy chains from two different antibodies. There is a need to provide better BsAbs that are easier to prepare, and have better clinical stability and efficacy.

SUMMARY

[0005] The present disclosure provides antibodies and antigen-binding fragments with modified Cκ and CH1 domains that still enable pairing of the Cκ and CH1 domains but have reduced pairing with CH1 and Cκ domains without the modifications. Such modifications can be particularly useful for preparing bispecific antibodies which two different pairs of Cκ and CH1 domains.

[0006] As demonstrated in the experimental examples, two groups of amino acids were identified as important interface residues which, when changed, can reduce or even disrupt the pairing of the Cκ and CH1 domains unless appropriate modifications are made to re-establish such interface.

[0007] One such group includes Val26 (Kabat numbering: Val133) and Phe11 (Kabat numbering: Phe118) of the Cκ domain and Leu11 (Kabat numbering: Leu124) of the CH1 domain. When one of these amino acids is substituted with Ala, for instance, the Cκ/CH1 pairing can be disrupted. Another example group includes Gln17 (Kabat numbering: 124) of Cκ and Phe9 (Kabat numbering: 122) of CH1.

[0008] Certain mutations at these interface residues, however, can restore the pairing, which is also demonstrated in the examples. One such example is Val26Trp (Cκ) with Leu11Trp (CH1). Further examples are shown in Table 1 and Table 2.

[0009] In one embodiment, provided is an antibody or antigen-binding fragment thereof, comprising a human CH1 fragment comprising a L11W substitution and a human Cκ fragment comprising a V26W substitution. Such an antibody or fragment can optionally include additional substitutions that further reduce the binding to the wild-type partner and/or enhance binding between the substituted fragments.

[0010] For instance, an additional pair of substitutions can be K101E in CH1 and D15K or D15H (D15K/H) in Cκ. Another pair of substitutions are K96D in CH1 and E16R in Cκ. Yet another example pair is K96E in CH1 and E16K in Cκ. Accordingly, in some embodiments, provided are antibody or antigen-binding fragment thereof, in which the CH1 fragment comprises substitutions L11W and K101E and the Cκ fragment comprises substitutions V26W and D15K/H; the CH1 fragment comprises substitutions L11W and K96D and the Cκ fragment comprises substitutions V26W and E16R; the CH1 fragment comprises substitutions L11W and K96E and the Cκ fragment comprises substitutions V26W and E16K; or the CH1 fragment comprises substitutions L11W and K96E and the Cκ fragment comprises substitutions V26W and E16R.

[0011] In one embodiment, provided is an antibody or antigen-binding fragment thereof, comprising a Cκ/CH1 pair, wherein the Cκ and CH1 fragments comprise amino acid residues selected from the group consisting of: (a) 26W in Cκ and 11K and 28N in CH1; (b) 11W and

26G in Cκ and 11W in CH1; (c) 26W in Cκ and 11W in CH1; (d) 17R in Cκ and 9D in CH1; (e) 17K in Cκ and 9D in CH1; and combinations thereof.

[0012] In some embodiments, the antibody or antigen-binding fragment thereof further comprises a second Cκ/CH1 pair. The second Cκ/CH1 pair can be wild-type or having a mutation group. The mutation group can be the same as in the first Cκ/CH1 pair but is preferable different such that there will not be mismatch between the pairs.

[0013] Another embodiment of the present disclosure provides an antibody or antigen-binding fragment thereof, comprising a Cκ domain comprising an amino acid modification at position V26 and/or F11, and a CH1 domain comprising an amino acid modification at position Leu11, wherein the modified amino acids interact with each other when the Cκ domain pairs with the CH1 domain. In some embodiments, the antibody or antigen-binding fragment thereof of claim 8, wherein the Cκ domain does not interact with a wild-type CH1 domain and the CH1 domain does not interact with a wild-type Cκ domain. In some embodiments, the modified amino acids are selected from Table 1.

[0014] Another embodiment provides an antibody or antigen-binding fragment thereof, comprising a Cκ domain comprising an amino acid modification at position Q17, and a CH1 domain comprising an amino acid modification at position F9, wherein the modified amino acids interact with each other when the Cκ domain pairs with the CH1 domain. In some embodiments, the Cκ domain does not interact with a wild-type CH1 domain and the CH1 domain does not interact with a wild-type Cκ domain. In some embodiments, the modified amino acids are selected from Table 2.

[0015] Also provided, in some embodiments, is a bispecific antibody comprising a first Cκ/CH1 pair and a second Cκ/CH1 pair, wherein the Cκ and CH1 fragments of the first pair comprise amino acid residues selected from the group consisting of: (a) 26W in Cκ and 11K and 28N in CH1; (b) 11W and 26G in Cκ and 11W in CH1; (c) 26W in Cκ and 11W in CH1; (d) 17R in Cκ and 9D in CH1; (e) 17K in Cκ and 9D in CH1; and combinations thereof, and the Cκ and CH1 fragments of the second pair are wild-type or comprise a different set of amino acid residues selected from (a)-(e).

BRIEF DESCRIPTION OF THE DRAWINGS

[0016] FIG. 1 shows the crystal structure of a pair of Cκ and CH1 domains (from 1CZ8) showing their interactions (the residues involved in hydrogen bond are colored in pink; salt bridge in yellow; hydrophobic interaction residues are sticks colored in blue or green).

[0017] FIG. 2 shows a few residues in the Cκ and CH1 domain that may be important for maintaining the interaction between the domains

[0018] FIG. 3 presents the picture of a reduced SDS-PAGE gel for ala/trp mutations for different interaction amino acid pairs.

[0019] FIG. 4A-4D show the pictures of reduced SDS-PAGE gels for various mutation pair analyzed in Example 3.

[0020] FIG. 5A-B present pcitures of reduced SDS-PAGE (5A) and non-reduced SDS-PAGE (5B) gels showing the binding between Cκ and CH1 domains.

[0021] FIG. 6A-C present gel images showing the binding between antibody heavy and light chains, some of which included mutations.

[0022] FIG. 7A-D illustrate the structures of a variaty of bispecific antibodies.

[0023] FIG. 8A-B present data to show the binding and functional potency of the tested bispecific antibodies to their respective binding targets.

DETAILED DESCRIPTION

Definitions

[0024] It is to be noted that the term "a" or "an" entity refers to one or more of that entity; for example, "an antibody," is understood to represent one or more antibodies. As such, the terms "a" (or "an"), "one or more," and "at least one" can be used interchangeably herein.

[0025] As used herein, the term "polypeptide" is intended to encompass a singular "polypeptide" as well as plural "polypeptides," and refers to a molecule composed of monomers (amino acids) linearly linked by amide bonds (also known as peptide bonds). The term "polypeptide" refers to any chain or chains of two or more amino acids, and does not

refer to a specific length of the product. Thus, peptides, dipeptides, tripeptides, oligopeptides, "protein," "amino acid chain," or any other term used to refer to a chain or chains of two or more amino acids, are included within the definition of "polypeptide," and the term "polypeptide" may be used instead of, or interchangeably with any of these terms. The term "polypeptide" is also intended to refer to the products of post-expression modifications of the polypeptide, including without limitation glycosylation, acetylation, phosphorylation, amidation, derivatization by known protecting/blocking groups, proteolytic cleavage, or modification by non- naturally occurring amino acids. A polypeptide may be derived from a natural biological source or produced by recombinant technology, but is not necessarily translated from a designated nucleic acid sequence. It may be generated in any manner, including by chemical synthesis.

[0026] The term "isolated" as used herein with respect to cells, nucleic acids, such as DNA or RNA, refers to molecules separated from other DNAs or RNAs, respectively, that are present in the natural source of the macromolecule. The term "isolated" as used herein also refers to a nucleic acid or peptide that is substantially free of cellular material, viral material, or culture medium when produced by recombinant DNA techniques, or chemical precursors or other chemicals when chemically synthesized. Moreover, an "isolated nucleic acid" is meant to include nucleic acid fragments which are not naturally occurring as fragments and would not be found in the natural state. The term "isolated" is also used herein to refer to cells or polypeptides which are isolated from other cellular proteins or tissues. Isolated polypeptides is meant to encompass both purified and recombinant polypeptides.

[0027] As used herein, the term "recombinant" as it pertains to polypeptides or polynucleotides intends a form of the polypeptide or polynucleotide that does not exist naturally, a non-limiting example of which can be created by combining polynucleotides or polypeptides that would not normally occur together.

[0028] "Homology" or "identity" or "similarity" refers to sequence similarity between two peptides or between two nucleic acid molecules. Homology can be determined by comparing a position in each sequence which may be aligned for purposes of comparison. When a position in the compared sequence is occupied by the same base or amino acid, then the molecules are homologous at that position. A degree of homology between sequences is a function of the number of matching or homologous positions shared by the sequences. An

"unrelated" or "non-homologous" sequence shares less than 40% identity, though preferably less than 25% identity, with one of the sequences of the present disclosure.

[0029] A polynucleotide or polynucleotide region (or a polypeptide or polypeptide region) has a certain percentage (for example, 60 %, 65 %, 70 %, 75 %, 80 %, 85 %, 90 %, 95 %, 98 % or 99 %) of "sequence identity" to another sequence means that, when aligned, that percentage of bases (or amino acids) are the same in comparing the two sequences. This alignment and the percent homology or sequence identity can be determined using software programs known in the art, for example those described in Ausubel *et al.* eds. (2007) Current Protocols in Molecular Biology. Preferably, default parameters are used for alignment. One alignment program is BLAST, using default parameters. In particular, programs are BLASTN and BLASTP, using the following default parameters: Genetic code = standard; filter = none; strand = both; cutoff = 60; expect = 10; Matrix = BLOSUM62; Descriptions = 50 sequences; sort by = HIGH SCORE; Databases = non-redundant, GenBank + EMBL + DDBJ + PDB + GenBank CDS translations + SwissProtein + SPupdate + PIR. Biologically equivalent polynucleotides are those having the above-noted specified percent homology and encoding a polypeptide having the same or similar biological activity.

[0030] The term "an equivalent nucleic acid or polynucleotide" refers to a nucleic acid having a nucleotide sequence having a certain degree of homology, or sequence identity, with the nucleotide sequence of the nucleic acid or complement thereof. A homolog of a double stranded nucleic acid is intended to include nucleic acids having a nucleotide sequence which has a certain degree of homology with or with the complement thereof. In one aspect, homologs of nucleic acids are capable of hybridizing to the nucleic acid or complement thereof. Likewise, "an equivalent polypeptide" refers to a polypeptide having a certain degree of homology, or sequence identity, with the amino acid sequence of a reference polypeptide. In some aspects, the sequence identity is at least about 70%, 75%, 80%, 85%, 90%, 95%, 98%, or 99%. In some aspects, the equivalent polypeptide or polynucleotide has one, two, three, four or five addition, deletion, substitution and their combinations thereof as compared to the reference polypeptide or polynucleotide. In some aspects, the equivalent sequence retains the activity (*e.g.*, epitope-binding) or structure (*e.g.*, salt-bridge) of the reference sequence.

[0031] Hybridization reactions can be performed under conditions of different "stringency". In general, a low stringency hybridization reaction is carried out at about 40°C in about 10 x

SSC or a solution of equivalent ionic strength/temperature. A moderate stringency hybridization is typically performed at about 50°C in about 6 x SSC, and a high stringency hybridization reaction is generally performed at about 60°C in about 1 x SSC. Hybridization reactions can also be performed under "physiological conditions" which is well known to one of skill in the art. A non-limiting example of a physiological condition is the temperature, ionic strength, pH and concentration of Mg^{2+} normally found in a cell.

[0032] A polynucleotide is composed of a specific sequence of four nucleotide bases: adenine (A); cytosine (C); guanine (G); thymine (T); and uracil (U) for thymine when the polynucleotide is RNA. Thus, the term "polynucleotide sequence" is the alphabetical representation of a polynucleotide molecule. This alphabetical representation can be input into databases in a computer having a central processing unit and used for bioinformatics applications such as functional genomics and homology searching. The term "polymorphism" refers to the coexistence of more than one form of a gene or portion thereof. A portion of a gene of which there are at least two different forms, i.e., two different nucleotide sequences, is referred to as a "polymorphic region of a gene". A polymorphic region can be a single nucleotide, the identity of which differs in different alleles.

[0033] The terms "polynucleotide" and "oligonucleotide" are used interchangeably and refer to a polymeric form of nucleotides of any length, either deoxyribonucleotides or ribonucleotides or analogs thereof. Polynucleotides can have any three-dimensional structure and may perform any function, known or unknown. The following are non-limiting examples of polynucleotides: a gene or gene fragment (for example, a probe, primer, EST or SAGE tag), exons, introns, messenger RNA (mRNA), transfer RNA, ribosomal RNA, ribozymes, cDNA, dsRNA, siRNA, miRNA, recombinant polynucleotides, branched polynucleotides, plasmids, vectors, isolated DNA of any sequence, isolated RNA of any sequence, nucleic acid probes and primers. A polynucleotide can comprise modified nucleotides, such as methylated nucleotides and nucleotide analogs. If present, modifications to the nucleotide structure can be imparted before or after assembly of the polynucleotide. The sequence of nucleotides can be interrupted by non-nucleotide components. A polynucleotide can be further modified after polymerization, such as by conjugation with a labeling component. The term also refers to both double- and single-stranded molecules. Unless otherwise specified or required, any embodiment of this disclosure that is a

polynucleotide encompasses both the double-stranded form and each of two complementary single-stranded forms known or predicted to make up the double-stranded form.

[0034] The term "encode" as it is applied to polynucleotides refers to a polynucleotide which is said to "encode" a polypeptide if, in its native state or when manipulated by methods well known to those skilled in the art, it can be transcribed and/or translated to produce the mRNA for the polypeptide and/or a fragment thereof. The antisense strand is the complement of such a nucleic acid, and the encoding sequence can be deduced therefrom.

[0035] As used herein, an "antibody" or "antigen-binding polypeptide" refers to a polypeptide or a polypeptide complex that specifically recognizes and binds to an antigen. An antibody can be a whole antibody and any antigen binding fragment or a single chain thereof. Thus the term "antibody" includes any protein or peptide containing molecule that comprises at least a portion of an immunoglobulin molecule having biological activity of binding to the antigen. Examples of such include, but are not limited to a complementarity determining region (CDR) of a heavy or light chain or a ligand binding portion thereof, a heavy chain or light chain variable region, a heavy chain or light chain constant region, a framework (FR) region, or any portion thereof, or at least one portion of a binding protein.

[0036] The terms "antibody fragment" or "antigen-binding fragment", as used herein, is a portion of an antibody such as $F(ab')_2$, $F(ab)_2$, Fab', Fab, Fv, scFv and the like. Regardless of structure, an antibody fragment binds with the same antigen that is recognized by the intact antibody. The term "antibody fragment" includes aptamers, spiegelmers, and diabodies. The term "antibody fragment" also includes any synthetic or genetically engineered protein that acts like an antibody by binding to a specific antigen to form a complex.

[0037] A "single-chain variable fragment" or "scFv" refers to a fusion protein of the variable regions of the heavy (V_H) and light chains (V_L) of immunoglobulins. In some aspects, the regions are connected with a short linker peptide of ten to about 25 amino acids. The linker can be rich in glycine for flexibility, as well as serine or threonine for solubility, and can either connect the N-terminus of the V_H with the C-terminus of the V_L, or vice versa. This protein retains the specificity of the original immunoglobulin, despite removal of the constant regions and the introduction of the linker. ScFv molecules are known in the art and are described, *e.g.*, in US patent 5,892,019.

[0038] The term antibody encompasses various broad classes of polypeptides that can be distinguished biochemically. Those skilled in the art will appreciate that heavy chains are classified as gamma, mu, alpha, delta, or epsilon (γ, μ, α, δ, ε) with some subclasses among them (*e.g.*, γ l- γ4). It is the nature of this chain that determines the "class" of the antibody as IgG, IgM, IgA IgG, or IgE, respectively. The immunoglobulin subclasses (isotypes) *e.g.*, IgG_1, IgG_2, IgG_3, IgG_4, IgG_5, etc. are well characterized and are known to confer functional specialization. Modified versions of each of these classes and isotypes are readily discernable to the skilled artisan in view of the instant disclosure and, accordingly, are within the scope of the instant disclosure. All immunoglobulin classes are clearly within the scope of the present disclosure, the following discussion will generally be directed to the IgG class of immunoglobulin molecules. With regard to IgG, a standard immunoglobulin molecule comprises two identical light chain polypeptides of molecular weight approximately 23,000 Daltons, and two identical heavy chain polypeptides of molecular weight 53,000-70,000. The four chains are typically joined by disulfide bonds in a "Y" configuration wherein the light chains bracket the heavy chains starting at the mouth of the "Y" and continuing through the variable region.

[0039] Antibodies, antigen-binding polypeptides, variants, or derivatives thereof of the disclosure include, but are not limited to, polyclonal, monoclonal, multispecific, human, humanized, primatized, or chimeric antibodies, single chain antibodies, epitope-binding fragments, *e.g.*, Fab, Fab' and $F(ab')_2$, Fd, Fvs, single-chain Fvs (scFv), single-chain antibodies, disulfide-linked Fvs (sdFv), fragments comprising either a VK or VH domain, fragments produced by a Fab expression library, and anti- idiotypic (anti-Id) antibodies (including, *e.g.*, anti-Id antibodies to LIGHT antibodies disclosed herein). Immunoglobulin or antibody molecules of the disclosure can be of any type (*e.g.*, IgG, IgE, IgM, IgD, IgA, and IgY), class (e.g., IgGl, IgG2, IgG3, IgG4, IgAl and IgA2) or subclass of immunoglobulin molecule.

[0040] Light chains are classified as either kappa or lambda (K, λ). Each heavy chain class may be bound with either a kappa or lambda light chain. In general, the light and heavy chains are covalently bonded to each other, and the "tail" portions of the two heavy chains are bonded to each other by covalent disulfide linkages or non-covalent linkages when the immunoglobulins are generated either by hybridomas, B cells or genetically engineered host

cells. In the heavy chain, the amino acid sequences run from an N-terminus at the forked ends of the Y configuration to the C-terminus at the bottom of each chain.

[0041] Both the light and heavy chains are divided into regions of structural and functional homology. The terms "constant" and "variable" are used functionally. In this regard, it will be appreciated that the variable domains of both the light (VK) and heavy (VH) chain portions determine antigen recognition and specificity. Conversely, the constant domains of the light chain (CK) and the heavy chain (CH1, CH2 or CH3) confer important biological properties such as secretion, transplacental mobility, Fc receptor binding, complement binding, and the like. By convention the numbering of the constant region domains increases as they become more distal from the antigen-binding site or amino- terminus of the antibody. The N-terminal portion is a variable region and at the C-terminal portion is a constant region; the CH3 and CK domains actually comprise the carboxy-terminus of the heavy and light chain, respectively.

[0042] As indicated above, the variable region allows the antibody to selectively recognize and specifically bind epitopes on antigens. That is, the VK domain and VH domain, or subset of the complementarity determining regions (CDRs), of an antibody combine to form the variable region that defines a three dimensional antigen-binding site. This quaternary antibody structure forms the antigen-binding site present at the end of each arm of the Y. More specifically, the antigen-binding site is defined by three CDRs on each of the VH and VK chains (*i.e.* CDR-H1, CDR-H2, CDR-H3, CDR-L1, CDR-L2 and CDR-L3). In some instances, *e.g.*, certain immunoglobulin molecules derived from camelid species or engineered based on camelid immunoglobulins, a complete immunoglobulin molecule may consist of heavy chains only, with no light chains. *See, e.g.*, Hamers-Casterman *et al.*, *Nature* 363:446-448 (1993).

[0043] In naturally occurring antibodies, the six "complementarity determining regions" or "CDRs" present in each antigen-binding domain are short, non-contiguous sequences of amino acids that are specifically positioned to form the antigen-binding domain as the antibody assumes its three dimensional configuration in an aqueous environment. The remainder of the amino acids in the antigen-binding domains, referred to as "framework" regions, show less inter-molecular variability. The framework regions largely adopt a β-sheet conformation and the CDRs form loops which connect, and in some cases form part of, the β -sheet structure. Thus, framework regions act to form a scaffold that provides for positioning

the CDRs in correct orientation by inter-chain, non-covalent interactions. The antigen-binding domain formed by the positioned CDRs defines a surface complementary to the epitope on the immunoreactive antigen. This complementary surface promotes the non-covalent binding of the antibody to its cognate epitope. The amino acids comprising the CDRs and the framework regions, respectively, can be readily identified for any given heavy or light chain variable region by one of ordinary skill in the art, since they have been precisely defined (*see* "Sequences of Proteins of Immunological Interest," Kabat, E., *et al.*, U.S. Department of Health and Human Services, (1983); and Chothia and Lesk, *J. MoI. Biol.*, 196:901-917 (1987)).

[0044] In the case where there are two or more definitions of a term which is used and/or accepted within the art, the definition of the term as used herein is intended to include all such meanings unless explicitly stated to the contrary. A specific example is the use of the term "complementarity determining region" ("CDR") to describe the non-contiguous antigen combining sites found within the variable region of both heavy and light chain polypeptides. This particular region has been described by Kabat *et al.*, U.S. Dept. of Health and Human Services, "Sequences of Proteins of Immunological Interest" (1983) and by Chothia *et al.*, *J. MoI. Biol.* 196:901-917 (1987), which are incorporated herein by reference in their entireties. The CDR definitions according to Kabat and Chothia include overlapping or subsets of amino acid residues when compared against each other. Nevertheless, application of either definition to refer to a CDR of an antibody or variants thereof is intended to be within the scope of the term as defined and used herein. The appropriate amino acid residues which encompass the CDRs as defined by each of the above cited references are set forth in the table below as a comparison. The exact residue numbers which encompass a particular CDR will vary depending on the sequence and size of the CDR. Those skilled in the art can routinely determine which residues comprise a particular CDR given the variable region amino acid sequence of the antibody.

	Kabat	**Chothia**
CDR-H1	31-35	26-32
CDR-H2	50-65	52-58
CDR-H3	95-102	95-102
CDR-L1	24-34	26-32
CDR-L2	50-56	50-52
CDR-L3	89-97	91-96

[0045] Kabat *et al.* also defined a numbering system for variable domain sequences that is applicable to any antibody. One of ordinary skill in the art can unambiguously assign this system of "Kabat numbering" to any variable domain sequence, without reliance on any experimental data beyond the sequence itself. As used herein, "Kabat numbering" refers to the numbering system set forth by Kabat *et al.*, U.S. Dept. of Health and Human Services, "Sequence of Proteins of Immunological Interest" (1983).

[0046] In addition to table above, the Kabat number system describes the CDR regions as follows: CDR-H1 begins at approximately amino acid 31 (*i.e.*, approximately 9 residues after the first cysteine residue), includes approximately 5-7 amino acids, and ends at the next tryptophan residue. CDR-H2 begins at the fifteenth residue after the end of CDR-H1, includes approximately 16-19 amino acids, and ends at the next arginine or lysine residue. CDR-H3 begins at approximately the thirty third amino acid residue after the end of CDR-H2; includes 3-25 amino acids; and ends at the sequence W-G-X-G, where X is any amino acid. CDR-L1 begins at approximately residue 24 (*i.e.*, following a cysteine residue); includes approximately 10-17 residues; and ends at the next tryptophan residue. CDR-L2 begins at approximately the sixteenth residue after the end of CDR-L1 and includes approximately 7 residues. CDR-L3 begins at approximately the thirty third residue after the end of CDR-L2 (*i.e.*, following a cysteine residue); includes approximately 7-11 residues and ends at the sequence F or W-G-X-G, where X is any amino acid.

[0047] Some other numbering systems include "IMGT numbering" and "IMGT exon numbering. For example, for constant domains CH1 and Cκ, the following table shows the correlation between the IMGT exon numbering system and the Kabat numbering system.

IMGT exon numbering and Kabat numbering for CH1

IMGT exon numbering	Kabat numbering	IMGT exon numbering	Kabat numbering	IMGT exon numbering	Kabat numbering
1	114	41	157	81	206
2	115	42	162	82	207
3	116	43	163	83	208
4	117	44	164	84	209
5	118	45	165	85	210
6	119	46	166	86	211
7	120	47	167	87	212
8	121	48	168	88	213
9	122	49	169	89	214
10	123	50	171	90	215
11	124	51	172	91	216
12	125	52	173	92	217
13	126	53	174	93	218
14	127	54	175	94	219
15	128	55	176	95	220
16	129	56	177	96	221
17	130	57	178	97	222
18	133	58	179	98	223
19	134	59	180		
20	135	60	182		
21	136	61	183		
22	137	62	184		
23	138	63	185		
24	139	64	186		
25	140	65	187		
26	141	66	188		
27	142	67	189		
28	143	68	190		
29	144	69	191		
30	145	70	192		
31	146	71	193		
32	147	72	194		
33	148	73	195		
34	149	74	196		
35	150	75	197		
36	151	76	198		
37	152	77	199		
38	153	78	200		
39	154	79	203		
40	156	80	205		

IMGT exon numbering and Kabat numbering for Cκ

IMGT exon numbering	Kabat numbering	IMGT exon numbering	Kabat numbering	IMGT exon numbering	Kabat numbering
1	108	41	148	81	188
2	109	42	149	82	189
3	110	43	150	83	190
4	111	44	151	84	191
5	112	45	152	85	192
6	113	46	153	86	193
7	114	47	154	87	194
8	115	48	155	88	195
9	116	49	156	89	196
10	117	50	157	90	197
11	118	51	158	91	198
12	119	52	159	92	199
13	120	53	160	93	200
14	121	54	161	94	201
15	122	55	162	95	202
16	123	56	163	96	203
17	124	57	164	97	204
18	125	58	165	98	205
19	126	59	166	99	206
20	127	60	167	100	207
21	128	61	168	101	208
22	129	62	169	102	209
23	130	63	170	103	210
24	131	64	171	104	211
25	132	65	172	105	212
26	133	66	173	106	213
27	134	67	174	107	214
28	135	68	175		
29	136	69	176		
30	137	70	177		
31	138	71	178		
32	139	72	179		
33	140	73	180		
34	141	74	181		
35	142	75	182		
36	143	76	183		
37	144	77	184		
38	145	78	185		
39	146	79	186		
40	147	80	187		

[0048] Antibodies disclosed herein may be from any animal origin including birds and mammals. Preferably, the antibodies are human, murine, donkey, rabbit, goat, guinea pig, camel, llama, horse, or chicken antibodies. In another embodiment, the variable region may be condricthoid in origin (*e.g.*, from sharks).

[0049] As used herein, the term "heavy chain constant region" includes amino acid sequences derived from an immunoglobulin heavy chain. A polypeptide comprising a heavy chain constant region comprises at least one of: a CH1 domain, a hinge (*e.g.*, upper, middle, and/or lower hinge region) domain, a CH2 domain, a CH3 domain, or a variant or fragment thereof. For example, an antigen-binding polypeptide for use in the disclosure may comprise a polypeptide chain comprising a CH1 domain; a polypeptide chain comprising a CH1 domain, at least a portion of a hinge domain, and a CH2 domain; a polypeptide chain comprising a CH1 domain and a CH3 domain; a polypeptide chain comprising a CH1 domain, at least a portion of a hinge domain, and a CH3 domain, or a polypeptide chain comprising a CH1 domain, at least a portion of a hinge domain, a CH2 domain, and a CH3 domain. In another embodiment, a polypeptide of the disclosure comprises a polypeptide chain comprising a CH3 domain. Further, an antibody for use in the disclosure may lack at least a portion of a CH2 domain (*e.g.*, all or part of a CH2 domain). As set forth above, it will be understood by one of ordinary skill in the art that the heavy chain constant region may be modified such that they vary in amino acid sequence from the naturally occurring immunoglobulin molecule.

[0050] The heavy chain constant region of an antibody disclosed herein may be derived from different immunoglobulin molecules. For example, a heavy chain constant region of a polypeptide may comprise a CH1 domain derived from an IgG_1 molecule and a hinge region derived from an IgG_3 molecule. In another example, a heavy chain constant region can comprise a hinge region derived, in part, from an IgG_1 molecule and, in part, from an IgG_3 molecule. In another example, a heavy chain portion can comprise a chimeric hinge derived, in part, from an IgG_1 molecule and, in part, from an IgG_4 molecule.

[0051] As used herein, the term "light chain constant region" includes amino acid sequences derived from antibody light chain. Preferably, the light chain constant region comprises at least one of a constant kappa domain or constant lambda domain.

[0052] A "light chain-heavy chain pair" refers to the collection of a light chain and heavy chain that can form a dimer through a disulfide bond between the CL domain of the light chain and the CH1 domain of the heavy chain.

[0053] As previously indicated, the subunit structures and three dimensional configuration of the constant regions of the various immunoglobulin classes are well known. As used herein,

the term "VH domain" includes the amino terminal variable domain of an immunoglobulin heavy chain and the term "CH1 domain" includes the first (most amino terminal) constant region domain of an immunoglobulin heavy chain. The CH1 domain is adjacent to the VH domain and is amino terminal to the hinge region of an immunoglobulin heavy chain molecule.

[0054] As used herein the term "CH2 domain" includes the portion of a heavy chain molecule that extends, *e.g.*, from about residue 244 to residue 360 of an antibody using conventional numbering schemes (residues 244 to 360, Kabat numbering system; and residues 231-340, EU numbering system; *see* Kabat *et al.*, U.S. Dept. of Health and Human Services, "Sequences of Proteins of Immunological Interest" (1983). The CH2 domain is unique in that it is not closely paired with another domain. Rather, two N-linked branched carbohydrate chains are interposed between the two CH2 domains of an intact native IgG molecule. It is also well documented that the CH3 domain extends from the CH2 domain to the C-terminal of the IgG molecule and comprises approximately 108 residues.

[0055] As used herein, the term "hinge region" includes the portion of a heavy chain molecule that joins the CH1 domain to the CH2 domain. This hinge region comprises approximately 25 residues and is flexible, thus allowing the two N-terminal antigen-binding regions to move independently. Hinge regions can be subdivided into three distinct domains: upper, middle, and lower hinge domains (Roux *et al.*, *J. Immunol* 161:4083 (1998)).

[0056] As used herein the term "disulfide bond" includes the covalent bond formed between two sulfur atoms. The amino acid cysteine comprises a thiol group that can form a disulfide bond or bridge with a second thiol group. In most naturally occurring IgG molecules, the CH1 and CK regions are linked by a disulfide bond and the two heavy chains are linked by two disulfide bonds at positions corresponding to 239 and 242 using the Kabat numbering system (position 226 or 229, EU numbering system).

[0057] As used herein, the term "chimeric antibody" will be held to mean any antibody wherein the immunoreactive region or site is obtained or derived from a first species and the constant region (which may be intact, partial or modified in accordance with the instant disclosure) is obtained from a second species. In certain embodiments the target binding region or site will be from a non-human source (*e.g.* mouse or primate) and the constant region is human.

[0058] As used herein, "percent humanization" is calculated by determining the number of framework amino acid differences (*i.e.*, non-CDR difference) between the humanized domain and the germline domain, subtracting that number from the total number of amino acids, and then dividing that by the total number of amino acids and multiplying by 100.

[0059] By "specifically binds" or "has specificity to," it is generally meant that an antibody binds to an epitope via its antigen-binding domain, and that the binding entails some complementarity between the antigen-binding domain and the epitope. According to this definition, an antibody is said to "specifically bind" to an epitope when it binds to that epitope, via its antigen-binding domain more readily than it would bind to a random, unrelated epitope. The term "specificity" is used herein to qualify the relative affinity by which a certain antibody binds to a certain epitope. For example, antibody "A" may be deemed to have a higher specificity for a given epitope than antibody "B," or antibody "A" may be said to bind to epitope "C" with a higher specificity than it has for related epitope "D."

Modified Cκ and CH1 domains

[0060] Bispecific antibodies (BsAbs), which target two antigens or epitopes, incorporate the specificities and properties of two distinct monoclonal antibodies (mAbs) into a single molecule. Mispairing may occur when there are two sets of paired VH-Ch1:VL-CL fragments. To avoid the mispairing of VH-CH1:VL-CL fragments derived from two distinct antibodies, a lot of methods have been used such as, Cross-Mab, common light chain, and FITIg.

[0061] An objective of the experimental examples was to introduce mutations into the Cκ and/or CH1 domain, in particular the human domains, to reduce mispairing. Preferably, the mutant Cκ can show good binding to the mutant CH1, but the mutant Cκ does not bind or has weak binding to the non-mutated CH1 domain and the mutant CH1 shows weak or no binding to the non-mutated Cκ.

[0062] First, important interface residues of human Cκ and CH1 were analyzed and five hotspots were discovered. To confirm the importance of these residues, mutations of each residue to alanine or tryptophan were prepared. Mutations at Gln17 of Cκ (Cκ_Q17) or Phe9 of CH1 (CH1_F9), and mutations at Val26 or Phe11 of Cκ (Cκ_V26_F11) or Leu11 of CH1

(CH1_L11) resulted in much decreased pairing of the light and heavy chains. These results confirmed that the groups Cκ_Q17/CH1_F9 (referred to as pair 1 in the examples) and Cκ_V26_F11/CH1_L11 (referred to as pair 2 in the examples) were important for the interaction of Cκ and CH1. Subsequently, mutations that could potentially restore the pairing were expressed and analyzed. Such modifications can be particularly useful for preparing bispecific antibodies which two different pairs of Cκ and CH1 domains.

[0063] For interface residues Cκ_V26_F11/CH1_L11 (and optionally L28), the following mutations are shown or contemplated to be able to restore the pairing of the Cκ and CH1 domains:

Table 1. Mutation Groups of Cκ at 26 and optionally at 11 with CH1 at 11 and optionally at 28

No.	Cκ (at 26 and/or 11)	CH1 (at 11 and/or 28)
1	26W	11W
2	26W	11K_and 28N
3	11W and 26G	11W
4	11W and 26G	11K and 28N
5	26F	11F
6	26W	11F
7	26F	11W
8	26L	11W
9	26M	11W
10	26E	11W
11	26W	11W and 28R
12	11A and 26W	11W

[0064] Likewise, for interface residues Cκ_Q17/CH1_F9, the following mutations are shown or contemplated to be able to restore the pairing of the Cκ and CH1 domains:

Table 2. Mutation Groups at Cκ 17/CH1 9

No.	Cκ (at 17)	CH1 (at 9)
1	17R	9D
2	17K	9D
3	17R	9E
4	17K	9E
5	17D	9R
6	17D	9K
7	17H	9I
8	17R	9H
9	17H	9H
10	17R	9P
11	17D	9H
12	17I	9H

13	17H	9M
14	17R	9Q
15	17H	9Q

[0065] As shown in Example 7, additional amino acid substitutions that disrupt one or more existing salt bridges in wild-type Cκ and CH1 domains and reestablish new ones can further improve the desired pairing specificity. The wild-type Cκ/CH1 pairs have salt bridges between CH1_K96 and Cκ_E16, between CH1_K101 and Cκ_D15, and between CH1_H51 and Cκ_D60. Each of these salt bridges can be suitable sites for substitutions.

[0066] For instance, in each of the salt bridges, the positively charged amino acid (e.g., K, R or H) can be substituted with a negatively charged amino acid (e.g., E or D), and the negatively amino acid (e.g., E or D) can be substituted with a positively charged amino acid (e.g., K, R, or H). One such example is CH1_K101E/Cκ_D15K or Cκ_D15H; another example is CH1_K96D/Cκ_E16R; another example is CH1_96E/Cκ_E16K; and another example is CH1_H51D/Cκ_D60K. These and other examples are illustrated in **Table 3**. Each of such substituted salt bridges can be used independently to prepare the new CH1/Cκ pairing, or in addition to any of the other substitutions described in the present disclosure.

Table 3. Disrupted and Reestablished Salt Bridges

No.	CH1	Cκ
1	K101E	D15H
2	K101E	D15K
3	K101E	D15R
4	K101D	D15H
5	K101D	D15K
6	K101D	D15R
7	K96D	E16R
8	K96E	E16K
9	K96D	E16K
10	K96E	E16R
11	K96D	E16H
12	K96E	E16H
13	H51D	D60K
14	H51D	D60R
15	H51D	D60H
16	H51E	D60K
17	H51E	D60R
16	H51E	D60H

[0067] In one embodiment, a disclosed antibody or antigen-binding fragment thereof includes a CH1 fragment having substitutions L11W and K101E and a Cκ fragment having substitutions V26W and D15K/H. In one embodiment, a disclosed antibody or antigen-

binding fragment thereof includes a CH1 fragment having substitutions L11W and K96D and a Cκ fragment having substitutions V26W and E16R. In one embodiment, a disclosed antibody or antigen-binding fragment thereof includes a CH1 fragment having substitutions L11W and K96E and a Cκ fragment having substitutions V26W and E16K.

[0068] These mutation groups can be useful for making mutated Cκ and CH1 domains that are able to bind each other, which cannot bind or have reduced binding to their wild type counterpart CH1 or Cκ domains. Such Cκ and CH1 domains can be incorporated into antibodies or antigen-binding fragments, in particular bispecific ones.

[0069] In one scenario, a bispecific antibody has a normal IgG structure which includes two light chain-heavy chain pairs. Each heavy chain includes a VH, CH1, CH2 and CH3 domains, and each light chain includes a VL and a CL (e.g., Cκ) domain. In accordance with one embodiment of the present disclosure, one of the Cκ/CH1 pairs includes a mutation group of the present disclosure and the other pair does not. In another embodiment, one of the Cκ/CH1 pairs includes a mutation group of the present disclosure and the other pair includes a different mutation group. In some embodiment, either of both of the pairs include two or more mutation groups (e.g., one group from Table 1 and another group from Table 2).

[0070] In another scenario, a bispecific antibody has a normal IgG structure which further is fused, at the C-terminus of the Fc fragment, to the N-termini of the VH's of a second Fab fragment. Such an antibody is illustrated in **FIG. 7A**. In accordance with one embodiment of the present disclosure, either of the Cκ/CH1 pairs at the N-terminal side of the Fc fragment or the Cκ/CH1 pairs at the C-terminal side of the Fc fragment includes a mutation group of the present disclosure and the other pairs do not. Furthermore, the mutation group can be included in both Cκ/CH1 pairs at the N or C-terminal side of the Fc fragment.

[0071] Yet in another embodiment, the bispecific antibody has a structure as illustrated in **FIG. 7B**. In this structure, each heavy chain and light chain includes two sets of concatenated Cκ/CH1 pairs. The mutation groups can be placed anywhere in this antibody so long as they favor the desired pairing. Another bispecific antibody, with a known knob-into-hole in the CH3 domains, is illustrated in **FIG. 7C**. Here, the mutation groups of the present disclosure can be inserted to either or both of the A and B Cκ/CH1 pairs. Yet other examples are illustrated in **FIG. 7D** which do not have CH2 or CH3 domains.

[0072] In one embodiment, the present disclosure provides an antibody or antigen-binding fragment thereof which includes a human Cκ/CH1 pair, wherein amino acid residue 26 of the Cκ domain is Trp and amino acid residue 11 of the CH1 domain is Trp. In some aspects, the antibody or antigen-binding fragment thereof further includes a second human Cκ/CH1 pair, wherein amino acid residue 26 of the second Cκ domain is not Trp and amino acid residue 11 of the second CH1 domain is not Trp. In some aspects, the antibody or antigen-binding fragment thereof further includes a heavy chain variable region, a light chain variable region, an Fc region, or the combination thereof.

[0073] In another embodiment, the present disclosure provides an antibody or antigen-binding fragment thereof, comprising a human Cκ domain comprising an amino acid modification at position Val26 and/or Phe11, and a human CH1 domain comprising an amino acid modification at position Leu11, wherein the modified amino acids interact with each other when the Cκ domain pairs with the CH1 domain. The amino modification, in some embodiments, is as compared to human IgG Cκ and CH1 domains. In some embodiments, the modified amino acids are selected from **Table 1.**

[0074] In some embodiments, the antibody or antigen-binding fragment thereof further includes a second Cκ/CH1 pair, wherein amino acid residue 26 of the second Cκ domain is Val and amino acid residue 11 of the second CH1 domain is Leu. In some aspects, amino acid residue 11 of the second Cκ domain is Phe.

[0075] In another embodiment, the present disclosure provides an antibody or antigen-binding fragment thereof, comprising a Cκ domain comprising an amino acid modification at position Gln17, and a CH1 domain comprising an amino acid modification at position Phe9, wherein the modified amino acids interact with each other when the Cκ domain pairs with the CH1 domain. The amino modification, in some embodiment, is as compared to human IgG Cκ and CH1 domains. In some embodiments, the modified amino acids are selected from **Table 2.**

[0076] In some embodiments, the antibody or antigen-binding fragment thereof further includes a second Cκ/CH1 pair, wherein amino acid residue 17 of the second Cκ domain is Gln and amino acid residue 9 of the second CH1 domain is Phe.

[0077] In some embodiments, the present disclosure provides an antibody or antigen-binding fragment thereof, which includes a mutation group of **Table 1** or a mutation group of **Table 2**. In some embodiments, the antibody or antigen-binding fragment thereof includes a mutation group of **Table 1** and a mutation group of **Table 2**. In some embodiments, the antibody or antigen-binding fragment thereof further includes a mutation group of **Table 3**.

[0078] the antibody or antigen-binding fragment thereof can be of any known class of antibodies, but is preferably of class IgG, including isotypes IgG1, IgG2, IgG3 and IgG4. The antibody or fragment thereof can be a chimeric antibody, a humanized antibody, or a fully human antibody.

Bispecific/Bifunctional Molecules

[0079] Bispecific antibodies are provided in some embodiments. In some embodiments, the bispecific antibody has a first specificity to a tumor antigen or a microorganism. In some embodiments, the bispecific antibody has a second specificity to an immune cell.

[0080] In some embodiments, the immune cell is selected from the group consisting of a T cell, a B cell, a monocyte, a macrophage, a neutrophil, a dendritic cell, a phagocyte, a natural killer cell, an eosinophil, a basophil, and a mast cell. Molecules on the immune cell which can be targeted include, for example, CD3, CD16, CD19, CD28, and CD64. Other examples include PD-1, CTLA-4, LAG-3 (also known as CD223), CD28, CD122, 4-1BB (also known as CD137), TIM3, OX-40 or OX40L, CD40 or CD40L, LIGHT, ICOS/ICOSL, GITR/GITRL, TIGIT, CD27, VISTA, B7H3, B7H4, HEVM or BTLA (also known as CD272), killer-cell immunoglobulin-like receptors (KIRs), and CD47. Specific examples of bispecificity include, without limitation, PD-L1/PD-1, PD-L1/LAG3, PD-L1/TIGIT, and PD-L1/CD47.

[0081] A "tumor antigen" is an antigenic substance produced in tumor cells, *i.e.*, it triggers an immune response in the host. Tumor antigens are useful in identifying tumor cells and are potential candidates for use in cancer therapy. Normal proteins in the body are not antigenic. Certain proteins, however, are produced or overexpressed during tumorigenesis and thus appear "foreign" to the body. This may include normal proteins that are well sequestered from the immune system, proteins that are normally produced in extremely small quantities,

proteins that are normally produced only in certain stages of development, or proteins whose structure is modified due to mutation.

[0082] An abundance of tumor antigens are known in the art and new tumor antigens can be readily identified by screening. Non-limiting examples of tumor antigens include EGFR, Her2, EpCAM, CD20, CD30, CD33, CD47, CD52, CD133, CD73, CEA, gpA33, Mucins, TAG-72, CIX, PSMA, folate-binding protein, GD2, GD3, GM2, VEGF, VEGFR, Integrin, $\alpha V\beta 3$, $\alpha 5\beta 1$, ERBB2, ERBB3, MET, IGF1R, EPHA3, TRAILR1, TRAILR2, RANKL, FAP and Tenascin.

[0083] Bifunctional molecules that include not just antibody or antigen binding fragment are also provided. As a tumor antigen targeting molecule, an antibody or antigen-binding fragment specific to PD-L1, such as those described here, can be combined with an immune cytokine or ligand optionally through a peptide linker. The linked immune cytokines or ligands include, but not limited to, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-10, IL-12, IL-13, IL-15, GM-CSF, TNF-α, CD40L, OX40L, CD27L, CD30L, 4-1BBL, LIGHT and GITRL. Such bi-functional molecules can combine the immune checkpoint blocking effect with tumor site local immune modulation.

Polynucleotides Encoding the Antibodies and Methods of Preparing the Antibodies

[0084] The present disclosure also provides isolated polynucleotides or nucleic acid molecules encoding the antibodies, variants or derivatives thereof of the disclosure. The polynucleotides of the present disclosure may encode the entire heavy and light chain variable regions of the antigen-binding polypeptides, variants or derivatives thereof on the same polynucleotide molecule or on separate polynucleotide molecules. Additionally, the polynucleotides of the present disclosure may encode portions of the heavy and light chain variable regions of the antigen-binding polypeptides, variants or derivatives thereof on the same polynucleotide molecule or on separate polynucleotide molecules.

[0085] Methods of making antibodies are well known in the art and described herein. In certain embodiments, both the variable and constant regions of the antigen-binding polypeptides of the present disclosure are fully human. Fully human antibodies can be made using techniques described in the art and as described herein. For example, fully human antibodies against a specific antigen can be prepared by administering the antigen to a

transgenic animal which has been modified to produce such antibodies in response to antigenic challenge, but whose endogenous loci have been disabled. Exemplary techniques that can be used to make such antibodies are described in U.S. patents: 6,150,584; 6,458,592; 6,420,140 which are incorporated by reference in their entireties.

[0086] In certain embodiments, the prepared antibodies will not elicit a deleterious immune response in the animal to be treated, *e.g.*, in a human. In one embodiment, antigen-binding polypeptides, variants, or derivatives thereof of the disclosure are modified to reduce their immunogenicity using art- recognized techniques. For example, antibodies can be humanized, primatized, deimmunized, or chimeric antibodies can be made. These types of antibodies are derived from a non-human antibody, typically a murine or primate antibody, that retains or substantially retains the antigen-binding properties of the parent antibody, but which is less immunogenic in humans. This may be achieved by various methods, including (a) grafting the entire non-human variable domains onto human constant regions to generate chimeric antibodies; (b) grafting at least a part of one or more of the non-human complementarity determining regions (CDRs) into a human framework and constant regions with or without retention of critical framework residues; or (c) transplanting the entire non-human variable domains, but "cloaking" them with a human-like section by replacement of surface residues. Such methods are disclosed in Morrison *et al.*, *Proc. Natl. Acad. Sci. USA* 57:6851-6855 (1984); Morrison *et al.*, *Adv. Immunol.* 44:65-92 (1988); Verhoeyen *et al.*, *Science* 239:1534-1536 (1988); Padlan, *Molec. Immun.* 25:489-498 (1991); Padlan, *Molec. Immun.* 31:169-217 (1994), and U.S. Pat. Nos.: 5,585,089, 5,693,761, 5,693,762, and 6,190,370, all of which are hereby incorporated by reference in their entirety.

[0087] De-immunization can also be used to decrease the immunogenicity of an antibody. As used herein, the term "de-immunization" includes alteration of an antibody to modify T-cell epitopes (*see, e.g.*, International Application Publication Nos.: WO/9852976 A1 and WO/0034317 A2). For example, variable heavy chain and variable light chain sequences from the starting antibody are analyzed and a human T-cell epitope "map" from each V region showing the location of epitopes in relation to complementarity-determining regions (CDRs) and other key residues within the sequence is created. Individual T-cell epitopes from the T-cell epitope map are analyzed in order to identify alternative amino acid substitutions with a low risk of altering activity of the final antibody. A range of alternative variable heavy and variable light sequences are designed comprising combinations of amino

acid substitutions and these sequences are subsequently incorporated into a range of binding polypeptides. Typically, between 12 and 24 variant antibodies are generated and tested for binding and/or function. Complete heavy and light chain genes comprising modified variable and human constant regions are then cloned into expression vectors and the subsequent plasmids introduced into cell lines for the production of whole antibody. The antibodies are then compared in appropriate biochemical and biological assays, and the optimal variant is identified.

[0088] The binding specificity of antigen-binding polypeptides of the present disclosure can be determined by *in vitro* assays such as immunoprecipitation, radioimmunoassay (RIA) or enzyme-linked immunoabsorbent assay (ELISA).

[0089] Alternatively, techniques described for the production of single-chain units (U.S. Pat. No. 4,694,778; Bird, *Science* 242:423-442 (1988); Huston *et al.*, *Proc. Natl. Acad. Sci. USA* 55:5879- 5883 (1988); and Ward *et al.*, *Nature* 334:544-554 (1989)) can be adapted to produce single-chain units of the present disclosure. Single-chain units are formed by linking the heavy and light chain fragments of the Fv region via an amino acid bridge, resulting in a single-chain fusion peptide. Techniques for the assembly of functional Fv fragments in *E. coli* may also be used (Skerra *et al.*, *Science* 242: 1038-1041 (1988)).

[0090] Examples of techniques which can be used to produce single-chain Fvs (scFvs) and antibodies include those described in U.S. Pat. Nos. 4,946,778 and 5,258,498; Huston *et al.*, *Methods in Enzymology* 203:46-88 (1991); Shu *et al.*, *Proc. Natl. Sci. USA* 90:1995-1999 (1993); and Skerra *et al.*, *Science* 240:1038-1040 (1988). For some uses, including *in vivo* use of antibodies in humans and *in vitro* detection assays, it may be preferable to use chimeric, humanized, or human antibodies. A chimeric antibody is a molecule in which different portions of the antibody are derived from different animal species, such as antibodies having a variable region derived from a murine monoclonal antibody and a human immunoglobulin constant region. Methods for producing chimeric antibodies are known in the art. *See, e.g.*, Morrison, *Science* 229:1202 (1985); Oi *et al.*, *BioTechniques* 4:214 (1986); Gillies *et al.*, *J. Immunol. Methods* 125:191-202 (1989); U.S. Pat. Nos. 5,807,715; 4,816,567; and 4,816397, which are incorporated herein by reference in their entireties.

[0091] Humanized antibodies are antibody molecules derived from a non-human species antibody that bind the desired antigen having one or more complementarity determining

regions (CDRs) from the non-human species and framework regions from a human immunoglobulin molecule. Often, framework residues in the human framework regions will be substituted with the corresponding residue from the CDR donor antibody to alter, preferably improve, antigen-binding. These framework substitutions are identified by methods well known in the art, *e.g.*, by modeling of the interactions of the CDR and framework residues to identify framework residues important for antigen-binding and sequence comparison to identify unusual framework residues at particular positions. (*See, e.g.*, Queen *et al.*, U.S. Pat. No. 5,585,089; Riechmann *et al.*, *Nature* 332:323 (1988), which are incorporated herein by reference in their entireties.) Antibodies can be humanized using a variety of techniques known in the art including, for example, CDR-grafting (EP 239,400; PCT publication WO 91/09967; U.S. Pat. Nos. 5,225,539; 5,530,101; and 5,585,089), veneering or resurfacing (EP 592,106; EP 519,596; Padlan, *Molecular Immunology* 28(4/5):489-498 (1991); Studnicka *et al.*, *Protein Engineering* 7(6):805-814 (1994); Roguska. *et al.*, *Proc. Natl. Sci. USA* 91:969-973 (1994)), and chain shuffling (U.S. Pat. No. 5,565,332, which is incorporated by reference in its entirety).

[0092] Completely human antibodies are particularly desirable for therapeutic treatment of human patients. Human antibodies can be made by a variety of methods known in the art including phage display methods using antibody libraries derived from human immunoglobulin sequences. *See also*, U.S. Pat. Nos. 4,444,887 and 4,716,111; and PCT publications WO 98/46645, WO 98/50433, WO 98/24893, WO 98/16654, WO 96/34096, WO 96/33735, and WO 91/10741; each of which is incorporated herein by reference in its entirety.

[0093] Human antibodies can also be produced using transgenic mice which are incapable of expressing functional endogenous immunoglobulins, but which can express human immunoglobulin genes. For example, the human heavy and light chain immunoglobulin gene complexes may be introduced randomly or by homologous recombination into mouse embryonic stem cells. Alternatively, the human variable region, constant region, and diversity region may be introduced into mouse embryonic stem cells in addition to the human heavy and light chain genes. The mouse heavy and light chain immunoglobulin genes may be rendered non-functional separately or simultaneously with the introduction of human immunoglobulin loci by homologous recombination. In particular, homozygous deletion of the JH region prevents endogenous antibody production. The modified embryonic stem cells

are expanded and microinjected into blastocysts to produce chimeric mice. The chimeric mice are then bred to produce homozygous offspring that express human antibodies. The transgenic mice are immunized in the normal fashion with a selected antigen, *e.g.*, all or a portion of a desired target polypeptide. Monoclonal antibodies directed against the antigen can be obtained from the immunized, transgenic mice using conventional hybridoma technology. The human immunoglobulin transgenes harbored by the transgenic mice rearrange during B-cell differentiation, and subsequently undergo class switching and somatic mutation. Thus, using such a technique, it is possible to produce therapeutically useful IgG, IgA, IgM and IgE antibodies. For an overview of this technology for producing human antibodies, *see* Lonberg and Huszar *Int. Rev. Immunol.* 73:65-93 (1995). For a detailed discussion of this technology for producing human antibodies and human monoclonal antibodies and protocols for producing such antibodies, *see, e.g.*, PCT publications WO 98/24893; WO 96/34096; WO 96/33735; U.S. Pat. Nos. 5,413,923; 5,625,126; 5,633,425; 5,569,825; 5,661,016; 5,545,806; 5,814,318; and 5,939,598, which are incorporated by reference herein in their entirety. In addition, companies such as Abgenix, Inc. (Freemont, Calif.) and GenPharm (San Jose, Calif.) can be engaged to provide human antibodies directed against a selected antigen using technology similar to that described above.

[0094] Completely human antibodies which recognize a selected epitope can also be generated using a technique referred to as "guided selection." In this approach a selected non-human monoclonal antibody, *e.g.*, a mouse antibody, is used to guide the selection of a completely human antibody recognizing the same epitope. (Jespers *et al.*, *Bio/Technology* 72:899-903 (1988). *See also*, U.S. Patent No. 5,565,332, which is incorporated by reference in its entirety.)

[0095] In another embodiment, DNA encoding desired monoclonal antibodies may be readily isolated and sequenced using conventional procedures (*e.g.*, by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of murine antibodies). The isolated and subcloned hybridoma cells serve as a preferred source of such DNA. Once isolated, the DNA may be placed into expression vectors, which are then transfected into prokaryotic or eukaryotic host cells such as *E. coli* cells, simian COS cells, Chinese Hamster Ovary (CHO) cells or myeloma cells that do not otherwise produce immunoglobulins. More particularly, the isolated DNA (which may be synthetic as described

herein) may be used to clone constant and variable region sequences for the manufacture antibodies as described in Newman *et al.*, U.S. Pat. No. 5,658,570, filed January 25, 1995, which is incorporated by reference herein. Essentially, this entails extraction of RNA from the selected cells, conversion to cDNA, and amplification by PCR using Ig specific primers. Suitable primers for this purpose are also described in U.S. Pat. No. 5,658,570. As will be discussed in more detail below, transformed cells expressing the desired antibody may be grown up in relatively large quantities to provide clinical and commercial supplies of the immunoglobulin.

[0096] Additionally, using routine recombinant DNA techniques, one or more of the CDRs of the antigen-binding polypeptides of the present disclosure, may be inserted within framework regions, *e.g.*, into human framework regions to humanize a non-human antibody. The framework regions may be naturally occurring or consensus framework regions, and preferably human framework regions (*see, e.g.,* Chothia *et al., J. Mol. Biol.* 278:457-479 (1998) for a listing of human framework regions). Preferably, the polynucleotide generated by the combination of the framework regions and CDRs encodes an antibody that specifically binds to at least one epitope of a desired polypeptide, *e.g.*, LIGHT. Preferably, one or more amino acid substitutions may be made within the framework regions, and, preferably, the amino acid substitutions improve binding of the antibody to its antigen. Additionally, such methods may be used to make amino acid substitutions or deletions of one or more variable region cysteine residues participating in an intrachain disulfide bond to generate antibody molecules lacking one or more intrachain disulfide bonds. Other alterations to the polynucleotide are encompassed by the present disclosure and within the skill of the art.

[0097] In addition, techniques developed for the production of "chimeric antibodies" (Morrison *et al.*, *Proc. Natl. Acad. Sci. USA*:851-855 (1984); Neuberger *et al.*, *Nature* 372:604-608 (1984); Takeda *et al.*, *Nature* 314:452-454 (1985)) by splicing genes from a mouse antibody molecule, of appropriate antigen specificity, together with genes from a human antibody molecule of appropriate biological activity can be used. As used herein, a chimeric antibody is a molecule in which different portions are derived from different animal species, such as those having a variable region derived from a murine monoclonal antibody and a human immunoglobulin constant region.

[0098] Yet another highly efficient means for generating recombinant antibodies is disclosed by Newman, *Biotechnology* 10: 1455-1460 (1992). Specifically, this technique results in the

generation of primatized antibodies that contain monkey variable domains and human constant sequences. This reference is incorporated by reference in its entirety herein. Moreover, this technique is also described in commonly assigned U.S. Pat. Nos. 5,658,570, 5,693,780 and 5,756,096 each of which is incorporated herein by reference.

[0099] Alternatively, antibody-producing cell lines may be selected and cultured using techniques well known to the skilled artisan. Such techniques are described in a variety of laboratory manuals and primary publications. In this respect, techniques suitable for use in the disclosure as described below are described in *Current Protocols in Immunology*, Coligan *et al.*, Eds., Green Publishing Associates and Wiley-Interscience, John Wiley and Sons, New York (1991) which is herein incorporated by reference in its entirety, including supplements.

[0100] Additionally, standard techniques known to those of skill in the art can be used to introduce mutations in the nucleotide sequence encoding an antibody of the present disclosure, including, but not limited to, site-directed mutagenesis and PCR-mediated mutagenesis which result in amino acid substitutions. Preferably, the variants (including derivatives) encode less than 50 amino acid substitutions, less than 40 amino acid subsitutions, less than 30 amino acid substitutions, less than 25 amino acid substitutions, less than 20 amino acid substitutions, less than 15 amino acid substitutions, less than 10 amino acid substitutions, less than 5 amino acid substitutions, less than 4 amino acid substitutions, less than 3 amino acid substitutions, or less than 2 amino acid substitutions relative to the reference variable heavy chain region, CDR-H1, CDR-H2, CDR-H3, variable light chain region, CDR-L1, CDR-L2, or CDR-L3. Alternatively, mutations can be introduced randomly along all or part of the coding sequence, such as by saturation mutagenesis, and the resultant mutants can be screened for biological activity to identify mutants that retain activity.

[0101] The present disclosure also provides pharmaceutical compositions. Such compositions comprise an effective amount of an antibody, and an acceptable carrier. In some embodiments, the composition further includes a second anticancer agent (e.g., an immune checkpoint inhibitor).

[0102] In a specific embodiment, the term "pharmaceutically acceptable" means approved by a regulatory agency of the Federal or a state government or listed in the U.S. Pharmacopeia or other generally recognized pharmacopeia for use in animals, and more particularly in

humans. Further, a "pharmaceutically acceptable carrier" will generally be a non-toxic solid, semisolid or liquid filler, diluent, encapsulating material or formulation auxiliary of any type.

[0103] The term "carrier" refers to a diluent, adjuvant, excipient, or vehicle with which the therapeutic is administered. Such pharmaceutical carriers can be sterile liquids, such as water and oils, including those of petroleum, animal, vegetable or synthetic origin, such as peanut oil, soybean oil, mineral oil, sesame oil and the like. Water is a preferred carrier when the pharmaceutical composition is administered intravenously. Saline solutions and aqueous dextrose and glycerol solutions can also be employed as liquid carriers, particularly for injectable solutions. Suitable pharmaceutical excipients include starch, glucose, lactose, sucrose, gelatin, malt, rice, flour, chalk, silica gel, sodium stearate, glycerol monostearate, talc, sodium chloride, dried skim milk, glycerol, propylene, glycol, water, ethanol and the like. The composition, if desired, can also contain minor amounts of wetting or emulsifying agents, or pH buffering agents such as acetates, citrates or phosphates. Antibacterial agents such as benzyl alcohol or methyl parabens; antioxidants such as ascorbic acid or sodium bisulfite; chelating agents such as ethylenediaminetetraacetic acid; and agents for the adjustment of tonicity such as sodium chloride or dextrose are also envisioned. These compositions can take the form of solutions, suspensions, emulsion, tablets, pills, capsules, powders, sustained-release formulations and the like. The composition can be formulated as a suppository, with traditional binders and carriers such as triglycerides. Oral formulation can include standard carriers such as pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate, etc. Examples of suitable pharmaceutical carriers are described in Remington's Pharmaceutical Sciences by E. W. Martin, incorporated herein by reference. Such compositions will contain a therapeutically effective amount of the antigen-binding polypeptide, preferably in purified form, together with a suitable amount of carrier so as to provide the form for proper administration to the patient. The formulation should suit the mode of administration. The parental preparation can be enclosed in ampoules, disposable syringes or multiple dose vials made of glass or plastic.

[0104] In an embodiment, the composition is formulated in accordance with routine procedures as a pharmaceutical composition adapted for intravenous administration to human beings. Typically, compositions for intravenous administration are solutions in sterile isotonic aqueous buffer. Where necessary, the composition may also include a solubilizing

agent and a local anesthetic such as lignocaine to ease pain at the site of the injection. Generally, the ingredients are supplied either separately or mixed together in unit dosage form, for example, as a dry lyophilized powder or water free concentrate in a hermetically sealed container such as an ampoule or sachette indicating the quantity of active agent. Where the composition is to be administered by infusion, it can be dispensed with an infusion bottle containing sterile pharmaceutical grade water or saline. Where the composition is administered by injection, an ampoule of sterile water for injection or saline can be provided so that the ingredients may be mixed prior to administration.

[0105] The compounds of the disclosure can be formulated as neutral or salt forms. Pharmaceutically acceptable salts include those formed with anions such as those derived from hydrochloric, phosphoric, acetic, oxalic, tartaric acids, etc., and those formed with cations such as those derived from sodium, potassium, ammonium, calcium, ferric hydroxides, isopropylamine, triethylamine, 2-ethylamino ethanol, histidine, procaine, etc.

EXAMPLES

Example 1: Cκ/CH1 Interface Interaction Analysis of Four Fab Fragments

[0106] This example analyzed a few antibody Fab fragments with respect to their Cκ/CH1 interface interactions.

Structure 1: Interface Interaction Analysis for Cκ and CH1 of Fab 1F8

[0107] 1F8 is a Fab molecule prepared from an antibody specific to human CD47. The complex crystal structure of the CD47 with anti-CD47 Fab 1F8 was conducted at a resolution of 3.1A in 2017 (the light chain had 219 amino acids, where the Cκ included amino acids 114-219; the heavy chain had 220 amino acids, where the CH included amino acids119-220).

[0108] In the interface between the Cκ and CH1 domains of this Fab fragment, there are a total of 32 residues from the CH domain and 35 residues from the Cκ domain. 1F8 has continuous residues between Ser14 and Gly20 in the CH domain. There is one more hydrogen bond formed between Lys16 main chain oxygen atom from the CH fragment and residue Lys100 from Cκ fragment, as compared to 4NYL (see structure 4 below). The hydrophobic interactions are similar to the other structures as shown below.

Hydrogen Bonds (distance cut-off: 3.5Å)

Cκ			CH1			Distance (Å)
Position	Residue	Atom Name	Position	Residue	Atom Name	
16	LYS	O	100	LYS	NZ	3.4
30	LYS	NZ	24	SER	OG	2.7
51	HIS	ND1	30	ASN	OD1	3.3
54	PRO	O	55	SER	OG	2.7
57	LEU	O	53	GLN	NE2	3.4
102	SER	OG	106	GLU	O	2.6

Notes:

1. HD between CH-Lys30 and Ser24 could be formed in the other three structures, as long as the NZ of Lys30 is rotated.
2. Extra HDs between CH-Lys16/CK-Lys100 and CH-Ser102/CK-Glu106 are formed because sequence difference than other 3 pdbs.

Salt Bridges between Cκ and CH1 of 1F8

CH1			Cκ			Distance (Å)
Position	Residue	Atom Name	Position	Residue	Atom Name	
96	LYS	NZ	16	GLU	OE2	3.1
101	LYS	NZ	15	ASP	OD2	3.8

Hydrophobic interface

CH1		Cκ		Notes
Position	Residue	Position	Residue	
9	PHE	17	GLN	Sandwich, more like Van der Waals
11	LEU	11,26	PHE, VAL	
12	ALA	11	PHE	
24	ALA	9,11	PHE	
53	PHE	28, 68,69	LEU, LEU, SER	
68	VAL	28	LEU	

* Hydrophobic contacts involved in hydrogen bonds and salt bonds too are excluded in this table

[0109] Free energy deviation analysis identified that some residues in 1F8 CH1 have stronger interactions with Cκ residues (see the first 10 residues in the table below, bolded).

Interfacing Residues in 1F8 CH1:

Position	Residue	Bond	ASA	BSA	DeltaG	Abs of DeltaG
53	**PHE**		**104.91**	**102.42**	**1.64**	**1.64**
9	**PHE**		**95.13**	**73.47**	**1.18**	**1.18**

11	**LEU**		**63.14**	**60.63**	**0.97**	**0.97**
56	**VAL**		**97.59**	**60.26**	**0.96**	**0.96**
30	**LYS**	**H**	**74.1**	**57.96**	**-0.85**	**0.85**
96	**LYS**	**S**	**71.12**	**24.42**	**-0.71**	**0.71**
28	**LEU**		**48.35**	**42.65**	**0.68**	**0.68**
24	**ALA**		**41.8**	**41.64**	**0.62**	**0.62**
68	**VAL**		**41.46**	**36.31**	**0.58**	**0.58**
54	**PRO**	**H**	**118.8**	**51.46**	**0.53**	**0.53**
16	LYS	H	190.06	97.08	0.44	0.44
19	SER		87.49	29.98	0.37	0.37
10	PRO		67.03	38.95	0.23	0.23
70	THR	H	74.01	32.39	-0.16	0.16
57	LEU	H	101.62	7.97	-0.09	0.09
51	HIS	H	125.59	86.42	0.08	0.08
58	GLN		49.39	19.38	0.08	0.08
17	SER	H	44.25	44.25	0.06	0.06
18	THR	H	54.47	19.11	-0.06	0.06
22	THR		61.97	7.01	-0.06	0.06
12	ALA		72.88	29.29	0.05	0.05
66	SER		30.01	25.56	-0.05	0.05
52	THR		60.18	4.28	-0.04	0.04
59	SER		129.9	3.35	-0.04	0.04
25	LEU		3.79	2.96	0.04	0.04
23	ALA		2.05	1.88	0.03	0.03
8	VAL		11.3	1.81	-0.02	0.02
65	LEU		13.88	1.01	0.02	0.02
14	SER		52.86	6.52	-0.01	0.01
15	SER		98.56	0.61	-0.01	0.01

Bond: bond type if formed hydrogen bond or salt bridge, H: hydrogen bond, S: salt bridge

ASA: accessible surface area

BSA: buried surface area

DeltaG: Change of Energy, positive involves more hydrophobic interaction while negative indicates more hydrophilic interaction

Abs of DeltaG: Absolute value of DeltaG, the table is sorted by this key. Residues (bold) with change of DeltaG above 0.5 can be regarded as the residues contribute more to stabilize the protein.

[0110] In the Cκ domain, seven residues are likely involved in interactions.

Interfacing residues in 1F8 Cκ:

Position	Residue	Bond	ASA	BSA	DeltaG	Abs of DeltaG
11	**PHE**		**103.34**	**103.03**	**1.65**	**1.65**
9	**PHE**		**85.86**	**83.98**	**1.34**	**1.34**
100	**LYS**	**H**	**86.5**	**41.41**	**-1.06**	**1.06**

57	**THR**		**76.88**	**61.43**	**0.93**	**0.93**
28	**LEU**		**47.22**	**45.38**	**0.73**	**0.73**
26	**VAL**		**42.67**	**42.67**	**0.68**	**0.68**
53	**GLN**	**H**	**153.38**	**81.8**	**-0.65**	**0.65**
14	SER		63.01	48.31	0.47	0.47
30	ASN	H	46.04	36.92	-0.44	0.44
102	PHE		43.9	22.09	0.35	0.35
12	PRO		78.85	40.42	0.31	0.31
16	GLU	S	132.97	48.56	-0.31	0.31
101	SER		64.98	27.71	-0.31	0.31
31	ASN		71.04	16.15	-0.25	0.25
73	THR	H	78.11	22.7	0.18	0.18
17	GLN		46.63	45.77	0.17	0.17
60	ASP		67.72	10.4	0.14	0.14
67	SER		21.05	20.24	0.13	0.13
69	SER		30.67	27.47	0.13	0.13
7	SER		56.42	8.61	0.12	0.12
54	GLU		92.77	11.21	-0.11	0.11
56	VAL		43.54	12.76	-0.1	0.1
71	THR		39.29	14.82	0.09	0.09
10	ILE	H	28.88	27.62	-0.07	0.07
58	GLU		156.59	7.29	0.05	0.05
55	SER	H	73.31	57.53	-0.04	0.04
24	SER	H	30.01	29.28	0.03	0.03
8	VAL		9.41	1.5	-0.02	0.02
68	LEU		7.95	1.41	0.02	0.02
20	SER		98.75	8.4	-0.01	0.01
22	THR		59.56	11.35	-0.01	0.01
103	ASN		47.6	0.87	-0.01	0.01

Bond: bond type if formed hydrogen bond or salt bridge, H: hydrogen bond, S: salt bridge

ASA: accessible surface area

BSA: buried surface area

DeltaG: Change of Energy, positive involves more hydrophobic interaction while negative indicates more hydrophilic interaction

Abs of DeltaG: Absolute value of DeltaG, the table is sorted by this key. Residues (bold) with change of DeltaG above 0.5 can be regarded as the residues contribute more to stabilize the protein.

Structure 2: Interface Interaction Analysis for Cκ and CH1 of 1CZ8

[0111] 1CZ8 (PDB ID **1CZ8**) is a Fab molecule prepared from an antibody specific to VEGF. The complex crystal structure of the VEGF and the Fab was conducted at a resolution of 2.4A in year 2000.

[0112] Amino acid residues formed three antiparallel beta sheets in CH domain and four antiparallel beta sheets in the Cκ domain. These beta sheets formed a face-to-face conformation in the interface. In the interface between Cκ and CH1 domains of this Fab fragment, there are totally 28 residues from CH and 30 residues from Cκdomain. There are three hydrogen bonds between the Cκ and CH1 domains. For example, in 1CZ8, CH residue His 51 and main chain oxygen atoms of Pro54 and Leu57 formed these three hydrogen bonds with Cκ residues Asn31, Ser55 and Gln53 respectively. These hydrogen binds are located on the one side of the interface.

[0113] The hydrophobic interactions are mainly located at the central and other side of the interface, between CH residues Phe9, Leu11, Phe53, Val68 and Cκ residues Gln17, Phe11, Val26, Phe69 and Val28. Two salt bridges were formed between C-term of CH residues Lys96 and Lys101 and Cκ residue Asp15 and Glu16 to stabilize the CH and Cκ complex structure on the other side of the interface (**FIG. 1**; residues involved in hydrogen bond colored in pink; salt bridge in yellow; hydrophobic interaction residues are sticks colored in blue or green).

Hydrogen Bonds (distance cut-off: 3.5Å)

CH1			Cκ			Distance (Å)
Position	Residue	Atom Name	Position	Residue	Atom Name	
51	HIS	NE2	31	ASN	OD1	2.86
54	PRO	O	55	SER	OG	2.6
57	LEU	O	53	GLN	NE2	2.9
Water-mediated hydrogen binding						
10	PRO	O	12	PRO	O	
58	GLN	OE1	24	SER	OG	
54	PRO	O	71	THR	OG1	

Salt Bridges between CH and Cκ

CH1			Cκ			Distance (Å)
Position	Residue	Atom Name	Position	Residue	Atom Name	
96	LYS	NZ	16	GLU*	OE1	3.0
101	LYS	NZ	15	ASP*	OD1	2.8

Hydrophobic interface (distance cut-off: 4Å)

CH1		Cκ		Notes
Position	Residue	Position	Residue	

9	PHE	17	Gln	
11	LEU	11,26	PHE, VAL	4A from Leu15 Ca atom
12	ALA	11	PHE	
24	ALA	11	PHE	Displaced
53	PHE	28,69	LEU, SER	Sandwich
68	VAL	28	LEU	Sandwich

Top 5 important interface residues for Cκ and CH1 interaction

CH1		Cκ	
Position	Residue	Position	Residue
51	HIS	31	ASN
57	LEU	53	GLN
9	PHE	17	GLN
53	PHE	69	SER
11	LEU	11,26	PHE, VAL

Note: Salt bridge residues are excluded

[0114] Free energy deviation analysis identified some residues in 1cz8 CH1 have stronger interactions with Cκ residues (see the first 9 residues in the table below, bolded).

Interfacing Residues: 1cz8 CH1

Position	Residue	bond	ASA	BSA	DeltaG	Abs of DeltaG
53	**PHE**		**103.02**	**99.71**	**1.6**	**1.6**
9	**PHE**		**96.3**	**77.27**	**1.24**	**1.24**
11	**LEU**		**64.71**	**61.37**	**0.98**	**0.98**
56	**VAL**		**93.45**	**56.39**	**0.9**	**0.9**
28	**LEU**		**48.79**	**44.61**	**0.71**	**0.71**
51	**HIS**	**H**	**114.24**	**93.31**	**0.68**	**0.68**
54	**PRO**	**H**	**120.4**	**53.11**	**0.59**	**0.59**
68	**VAL**		**35.98**	**34.81**	**0.56**	**0.56**
24	**ALA**		**53.89**	**51.35**	**0.55**	**0.55**
70	THR		64.58	33.59	0.43	0.43
96	LYS	S	63.68	16.19	-0.39	0.39
101	LYS	S	231.91	46.46	-0.39	0.39
30	LYS		62.59	47.59	0.29	0.29
10	PRO		66.28	37.49	0.26	0.26
12	ALA		47.32	20.55	-0.2	0.2
57	LEU	H	101.85	12.03	-0.14	0.14
66	SER		28.08	23.48	0.13	0.13
58	GLN		41.61	13.28	0.11	0.11
8	VAL		16.24	6.24	-0.07	0.07
25	LEU		3.82	3.49	0.06	0.06
23	ALA		14.88	3.31	0.05	0.05
59	SER		132.12	3.5	-0.04	0.04
52	THR		59.64	4.29	-0.03	0.03
22	THR		97.5	16.73	0.02	0.02

64	SER		11.46	1.84	-0.02	0.02
13	PRO		5.85	0.5	0.01	0.01
14	SER		149.42	0.94	0.01	0.01

Bond: bond type if formed hydrogen bond or salt bridge, H: hydrogen bond, S: salt bridge

ASA: accessible surface area

BSA: buried surface area

DeltaG: Change of Energy, positive involves more hydrophobic interaction while negative indicates more hydrophilic interaction

Abs of DeltaG: Absolute value of DeltaG, the table is sorted by this key. Residues (bold) with change of DeltaG above 0.5 can be regarded as the residues contribute more to stabilize the protein.

[0115] In the Cκ domain, five residues are likely involved in interactions.

Interfacing Residues: 1cz8 Cκ

Position	Residue	bond	ASA	BSA	DeltaG	Abs of DeltaG
11	**PHE**		**100.45**	**95.29**	**1.52**	**1.52**
9	**PHE**		**99.99**	**59.32**	**0.95**	**0.95**
28	**LEU**		**48.88**	**48.38**	**0.77**	**0.77**
26	**VAL**		**46.36**	**45.71**	**0.73**	**0.73**
53	**GLN**	**H**	**152.03**	**80.54**	**-0.63**	**0.63**
14	SER		62.19	48.96	0.49	0.49
30	ASN		45.99	39.43	-0.49	0.49
16	GLU	S	133.88	61.31	-0.46	0.46
15	ASP	S	118.39	37.99	-0.36	0.36
69	SER		37.96	30.45	0.3	0.3
31	ASN	H	71	17.38	-0.27	0.27
67	SER		20.55	20.55	0.18	0.18
17	GLN		55.44	53.37	0.16	0.16
56	VAL		64.32	17.9	-0.14	0.14
71	THR		44.12	16.45	0.12	0.12
60	ASP		61.58	15.19	-0.11	0.11
54	GLU		92.25	12.17	-0.1	0.1
22	THR		65.41	8.27	0.07	0.07
57	THR		59.04	44.28	-0.07	0.07
68	LEU		7.25	2.72	0.04	0.04
20	SER		84.08	5.75	-0.02	0.02
58	GLU		146.27	4.53	0.02	0.02
10	ILE		25.87	0.86	-0.01	0.01
13	PRO		12.65	1.66	-0.01	0.01
24	SER		31.09	30.1	0.01	0.01
55	SER	H	71.35	56.44	-0.01	0.01
73	THR		81.21	12.51	-0.01	0.01

Bond: bond type if formed hydrogen bond or salt bridge, H: hydrogen bond, S: salt bridge

ASA: accessible surface area

BSA: buried surface area

DeltaG: Change of Energy, positive involves more hydrophobic interaction while negative indicates more hydrophilic interaction

Abs of DeltaG: Absolute value of DeltaG, the table is sorted by this key. Residues (bold) with change of DeltaG above 0.5 can be regarded as the residues contribute more to stabilize the protein.

Structure 3: Interface Interaction Analysis for Cκ and CH1 of 1L7I

[0116] 1L7I is a known Fab molecule (PDB ID: 1L7I) targeting ErbB2. The crystal Structure of this anti-ErbB2 Fab2C4 was resolved at 1.8A in year 2002.

[0117] In the interface between Cκ and CH1 domain of this Fab fragment (PDB ID 1L7i), there are total 33 residues from CH and 35 residues from Cκ domain.

[0118] Hydrogen Bonds of 1L7i (distance cut-off: 3.5Å)

Cκ			**CH1**			**Distance (Å)**
Position	Residue	Atom Name	Position	Residue	Atom Name	
16	LYS	O	10	ILE	N	3.16
16	LYS	NZ	101	SER	O	2.99
51	HIS	ND1	31	ASN	OD1	3.2
54	PRO	O	55	SER	OG	2.7
57	LEU	O	53	GLN	NE2	2.9
Water-mediated hydrogen binding						
10	PRO	O	12	PRO	O	
12	ALA	O	10	ILE	O	
54	PRO	O	71	THR	OG1	

Salt Bridges between CK and CH of 1L7i

CH1			**Cκ**			**Distance (Å)**
Position	Residue	Atom Name	Position	Residue	Atom Name	
96	LYS	NZ	16	GLU	OE1, OE2	3.4-2.7

Note: C-term residues Cys 103 of CH and Cys 107 CK formed a disulfide bridge which broke the salt bridge between CH residue Lys101 and Ck residue Asp15 which was seen in other structures.

Hydrophobic interface of 1L7i

Cκ			**CH1**			**Distance (Å)**
Position	Residue	Atom Name	Position	Residue	Atom Name	
16	LYS	O	100	LYS	NZ	3.4
30	LYS	NZ	24	SER	OG	2.7

51	HIS	ND1	30	ASN	OD1	3.3
54	PRO	O	55	SER	OG	2.7
57	LEU	O	53	GLN	NE2	3.4
102	SER	OG	106	GLU	O	2.6

[0119] Free energy deviation analysis identified some residues in 1L7i CH1 have stronger interactions with Cκ residues (see the first 12 residues in the table below, bolded).

Interfacing Residues: 1L7i CH1

Position	Residue	bond	ASA	BSA	DeltaG	Abs of DeltaG
103	**CYS**		**113.02**	**79.88**	**2.31**	**2.31**
53	**PHE**		**104.25**	**101.74**	**1.63**	**1.63**
9	**PHE**		**99.04**	**80.13**	**1.28**	**1.28**
101	**LYS**	**S**	**141.67**	**60.98**	**-1.2**	**1.2**
56	**VAL**		**92.31**	**59.34**	**0.95**	**0.95**
11	**LEU**		**61.26**	**57.09**	**0.91**	**0.91**
54	**PRO**	**H**	**116.04**	**53.67**	**0.73**	**0.73**
28	**LEU**		**50.04**	**45.19**	**0.72**	**0.72**
17	**SER**		**37.12**	**37.12**	**0.55**	**0.55**
68	**VAL**		**34.63**	**33.97**	**0.54**	**0.54**
24	**ALA**		**33.93**	**33.93**	**0.52**	**0.52**
96	**LYS**	**S**	**69.62**	**16.57**	**-0.52**	**0.52**
70	THR		59.2	34.05	0.42	0.42
10	PRO		57.58	41.55	0.33	0.33
30	LYS		64.74	49.18	0.29	0.29
58	GLN		48.94	23.52	0.26	0.26
16	LYS	H	154.48	98.09	0.21	0.21
12	ALA		37.27	23.91	-0.17	0.17
66	SER		26.68	23.26	0.14	0.14
22	THR		61.32	9.4	0.13	0.13
102	SER		106.39	12.64	-0.12	0.12
57	LEU		109.6	10.1	-0.11	0.11
18	THR		47.02	7.72	-0.09	0.09
19	SER		89.59	40.33	-0.07	0.07
25	LEU		4.95	4.61	0.07	0.07
15	SER		83.74	3.93	-0.04	0.04

14	SER		15.02	4.4	-0.03	0.03
51	HIS		111.16	79.43	0.02	0.02
52	THR		62.51	3.83	-0.02	0.02
23	ALA		0.33	0.33	0.01	0.01
59	SER		127.52	1.31	-0.01	0.01
64	SER		9.13	0.61	-0.01	0.01

Bond: bond type if formed hydrogen bond or salt bridge, H: hydrogen bond, S: salt bridge

ASA: accessible surface area

BSA: buried surface area

DeltaG: Change of Energy, positive involves more hydrophobic interaction while negative indicates more hydrophilic interaction

Abs of DeltaG: Absolute value of DeltaG, the table is sorted by this key. Residues (bold) with change of DeltaG above 0.5 can be regarded as the residues contribute more to stabilize the protein.

[0120] In the Cκ domain, nine residues are likely involved in interactions.

Interfacing Residues: 1L7i Cκ

Position	Residue	bond	ASA	BSA	DeltaG	Abs of DeltaG
11	**PHE**		**105.85**	**105.73**	**1.68**	**1.68**
9	**PHE**		**89.04**	**88.03**	**1.41**	**1.41**
100	**LYS**	**H**	**85.84**	**42.05**	**-1.08**	**1.08**
57	**THR**		**74.5**	**58.15**	**0.89**	**0.89**
107	**CYS**	**S**	**101.84**	**64.95**	**0.89**	**0.89**
28	**LEU**		**48.03**	**47.86**	**0.77**	**0.77**
26	**VAL**		**44.53**	**44.19**	**0.71**	**0.71**
53	**GLN**		**151.85**	**79.95**	**-0.58**	**0.58**
12	**PRO**		**56.22**	**48.12**	**0.54**	**0.54**
30	ASN		43.79	36.72	-0.44	0.44
16	GLU	S	108.76	56.23	-0.4	0.4
14	SER		53.87	41.73	0.39	0.39
69	SER		41	33.43	0.33	0.33
31	ASN		78.35	17.4	-0.27	0.27
101	SER		57.64	22.68	-0.26	0.26
102	PHE		21.99	16.51	0.26	0.26
73	THR		63.23	17.38	0.15	0.15
60	ASP		61.68	11.2	-0.14	0.14
7	SER		48.66	8.03	0.13	0.13
67	SER		16.14	16.14	0.13	0.13
56	VAL		37.56	12.43	-0.12	0.12
106	GLU		136	18.91	-0.12	0.12
55	SER	H	69.61	59.4	0.11	0.11
13	PRO		14.76	6.87	-0.08	0.08
17	GLN		49.85	48.28	0.08	0.08

24	SER		24.37	22.04	0.08	0.08
54	GLU		96.84	10.59	-0.07	0.07
8	VAL		15.41	5.63	-0.06	0.06
71	THR		41.6	14.33	0.06	0.06
58	GLU		156.39	9.76	0.04	0.04
10	ILE	H	31.71	31.59	0.03	0.03
15	ASP		107.21	9.87	0.03	0.03
68	LEU		4.61	1.93	0.03	0.03
22	THR		52.89	8.19	0.02	0.02
20	SER		84.54	13.16	0.01	0.01

Bond: bond type if formed hydrogen bond or salt bridge, H: hydrogen bond, S: salt bridge
ASA: accessible surface area
BSA: buried surface area
DeltaG: Change of Energy, positive involves more hydrophobic interaction while negative indicates more hydrophilic interaction
Abs of DeltaG: Absolute value of DeltaG, the table is sorted by this key. Residues (bold) with change of DeltaG above 0.5 can be regarded as the residues contribute more to stabilize the protein.

Structure 4: Interface Interaction Analysis for Cκ and CH1 of 4NYL

[0121] The fourth structure being studied was 4NYL, a known Fab molecule (PDB ID: 4NYL), targeting TNFa. The crystal structure of the adalimumab FAB fragment was resolved at 2.8A in year 2014 (solved with a relative high Rfree (Rfree=35.8%/R=27.5), which means that the structure is not suitable for detailed analysis). *Adalimumab* is antibody against TNFa, used to treat patients with rheumatoid arthritis, psoriatic arthritis and ankylosing spondylitis, and children with juvenile idiopathic arthritis. In the interface between Cκ and CH1 domain of adalimumab Fab fragment (PDB ID 4NYL), there are total 24 residues from CH1 and 28 residues from CK domain.

[0122] 4NYL has the same hydrogen bond and hydrophobic interaction as that in 1CZ8. Due to the lack of C-term Ch residues, only one salt bridge was formed between C-term of CH residue Lys96 and CK residue Glu15.

Hydrogen Bonds of 4NYL (distance cut-off: 3.5Å)

CH1			**Cκ+Vκ**			**Distance (Å)**
Position	Residue	Atom Name	Position	Residue	Atom Name	
51	HIS	NE2	30	ASN	OD1	3.22
54	PRO	O	55	SER	OG	2.7
57	LEU	O	53	GLN	OE1	2.97

Note: due to resolution limit, no water mediated hydrogen bonds are found.

Salt Bridges between CK and CH of 4NYL

CH1			Cκ			Distance (Å)
Position	Residue	Atom Name	Position	Residue	Atom Name	
96	LYS	NZ	16	GLU	OE1, OE2	3.4-2.7

Note**:** As 4NYL has C-term residues 100-103 missing, so salt bridge between CH-Lys101 and CK-Asp15 is missing.

Hydrophobic interface of 4NYL

CH1		Cκ		Notes
Position	Residue	Position	Residue	
9	PHE	17	GLN	Sandwich
11	LEU	11, 26	PHE, VAL	Sandwich
12	ALA	11	PHE	displace
24	ALA	9,11,28	PHE, LEU, LEU	Sandwich
68	VAL	28	LEU	Sandwich

[0123] Free energy deviation analysis identified some residues in 4NYL CH1 have stronger interactions with Cκ residues (see the first nine residues in the table below, bolded).

Interfacing Residues: 4NYL CH1

Position	Residue	bond	ASA	BSA	DeltaG	Abs of DeltaG
53	**PHE**		**96.83**	**95.9**	**1.53**	**1.53**
9	**PHE**		**97.57**	**74.98**	**1.2**	**1.2**
11	**LEU**		**67.89**	**65.22**	**1.04**	**1.04**
56	**VAL**		**102.16**	**64.24**	**1.03**	**1.03**
28	**LEU**		**56.92**	**51.23**	**0.82**	**0.82**
54	**PRO**	**H**	**117.72**	**48.38**	**0.65**	**0.65**
68	**VAL**		**38.86**	**38.35**	**0.61**	**0.61**
24	**ALA**		**56.65**	**54.38**	**0.59**	**0.59**
51	**HIS**	**H**	**109.04**	**76.51**	**0.54**	**0.54**
70	THR		65.96	30.78	0.47	0.47
12	ALA		65.59	34.43	-0.27	0.27
10	PRO		58.21	35.55	0.21	0.21
96	LYS		71.02	8.03	0.13	0.13
13	PRO		110.53	7.16	0.11	0.11
58	GLN		45.51	21.8	0.11	0.11
52	THR		68.63	7.32	-0.08	0.08
57	LEU	H	105.07	6.99	-0.08	0.08
25	LEU		10.31	4.61	0.07	0.07
66	SER		27.04	21.25	0.07	0.07
23	ALA		20.19	3.62	0.06	0.06
22	THR		99.8	17.73	0.04	0.04

59	SER		129.22	4.43	-0.04	0.04
30	LYS		68.02	48.93	-0.03	0.03
64	SER		15.79	1.32	-0.01	0.01

Bond: bond type if formed hydrogen bond or salt bridge, H: hydrogen bond, S: salt bridge

ASA: accessible surface area

BSA: buried surface area

DeltaG: Change of Energy, positive involves more hydrophobic interaction while negative indicates more hydrophilic interaction

Abs of DeltaG: Absolute value of DeltaG, the table is sorted by this key. Residues (bold) with change of DeltaG above 0.5 can be regarded as the residues contribute more to stabilize the protein.

[0124] In the Cκ domain, seven residues are likely involved in interactions.

Interfacing Residues: 4NYL Cκ

Position	Residue	bond	ASA	BSA	DeltaG	Abs of DeltaG
11	**PHE**		**94.4**	**92.85**	**1.49**	**1.49**
9	**PHE**		**98.42**	**57.04**	**0.91**	**0.91**
28	**LEU**		**53.03**	**53.03**	**0.85**	**0.85**
57	**THR**		**77.07**	**52.33**	**0.81**	**0.81**
26	**VAL**		**46.03**	**45.87**	**0.73**	**0.73**
53	**GLN**	**H**	**146.87**	**74.61**	**-0.59**	**0.59**
30	**ASN**		**53.19**	**42.51**	**-0.51**	**0.51**
14	SER		73.33	53.83	0.41	0.41
16	GLU		81.87	23.61	0.36	0.36
69	SER		36.91	32.06	0.36	0.36
31	ASN	H	68.16	17.44	-0.21	0.21
22	THR		53.37	11.88	0.19	0.19
67	SER		17.29	16.83	0.15	0.15
20	SER		77.72	8.53	0.14	0.14
12	PRO		72.19	25.45	0.12	0.12
56	VAL		66.37	16.66	-0.12	0.12
60	ASP		61.84	6.5	-0.11	0.11
73	THR		69.46	16.93	0.1	0.1
17	GLN		47.27	41.79	0.08	0.08
24	SER		37.71	35.01	0.05	0.05
54	GLU		93.02	10.6	-0.05	0.05
58	GLU		154.24	3.58	0.05	0.05
10	ILE		33.1	3.56	-0.04	0.04
55	SER	H	70.17	60.32	0.04	0.04
13	PRO		16.67	1.84	0.03	0.03

68	LEU		7.35	1.92	0.03	0.03
71	THR		45.38	15.86	0.01	0.01

Bond: bond type if formed hydrogen bond or salt bridge, H: hydrogen bond, S: salt bridge

ASA: accessible surface area

BSA: buried surface area

DeltaG: Change of Energy, positive involves more hydrophobic interaction while negative indicates more hydrophilic interaction

Abs of DeltaG: Absolute value of DeltaG, the table is sorted by this key. Residues (bold) with change of DeltaG above 0.5 can be regarded as the residues contribute more to stabilize the protein.

Interface analysis for CH1_Cκ of 1cz8, 4nyl, 1l7i, hCD47-1_1F8

[0125] Interface analysis for the above four structures includes salt bridge, hydrogen bond and hydrophobic interaction. All of the DeltaG were calculated and the amino acids were ranked by DeltaG. For each structure, Top10 pairs were chosen for further analysis. The analysis focused on hydrophobic interaction regardless of other interactions. Then Top5 pairs were selected for lead candidates.

Sequence recoding of CH1

Recoding		1cz8	1l7i	4nyl	1F8
1	**ALA**	ALA 124	ALA 114	ALA 122	ALA 119
2	**SER**	SER 125	SER 115	SER 123	SER 120
3	**THR**	THR 126	THR 116	THR 124	THR 121
4	**LYS**	LYS 127	LYS 117	LYS 125	LYS 122
5	**GLY**	GLY 128	GLY 118	GLY 126	GLY 123
6	**PRO**	PRO 129	PRO 119	PRO 127	PRO 124
7	**SER**	SER 130	SER 120	SER 128	SER 125
8	**VAL**	VAL 131	VAL 121	VAL 129	VAL 126
9	**PHE**	PHE 132	PHE 122	PHE 130	PHE 127
10	**PRO**	PRO 133	PRO 123	PRO 131	PRO 128
11	**LEU**	LEU 134	LEU 124	LEU 132	LEU 129
12	**ALA**	ALA 135	ALA 125	ALA 133	ALA 130
13	**PRO**	PRO 136	PRO 126	PRO 134	PRO 131
14	**SER**	SER 137	SER 127	/	SER 132
15	**SER**	/	SER 128	/	SER 133
16	**LYS**	/	LYS 129	/	LYS 134
17	**SER**	/	SER 130	/	SER 135
18	**THR**	/	THR 131	/	THR 136
19	**SER**	/	SER 132	/	SER 137
20	**GLY**	/	GLY 133	/	GLY 138
21	**GLY**	GLY 144	GLY 134	GLY 142	GLY 139
22	**THR**	THR 145	THR 135	THR 143	THR 140
23	**ALA**	ALA 146	ALA 136	ALA 144	ALA 141

24	**ALA**	ALA 147	ALA 137	ALA 145	ALA 142
25	**LEU**	LEU 148	LEU 138	LEU 146	LEU 143
26	**GLY**	GLY 149	GLY 139	GLY 147	GLY 144
27	**CYS**	CYS 150	CYS 140	CYS 148	CYS 145
28	**LEU**	LEU 151	LEU 141	LEU 149	LEU 146
29	**VAL**	VAL 152	VAL 142	VAL 150	VAL 147
30	**LYS**	LYS 153	LYS 143	LYS 151	LYS 148
31	**ASP**	ASP 154	ASP 144	ASP 152	ASP 149
32	**TYR**	TYR 155	TYR 145	TYR 153	TYR 150
33	**PHE**	PHE 156	PHE 146	PHE 154	PHE 151
34	**PRO**	PRO 157	PRO 147	PRO 155	PRO 152
35	**GLU**	GLU 158	GLU 148	GLU 156	GLU 153
36	**PRO**	PRO 159	PRO 149	PRO 157	PRO 154
37	**VAL**	VAL 160	VAL 150	VAL 158	VAL 155
38	**THR**	THR 161	THR 151	THR 159	THR 156
39	**VAL**	VAL 162	VAL 152	VAL 160	VAL 157
40	**SER**	SER 163	SER 153	SER 161	SER 158
41	**TRP**	TRP 164	TRP 154	TRP 162	TRP 159
42	**ASN**	ASN 165	ASN 155	ASN 163	ASN 160
43	**SER**	SER 166	SER 156	SER 164	SER 161
44	**GLY**	GLY 167	GLY 157	GLY 165	GLY 162
45	**ALA**	ALA 168	ALA 158	ALA 166	ALA 163
46	**LEU**	LEU 169	LEU 159	LEU 167	LEU 164
47	**THR**	THR 170	THR 160	THR 168	THR 165
48	**SER**	SER 171	SER 161	SER 169	SER 166
49	**GLY**	GLY 172	GLY 162	GLY 170	GLY 167
50	**VAL**	VAL 173	VAL 163	VAL 171	VAL 168
51	**HIS**	HIS 174	HIS 164	HIS 172	HIS 169
52	**THR**	THR 175	THR 165	THR 173	THR 170
53	**PHE**	PHE 176	PHE 166	PHE 174	PHE 171
54	**PRO**	PRO 177	PRO 167	PRO 175	PRO 172
55	**ALA**	ALA 178	ALA 168	ALA 176	ALA 173
56	**VAL**	VAL 179	VAL 169	VAL 177	VAL 174
57	**LEU**	LEU 180	LEU 170	LEU 178	LEU 175
58	**GLN**	GLN 181	GLN 171	GLN 179	GLN 176
59	**SER**	SER 182	SER 172	SER 180	SER 177
60	**SER**	SER 183	SER 173	SER 181	SER 178
61	**GLY**	GLY 184	GLY 174	GLY 182	GLY 179
62	**LEU**	LEU 185	LEU 175	LEU 183	LEU 180
63	**TYR**	TYR 186	TYR 176	TYR 184	TYR 181
64	**SER**	SER 187	SER 177	SER 185	SER 182
65	**LEU**	LEU 188	LEU 178	LEU 186	LEU 183
66	**SER**	SER 189	SER 179	SER 187	SER 184
67	**SER**	SER 190	SER 180	SER 188	SER 185
68	**VAL**	VAL 191	VAL 181	VAL 189	VAL 186
69	**VAL**	VAL 192	VAL 182	VAL 190	VAL 187
70	**THR**	THR 193	THR 183	THR 191	THR 188
71	**VAL**	VAL 194	VAL 184	VAL 192	VAL 189

72	**PRO**	PRO 195	PRO 185	PRO 193	PRO 190
73	**SER**	SER 196	SER 186	SER 194	SER 191
74	**SER**	SER 197	SER 187	SER 195	SER 192
75	**SER**	SER 198	SER 188	SER 196	SER 193
76	**LEU**	LEU 199	LEU 189	LEU 197	LEU 194
77	**GLY**	GLY 200	GLY 190	GLY 198	GLY 195
78	**THR**	THR 201	THR 191	THR 199	THR 196
79	**GLN**	GLN 202	GLN 192	GLN 200	GLN 197
80	**THR**	THR 203	THR 193	THR 201	THR 198
81	**TYR**	TYR 204	TYR 194	TYR 202	TYR 199
82	**ILE**	ILE 205	ILE 195	ILE 203	ILE 200
83	**CYS**	CYS 206	CYS 196	CYS 204	CYS 201
84	**ASN**	ASN 207	ASN 197	ASN 205	ASN 202
85	**VAL**	VAL 208	VAL 198	VAL 206	VAL 203
86	**ASN**	ASN 209	ASN 199	ASN 207	ASN 204
87	**HIS**	HIS 210	HIS 200	HIS 208	HIS 205
88	**LYS**	LYS 211	LYS 201	LYS 209	LYS 206
89	**PRO**	PRO 212	PRO 202	PRO 210	PRO 207
90	**SER**	SER 213	SER 203	SER 211	SER 208
91	**ASN**	ASN 214	ASN 204	ASN 212	ASN 209
92	**THR**	THR 215	THR 205	THR 213	THR 210
93	**LYS**	LYS 216	LYS 206	LYS 214	LYS 211
94	**VAL**	VAL 217	VAL 207	VAL 215	VAL 212
95	**ASP**	ASP 218	ASP 208	ASP 216	ASP 213
96	**LYS**	LYS 219	LYS 209	LYS 217	LYS 214
97	**LYS**	LYS 220	LYS 210	LYS 218	LYS 215
98	**VAL**	VAL 221	VAL 211	VAL 219	VAL 216
99	**GLU**	GLU 222	GLU 212	GLU 220	GLU 217
100	**PRO**	PRO 223	PRO 213		PRO 218
101	**LYS**	LYS 224	LYS 214		LYS 219
102	**SER**		SER 215		SER 220
103	**CYS**		CYS 216		

Sequence recoding of Cκ

Recoding		**1cz8**	**1l7i**	**4nyl**	**1F8**
1	**ARG**	ARG 108	ARG 108	ARG 108	ARG 114
2	**THR**	THR 109	THR 109	THR 109	THR 115
3	**VAL**	VAL 110	VAL 110	VAL 110	VAL 116
4	**ALA**	ALA 111	ALA 111	ALA 111	ALA 117
5	**ALA**	ALA 112	ALA 112	ALA 112	ALA 118
6	**PRO**	PRO 113	PRO 113	PRO 113	PRO 119
7	**SER**	SER 114	SER 114	SER 114	SER 120
8	**VAL**	VAL 115	VAL 115	VAL 115	VAL 121
9	**PHE**	PHE 116	PHE 116	PHE 116	PHE 122
10	**ILE**	ILE 117	ILE 117	ILE 117	ILE 123
11	**PHE**	PHE 118	PHE 118	PHE 118	PHE 124

12	**PRO**	PRO 119	PRO 119	PRO 119	PRO 125
13	**PRO**	PRO 120	PRO 120	PRO 120	PRO 126
14	**SER**	SER 121	SER 121	SER 121	SER 127
15	**ASP**	ASP 122	ASP 122	ASP 122	ASP 128
16	**GLU**	GLU 123	GLU 123	GLU 123	GLU 129
17	**GLN**	GLN 124	GLN 124	GLN 124	GLN 130
18	**LEU**	LEU 125	LEU 125	LEU 125	LEU 131
19	**LYS**	LYS 126	LYS 126	LYS 126	LYS 132
20	**SER**	SER 127	SER 127	SER 127	SER 133
21	**GLY**	GLY 128	GLY 128	GLY 128	GLY 134
22	**THR**	THR 129	THR 129	THR 129	THR 135
23	**ALA**	ALA 130	ALA 130	ALA 130	ALA 136
24	**SER**	SER 131	SER 131	SER 131	SER 137
25	**VAL**	VAL 132	VAL 132	VAL 132	VAL 138
26	**VAL**	VAL 133	VAL 133	VAL 133	VAL 139
27	**CYS**	CYS 134	CYS 134	CYS 134	CYS 140
28	**LEU**	LEU 135	LEU 135	LEU 135	LEU 141
29	**LEU**	LEU 136	LEU 136	LEU 136	LEU 142
30	**ASN**	ASN 137	ASN 137	ASN 137	ASN 143
31	**ASN**	ASN 138	ASN 138	ASN 138	ASN 144
32	**PHE**	PHE 139	PHE 139	PHE 139	PHE 145
33	**TYR**	TYR 140	TYR 140	TYR 140	TYR 146
34	**PRO**	PRO 141	PRO 141	PRO 141	PRO 147
35	**ARG**	ARG 142	ARG 142	ARG 142	ARG 148
36	**GLU**	GLU 143	GLU 143	GLU 143	GLU 149
37	**ALA**	ALA 144	ALA 144	ALA 144	ALA 150
38	**LYS**	LYS 145	LYS 145	LYS 145	LYS 151
39	**VAL**	VAL 146	VAL 146	VAL 146	VAL 152
40	**GLN**	GLN 147	GLN 147	GLN 147	GLN 153
41	**TRP**	TRP 148	TRP 148	TRP 148	TRP 154
42	**LYS**	LYS 149	LYS 149	LYS 149	LYS 155
43	**VAL**	VAL 150	VAL 150	VAL 150	VAL 156
44	**ASP**	ASP 151	ASP 151	ASP 151	ASP 157
45	**ASN**	ASN 152	ASN 152	ASN 152	ASN 158
46	**ALA**	ALA 153	ALA 153	ALA 153	ALA 159
47	**LEU**	LEU 154	LEU 154	LEU 154	LEU 160
48	**GLN**	GLN 155	GLN 155	GLN 155	GLN 161
49	**SER**	SER 156	SER 156	SER 156	SER 162
50	**GLY**	GLY 157	GLY 157	GLY 157	GLY 163
51	**ASN**	ASN 158	ASN 158	ASN 158	ASN 164
52	**SER**	SER 159	SER 159	SER 159	SER 165
53	**GLN**	GLN 160	GLN 160	GLN 160	GLN 166
54	**GLU**	GLU 161	GLU 161	GLU 161	GLU 167
55	**SER**	SER 162	SER 162	SER 162	SER 168

56	**VAL**	VAL 163	VAL 163	VAL 163	VAL 169
57	**THR**	THR 164	THR 164	THR 164	THR 170
58	**GLU**	GLU 165	GLU 165	GLU 165	GLU 171
59	**GLN**	GLN 166	GLN 166	GLN 166	GLN 172
60	**ASP**	ASP 167	ASP 167	ASP 167	ASP 173
61	**SER**	SER 168	SER 168	SER 168	SER 174
62	**LYS**	LYS 169	LYS 169	LYS 169	LYS 175
63	**ASP**	ASP 170	ASP 170	ASP 170	ASP 176
64	**SER**	SER 171	SER 171	SER 171	SER 177
65	**THR**	THR 172	THR 172	THR 172	THR 178
66	**TYR**	TYR 173	TYR 173	TYR 173	TYR 179
67	**SER**	SER 174	SER 174	SER 174	SER 180
68	**LEU**	LEU 175	LEU 175	LEU 175	LEU 181
69	**SER**	SER 176	SER 176	SER 176	SER 182
70	**SER**	SER 177	SER 177	SER 177	SER 183
71	**THR**	THR 178	THR 178	THR 178	THR 184
72	**LEU**	LEU 179	LEU 179	LEU 179	LEU 185
73	**THR**	THR 180	THR 180	THR 180	THR 186
74	**LEU**	LEU 181	LEU 181	LEU 181	LEU 187
75	**SER**	SER 182	SER 182	SER 182	SER 188
76	**LYS**	LYS 183	LYS 183	LYS 183	LYS 189
77	**ALA**	ALA 184	ALA 184	ALA 184	ALA 190
78	**ASP**	ASP 185	ASP 185	ASP 185	ASP 191
79	**TYR**	TYR 186	TYR 186	TYR 186	TYR 192
80	**GLU**	GLU 187	GLU 187	GLU 187	GLU 193
81	**LYS**	LYS 188	LYS 188	LYS 188	LYS 194
82	**HIS**	HIS 189	HIS 189	HIS 189	HIS 195
83	**LYS**	LYS 190	LYS 190	LYS 190	LYS 196
84	**VAL**	VAL 191	VAL 191	VAL 191	VAL 197
85	**TYR**	TYR 192	TYR 192	TYR 192	TYR 198
86	**ALA**	ALA 193	ALA 193	ALA 193	ALA 199
87	**CYS**	CYS 194	CYS 194	CYS 194	CYS 200
88	**GLU**	GLU 195	GLU 195	GLU 195	GLU 201
89	**VAL**	VAL 196	VAL 196	VAL 196	VAL 202
90	**THR**	THR 197	THR 197	THR 197	THR 203
91	**HIS**	HIS 198	HIS 198	HIS 198	HIS 204
92	**GLN**	GLN 199	GLN 199	GLN 199	GLN 205
93	**GLY**	GLY 200	GLY 200	GLY 200	GLY 206
94	**LEU**	LEU 201	LEU 201	LEU 201	LEU 207
95	**SER**	SER 202	SER 202	SER 202	SER 208
96	**SER**	SER 203	SER 203	SER 203	SER 209
97	**PRO**	PRO 204	PRO 204	PRO 204	PRO 210
98	**VAL**	VAL 205	VAL 205	VAL 205	VAL 211
99	**THR**	THR 206	THR 206	THR 206	THR 212

100	**LYS**	LYS 207	LYS 207	LYS 207	LYS 213
101	**SER**	SER 208	SER 208	SER 208	SER 214
102	**PHE**	PHE 209	PHE 209	PHE 209	PHE 215
103	**ASN**	ASN 210	ASN 210	ASN 210	ASN 216
104	**ARG**	ARG 211	ARG 211	ARG 211	ARG 217
105	**GLY**	GLY 212	GLY 212		GLY 218
106	**GLU**	GLU 213	GLU 213		GLU 219
107	**CYS**		CYS 214		

Summary table of top Free energy residues of 1cz8,4nyl,1l7i and 1F8

1cz8			**4nyl**			**1l7i**			**1F8**		
53	PHE		53	PHE		103	**CYS**		53	PHE	**CH1**
9	PHE		9	PHE		53	PHE		9	PHE	
11	LEU		11	LEU		9	PHE		11	LEU	
56	VAL		56	VAL		**101**	**LYS**		56	VAL	
28	LEU		28	LEU		56	VAL		**30**	**LYS**	
51	HIS		54	PRO		11	LEU		96	LYS	
54	PRO		68	VAL		54	PRO		28	LEU	
68	VAL		24	ALA		28	LEU		24	ALA	
24	ALA		51	HIS		**17**	**SER**		68	VAL	
						68	VAL		54	PRO	
						24	ALA				
						96	LYS				
11	PHE		11	PHE		11	PHE		11	PHE	**Cκ**
9	PHE		9	PHE		9	PHE		9	PHE	
28	LEU		28	LEU		100	LYS		100	LYS	
26	VAL		57	THR		57	THR		57	THR	
53	GLN		26	VAL		**107**	**CYS**		28	LEU	
14	**SER**		53	GLN		28	LEU		26	VAL	
30	ASN		30	ASN		26	VAL		53	GLN	
						53	GLN				
						12	**PRO**				

Bold: Unique residues

Underlined: Low homologous residues

No marking: Conserved residues

Residues with most stabilizing effects

1cz8			4nyl			1l7i			1F8	
CH										
9	PHE		9	PHE					9	PHE
51	PHE		11	LEU						
53	PHE		53	PHE					53	PHE
			54	PRO		103	CYS			
CK										
			9	PHE		9	PHE		9	PHE
11	PHE		11	PHE		11	PHE		11	PHE

Five important interface residues for C_K and CH1 interaction (based on structure and free energy)

CH1		Cκ	
Position	Residue	Position	Residue
9	PHE	17	GLN
11	LEU	11, 26	PHE, VAL
24	ALA	9,11	PHE
51	HIS	31	ASN
53	PHE	69	SER

Note: Salt bridge residues are excluded

Example 2: Discovery of Important Interface Residues for Cκ and CH1 Interaction

[0126] Based on the interface analysis of Cκ and CH1, this example summarized the top important interface residues for Cκ and CH1 interaction (see **FIG. 2** and Table 4 below).

Table 4. Residue Pairs Impacting Cκ/CH1 Interaction

CH1		Cκ		Pair No.
Position	Residue	Position	Residue	
9	PHE	17	GLN	1
11	LEU	11, 26	PHE, VAL	2
24	ALA	9, 11	PHE	3
51	HIS	31	ASN	4
53	PHE	69	SER	5

Note: Salt bridge residues are excluded

[0127] From the table above, alanine or tryptophan single mutations were used to test each interface residue. IgG(-Fv) without VH and VL was constructed and expressed for Ala and Trp screening. The mutation list is listed as below.

Alanine screening

Name	Description	Cκ	CH1
Cκ/CH1_001	Cκ/CH1	WT	WT
Cκ/CH1_002	Cκ_L28Y _S69W/CH1_H51A_ F53G	L28Y _S69W	H51A_ F53G
Cκ/CH1_003	Cκ/CH1_H51A_ F53G	WT	H51A_ F53G
Cκ/CH1_004	Cκ/CH1_ D31K_F53T_V68F	WT	D31K_F53T_V68F
Cκ/CH1_005	Cκ/CH1_F9A_F53A	WT	F9A_F53A
Cκ/CH1_006	Cκ _F9G_F11A_K100A/CH1	F9G_F11A_K100A	WT
Cκ/CH1_007	Cκ _F11A/CH1	F11A	WT
Cκ/CH1_008	Cκ _F9A_F11A/CH1	F9A_F11A	WT
Cκ/CH1_009	Cκ _F11A_K100A/CH1	F11A_K100A	WT
Cκ/CH1_010	Cκ _F9A_K100A/CH1	F9A_K100A	WT
Cκ/CH1_011	Cκ/CH1_F9A_ L11A	WT	F9A_ L11A
Cκ/CH1_012	Cκ/CH1_L11A_ F53A	WT	L11A_ F53A
Cκ/CH1_013	Cκ/CH1_F9A	WT	F9A
Cκ/CH1_014	Cκ/CH1_L11A	WT	L11A
Cκ/CH1_015	Cκ_F9A/CH1	F9A	WT
Cκ/CH1_016	Cκ_F9A_F11M/CH1	F9A_F11M	WT
Cκ/CH1_017	Cκ/CH1_A24F	WT	A24F
Cκ/CH1_018	Cκ/CH1_A24L	WT	A24L
Cκ/CH1_019	Cκ_F9A_F11A/CH1_A24F	F9A_F11A	A24F
Cκ/CH1_020	Cκ_F9A_F11A/CH1_A24L	F9A_F11A	A24L
Cκ/CH1_021	Cκ_F9A_F11M/CH1_A24F	F9A_F11M	A24F
Cκ/CH1_022	Cκ_F9A_F11M/CH1_A24L	F9A_F11M	A24L
Cκ/CH1_023	Cκ_V26A/CH1	V26A	WT
Cκ/CH1_024	Cκ _V26A_F11A/CH1	V26A_F11A	WT
Cκ/CH1_025	Cκ/CH1_L11F_L28G	WT	L11F_L28G
Cκ/CH1_026	Cκ_V26A/CH1_L11F_L28G	V26A	L11F_L28G
Cκ/CH1_027	Cκ_V26A_F11A/CH1_L11F_L28G	V26A_F11A	L11F_L28G

Tryptophan screening

Name	Description	Cκ	CH1
Cκ/CH1_028	Cκ/CH1_A24W	WT	A24W
Cκ/CH1_029	Cκ/CH1_L11F	WT	L11F
Cκ/CH1_030	Cκ/CH1_L11W	WT	L11W
Cκ/CH1_031	Cκ_F9A_F11A/CH1_L11F_A24F	F9A_F11A	L11F_A24F
Cκ/CH1_032	Cκ_V26W/CH1	V26W	WT

[0128] As shown in the SDS-PAGE image of **FIG. 3**, for Pair 2 (Cκ_F11_V26 and CH1_L11), the two mutants Cκ_F11A/CH1 and Cκ_V26A/CH1 greatly interrupted the interaction of Cκ and CH1; the two mutants Cκ_V26W/CH1 and Cκ /CH1_L11W also disrupted the interaction(FIG4). Mutations Cκ/CH1_L11A and Cκ/CH1_F9A (from Pair 1) also disrupted the interaction. Mutants Cκ_F9A/CH1, Cκ/CH1_A24F and Cκ/CH1_A24L, by contrast, did not affect the interaction of Cκ and CH1. This suggests that Pair 3 (Cκ_F9 and CH1_A24) is not important for the binding of Cκ and CH1.

Pair No.	CH1	Cκ	Important
Pair 1	Phe9	Gln17	Yes
Pair 2	Leu11	Phe11, Val26	Yes
Pair 3	Ala24	Phe9	No

Example 3: Mutation Pair Development for Pair 1 by Discovery Studio

[0129] Upon identification of residue pairs that are important for maintaining the interaction between Cκ and CH1, this example tested mutation pairs that establish new interactions. The rationale of this development is that: mutant Cκ can show good binding to mutant CH1; but mutant Cκ does not bind or weakly bind to wild type CH1 and mutant CH1 show weak or no binding to wild type Cκ.

Mutation development for Pair 1

[0130] The residues in Pair 1 are Cκ_Q17 and CH1_F9 (Table 4). These mutations of Cκ/ CH1_033 to 050 were designed and analyzed by the inventors. Cκ/ CH1_051-066 mutation pairs were developed by a software program, Discovery Studio (DS), to design random mutations for this site. It generated eight pairs for Cκ_Q17 and CH1_F9 as listed below.

Mutation	Mutation energy	Effect	VDW	Electro-static	Entropy	Non-polar
H:PHE9>ILE.L:GLN17>HIS	0.03	Neutral	0.15	0.03	-0.07	0
H:PHE9>HIS.L:GLN17>ARG	0.07	Neutral	-1.42	1.4	0.09	0
H:PHE9>LYS.L:GLN17>ASP	0.09	Neutral	2.15	-3.23	0.72	0
H:PHE9>HIS.L:GLN17>HIS	0.19	Neutral	0.16	-0.07	0.17	0
H:PHE9>PRO.L:GLN17>ARG	0.25	Neutral	0.13	1.33	-0.55	0
H:PHE9>MET.L:GLN17>HIS	0.29	Neutral	0.12	0.09	0.21	0
H:PHE9>GLN.L:GLN17>ARG	0.3	Neutral	-0.42	1.89	-0.49	0

H:PHE9>GLN.L:GLN17>HIS	0.33	Neutral	-0.46	-0.12	0.7	0

Notes: Mutation energy: energy difference after mutation; low value means more stable ; VDW: Van der Waals

Cκ/CH1_033	Cκ_Q17R/CH1	Q17R	WT
Cκ/CH1_034	Cκ_Q17K/CH1	Q17K	WT
Cκ/CH1_035	Cκ_Q17D/CH1	Q17D	WT
Cκ/CH1_036	Cκ_Q17E/CH1	Q17E	WT
Cκ/CH1_037	Cκ/CH1_F9R	WT	F9R
Cκ/CH1_038	Cκ/CH1_F9K	WT	F9K
Cκ/CH1_039	Cκ/CH1_F9D	WT	F9D
Cκ/CH1_040	Cκ/CH1_F9E	WT	F9E
Cκ/CH1_041	Cκ_F11E_V26A/CH1_L11R	F11E_V26A	L11R
Cκ/CH1_042	Cκ_Q17K_F11K_V26A/CH1_F9E_L11E	Q17K_F11K_V26A	F9E_L11E
Cκ/CH1_043	Cκ_Q17R/CH1_F9D	Cκ_Q17R	CH1_F9D
Cκ/CH1_044	Cκ_Q17K/CH1_F9D	Cκ_Q17K	CH1_F9D
Cκ/CH1_045	Cκ_Q17R/CH1_F9E	Cκ_Q17R	CH1_F9E
Cκ/CH1_046	Cκ_Q17K/CH1_F9E	Cκ_Q17K	CH1_F9E
Cκ/CH1_047	Cκ_Q17D/CH1_F9R	Cκ_Q17D	CH1_F9R
Cκ/CH1_048	Cκ_Q17D/CH1_F9K	Cκ_Q17D	CH1_F9K
Cκ/CH1_049	Cκ/CH1_F9D_L11A	Cκ	CH1_F9D_L11A
Cκ/CH1_050	Cκ_Q17K/CH1_F9D_L11A	Cκ_Q17K	CH1_F9D_L11A
Cκ/CH1_051	Cκ_Q17H/CH1_F9I	Cκ_Q17H	CH1_F9I
Cκ/CH1_052	Cκ_Q17R/CH1_F9H	Cκ_Q17R	CH1_F9H
Cκ/CH1_053	Cκ_Q17H/CH1_F9H	Cκ_Q17H	CH1_F9H
Cκ/CH1_054	Cκ_Q17R/CH1_F9P	Cκ_Q17R	CH1_F9P
Cκ/CH1_055	Cκ_Q17D/CH1_F9H	Cκ_Q17D	CH1_F9H
Cκ/CH1_056	Cκ_Q17I/CH1_F9H	Cκ_Q17I	CH1_F9H
Cκ/CH1_057	Cκ_Q17H/CH1_F9M	Cκ_Q17H	CH1_F9M
Cκ/CH1_058	Cκ_Q17R/CH1_F9Q	Cκ_Q17R	CH1_F9Q
Cκ/CH1_059	Cκ_Q17H/CH1_F9Q	Cκ_Q17H	CH1_F9Q
Cκ/CH1_060	Cκ_Q17H/CH1	Cκ_Q17H	CH1
Cκ/CH1_061	Cκ_Q17I/CH1	Cκ_Q17I	CH1
Cκ/CH1_062	Cκ/CH1_F9I	Cκ	CH1_F9I
Cκ/CH1_063	Cκ/CH1_F9H	Cκ	CH1_F9H
Cκ/CH1_064	Cκ/CH1_F9P	Cκ	CH1_F9P
Cκ/CH1_065	Cκ/CH1_F9M	Cκ	CH1_F9M
Cκ/CH1_066	Cκ/CH1_F9Q	Cκ	CH1_F9Q

[0131] Two good mutation pairs are listed below:

Mutation ID	**Position Numbering**	**Kabat Numbering**
Cκ/CH1_043	Cκ_Q17R/CH1_F9D	Cκ_Q124R/CH1_F122D
Cκ/CH1_044	Cκ_Q17K/CH1_F9D	Cκ_Q124K/CH1_F122D

Example 4: Mutation Pair Development for Pair 2 by Discovery Studio

[0132] For Pair 2, alanine/tryptophan single mutations were tested for each interface residue. IgG(-Fv) without VH and VL was constructed and expressed for Ala and Trp screening. This example used Discovery Studio to design random mutations for this site.

[0133] Three good mutation pairs are Cκ/CH1_072, Cκ/CH1_079 and Cκ/CH1_107 listed below:

Mutation ID	Position Numbering	Kabat Numbering
Cκ/CH1_072	Cκ_V26W/CH1_L11K_L28N	Cκ_V133W/CH1_L124K_L141N
Cκ/CH1_079	Cκ_F11W_V26G/CH1_L11W	Cκ_F118W_V133G/CH1_L124W
Cκ/CH1_107	Cκ_V26W/CH1_L11W	Cκ_V133W/CH1_L124W

Mutation development for Pair 2

[0134] The important residues for Pair 2 are Cκ_F11_V26 and CH1_L11_L28 (see Table 4). The strategy of mutation development for this hot spot is to fix mutation V26W or L11W. This example also tested introducing saturated point mutations for Cκ_F11_V26 and CH1_L11_L28; then applying DS to calculate all potent mutations.

[0135] Strategy I: with fixed mutation V26W, random point mutations were introduced into CH1_L11_L28; then DS software was used to generate some mutation pairs for this site. Some preferable mutation pairs are listed as below.

Mutation	Mutation energy	Effect	VDW	Electro-static	Entropy	Non-polar
H:LEU11>VAL.L:VAL26>TRP	-0.14	Neutral	-0.31	0.11	-0.04	0
H:LEU11>ASN.L:VAL26>TRP	-0.08	Neutral	-0.56	0.29	0.06	0
H:LEU11>MET.L:VAL26>TRP	-0.03	Neutral	-0.46	0.32	0.04	0
H:LEU11>ILE.L:VAL26>TRP	0.18	Neutral	-0.01	0.3	0.04	0
H:LEU11>SER.L:VAL26>TRP	0.31	Neutral	-0.83	0.58	0.49	0
H:LEU11>GLU.L:VAL26>TRP	0.38	Neutral	-1.76	2.45	0.04	0
H:LEU11>GLY.L:VAL26>TRP	0.41	Neutral	0.16	0.18	0.27	0

Mutation	Mutation energy	Effect	VDW	Electro-static	Entropy	Non-polar
H:LEU28>SER.L:VAL26>TRP	-0.54	Stabilizing	-2.58	0.34	0.66	0
H:LEU28>GLU.L:VAL26>TRP	-0.5	Neutral	-4.5	3.13	0.21	0
H:LEU28>ASN.L:VAL26>TRP	-0.43	Neutral	-2.26	0.37	0.58	0
H:LEU28>CYS.L:VAL26>TRP	-0.21	Neutral	-1.49	0.12	0.54	0
H:LEU28>THR.L:VAL26>TRP	-0.09	Neutral	-1.22	0.3	0.42	0
H:LEU28>VAL.L:VAL26>TRP	-0.06	Neutral	-1.14	0.16	0.49	0
H:LEU28>ALA.L:VAL26>TRP	0.1	Neutral	-1.04	0.12	0.64	0
H:LEU28>ASP.L:VAL26>TRP	0.38	Neutral	-2.6	2.36	0.57	0

Mutation	Mutation energy	Effect	VDW	Electro-static	Entropy	Non-polar
H:LEU11>ILE.H:LEU28>PHE.L:VAL26>TRP	-2.85	Stabilizing	-6.21	0.1	0.23	0
H:LEU11>ARG.H:LEU28>PRO.L:VAL26>TRP	-2.38	Stabilizing	-7.66	0.45	1.39	0
H:LEU11>ILE.H:LEU28>GLN.L:VAL26>TRP	-1.86	Stabilizing	-4.73	0.48	0.3	0
H:LEU11>ARG.H:LEU28>GLY.L:VAL26>TRP	-1.64	Stabilizing	-6.13	0.44	1.37	0
H:LEU11>ARG.H:LEU28>ASP.L:VAL26>TRP	-1.46	Stabilizing	-7.85	2.67	1.29	0

H:LEU11>LYS.H:LEU28>ASN.L:VAL26>TRP	-1.33	Stabilizing	-5.94	1.42	1.06	0
H:LEU11>THR.H:LEU28>HIS.L:VAL26>TRP	-1.32	Stabilizing	-4.18	0.55	0.56	0
H:LEU11>LEU.H:LEU28>THR.L:VAL26>TRP	-1.17	Stabilizing	-3.35	0.42	0.33	0
H:LEU11>ALA.H:LEU28>ARG.L:VAL26>TRP	-1.16	Stabilizing	-4.55	-0.57	1.59	0
H:LEU11>GLU.H:LEU28>GLN.L:VAL26>TRP	-1.12	Stabilizing	-5.43	2.65	0.31	0

[0136] Strategy 2: with fixed mutation L11W, random point mutations were introduced into Cκ_F11_V26; then the DS software was used to generate some mutation pairs for this site. Some preferable mutation pairs are listed as below.

Mutation	Mutation energy	Effect	VDW	Electro-static	Entropy	Non-polar
H:LEU11>TRP.L:VAL26>LEU	-2.24	Stabilizing	-4.78	0.32	-0.01	0
H:LEU11>TRP.L:VAL26>MET	-1.89	Stabilizing	-4.2	0.19	0.13	0
H:LEU11>TRP.L:VAL26>TRP	-1.38	Stabilizing	-3.01	0.58	-0.19	0
H:LEU11>TRP.L:VAL26>GLU	-1.21	Stabilizing	-3.88	0.87	0.34	0
H:LEU11>TRP.L:VAL26>LYS	-0.9	Stabilizing	-5.72	3.44	0.27	0
H:LEU11>TRP.L:VAL26>CYS	-0.84	Stabilizing	-1.95	0.12	0.09	0
H:LEU11>TRP.L:VAL26>SER	-0.68	Stabilizing	-2.65	0.42	0.49	0
H:LEU11>TRP.L:VAL26>ALA	-0.6	Stabilizing	-1.65	0.02	0.25	0
H:LEU11>TRP.L:VAL26>GLY	-0.26	Neutral	-1.66	0.16	0.56	0
H:LEU11>TRP.L:VAL26>PRO	-0.19	Neutral	-0.91	0.1	0.25	0

Mutation	Mutation energy	Effect	VDW	Electro-static	Entropy	Non-polar
H:LEU11>TRP.L:PHE11>HIS	-0.3	Neutral	-1.46	0.14	0.41	0

Mutation	Mutation energy	Effect	VDW	Electro-static	Entropy	Non-polar
H:LEU11>TRP.L:PHE11>TRP.L:VAL26>LYS	-1.94	Stabilizing	-7.43	3.17	0.22	0
H:LEU11>TRP.L:PHE11>HIS.L:VAL26>ARG	-1.68	Stabilizing	-7.35	2.56	0.81	0
H:LEU11>TRP.L:PHE11>TRP.L:VAL26>GLY	-1.64	Stabilizing	-4.45	0.07	0.63	0
H:LEU11>TRP.L:PHE11>HIS.L:VAL26>LEU	-1.58	Stabilizing	-4	0.3	0.31	0
H:LEU11>TRP.L:PHE11>ARG.L:VAL26>TYR	-1.56	Stabilizing	-6.43	2.37	0.54	0
H:LEU11>TRP.L:PHE11>ARG.L:VAL26>GLU	-1.34	Stabilizing	-6.03	1.87	0.84	0
H:LEU11>TRP.L:PHE11>HIS.L:VAL26>MET	-1.25	Stabilizing	-3.3	0.07	0.42	0
H:LEU11>TRP.L:PHE11>HIS.L:VAL26>TRP	-1.21	Stabilizing	-3.06	0.33	0.18	0
H:LEU11>TRP.L:PHE11>LEU.L:VAL26>ARG	-1.16	Stabilizing	-5.72	2.42	0.56	0

H:LEU11>TRP.L:PHE11>ARG.L:VAL26>LEU	-1.09	Stabilizing	-5.83	2.2	0.82	0

[0137] Strategy 3: saturated point mutations were introduced for Cκ_F11_V26 and CH1_L11_L28; then DS was used to calculate all potent mutations. It generated 23 preferable mutation pairs listed below.

Mutation	Mutation energy	Effect	VDW	Electro-static	Entropy	Non-polar
H:LEU11>VAL.L:VAL26>TRP	-0.14	Neutral	-0.31	0.11	-0.04	0
H:LEU11>ASN.L:VAL26>TRP	-0.08	Neutral	-0.56	0.29	0.06	0
H:LEU11>MET.L:VAL26>TRP	-0.03	Neutral	-0.46	0.32	0.04	0
H:LEU11>MET.L:VAL26>GLU	0.14	Neutral	-0.04	-0.17	0.28	0
H:LEU11>ASN.L:VAL26>GLU	0.16	Neutral	-0.65	0.14	0.47	0
H:LEU11>ILE.L:VAL26>TRP	0.18	Neutral	-0.01	0.3	0.04	0
H:LEU11>PRO.L:VAL26>GLU	0.28	Neutral	0.39	-0.52	0.39	0
H:LEU11>MET.L:VAL26>LEU	0.3	Neutral	0.78	0.05	-0.13	0
H:LEU11>SER.L:VAL26>TRP	0.31	Neutral	-0.83	0.58	0.49	0
H:LEU11>VAL.L:VAL26>GLU	0.34	Neutral	0.22	-0.32	0.44	0
H:LEU11>GLU.L:VAL26>TRP	0.38	Neutral	-1.76	2.45	0.04	0
H:LEU11>GLY.L:VAL26>TRP	0.41	Neutral	0.16	0.18	0.27	0
H:LEU11>ILE.L:VAL26>GLU	0.43	Neutral	0.38	-0.22	0.4	0
H:LEU11>MET.L:VAL26>MET	0.44	Neutral	0.91	0.01	-0.02	0
H:LEU28>SER.L:VAL26>TRP	-0.54	Stabilizing	-2.58	0.34	0.66	0
H:LEU28>GLU.L:VAL26>TRP	-0.5	Neutral	-4.5	3.13	0.21	0
H:LEU28>ASN.L:VAL26>TRP	-0.43	Neutral	-2.26	0.37	0.58	0
H:LEU28>CYS.L:VAL26>TRP	-0.21	Neutral	-1.49	0.12	0.54	0
H:LEU28>THR.L:VAL26>TRP	-0.09	Neutral	-1.22	0.3	0.42	0
H:LEU28>VAL.L:VAL26>TRP	-0.06	Neutral	-1.14	0.16	0.49	0
H:LEU28>ALA.L:VAL26>TRP	0.1	Neutral	-1.04	0.12	0.64	0
H:LEU28>ASP.L:VAL26>TRP	0.38	Neutral	-2.6	2.36	0.57	0
H:LEU28>VAL.L:VAL26>GLU	0.42	Neutral	-0.19	-0.07	0.62	0

[0138] Based on the above the mutation pairs, for Pair 1, all of the mutation pairs were analyzed by SDS-PAGE (Reduced and Non-Reduced, **FIG. 4A-D**); for pair 2, some potent mutation pairs with the lowest free energy were chosen for analysis. Among the all mutation pairs, three mutation pairs Cκ/CH1_107 are more potent. The results can be comparable to published mutation pair. IgG(-Fv) without VH and VL was constructed and expressed for each mutation pair. Mutation list is listed as below.

[0139] Three good mutation pairs are Cκ/CH1_072, Cκ/CH1_079 and Cκ/CH1_107 listed below:

Cκ/CH1_072	Cκ_V26W/CH1_L11K_L28N
Cκ/CH1_079	Cκ_F11W_V26G/CH1_L11W
Cκ/CH1_107	Cκ_V26W/CH1_L11W

[0140] As shown in the SDS-PAGE gel pictures in **FIG. 5A-5B**, mutation pair Cκ_V26W/CH1_L11W re-established binding between Cκ and CH1 (Cκ_L28Y_S69W/CH1_H51A_F53G was used as control).

Cκ/CH1_067	Cκ_V26W/CH1_L11I_L28F	Cκ_V26W	CH1_L11I_L28F
Cκ/CH1_068	Cκ_V26W/CH1_L11R_L28P	Cκ_V26W	CH1_L11R_L28P
Cκ/CH1_069	Cκ_V26W/CH1_L11I_L28Q	Cκ_V26W	CH1_L11I_L28Q
Cκ/CH1_070	Cκ_V26W/CH1_L11R_L28G	Cκ_V26W	CH1_L11R_L28G
Cκ/CH1_071	Cκ_V26W/CH1_L11R_L28D	Cκ_V26W	CH1_L11R_L28D
Cκ/CH1_072	Cκ_V26W/CH1_L11K_L28N	Cκ_V26W	CH1_L11K_L28N
Cκ/CH1_073	Cκ_V26W/CH1_L11T_L28H	Cκ_V26W	CH1_L11T_L28H
Cκ/CH1_074	Cκ_V26W/CH1_L28T	Cκ_V26W	CH1_L28T
Cκ/CH1_075	Cκ_V26W/CH1_L11A_L28R	Cκ_V26W	CH1_L11A_L28R
Cκ/CH1_076	Cκ_V26W/CH1_L11E_L28Q	Cκ_V26W	CH1_L11E_L28Q
Cκ/CH1_077	Cκ_F11W_V26K/CH1_L11W	Cκ_F11W_V26K	CH1_L11W
Cκ/CH1_078	Cκ_F11H_V26R/CH1_L11W	Cκ_F11H_V26R	CH1_L11W
Cκ/CH1_079	Cκ_F11W_V26G/CH1_L11W	Cκ_F11W_V26G	CH1_L11W
Cκ/CH1_080	Cκ_F11H_V26L/CH1_L11W	Cκ_F11H_V26L	CH1_L11W
Cκ/CH1_081	Cκ_F11R_V26Y/CH1_L11W	Cκ_F11R_V26Y	CH1_L11W
Cκ/CH1_082	Cκ_F11R_V26E/CH1_L11W	Cκ_F11R_V26E	CH1_L11W
Cκ/CH1_083	Cκ_F11H_V26M/CH1_L11W	Cκ_F11H_V26M	CH1_L11W
Cκ/CH1_084	Cκ_F11H_V26W/CH1_L11W	Cκ_F11H_V26W	CH1_L11W
Cκ/CH1_085	Cκ_F11L_V26R/CH1_L11W	Cκ_F11L_V26R	CH1_L11W
Cκ/CH1_086	Cκ_F11R_V26L/CH1_L11W	Cκ_F11R_V26L	CH1_L11W
Cκ/CH1_087	Cκ/CH1_L11I_L28F	Cκ	CH1_L11I_L28F
Cκ/CH1_088	Cκ/CH1_L11R_L28P	Cκ	CH1_L11R_L28P
Cκ/CH1_089	Cκ/CH1_L11I_L28Q	Cκ	CH1_L11I_L28Q
Cκ/CH1_090	Cκ/CH1_L11R_L28G	Cκ	CH1_L11R_L28G
Cκ/CH1_091	Cκ/CH1_L11R_L28D	Cκ	CH1_L11R_L28D
Cκ/CH1_092	Cκ/CH1_L11K_L28N	Cκ	CH1_L11K_L28N
Cκ/CH1_093	Cκ/CH1_L11T_L28H	Cκ	CH1_L11T_L28H
Cκ/CH1_094	Cκ/CH1_L28T	Cκ	CH1_L28T
Cκ/CH1_095	Cκ/CH1_L11A_L28R	Cκ	CH1_L11A_L28R
Cκ/CH1_096	Cκ/CH1_L11E_L28Q	Cκ	CH1_L11E_L28Q
Cκ/CH1_097	Cκ_F11W_V26K/CH1	Cκ_F11W_V26K	CH1
Cκ/CH1_098	Cκ_F11H_V26R/CH1	Cκ_F11H_V26R	CH1
Cκ/CH1_099	Cκ_F11W_V26G/CH1	Cκ_F11W_V26G	CH1
Cκ/CH1_100	Cκ_F11H_V26L/CH1	Cκ_F11H_V26L	CH1
Cκ/CH1_101	Cκ_F11R_V26Y/CH1	Cκ_F11R_V26Y	CH1
Cκ/CH1_102	Cκ_F11R_V26E/CH1	Cκ_F11R_V26E	CH1
Cκ/CH1_103	Cκ_F11H_V26M/CH1	Cκ_F11H_V26M	CH1
Cκ/CH1_104	Cκ_F11H_V26W/CH1	Cκ_F11H_V26W	CH1
Cκ/CH1_105	Cκ_F11L_V26R/CH1	Cκ_F11L_V26R	CH1
Cκ/CH1_106	Cκ_F11R_V26L/CH1	Cκ_F11R_V26L	CH1
Cκ/CH1_107	Cκ_V26W/CH1_L11W	Cκ_V26W	CH1_L11W

Example 5: Mutation pair Cκ_V26W/CH1_L11W improvement by Discovery Studio

[0141] Strategy 4: With fixed mutation Cκ_V26W and CH1_L11W, saturated point mutations were introduced for Cκ_F11 and CH1_L28; then DS was used to calculate all potent mutations. It generated 23 preferable mutation pairs listed below.

mutation	mutation energy	effect	VDW	ELECTROSTATIC	ENTROPY	non-polar	double mutation
H:LEU11>TRP.L:PHE11>HIS.L:VAL26>TRP	-1.21	STABILIZING	-3.06	0.33	0.18	0	1.17
H:LEU11>TRP.L:PHE11>ALA.L:VAL26>TRP	-0.27	NEUTRAL	-0.35	-0.18	0	0	4.52
H:LEU11>TRP.H:LEU28>ARG.L:VAL26>TRP	-0.78	STABILIZING	-3.18	0.44	0.67	0	0.72
H:LEU11>TRP.H:LEU28>PRO.L:VAL26>TRP	0.42	NEUTRAL	-0.63	0.6	0.49	0	2.86
H:LEU11>TRP.H:LEU28>ARG.L:PHE11>CYS.L:VAL26>TRP	-0.34	NEUTRAL	-2.12	-0.22	0.94	0	H:0.72 L:4.06
H:LEU11>TRP.H:LEU28>ARG.L:PHE11>ILE.L:VAL26>TRP	-0.25	NEUTRAL	-1.76	0.41	0.48	0	H:0.72 L:2.87
H:LEU11>TRP.H:LEU28>ARG.L:PHE11>PRO.L:VAL26>TRP	-0.14	NEUTRAL	-1.77	-0.07	0.89	0	H:0.72 L:3.18
H:LEU11>TRP.H:LEU28>ARG.L:PHE11>ARG.L:VAL26>TRP	-0.06	NEUTRAL	-5.26	2.92	1.26	0	H:0.72 L:3.37
H:LEU11>TRP.H:LEU28>ARG.L:PHE11>MET.L:VAL26>TRP	0.43	NEUTRAL	-1.35	0.13	1.18	0	H:0.72 L:2.97

Example 6: Mutation Pair Development

[0142] For Pair 2, alanine/tryptophan single mutations were tested for each interface residue. IgG(-Fv) without VH and VL was constructed and expressed for Ala and Trp screening. Mutation list is listed as below.

Name	Description	Cκ	CH1
Cκ/CH1_001	Cκ/CH1	WT	WT
Cκ/CH1_002	Cκ_L28Y_S69W/CH1_H51A_F53G	L28Y_S69W	H51A_F53G
Cκ/CH1_003	Cκ/CH1_H51A_F53G	WT	H51A_F53G
Cκ/CH1_004	Cκ/CH1_D31K_F53T_V68F	WT	D31K_F53T_V68F
Cκ/CH1_005	Cκ/CH1_F9A_F53A	WT	F9A_F53A
Cκ/CH1_006	Cκ_F9G_F11A_K100A/CH1	F9G_F11A_K100A	WT
Cκ/CH1_007	Cκ_F11A/CH1	F11A	WT
Cκ/CH1_008	Cκ_F9A_F11A/CH1	F9A_F11A	WT
Cκ/CH1_009	Cκ_F11A_K100A/CH1	F11A_K100A	WT
Cκ/CH1_010	Cκ_F9A_K100A/CH1	F9A_K100A	WT
Cκ/CH1_011	Cκ/CH1_F9A_L11A	WT	F9A_L11A
Cκ/CH1_012	Cκ/CH1_L11A_F53A	WT	L11A_F53A
Cκ/CH1_013	Cκ/CH1_F9A	WT	F9A
Cκ/CH1_014	Cκ/CH1_L11A	WT	L11A
Cκ/CH1_015	Cκ_F9A/CH1	F9A	WT

Cκ/CH1_016	Cκ_F9A_F11M/CH1	F9A_F11M	WT
Cκ/CH1_017	Cκ/CH1_A24F	WT	A24F
Cκ/CH1_018	Cκ/CH1_A24L	WT	A24L
Cκ/CH1_019	Cκ_F9A_F11A/CH1_A24F	F9A_F11A	A24F
Cκ/CH1_020	Cκ_F9A_F11A/CH1_A24L	F9A_F11A	A24L
Cκ/CH1_021	Cκ_F9A_F11M/CH1_A24F	F9A_F11M	A24F
Cκ/CH1_022	Cκ_F9A_F11M/CH1_A24L	F9A_F11M	A24L
Cκ/CH1_023	Cκ_V26A/CH1	V26A	WT
Cκ/CH1_024	Cκ_V26A_F11A/CH1	V26A_F11A	WT
Cκ/CH1_025	Cκ/CH1_L11F_L28G	WT	L11F_L28G
Cκ/CH1_026	Cκ_V26A/CH1_L11F_L28G	V26A	L11F_L28G
Cκ/CH1_027	Cκ_V26A_F11A/CH1_L11F_L28G	V26A_F11A	L11F_L28G
Cκ/CH1_028	Cκ/CH1_A24W	WT	A24W
Cκ/CH1_029	Cκ/CH1_L11F	WT	L11F
Cκ/CH1_030	Cκ/CH1_L11W	WT	L11W
Cκ/CH1_031	Cκ_F9A_F11A/CH1_L11F_A24F	F9A_F11A	L11F_A24F
Cκ/CH1_032	Cκ_V26W/CH1	V26W	WT

Example 7: Alteration of Salt Bridges

[0143] The interface interaction analysis for Ck and CH1 in example 1 has shown that the common salt bridge between CH1 and Ck of 1F8, 1CZ8, 1L7I and 4NYL is below:

CH1			**Cκ**			**Distance (Å)**
Position	Residue	Atom Name	Position	Residue	Atom Name	
96	LYS	NZ	16	GLU	OE2	2.7-3.4

[0144] There is one more salt bridge in 1F8 and 1CZ8:

CH1			**Cκ**			**Distance (Å)**
Position	Residue	Atom Name	Position	Residue	Atom Name	
101	LYS	NZ	15	ASP	OD2	2.7-3.8

[0145] Therefore, this example focused on CH1 and Ck of 1F8 with two salt bridges and utilized the Discovery Studio to design new salt bridge pairs within CH1 and Ck that disfavor the binding of mutated CH1 or Ck to their WT counterpart and rebuild the binding between the mutated CH and Ck with a new salt bridge.

[0146] The design on the salt bridge CH1_LYS96 and Ck_GLU16. As shown in the below table, two pairs showed to stabilize $CH1^{mut}$ and Ck^{mut} with new salt bridge:

CH1: LYS96>ASP mutation and Ck: GLU16>ARG mutation;

CH1: LYS96>GLU mutation and Ck: GLU16>ARG mutation;

Mutation	Mutation Energy	Effect	VDW	Electro static	Entropy	Non-polar	Single Mutation
H:LYS96>ASP.L:GLU16>ARG	-1.06	Stabilizing	-2.86	1.02	-0.16	0	1.02 1.32
H:LYS96>GLU.L:GLU16>ARG	-0.52	Stabilizing	-2.38	0.98	0.21	0	1.28 1.32
H:LYS96>ASP.L:GLU16>LYS	-0.41	Neutral	-2.06	1.08	0.09	0	1.02 1.84
H:LYS96>GLU.L:GLU16>HIS	0.12	Neutral	-0.24	1.74	-0.72	0	1.28 0.79
H:LYS96>GLU.L:GLU16>LYS	0.26	Neutral	-1.6	1.43	0.39	0	1.28 1.84

[0147] Discovery Studio was further used to find new salt bridge that could be in synergy with new Cκ_V26W and CH1_L11W to disfavor the binding of mutated CH1 or Ck to their WT counterpart and rebuild the binding between the mutated CH and Cκ. As shown in the below tables: three pairs showed to stabilize $CH1^{mut}$ and Ck^{mut} with in synergy with Cκ_V26W and CH1_L11W:

CH1: LEU11>TRP; LYS96>GLU mutation and Ck: GLU16>LYS; VAL26>TRP mutation

CH1: LEU11>TRP; LYS96>GLU mutation and Ck: GLU16>ARG; VAL26>TRP mutation

CH1: LEU11>TRP; LYS101>GLU mutation and Ck: ASP15>LYS; VAL26>TRP mutation

Table 5: Mutations in CH1_K96/Cκ_E16

Mutation	Mutation Energy	Effect	VDW	Electro-static	Entropy	Non-polar	Double mutation
H:LEU11>TRP.H:LYS96>GLU.L:GLU16>LYS.L:VAL26>TRP	-1.06	Stabilizing	-4.09	1.61	0.2	0	-0.34 3.39
H:LEU11>TRP.H:LYS96>GLU.L:GLU16>ARG.L:VAL26>TRP	-0.57	Stabilizing	-1.6	1.94	-0.84	0	-0.34 2.03
H:LEU11>TRP.H:LYS96>GLU.L:GLU16>HIS.L:VAL26>TRP	-0.5	Neutral	-0.79	2.11	-1.32	0	-0.34 1.11

Table 6: Mutations in CH1_K101/Cκ_D15

mutation	Mutation Energy	Effect	VDW	Electro-static	Entropy	non-polar	Double mutation
H:LEU11>TRP.H:LYS101>GLU L:ASP15>LYS.L:VAL26>TRP	-0.65	Stabilizing	-2.28	2.18	-0.68	0	0.58 1.57
H:LEU11>TRP.H:LYS101>GLU.L:ASP15>HIS.L:VAL26>TRP	-0.06	Neutral	-1.66	2.08	-0.31	0	0.58 1.66
H:LEU11>TRP.H:LYS101>ASP.L:ASP15>LYS.L:VAL26>TRP	0.27	Neutral	1.33	1.91	-1.53	0	0.62 1.57
H:LEU11>TRP.H:LYS101>ASP.L:ASP15>ARG.L:VAL26>TRP	0.44	Neutral	1.46	1.96	-1.44	0	0.62 1

Example 8: Testing of Altered Salt Bridges

[0148] Plasmids containing polynucleotides encoding CH1-CH2-CH3 or Cκ were constructed. Mutations were introduced in some of the domains as listed below.

[0149] Plasmids were transiently transfected into 293F cells for protein expression. The proteins were purified by protein A columns and anti-FLAG affinity gel, and the purified proteins were analyzed by SDS-PAGE (5 μg per lane). As protein A binds to the heavy chain only, the density of the light chains indicated strength of binding between the heavy chain and the light chain.

[0150] In the first batch, 13 antibodies were tested. The mutations included in these antibodies are listed in **Table 7**.

Table 7. Test antibodies with mutations

No.	Protein name	CH1-CH2CH3	Cκ
1	Cκ/CH1_001	WT	WT
2	Cκ/CH1_200	WT	E16R
3	Cκ/CH1_201	K96D	WT
4	Cκ/CH1_202	K96E	WT
5	Cκ/CH1_203	K96D	E16R
6	Cκ/CH1_204	K96E	E16R
7	Cκ/CH1_107	L11W	V26W
8	Cκ/CH1_205	L11W; K96E	WT
9	Cκ/CH1_206	WT	E16K; V26W
10	Cκ/CH1_207	L11W; K96E	E16K; V26W
11	Cκ/CH1_208	L11W; K96D	WT
12	Cκ/CH1_209	WT	V26W; E16R
13	Cκ/CH1_210	L11W; K96D	V26W; E16R

[0151] The results are shown in **FIG. 6A**. Good bindings were observed for Cκ/CH1_001 (wild-type) and Cκ/CH1_107 (L11W in CH1 and V26W in Cκ). Cκ/CH1_203 included a positive-to-negative and negative-to-positive mutation pair that disrupted the wild-type salt bridge (K96-E16). The binding in Cκ/CH1_210 (L11W and K96D in CH1 and V26W and E16R in Cκ) was markedly stronger than that between K96D and E16R. Each of the mutant chains, by contrast, more clearly failed to bind to the wild-type counterpart (see, Cκ/CH1_208 and Cκ/CH1_209).

[0152] The mutant chains in Cκ/CH1_207, CH1 with L11W and K96E, and Cκ with E16K and V26W also exhibited more binding within mutants than their wild-type counterparts (see, Cκ/CH1_205 and Cκ/CH1_206).

[0153] In the second batch, seven antibodies were tested. The mutations included in these antibodies are listed in **Table 8.**

Table 8. Test antibodies with mutations

	Protein name	CH1-CH2CH3	Ck
1	Cκ/CH1_001	Wt	Wt
2	Cκ/CH1_211	Wt	E16K
3	Cκ/CH1_202	K96E	Wt
4	Cκ/CH1_212	K96E	E16K
5	Cκ/CH1_205	L11W, K96E	Wt
6	Cκ/CH1_209	Wt	E16R,V26W
7	Cκ/CH1_213	L11W, K96E	E16R,V26W

[0154] The results are shown in **FIG. 6B**. The mutant chains in Cκ/CH1_213, CH1 with L11W and K96E, and Cκ with E16R and V26W exhibited more binding within mutants than their wild-type counterparts (see, Cκ/CH1_205 and Cκ/CH1_206).

[0155] In the third batch, fifteen antibodies were tested. The mutations included in these antibodies are listed in **Table 9**.

Table 9. Test antibodies with mutations

No.	Protein name	CH1-CH2CH3	Cκ
1	Cκ/CH1_001	WT	WT
2	Cκ/CH1_030	L11W	WT
3	Cκ/CH1_032	WT	V26W
4	Cκ/CH1_107	L11W	V26W
5	Cκ/CH1_201	K96D	WT
6	Cκ/CH1_214	WT	C16R, Q17A
7	Cκ/CH1_217	K96D	E16R, Q17A
8	Cκ/CH1_208	L11W, K96D	WT
9	Cκ/CH1_225	WT	E16R, Q17A, V26W
10	Cκ/CH1_226	L11W, K96D	E16R, Q17A, V26W
11	Cκ/CH1_221	WT	D15K, V26W
12	Cκ/CH1_222	WT	D15H, V26W
13	Cκ/CH1_220	L11W, K101E	WT

14	Cκ/CH1_223	L11W, K101E	D15K, V26W
15	Cκ/CH1_224	L11W, K101E	D15H, V26W

[0156] As shown in **FIG. 6C**, the reestablished salt bridges in Cκ/CH1_223 (K101E-D15K) and Cκ/CH1_224 (K101E-D15H) resulted in strong interactions between the mutated heavy and light chains, and each of them individually was more clearly unable to bind the wild-type counterpart as compared with Cκ/CH1_107 (L11W in CH1 and V26W in Cκ). The strong binding between the mutants, as shown in the figure, is also based on the hydrophobic interaction between L11W and V26W. In other words, the synergy between the hydrophobic interaction and the new salt bridge brings about strong binding and high specificity which will be useful for design of multi-specific antibodies.

Example 9: Bi-specific antibody construction

[0157] To further evaluate the effect of CH1/Ck mutations on light chain mismatch, we used IgG like heterodimer bi-specific format by using DE/EE mutations in CH3 domain (J. Biol. Chem. (2017) 292(35) 14706–14717). We constructed bi-specific antibodies by using the PDL1/CD73 pair.

[0158] The PDL1/CD73 pair design is described in the table below:

Protein	B5021		B5022		B5023		B5024	
CH3	DE/KK		DE/KK		DE/KK		DE/KK	
Fab	PDL1	CD73	PDL1	CD73	PDL1	CD73	PDL1	CD73
CH1	K96D	L11W	K96D	WT	L11W/K96D	WT	L11W/K96E	WT
Ck	E16K	V26W	E16K	WT	V26W/E16R	WT	V26W/E16K	WT

[0159] As shown in **FIG. 8A**, all the designed pairs didn't affect the PDL1 part binding by ELISA, while the binding potency of CD47 was impaired. B5024 Cκ/CH1_207 mutations (CH1:L11W/K96E; Cκ: E16K/V26W) and B5023 Cκ/CH1_210 mutations (CH1:L11W/K96D; Cκ: E16R/V26W) can restore the CD73 part antigen binding by ELISA. In addition, the PDL1 singling assay and CD73 enzymatic activity assay showed similar pattern with ELISA binding (**FIG. 8B**). In this regard, all the PDL1 part showed similar PDL1 antagonism activity and only B5024 and B5023 showed potent CD73 antagonist activity. In this pair, the light chain of PDL1 significantly impaired the function of CD73 arm, while CD73 light chain has little effect on PDL1 arm. Both Cκ/CH1_207 and

Cκ/CH1_210 mutations can restore the function of CD73 and didn't affect the PDL1 arm, suggesting CH1/Ck mutations can prevent the light chain mismatch.

* * *

[0160] The present disclosure is not to be limited in scope by the specific embodiments described which are intended as single illustrations of individual aspects of the disclosure, and any compositions or methods which are functionally equivalent are within the scope of this disclosure. It will be apparent to those skilled in the art that various modifications and variations can be made in the methods and compositions of the present disclosure without departing from the spirit or scope of the disclosure. Thus, it is intended that the present disclosure cover the modifications and variations of this disclosure provided they come within the scope of the appended claims and their equivalents.

[0161] All publications and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference

CLAIMS

What is claimed is:

1. An antibody or antigen-binding fragment thereof, comprising a first human CH1 fragment comprising a L11W substitution, a first human Cκ fragment comprising a V26W substitution, a second human CH1 fragment that does not include the L11W substitution, and a second human Cκ fragment that does not include the V26W substitution.

2. The antibody or antigen-binding fragment thereof of claim 1, wherein the first CH1 fragment comprises substitutions L11W and K101E and the first Cκ fragment comprises substitutions V26W and D15K/H.

3. The antibody or antigen-binding fragment thereof of claim 1, wherein the first CH1 fragment comprises substitutions L11W and K96D and the first Cκ fragment comprises substitutions V26W and E16R.

4. The antibody or antigen-binding fragment thereof of claim 1, wherein the first CH1 fragment comprises substitutions L11W and K96E and the first Cκ fragment comprises substitutions V26W and E16K.

5. The antibody or antigen-binding fragment thereof of claim 1, wherein the first CH1 fragment comprises substitutions L11W and K96E and the first Cκ fragment comprises substitutions V26W and E16R.

6. The antibody or antigen-binding fragment thereof of any one of claims 1-5, wherein the second human CH1 and the second human Cκ fragments are wild-type.

7. The antibody or antigen-binding fragment thereof of any one of claims 1-6, further comprising a heavy chain variable region, a light chain variable region, an Fc region, or the combination thereof.

8. The antibody or antigen-binding fragment thereof of claim 7, which is of class IgG.

9. The antibody or antigen-binding fragment thereof of claim 8, wherein the isotype is IgG1, IgG2, IgG3 or IgG4.

10. A bispecific antibody comprising a first CH1/Cκ pair and a second CH1/Cκ pair, wherein the CH1 and Cκ fragments of the first pair comprise amino acid substitutions L11W in CH1 and V26W in Cκ, and the CH1 and Cκ fragments of the second pair do not include the L11W and V26W substitutions.

11. The bispecific antibody of claim 10, wherein the CH1 and Cκ fragments of the first pair further comprise substitutions selected from the group consisting of (a) K101E in CH1 and D15K/H in Cκ, (b) K96D in CH1 and E16R in Cκ, and (c) K96E in CH1 and E16K in Cκ, and the CH1 and Cκ fragments of the second pair do not include the substitutions of (a)-(c).

12. A composition comprising the antibody or antigen-binding fragment thereof of any one of claims 1-11 and a pharmaceutically acceptable carrier.

13. An isolated cell comprising one or more polynucleotide encoding the antibody or antigen-binding fragment thereof of any one of claims 1-11.