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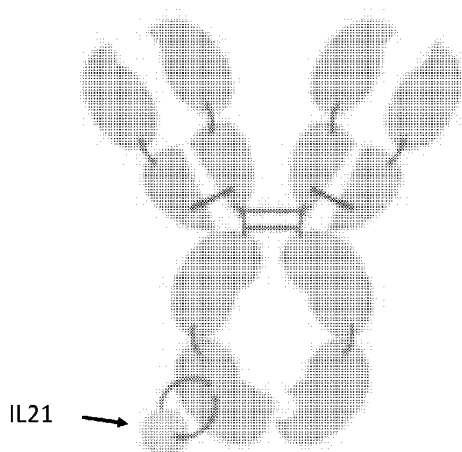
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Tiragolumab-IL21-3A-knob

FIG. 1

(57) Abstract: Provided are protein complexes targeting IL21 pathway and methods of use thereof. In some embodiments, the protein complexes also target TIGIT.

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PROTEIN COMPLEXES TARGETING IL21 PATHWAY

CLAIM OF PRIORITY

This application claims the benefit of PCT Patent Application No. PCT/CN2021/117918, filed on September 13, 2021. The entire contents of the foregoing application are incorporated
5 herein by reference.

TECHNICAL FIELD

This disclosure relates to protein complexes targeting IL21 pathway and methods of use thereof.

BACKGROUND

According to World Health Organization, cancer is the second leading cause of death globally, accounting for an estimated 9.6 million deaths in 2018. Lung, prostate, colorectal, stomach and liver cancer are the most common types of cancer in men, while breast, colorectal, lung, cervical and thyroid cancer are the most common among women.

Recent clinical and commercial success of checkpoint inhibitors has created great interest
15 in immunotherapies. These checkpoint inhibitors (e.g., anti-PD-1 antibodies) have changed the treatment landscape of many cancers. However, response rate remains relatively low in many cases. There is a need to develop immunotherapies that can further overcome tumor-induced immune suppression while minimizing toxicity of these therapies.

SUMMARY

The present disclosure provides protein complexes targeting TIGIT and IL21R, which can be used for cancer treatment. The protein complex can include an anti-TIGIT antibody fused with IL21 (e.g., human or mouse IL21). In some embodiments, the anti-TIGIT antibody can block the interaction between TIGIT and a TIGIT ligand (e.g., CD155 or CD112), thereby
20 increasing immune response by inhibiting the immunosuppressive signal. Meanwhile, IL21 can further boost the immune response by activating IL21 receptor. Particularly, fusion of IL21 to an anti-TIGIT antibody can reduce the toxicity, increase stability, and enhance the function of IL21.

In some embodiments, the protein complexes described herein can activate T cells in tumor microenvironment by specifically targeting T cells expressing both TIGIT and IL21R.

In one aspect, the disclosure is related to a protein complex comprising a targeting moiety fused with an immunomodulatory moiety, in some embodiments, (a) the targeting moiety specifically binds to T cell immunoreceptor with Ig and ITIM domains (TIGIT); and (b) the immunomodulatory moiety specifically binds to interleukin-21 receptor (IL21R).

In some embodiments, the immunomodulatory moiety comprises an antibody or antigen-binding fragment, a single chain variable fragment (scFv), a Fc-containing polypeptide, or a fusion protein that specifically binds to IL21R.

In some embodiments, the immunomodulatory moiety is an IL21R agonist.

In some embodiments, the immunomodulatory moiety comprises an IL21 polypeptide. In some embodiments, the IL21 is a human IL21 polypeptide.

In some embodiments, the targeting moiety comprises an antibody or antigen-binding fragment, a single chain variable fragment (scFv), a Fc-containing polypeptide, or a fusion protein that specifically binds to TIGIT.

In some embodiments, the targeting moiety comprises a full-length antibody.

In some embodiments, the targeting moiety blocks the interaction between TIGIT and a TIGIT ligand (e.g., CD155 or CD112).

In some embodiments, targeting moiety has a K_D of less than 1×10^{-8} M, less than 1×10^{-9} M, less than 1×10^{-10} M, or less than 5×10^{-11} M with TIGIT (e.g., less than 1×10^{-9} M). In some embodiments, immunomodulatory moiety has a K_D of less than 1×10^{-7} M, 1×10^{-8} M, less than 5×10^{-9} M, or less than 2×10^{-9} M with IL21R.

In some embodiments, the targeting moiety comprises a polypeptide, in some embodiments, the immunomodulatory moiety is fused to the N-terminus or the C-terminus of the polypeptide. In some embodiments, the immunomodulatory moiety comprises a polypeptide, in some embodiments, the targeting moiety is fused to the N-terminus or the C-terminus of the polypeptide.

In some embodiments, the targeting moiety comprises a polypeptide comprising a CH3 domain, in some embodiments, the immunomodulatory moiety is fused to the CH3 domain.

In some embodiments, the targeting moiety and the immunomodulatory moiety are fused to a scaffold protein (e.g., an albumin).

In some embodiments, the protein complex comprises a bispecific antibody, in some embodiments, the bispecific antibody binds to TIGIT and IL21R.

In some embodiments, the protein complex comprises two or more immunomodulatory moieties that specifically bind to IL21R.

5 In some embodiments, the protein complex comprises two or more targeting moieties that specifically bind to TIGIT.

In some embodiments, the protein complex comprises an Fc comprising two CH3 domains.

10 In some embodiments, the immunomodulatory moiety is linked to a CH3 domain of the two CH3 domains in the Fc. In some embodiments, the immunomodulatory moiety is linked to the C-terminus of a CH3 domain of the two CH3 domains. In some embodiments, the immunomodulatory moiety is fused to a CH3 domain of the two CH3 domains in the Fc at a region from position 344 to position 382 of the CH3 domain according to EU numbering. In some embodiments, the immunomodulatory moiety is inserted to a CH3 domain of the two CH3 domains in the Fc between position 359 and position 360 of the CH3 domain according to EU numbering.

15 In some embodiments, the targeting moiety is linked to a CH3 domain of the two CH3 domains in the Fc. In some embodiments, the targeting moiety is linked to the C-terminus of a CH3 domain of the two CH3 domains in the Fc. In some embodiments, the targeting moiety is fused to a CH3 domain of the two CH3 domains in the Fc at a region from position 344 to position 382 of the CH3 domain according to EU numbering. In some embodiments, the targeting moiety comprises a scFv (e.g., a scFv targeting TIGIT).

20 In some embodiments, the protein complex comprises two light chains. In some embodiments, the immunomodulatory moiety is linked to one of the two light chains. In some embodiments, the targeting moiety is linked to one of the two light chains.

25 In some embodiments, the protein complex further comprises a targeting moiety targeting PD-1.

30 In some embodiments, the protein complex further comprises an interferon (e.g., IFN α 1, IFN α 2, IFN α 3, IFN α 4, and/or IFN γ). In some embodiments, the interferon is linked to the protein complex by a linker sequence.

In some embodiments, the protein complex further comprises a cytokine (e.g., IL2, IL7, IL15, IL18, and/or IL12). In some embodiments, the targeting moiety targeting PD-1 and/or cytokine are linked to the protein complex by a linker sequence.

In some embodiments, the protein complex comprises a linker sequence (GGGGS)_n, in some embodiments, n can be 1, 2, 3, 4, 5, 6, 7 or 8.

In some embodiments, the targeting moiety comprises an anti-TIGIT antibody or antigen-binding fragment thereof. In some embodiments, the anti-TIGIT antibody or antigen-binding fragment thereof is an IgG (e.g., IgG1, IgG2 or IgG4). In some embodiments, the anti-TIGIT antibody or antigen-binding fragment thereof is an IgG-like molecule.

In some embodiments, the targeting moiety comprises a heavy chain variable region (VH) comprising an amino acid sequence that is at least 90% identical to SEQ ID NO: 1, 12, 14, 16, or 18; and a light chain variable region (VL) comprising an amino acid sequence that is at least 90% identical to SEQ ID NO: 2, 13, 15, 17, or 19; or the immunomodulatory moiety comprises a sequence that is at least 80% identical to SEQ ID NO: 9 or SEQ ID NO: 10.

In one aspect, the disclosure provides a protein complex comprising an Fc, and one or more IL21 polypeptides, wherein the one or more IL21 polypeptides are fused to the Fc.

In one aspect, the disclosure is related to a method of treating a subject having cancer, the method comprising administering a therapeutically effective amount of a composition comprising the protein complex as described herein, to the subject. In some embodiments, the subject has at least one tumor infiltrating immune cells expressing TIGIT. In some embodiments, the cancer is resistant to anti-PD-1 antibody treatment and/or anti-PD-L1 antibody treatment and/or anti-TIGIT antibody treatment; and/or a chemotherapy. In some embodiments, the method further comprises administering an effective amount of an anti-PD-1 antibody or an anti-PD-L1 antibody to the subject.

In one aspect, the disclosure is related to a method of increasing immune response in tumor microenvironment in a subject, the method comprising administering a therapeutically effective amount of a composition comprising the protein complex as described herein to the subject.

In one aspect, the disclosure is related to a method of activating T cells in tumor microenvironment of a subject, the method comprising administering a therapeutically effective amount of a composition comprising the protein complex as described herein to the subject.

In one aspect, the disclosure is related to a method of increasing IL21 stability or enhancing IL21 function when delivering IL21 into a subject, the method comprising administering a therapeutically effective amount of a composition comprising the protein complex as described herein, to the subject.

5 In one aspect, the disclosure is related to a method of reducing the toxicity of IL21 when delivering IL21 into a subject, the method comprising fusing IL21 to an anti-TIGIT antibody.

In one aspect, the disclosure is related to a method of increasing the therapeutic effect of an anti-TIGIT antibody, comprising fusing IL21 to the anti-TIGIT antibody. In one aspect, the disclosure is related to a method of reducing the toxicity of IL21, comprising fusing IL21 to the
10 anti-TIGIT antibody.

In some embodiments, the IL21 is mouse IL21 or human IL21. In some embodiments, the anti-TIGIT antibody is any of the anti-TIGIT antibodies described herein.

In one aspect, the disclosure is related to an isolated molecule comprising the protein complex as described herein; covalently bound to a therapeutic agent. In some embodiments, the
15 therapeutic agent is a cytotoxic or cytostatic agent.

In one aspect, the disclosure is related to a pharmaceutical composition comprising the protein complex as described herein; and a pharmaceutically acceptor carrier.

In one aspect, the disclosure is related to a nucleic acid encoding the protein complex of as described herein.

20 In one aspect, the disclosure is related to a vector comprising the nucleic acid as described herein.

In one aspect, the disclosure is related to a host cell comprising the nucleic acid as described herein.

In one aspect, the disclosure is related to a method for producing a protein complex, the
25 method comprising culturing the host cell as described herein under conditions suitable to produce the protein complex.

As used herein, the term “protein complex” refers to a group of associated polypeptides. These polypeptides may have different or the same functions. They can associate with each other by non-covalent interactions or covalent bonds (e.g., peptide bonds or disulfide bonds). A protein
30 complex can have 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more than 10 polypeptide chains.

As used herein, the term “antibody” or “immunoglobulin” refers to any antigen-binding molecule that contains at least one (e.g., one, two, three, four, five, or six) complementary determining region (CDR) (e.g., any of the three CDRs from an immunoglobulin light chain or any of the three CDRs from an immunoglobulin heavy chain) and is capable of specifically binding to an epitope. Non-limiting examples of antibodies include: monoclonal antibodies, polyclonal antibodies, multi-specific antibodies (e.g., bi-specific antibodies), single-chain antibodies, single domain antibodies, nanobodies, chimeric antibodies, human antibodies, and humanized antibodies. In some embodiments, an antibody contains an Fc region of a human antibody. The term antibody also includes derivatives, e.g., bi-specific antibodies, single-chain antibodies, scFv, diabodies, linear antibodies, and multi-specific antibodies formed from antibody fragments.

As used herein, the term “IgG-like” refers to a molecule that is largely similar to an IgG antibody. Non-limiting examples of IgG-like molecules include an IgG antibody with one or more non-native polypeptides that are added to the IgG antibody. In some embodiments, the protein complex as described herein is an IgG-like molecule. In some embodiments, an IgG-like molecule is derived from a modification of IgG (e.g., IgG1, IgG2, IgG3, or IgG4).

As used herein, the term “antigen-binding fragment” refers to a portion of a full-length antibody, wherein the portion of the antibody is capable of specifically binding to an antigen. In some embodiments, the antigen-binding fragment contains at least one variable domain (e.g., a variable domain of a heavy chain or a variable domain of light chain). Non-limiting examples of antibody fragments include, e.g., Fab, Fab', F(ab')₂, Fv fragments, and VHH (single domain antibody or the variable domain of a heavy chain antibody).

As used herein, the term “full-length antibody” refers to an antibody that has an essentially intact structure as compared to a wild-type antibody. In some embodiments, a full-length antibody is an antibody having two full-length heavy chains and two full-length light chains. In some embodiments, the full-length antibody can have insertions, deletions, or replacements, provided that the essential structure of all variable regions and constant regions in the antibody are retained.

As used herein, the term “human antibody” refers to an antibody that is encoded by an endogenous nucleic acid (e.g., rearranged human immunoglobulin heavy or light chain locus) present in a human. In some embodiments, a human antibody is collected from a human or

produced in a human cell culture (e.g., human hybridoma cells). In some embodiments, a human antibody is produced in a non-human cell (e.g., a mouse or hamster cell line).

As used herein, the term “chimeric antibody” refers to an antibody that contains a sequence present in at least two different antibodies (e.g., antibodies from two different mammalian species such as a human and a mouse antibody). A non-limiting example of a chimeric antibody is an antibody containing the variable domain sequences (e.g., all or part of a light chain and/or heavy chain variable domain sequence) of a non-human (e.g., mouse) antibody and the constant domains of a human antibody.

As used herein, the term “humanized antibody” refers to a non-human antibody which contains minimal sequence derived from a non-human (e.g., mouse) immunoglobulin and contains sequences derived from a human immunoglobulin. In non-limiting examples, humanized antibodies are human antibodies (recipient antibody) in which hypervariable (e.g., CDR) region residues of the recipient antibody are replaced by hypervariable (e.g., CDR) region residues from a non-human antibody (e.g., a donor antibody), e.g., a mouse, rat, or rabbit antibody, having the desired specificity, affinity, and capacity. In some embodiments, the Fv framework residues of the human immunoglobulin are replaced by corresponding non-human (e.g., mouse) immunoglobulin residues. In some embodiments, humanized antibodies may contain residues which are not found in the recipient antibody or in the donor antibody. These modifications can be made to further refine antibody performance.

As used herein, the term “single-chain antibody” refers to a single polypeptide that contains at least two immunoglobulin variable domains (e.g., a variable domain of a mammalian immunoglobulin heavy chain or light chain) that is capable of specifically binding to an antigen. Non-limiting examples of single-chain antibodies are described herein.

As used herein, the term “multi-specific antibody” refers to an antibody that can specifically bind to two or more different antigens or epitopes. In some embodiments, a multi-specific antibody is a bispecific antibody.

As used herein, the term “IL21 polypeptide” refers to IL21 and its variants, wherein these variants can interact with IL21R and retains one or more IL21 functions. In some embodiments, the IL21 polypeptide is a wild type IL21. In some embodiments, the IL21 polypeptide has at least 1, 2, 3, 4, or 5 mutations. In some embodiments, the IL21 polypeptide has no more than 1, 2, 3, 4, or 5 mutations. In some embodiments, the IL21 polypeptide is human IL21.

As used herein, when referring to an antibody, the phrases “specifically binding” and “specifically binds” mean that the antibody interacts with its target molecule (e.g., TIGIT) preferably to other molecules, because the interaction is dependent upon the presence of a particular structure (i.e., the antigenic determinant or epitope) on the target molecule; in other words, the reagent is recognizing and binding to molecules that include a specific structure rather than to all molecules in general. An antibody that specifically binds to the target molecule may be referred to as a target-specific antibody. For example, an antibody that specifically binds to a TIGIT molecule may be referred to as a TIGIT-specific antibody or an anti-TIGIT antibody.

As used herein, the terms “subject” and “patient” are used interchangeably throughout the specification and describe an animal, human or non-human, to whom treatment according to the methods of the present invention is provided. Veterinary and non-veterinary applications are contemplated by the present invention. Human patients can be adult humans or juvenile humans (e.g., humans below the age of 18 years old). In addition to humans, patients include but are not limited to mice, rats, hamsters, guinea-pigs, rabbits, ferrets, cats, dogs, and primates. Included are, for example, non-human primates (e.g., monkey, chimpanzee, gorilla, and the like), rodents (e.g., rats, mice, gerbils, hamsters, ferrets, rabbits), lagomorphs, swine (e.g., pig, miniature pig), equine, canine, feline, bovine, and other domestic, farm, and zoo animals.

As used herein, the terms “polypeptide,” “peptide,” and “protein” are used interchangeably to refer to polymers of amino acids of any length of at least two amino acids.

As used herein, the terms “polynucleotide,” “nucleic acid molecule,” and “nucleic acid sequence” are used interchangeably herein to refer to polymers of nucleotides of any length of at least two nucleotides, and include, without limitation, DNA, RNA, DNA/RNA hybrids, and modifications thereof.

As used herein, the term “fusion” or “fused” when used with respect to amino acid sequences (e.g. peptide, polypeptide or protein) refers to combination of two or more amino acid sequences, for example by chemical bonding or recombinant means, into a single amino acid sequence. A fusion amino acid sequence can be produced by genetic recombination of two encoding polynucleotide sequences, and can be expressed by a method of introducing a construct containing the recombinant polynucleotides into a host cell.

As used herein, the term “linked” when used with respect to amino acid sequences (e.g. peptide, polypeptide or protein) refers to combination of two or more amino acid sequences by chemical bonds (e.g., a peptide bond, a disulfide bond, bis-sulfone linker).

As used herein, the term “about” or “approximately” refers to a quantity, level, value, number, frequency, percentage, dimension, size, amount, weight or length that varies by as much as 30, 25, 20, 15, 10, 9, 8, 7, 6, 5, 4, 3, 2 or 1% to a reference quantity, level, value, number, frequency, percentage, dimension, size, amount, weight or length. In some embodiments, the terms “about” or “approximately” when preceding a numerical value or a level indicates the value plus or minus a range of 15%, 10%, 5%, or 1%. The term “about the same level” indicates the level plus or minus a range of 15%, 10%, 5%, or 1%.

As used herein, the term “operably link” or “operably linked” refers to a juxtaposition, with or without a spacer or linker sequence, of two or more biological sequences of interest in such a way that they are in a relationship permitting them to function in an intended manner. When used with respect to polypeptides, it is intended to mean that the polypeptide sequences are linked in such a way that permits the linked product to have the intended biological function. For example, an antibody variable region may be operably linked to a constant region so as to provide for a stable product with antigen-binding activity. The term can also be used with respect to polynucleotides. For one instance, when a polynucleotide encoding a polypeptide is operably linked to a regulatory sequence (e.g., promoter, enhancer, silencer sequence, etc.), it is intended to mean that the polynucleotide sequences are linked in such a way that permits regulated expression of the polypeptide from the polynucleotide.

As used herein, the term “binding partner” refers to a member of a pair of molecules capable of recognizing a specific structural aspect of another molecule, wherein the binding partners interact with each other by means of a specific, noncovalent or covalent interaction. Examples of such binding partners and corresponding molecules or compositions include, but are not limited to, any of the class of immune-type binding pairs, such as antigen/antibody; and also any of the class of nonimmune-type binding pairs, such as ligand/receptor, biotin/avidin, biotin/streptavidin, digoxigenin/anti-digoxigenin F(ab')₂, folic acid/folate binding protein, complementary nucleic acid segments, protein A or G/immunoglobulins, lectin/carbohydrate, substrate/enzyme, inhibitor/enzyme, or virus/cellular receptor.

As used herein, a “targeting moiety” refers to a molecule that has the ability to localize and bind to a specific molecule or cellular component. The targeting moiety can be an antibody, antibody fragment, scFv, Fc-containing polypeptide, fusion antibody, polypeptide, peptide, aptamer, ligand, nucleic acid, or any combination thereof. In some embodiments, a targeting moiety can bind to a molecule present in a cell or tissue. In some embodiments, the targeting moiety can bind to a molecule in a diseased cell or tissue, e.g., a cancer cell or tumor. In some embodiments, the targeting molecule can bind to a normal cell or tissue, e.g., an immune cell such as T cell. In some embodiments, the targeting moiety can bind to a cellular or extracellular molecule that modulates the immune response. In some embodiments, the targeting moiety binds to TIGIT.

As used herein, an “immunomodulatory moiety” refers to a ligand, peptide, polypeptide, or Fc-containing polypeptide that binds a specific component of an immune cell (e.g., T cell, regulatory T cell, myeloid suppressor cell, or dendritic cell) and modulates the number or function of immune cells. In some embodiments, the “immunomodulatory moiety” specifically binds a cytokine, cytokine receptor, co-stimulatory molecule, or co-inhibitory molecule that modulates the immune system. In some embodiments, the immunomodulatory moiety specifically binds to IL21R. In some embodiments, the immunomodulatory moiety is an agonist that increases the function of the bound molecule.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Methods and materials are described herein for use in the present invention; other, suitable methods and materials known in the art can also be used. The materials, methods, and examples are illustrative only and not intended to be limiting. All publications, patent applications, patents, sequences, database entries, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present specification, including definitions, will control.

Other features and advantages of the invention will be apparent from the following detailed description and figures, and from the claims.

DESCRIPTION OF DRAWINGS

FIG. 1 shows a schematic structure of Tiragolumab-IL21-3A-knob, in which IL21 is fused at the 3A site.

FIG. 2 shows non-reducing SDS-PAGE results of Tiragolumab-IL21-3A2-knob. M is a protein marker. Tiragolumab-IL21-3A2-knob is at lane 3. Lanes 1 and 2 show bands of irrelevant proteins.

FIG. 3 is a graph showing body weight over time of the hTIGIT mice that were injected with mouse colon cancer cells MC38, and were treated with Tiragolumab-IgG1 at 3 mg/kg (G2), Tiragolumab-IgG1 at 10 mg/kg (G3), Tiragolumab-IL21-3A2-knob at 3.28 mg/kg (G4), and Tiragolumab-IL21-3A2-knob at 11 mg/kg (G5). PBS was injected as a control (G1).

FIG. 4 is a graph showing body weight change over time of the hTIGIT mice that were injected with mouse colon cancer cells MC38, and were treated with Tiragolumab-IgG1 at 3 mg/kg (G2), Tiragolumab-IgG1 at 10 mg/kg (G3), Tiragolumab-IL21-3A2-knob at 3.28 mg/kg (G4), and Tiragolumab-IL21-3A2-knob at 11 mg/kg (G5). PBS was injected as a control (G1).

FIG. 5 is a graph showing average tumor volume in different groups of the hTIGIT mice that were injected with mouse colon cancer cells MC38, and were treated with Tiragolumab-IgG1 at 3 mg/kg (G2), Tiragolumab-IgG1 at 10 mg/kg (G3), Tiragolumab-IL21-3A2-knob at 3.28 mg/kg (G4), and Tiragolumab-IL21-3A2-knob at 11 mg/kg (G5). PBS was injected as a control (G1).

FIG. 6 shows sequences discussed in the disclosure.

DETAILED DESCRIPTION

T cell immunoglobulin and ITIM domain (TIGIT) is an inhibitory receptor expressed on lymphocytes. It comprises an extracellular immunoglobulin variable domain, a type I transmembrane domain, and a short intracellular domain. The intracellular domain also includes an immunoreceptor tyrosine inhibitory motif (ITIM) and an immunoglobulin tyrosine tail (ITT)-like motif. TIGIT was first discovered in 2009 and has become a major emerging target for cancer immunotherapy.

TIGIT interacts with CD155 expressed on antigen-presenting cells or tumor cells to down-regulate the functions of T cells and natural killer cells. It has become a key inhibitor of anti-tumor response and can block multiple steps in the cancer immune cycle. Preclinical studies

have shown that TIGIT blockade can prevent various solid cancers and hematological cancers. Several monoclonal antibodies that block the inhibitory activity of human TIGIT have been developed. Clinical trials are ongoing to study TIGIT blockade as a monotherapy or in combination with anti-PD-1/PD-L1 monoclonal antibodies to treat patients with advanced solid malignancies.

Studies have shown that anti-TIGIT antibody alone cannot sufficiently inhibit the growth of subcutaneous tumors in mice. Thus, there is a need to combine the anti-TIGIT antibody with other therapies. Currently, it is commonly used in combination with the PD-1/PD-L1 pathway therapy (e.g., an anti-PD-L1 antibody). As a new type of cancer immunotherapy target, TIGIT has been studied for nearly a decade, and its biological function is still not well understood.

IL21 is a cytokine with anti-tumor effect. This 4 α -helix bundle cytokine produced by CD4⁺ T cells and natural killer T cells can play a multi-functional function by stimulating the differentiation of CD8⁺ T cells and inhibiting the production of Tregs. The half-life of IL21 is very short, and for most patients, intratumoral injection of recombinant IL21 is extremely difficult.

This disclosure is based on, *in part*, the unexpected discovery that IL21 can be fused to an anti-TIGIT antibody (e.g., Tiragolumab). The protein complex can greatly improve the *in vivo* tumor inhibition efficacy relative to an unmodified anti-TIGIT antibody. As such, the protein complex can greatly increase the immune response in the tumor microenvironment, and further increase the therapeutic efficacy of the antibody that targets TIGIT.

T cell immunoreceptor with Ig and ITIM domains (TIGIT)

TIGIT is a type I transmembrane protein expressed on the surface of T cells and NK cells. It has immunoglobulin domain, transmembrane region and immunoreceptor protein tyrosine inhibitory motif. It is an immunosuppressive co-stimulatory molecule. Immunotherapy is an important area in tumor research. Clinical studies have shown that the treatment targeting the inhibitory receptors of T cells can have significant treatment effect. It has been shown in a lot of studies that TIGIT can be used as a potential target for tumor immunotherapy. When receiving the stimulation from an anti-TIGIT agonistic monoclonal antibody, TIGIT, as a receptor, is able to inhibit the activity of T cells and NK cells. TIGIT can also act as a ligand functioning on the

dendritic cell (DC) surface of the poliovirus receptor (PVR), promote DC secretion of IL-10, and thus inhibiting the immune response.

TIGIT is highly expressed in chronic viral infections and in cancers. When compared with normal tissue, the ratio of TIGIT : T3 increases in T cells in cancer tissues, indicating that TIGIT is up-regulated in tumor-infiltrating T cells. Therefore, anti-TIGIT antibodies can be used in cancer treatment. Although the inhibition of PD-L1 or TIGIT alone does not yield good result, but an inhibition of both at the same time can significantly improve CD8-mediated inhibition of tumor proliferation. More importantly, only when PD-L1 and TIGIT are inhibited at the same time, IFN and TNF expression can be induced, which may be the reason for using anti-TIGIT antibody in combination with other drugs.

TIGIT was able to negatively regulate the immune response of T cells in the autoimmune response. In the TIGIT-deficient mouse models, T cells have higher reproductive capacity and can produce more pro-inflammatory cytokines. In the disease model of collagen-induced arthritis, soluble TIGIT-Fc protein can significantly inhibit the deterioration of the disease. In addition, blocking the function of anti-TIGIT will accelerate the occurrence of the disease. Therefore, TIGIT can negatively regulate the immune response of T cells, and thus participate in the inhibition of autoimmune diseases. In addition, TIGIT ligands CD155 and CD112 are overexpressed in some tumor cells, such as colorectal cancer, gastric cancer, neuroblastoma and so on. TIGIT binds to its ligand to inhibit the immune response of T cells, leading to tumor cell escape.

Both TIGIT and PD-1 have been shown to be over expressed on tumor antigen-specific (TA-specific) CD8⁺ T cells and CD8⁺ tumor infiltrating lymphocytes (TILs) from individuals with melanoma. Blockade of TIGIT and PD-1 led to increased cell proliferation, cytokine production, and degranulation of TA-specific CD8⁺ T cells and TIL CD8⁺ T cells. It can be considered an immune checkpoint.

Protein complex targeting IL21 pathway

IL21 is a member of the common gamma chain (γ c) family of cytokines and is expressed by multiple immune cell types, with activated CD4⁺ T cells, including T follicular helper (TFH) cells and natural killer (NK) T cells, being the major sources of this cytokine. The induction of

IL21 in activated CD4⁺ T cells is mediated by c-Maf *in vitro* and *in vivo*, whereas the expression of c-Maf in CD4⁺ T cells is regulated by IL-6 or IL-27.

The biological functions of IL21 are mediated by binding to its corresponding receptor, IL21R. IL21R is expressed by a wide range of immune cells, including T cells, B cells, NK cells, DCs and macrophages as well as non-immune cells, including epithelial cells and keratinocytes. The ubiquitous expression of the IL21R may explain the broad biological functions of IL21 on the cells of hemopoietic and non-hemopoietic origins. The IL21/IL21R signaling activates the Janus kinase (JAK1/3)-signal transducer and activator of transcription (STAT) signaling pathway. Accordingly, the phosphorylated STAT proteins are dimerized and translocated into the nucleus, where they bind to interferon (IFN)- γ -activated sequence (GAS) elements and initiate a gene transcription profile. IL21 exerts its regulatory functions on the target cells predominantly via the activation of STAT3 but it also recruits STAT1 and STAT5. The cascade of signaling events downstream of the IL21-induced activation of STAT3 is well-characterized, however, what is still unclear is how the activation of STAT1 by IL21 regulates the expression of the downstream IL21 target genes. Interestingly, the IL21-induced activation of STAT1 leads to the augmented expression of Tbx21 and Ifng genes.

TFH cells are considered one of the major sources of the IL21 production. These cells are a specialized subset of CD4⁺ T cells that can promote T cell-dependent humoral immune responses. Multiple signaling pathways contribute to the differentiation and the development of TFH cells, among which IL-6 and the inducible T-cell costimulator (ICOS) ligand or ICOSL (CD275) have been shown to be important in the early differentiation of these cells in the mouse. TFH cells are identified by several surface markers, including CXCR5, ICOS, PD-1 and, in addition to IL21, these cells canonically secrete C-X-C motif chemokine 13 (CXCL13) and IL-4. CXCL13 is expressed predominantly by non-TFH cell sources (i.e., stromal cells) in mice, whereas TFH cells are the major source of this molecule in humans. The transcription factor, B-cell lymphoma 6 (Bcl6) is required for the differentiation of TFH cells, however, other transcription factors have been shown to regulate the differentiation of TFH cells via the induction of Bcl6.

Germinal centers (GCs) are specialized locations in the secondary lymphoid tissues within which B cells proliferation, antibody somatic hypermutation and the affinity maturation (i.e., generation and selection of B cells with high affinity antibody secretion) occur. Severe

impairment in B cells response to antigen of protein origin, a significant reduction in plasma cell formation in both the spleen and the bone marrow were observed in mice lacking IL21 or its cognate receptor, IL21R, IL21^{-/-}, and IL21R^{-/-} mice, respectively. It has been proposed that the IL21/IL21R signaling regulates the Bcl6 expression by B cells and the TFH-derived IL21 promotes the transition of the peri-follicular pre-GC B cell to the intrafollicular phase. In addition to its role in the regulation of GC responses, earlier reports have identified IL21/IL21R signaling as a switch factor for the secretion of all IgG subclasses, especially IgG1 and IgG3, as well as IgA by human splenic or peripheral naïve CD19⁺ B cells. The B cell-intrinsic signaling via IL21/IL21R axis and the downstream STAT3 signaling have been accounted for the generation and the development of long-lived antibody responses. Consistent with these findings, mice lacking the IL21R (IL21R^{-/-}) failed to expand T cell-dependent, antigen-specific memory B cells and plasma cells. Altogether, these observations demonstrate diverse biological functions for the IL21/IL21R axis in B cell-mediated immunity, spanning from the generation and the development of GCs to B cell proliferation, the antibody affinity maturation and the generation and the expansion of memory B cells.

IL21 can also regulate the effector function (e.g., cytokine production) of T cell subsets alone or in synergy with other cytokines. IL21 exhibits synergy with IL-15 for the enhancement of the T-bet expression and with IL-12 for induction of STAT4-dependent DNA binding in NK cells and T cells and the subsequent augmentation of TH1-polarized immune response, as evidenced by enhanced IFN- γ production. IL21 also showed synergy with IFN- γ to induce the optimal expression of a certain set of signature interferon-stimulated genes (ISGs) in humans or mice. Interestingly, this effect on ISG expression was independent of STAT-3. These findings exemplify the breadth and the complexity of the IL21/IL21R signaling axis across species and in diverse biological settings.

The function of IL21, and the details of some of these clinical trials are described, e.g., in Solaymani-Mohammadi, S. et al., "Interleukin (IL)-21 in inflammation and immunity during parasitic diseases." *Frontiers in cellular and infection microbiology* 9 (2019): 401; and Leonard, W. J. et al., "IL21 signaling in immunity." *F1000Research* 5 (2016); each of which is incorporated herein by reference in its entirety.

This disclosure relates to protein complexes targeting IL21R. The disclosure demonstrates that the protein complex as described herein can activate T cells in a tumor

microenvironment. Particularly, due to its specific binding to TIGIT, the protein complex as described herein can deliver IL21 to the tumor microenvironment when it is administered to a subject. In addition, the protein complex can effectively activate the IL21 signaling pathway in T cells that are in close proximity of tumor cells. In one aspect, the protein complex can further
5 block the interaction between TIGIT and a TIGIT ligand (e.g., CD155 or CD112), thereby further increasing the immune response in the tumor microenvironment.

In some embodiments, fusion of IL21 to the anti-TIGIT antibody can increase the half-life of the fused IL21 (e.g., by at least 10%, at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 1-fold, at least 2-fold, at least 3-
10 fold, at least 4-fold, at least 5-fold, at least 10-fold, at least 20-fold, at least 50-fold, or at least 100-fold) as compared to that of a wildtype IL21, when administered to a subject.

In one aspect, the disclosure provides a protein complex comprising (a) a targeting moiety; and (b) an immunomodulatory moiety specifically binds to interleukin-21 receptor (IL21R). In some embodiments, the targeting moiety specifically binds to an immune checkpoint
15 molecule (e.g., TIGIT).

In one aspect, the disclosure also provides a protein complex that comprises (a) a first portion (e.g., a functional domain, a functional unit, or an immunomodulatory moiety) targeting Interleukin-21 receptor (IL21R); and (b) a second portion (e.g., a functional domain, a functional unit, or a targeting moiety) targeting TIGIT pathway. As used herein, a “functional domain”
20 refers to a distinct functional and structural unit in a protein. A functional domain can vary in length, but it generally has between about 50 amino acids to about 250 amino acids in length. In some embodiments, a functional domain is a segment of a polypeptide. In some embodiments, a functional domain can be formed by multiple segments of one polypeptide (e.g., a VH-VL pair in scFv) or multiple segments from different polypeptides (e.g., a VH-VL pair in Fab). In some
25 embodiments, a targeting moiety comprises a functional domain. In some embodiments, an immunomodulatory moiety comprises a functional domain.

The protein complex can have 1, 2, 3, 4, 5, 6 or more than 6 functional domains or targeting moieties that target TIGIT pathway. In some embodiments, the protein complex can have 1, 2, 3, 4, 5, 6 or more than 6 functional domains or immunomodulatory moieties that target
30 IL21R. In some embodiments, the ratio of the portions that target TIGIT pathway to the portions that target IL21R is 4:1, 2:1, 1:1, 1:2, or 1:4.

In some embodiments, the functional domains or moieties targeting TIGIT pathway can block the interaction between TIGIT and a TIGIT ligand. In some embodiments, the functional domains or moieties targeting TIGIT pathway cannot block the interaction between TIGIT and a TIGIT ligand (e.g., a non-blocking anti-TIGIT antibody). In some embodiments, the functional domain or moiety targeting TIGIT pathway is a full-length antibody or antigen binding fragment. In some embodiments, the functional domain or moiety targeting TIGIT pathway is an anti-TIGIT antibody.

In some embodiments, the functional domain or the immunomodulatory moiety targeting IL21R is a IL21 polypeptide (e.g., human IL21). In some embodiments, the IL21 polypeptide is a variant (e.g., a fusion protein or a truncated protein thereof) of IL21. In some embodiments, the IL21 variant includes at least 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 mutations. In some embodiments, the IL21 variant maintains a partial or full function of wild-type IL21. In some embodiments, the IL21 variant comprises a sequence that is at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical to SEQ ID NO: 9 or 10. In some embodiments, the IL21 can be modified, e.g., to improve the *in vivo* efficacy of the protein complex for treating cancer. In some embodiments, the IL21 can be modified to reduce its activity (e.g., to less than 90%, less than 80%, less than 70%, less than 60%, or less than 50%). In some embodiments, the IL21 polypeptide does not comprise a signal peptide. In some embodiments, the functional domain or the immunomodulatory moiety targeting IL21R is an anti-IL21R antibody or antigen binding fragment thereof (e.g., scFV or VHH). In some embodiments, the anti-IL21R antibody is an agonist anti-IL21R antibody.

In some embodiments, the immunomodulatory moiety described herein binds to IL21 receptor (e.g., human or mouse IL21 receptor) with a KD value less than 1×10^{-6} M, less than 9×10^{-7} M, less than 8×10^{-7} M, less than 7×10^{-7} M, less than 6×10^{-7} M, less than 5×10^{-7} M, less than 4×10^{-7} M, less than 3×10^{-7} M, less than 2×10^{-7} M, less than 1×10^{-7} M, less than 9×10^{-8} M, less than 8×10^{-8} M, less than 7×10^{-8} M, less than 6×10^{-8} M, less than 5×10^{-8} M, less than 4×10^{-8} M, less than 3×10^{-8} M, less than 2×10^{-8} M, less than 1×10^{-8} M, less than 9×10^{-9} M, less than 8×10^{-9} M, less than 7×10^{-9} M, less than 6×10^{-9} M, less than 5×10^{-9} M, less than 4×10^{-9} M, less than 3×10^{-9} M, less than 2×10^{-9} M, less than 1×10^{-9} M, less than 1×10^{-10} M, or less than 1×10^{-11} M. In some embodiments, the immunomodulatory moiety

described herein binds to IL21 receptor (e.g., human or mouse IL21 receptor) with a KD value greater than 1×10^{-7} M, greater than 9×10^{-8} M, greater than 8×10^{-8} M, greater than 7×10^{-8} M, greater than 6×10^{-8} M, greater than 5×10^{-8} M, greater than 4×10^{-8} M, greater than 3×10^{-8} M, greater than 2×10^{-8} M, greater than 1×10^{-8} M, greater than 9×10^{-9} M, greater than 8×10^{-9} M, greater than 7×10^{-9} M, greater than 6×10^{-9} M, greater than 5×10^{-9} M, greater than 4×10^{-9} M, greater than 3×10^{-9} M, greater than 2×10^{-9} M, greater than 1×10^{-9} M, greater than 1×10^{-10} M, or greater than 1×10^{-11} M. In some embodiments, the KD is 1×10^{-6} M $\sim 1 \times 10^{-7}$ M, 1×10^{-7} M $\sim 1 \times 10^{-8}$ M or 1×10^{-8} M $\sim 1 \times 10^{-9}$ M.

In some embodiments, the immunomodulatory moiety and the targeting moiety are linked (e.g., fused) together, optionally through a linker sequence. In some embodiments, the immunomodulatory moiety and the targeting moiety are linked to a third functional domain or moiety, such as a protein scaffold (e.g., Fc, albumin). In some embodiments, they can be linked to the third functional domain or moiety through a linker sequence. In some embodiments, the immunomodulatory moiety and the targeting moiety are linked to a nanoparticle.

In some embodiments, the functional domain or moiety targeting IL21R is fused to the heavy chain at a region from position 344 to position 382 of the heavy chain CH3 domain according to EU numbering, or is linked (e.g., fused) to the N-terminus or C-terminus of a heavy chain or a light chain.

In some embodiments, the protein complex comprises (a) a first portion (e.g., an immunomodulatory moiety) targeting interleukin 21 receptor (IL21R); and (b) a second portion (e.g., a targeting moiety) targeting TIGIT pathway. The first portion (e.g., an immunomodulatory moiety) can be linked or fused to a heavy chain or a portion thereof (e.g., CH3, or Fc) by various ways as described herein. In some embodiments, an IL21 polypeptide (e.g., a human or mouse IL21, or their variants) is fused to the heavy chain at a region from position 344 to position 382 of the heavy chain CH3 domain according to EU numbering, or is linked to the N-terminus or C-terminus of a heavy chain or a light chain.

In some embodiments, the protein complex can further include a cytokine (e.g., IL2, IL7, IL15, IL18, and/or IL12) and/or an interferon (e.g., IFNa1, IFNa2, IFNa3, IFNa4, and/or IFN γ). The cytokine or interferon can be linked or fused to the protein complex, e.g., linked or fused to the targeting moiety, the immunomodulatory moiety, or the protein scaffold, directly or indirectly through a linker sequence.

As it is difficult to perform intratumoral injection of recombinant IL21, the present disclosure also provides a protein complex comprising IL21 that can be enriched in a tumor microenvironment. The effects of IL21 is largely limited to the tumor microenvironment, thereby reducing the undesirable or non-specific stimulation by IL21 on the entire immune system. In one aspect, the present disclosure provides a fusion protein comprising IL21, wherein the fusion protein is engineered to target an immune checkpoint molecule.

In some embodiments, the fusion protein comprises an Fc region or a full-length antibody, e.g., anti-TIGIT antibody. In some embodiments, IL21 is linked to a region from position 344 to position 382 of a CH3 domain of the Fc region according to EU numbering. In some

embodiments, the anti-TIGIT antibody or antigen-binding fragment thereof comprises a VHH. Without wishing to be bound by theory, it is also hypothesized that the protein complex targeting both TIGIT and IL21R can generate a synergistic effect to reduce tumor growth in the tumor microenvironment. For example, the protein complex can block the interaction between TIGIT and a TIGIT ligand (e.g., CD155 or CD112), meanwhile activating the IL21 signaling pathway in T cells, thereby increasing the immune response in the tumor microenvironment.

In some embodiments, the protein complex targeting both TIGIT and IL21R as described herein has a better tumor growth inhibition effect when administered to a subject, as compared to that of a monotherapy using the anti-TIGIT antibody that forms the protein complex.

In some embodiments, the protein complex targeting both TIGIT and IL21R as described herein has a better tumor growth inhibition effect when administered to a subject, as compared to that of a protein complex having a similar structure targeting a different immune checkpoint molecule (e.g., PD-1 or PD-L1) and IL21R.

In one aspect, the disclosure provides a protein complex comprising or consisting of an anti-TIGIT antibody or antigen-binding fragment thereof, and an IL21. In some embodiments, the anti-TIGIT antibody or antigen-binding fragment thereof is an immunoglobulin (e.g., IgM, IgA, IgE, IgD, or IgG). In some embodiments, the protein complex comprises 1, 2, 3, 4, 5, 6 or more than 6 IL21 functional domains or immunomodulatory moieties. These IL21 functional domains or immunomodulatory moieties can be linked to N terminal or C terminal of a heavy chain and/or a light chain. In some embodiments, the IL21 functional domains or immunomodulatory moieties can be linked to the CH3 domain in the anti-TIGIT antibody or antigen-binding fragment thereof.

In some embodiments, the anti-TIGIT antibody or antigen-binding fragment thereof comprises or consists of: a first heavy chain polypeptide comprising a first heavy chain CH3 domain, a second heavy chain polypeptide comprising a second heavy chain CH3 domain, a first light chain polypeptide, and a second light chain polypeptide; wherein the first heavy chain polypeptide and the first light chain polypeptide associate with each other, forming a first antigen-binding region targeting TIGIT; wherein the second heavy chain polypeptide and the second light chain polypeptide associate with each other, forming a second antigen-binding region targeting TIGIT.

In some embodiments, IL21 is fused to the CH3 domain of the first heavy chain polypeptide and/or the second heavy chain polypeptide. In some embodiments, IL21 is linked to the C terminal of the first heavy chain polypeptide and/or the second heavy chain polypeptide. In some embodiments, IL21 is linked to the C terminal of the first light chain polypeptide and/or the second light chain polypeptide.

In some embodiments, IL21 is linked to the anti-TIGIT antibody or antigen-binding fragment thereof with a linker sequence as described herein.

In some embodiments, IL21 has a sequence that is at least 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% identical to SEQ ID NO: 9 or 10.

In some embodiments, at least two functional domains or immunomodulatory moieties (e.g., 2, 3, 4, 5, or 6 IL21) are linked to the anti-TIGIT antibody or antigen-binding fragment thereof.

In some embodiments, one or more additional cytokine functional domains (e.g., IL2, IL7, IL15, IL18, and/or IL12) and/or one or more interferon functional domains (e.g., IFN α 1, IFN α 2, IFN α 3, IFN α 4, and/or IFN γ) are linked to the anti-TIGIT antibody or antigen-binding fragment thereof.

In one aspect, the disclosure provides a modified polypeptide comprising a CH3 domain. In some embodiments, the polypeptide comprises a first portion of CH3; a region comprising an interleukin 21 polypeptide (e.g., a human or mouse IL21); and a second portion of CH3. In some embodiments, the first portion of CH3 comprises amino acid residues 341-343 (e.g., 341-359) of a CH3 domain according to EU numbering, and the second portion of CH3 comprises amino acid residues 383-447 (e.g., 360-447) of a CH3 domain according to EU numbering. In some embodiments, the modified polypeptide further comprises a CH2 domain. In some embodiments,

two of these modified polypeptides associate with each other, forming an Fc-like structure. In some embodiments, the Fc-like structure only has one IL21 functional domain.

In one aspect, the disclosure further provides a fusion heavy chain polypeptide, wherein the fusion heavy chain polypeptide comprises, e.g., from N-terminus to C-terminus: a first region comprising VH, CH1, CH2, and CH3; and a second region comprising an interleukin 21 polypeptide. In some embodiments, the first region is linked to the second region through a linker sequence (e.g., any one of the linker sequences described herein).

In one aspect, the disclosure provides a protein complex comprising or consisting of an Fc, and one or more IL21 polypeptides, wherein the one or more IL21 polypeptides are fused to the Fc. In some embodiments, the IL21 polypeptide is fused to the C terminal of Fc. In some embodiments, the IL21 polypeptide is fused to the N terminal of Fc. In some embodiments, the protein complex does not have antigen binding domains or Fab.

Modified immunoglobulins

In one aspect, the disclosure provides a protein complex that comprises (a) a first portion (e.g., a functional domain, a functional unit, or an immunomodulatory moiety) targeting IL21 pathway; and (b) a second portion (e.g., a functional domain, a functional unit, or a targeting moiety) targeting TIGIT pathway. In some embodiments, the protein complex can comprise an antibody or a portion thereof (e.g., Fc). The first portion (e.g., an immunomodulatory moiety) and the second portion (e.g., a targeting moiety) can be linked to the antibody or a portion thereof (e.g., Fc) by various ways.

In general, antibodies (also called immunoglobulins) are made up of two classes of polypeptide chains, light chains and heavy chains. A non-limiting examples of antibody of the present disclosure can be an intact, four immunoglobulin chain antibody comprising two heavy chains and two light chains. The heavy chains, which each contain one variable domain (or variable region, V_H) and multiple constant domains (or constant regions), bind to one another via disulfide bonding within their constant domains to form the “stem” of the antibody. The light chains, which each contain one variable domain (or variable region, V_L) and one constant domain (or constant region), each bind to one heavy chain via disulfide binding. The variable region of each light chain is aligned with the variable region of the heavy chain to which it is bound. The variable regions of both the light chains and heavy chains contain three

hypervariable regions sandwiched between more conserved framework regions (FR). These hypervariable regions, known as the complementary determining regions (CDRs), form loops that comprise the antigen binding surface of the antibody. The four framework regions largely adopt a beta-sheet conformation and the CDRs form loops connecting the beta-sheet structure, and in some cases forming part of, the beta-sheet structure. The CDRs in each chain are held in close proximity by the framework regions and, with the CDRs from the other chain, contribute to the formation of the antigen-binding region.

The heavy chain has four to five domains, depending on the isotype, including a variable (VH) domain and several constant (CH) domains: three CH domains (CH1, CH2, CH3) in IgG, IgA and IgD and four CH domains (CH1, CH2, CH3, CH4) in IgM and IgE. The antigen-binding fragment (Fab) is formed by the light chain (VL and CL) and the first two domains of the heavy chain (VH and CH1) and is specifically involved in antigen binding. The Ig Fc (fragment crystallizable) portion is formed by the CH2 and CH3 constant domains, and optionally with CH4 constant domain, from each heavy chain. The Fc region ensures that each antibody generates an appropriate immune response for a given antigen, by binding to a specific class of Fc receptors, and other immune molecules, such as complement proteins. By doing this, it mediates different physiological effects, including recognition of opsonized particles (binding to FcγR), lysis of cells (binding to complement), and degranulation of mast cells, basophils, and eosinophils (binding to FcεR).

In some embodiments, the heavy chain described herein can be a heavy chain variant, e.g., a heavy chain variant without the CH1 domain; or a heavy chain variant with at least the CH3 domain (e.g., CH2 and CH3 domains).

All domains in immunoglobulins have a similar structure and are constructed from two β sheets. The sheets are linked by a disulfide bridge and together form a roughly barrel-shaped structure, known as a β barrel. The distinctive folded structure of the immunoglobulin protein domain is known as the immunoglobulin fold. The constant domains are built up from seven β strands arranged such that four strands form one β sheet and three strands form a second sheet. The loops connecting the β strands are relatively short and, as a result, a majority of the residues of the domain are contained in the two β sheets. These strands include A-strand, B-strand, C-strand, D-strand, E-strand, F-Strand, and G-strand. The sequence connecting the β strands include AB-turn, BC-loop, CD-strand, DE-turn, and EF-turn. A detailed description of the

structure of the constant domain can be found e.g., in Lefranc et al., "IMGT® and 30 years of Immunoinformatics Insight in antibody V and C domain structure and function." *Antibodies* 8.2 (2019): 29, which is incorporated herein by reference in its entirety.

In some embodiments, the protein complex can comprise an antibody (IgM, IgA, IgE, IgD, IgG, IgG1, IgG2a, IgG2b, IgG3) or a portion thereof (e.g., Fc). The functional domains, immunomodulatory moieties, and/or targeting moieties can be linked to the antibody or a portion thereof (e.g., Fc) by various ways. In some embodiments, the functional domains, immunomodulatory moieties, and/or targeting moieties can comprise an antibody or a portion thereof (e.g., Fc, Fab, scFv, VHH).

In some embodiments, the functional domains, immunomodulatory moieties, and/or targeting moieties can be linked to the C terminal of an Fc. In some embodiments, the functional domains, immunomodulatory moieties, and/or targeting moieties can be linked to the N terminal of an Fc, e.g., they can be linked to CH2 domain through an optional hinge region or a linker sequence. In some embodiments, the functional domains, immunomodulatory moieties, and/or targeting moieties can be linked to the C terminal of an Fc, e.g., through a linker sequence. In some embodiments, the functional domains, immunomodulatory moieties, and/or targeting moieties can be linked to the N terminal of a light chain in the protein complex or the C terminal of a light chain in the protein complex, e.g., through a linker sequence.

In some embodiments, the functional domains, immunomodulatory moieties, and/or targeting moieties can be fused to a particular region in the Fc region. In some embodiments, a non-native polypeptide is fused to a particular region in the Fc region in the protein complex. As used herein, a "non-native" polypeptide refers to a polypeptide which cannot be found in the Fc region of a wild-type immunoglobulin. This particular region in the present disclosure is referred as the "3A site." The 3A site is located in the CH3 domain, and starts from position 344 to position 382 (EU numbering). The fusion of a non-native polypeptide can provide superior results. As compared to some other modified immunoglobulins, the immunoglobulins with this modification is very stable, and the immunoglobulins with a non-native polypeptide fused at this site can be expressed at a high level and they do not form aggregates. The property of this fusion site is also unexpected, as the 3A site is located in the A-strand and B-strand, which seems to be important for the function and stability of the CH3 domain.

In some embodiments, the modification is made to IgG, IgM, IgD, IgE, or IgA. Thus, in one aspect, the disclosure provides a modified Fc region or a polypeptide complex comprising a first polypeptide comprising a first heavy chain CH3 domain, and a second polypeptide comprising a second heavy chain CH3 domain. The two polypeptides interact with each other and can form a homodimer or a heterodimer. One or two non-native polypeptides can be fused to the heavy chain CH3 domains of the one or two polypeptides at the 3A site. The 3A site starts from position 344 to position 382 (EU numbering). In some embodiments, the functional domains, immunomodulatory moieties, or targeting moieties replaces 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, or 39 amino acids at the fusion site or is inserted between any of the two amino acids at this fusion site. In some embodiments, when a non-native polypeptide is inserted between two non-consecutive amino acids at the fusion site, it also replaces all amino acids between the two non-consecutive amino acids. In some embodiments, the non-native polypeptide is linked to two amino acid residues of the heavy chain CH3 domain of the modified immunoglobulin. The two amino acid residues can be consecutive or non-consecutive.

In some embodiments, the functional domains, immunomodulatory moieties, and/or targeting moieties are linked to two amino acid residues of the heavy chain CH3 domain of a modified immunoglobulin. In some embodiments, the two residues are selected from any two of positions 343, 344, 345, 346, 347, 348, 349, 350, 351, 352, 353, 354, 355, 356, 357, 358, 359, 360, 361, 362, 363, 364, 365, 366, 367, 368, 369, 370, 371, 372, 373, 374, 375, 376, 377, 378, 379, 380, 381, 382, and 383 of the heavy chain CH3 domain according to EU numbering. The functional domains, immunomodulatory moieties, and/or targeting moieties are linked to a starting amino acid and an ending amino acid in the heavy chain CH3 domain. The present disclosure also provides all different combinations of the starting amino acid and the ending amino acid at the 3A site. For example, if the functional domains, immunomodulatory moieties, and/or targeting moieties are linked to the amino acid residues located at position 343 and 383, the entire 3A site (positions 344-382) is replaced by the functional domains, immunomodulatory moieties, and/or targeting moieties. If the functional domains, immunomodulatory moieties, and/or targeting moieties are linked to two consecutive amino acid residues, e.g., located at position 359 and 360, the functional domains, immunomodulatory moieties, and/or targeting moieties are inserted between the two consecutive amino acid residues. The specific

combinations of any two amino residues at positions 343, 344, 345, 346, 347, 348, 349, 350, 351, 352, 353, 354, 355, 356, 357, 358, 359, 360, 361, 362, 363, 364, 365, 366, 367, 368, 369, 370, 371, 372, 373, 374, 375, 376, 377, 378, 379, 380, 381, 382, and 383 of the heavy chain CH3 domain (EU numbering) are provided.

In some embodiments, the starting amino acid is selected from 343, 344, 345, 346, 347, 348, 349, 350, 351, 352, 353, 354, 355, or 356. In some embodiments, the starting amino acid is selected from 357, 358, 359, 360, 361, or 362. In some embodiments, the ending amino acid is selected from 358, 359, 360, 361, 362, 363, 364, 365, 366, 367, 368, 369, 370, 371, 372, 373, 374, 375, 376, 377, 378, 379, 380, 381, 382, or 383. In some embodiments, the ending amino acid is selected from 358, 359, 360, 361, 362, or 363.

In some embodiments, the fusion site is located at a region from position 351 to 362 (EU numbering). In some embodiments, the functional domains, immunomodulatory moieties, and/or targeting moieties replace 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, or all amino acids (e.g., 351-362) at the fusion site, or are inserted between any of the two amino acids at this fusion site, e.g., inserted at the position 351-352, 352-353, 353-354, 354-355, 355-356, 356-357, 357-358, 358-359, 359-360, 360-361, or 361-362.

In some embodiments, the two residues are selected from any two of positions 350, 351, 352, 353, 354, 355, 356, 357, 358, 359, 360, 361, 362, and 363 of the heavy chain CH3 domain according to EU numbering. The combinations of these starting amino acids and the ending amino acids are provided in the table below. For example, if the functional domains, immunomodulatory moieties, and/or targeting moieties are linked to the amino acid residues located at position 350 and 363, the amino acids in this fusion site (positions 351-362) are replaced by the functional domains, immunomodulatory moieties, and/or targeting moieties. If the functional domains, immunomodulatory moieties, and/or targeting moieties are linked to two consecutive amino acid residues, e.g., located at position 359 and 360, the functional domains, immunomodulatory moieties, and/or targeting moieties are inserted between the two consecutive amino acid residues.

Table 1.

Start	End	Comment	Start	End	Comment	Start	End	Comment
350	351	Insert	352	359	Insert/delete	355	363	Insert/delete

350	352	Insert/delete	352	360	Insert/delete	356	357	Insertion
350	353	Insert/delete	352	361	Insert/delete	356	358	Insert/delete
350	354	Insert/delete	352	362	Insert/delete	356	359	Insert/delete
350	355	Insert/delete	352	363	Insert/delete	356	360	Insert/delete
350	356	Insert/delete	353	354	Insertion	356	361	Insert/delete
350	357	Insert/delete	353	355	Insert/delete	356	362	Insert/delete
350	358	Insert/delete	353	356	Insert/delete	356	363	Insert/delete
350	359	Insert/delete	353	357	Insert/delete	357	358	Insertion
350	360	Insert/delete	353	358	Insert/delete	357	359	Insert/delete
350	361	Insert/delete	353	359	Insert/delete	357	360	Insert/delete
350	362	Insert/delete	353	360	Insert/delete	357	361	Insert/delete
350	363	Insert/delete	353	361	Insert/delete	357	362	Insert/delete
351	352	Insertion	353	362	Insert/delete	357	363	Insert/delete
351	353	Insert/delete	353	363	Insert/delete	358	359	Insertion
351	354	Insert/delete	354	355	Insertion	358	360	Insert/delete
351	355	Insert/delete	354	356	Insert/delete	358	361	Insert/delete
351	356	Insert/delete	354	357	Insert/delete	358	362	Insert/delete
351	357	Insert/delete	354	358	Insert/delete	358	363	Insert/delete
351	358	Insert/delete	354	359	Insert/delete	359	360	Insertion
351	359	Insert/delete	354	360	Insert/delete	359	361	Insert/delete
351	360	Insert/delete	354	361	Insert/delete	359	362	Insert/delete
351	361	Insert/delete	354	362	Insert/delete	359	363	Insert/delete
351	362	Insert/delete	354	363	Insert/delete	360	361	Insertion
351	363	Insert/delete	355	356	Insertion	360	362	Insert/delete
352	353	Insertion	355	357	Insert/delete	360	363	Insert/delete
352	354	Insert/delete	355	358	Insert/delete	361	362	Insertion
352	355	Insert/delete	355	359	Insert/delete	361	363	Insert/delete
352	356	Insert/delete	355	360	Insert/delete	362	363	Insert
352	357	Insert/delete	355	361	Insert/delete			
352	358	Insert/delete	355	362	Insert/delete			

In some embodiments, the fusion site is located at a region from 358 to 362 (EU numbering). In some embodiments, the functional domains or the moieties can replace 1, 2, 3, 4, 5 or all amino acids at the fusion site or is inserted between any of the two amino acids at this fusion site, e.g., inserted at the position 358-359, 359-360, 360-361, or 361-362.

In some embodiments, the two residues are selected from any two of positions 357, 358, 359, 360, 361, 362, and 363 of the heavy chain CH3 domain according to EU numbering. The

combinations of these starting amino acids and the ending amino acids are provided in the table below.

Table 2.

Starting	Ending	Comment	Starting	Ending	Comment
357	358	Insert	359	360	Insert
357	359	Insert/delete	359	361	Insert/delete
357	360	Insert/delete	359	362	Insert/delete
357	361	Insert/delete	359	363	Insert/delete
357	362	Insert/delete	360	361	Insert
357	363	Insert/delete	360	362	Insert/delete
358	359	Insert	360	363	Insert/delete
358	360	Insert/delete	361	362	Insert
358	361	Insert/delete	361	363	Insert/delete
358	362	Insert/delete	362	363	Insert
358	363	Insert/delete			

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Thus, in some embodiments, the two residues are positions 357 and 358 of the heavy chain CH3 domain according to EU numbering. In some embodiments, the two residues are positions 357 and 359 of the heavy chain CH3 domain according to EU numbering. In some embodiments, the two residues are positions 357 and 360 of the heavy chain CH3 domain according to EU numbering. In some embodiments, the two residues are positions 357 and 361 of the heavy chain CH3 domain according to EU numbering. In some embodiments, the two residues are positions 357 and 362 of the heavy chain CH3 domain according to EU numbering. In some embodiments, the two residues are positions 357 and 363 of the heavy chain CH3 domain according to EU numbering. In some embodiments, the two residues are positions 358 and 359 of the heavy chain CH3 domain according to EU numbering. In some embodiments, the two residues are positions 358 and 360 of the heavy chain CH3 domain according to EU numbering. In some embodiments, the two residues are positions 358 and 361 of the heavy chain CH3 domain according to EU numbering. In some embodiments, the two residues are positions 358 and 362 of the heavy chain CH3 domain according to EU numbering. In some embodiments, the two residues are positions 358 and 363 of the heavy chain CH3 domain according to EU numbering. In some embodiments, the two residues are positions 359 and 360 of the heavy chain CH3 domain according to EU numbering. In some embodiments, the two residues are positions

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359 and 361 of the heavy chain CH3 domain according to EU numbering. In some embodiments, the two residues are positions 359 and 362 of the heavy chain CH3 domain according to EU numbering. In some embodiments, the two residues are positions 359 and 363 of the heavy chain CH3 domain according to EU numbering. In some embodiments, the two residues are positions 360 and 361 of the heavy chain CH3 domain according to EU numbering. In some embodiments, the two residues are positions 360 and 362 of the heavy chain CH3 domain according to EU numbering. In some embodiments, the two residues are positions 360 and 363 of the heavy chain CH3 domain according to EU numbering. In some embodiments, the two residues are positions 361 and 362 of the heavy chain CH3 domain according to EU numbering. In some embodiments, the two residues are positions 361 and 363 of the heavy chain CH3 domain according to EU numbering. In some embodiments, the two residues are positions 362 and 363 of the heavy chain CH3 domain according to EU numbering.

In some embodiments, IL21 (e.g., human IL21 or mouse IL21) is inserted at the 3A site of an anti-TIGIT antibody (e.g., Tiragolumab). Specifically, fusion protein Tiragolumab-IL21-3A1-knob can be obtained by inserting the IL21 between position 358 and position 359 (according to EU numbering) within the 3A site of the knob heavy chain. Fusion protein Tiragolumab-IL21-3A2-knob can be obtained by inserting the IL21 between position 359 and position 360 (according to EU numbering) within the 3A site of the knob heavy chain. Fusion protein Tiragolumab-IL21-3A3-knob can be obtained by inserting the IL21 between position 360 and position 361 (according to EU numbering) within the 3A site of the knob heavy chain. Fusion protein Tiragolumab-IL21-3A4-knob can be obtained by inserting the IL21 between position 361 and position 362 (according to EU numbering) within the 3A site of the knob heavy chain. In some embodiments, Tiragolumab-IL21-3A-knob can be selected from any one of Tiragolumab-IL21-3A1-knob, Tiragolumab-IL21-3A2-knob, Tiragolumab-IL21-3A3-knob, and Tiragolumab-IL21-3A4-knob. In some embodiments, knobs-into-holes (KIH) mutations are introduced to the Tiragolumab-IgG1 antibody to generate a knob heavy chain and a hole heavy chain.

The fusion of the functional domains, immunomodulatory moieties, and/or targeting moieties at the 3A site does not interfere with the antigen-binding site of the immunoglobulins. In addition, the fused functional domains, immunomodulatory moieties, or targeting moieties can maintain its bioactivity. Moreover, the modification at the 3A site does not significantly affect

the binding affinity of Fc to FcγRIIA, FcγRIIIA, FcγRIIIB, or FcRn receptors. In some embodiments, the binding affinities of the modified Fc to FcγRIIA, FcγRIIIA, FcγRIIIB, or FcRn receptors are about the same as compared to the same immunoglobulins before any modifications. In some embodiments, the binding affinities of the modified Fc to FcγRIIA, FcγRIIIA, FcγRIIIB, or FcRn receptors are higher (e.g., at least 10%, 20%, 30%, 40%, or 50%) as compared to the same immunoglobulins before any modifications. In some embodiments, the binding affinities of the modified Fc to FcγRIIA, FcγRIIIA, FcγRIIIB, or FcRn receptors are lower (e.g., no more than 10%, 20%, 30%, 40%, or 50% lower) as compared to the same immunoglobulins before any modifications.

In some embodiments, the protein complex has at least one of antibody-dependent cell cytotoxicity (ADCC), antibody-dependent cellular phagocytosis (ADCP), complement dependent cytotoxicity (CDC) or apoptotic activity. In some embodiments, when the fused polypeptide at the 3A site interacts with a target protein, the Fc will not bind FcγRIIA, FcγRIIIA, or FcγRIIIB receptors because of steric effects. Thus, when the fused polypeptide at the 3A site interacts with a target protein, the ADCC, ADCP, and CDC effects are reduced.

Moreover, the fusion of the functional domains, immunomodulatory moieties, or targeting moieties at the 3A site does not interfere with the function of the functional domains, immunomodulatory moieties, or targeting moieties. Particularly, the functional domains, immunomodulatory moieties, or targeting moieties in the modified immunoglobulins can have about the same or even a higher level of biological activity as compared to an isolated functional domains, immunomodulatory moieties, or targeting moieties. In some embodiments, the biological activity of the functional domains, immunomodulatory moieties, or targeting moieties in the modified immunoglobulins can be at least or about 85%, 90%, 95%, or 100% of the isolated functional domains, immunomodulatory moieties, or targeting moieties.

The fusion of the functional domains, immunomodulatory moieties, or targeting moieties at the 3A site does not interfere with the binding affinity of the modified immunoglobulins. Particularly, the modified immunoglobulins can have about the same or even a higher level of binding affinity as compared to the parent immunoglobulins. As used herein, the term “parent” molecule refers to a molecule before any non-native peptides as described herein is fused to the molecule or any other modifications are made to the molecule. In some embodiments, the binding affinity can be at least or about 85%, 90%, 95%, or 100% of the parent immunoglobulins.

The fusion of the functional domains, immunomodulatory moieties, or targeting moieties at the 3A site does not interfere with the expression level and does not reduce expression yield. Particularly, the protein complex has a very high expression level. In some embodiments, the expression level can be at least or about 50%, 60%, 70%, 80%, 90%, or 100% higher than the expression level of a similar antibody (e.g., a bispecific antibody that binds to the same targets) or an immunocytokine (e.g., an antibody with a cytokine that is linked at the C-terminal of the Fc). In some embodiments, the modified immunoglobulins do not form aggregates easily. In some embodiments, the percentage of aggregates in the purified form (e.g., after purified by Protein A chromatography) is less than 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2% or 1%.

The protein complex can have various forms or formats. In some embodiments, the protein complex or the targeting moiety can comprise an intact immunoglobulin molecule (e.g., IgG1, IgG2a, IgG2b, IgG3, IgM, IgD, IgE, IgA). The IgG subclasses (IgG1, IgG2, IgG3, and IgG4) are highly conserved, differ in their constant region, particularly in their hinges and upper CH2 domains. The EU numbering of these immunoglobulins, the sequences and differences of the IgG subclasses are known in the art, and are described, e.g., in Vidarsson, et al, "IgG subclasses and allotypes: from structure to effector functions." *Frontiers in immunology* 5 (2014); Irani, et al. "Molecular properties of human IgG subclasses and their implications for designing therapeutic monoclonal antibodies against infectious diseases." *Molecular immunology* 67.2 (2015): 171-182; Shakib, Farouk, ed. *The human IgG subclasses: molecular analysis of structure, function and regulation*. Elsevier, 2016; Lefranc et al., "IMGT® and 30 years of Immunoinformatics Insight in antibody V and C domain structure and function." *Antibodies* 8.2 (2019): 29; each of which is incorporated herein by reference in its entirety.

The protein complex can comprise an immunoglobulin molecule that is derived from any species (e.g., human, rodent, rat, mouse, camelid, dog, horn shark, *Xenopus laevis*, rhesus monkey, cat, rabbit). Antibodies disclosed herein also include, but are not limited to, polyclonal, monoclonal, monospecific, polyspecific antibodies, and chimeric antibodies that include an immunoglobulin binding domain fused to another polypeptide. The term "antigen binding domain" or "antigen binding fragment" is a portion of an antibody that retains specific binding activity of the intact antibody, i.e., any portion of an antibody that is capable of specific binding to an epitope on the intact antibody's target molecule. It includes, e.g., Fab, Fab', F(ab')₂, VHH, and variants of these fragments. Thus, in some embodiments, an antibody or an antigen binding

fragment thereof can be, e.g., a scFv, a Fv, a Fd, a dAb, a VHH, a bispecific antibody, a bispecific scFv, a diabody, a linear antibody, a single-chain antibody molecule, a multi-specific antibody formed from antibody fragments, and any polypeptide that includes a binding domain which is, or is homologous to, an antibody binding domain. Non-limiting examples of antigen binding domains include, e.g., the heavy chain and/or light chain CDRs of an intact antibody, the heavy and/or light chain variable regions of an intact antibody, full-length heavy or light chains of an intact antibody, or an individual CDR from either the heavy chain or the light chain of an intact antibody.

Fragments of antibodies are suitable for use in the protein complex described herein are also provided. The Fab fragment contains a variable and constant domain of the light chain and a variable domain and the first constant domain (CH1) of the heavy chain. F(ab')₂ antibody fragments comprise a pair of Fab fragments which are generally covalently linked near their carboxy termini by hinge cysteines between them. Other chemical couplings of antibody fragments are also known in the art.

In some embodiments, the heavy chain of the antibody can be of any isotype including IgM, IgG, IgE, IgA, or IgD or sub-isotype including IgG1, IgG2, IgG2a, IgG2b, IgG3, IgG4, IgE1, IgE2, etc. The light chain can be a kappa light chain or a lambda light chain. An antibody can comprise two identical copies of a light chain and two identical copies of a heavy chain.

In some embodiments, the protein complex can have a format as shown in the table below. In some embodiments, a functional domain, an immunomodulatory moiety, or a targeting moiety is linked to a format as shown in the table below. Detailed descriptions of these antibody formats can be found, e.g., in Brinkmann, et al., "The making of bispecific antibodies." MAb. Vol. 9. No. 2. Taylor & Francis, 2017, which is incorporated herein by reference in the entirety.

Table 3.

Bispecific antibody conjugates	IgG2	Appended & Fc-modified IgGs	IgG(kih)-Fv
	CovX-Body		IgG(HA-TF-Fv
Hybrid bispecific IgGs	IgG		IgG(kih)-scFab
	Mouse/rat chimeric IgG		scFab-Fc(kih)-scFv2
	κ/λ-body common HC		scFab-Fc(kih)-scFv
Non-immunoglobulin fusion proteins	DNL-Fab ₂ -IgG-Cytokine ₂		Half DVD-Ig
Fc-modified IgGs	IgG(kih)		DVD-Ig(four-in-one)

	IgG(kih) common LC	Modified Fc and CH3 fusion proteins	CrossMab-Fab
	ZW1 IgG common LC		scFv-Fc(kih)
	Biclonics common LC		scFv-Fc(CH3 charge pairs)
	CrossMab (IgG-kih)		scFv-Fc(EW-RVT)
	scFab-IgG(kih)		scFv-Fc(HA-TF)
	orthogonal Fab IgG(kih)		scFv-Fc(SEEDbody)
	DuetMab		taFv-Fc(kih)
	CH3 charge pairs + CH1/CL charge pairs		scFv-Fc(kih)-scFv
	hinge/CH3 charge pairs		Fab-Fc(kih)-scFv
	Duobody		Fab-scFv-Fc(BEAT)
	four-in-one CrossMab (kih)		Fab- scFv-Fc(SEEDbody)
	LUZ-Y common LC		DART-Fc
	LUZ-Y scFab-IgG		scFv-CH3(kih)
	FcFc		TriFabs
Appended IgGs-LC fusions	IgG-scFv(LC)	Appended IgGs-HC fusions	IgG-HC-scFv
	ScFv(LC)-IgG		IgG-dAb
	dAb-IgG		IgG-taFv
Appended IgGs-HC&LC fusions	DVD-Ig		IgG-CrossFab
	TVD-Ig		IgG-orthogonal Fab
	CODV-Ig		IgG-(CαCβ)Fab
	scFv4-IgG		scFv-HC-IgG
	Zybody		tandem Fab-IgG(orthogonal Fab)
Fc fusions	DL-diabody	Modified IgGs	Fab-IgG(CαCβFab)
	scDb-Fc		Fab-IgG(CR3)
	taFv-Fc		Fab-hinge-IgG(CR3)
	scFv-Fc-scFv		DAF(two-in-one-IgG)
	HCAb-VHH	CH3 fusinos	DutaMab
	Fab-scFv-Fc		Di-diabody
	scFv4-Ig		scDb-CH3
Non-immunoglobulin fusions	DNL-Fab ₄ -IgG	-	-

In some embodiments, the protein complex comprises a bi-specific antibody. Bi-specific antibodies can be made by engineering the interface between a pair of antibody molecules to maximize the percentage of heterodimers that are recovered from recombinant cell culture. In some embodiments, the protein complex targets both TIGIT and IL21R. For example, the interface can contain at least a part of the CH3 domain of an antibody constant domain. In this method, one or more small amino acid side chains from the interface of the first antibody

molecule are replaced with larger side chains (e.g., tyrosine or tryptophan). Compensatory “cavities” of identical or similar size to the large side chain(s) are created on the interface of the second antibody molecule by replacing large amino acid side chains with smaller ones (e.g., alanine or threonine). This provides a mechanism for increasing the yield of the heterodimer over other unwanted end-products such as homodimers. This method is described, e.g., in WO 96/27011, which is incorporated by reference in its entirety. While the modification is made to Fc region, the present disclosure also shows that the modification is compatible with knobs-in-holes. The “knobs into holes” approach introduces a mutation for an amino acid with a large sidechain in one heavy chain, and a mutation for an amino acid with a small sidechain in the other heavy chain. Thus, the same heavy chains are less likely to associate with each other and the two different heavy chains have a higher chance to associate with each other. The “knobs into holes” approaches are described, e.g., in Ridgway, John BB, Leonard G. Presta, and Paul Carter. “‘Knobs-into-holes’ engineering of antibody CH3 domains for heavy chain heterodimerization.” *Protein Engineering, Design and Selection* 9.7 (1996), which is incorporated herein by reference in its entirety. In some embodiments, one or more amino acid residues in the CH3 domain of the IgG are substituted. In some embodiments, one heavy chain has one or more of the following substitutions Y349C and T366W. The other heavy chain can have one or more the following substitutions E356C, T366S, L368A, and Y407V. In some embodiments, one heavy chain has a T366Y (knob) substitution, and the other heavy chain has a Y407T (hole) substitution. In some embodiments, one heavy chain has a T366Y (knob) substitution, and the other heavy chain has one, two, or three of these substitutions T366S, L368A, Y407V (hole).

In some embodiments, the protein complex comprises an antibody with various formats, such as DVD-Ig, CrossMab, BiTE etc. In some embodiments, the protein complex comprises a targeting moiety and an immunomodulatory moiety. The targeting moiety and the immunomodulatory moiety, together with some optional moieties, can form an antibody with various formats. In some embodiments, the targeting moiety and the immunomodulatory moiety are linked to an antibody with various formats.

These formats are described in e.g., Spiess et al., “Alternative molecular formats and therapeutic applications for bispecific antibodies.” *Molecular immunology* 67.2 (2015): 95-106, which is incorporated herein by reference in its entirety. In certain embodiments, the bispecific

polypeptide complex as provided herein is based on a bispecific format selected from Triomabs; hybrid hybridoma (quadroma); Multispecific anticalin platform (Pieris); Diabodies; Single chain diabodies; Tandem single chain Fv fragments; TandAbs, Trispecific Abs; Darts (dual affinity retargeting; MacroGenics); Bispecific Xmabs (Xencor); Bispecific T cell engagers (Bites; Amgen; 5 55 kDa); Triplebodies; Tribody (Fab-scFv) Fusion Protein (Creative Biolabs) multifunctional recombinant antibody derivatives; Duobody platform (Genmab); Dock and lock platform; Humanized bispecific IgG antibody (REGN1979) (Regeneron); Mab2 bispecific antibodies (F-Star); DVD-Ig (dual variable domain immunoglobulin) (Abbvie); kappa-lambda bodies; TBTI (tetravalent bispecific tandem Ig); and CrossMab. In certain embodiments, the bispecific 10 polypeptide complex as provided herein is based on the format of a “whole” antibody, such as whole IgG or IgG-like molecules, and small recombinant formats, such as tandem single chain variable fragment molecules (taFvs), diabodies (Dbs), single chain diabodies (scDbs) and various other derivatives of these, and BiTE (bispecific T cell engager).

“MAb-Fv” or “IgG-Fv” refers to a fusion protein formed by fusion of VH to the C- 15 terminus of one Fc chain and the VL domain either expressed separately or fused to the C-terminus of the other resulted in a bispecific, trivalent IgG-Fv (mAb-Fv) fusion protein, with the Fv stabilized by an interdomain disulphide bond.

“ScFab-Fc-scFv2” and “ScFab-Fc-scFv” refer to a fusion protein formed by fusion of a single-chain Fab with Fc and disulphide-stabilized Fv domains.

20 “Appended IgG” refers to a fusion protein with a Fab arm fused to an IgG to form the format of bispecific (Fab) 2-Fc. It can form a “IgG-Fab” or a “Fab-IgG”, with a Fab fused to the C-terminus or N-terminus of an IgG molecule with or without a connector.

“DVD-Ig” refers to a dual-variable-domain antibody that is formed by fusion of an additional VH domain and VL domain of a second specificity to an IgG heavy chain and light 25 chain. “CODV-Ig” refers to a related format where the two VH and two VL domains are linked in a way that allows crossover pairing of the variable VH-VL domains, which are arranged either (from N-to C-terminus) in the order VH A-VH B and VL B-VL A, or in the order HV B-HV A and VL A-VL B.

A “CrossMab” refers to a technology of pairing of unmodified light chain with the 30 corresponding unmodified heavy chain and pairing of the modified light chain with the

corresponding modified heavy chain, thus resulting an antibody with reduced mispairing in the light chain.

A “BiTE” is a bispecific T-cell engager molecule, comprising a first scFv with a first antigen specificity in the VL-VL orientation linked to a second scFv with a second specificity in the VH-VL orientation.

In some embodiments, the protein complex comprises an Fc. The Fc region can be modified to provide desired effector functions or serum half-life.

Any of the protein complex described herein can be conjugated to a stabilizing molecule (e.g., a molecule that increases the half-life of the modified immunoglobulin in a subject or in solution). Non-limiting examples of stabilizing molecules include: a polymer (e.g., a polyethylene glycol) or a protein (e.g., serum albumin, such as human serum albumin). The conjugation of a stabilizing molecule can increase the half-life or extend the biological activity of an antibody or an antigen-binding fragment *in vitro* (e.g., in tissue culture or when stored as a pharmaceutical composition) or *in vivo* (e.g., in a human).

In some embodiments, the protein complex described herein can be conjugated to a therapeutic agent. In some embodiments, the therapeutic agent is a cytotoxic or cytostatic agent (e.g., cytochalasin B, gramicidin D, ethidium bromide, emetine, mitomycin, etoposide, tenoposide, vincristine, vinblastine, colchicin, doxorubicin, daunorubicin, dihydroxy anthracin, maytansinoids such as DM-1 and DM-4, mitoxantrone, mithramycin, actinomycin D, 1-dehydrotestosterone, glucocorticoids, procaine, tetracaine, lidocaine, propranolol, puromycin, epirubicin, and cyclophosphamide and analogs).

In some embodiments, the therapeutic agent can be linked to the functional domains, immunomodulatory moieties, or targeting moieties as described herein. For example, one or more therapeutic agents can be covalently linked to one or more amino acids (e.g., side chains) of the functional domains, immunomodulatory moieties, or targeting moieties (e.g., fused to different fusion sites as described herein).

In some embodiments, the protein complex comprises a modified immunoglobulin. Various polypeptides can be fused to modified immunoglobulins. The polypeptides can be fused to any sites described herein (e.g., the 3A site) and/or the C-terminal of the heavy chain. As shown in the present disclosure, after the polypeptide is fused to the Fc region, the fused polypeptide can adopt a proper conformation and maintain its bioactivity. In some embodiments,

at least 1, 2, 3, 4, 5, or 6 polypeptides can be fused to the modified immunoglobulin. In some embodiments, a polypeptide is fused to a heavy chain CH3 domain of a modified immunoglobulin from one or more amino acid residues. In some embodiments, the polypeptide is fused to one amino acid residue of the heavy chain CH3 domain. In some embodiments, the polypeptide is fused to the C-terminal amino acid residue of the heavy chain CH3 domain.

The disclosure further provides fusion proteins comprising a targeting moiety and an immunomodulatory moiety as described herein. As used herein, the term “fusion protein” in the present disclosure refers to a molecule comprising two or more proteins or the fragments thereof which are linked by the covalent bond via their respective main chains of the peptides, and more preferably, the fusion protein is generated by the genetic expression of the polynucleotide molecules encoding these proteins. In some embodiments, the fusion protein comprises an immunoglobulin domain. In some embodiments, the fusion protein is an Fc-fusion protein.

In one aspect, the protein complex comprises or is a modified antibody. In some embodiments, the modified antibody comprises or consists of two fusion heavy chain polypeptides and two light chain polypeptides. In some embodiments, the two fusion heavy chain polypeptides are identical. In some embodiments, the two fusion heavy chain polypeptides are different. In some embodiments, the modified antibody comprises or consists of a fusion heavy chain polypeptide, a heavy chain polypeptide, and two light chain polypeptides.

In some embodiments, the fusion heavy chain polypeptide comprises or consists of, e.g., preferably from N-terminus to C-terminus: a heavy chain variable region (VH), a CH1 domain, a CH2 domain, a first portion of a CH3 domain, an optional first linker sequence, an interleukin 21 polypeptide, an optional second linker sequence, and a second portion of a CH3 domain. In some embodiments, the first portion of the CH3 domain comprises amino acid residues 341-343, 341-350, 341-357, or 341-359 of the CH3 domain according to EU numbering. In some embodiments, the second portion of the CH3 domain comprises amino acid residues 383-447, 363-447, or 360-447 of the CH3 domain according to EU numbering. In some embodiments, the optional first linker sequence and/or the optional second linker sequence are identical to any of the linker sequences described herein (e.g., SEQ ID NO: 3 or SEQ ID NO: 8)

In some embodiments, the fusion heavy chain polypeptide and/or the heavy chain polypeptide comprise an IgG1 constant region. In some embodiments, the last lysine residue in of the fusion heavy chain polypeptide and/or the heavy chain polypeptide is mutated (e.g., to

alanine) in order to reduce hydrolysis rate (e.g., by at least 10%, at least 20%, at least 30%, at least 40%, or at least 50%). In some embodiments, the light chain polypeptide comprises a light chain constant region (CL). In some embodiments, the CL comprises a sequence that is at least 80%, at least 85%, at least 90%, at least 95%, or 100% identical to SEQ ID NO: 11.

5 In some embodiments, the interleukin 21 polypeptide is a mouse IL21. In some embodiments, the IL21 comprises a sequence that is at least 80%, at least 85%, at least 90%, at least 95%, or 100% identical to SEQ ID NO: 9. In some embodiments, the IL21 is a human IL21 (hIL21). In some embodiments, the hIL21 comprises a sequence that is at least 80%, at least 85%, at least 90%, at least 95%, or 100% identical to SEQ ID NO: 10.

10 In some embodiments, the fusion heavy chain polypeptide comprises or consists of, e.g., preferably from N-terminus to C-terminus: a VH, a CH1 domain, a CH2 domain, a first portion of a CH3 domain, an optional first linker sequence, an interleukin 21 polypeptide, an optional second linker sequence, a second portion of a CH3 domain, an optional third linker sequence, and a cytokine.

15 In some embodiments, the cytokine is IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13, IL-14, IL-15, IL-16, IL-17, IL-18, IL-19, IL-20, IL-21, IL-22, IL-23, IL-24, IL-25, IL-26, IL-27, IL-28, IL-29, IL-30, IL-31, IL-32, IL-33, IL-35, or IL-36. In some embodiments, the fusion heavy chain polypeptide comprises or consists of, e.g., preferably from N-terminus to C-terminus: a VH, a CH1 domain, a CH2 domain, a first portion of a CH3 domain,
20 an optional first linker sequence, an interleukin 21 polypeptide, an optional second linker sequence, a second portion of a CH3 domain, an optional third linker sequence, and an interferon (IFN). In some embodiments, the optional third linker sequence is identical to any of the linker sequences described herein. In some embodiments, the interferon is an IFN- α , IFN- β , IFN- ϵ , IFN- κ , IFN- τ , IFN- δ , IFN- ζ , IFN- ω , or IFN- γ . In some embodiments, the interferon is IFNa1, IFNa2, IFNa4, IFNa5, IFNa6, IFNa7, IFNa8, IFNa10, IFNa13, IFNa14, IFNa16, IFNa17, or
25 IFNa21. In some embodiments, the interferon is mouse IFNa4 (mIFNa4). In some embodiments, the interferon is human IFNa4 (hIFNa4). In some embodiments, the interferon is human IFNa2 (hIFNa2).

30 In some embodiments, the modified antibody comprises one or more knobs-into-holes (KIH) modifications. The KIH modifications include mutations at position S354C, T366W, Y349C, T366S, L368A, Y407V according EU numbering.

In some embodiments, the modified antibody comprises or consists of a fusion heavy chain polypeptide, a heavy chain polypeptide, and two light chain polypeptides. In some embodiments, the fusion heavy chain polypeptide comprises one or more hole mutations and the heavy chain polypeptide comprises one or more corresponding knob mutations. In some
5 embodiments, the fusion heavy chain polypeptide comprises one or more knob mutations and the heavy chain polypeptide comprises one or more corresponding hole mutations. In some embodiments, IL21 is fused to the fusion heavy chain polypeptide. In some embodiments, IL21 is not fused to the heavy chain polypeptide. In some embodiments, the fusion heavy chain polypeptide comprises a sequence that is at least 80%, at least 85%, at least 90%, at least 95%, or
10 100% identical to SEQ ID NO: 4, 20, 23, 26, or 29. In some embodiments, the heavy chain polypeptide comprises a sequence that is at least 80%, at least 85%, at least 90%, at least 95%, or 100% identical to SEQ ID NO: 5, 21, 24, 27, or 30. In some embodiments, the light chain polypeptide comprises a sequence that is at least 80%, at least 85%, at least 90%, at least 95%, or 100% identical to SEQ ID NO: 6, 22, 25, 28, or 31.

15 In some embodiments, the fusion heavy chain polypeptide comprises a sequence that is at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identical to SEQ ID NO: 4, the heavy chain polypeptide comprises a sequence that is at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identical to SEQ ID NO: 5, and the light chain polypeptide comprises a sequence that is at least 80%, 85%, 90%, 91%, 92%,
20 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identical to SEQ ID NO: 6. In some embodiments, the fusion heavy chain polypeptide comprises a sequence that is at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identical to SEQ ID NO: 20, the heavy chain polypeptide comprises a sequence that is at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identical to SEQ ID NO: 21, and the light
25 chain polypeptide comprises a sequence that is at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identical to SEQ ID NO: 22. In some embodiments, the fusion heavy chain polypeptide comprises a sequence that is at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identical to SEQ ID NO: 23, the heavy chain polypeptide comprises a sequence that is at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%,
30 96%, 97%, 98%, 99%, or 100% identical to SEQ ID NO: 24, and the light chain polypeptide comprises a sequence that is at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%,

98%, 99%, or 100% identical to SEQ ID NO: 25. In some embodiments, the fusion heavy chain polypeptide comprises a sequence that is at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identical to SEQ ID NO: 26, the heavy chain polypeptide comprises a sequence that is at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identical to SEQ ID NO: 27, and the light chain polypeptide comprises a sequence that is at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identical to SEQ ID NO: 28. In some embodiments, the fusion heavy chain polypeptide comprises a sequence that is at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identical to SEQ ID NO: 29, the heavy chain polypeptide comprises a sequence that is at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identical to SEQ ID NO: 30, and the light chain polypeptide comprises a sequence that is at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identical to SEQ ID NO: 31.

In some embodiments, the modified antibody comprises or consists of a first fusion heavy chain polypeptide, a second fusion heavy chain polypeptide, and two light chain polypeptides. In some embodiments, the first fusion heavy chain polypeptide comprises one or more hole mutations and the second fusion heavy chain polypeptide comprises one or more corresponding knob mutations.

In some embodiments, the fusion heavy chain polypeptide comprises or consists of, e.g., preferably from N-terminus to C-terminus, a VH, a CH1 domain, a CH2 domain, a CH3 domain, an optional linker sequence, and an interleukin 21 polypeptide. In some embodiments, the optional linker sequence is identical to any of the linker sequences described herein.

In one aspect, the disclosure is related to a modified antibody. In some embodiments, the modified antibody comprises or consists of two heavy chain polypeptides and two fusion light chain polypeptides. In some embodiments, the two fusion light chain polypeptides are identical. In some embodiments, the two fusion light chain polypeptides are different. In some embodiments, the modified antibody comprises or consists of two heavy chain polypeptides, a fusion light chain polypeptide, and a light chain polypeptide.

In some embodiments, the fusion light chain polypeptide comprises or consists of, e.g., preferably from N-terminus to C-terminus: a light chain variable region (VL), a light chain constant region (CL), an optional linker sequence, and an interleukin 21 polypeptide. In some

embodiments, the optional linker sequence is identical to any of the linker sequences described herein. In some embodiments, the modified antibody comprises or consists of a structure as shown in **FIG. 1**.

5 Targeting moieties

The targeting moiety can specifically bind to various antigens. In some embodiments, the antigen is an immune checkpoint molecule. Immune checkpoints are regulators of the immune system. In some embodiments, the immune checkpoint molecule is PD-1, PD-L1, PD-L2, CTLA-4, B7-H3, TIM-3, LAG-3, VISTA, ICOS, 4-1BB, OX40, GITR, or CD40. In some
 10 embodiments, the antigen is programmed cell death protein 1 (PD-1), cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), Lymphocyte Activating 3 (LAG-3), B And T Lymphocyte Associated (BTLA), Programmed Cell Death 1 Ligand 1 (PD-L1), CD27, CD28, CD40, CD47, CD137, CD154, T-Cell Immunoreceptor With Ig And ITIM Domains (TIGIT), Glucocorticoid-Induced TNFR-Related Protein (GITR), T-cell immunoglobulin and mucin-domain containing-3
 15 (TIM-3), or TNF Receptor Superfamily Member 4 (TNFRSF4 or OX40). In some embodiments, the immune checkpoint molecule is TIGIT.

In some embodiments, the antigen is a tumor-associated antigen. As used herein, the term “tumor-associated antigen” refers to an antigen that is or can be presented on a tumor cell surface. In some embodiments, the tumor associated antigens can be exclusively expressed on tumor cells
 20 or may represent a tumor specific mutation compared to non-tumor cells. In some embodiments, the tumor associated antigens can be found in both tumor cells and non-tumor cells, but is overexpressed on tumor cells when compared to non-tumor cells or are accessible for antibody binding in tumor cells due to the less compact structure of the tumor tissue compared to non-tumor tissue. In some embodiments the tumor associated antigen is located on the vasculature of
 25 a tumor. Illustrative examples of a tumor associated surface antigen are CD10, CD19, CD20, CD22, CD21, CD22, CD25, CD30, CD33, CD34, CD37, CD44v6, CD45, CD133, Fms-like tyrosine kinase 3 (FLT-3, CD135), chondroitin sulfate proteoglycan 4 (CSPG4, melanoma-associated chondroitin sulfate proteoglycan), Epidermal growth factor receptor (EGFR), Her2, Her2neu, Her3, IGFR, IL3R, fibroblast activating protein (FAP), CDCP1, Derlin1, Tenascin,
 30 frizzled 1-10, the vascular antigens VEGFR2 (KDR/FLK1), VEGFR3 (FLT4, CD309), PDGFR-alpha (CD140a), PDGFR-beta (CD140b) Endoglin, CLEC14, Tem1-8, and Tie2.

Further examples may include A33, CAMPATH-1 (CDw52), Carcinoembryonic antigen (CEA), Carboanhydrase IX (MN/CA IX), de2-7 EGFR, EGFRvIII, EpCAM, Ep-CAM, Folate-binding protein, G250, Fms-like tyrosine kinase 3 (FLT-3, CD135), c-Kit (CD117), CSF1R (CD115), HLA-DR, IGFR, IL-2 receptor, IL3R, MCSP (Melanoma-associated cell surface chondroitin sulfate proteoglycane), Muc-1, Prostate-specific membrane antigen (PSMA), Prostate stem cell antigen (PSCA), Prostate specific antigen (PSA), and TAG-72. In some embodiments, the tumor associated antigen is PD-L1.

In some embodiments, the targeting moiety described herein binds to an immune checkpoint molecule (e.g., any of the immune checkpoint molecules described herein) or a tumor-associated antigen (any of the tumor-associated antigens described herein) with a KD value less than 2×10^{-7} M, less than 1×10^{-7} M, less than 9×10^{-8} M, less than 8×10^{-8} M, less than 7×10^{-8} M, less than 6×10^{-8} M, less than 5×10^{-8} M, less than 4×10^{-8} M, less than 3×10^{-8} M, less than 2×10^{-8} M, less than 1×10^{-8} M, less than 9×10^{-9} M, less than 8×10^{-9} M, less than 7×10^{-9} M, less than 6×10^{-9} M, less than 5×10^{-9} M, less than 4×10^{-9} M, less than 3×10^{-9} M, less than 2×10^{-9} M, less than 1×10^{-9} M, less than 9×10^{-10} M, less than 8×10^{-10} M, less than 7×10^{-10} M, less than 6×10^{-10} M, less than 5×10^{-10} M, less than 4×10^{-10} M, less than 3×10^{-10} M, less than 2×10^{-10} M, less than 1×10^{-10} M, less than 9×10^{-11} M, less than 8×10^{-11} M, less than 7×10^{-11} M, less than 6×10^{-11} M, less than 5×10^{-11} M, less than 4×10^{-11} M, less than 3×10^{-11} M, less than 2×10^{-11} M, or less than 1×10^{-11} M. In some embodiments, the KD is 1×10^{-7} M $\sim 1 \times 10^{-8}$ M, 1×10^{-8} M $\sim 1 \times 10^{-9}$ M, 10^{-9} M $\sim 1 \times 10^{-10}$ M, 10^{-10} M $\sim 1 \times 10^{-11}$ M, or 10^{-11} M $\sim 1 \times 10^{-12}$ M. The KD value can be determined using methods known in the art, e.g., surface plasmon resonance (SPR) or biolayer interferometry (BLI).

In some embodiments, the binding affinity (e.g., as measured by affinity constant (K_a or $1/KD$) or K_{on}) of the targeting moiety (e.g., an anti-TIGIT antibody) when it binds to its target antigen (e.g., TIGIT) is at least 1-fold, 2-fold, 3-fold, 4-fold, 5-fold, 6-fold, 7-fold, 8-fold, 9-fold, 10-fold, 20-fold, 30-fold, 40-fold, 50-fold, 60-fold, 70-fold, 80-fold, 90-fold, 100-fold, 200-fold, 300-fold, 400-fold, 500-fold, 600-fold, 700-fold, 800-fold, 900-fold, 1000-fold, 2000-fold, 3000-fold, 4000-fold, 5000-fold, 6000-fold, 7000-fold, 8000-fold, 9000-fold, or 10000-fold stronger than the binding affinity of the immunomodulatory moiety described herein (e.g., human or mouse IL21) when it binds to IL21 receptor (e.g., human or mouse IL21 receptor). In some embodiments, the protein complex (e.g., any of the protein complexes described herein) has a

higher binding affinity to the target antigen (e.g., TIGIT) than IL21 receptor. While not intending to be bound by any theory, it is believed that the binding of the protein complex (e.g., any of the protein complexes described herein) to its target cells (e.g., T cells) is driven by the presence of the target antigen (e.g., TIGIT) on the surface of target cells, due to its higher binding affinity to the target antigen (e.g., TIGIT). As a result, the protein complex (e.g., any of the protein complexes described herein) binds to the target antigen first, reducing the chance that IL21 randomly activates IL21R on non-target cells, thereby reducing the toxicity of IL21. Thus, in one aspect, the disclosure provides a method of reducing the toxicity of IL21, comprising fusing IL21 to an antibody that has a much higher binding affinity to a target antigen as compared to the binding affinity of IL21 to IL21R.

In one aspect, the disclosure provides a protein complex comprising (a) a first targeting moiety; (b) a second targeting moiety; and (c) an immunomodulatory moiety specifically binds to interleukin-21 receptor (IL21R). In some embodiments, the first targeting moiety specifically binds to a first immune checkpoint molecule (e.g., TIGIT). In some embodiments, the second targeting moiety specifically binds to a second immune checkpoint molecule (e.g., PD-1). In some embodiments, IL21 is fused to an anti-TIGIT/PD1 bispecific antibody (e.g., at 3A site).

In one aspect, the disclosure provides a protein complex comprising (a) a first targeting moiety; (b) a second targeting moiety; and (c) an immunomodulatory moiety specifically binds to interleukin-21 receptor (IL21R). In some embodiments, the first targeting moiety specifically binds to a first immune checkpoint molecule (e.g., TIGIT). In some embodiments, the second targeting moiety specifically binds to a tumor-associated antigen.

In some embodiments, the functional domain, the targeting moiety, and/or the immunomodulatory moiety comprises an antibody or antigen binding fragment thereof. The binding affinity of an antibody or antigen binding fragment to the antigen is determined by CDRs. They are part of the variable chains in immunoglobulins (antibodies) and T cell receptors. Methods for identifying the CDR regions of an antibody by analyzing the amino acid sequence of the antibody are well known, and a number of definitions of the CDRs are commonly used. The Kabat definition is based on sequence variability, and the Chothia definition is based on the location of the structural loop regions. These methods and definitions are described in, e.g., Martin, "Protein sequence and structure analysis of antibody variable domains," Antibody engineering, Springer Berlin Heidelberg, 2001. 422-439; Abhinandan, et al. "Analysis and

improvements to Kabat and structurally correct numbering of antibody variable domains," Molecular immunology 45.14 (2008): 3832-3839; Wu, T.T. and Kabat, E.A. (1970) J. Exp. Med. 132: 211-250; Martin et al., Methods Enzymol. 203:121-53 (1991); Morea et al., Biophys Chem. 68(1-3):9-16 (Oct. 1997); Morea et al., J Mol Biol. 275(2):269-94 (Jan .1998); Chothia et al.,
5 Nature 342(6252):877-83 (Dec. 1989); Ponomarenko and Bourne, BMC Structural Biology 7:64 (2007); each of which is incorporated herein by reference in its entirety.

The CDRs are important for recognizing an epitope of an antigen. As used herein, an "epitope" is the smallest portion of a target molecule capable of being specifically bound by the antigen binding domain of an antibody. The minimal size of an epitope may be about three, four,
10 five, six, or seven amino acids, but these amino acids need not be in a consecutive linear sequence of the antigen's primary structure, as the epitope may depend on an antigen's three-dimensional configuration based on the antigen's secondary and tertiary structure.

In addition, the functional domains, immunomodulatory moieties, or targeting moieties can be fused to various modified immunoglobulins, antibodies, antibody-like, or IgG-like
15 molecules.

In some embodiments, the protein complex or the targeting moiety comprises an antibody and antigen-binding fragment thereof that specifically binds to TIGIT. In some embodiments, the antibody and antigen-binding fragment described herein can block TIGIT interaction with a TIGIT ligand (e.g., CD155 or CD112), thereby increasing immune response. In some
20 embodiments, the antibody or antigen-binding fragment described herein is capable of binding to TIGIT without blocking its interaction with a TIGIT ligand. In some embodiments, the antibody or antigen-binding fragment thereof is an agonist. In some embodiments, the antibody or antigen-binding fragment thereof is an antagonist. The disclosure provides, e.g., anti-TIGIT antibody Tiragolumab.

25 The amino acid sequence for heavy chain variable region and light variable region of Tiragolumab are also provided. In some embodiments, the amino acid sequence for the heavy chain variable region of Tiragolumab is set forth in SEQ ID NO: 1. In some embodiments, the amino acid sequence for the light chain variable region of Tiragolumab is set forth in SEQ ID NO: 2. The heavy chain variable region sequence can be paired with the light chain variable
30 region sequence, forming an antigen-binding site that binds to TIGIT.

The disclosure also provides antibodies or antigen-binding fragments thereof that bind to TIGIT. The antibodies or antigen-binding fragments thereof contain a heavy chain variable region (VH) comprising or consisting of an amino acid sequence that is at least 80%, 85%, 90%, or 95% identical to SEQ ID NO: 1, and a light chain variable region (VL) comprising or
5 consisting of an amino acid sequence that is at least 80%, 85%, 90%, or 95% identical to SEQ ID NO: 2.

The disclosure also provides nucleic acid comprising a polynucleotide encoding a polypeptide comprising an immunoglobulin heavy chain or an immunoglobulin light chain. When the polypeptides are paired with corresponding polypeptide (e.g., a corresponding heavy
10 chain variable region or a corresponding light chain variable region), the paired polypeptides bind to TIGIT (e.g., human TIGIT).

In some embodiments, the anti-TIGIT antibody has an IgG1 or IgG4 subtype. In some embodiments, the anti-TIGIT antibody described herein is Tiragolumab (VH: SEQ ID NO: 1; VL: SEQ ID NO: 2). Details of Tiragolumab can be found, e.g., in PCT Application No.

15 PCT/US2019/019603, which is incorporated herein by reference in its entirety. In some embodiments, the anti-TIGIT antibody described herein is Vibostolimab (VH: SEQ ID NO: 12; VL: SEQ ID NO: 13). In some embodiments, the anti-TIGIT antibody described herein is Etigilimab (VH: SEQ ID NO: 14; VL: SEQ ID NO: 15). In some embodiments, the anti-TIGIT antibody described herein is Domvanalimab (VH: SEQ ID NO: 16; VL: SEQ ID NO: 17). In
20 some embodiments, the anti-TIGIT antibody described herein is Ociperlimab (VH: SEQ ID NO: 18; VL: SEQ ID NO: 19). In some embodiments, the anti-TIGIT antibody described herein includes a VH that is at least at least 80%, at least 85%, at least 90%, at least 95%, or 100% identical to SEQ ID NO: 1, and a VL that is at least at least 80%, at least 85%, at least 90%, at least 95%, or 100% identical to SEQ ID NO: 2. In some embodiments, the anti-TIGIT antibody
25 described herein includes a VH that is at least at least 80%, at least 85%, at least 90%, at least 95%, or 100% identical to SEQ ID NO: 12, and a VL that is at least at least 80%, at least 85%, at least 90%, at least 95%, or 100% identical to SEQ ID NO: 13. In some embodiments, the anti-TIGIT antibody described herein includes a VH that is at least at least 80%, at least 85%, at least 90%, at least 95%, or 100% identical to SEQ ID NO: 14, and a VL that is at least at least 80%, at
30 least 85%, at least 90%, at least 95%, or 100% identical to SEQ ID NO: 15. In some embodiments, the anti-TIGIT antibody described herein includes a VH that is at least at least

80%, at least 85%, at least 90%, at least 95%, or 100% identical to SEQ ID NO: 16, and a VL that is at least at least 80%, at least 85%, at least 90%, at least 95%, or 100% identical to SEQ ID NO: 17. In some embodiments, the anti-TIGIT antibody described herein includes a VH that is at least at least 80%, at least 85%, at least 90%, at least 95%, or 100% identical to SEQ ID NO: 18, and a VL that is at least at least 80%, at least 85%, at least 90%, at least 95%, or 100% identical to SEQ ID NO: 19.

In some embodiments, the anti-TIGIT antibodies and antigen-binding fragments can also be antibody variants (including derivatives and conjugates) of antibodies or antibody fragments and multi-specific (e.g., bi-specific) antibodies or antibody fragments. Additional antibodies provided herein are polyclonal, monoclonal, multimeric, multispecific (e.g., bi-specific), human antibodies, chimeric antibodies (e.g., human-mouse chimera), single-chain antibodies, intracellularly-made antibodies (i.e., intrabodies), and antigen-binding fragments thereof. The antibodies or antigen-binding fragments thereof can be of any type (e.g., IgG, IgE, IgM, IgD, IgA, and IgY), class (e.g., IgG1, IgG2, IgG3, IgG4, IgA1, and IgA2), or subclass. In some embodiments, the antibody or antigen-binding fragment thereof is an IgG antibody or antigen-binding fragment thereof. In some embodiments, the antigen-binding fragment is a scFv or a Fab. Generally, the scFv polypeptide further comprises a polypeptide linker between the VH and VL domains, which enables the scFv to form the desired structure for antigen binding. The Fab fragment contains a variable and constant domain of the light chain and a variable domain and the first constant domain (CH1) of the heavy chain. F(ab')₂ antibody fragments comprise a pair of Fab fragments which are generally covalently linked near their carboxy termini by hinge cysteines between them. Other chemical couplings of antibody fragments are also known in the art.

Linker sequence

The functional domain, the immunomodulatory moiety (e.g., IL21), and/or the targeting moiety can be fused to any protein complex as described herein or fused with each other (e.g., the N terminal or the C terminal of a heavy chain, or the N terminal or the C terminal of a light chain, or the 3A site in the CH3 domain) with or without a linker sequence. In some embodiments, the linker peptide is optional, i.e., the two regions that are linked together can be directly linked by a peptide bond.

In some embodiments, the linker sequence comprises at least or about 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 30, 40, or 50 amino acid residues. In some embodiments, the linker sequence comprises at least or about 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 20, 25, 30, or 40 glycine residues. In some embodiments, the linker sequence comprises at least or about 1, 2, 3, 4, 5, 6, 7, or 8 serine residues. In some embodiments, the linker sequence consists of both glycine and serine residues. In some embodiments, the linker sequence comprises or consists of a sequence that is at least or about 70%, at least or about 75%, at least or about 80%, at least or about 85%, at least or about 90%, at least or about 95%, at least or about 99%, or 100% identical to any SEQ ID NO: 3 or 8. In some embodiments, the linker sequence comprises at least 1, 2, 3, 4, 5, or 6 repeats of GGGGS (SEQ ID NO: 8). In some embodiments, the linker sequence has no more than 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 30, 40, or 50 amino acid residues.

In one aspect, a polypeptide is fused to a heavy chain CH3 domain. In some embodiments, the inserted polypeptide includes an N-terminal linker sequence and a C-terminal linker sequence. As used herein, the term “N-terminal linker sequence” refers to a linker sequence that is located at the N-terminal of the inserted or fused polypeptide. As used herein, the term “C-terminal linker sequence” refers to a linker sequence that is located at the C-terminal of the inserted or fused polypeptide. The N-terminal linker sequence and the C-terminal linker sequence can be the same or different, and can comprise or consist of any linker sequences as described herein.

Methods of making protein complexes

The present disclosure also provides recombinant vectors (e.g., an expression vectors) that include an isolated polynucleotide disclosed herein (e.g., a polynucleotide that encodes a polypeptide disclosed herein), host cells into which are introduced the recombinant vectors (i.e., such that the host cells contain the polynucleotide and/or a vector comprising the polynucleotide), and the production of protein complex by recombinant techniques.

As used herein, a “vector” is any construct capable of delivering one or more polynucleotide(s) of interest to a host cell when the vector is introduced to the host cell. An “expression vector” is capable of delivering and expressing the one or more polynucleotide(s) of interest as an encoded polypeptide in a host cell into which the expression vector has been

introduced. Thus, in an expression vector, the polynucleotide of interest is positioned for expression in the vector by being operably linked with regulatory elements such as a promoter, enhancer, and/or a poly-A tail, either within the vector or in the genome of the host cell at or near or flanking the integration site of the polynucleotide of interest such that the polynucleotide of interest will be translated in the host cell introduced with the expression vector.

A vector can be introduced into the host cell by methods known in the art, e.g., electroporation, chemical transfection (e.g., DEAE-dextran), transformation, transfection, and infection and/or transduction (e.g., with recombinant virus). Thus, non-limiting examples of vectors include viral vectors (which can be used to generate recombinant virus), naked DNA or RNA, plasmids, cosmids, phage vectors, and DNA or RNA expression vectors associated with cationic condensing agents.

The expression vectors can include at least one selectable marker. Such markers include e.g., dihydrofolate reductase or neomycin resistance for eukaryotic cell culture and tetracycline or ampicillin resistance genes for culturing in *E. coli* and other bacteria. Representative examples of appropriate hosts include, but are not limited to, bacterial cells, such as *E. coli*, *Streptomyces*, and *Salmonella typhimurium* cells; fungal cells, such as yeast cells; insect cells such as *Drosophila* S2 and *Spodoptera* Sf9 cells; animal cells such as CHO, COS, Bowes melanoma, and HK 293 cells; and plant cells. Appropriate culture mediums and conditions for the host cells described herein are known in the art.

For secretion of the translated protein into the lumen of the endoplasmic reticulum, into the periplasmic space or into the extracellular environment, appropriate secretion signals may be incorporated into the expressed polypeptide. The signals may be endogenous to the polypeptide or they may be heterologous signals.

In some embodiments, a covalent modification can be made to the protein complex. These covalent modifications can be made by chemical or enzymatic synthesis, or by enzymatic or chemical cleavage. Other types of covalent modifications can be introduced into the molecule by reacting targeted amino acid residues in the protein complex with an organic derivatization agent that is capable of reacting with selected side chains or the N- or C-terminal residues.

In some embodiments, the protein complex can comprises an antibody having a carbohydrate structure that lacks fucose attached (directly or indirectly) to an Fc region. For example, the amount of fucose in such antibody composition may be from 1% to 80%, from 1%

to 65%, from 5% to 65% or from 20% to 40%. The amount of fucose is determined by calculating the average amount of fucose within the sugar chain at Asn297, relative to the sum of all glycostructures attached to Asn 297 (e.g. complex, hybrid and high mannose structures) as measured by MALDI-TOF mass spectrometry, as described in WO 2008/077546, for example.

5 Asn297 refers to the asparagine residue located at about position 297 in the Fc region (Eu numbering of Fc region residues; or position 314 in Kabat numbering); however, Asn297 may also be located about ± 3 amino acids upstream or downstream of position 297, i.e., between positions 294 and 300, due to minor sequence variations in antibodies. Such fucosylation variants may have improved ADCC function. In some embodiments, to reduce glycan
10 heterogeneity, the Fc region of the antibody can be further engineered to replace the Asparagine at position 297 with Alanine (N297A). In some embodiments, to facilitate production efficiency by avoiding Fab-arm exchange, the Fc region of the antibodies was further engineered to replace the serine at position 228 (EU numbering) of IgG4 with proline (S228P). A detailed description regarding S228 mutation is described, e.g., in Silva et al. "The S228P mutation prevents in vivo
15 and in vitro IgG4 Fab-arm exchange as demonstrated using a combination of novel quantitative immunoassays and physiological matrix preparation." *Journal of Biological Chemistry* 290.9 (2015): 5462-5469, which is incorporated by reference in its entirety.

In one aspect, the disclosure provides a vector that comprises a sequence encoding a knob heavy chain, wherein IL21 is fused to the knob heavy chain; a vector that comprises a sequence
20 encoding a hole heavy chain; and/or a vector that comprises a sequence encoding a light chain. In some embodiments, the knob heavy chain and hole heavy chain are encoded by the same vector. In some embodiments, the knob heavy chain, the hole heavy chain, and the light chain are encoded by the same vector. In some embodiments, an interferon (e.g., IFN α 4) is further fused to the heavy chain.

25 The disclosure also provides a nucleic acid sequence that is at least 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% identical to any nucleotide sequence as described herein, and an amino acid sequence that is at least 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%,
30 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% identical to any amino acid sequence as described herein.

In some embodiments, the disclosure relates to nucleotide sequences encoding any peptides that are described herein, or any amino acid sequences that are encoded by any nucleotide sequences as described herein. In some embodiments, the nucleic acid sequence is less than 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 150, 200, 250, 300, 350, 400, 500, or 600 nucleotides. In some embodiments, the amino acid sequence is less than 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 250, 300, 350, 400 or 500 amino acid residues.

In some embodiments, the amino acid sequence (i) comprises an amino acid sequence; or (ii) consists of an amino acid sequence, wherein the amino acid sequence is any one of the sequences as described herein.

In some embodiments, the nucleic acid sequence (i) comprises a nucleic acid sequence; or (ii) consists of a nucleic acid sequence, wherein the nucleic acid sequence is any one of the sequences as described herein.

To determine the percent identity of two amino acid sequences, or of two nucleic acid sequences, the sequences are aligned for optimal comparison purposes (e.g., gaps can be introduced in one or both of a first and a second amino acid or nucleic acid sequence for optimal alignment and non-homologous sequences can be disregarded for comparison purposes). The amino acid residues or nucleotides at corresponding amino acid positions or nucleotide positions are then compared. When a position in the first sequence is occupied by the same amino acid residue or nucleotide as the corresponding position in the second sequence, then the molecules are identical at that position (as used herein amino acid or nucleic acid “identity” is equivalent to amino acid or nucleic acid “homology”). The percent identity between the two sequences is a function of the number of identical positions shared by the sequences, taking into account the number of gaps, and the length of each gap, which need to be introduced for optimal alignment of the two sequences. For example, the comparison of sequences and determination of percent identity between two sequences can be accomplished using a Blossum 62 scoring matrix with a gap penalty of 12, a gap extend penalty of 4, and a frameshift gap penalty of 5.

Methods of treatment

The protein complex as described herein can be used for various therapeutic purposes. In one aspect, the disclosure provides methods for treating a cancer in a subject, methods of

reducing the rate of the increase of volume of a tumor in a subject over time, methods of reducing the risk of developing a metastasis, or methods of reducing the risk of developing an additional metastasis in a subject. In some embodiments, the treatment can halt, slow, retard, or inhibit progression of a cancer. In some embodiments, the treatment can result in the reduction of
5 in the number, severity, and/or duration of one or more symptoms of the cancer in a subject.

As used herein, the term “cancer” refers to cells having the capacity for autonomous growth. Examples of such cells include cells having an abnormal state or condition characterized by rapidly proliferating cell growth. The term is meant to include cancerous growths, e.g., tumors; oncogenic processes, metastatic tissues, and malignantly transformed cells, tissues, or
10 organs, irrespective of histopathologic type or stage of invasiveness. Also included are malignancies of the various organ systems, such as respiratory, cardiovascular, renal, reproductive, hematological, neurological, hepatic, gastrointestinal, and endocrine systems; as well as adenocarcinomas which include malignancies such as most colon cancers, renal-cell carcinoma, prostate cancer and/or testicular tumors, non-small cell carcinoma of the lung, and
15 cancer of the small intestine. Cancer that is “naturally arising” includes any cancer that is not experimentally induced by implantation of cancer cells into a subject, and includes, for example, spontaneously arising cancer, cancer caused by exposure of a patient to a carcinogen(s), cancer resulting from insertion of a transgenic oncogene or knockout of a tumor suppressor gene, and cancer caused by infections, e.g., viral infections. The term “carcinoma” is art recognized and
20 refers to malignancies of epithelial or endocrine tissues. The term also includes carcinosarcomas, which include malignant tumors composed of carcinomatous and sarcomatous tissues. An “adenocarcinoma” refers to a carcinoma derived from glandular tissue or in which the tumor cells form recognizable glandular structures. The term “sarcoma” is art recognized and refers to malignant tumors of mesenchymal derivation. The term “hematopoietic neoplastic disorders”
25 includes diseases involving hyperplastic/neoplastic cells of hematopoietic origin. A hematopoietic neoplastic disorder can arise from myeloid, lymphoid or erythroid lineages, or precursor cells thereof.

In one aspect, the disclosure features methods that include administering a therapeutically effective amount of a protein complex disclosed herein to a subject in need thereof (e.g., a
30 subject having, or identified or diagnosed as having, a cancer), e.g., breast cancer (e.g., triple-negative breast cancer), carcinoid cancer, cervical cancer, endometrial cancer, glioma, head and

neck cancer, liver cancer, lung cancer, small cell lung cancer, lymphoma, melanoma, ovarian cancer, pancreatic cancer, prostate cancer, renal cancer, colorectal cancer, gastric cancer, testicular cancer, thyroid cancer, bladder cancer, urethral cancer, or hematologic malignancy. In some embodiments, the cancer is unresectable melanoma or metastatic melanoma, non-small cell lung carcinoma (NSCLC), small cell lung cancer (SCLC), bladder cancer, or metastatic hormone-refractory prostate cancer. In some embodiments, the subject has a solid tumor. In some embodiments, the cancer is squamous cell carcinoma of the head and neck (SCCHN), renal cell carcinoma (RCC), triple-negative breast cancer (TNBC), or colorectal carcinoma. In some embodiments, the subject has Hodgkin's lymphoma. In some embodiments, the subject has triple-negative breast cancer (TNBC), gastric cancer, urothelial cancer, Merkel-cell carcinoma, or head and neck cancer. In some embodiments, the cancer is melanoma, pancreatic carcinoma, mesothelioma, hematological malignancies, especially Non-Hodgkin's lymphoma, lymphoma, chronic lymphocytic leukemia, or advanced solid tumors. In some embodiments, the cancer is PD-L1 positive metastatic non-small cell lung cancer.

In some embodiments, the compositions and methods disclosed herein can be used for treatment of patients at risk for a cancer. Patients with cancer can be identified with various methods known in the art.

In some embodiments, the cancer expresses PD-L1. In some embodiments, the cancer is resistant to the anti-PD-1 antibody treatment or the anti-PD-L1 antibody treatment. In some embodiments, the subject is not responsive to the anti-TIGIT antibody treatment or IL21 treatment. In some embodiments, the IL21 in the protein complex can activate tumor-infiltrating lymphocyte effector functions.

In some embodiments, the subject is not responsive to the immunotherapy. The immune-desert and immune-excluded phenotypes are known as cold tumors (non-inflamed), and the density of CD8⁺ T cells in the tumors is low. As the protein complex can increase immune response in the tumor microenvironment, the protein complex can be used to treat patients who are not responsive to the immunotherapy.

Thus, in one aspect, the disclosure further provides methods of increasing immune response in tumor microenvironment and methods of activating T cells in tumor microenvironment. These methods involve administering a therapeutically effective amount of the protein complex as described herein to the subject.

As used herein, by an “effective amount” is meant an amount or dosage sufficient to effect beneficial or desired results including halting, slowing, retarding, or inhibiting progression of a disease, e.g., a cancer or an autoimmune disease. An effective amount will vary depending upon, e.g., an age and a body weight of a subject to which the therapeutic agent is to be administered, a severity of symptoms and a route of administration, and thus administration can be determined on an individual basis.

An effective amount can be administered in one or more administrations. By way of example, an effective amount of a protein complex is an amount sufficient to ameliorate, stop, stabilize, reverse, inhibit, slow and/or delay progression of an autoimmune disease or a cancer in a patient or is an amount sufficient to ameliorate, stop, stabilize, reverse, slow and/or delay proliferation of a cell (e.g., a biopsied cell, any of the cancer cells described herein, or cell line (e.g., a cancer cell line)) *in vitro*. As is understood in the art, an effective amount of the protein complex may vary, depending on, *inter alia*, patient history as well as other factors such as the type (and/or dosage) of the therapeutic agent used.

Effective amounts and schedules for administering the protein complex, and/or compositions disclosed herein may be determined empirically, and making such determinations is within the skill in the art. Those skilled in the art will understand that the dosage that must be administered will vary depending on, for example, the mammal that will receive the protein complex, and/or compositions disclosed herein, the route of administration, the particular type of protein complex, and/or compositions disclosed herein used and other drugs being administered to the mammal.

The dosage of an effective amount of the protein complex can be 0.01 mg/kg to 100 mg/kg (mg per kg of patient weight). In some embodiments, the dosage can be less than 100 mg/kg, 10 mg/kg, 9 mg/kg, 8 mg/kg, 7 mg/kg, 6 mg/kg, 5 mg/kg, 4 mg/kg, 3 mg/kg, 2 mg/kg, 1 mg/kg, 0.5 mg/kg, or 0.1 mg/kg. In some embodiments, the dosage can be greater than 10 mg/kg, 9 mg/kg, 8 mg/kg, 7 mg/kg, 6 mg/kg, 5 mg/kg, 4 mg/kg, 3 mg/kg, 2 mg/kg, 1 mg/kg, 0.5 mg/kg, 0.1 mg/kg, 0.05 mg/kg, or 0.01 mg/kg. In some embodiments, the dosage is about 10 mg/kg, 9 mg/kg, 8 mg/kg, 7 mg/kg, 6 mg/kg, 5 mg/kg, 4 mg/kg, 3 mg/kg, 2 mg/kg, 1 mg/kg, 0.9 mg/kg, 0.8 mg/kg, 0.7 mg/kg, 0.6 mg/kg, 0.5 mg/kg, 0.4 mg/kg, 0.3 mg/kg, 0.2 mg/kg, or 0.1 mg/kg.

In any of the methods described herein, the protein complex, or pharmaceutical composition, optionally, at least one additional therapeutic agent can be administered to the

subject at least once a week (e.g., once a week, twice a week, three times a week, four times a week, once a day, twice a day, or three times a day). In some embodiments, the protein complex and at least one additional therapeutic agent are administered in the same composition (e.g., a liquid composition). In some embodiments, the protein complex and the at least one additional therapeutic agent are administered in two different compositions (e.g., a liquid composition containing the protein complex and a solid oral composition containing at least one additional therapeutic agent). In some embodiments, the at least one additional therapeutic agent is administered as a pill, tablet, or capsule. In some embodiments, the at least one additional therapeutic agent is administered in a sustained-release oral formulation.

In some embodiments, the one or more additional therapeutic agents can be administered to the subject prior to, or after administering the protein complex. In some embodiments, the one or more additional therapeutic agents and the protein complex are administered to the subject such that there is an overlap in the bioactive period of the one or more additional therapeutic agents and the protein complex in the subject.

In some embodiments, the subject can be administered the protein complex over an extended period of time (e.g., over a period of at least 1 week, 2 weeks, 3 weeks, 1 month, 2 months, 3 months, 4 months, 5 months, 6 months, 7 months, 8 months, 9 months, 10 months, 11 months, 12 months, 1 year, 2 years, 3 years, 4 years, or 5 years). A skilled medical professional may determine the length of the treatment period using any of the methods described herein for diagnosing or following the effectiveness of treatment (e.g., the observation of at least one symptom of cancer).

In some embodiments, one or more additional therapeutic agents can be administered to the subject. The additional therapeutic agent can comprise one or more inhibitors selected from the group consisting of an inhibitor of B-Raf, an EGFR inhibitor, an inhibitor of a MEK, an inhibitor of ERK, an inhibitor of K-Ras, an inhibitor of c-Met, an inhibitor of anaplastic lymphoma kinase (ALK), an inhibitor of a phosphatidylinositol 3-kinase (PI3K), an inhibitor of an Akt, an inhibitor of mTOR, a dual PI3K/mTOR inhibitor, an inhibitor of Bruton's tyrosine kinase (BTK), and an inhibitor of Isocitrate dehydrogenase 1 (IDH1) and/or Isocitrate dehydrogenase 2 (IDH2). In some embodiments, the additional therapeutic agent is an inhibitor of indoleamine 2,3-dioxygenase-1 (IDO1) (e.g., epacadostat). In some embodiments, the additional therapeutic agent can comprise one or more inhibitors selected from the group

consisting of an inhibitor of HER3, an inhibitor of LSD1, an inhibitor of MDM2, an inhibitor of BCL2, an inhibitor of CHK1, an inhibitor of activated hedgehog signaling pathway, and an agent that selectively degrades the estrogen receptor.

In some embodiments, the additional therapeutic agent can comprise one or more
5 therapeutic agents selected from the group consisting of Trabectedin, nab-paclitaxel, Trebananib, Pazopanib, Cediranib, Palbociclib, everolimus, fluoropyrimidine, IFL, regorafenib, Reolysin, Alimta, Zykadia, Sutent, temsirolimus, axitinib, everolimus, sorafenib, Votrient, Pazopanib, IMA-901, AGS-003, cabozantinib, Vinflunine, an Hsp90 inhibitor, Ad-GM-CSF, Temazolomide, IL-2, IFNa, vinblastine, Thalomid, dacarbazine, cyclophosphamide, lenalidomide, azacytidine,
10 lenalidomide, bortezomid, amrubicine, carfilzomib, pralatrexate, and enzastaurin.

In some embodiments, the additional therapeutic agent can comprise one or more therapeutic agents selected from the group consisting of an adjuvant, a TLR agonist, tumor necrosis factor (TNF) alpha, IL-1, HMGB1, an IL-10 antagonist, an IL-4 antagonist, an IL-13 antagonist, an IL-17 antagonist, an HVEM antagonist, an ICOS agonist, a treatment targeting
15 CX3CL1, a treatment targeting CXCL9, a treatment targeting CXCL10, a treatment targeting CCL5, an LFA-1 agonist, an ICAM1 agonist, and a Selectin agonist. In some embodiments, carboplatin, nab-paclitaxel, paclitaxel, cisplatin, pemetrexed, gemcitabine, FOLFOX, or FOLFIRI are administered to the subject.

In some embodiments, the additional therapeutic agent is an anti-OX40 antibody, an anti-
20 PD-1 antibody, an anti-PD-L1 antibody, an anti-PD-L2 antibody, an anti-LAG-3 antibody, an anti-TIGIT antibody, an anti-BTLA antibody, an anti-CTLA-4 antibody, or an anti-GITR antibody.

In some embodiments, the additional therapeutic agent is an immunotherapy, e.g., a blocking anti-PD-1 antibody (e.g., Pembrolizumab, Nivolumab, or Cemiplimab) or a block anti-
25 PD-L1 antibody (e.g., Atezolizumab, Avelumab, or Durvalumab).

Pharmaceutical Compositions

Also provided herein are pharmaceutical compositions that contain the protein complex described herein. The pharmaceutical compositions may be formulated in any manner known in
30 the art.

A “pharmaceutically acceptable carrier” refers to an ingredient in a pharmaceutical formulation, other than an active ingredient, which is bioactivity acceptable and nontoxic to a subject. Pharmaceutical acceptable carriers for use in the pharmaceutical compositions disclosed herein may include, for example, pharmaceutically acceptable liquid, gel, or solid carriers, aqueous vehicles, nonaqueous vehicles, antimicrobial agents, isotonic agents, buffers, antioxidants, anesthetics, suspending/dispersing agents, sequestering or chelating agents, diluents, adjuvants, excipients, or non-toxic auxiliary substances, other components known in the art, or various combinations thereof. Some suitable components may include, for example, antioxidants, fillers, binders, disintegrants, buffers, preservatives, lubricants, flavorings, thickeners, coloring agents, emulsifiers or stabilizers such as sugars and cyclodextrins. Suitable antioxidants may include, for example, methionine, ascorbic acid, EDTA, sodium thiosulfate, platinum, catalase, citric acid, cysteine, thioglycerol, thioglycolic acid, thiosorbitol, butylated hydroxyanisole, butylated hydroxytoluene, and/or propyl gallate. As disclosed herein, inclusion of one or more antioxidants such as methionine in a pharmaceutical composition provided herein decreases oxidation of the polypeptide complex or the bispecific polypeptide complex. This reduction in oxidation prevents or reduces loss of binding affinity, thereby improving protein stability and maximizing shelf-life. Therefore, in certain embodiments, compositions are provided that comprise the polypeptide complex or the bispecific polypeptide complex disclosed herein and one or more antioxidants such as methionine.

Pharmaceutical compositions are also formulated to be compatible with their intended route of administration (e.g., intravenous, intraarterial, intramuscular, intradermal, subcutaneous, or intraperitoneal). Preparations of the compositions can be formulated and enclosed in ampules, disposable syringes, or multiple dose vials. Where required (as in, for example, injectable formulations), proper fluidity can be maintained by, for example, the use of a coating, such as lecithin, or a surfactant. Absorption of the protein complex can be prolonged by including an agent that delays absorption (e.g., aluminum monostearate and gelatin). Alternatively, controlled release can be achieved by implants and microencapsulated delivery systems, which can include biodegradable, biocompatible polymers (e.g., ethylene vinyl acetate, polyanhydrides, polyglycolic acid, collagen, polyorthoesters, and polylactic acid).

Compositions containing one or more of any of the protein complex described herein can be formulated for parenteral (e.g., intravenous, intraarterial, intramuscular, intradermal,

subcutaneous, or intraperitoneal) administration in dosage unit form (i.e., physically discrete units containing a predetermined quantity of active compound for ease of administration and uniformity of dosage).

Pharmaceutical compositions for parenteral administration are preferably sterile and substantially isotonic and manufactured under Good Manufacturing Practice (GMP) conditions. Pharmaceutical compositions can be provided in unit dosage form (i.e., the dosage for a single administration). For injection, protein complex can be formulated in aqueous solutions, preferably in physiologically-compatible buffers to reduce discomfort at the site of injection.

Exemplary doses include milligram or microgram amounts of any of the protein complex described herein per kilogram of the subject's weight (e.g., about 1 µg/kg to about 500 mg/kg; about 100 µg/kg to about 500 mg/kg; about 100 µg/kg to about 50 mg/kg; about 10 µg/kg to about 5 mg/kg; about 10 µg/kg to about 0.5 mg/kg; about 1 µg/kg to about 50 µg/kg; about 1 mg/kg to about 10 mg/kg; or about 1 mg/kg to about 5 mg/kg). While these doses cover a broad range, one of ordinary skill in the art will understand that therapeutic agents, including protein complex, vary in their potency, and effective amounts can be determined by methods known in the art. Typically, relatively low doses are administered at first, and the attending health care professional or veterinary professional (in the case of therapeutic application) or a researcher (when still working at the development stage) can subsequently and gradually increase the dose until an appropriate response is obtained. In addition, it is understood that the specific dose level for any particular subject will depend upon a variety of factors including the activity of the specific compound employed, the age, body weight, general health, gender, and diet of the subject, the time of administration, the route of administration, the rate of excretion, and the half-life of the protein complex *in vivo*.

The pharmaceutical compositions can be included in a container, pack, or dispenser together with instructions for administration. The disclosure also provides methods of manufacturing the protein complex for various uses as described herein.

EXAMPLES

The invention is further described in the following examples, which do not limit the scope of the invention described in the claims.

Example 1. IL21 fused to anti-TIGIT antibody Tiragolumab

Plasmids were constructed to express a fusion protein that comprises a mouse interleukin 21 (IL21), fused at the 3A site in one of the two heavy chains of Tiragolumab, as shown in **FIG. 1**. Specifically, knobs-into-holes (KIH) mutations were introduced to the Tiragolumab-IgG1 antibody, and IL21 was inserted between position 359 and position 360 (according to EU numbering) to the knob heavy chain. The N-terminus of IL21 was linked to position 359 of Tiragolumab-IgG1 knob heavy chain via GGGGSGGGGS (SEQ ID NO: 3), and the C-terminus of IL21 was linked to position 360 of Tiragolumab-IgG1 knob heavy chain via GGGGSGGGGS (SEQ ID NO: 3).

The modified Tiragolumab-IgG1 antibody fused with IL21 was named Tiragolumab-IL21-3A2-knob. Sequence of the knob heavy chain of Tiragolumab-IL21-3A2-knob is set forth in SEQ ID NO: 4; sequence of the hole heavy chain of Tiragolumab-IL21-3A2-knob is set forth in SEQ ID NO: 5; and sequence of the light chain of Tiragolumab-IL21-3A2-knob is set forth in SEQ ID NO: 6.

Unless otherwise specified in the following experiments, the antibodies used were all purified by a protein A column using the AKTA™ chromatography system.

Non-reducing SDS-PAGE (sodium dodecyl sulphate - polyacrylamide gel electrophoresis) was performed to determine the purity using a 4-12% acrylamide gel. The protein samples were prepared as follows. 2.4 µl of the protein sample (2.9 mg/mL) was mixed with 6 µl Tris-Glycine SDS Sample Buffer (2×) (Thermo, Cat# LC2676) and 3.6 µl distilled water. The mixture was then boiled for 2 minutes and instantly centrifuged before loading. As shown in **FIG. 2**, Tiragolumab-IL21-3A2-knob showed a single band with correct molecular weight (lane 3).

Example 2. Verification of *in vitro* functions***Determination of binding affinity to TIGIT***

The binding affinity between purified His-tagged human TIGIT (hTIGIT-His; ACRO Biosystems, Cat# TIT-H52H3) and Tiragolumab-IgG1 or Tiragolumab-IL21-3A2-knob were measured by surface plasmon resonance (SPR) using Biacore™ (Biacore Inc., Piscataway N.J.) T200 biosensor equipped with pre-immobilized protein A sensor chips.

HBS-EP+ buffer (10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 150 mM NaCl, 3 mM ethylenediaminetetraacetic acid (EDTA) and 0.05% P20, pH 7.4) was

diluted from HBS-EP+ buffer (10×) as the running buffer throughout the experiment. The fusion protein Tiragolumab-IL21-3A2-knob and antibody Tiragolumab-IgG1 (1 µg/mL) were injected into Biacore™ T200 biosensor at 10 µL/min for 50 seconds to achieve to a desired protein density (about 100 RU). His-tagged human TIGIT (hTIGIT-His) at a concentration of 100 nM was then injected at 30 µL/min for 180 seconds. Dissociation was monitored for 600 seconds. The chip was regenerated after the last injection of each titration with glycine (pH 2.0, 30 µL/min for 12 seconds). As a person of ordinary skill in the art would understand, the same method with appropriate adjustments for parameters (e.g., fusion protein concentration) was performed for each tested fusion protein. The results for the tested fusion proteins are shown in the table below.

Table 4.

Ligand	Analysis	kon (1/Ms)	koff (1/s)	KD (M)	Rmax (RU)
Tiragolumab-IgG1	hTIGIT-His	1.83E+06	7.44E-05	4.06E-11	21.1
Tiragolumab-IL21-3A2-knob	hTIGIT-His	1.48E+06	5.76E-05	3.88E-11	25.0

Kinetic association rates (kon) and dissociation rates (koff) were obtained simultaneously by fitting the data globally to a 1:1 Langmuir binding model (Karlsson, R. Roos, H. Fagerstam, L. Petersson, B., 1994. Methods Enzymology 6. 99-110) using Biacore™ T200 Evaluation Software 3.0. Affinities were calculated from the quotient of the kinetic rate constants (KD=koff/kon). The results showed that the tested fusion protein Tiragolumab-IL21-3A2-knob and antibody Tiragolumab-IgG1 can bind to hTIGIT-His with comparable binding affinities.

Determination of binding affinity to IL21R

The binding affinity between purified His-tagged recombinant mouse IL21R protein (mIL21R-His; Sino Biological Inc., Cat# 51184-M08H) and Tiragolumab-IL21-3A2-knob was measured by SPR using Biacore™ (Biacore Inc., Piscataway N.J.) T200 biosensor equipped with pre-immobilized protein A sensor chips. The experiment was performed by a similar method as described above. The results for the tested fusion proteins are shown in the table below.

Table 5.

Ligand	Analysis	kon (1/Ms)	koff (1/s)	KD (M)	Rmax (RU)
Tiragolumab-IL21-3A2-knob	mIL21R-His	4.65E+05	7.71E-04	1.66E-09	12.6

Example 3. *In vivo* pharmacological validation

A hTIGIT mouse model (obtained from Biocytogen Pharmaceuticals (Beijing) Co., Ltd. Cat#: 110017) was engineered to express a chimeric TIGIT protein. The chimeric TIGIT protein (SEQ ID NO: 7) includes a replacement of a portion of the extracellular region of the mouse TIGIT protein with the corresponding human TIGIT extracellular region.

The TIGIT gene humanized mice (7-8 weeks) were subcutaneously injected with mouse colon cancer cell MC38 ($5 \times 10^5/100 \mu\text{l}$ PBS), and when the tumor volume grew to about 100-150 mm³, the mice were divided to a control group and four treatment groups based on tumor size (6 mice per group). The treatment groups were randomly selected for anti-human TIGIT antibody Tiragolumab-IgG1 treatment (3 mg/kg (G2) or 10 mg/kg (G3)), or fusion protein Tiragolumab-IL21-3A2-knob treatment (3.28 mg/kg (G4) or 11 mg/kg (G5)). The molar dosage (e.g., mole/kg) of Tiragolumab-IL21-3A2-knob was equal to that of Tiragolumab-IgG1. The control group mice were injected with an equal volume of phosphate buffer saline (PBS). The frequency of administration was twice a week (4 times of administrations in total). The tumor volume was measured twice a week and the body weight of the mice was weighed as well. Euthanasia was performed when the tumor volume of the mouse reached 3000 mm³.

Table 6.

Group	No. of mice	Antibodies	Dosage (mg/kg)	Route	Frequency	Total No. of administration
G1	6	PBS	-	i.p.	BIW	6
G2	6	Tiragolumab-IgG1	3mg/kg	i.p.	BIW	6
G3	6	Tiragolumab-IgG1	10mg/kg	i.p.	BIW	6
G4	6	Tiragolumab-IL21-3A2-knob	3.28mg/kg	i.p.	BIW	6
G5	6	Tiragolumab-IL21-3A2-knob	11mg/kg	i.p.	BIW	6

The injected volume was calculated based on the weight of the mouse. The length of the long axis and the short axis of the tumor were measured and the volume of the tumor was calculated as $0.5 \times (\text{long axis}) \times (\text{short axis})^2$. The weight of the mice was also measured twice a week. The tumor growth inhibition percentage (TGI%) was calculated using the following formula: $\text{TGI} (\%) = [1 - (\text{Ti} - \text{T}_0) / (\text{Vi} - \text{V}_0)] \times 100$. Ti is the average tumor volume in the treatment group on day i. T₀ is the average tumor volume in the treatment group on day zero. Vi is the

average tumor volume in the control group on day i . V_0 is the average tumor volume in the control group on day zero. T-test was performed for statistical analysis. A TGI% higher than 60% indicates clear suppression of tumor growth. $P < 0.05$ is a threshold to indicate significant difference.

Overall, the mice in each group were healthy. The body weight of all the treatment and control group mice increased, and the body weight were not obviously different from each other (**FIG. 3** and **FIG. 4**). As shown in **FIG. 5**, the tumor in the control group continued growing during the experimental period. When compared with the control group mice, the tumor volumes of the treatment group mice were smaller. Thus, the anti-human TIGIT antibody Tiragolumab-IgG1 and the fusion protein Tiragolumab-IL21-3A2-knob were well tolerated, and inhibited the tumor growth in mice.

The table below summarizes the results for this experiment, including the tumor volumes on the day of grouping (day 0), 14 days after the grouping (day 14), and at the end of the experiment (day 21); the number of tumor-free mice; the survival rate of the mice; the Tumor Growth Inhibition value (TGI_{TV}); and the statistical differences (P value) of body weight and tumor volume between the treatment and control groups.

Table 7.

		Tumor volume(mm ³)			Tumor-free	Survival	TGI _{TV} %	P value	
		Day 0	Day 14	Day 21				Body weight	Tumor Volume
Control	G1	123±3	1396±157	2550±351	0	5/6	N/A	NA	N/A
Treatment groups	G2	123±4	1101±95	1657±202	0	6/6	36.8	0.222	0.047
	G3	123±4	976±167	1579±306	0	6/6	40.0	1,000	0.065
	G4	123±4	527±187	837±322	1	6/6	70.5	0.510	0.006
	G5	123±5	426±110	613±165	1	6/6	79.8	0.001	< 0.001

At the end of the experiment (day 21), the body weight of the mice in each group increased and there was no significant difference between the treatment group mice (except G5 group mice) and control group mice. Although the body weight of the G5 group mice was lower than that of the G1 group mice, the G5 group mice continued to gain weight throughout the experimental period, and the body weight increased by about 10% at the end of the experiment. No obvious difference in body weight change was observed. Considering that the mice in the G5

group had a lower tumor volume at the end of the experiment, the difference in body weight between the G5 group mice and the G1 group mice was mainly due to the tumor weight.

As shown in **FIG. 5**, the tumor volumes of all treatment group mice (G2-G5) were smaller than those of the control group mice (G1). The results also showed that the anti-human TIGIT antibody Tiragolumab-IgG1 and the fusion protein Tiragolumab-IL21-3A2-knob had different tumor inhibitory effects, which was dosage-dependent. Under the same condition (e.g., administration dosage and frequency), the inhibitory effects of the fusion protein (G4 and G5) were better than those of the anti-PD-L1 antibody (G2 and G3). This indicates that fusion of IL21 to TIGIT antibody can improve the *in vivo* efficacy of the TIGIT antibody. The above results showed that the fusion protein (Tiragolumab-IL21-3A2-knob) exhibited significantly better tumor growth inhibitory effect as compared to that of Tiragolumab-IgG1 in TIGIT humanized mice. In addition, the fusion protein had no obvious toxic effects in mice.

Example 4. IL21 fused to different anti-TIGIT antibodies

Multiple anti-TIGIT monoclonal antibodies are currently in clinical stages, including Vibostolimab (VH: SEQ ID NO: 12; VL: SEQ ID NO: 13) developed by Merck & Co.; Etigilimab (VH: SEQ ID NO: 14; VL: SEQ ID NO: 15) developed by Mereo BioPharma Group Ltd.; Domvanalimab (VH: SEQ ID NO: 16; VL: SEQ ID NO: 17) developed by Arcus Biosciences Inc.; and Ociperlimab (VH: SEQ ID NO: 18; VL: SEQ ID NO: 19) developed by BeiGene Co. Ltd..

In one experiment, the variable regions of these antibodies can be used to replace the variable regions of Tiragolumab-IL21-3A2-knob according to their sequences. The resulting protein complexes are named Vibostolimab-IL21-3A2-knob (full-length knob chain: SEQ ID NO: 20; full-length hole chain: SEQ ID NO: 21; full-length light chain: SEQ ID NO: 22), Etigilimab-IL21-3A2-knob (full-length knob chain: SEQ ID NO: 23; full-length hole chain: SEQ ID NO: 24; full-length light chain: SEQ ID NO: 25), Domvanalimab-IL21-3A2-knob (full-length knob chain: SEQ ID NO: 26; full-length hole chain: SEQ ID NO: 27; full-length light chain: SEQ ID NO: 28), and Ociperlimab-IL21-3A2-knob (full-length knob chain: SEQ ID NO: 29; full-length hole chain: SEQ ID NO: 30; full-length light chain: SEQ ID NO: 31), respectively.

In addition, anti-TIGIT monoclonal antibodies can be fused to human IL21, and the resulting protein complexes are named Tiragolumab-hIL21-3A2-knob, Vibostolimab-hIL21-

3A2-knob, Etigilimab-hIL21-3A2-knob, Domvanalimab-hIL21-3A2-knob and Ociperlimab-hIL21-3A2-knob, respectively.

Similar to the experiments described in Example 1, non-reducing SDS-PAGE can be used to determine the purity of the above protein complexes using a 4-12% acrylamide gel. A single band with correct molecular weight indicates successful expression of the protein complexes.

Size-Exclusion Ultra Performance Liquid Chromatography (SEC-UPLC) can be further performed to detect the purity changes of the protein complex. For example, Agilent 1290 chromatograph system (connected with XBridge™ Protein BEH SEC column (200 Å, Waters Corporation)) can be used. The proteins samples can be diluted to 1 mg/mL with purified water. The following parameters are used: mobile phase: 100 mmol/L phosphate buffer (PB) (pH 7.4) + 0.2 mol/L NaCl + 10% acetonitrile; flow rate: 1.8 mL/min; column temperature: 25 °C; detection wavelength: 280 nm; injection volume: 10 µg; sample tray temperature: 6 °C; and running time: 7 minutes.

Similar to the results described above, it is expected that the protein complexes described herein can be expressed with a high purify (e.g., above 90% pure).

Example 5. Determination of binding affinity to IL21R and TIGIT

Similar to the binding affinity determination experiments described in Example 2, the binding affinity between purified His-tagged human TIGIT and Vibostolimab-IL21-3A2-knob, Etigilimab-IL21-3A2-knob, Domvanalimab-IL21-3A2-knob, Ociperlimab-IL21-3A2-knob, Tiragolumab-hIL21-3A2-knob, Vibostolimab-hIL21-3A2-knob, Etigilimab-hIL21-3A2-knob, Domvanalimab-hIL21-3A2-knob or Ociperlimab-hIL21-3A2-knob can be measured by surface plasmon resonance using Biacore™ T200 biosensor equipped with pre-immobilized protein A sensor chips.

The binding affinity detection between His-tagged recombinant mouse IL21R protein and Vibostolimab-IL21-3A2-knob, Etigilimab-IL21-3A2-knob, Domvanalimab-IL21-3A2-knob or Ociperlimab-IL21-3A2-knob can be determined using the methods described in Example 2. Similarly, the binding affinity detection between His-tagged recombinant human IL21R protein (ACRO Biosystems Inc., Cat# ILR-H5226) and Tiragolumab-hIL21-3A2-knob, Vibostolimab-

hIL21-3A2-knob, Etigilimab-hIL21-3A2-knob, Domvanalimab-hIL21-3A2-knob, or Ociperlimab-hIL21-3A2-knob can be determined using the methods described in Example 2.

Similar to the results described above, it is expected that the protein complexes described herein can achieve high binding affinities to IL21R and TIGIT. For example, the K_D value of the protein complexes binding to TIGIT can be less than 1×10^{-7} M, less than 1×10^{-8} M, less than 1×10^{-9} M, less than 1×10^{-10} M, or less than 1×10^{-11} M; the K_D value of the protein complexes binding to IL21 receptor (e.g., human or mouse IL21 receptor) can be less than 1×10^{-7} M, less than 1×10^{-8} M, less than 1×10^{-9} M, less than 1×10^{-10} M, or less than 1×10^{-11} M.

Example 6. Anti-Tumor Activity in hTIGIT model

Similar to the *in vivo* pharmacological validation experiments described in Example 3, the protein complexes Vibostolimab-IL21-3A2-knob, Etigilimab-IL21-3A2-knob, Domvanalimab-IL21-3A2-knob and Ociperlimab-IL21-3A2-knob can be tested for their effect on tumor growth in a hTIGIT mouse model of colon carcinoma.

Specifically, about 5×10^5 MC38 cells can be injected subcutaneously in hTIGIT mice, and when the tumor volume grows to about 100-150 mm³, the mice are placed to different groups based on tumor size (6 mice per group). The treatment groups are randomly selected for Vibostolimab-IL21-3A2-knob, Etigilimab-IL21-3A2-knob, Domvanalimab-IL21-3A2-knob or Ociperlimab-IL21-3A2-knob treatment. The control group mice are injected with PBS. The tumor volume can be measured twice a week and the body weight of the mice is weighed as well. Euthanasia is performed when the tumor volume of the mouse reaches 3000 mm³. An increase of body weight indicates that the tested proteins are tolerated and are not obviously toxic to the mice.

In a similar experiment, Tiragolumab-hIL21-3A2-knob, Vibostolimab-hIL21-3A2-knob, Etigilimab-hIL21-3A2-knob, Domvanalimab-hIL21-3A2-knob and Ociperlimab-hIL21-3A2-knob can be tested for their effect on tumor growth in a hTIGIT/IL21R mouse model of colon carcinoma. The hTIGIT/IL21R mouse model can be obtained by breeding hTIGIT mice with IL21R gene humanized mice (hIL21R mice). The hIL21R mice are engineered to express a chimeric IL21R protein (SEQ ID NO: 32) wherein a part of the extracellular region of the mouse IL21R protein is replaced with the corresponding human IL21R extracellular region. Detailed

descriptions regarding the humanized IL21R mouse model can be found in CN112501204B, which is incorporated herein by reference in its entirety.

Specifically, about 5×10^5 MC38 cells can be injected subcutaneously in hTIGIT/hIL21R mice, and when the tumor volume grows to about 100-150 mm³, the mice are placed to different groups based on tumor size (6 mice per group). The treatment groups are randomly selected for Tiragolumab-hIL21-3A2-knob, Vibostolimab-hIL21-3A2-knob, Etigilimab-hIL21-3A2-knob, Domvanalimab-hIL21-3A2-knob, or Ociperlimab-hIL21-3A2-knob treatment. The control group mice are injected with PBS. The tumor volume is measured twice a week and the body weight of the mice is weighed as well. Euthanasia is performed when the tumor volume of the mouse reaches 3000 mm³. An increase of body weight indicates that the tested proteins are tolerated and are not obviously toxic to the mice. A high TGI% (e.g., greater than 60%) indicates robust tumor inhibitory effects in the treatment groups.

Similar to the results described above, it is expected that the protein complexes described herein can also exhibit robust tumor inhibitory effects (e.g., TGI% greater than 50%, 60%, 70%, 80%, 90% or 100%).

OTHER EMBODIMENTS

It is to be understood that while the invention has been described in conjunction with the detailed description thereof, the foregoing description is intended to illustrate and not limit the scope of the invention, which is defined by the scope of the appended claims. Other aspects, advantages, and modifications are within the scope of the following claims.

WHAT IS CLAIMED IS:

1. A protein complex comprising a targeting moiety fused with an immunomodulatory moiety, wherein (a) the targeting moiety specifically binds to T cell immunoreceptor with Ig and ITIM domains (TIGIT); and (b) the immunomodulatory moiety specifically binds to interleukin-21 receptor (IL21R).
2. The protein complex of claim 1, wherein the immunomodulatory moiety comprises an antibody or antigen-binding fragment, a single chain variable fragment (scFv), a Fc-containing polypeptide, or a fusion protein that specifically binds to IL21R.
3. The protein complex of claim 1 or 2, wherein the immunomodulatory moiety is an IL21R agonist.
4. The protein complex of any one of claims 1-3, wherein the immunomodulatory moiety comprises an IL21 polypeptide.
5. The protein complex of claim 4, wherein the IL21 is a human IL21 polypeptide.
6. The protein complex of any one of claims 1-5, wherein the targeting moiety comprises an antibody or antigen-binding fragment, a single chain variable fragment (scFv), a Fc-containing polypeptide, or a fusion protein that specifically binds to TIGIT.
7. The protein complex of any one of claims 1-6, wherein the targeting moiety comprises a full-length antibody.
8. The protein complex of any one of claims 1-7, wherein targeting moiety has a KD of less than 1×10^{-8} M, less than 1×10^{-9} M, less than 1×10^{-10} M, or less than 5×10^{-11} M with TIGIT (e.g., less than 1×10^{-9} M).

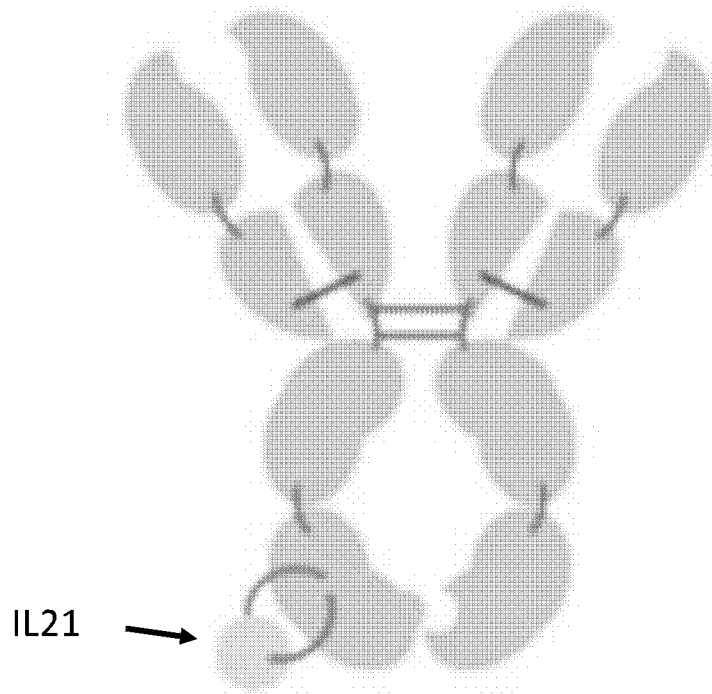
9. The protein complex of any one of claims 1-8, wherein immunomodulatory moiety has a KD of less than 1×10^{-7} M, 1×10^{-8} M, less than 5×10^{-9} M, or less than 2×10^{-9} M with IL21R.
10. The protein complex of any one of claims 1-9, wherein the targeting moiety comprises a polypeptide, wherein the immunomodulatory moiety is fused to the N-terminus or the C-terminus of the polypeptide.
11. The protein complex of any one of claims 1-9, wherein the immunomodulatory moiety comprises a polypeptide, wherein the targeting moiety is fused to the N-terminus or the C-terminus of the polypeptide.
12. The protein complex of any one of claims 1-9, wherein the targeting moiety comprises a polypeptide comprising a CH3 domain, wherein the immunomodulatory moiety is fused to the CH3 domain.
13. The protein complex of any one of claims 1-9, wherein the targeting moiety and the immunomodulatory moiety are fused to a scaffold protein (e.g., an albumin).
14. The protein complex of any one of claims 1-9, wherein the protein complex comprises a bispecific antibody, wherein the bispecific antibody binds to TIGIT and IL21R.
15. The protein complex of any one of claims 1-14, wherein the protein complex comprises two or more immunomodulatory moieties that specifically bind to IL21R.
16. The protein complex of any one of claims 1-15, wherein the protein complex comprises two or more targeting moieties that specifically bind to TIGIT.
17. The protein complex of any one of claims 1-16, wherein the protein complex comprises an Fc comprising two CH3 domains.

18. The protein complex of claim 17, wherein the immunomodulatory moiety is linked to a CH3 domain of the two CH3 domains in the Fc.
19. The protein complex of claim 17, wherein the immunomodulatory moiety is linked to the C-terminus of a CH3 domain of the two CH3 domains.
20. The protein complex of claim 17, wherein the immunomodulatory moiety is fused to a CH3 domain of the two CH3 domains in the Fc at a region from position 344 to position 382 of the CH3 domain according to EU numbering.
21. The protein complex of claim 20, wherein the immunomodulatory moiety is inserted to a CH3 domain of the two CH3 domains in the Fc between position 359 and position 360 of the CH3 domain according to EU numbering.
22. The protein complex of claim 17, wherein the targeting moiety is linked to a CH3 domain of the two CH3 domains in the Fc.
23. The protein complex of claim 17, wherein the targeting moiety is linked to the C-terminus of a CH3 domain of the two CH3 domains in the Fc.
24. The protein complex of claim 17, wherein the targeting moiety is fused to a CH3 domain of the two CH3 domains in the Fc at a region from position 344 to position 382 of the CH3 domain according to EU numbering.
25. The protein complex of claim 17, wherein the targeting moiety comprises a scFv (e.g., a scFv targeting TIGIT).
26. The protein complex of any one of claims 1-25, wherein the protein complex comprises two light chains.

27. The protein complex of claim 26, wherein the immunomodulatory moiety is linked to one of the two light chains.
28. The protein complex of claim 26, wherein the targeting moiety is linked to one of the two light chains.
29. The protein complex of any one of claims 1-28, wherein the protein complex further comprises a targeting moiety targeting PD-1.
30. The protein complex of any one of claims 1-29, wherein the protein complex further comprises a cytokine (e.g., IL2, IL7, IL15, IL18, and/or IL12).
31. The protein complex of claim 29 or 30, wherein the targeting moiety targeting PD-1 and/or cytokine are linked to the protein complex by a linker sequence.
32. The protein complex of any one of claims 1-31, wherein the protein complex comprises a linker sequence (GGGS)_n, wherein n can be 1, 2, 3, 4, 5, 6, 7 or 8.
33. The protein complex of any one of claims 1-32, wherein the targeting moiety comprises an anti-TIGIT antibody or antigen-binding fragment thereof.
34. The protein complex of claim 33, wherein the anti-TIGIT antibody or antigen-binding fragment thereof is an IgG-like molecule.
35. The protein complex of any one of claims 1-34, wherein the targeting moiety comprises a heavy chain variable region (VH) comprising an amino acid sequence that is at least 90% identical to SEQ ID NO: 1, 12, 14, 16, or 18; and a light chain variable region (VL) comprising an amino acid sequence that is at least 90% identical to SEQ ID NO: 2, 13, 15, 17, or 19; or the immunomodulatory moiety comprises a sequence that is at least 80% identical to SEQ ID NO: 9 or SEQ ID NO: 10.

36. A method of treating a subject having cancer, the method comprising administering a therapeutically effective amount of a composition comprising the protein complex of any one of claims 1-35, to the subject.
37. The method of claim 36, wherein the subject has at least one tumor infiltrating immune cells expressing TIGIT.
38. The method of claim 36 or 37, wherein the cancer is resistant to anti-PD-1 antibody treatment and/or anti-PD-L1 antibody treatment and/or anti-TIGIT antibody treatment; and/or a chemotherapy.
39. The method of any one of claims 36-38, further comprising administering an effective amount of an anti-PD-1 antibody or an anti-PD-L1 antibody to the subject.
40. A method of increasing immune response in tumor microenvironment in a subject, the method comprising administering a therapeutically effective amount of a composition comprising the protein complex of any one of claims 1-35 to the subject.
41. A method of activating T cells in tumor microenvironment of a subject, the method comprising administering a therapeutically effective amount of a composition comprising the protein complex of any one of claims 1-35 to the subject.
42. A method of increasing IL21 stability or enhancing IL21 function when delivering IL21 into a subject, the method comprising administering a therapeutically effective amount of a composition comprising the protein complex of any one of claims 1-35, to the subject.
43. A method of reducing the toxicity of IL21 when delivering IL21 into a subject, the method comprising administering a therapeutically effective amount of a composition comprising the protein complex of any one of claims 1-35, to the subject.

44. A method of increasing the therapeutic effect of an anti-TIGIT antibody or reducing the toxicity of IL21, comprising:
fusing IL21 to the anti-TIGIT antibody.
45. An isolated molecule comprising the protein complex of any one of claims 1-35; covalently bound to a therapeutic agent.
46. The isolated molecule of claim 45, wherein the therapeutic agent is a cytotoxic or cytostatic agent.
47. A pharmaceutical composition comprising the protein complex of any one of claims 1-35; and a pharmaceutically acceptor carrier.
48. A nucleic acid encoding the protein complex of any one of claims 1-35.
49. A vector comprising the nucleic acid of claim 48.
50. A host cell comprising the nucleic acid of claim 48.
51. A method for producing a protein complex, the method comprising culturing the host cell of claim 50 under conditions suitable to produce the protein complex.



Tiragolumab-IL21-3A-knob

FIG. 1

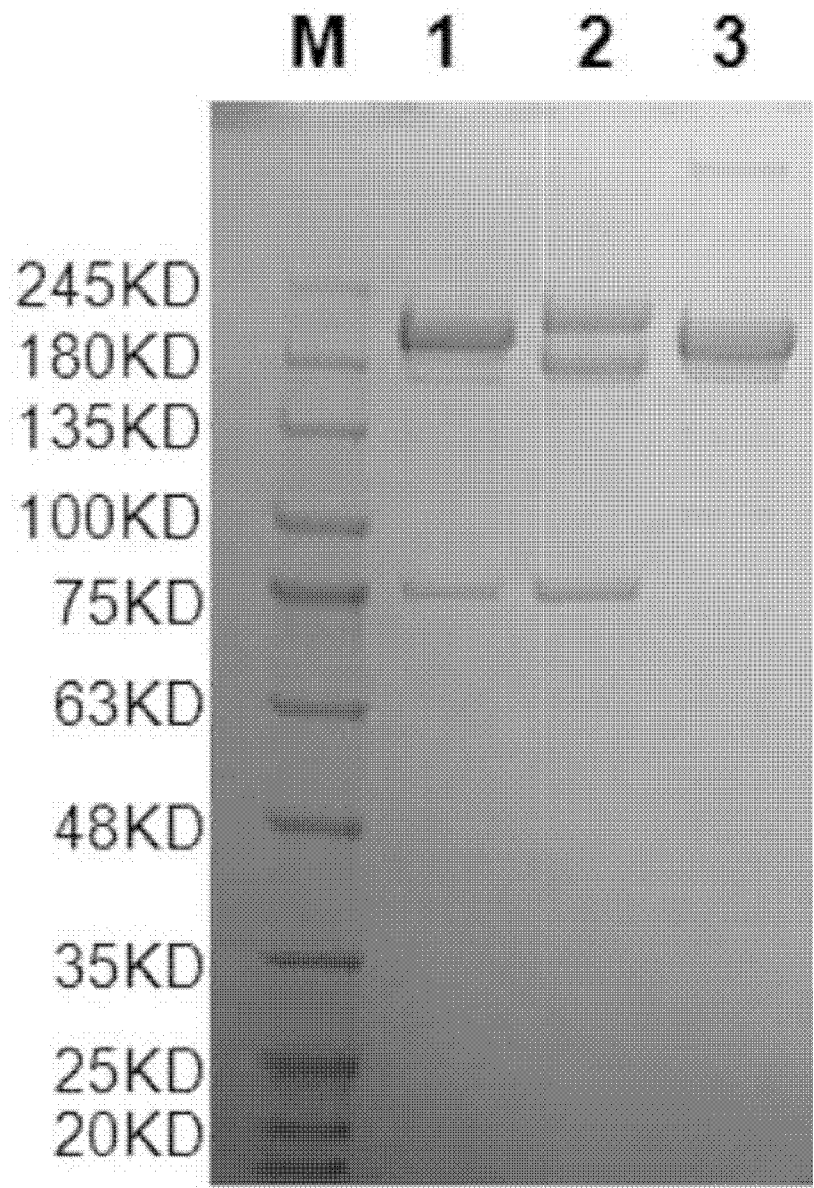


FIG. 2

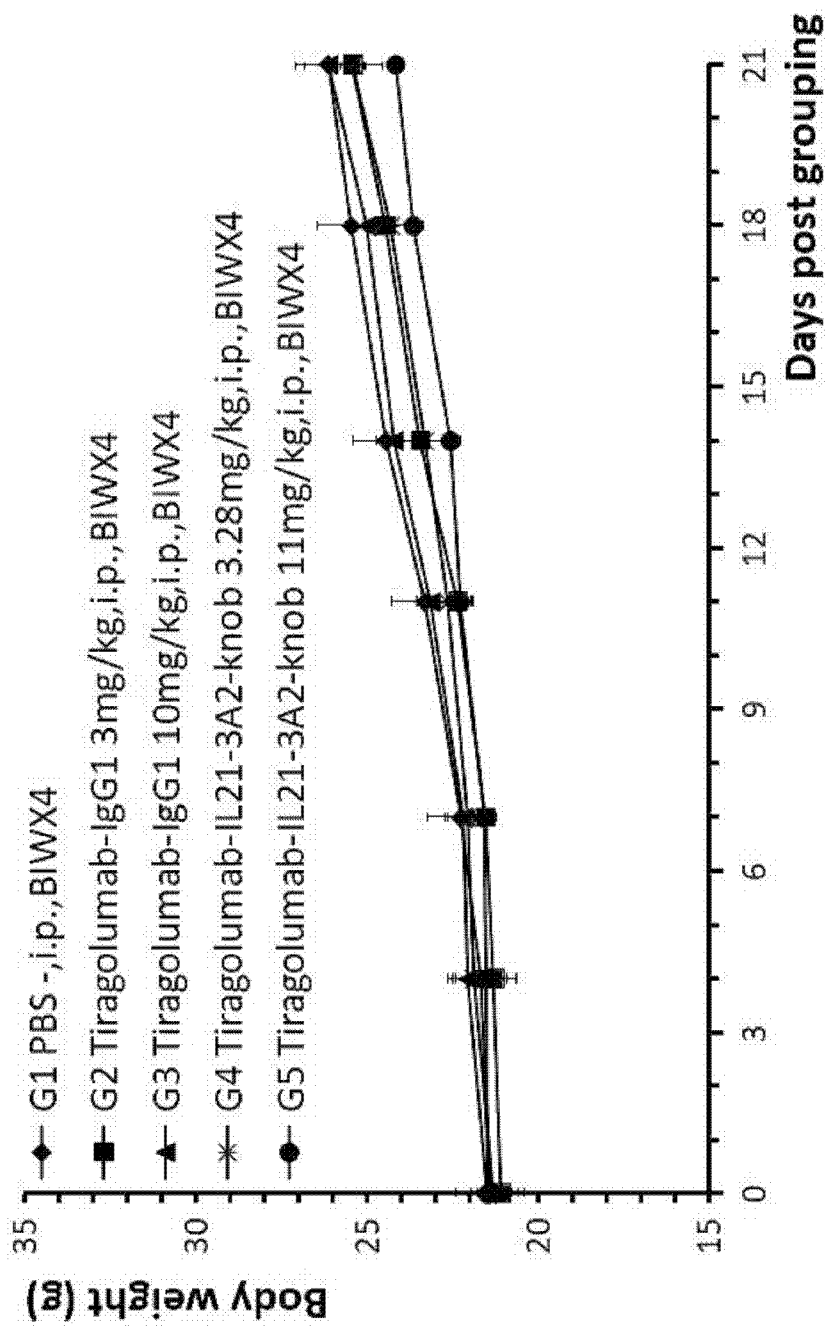


FIG. 3

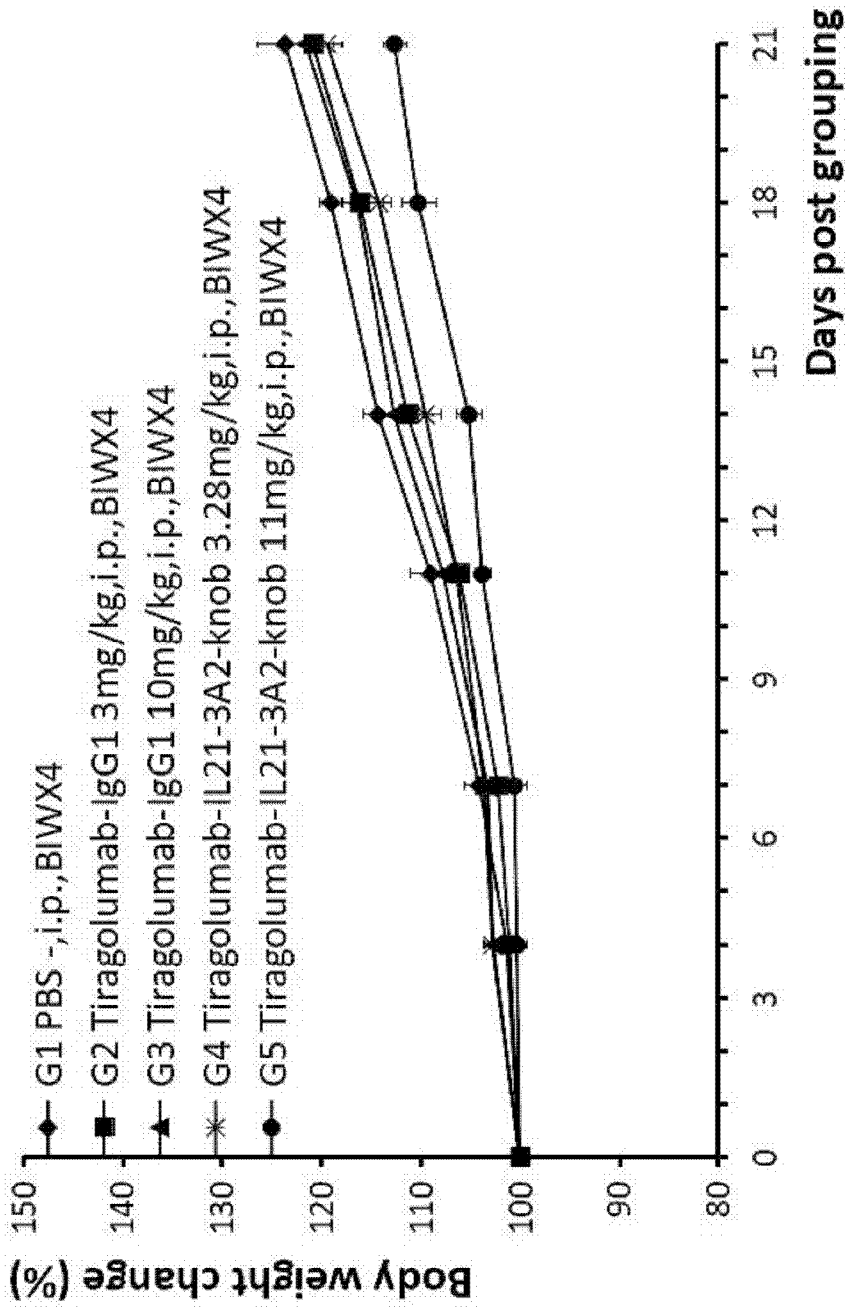


FIG. 4

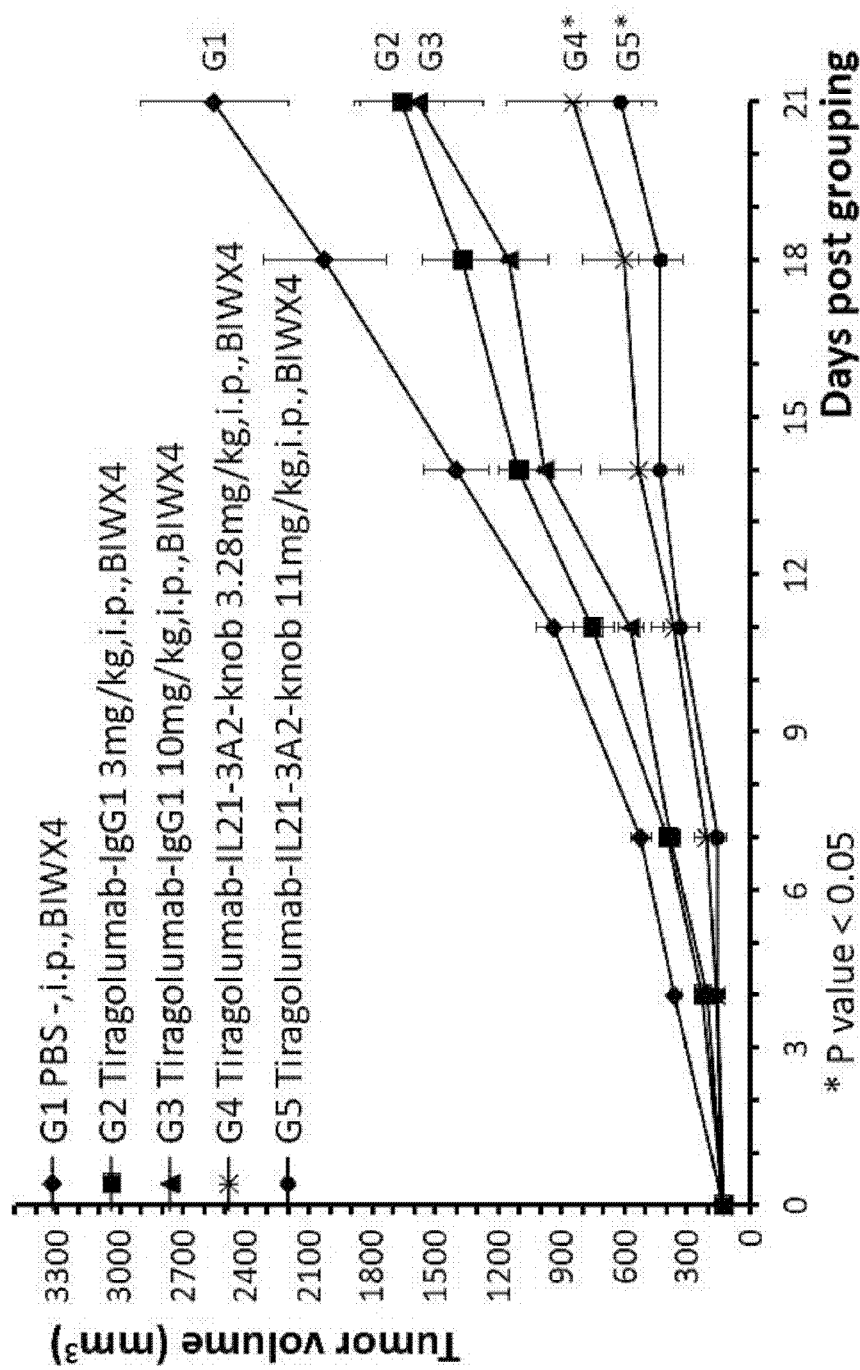


FIG. 5

FIG. 6

SEQ ID NO	Sequence	Name
1	EVQLQQSGPGLVKPSQTLSTCAISGDSVSSNSAAWNWIQSPSR GLEWLGKTYRFRKWYSDYAVSVKGRITINPDTSKNQFSLQLNSVT PEDTAVFYCTRESTTYYDLLAGPFDYWGQGTLLTVSS	Tiragolumab heavy chain variable region (VH)
2	DIVMTQSPDSLAVSLGERATINCKSSQTVLYSSNNKKYLAWYQQK PGQPPNLLIYWASTRESGVPDRFSGSGSGTDFTLTISLQAEDVA VYYCQQYYSTPFTFGPGTKVEIK	Tiragolumab light chain variable region (VL)
3	GGGSGGGGS	LINKER
4	EVQLQQSGPGLVKPSQTLSTCAISGDSVSSNSAAWNWIQSPSR GLEWLGKTYRFRKWYSDYAVSVKGRITINPDTSKNQFSLQLNSVT PEDTAVFYCTRESTTYYDLLAGPFDYWGQGTLLTVSSASTKGPSVF PLAPSSKSTSGGTAALGCLVKDYFPEPVTWNSGALTSGVHTFP AVLQSSGLYSLSSVTVPSSSLGTQTYICNVNHKPSNTKVDKKVE PKSCDKHTHTCPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVTC VVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVL TVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTL PPCREEMTGGGSGGGGSHKSSPQGPDRLLIRLRLIDIVEQLKI YENDLDPELLSAPQDVKGHCHEAFAFCFQKAKLKPSNPGNNKTFI IDLVAQLRRRLPARRGKKQKHIACPCSDSYEKRTPKFELERLK WLLQKMIHQHLSGGGSGGGGSKNQVSLWCLVKGFYPSDIAVEWE SNGQOPENNYKTTTPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSV MHEALHNHYTQKSLSLSPGK	Full-length knob chain (with fused IL21) of Tiragolumab-IL21-3A2-knob
5	EVQLQQSGPGLVKPSQTLSTCAISGDSVSSNSAAWNWIQSPSR GLEWLGKTYRFRKWYSDYAVSVKGRITINPDTSKNQFSLQLNSVT PEDTAVFYCTRESTTYYDLLAGPFDYWGQGTLLTVSSASTKGPSVF PLAPSSKSTSGGTAALGCLVKDYFPEPVTWNSGALTSGVHTFP AVLQSSGLYSLSSVTVPSSSLGTQTYICNVNHKPSNTKVDKKVE PKSCDKHTHTCPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVTC VVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVL TVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVCTL PPSREEMTKNQVSLSCAVKGFYPSDIAVEWESNGQOPENNYKTTTP VLDSDGSFFLVSKLTVDKSRWQQGNVFSCSV MHEALHNHYTQKSLSLSPGK	Full-length hole chain of Tiragolumab-IL21-3A2-knob
6	DIVMTQSPDSLAVSLGERATINCKSSQTVLYSSNNKKYLAWYQQK PGQPPNLLIYWASTRESGVPDRFSGSGSGTDFTLTISLQAEDVA VYYCQQYYSTPFTFGPGTKVEIKRTVAAPSVEIFPPSDEQLKSGT ASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSSTYS LSSTLTLSKADYEEKHKVYACEVTHQGLSSPVTKSFNRGEC	Full-length light chain of Tiragolumab-IL21-3A2-knob
7	MHGWL L V V V Q G L I Q A A F L A T G A T T G T I E T T G N I S A E K G G S I I L Q C H L S S T T A Q V T Q V N W E Q Q D Q L L A I C N A D L G W H I S P S F K D R V A P G P G L G L T L Q S L T V N D T G E Y F C I Y H T Y P D G T Y T G R I F L E V L E S S V A Q F Q T A P L G G T M A A V L G L I C L M V T G V T V L A R K K S I R M H S I E S G L G R T E A E P Q E W N L R S L S P G S P V Q T Q T A P A G P C G E Q A E D D Y A D P Q E Y F N V L S Y R S L E S F I A V S K T G	chimeric TIGIT protein
8	GGGGS	LINKER2

9	HKSSPQGPDRLLIRLRHLIDIVEQLKIYENDLDPCELLSAPQDVKG HCEHAFAFACFQKAKLKPSNPGNNKTFIIDLVAQLRRRLPARRGGK KQKHIACPCSDSYEKRTPKFELERLKWLLQKMIHQHLS	Mouse IL21
10	HKSSSQGQDRHMIRMRLIDIVDQLKNYVNDLVPEFLPAPEDVET NCEWSAFSCFQKAQLKSANTGNNERIINVSICKLKRKPPSTNAGR RQKHRLTCPSCDSYEKKPPKEFLERFKSLQKMIHQHLSRTHGS EDS	Human IL21
11	RTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDN ALQSGNSQESVTEQDSKSTYLSSTLTLSKADYEKHKVYACEVT HQGLSSPVTKSFNRGEC	kappa light chain constant region (CL)
12	EVQLVQSGAEVKKPGSSVKVSKASGYTFSSYVMHWVRQAPGQGL EWIGYIDPYNDGAKYAQKFQGRVTLTSDKSTSTAYMELSSLRSED TAVYYCARGGPYGWYFDVWGQGTTVTVSS	Vibostolimab VH
13	DIQMTQSPSSLSASVGDRTITCRASEHIYSYLSWYQQKPGKVPK LLIYNAAKTLAEGVPSRFSGSGSGTDFTLTITSSLPEDVATYYCQH HFGSPLTFGQGTREIK	Vibostolimab VL
14	QVQLQESGPGLVKPSSETLSLTCAVSGYSITSDYAWNWIROPKKG LEWIGYISYSGSTSYNPSLRSRVTISRDTSKNQFFLKLSSVTAAD TAVYYCARRQVGLGFAYWGQGTTLTVSS	Etigilimab VH
15	DIQMTQSPSSLSASVGDRTITCKASQDVSTAVAWYQQKPGKAPK LLIYSASYRYTGVPSRFSGSGSGTDFTTITSSLPEDIATYYCQQ HYSTPWTFGQGTKVEIK	Etigilimab VL
16	EVQLVESGGGLVQPGGSLRLSCAASGFTFSNFGMHWRQAPGKGL EWVAFISSGSSSIYYADTVKGRFTISRDNANKSLYLQMNSLRAED TAVYYCARMRLDYYAMDYWGQGTMTVTVSS	Domvanalimab VH
17	DIQMTQSPSSLSASVGDRTITCRASKSISKYLAWYQQKPGKAPK LLIYSGSTLQSGVPSRFSGSGSGTDFTLTITSSLPEDFATYYCQQ HNEYPTWTFGGGTKVEIK	Domvanalimab VL
18	EVQLVESGGGLVQPGGSLRLSCAASGFTFSYYMYWVRQAPGKGL EWVAYITKGGGSTYYPDSVKGRFTISRDNANKNTLYLQMNSLRAED TAVYYCARQTNVDFTMDYWGQGTTLTVSS	Ociperlimab VH
19	EIVMTQSPATLSVSPGERATLSCKASQDVGTSAWYQQKPGQAPR LLIYWASARHTGIPARFSGSGSGTEFTLTITSSLQSEDFAVYYCQQ YSSYPLTFGGGTKVEIK	Ociperlimab VL
20	EVQLVQSGAEVKKPGSSVKVSKASGYTFSSYVMHWVRQAPGQGL EWIGYIDPYNDGAKYAQKFQGRVTLTSDKSTSTAYMELSSLRSED TAVYYCARGGPYGWYFDVWGQGTTVTVSSASTKGPSVFPLAPSSK STSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSG LYSLSSVTVTPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKT HTCPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSH EDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDW LNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPCREEM TGGGSGGGGSHKSSPQGPDRLLIRLRHLIDIVEQLKIYENDLDP ELLSAPQDVKGHCEHAFAFACFQKAKLKPSNPGNNKTFIIDLVAQL RRRLPARRGGKKQKHIACPCSDSYEKRTPKFELERLKWLLQKMI HQHLSGGGGSGGGGSKNQVSLWCLVKGFYPSDIAVEWESNGQPEN NYKTTTPVLDSDGSFFLYSKLTVDKSRWQQGNVFCSSVMHEALHN HYTQKSLSLSPGK	Full-length knob chain (with fused IL21) of Vibostolimab- IL21-3A2-knob
21	EVQLVQSGAEVKKPGSSVKVSKASGYTFSSYVMHWVRQAPGQGL EWIGYIDPYNDGAKYAQKFQGRVTLTSDKSTSTAYMELSSLRSED TAVYYCARGGPYGWYFDVWGQGTTVTVSS	Full-length hole chain of Vibostolimab-

	ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGA LTSGVHTFPAVLQSSGLYSLSSVTVPSSSLGTQTYICNVNHKPS NTKVDKKVEPKSCDKTHTCPPCPAPEAAGGPSVFLFPPKPKDTLM ISRTPEVTCVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYN STYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQ PREPQVCTLPSSREEMTKNQVSLSCAVKGFYPSDIAVEWESNGQP ENNYKTTPPVLDSDGSFFLVSKLTVDKSRWQQGNVFCSCVMHEAL HNHYTQKSLSLSPGK	IL21-3A2-knob
22	DIQMTQSPSSLSASVGDRVTITCRASEHIYSYLSWYQQKPGKVPK LLIYNAKTLAEGVPSRFSGSGSGTDFTLTITSSSLQPEDVATYYCQH HFGSPLTFGQGTRELEIKRTVAAPSVFIFPPSDEQLKSGTASVVCL LNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKSTYLSSTLT LSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC	Full-length light chain of Vibostolimab- IL21-3A2-knob
23	QVQLQESGPGLVKPKSETLSLTCAVSGYSITSDYAWNWIROP LEWIGYISYSGSTSYNPSLRSRVTISRDTSKNQFFLKLSSVTAAD TAVYYCARRQVGLGFAYWGQGTLLTVSS ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGA LTSGVHTFPAVLQSSGLYSLSSVTVPSSSLGTQTYICNVNHKPS NTKVDKKVEPKSCDKTHTCPPCPAPEAAGGPSVFLFPPKPKDTLM ISRTPEVTCVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYN STYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQ PREPQVYTLPPCREEMTGGGGSGGGGSHKSSPQGPDRLLIRLRHL IDIVEQLKIYENDLDPELLSAPQDVKGHCHEAFAFCQKAKLKPS NPGNNKTFIIDLVAQLRRRLPARRGKKQKHIACPCSDSYEKRT PKEFLERLKWLLQKMIHQHLSGGGGSGGGGSKNQVSLWCLVKGFY PSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQ QGNVFCSCVMHEALHNHYTQKSLSLSPGK	Full-length knob chain (with fused IL21) of Etigilimab- IL21-3A2-knob
24	QVQLQESGPGLVKPKSETLSLTCAVSGYSITSDYAWNWIROP LEWIGYISYSGSTSYNPSLRSRVTISRDTSKNQFFLKLSSVTAAD TAVYYCARRQVGLGFAYWGQGTLLTVSS ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGA LTSGVHTFPAVLQSSGLYSLSSVTVPSSSLGTQTYICNVNHKPS NTKVDKKVEPKSCDKTHTCPPCPAPEAAGGPSVFLFPPKPKDTLM ISRTPEVTCVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYN STYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQ PREPQVCTLPSSREEMTKNQVSLSCAVKGFYPSDIAVEWESNGQP ENNYKTTPPVLDSDGSFFLVSKLTVDKSRWQQGNVFCSCVMHEAL HNHYTQKSLSLSPGK	Full-length hole chain of Etigilimab- IL21-3A2-knob
25	DIQMTQSPSSLSASVGDRVTITCKASQDVSTAVAWYQQKPGKAPK LLIYSASYRYTGVPSSRFSGSGSGTDFTFTITSSSLQPEDVATYYCQQ HYSTPWTFGQGTKEIKRTVAAPSVFIFPPSDEQLKSGTASVVCL LNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKSTYLSNTLT LSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC	Full-length light chain of Etigilimab- IL21-3A2-knob
26	EVQLVESGGGLVQPGGSLRLSCAASGFTFSNFGMHWRQAPGKGL EWVAFISSGSSSIYYADTVKGRFTISRDNAKNSLYLQMNSLRAED TAVYYCARMRLDYYAMDYWGQGTMTVTVSS ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGA	Full-length knob chain (with fused IL21) of Domvanalimab- IL21-3A2-knob

	LTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPS NTKVDKKVEPKSCDKTHTCPPCPAPEAAGGPSVFLFPPKPKDTLM ISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYN STYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQ PREPQVYTLPPCREEMTGGGSGGGGSHKSSPQGPDRLLIRLRHL IDIVEQLKIYENDLDPELLSAPQDVKGHCHEAAAFACFQKAKLKPS NPGNNKTFIIDLVAQLRRRLPARRGGKKQKHIACPCSDSYEKRT PKEFLERLKWLLQKMIHQHLSGGGSGGGGSKNQVSLWCLVKGFY PSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQ QGNVFSCSVMHEALHNHYTQKSLSLSPGK	
27	EVQLVESGGGLVQPGGSLRLSCAASGFTFSNFGMHWRQAPGKGL EWVAFISSGSSSIYYADTVKGRFTISRDNKNSLYLQMNSLRAED TAVYYCARMRLDYYAMDYWGQGTMTVTVSS ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGA LTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPS NTKVDKKVEPKSCDKTHTCPPCPAPEAAGGPSVFLFPPKPKDTLM ISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYN STYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQ PREPQVCTLPSSREEMTKNQVSLSCAVKGFYPSDIAVEWESNGQP ENNYKTTPPVLDSDGSFFLVSKLTVDKSRWQQGNVFSCSVMHEAL HNHYTQKSLSLSPGK	Full-length hole chain of Domvanalimab- IL21-3A2-knob
28	DIQMTQSPSSLSASVGDRVTITCRASKSISKYLAWYQQKPGKAPK LLIYSGSTLQSGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQ HNEYPTWFGGGTKVEIKRTVAAPSVFIFPPSDEQLKSGTASVVCL LNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSYSTLSSTLT LSKADYEEKHKVYACEVTHQGLSSPVTKSFNRGEC	Full-length light chain of Domvanalimab- IL21-3A2-knob
29	EVQLVESGGGLVQPGGSLRLSCAASGFTFSDDYYMYWVRQAPGKGL EWVAYITKGGGSTYYPD SVKGRFTISRDNKNTLYLQMNSLRAED TAVYYCARQTNYDFTMDYWGQGT LVTVSS ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGA LTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPS NTKVDKKVEPKSCDKTHTCPPCPAPEAAGGPSVFLFPPKPKDTLM ISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYN STYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQ PREPQVYTLPPCREEMTGGGSGGGGSHKSSPQGPDRLLIRLRHL IDIVEQLKIYENDLDPELLSAPQDVKGHCHEAAAFACFQKAKLKPS NPGNNKTFIIDLVAQLRRRLPARRGGKKQKHIACPCSDSYEKRT PKEFLERLKWLLQKMIHQHLSGGGSGGGGSKNQVSLWCLVKGFY PSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQ QGNVFSCSVMHEALHNHYTQKSLSLSPGK	Full-length knob chain (with fused IL21) of Ociperlimab- IL21-3A2-knob
30	EVQLVESGGGLVQPGGSLRLSCAASGFTFSDDYYMYWVRQAPGKGL EWVAYITKGGGSTYYPD SVKGRFTISRDNKNTLYLQMNSLRAED TAVYYCARQTNYDFTMDYWGQGT LVTVSS ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGA LTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPS NTKVDKKVEPKSCDKTHTCPPCPAPEAAGGPSVFLFPPKPKDTLM ISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYN STYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQ	Full-length hole chain of Ociperlimab- IL21-3A2-knob

	PREPQVCTLPPSREEMTKNQVSLSCAVKGFYPSDIAVEWESNGQP ENNYKTTPPVLDSDGSFFLVSKLTVDKSRWQQGNVFSCSVMEAL HNHYTQKSLSLSPGK	
31	EIVMTQSPATLSVSPGERATLSCKASQDVGTSVAWYQQKPGQAPR LLIYWASARHTGIPARFSGSGSGTEFTLTISSLQSEDFAVYYCQQ YSSYPLTFGGGKVEIKRTVAAPSVFIFPPSDEQLKSGTASVVCL LNNFYPREAKVQAKVDNALQSGNSQESVTEQDSKDSTYLSSTLT LSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC	Full-length light chain of Ociperlimab- IL21-3A2-knob
32	MPRGPVAALLLLILHGAWSCLDLVCYTDYLQTVICILEMWNLHPS TLTLTWQDQYEELKDEATSCSLHRSAHNATHATYTCHMDVFHFMA DDIFSVNITDQSGNYSQECGSFLLAESIKPAPPFNVTVTFSGQYN ISWRSDYEDPAFYMLKGKLQYELQYRNRGDPWAVSPRRKLISVDS RSVSLLPLEFRKDSSYELQVRAGPMPGSSYQGTWSEWSDPVIFQT QAGEPEAGWDPHMLLLLAFLIIIVLFVFMGLKIHLPWRLWKKIWAPV PTPESFFQPLYREHSGNFKKWVNTPFTASSIELVPQSSTTTTSALH LSLYPAKEKKFPGLPGLLEEQLCECDGMSEPGHWCIIPLAAGQAVSA YSEERDRPYGLVSIDTVTVGDAEGLCVWPCSCEDDGYPAMNLDAG RESGPNSEDLLLVTDP AFLSCGCVSGSLRLGGSPGSLLDRLRLS FAKEGDWTADPTWRTGSPGGGSESEAGSPGLMDTDFDSGFAGSD CGSPVETDEGPPRSYLRQWVVRTPPPVDSGAQSS	Chimeric IL21R protein

INTERNATIONAL SEARCH REPORT

International application No.

PCT/CN2022/118470

A. CLASSIFICATION OF SUBJECT MATTER

C07K 16/28(2006.01)i; A61K 45/06(2006.01)i; A61K 39/395(2006.01)i

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C07K; A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

CNTXT,WPABS,WPABSC,VEN,VCN,DWPI,EPTXT, USTXT,PUBMED, CNKI,BAIDU,BING:TIGIT, antibody,IL21, fusion, fuse,IL21R, tumor,cancer,stability,toxicity

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
PX	LIU, H.C.et al. "An engineered IL-21 with half-life extension enhances anti-tumor immunity as a monotherapy or in combination with PD-1 or TIGIT blockade" <i>International Immunopharmacology</i> , Vol. 101, 01 November 2021 (2021-11-01), Article 108307 Pages 1-16	1-51
Y	SHEN, S.L.et al. "Engineered IL-21 Cytokine Muteins Fused to Anti-PD-1 Antibodies Can Improve CD8+ T Cell Function and Anti-tumor Immunity" <i>ORIGINAL RESEARCH</i> , Vol. 11, 08 May 2020 (2020-05-08), Article 832 Pages 1-14	1-51
Y	US 2016355589 A1 (erck Sharp & Dohme Corp) 08 December 2016 (2016-12-08) claims 1-22	1-51
Y	CN 111744007 A (IANGSU HENGRUI MEDICINE CO., LTD. et al) 09 October 2020 (2020-10-09) claims 1-16	1-51
Y	CN 109734806 A (NHUI ANKE BIOTECHNOLOGY GROUP CO., LTD) 10 May 2019 (2019-05-10) claims 1-10	1-51



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

28 November 2022

Date of mailing of the international search report

14 December 2022

Name and mailing address of the ISA/CN

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100088, China

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GUAN,Bing

Facsimile No. (86-10)62019451

Telephone No. (86-10)53961945

INTERNATIONAL SEARCH REPORT

International application No.

PCT/CN2022/118470

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	CN 110818795 A (HANGHAI HENLIUS BIOTECH CO.,LTD) 21 February 2020 (2020-02-21) claims 1-18	1-51

INTERNATIONAL SEARCH REPORT

International application No.

PCT/CN2022/118470

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:
- a. ☒ forming part of the international application as filed:
- ☒ in the form of an Annex C/ST.25 text file.
- ☐ on paper or in the form of an image file.
- b. ☐ furnished together with the international application under PCT Rule 13*ter*.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.
- c. ☐ furnished subsequent to the international filing date for the purposes of international search only:
- ☐ in the form of an Annex C/ST.25 text file (Rule 13*ter*.1(a)).
- ☐ on paper or in the form of an image file (Rule 13*ter*.1(b) and Administrative Instructions, Section 713).
2. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.
3. Additional comments:
- [1] The sequence listing is actually filed in the form of an Annex C/ST.26 XML file.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/CN2022/118470

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: **36-44**
because they relate to subject matter not required to be searched by this Authority, namely:
 - [1] The subject matter of claims 36-44 relates to a method of treating a subject having cancer, a method of increasing immune response or activating T cells in tumor microenvironment in a subject, a method of increasing IL21 stability or enhancing IL21 function reducing the toxicity of IL21 when delivering IL21 into a subject, or a method of increasing the therapeutic effect of an anti-TIGIT antibody or reducing the toxicity of IL21, and does not warrant an international search according to the criteria set out in PCT Rule 39.1(iv). An international search is still carried out on the basis of the use of the protein complex for manufacturing of medicament.
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.

PCT/CN2022/118470

Patent document cited in search report			Publication date (day/month/year)	Patent family member(s)			Publication date (day/month/year)
US	2016355589	A1	08 December 2016	KR	20170041272	A	14 April 2017
				EP	3183267	A1	28 June 2017
				MX	2017002229	A	09 May 2017
				CL	2017000310	A1	18 August 2017
				DO	P2017000046	A	31 August 2017
				CL	2019001926	A1	29 November 2019
				JP	2022105108	A	12 July 2022
				NI	201700019	A	08 March 2017
				EA	201790413	A1	31 August 2017
				BR	112017003108	A2	05 December 2017
				AU	2015305754	A1	23 February 2017
				CA	2957722	A1	25 February 2016
				JO	3664	B1	27 August 2020
				US	2020270346	A1	27 August 2020
				US	2018066055	A1	08 March 2018
				TN	2017000024	A1	04 July 2018
				AU	2019200426	A1	07 February 2019
				AP	201709765	A0	28 February 2017
				TW	201609813	A	16 March 2016
				CN	113583131	A	02 November 2021
				JP	2020162614	A	08 October 2020
				TW	202144422	A	01 December 2021
				WO	2016028656	A1	25 February 2016
				US	2017198042	A1	13 July 2017
				AU	2021202360	A1	13 May 2021
				GT	201700033	A	30 September 2019
				JO	P20200192	A1	16 June 2017
				IL	250583	D0	30 April 2017
				PH	12017500296	A1	28 June 2017
				GE	P20217331	B	10 December 2021
				JP	2017532007	A	02 November 2017
				SG	11201701161Q	A	30 March 2017
				MA	40475	A	28 June 2017
				CR	20170060	A	18 April 2017
				PE	20170289	A1	05 April 2017
				UA	122395	C2	10 November 2020
				CN	107148430	A	08 September 2017
				MD	20170032	A2	31 August 2017
				CO	2017001561	A2	19 May 2017
				KR	20190133078	A	29 November 2019
				AP	201709765	D0	28 February 2017
CN	111744007	A	09 October 2020	None			
CN	109734806	A	10 May 2019	None			
CN	110818795	A	21 February 2020	WO	2021139777	A1	15 July 2021
				CA	3164281	A1	15 July 2021
				EP	4089115	A1	16 November 2022
				AU	2021206420	A1	01 September 2022
				KR	20220131258	A	27 September 2022
				BR	112022013545	A2	06 September 2022