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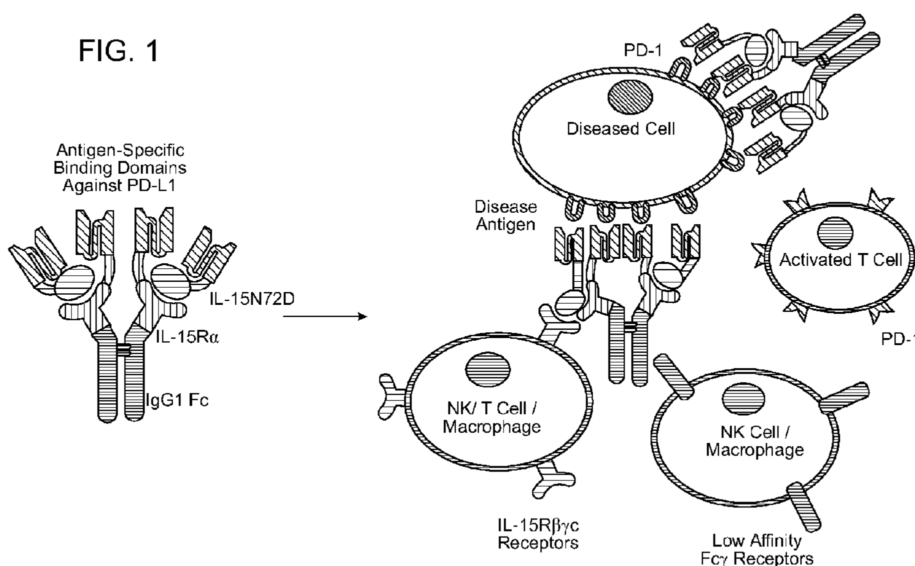
(54) **Title:** MULTIMERIC IL-15-BASED MOLECULES



(57) **Abstract:** The invention features multi- specific protein complexes with one domain comprising IL-15 or a functional variant and a binding domain specific to a disease antigen, immune checkpoint or signaling molecule.

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MULTIMERIC IL-15-BASED MOLECULES

CROSS REFERENCE TO RELATED APPLICATIONS

This Application claims the benefit of U.S. Provisional Application 62/513,964 filed on June 1, 2017 and U.S. Provisional Application 62/411,216 filed on October 21, 2016. The entire contents of these applications are incorporated herein by reference in their entirety.

FIELD OF THE INVENTION

This invention relates generally to the field of multimeric fusion molecules.

BACKGROUND OF THE INVENTION

Prior to the invention described herein, there was a pressing need to develop new strategies to target various effector molecules to a disease site to provide therapeutic benefit without the side effects associated with non-specific immune activity.

SUMMARY OF THE INVENTION

The invention is based, at least in part, on the surprising discovery that multi-specific IL-15-based protein complexes enhance the stimulation of immune cells and promote their activity against disease cells, thereby resulting in reduction or prevention of disease. These IL-15-based protein complexes also show increased binding to disease and target antigens. Provided herein are multi-specific protein complexes with one domain comprising IL-15 or a functional variant and a binding domain specific to a disease antigen, immune checkpoint or signaling molecule. Specifically, described herein are protein complexes comprising an IL-15N72D:IL-15RαSu-Ig Fc scaffold fused to binding domains that recognize programmed death ligand 1 (PD-L1), programmed death 1 (PD-1), cytotoxic T-lymphocyte associated protein 4 (CTLA-4), cluster of differentiation 47 (CD47), T-cell immunoglobulin and mucin-domain containing-3 (TIM-3, TIM3) or glucocorticoid-induced tumor necrosis factor receptor (TNFR) family related gene (GITR). These complexes induce NK and T cell responses via IL-15 activity and further augment immune responses through immune checkpoint blockade via the anti-PD-L1, PD-1, CTLA-4, CD47, TIM3 or GITR binding domains (FIG. 1). In some cases, these complexes also

recognize antigens, such as PD-L1, single stranded deoxyribonucleic acid (ssDNA), CD20, human epidermal growth factor receptor 2 (HER2), epidermal growth factor receptor (EGFR), CD19, CD38, CD52, disialoganglioside (GD2), CD33, Notch1, intercellular adhesion molecule 1 (ICAM-1), tissue factor or HIV envelope, expressed on disease cells and stimulate antibody-dependent cell-mediated cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) against the disease cell via the Fc binding domain.

Provided is an isolated soluble fusion protein complex comprising at least two soluble proteins. For example, the first protein comprises an interleukin-15 (IL-15) polypeptide, e.g., a variant IL-15 polypeptide comprising an N72D mutation (IL-15N72D). The second protein comprises a soluble IL-15 receptor alpha sushi-binding domain (IL-15RαSu) fused to an immunoglobulin Fc domain (IL-15RαSu/Fc). A third component of the isolated soluble fusion protein complex comprises a binding domain that recognizes a disease antigen, immune checkpoint molecule or a signaling molecule, e.g., PD-L1, PD-1, CTLA-4, CD47, TIM3 or GITR, wherein the binding domain is fused to the either the IL-15N72D or the IL-15RαSu/Fc protein. In some cases, these binding domains are fused to both the IL-15N72D and IL-15RαSu/Fc proteins. In other cases, one of these binding domains is fused to the IL-15N72D or the IL-15RαSu/Fc proteins and a second binding domain, i.e. specific to an immune checkpoint or signaling molecule or a disease antigen, is fused to the other protein. In one aspect, the disease antigen is associated with neoplasia, infectious disease, or autoimmune disease. In some cases, the first or second soluble protein further comprises a binding domain that recognizes a disease antigen, e.g., PD-L1, ssDNA, CD20, HER2, EGFR, CD19, CD38, CD52, GD2, CD33, Notch1, intercellular adhesion molecule 1 (ICAM-1), tissue factor or HIV envelope or other known antigens, expressed on disease cells. Alternatively, either the IL-15N72D or the IL-15RαSu/Fc protein comprise the binding domain specific to a disease antigen, immune checkpoint or signaling molecule and the other protein (IL-15RαSu/Fc or IL-15N72D protein, respectively) do not comprise an additional fused binding domain. The IL-15N72D domain of the first protein binds to the soluble IL-15RαSu domain of the second protein to form a soluble fusion protein complex. An exemplary fusion protein complex comprises an anti-PD-L1 antibody covalently linked to an IL-15N72D and/or an IL-15RαSu/Fc fusion protein (FIG. 1 and FIG. 2). Alternatively, the first protein comprises an anti-PD-L1 antibody covalently linked to a soluble IL-15 receptor alpha sushi-binding domain (IL-15RαSu) fused to an immunoglobulin Fc domain

whereas the second protein comprises a binding domain that recognizes disease antigens covalently linked and a variant interleukin-15 (IL-15) polypeptide comprising an N72D mutation (IL-15N72D).

In some cases, the binding domain comprises a single chain antibody (scAb or scFv) wherein an immunoglobulin light chain variable domain is covalently linked to an immunoglobulin heavy chain variable domain by a polypeptide linker sequence. Alternatively, the binding domain comprises a soluble or extracellular ligand or receptor domain capable of acting as an immune checkpoint inhibitor or immune agonist.

Exemplary first proteins comprise the amino acid sequences set forth in SEQ ID NOs: 2, 6, 10, 18, 20, 24, 28, 32, or 38. Exemplary second proteins comprise the amino acid sequences set forth in SEQ ID NOs: 4, 8, 12, 14, 16, 22, 26, 30, 34, 36, 40, 42, 44, 46, 51, 52, 53, or 54. Exemplary nucleic acid sequences encoding the first protein comprise the sequences set forth in SEQ ID NOs: 1, 5, 9, 17, 19, 23, 27, 31 or 37. Exemplary nucleic acid sequences encoding the second protein comprise the sequences set forth in SEQ ID NOs: 3, 7, 11, 13, 15, 21, 25, 29, 33, 35, 39, 41, 43, 45, 47, 48, 49, or 50. In one aspect, the nucleic acid sequence(s) further comprises a promoter, translation initiation signal, and leader sequence operably linked to the sequence encoding the fusion protein. Also provided are DNA vector(s) comprising the nucleic acid sequences described herein. For example, the nucleic acid sequence is in a vector for replication, expression, or both.

Also provided is a soluble fusion protein complex comprising a first soluble fusion protein complex covalently linked to a second soluble fusion protein complex. For example, the soluble fusion protein complexes of the invention are multimerized, e.g., dimerized, trimerized, or otherwise multimerized (e.g., 4 complexes, 5 complexes, etc.). For example, the multimers are homomultimers or heteromultimers. The soluble fusion protein complexes are joined by covalent bonds, e.g., disulfide bonds, chemical cross-linking agents. In some cases, one soluble fusion protein is covalently linked to another soluble fusion protein by a disulfide bond linking the Fc domain of the first soluble protein to the Fc domain of the second soluble protein.

The Fc domain or functional fragment thereof includes an Fc domain selected from the group consisting of IgG Fc domain, human IgG1 Fc domain, human IgG2 Fc domain, human IgG3 Fc domain, human IgG4 Fc domain, IgA Fc domain, IgD Fc domain, IgE Fc domain, and IgM Fc domain; mouse IgG2A domain, or any combination thereof. Optionally, the Fc domain

includes an amino acid change that results in an Fc domain with altered complement or Fc receptor binding properties or altered dimerization or glycosylation profiles. Amino acid changes to produce an Fc domain with altered complement or Fc receptor binding properties or altered dimerization or glycosylation profiles are known in the art. For example, a substitution of leucine residues at positions 234 and 235 of the IgG1 CH2 (numbering based on antibody consensus sequence) (i.e., … P E L L G G …) with alanine residues (i.e., … P E A A G G …) results in a loss of Fc gamma receptor binding, whereas the substitution of the lysine residue at position 322 of the IgG1 CH2 (numbering based on antibody consensus sequence) (i.e., … K C K S L …) with an alanine residue (i.e., … K C A S L …) results in a loss of complement activation. In some examples, such mutations are combined.

In some aspects, the binding domain is covalently linked to an IL-15 polypeptide (or functional fragment thereof) by a polypeptide linker sequence. Similarly, the binding domain is covalently linked to an IL-15Rα polypeptide (or functional fragment thereof) by polypeptide linker sequence. Optionally, the IL-15Rα polypeptide (or functional fragment thereof) is covalently linked to the Fc domain (or functional fragment thereof) by polypeptide linker sequence. Each polypeptide linker sequence can be selected independently. Optionally, the polypeptide linker sequences are the same. Alternatively, they are different.

Optionally, the soluble fusion protein complexes of the invention are provided wherein at least one of the soluble fusion proteins comprise a detectable label. Detectable labels include, but are not limited to, biotin, streptavidin, an enzyme, or catalytically active fragment thereof, a radionuclide, a nanoparticle, a paramagnetic metal ion, or a fluorescent, phosphorescent, or chemiluminescent molecule, or any combination thereof.

In some embodiments, a nucleic acid sequence encoding a first soluble protein comprises the sequence set forth in one of SEQ ID NOS: 1, 5, 9, 17, 19, 23, 27, 31 or 37. In some embodiments, a nucleic acid sequence encoding the second soluble protein comprises the sequence set forth in one of SEQ ID NOS: 3, 7, 11, 13, 15, 21, 25, 29, 33, 35, 39, 41, 43, 45, 47, 48, 49 or 50.

In some embodiments, a nucleic acid sequence comprises SEQ ID NOS. 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 35, 39, 41, 43,45, 47, 48, 49 or 50.

The nucleic acid sequences further comprise a promoter, translation initiation signal, and leader sequence operably linked to the sequence encoding the soluble protein.

In other embodiments, a peptide comprises SEQ ID NOS: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, 51, 52, 53 or 54.

The invention provides method for making the soluble fusion protein complexes of the invention. The method includes the steps of: a) introducing into a first host cell a DNA vector with appropriate control sequences encoding the first protein, b) culturing the first host cell in media under conditions sufficient to express the first protein in the cell or the media; c) purifying the first protein from the host cells or media, d) introducing into a second host cell a DNA vector with appropriate control sequences encoding the second protein, e) culturing the second host cell in media under conditions sufficient to express the second protein in the cell or the media; and f) purifying the second protein from the host cells or media, and g) mixing the first and second proteins under conditions sufficient to allow binding between IL-15 domain of a first protein and the soluble IL-15Rα domain of a second protein to form the soluble fusion protein complex.

In some cases, the method further includes mixing the first and second protein under conditions sufficient to allow formation of a disulfide bond between the polypeptides expressed from the expression vectors.

Alternatively, methods for making soluble fusion protein complexes of the invention are carried out by a) introducing into a host cell a DNA vector with appropriate control sequences encoding the first protein and a DNA vector with appropriate control sequences encoding the second protein, b) culturing the host cell in media under conditions sufficient to express the proteins in the cell or the media and allow association between IL-15 domain of a first protein and the soluble IL-15Rα domain of a second protein to form the soluble fusion protein complex; and c) purifying the soluble fusion protein complex from the host cells or media.

In one aspect, the method further includes mixing the first and second protein under conditions sufficient to allow formation of a disulfide bond between the polypeptides expressed from the expression vectors.

Also provided are methods for making soluble fusion protein complexes comprising a) introducing into a host cell a DNA vector with appropriate control sequences encoding the first and second proteins, b) culturing the host cell in media under conditions sufficient to express the proteins in the cell or the media and allow association between IL-15 domain of a first protein and the soluble IL-15Rα domain of a second protein to form the soluble fusion

protein complex, and to allow formation of a disulfide bond between the polypeptides; and c) purifying the soluble fusion protein complex from the host cells or media.

Optionally, the method further includes mixing the first and second protein under conditions sufficient to allow formation of a disulfide bond between the polypeptides expressed from the expression vectors.

Methods for treating a neoplasia, infectious disease, or autoimmune disease in a subject in need thereof are carried out by administering to a subject an effective amount of a pharmaceutical composition comprising a soluble fusion protein complex described herein, e.g., a soluble anti-PD-L1 scAb/IL-15N72D:anti-PD-L1 scAb/IL-15RαSu/Fc fusion protein complex, thereby treating the neoplasia, infectious disease, or autoimmune disease. For example, methods for treating solid or hematological malignancies in a subject in need thereof are carried out by administering to a subject an effective amount of a pharmaceutical composition comprising a soluble anti-human PD-L1 scAb/huIL-15N72D:anti-human PD-L1 scAb/huIL-15RαSu/huIgG1 Fc fusion protein complex, thereby treating the malignancy. Exemplary anti-human PD-L1 scAb/huIL-15N72D proteins comprise the amino acid sequences set forth in SEQ ID NOs: 2 and 6. Exemplary anti-human PD-L1 scAb/huIL-15RαSu/huIgG1 Fc proteins comprise the amino acid sequences set forth in SEQ ID NOs: 4 and 8.

Suitable neoplasias for treatment with the methods described herein include a glioblastoma, prostate cancer, acute myeloid leukemia, B-cell neoplasm, multiple myeloma, B-cell lymphoma, B cell non-Hodgkin's lymphoma, Hodgkin's lymphoma, chronic lymphocytic leukemia, acute myeloid leukemia, cutaneous T-cell lymphoma, T-cell lymphoma, a solid tumor, urothelial/bladder carcinoma, melanoma, lung cancer, renal cell carcinoma, breast cancer, gastric and esophageal cancer, head and neck cancer, prostate cancer, pancreatic cancer, colorectal cancer, ovarian cancer, non-small cell lung carcinoma, and squamous cell head and neck carcinoma.

An exemplary infection for treatment using the methods described herein is infection with human immunodeficiency virus (HIV). Exemplary nucleic acid sequences include: SEQ ID NOS: 47, 48, 49 or 50. Exemplary amino acid sequences include: SEQ ID NOS: 51, 52, 53 or 54. The methods described herein are also useful to treat bacterial infections (e.g., gram positive or gram negative bacteria) (Oleksiewicz et al. 2012. Arch Biochem Biophys. 526:124-31). An exemplary autoimmune disease for treatment using the methods described herein is an

autoimmune disease mediated by B cells. Such autoimmune diseases include rheumatoid arthritis, multiple sclerosis, idiopathic thrombocytopaenia, IgM-mediated polyneuropathy, Factor VIII deficiency, systemic lupus erythematosus, Sjögren's syndrome, inflammatory myositis, pemphigus vulgaris, neuromyelitis optica, ANCA-associated vasculitis, chronic inflammatory demyelinating polyneuropathy, autoimmune anemias, pure red cell aplasia, thrombotic thrombocytopenic purpura (TTP), idiopathic thrombocytopenic purpura (ITP), Evans syndrome, vasculitis (for example granulomatosis with polyangiitis, formerly Wegener's), bullous skin disorders (for example pemphigus, pemphigoid), type 1 diabetes mellitus, anti-NMDA receptor encephalitis and Devic's disease, Graves' ophthalmopathy, autoimmune pancreatitis, Opsoclonus myoclonus syndrome (OMS), and IgG4-related disease.

The pharmaceutical composition comprising a fusion protein complex is administered in an effective amount. For example, an effective amount of the pharmaceutical composition is between about 1 μg/kg and 100 μg/kg, e.g., 1, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, or 100 μg/kg. Alternatively, TxM complex is administered as a fixed dose or based on body surface area (i.e., per m^2).

The pharmaceutical composition comprising the fusion protein complex is administered at least one time per month, e.g., twice per month, once per week, twice per week, once per day, twice per day, every 8 hours, every 4 hours, every 2 hours, or every hour. Suitable modes of administration for the pharmaceutical composition include systemic administration, intravenous administration, local administration, subcutaneous administration, intramuscular administration, intratumoral administration, inhalation, and intraperitoneal administration.

Preferably, the fusion protein complex increases serum levels of interferon gamma (IFN-γ), and/or stimulates $CD4^+$ and $CD8^+$ T cells and NK cells to kill diseased cells or tumor cells in a subject.

In certain aspects of the soluble fusion protein complexes of the invention, the IL-15 polypeptide is an IL-15 variant having a different amino acid sequence than native IL-15 polypeptide. The human IL-15 polypeptide is referred to herein as huIL-15, hIL-15, huIL15, hIL15, IL-15 wild type (wt), and variants thereof are referred to using the native amino acid, its position in the mature sequence and the variant amino acid. For example, huIL15N72D refers to human IL-15 comprising a substitution of N to D at position 72. In one aspect, the IL-15 variant functions as an IL-15 agonist as demonstrated, e.g., by increased binding activity for the IL-

15/IL-2 $\beta\gamma_C$ receptors (IL-15R) compared to the native IL-15 polypeptide. Alternatively, the IL-15 variant functions as an IL-15 antagonist as demonstrated by e.g., decreased binding activity for the IL-15R compared to the native IL-15 polypeptide.

Methods for killing a target cell are carried out by a) contacting a plurality of cells with a soluble fusion protein complex of the invention, wherein the plurality of cells further include immune cells bearing the IL-15R chains recognized by the IL-15 domain, or immune cells bearing checkpoint or signaling molecules modulated by the checkpoint inhibitor or immune agonist binding domains, and the target disease cells; b) activating the immune cells via the IL-15R or signaling molecules or via blockade of the checkpoint molecules; and c) killing the target disease cells by the activated immune cells. For example, the target disease cells are tumor cells, autoimmune cells, or virally infected cells. In some cases, the binding domain comprises an anti-PD-L1 antibody.

Methods for killing a target cell further comprise a) contacting a plurality of cells with a soluble fusion protein complex of the invention, wherein the plurality of cells further include immune cells bearing Fc receptor chains recognized by the Fc domain, and the target disease cells bearing an antigen recognized by binding domain such as an antigen-specific scAb; b) forming a specific binding complex (bridge) between the antigen on the target disease cells and Fc receptor chains on the immune cells sufficient to bind and activate the immune cells; and c) killing the target disease cells by the bound activated immune cells. For example, the target disease cells are tumor cells, autoimmune cells, or virally infected cells. In some cases, the binding domain comprises an anti-PD-L1 antibody.

Also provided are methods for preventing or treating disease in a patient, the method including the steps of: a) administering to the patient a soluble fusion protein complex of the invention; b) activating the immune cells in the patient; and c) damaging or killing the disease cells via the activated immune cells sufficient to prevent or treat the disease in the patient.

The invention also provides methods for preventing or treating disease in a patient in which the diseased cells, the method including the steps of: a) mixing immune cells bearing IL-15R chains or checkpoint or signaling molecules with a soluble fusion protein complex of the invention; b) activating the immune cells; c) administering to the patient the activated immune cells; and d) damaging or killing the disease cells via the activated immune cells sufficient to prevent or treat the disease in the patient.

Administration of the fusion protein complexes of the invention induces an immune response in a subject. For example, administration of the fusion protein complexes of the invention induces an immune response against cells associated with neoplasia, infectious disease, or autoimmune disease. In one aspect, the fusion protein complex of the invention increases immune cell proliferation.

The invention provides methods of stimulating immune responses in a mammal by administering to the mammal an effective amount of the soluble fusion protein complex of the invention. The invention also provides methods of suppressing immune responses in a mammal by administering to the mammal an effective amount of the soluble fusion protein complex of any one of the invention.

Unless defined otherwise, all technical and scientific terms used herein have the meaning commonly understood by a person skilled in the art to which this invention belongs. The following references provide one of skill with a general definition of many of the terms used in this invention: Singleton et al., Dictionary of Microbiology and Molecular Biology (2nd ed. 1994); The Cambridge Dictionary of Science and Technology (Walker ed., 1988); The Glossary of Genetics, 5th Ed., R. Rieger et al. (eds.), Springer Verlag (1991); and Hale & Marham, The Harper Collins Dictionary of Biology (1991). As used herein, the following terms have the meanings ascribed to them below, unless specified otherwise.

By “agent” is meant a peptide, nucleic acid molecule, or small compound.

By “TxM” is meant a complex comprising an IL-15N72D:IL-15RαSu/Fc scaffold linked to a binding domain (FIG. 2). An exemplary TxM is an IL-15N72D:IL-15RαSu complex comprising a fusion to a binding domain that specifically recognizes PD-L1 (PD-L1 TxM).

By “ameliorate” is meant decrease, suppress, attenuate, diminish, arrest, or stabilize the development or progression of a disease.

By “analog” is meant a molecule that is not identical, but has analogous functional or structural features. For example, a polypeptide analog retains the biological activity of a corresponding naturally-occurring polypeptide, while having certain biochemical modifications that enhance the analog's function relative to a naturally occurring polypeptide. Such biochemical modifications could increase the analog's protease resistance, membrane permeability, or half-life, without altering, for example, ligand binding. An analog may include an unnatural amino acid.

The term "binding domain" is intended to encompass an antibody, single chain antibody, Fab, Fv, T-cell receptor binding domain, ligand binding domain, receptor binding domain, or other antigen-specific polypeptides known in the art.

The invention includes antibodies or fragments of such antibodies, so long as they exhibit the desired biological activity. Also included in the invention are chimeric antibodies, such as humanized antibodies. Generally, a humanized antibody has one or more amino acid residues introduced into it from a source that is non-human. Humanization can be performed, for example, using methods described in the art, by substituting at least a portion of a rodent complementarity-determining region for the corresponding regions of a human antibody.

The term "antibody" or "immunoglobulin" is intended to encompass both polyclonal and monoclonal antibodies. The preferred antibody is a monoclonal antibody reactive with the antigen. The term "antibody" is also intended to encompass mixtures of more than one antibody reactive with the antigen (e.g., a cocktail of different types of monoclonal antibodies reactive with the antigen). The term "antibody" is further intended to encompass whole antibodies, biologically functional fragments thereof, single-chain antibodies, and genetically altered antibodies such as chimeric antibodies comprising portions from more than one species, bifunctional antibodies, antibody conjugates, humanized and human antibodies. Biologically functional antibody fragments, which can also be used, are those peptide fragments derived from an antibody that are sufficient for binding to the antigen. "Antibody" as used herein is meant to include the entire antibody as well as any antibody fragments (e.g. $F(ab')_2$, Fab', Fab, Fv) capable of binding the epitope, antigen, or antigenic fragment of interest.

By "binding to" a molecule is meant having a physicochemical affinity for that molecule.

"Detect" refers to identifying the presence, absence, or amount of the analyte to be detected.

By "disease" is meant any condition or disorder that damages or interferes with the normal function of a cell, tissue, or organ. Examples of diseases include neoplasias, autoimmune diseases and viral infections.

By the terms "effective amount" and "therapeutically effective amount" of a formulation or formulation component is meant a sufficient amount of the formulation or component, alone or in a combination, to provide the desired effect. For example, by "an effective amount" is meant an amount of a compound, alone or in a combination, required to ameliorate the

symptoms of a disease relative to an untreated patient. The effective amount of active compound(s) used to practice the present invention for therapeutic treatment of a disease varies depending upon the manner of administration, the age, body weight, and general health of the subject. Ultimately, the attending physician or veterinarian will decide the appropriate amount and dosage regimen. Such amount is referred to as an "effective" amount.

By "fragment" is meant a portion of a polypeptide or nucleic acid molecule. This portion contains, preferably, at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, or 90% of the entire length of the reference nucleic acid molecule or polypeptide. For example, a fragment may contain 10, 20, 30, 40, 50, 60, 70, 80, 90, or 100, 200, 300, 400, 500, 600, 700, 800, 900, or 1000 nucleotides or amino acids. However, the invention also comprises polypeptides and nucleic acid fragments, so long as they exhibit the desired biological activity of the full-length polypeptides and nucleic acid, respectively. A nucleic acid fragment of almost any length is employed. For example, illustrative polynucleotide segments with total lengths of about 10,000, about 5,000, about 3,000, about 2,000, about 1,000, about 500, about 200, about 100, about 50 base pairs in length (including all intermediate lengths) are included in many implementations of this invention. Similarly, a polypeptide fragment of almost any length is employed. For example, illustrative polypeptide segments with total lengths of about 10,000, about 5,000, about 3,000, about 2,000, about 1,000, about 5,000, about 1,000, about 500, about 200, about 100, or about 50 amino acids in length (including all intermediate lengths) are included in many implementations of this invention.

The terms "isolated", "purified", or "biologically pure" refer to material that is free to varying degrees from components which normally accompany it as found in its native state. "Isolate" denotes a degree of separation from original source or surroundings. "Purify" denotes a degree of separation that is higher than isolation.

A "purified" or "biologically pure" protein is sufficiently free of other materials such that any impurities do not materially affect the biological properties of the protein or cause other adverse consequences. That is, a nucleic acid or peptide of this invention is purified if it is substantially free of cellular material, viral material, or culture medium when produced by recombinant DNA techniques, or chemical precursors or other chemicals when chemically synthesized. Purity and homogeneity are typically determined using analytical chemistry techniques, for example, polyacrylamide gel electrophoresis or high-performance liquid

chromatography. The term "purified" can denote that a nucleic acid or protein gives rise to essentially one band in an electrophoretic gel. For a protein that can be subjected to modifications, for example, phosphorylation or glycosylation, different modifications may give rise to different isolated proteins, which can be separately purified.

Similarly, by "substantially pure" is meant a nucleotide or polypeptide that has been separated from the components that naturally accompany it. Typically, the nucleotides and polypeptides are substantially pure when they are at least 60%, 70%, 80%, 90%, 95%, or even 99%, by weight, free from the proteins and naturally-occurring organic molecules with they are naturally associated.

By "isolated nucleic acid" is meant a nucleic acid that is free of the genes which flank it in the naturally-occurring genome of the organism from which the nucleic acid is derived. The term covers, for example: (a) a DNA which is part of a naturally occurring genomic DNA molecule, but is not flanked by both of the nucleic acid sequences that flank that part of the molecule in the genome of the organism in which it naturally occurs; (b) a nucleic acid incorporated into a vector or into the genomic DNA of a prokaryote or eukaryote in a manner, such that the resulting molecule is not identical to any naturally occurring vector or genomic DNA; (c) a separate molecule such as a cDNA, a genomic fragment, a fragment produced by polymerase chain reaction (PCR), or a restriction fragment; and (d) a recombinant nucleotide sequence that is part of a hybrid gene, i.e., a gene encoding a fusion protein. Isolated nucleic acid molecules according to the present invention further include molecules produced synthetically, as well as any nucleic acids that have been altered chemically and/or that have modified backbones. For example, the isolated nucleic acid is a purified cDNA or RNA polynucleotide. Isolated nucleic acid molecules also include messenger ribonucleic acid (mRNA) molecules.

By an "isolated polypeptide" is meant a polypeptide of the invention that has been separated from components that naturally accompany it. Typically, the polypeptide is isolated when it is at least 60%, by weight, free from the proteins and naturally-occurring organic molecules with which it is naturally associated. Preferably, the preparation is at least 75%, more preferably at least 90%, and most preferably at least 99%, by weight, a polypeptide of the invention. An isolated polypeptide of the invention may be obtained, for example, by extraction from a natural source, by expression of a recombinant nucleic acid encoding such a polypeptide;

or by chemically synthesizing the protein. Purity can be measured by any appropriate method, for example, column chromatography, polyacrylamide gel electrophoresis, or by HPLC analysis.

By “marker” is meant any protein or polynucleotide having an alteration in expression level or activity that is associated with a disease or disorder.

By “neoplasia” is meant a disease or disorder characterized by excess proliferation or reduced apoptosis. Illustrative neoplasms for which the invention can be used include, but are not limited to leukemias (e.g., acute leukemia, acute lymphocytic leukemia, acute myelocytic leukemia, acute myeloblastic leukemia, acute promyelocytic leukemia, acute myelomonocytic leukemia, acute monocytic leukemia, acute erythroleukemia, chronic leukemia, chronic myelocytic leukemia, chronic lymphocytic leukemia), polycythemia vera, lymphoma (Hodgkin's disease, non-Hodgkin's disease), Waldenstrom's macroglobulinemia, heavy chain disease, and solid tumors such as sarcomas and carcinomas (e.g., fibrosarcoma, myxosarcoma, liposarcoma, chondrosarcoma, osteogenic sarcoma, chordoma, angiosarcoma, endotheliosarcoma, lymphangiosarcoma, lymphangioendotheliosarcoma, synovioma, mesothelioma, Ewing's tumor, leiomyosarcoma, rhabdomyosarcoma, colon carcinoma, pancreatic cancer, breast cancer, ovarian cancer, prostate cancer, squamous cell carcinoma, basal cell carcinoma, adenocarcinoma, sweat gland carcinoma, sebaceous gland carcinoma, papillary carcinoma, papillary adenocarcinomas, cystadenocarcinoma, medullary carcinoma, bronchogenic carcinoma, renal cell carcinoma, hepatoma, nile duct carcinoma, choriocarcinoma, seminoma, embryonal carcinoma, Wilm's tumor, cervical cancer, uterine cancer, testicular cancer, lung carcinoma, small cell lung carcinoma, bladder carcinoma, epithelial carcinoma, glioma, glioblastoma multiforme, astrocytoma, medulloblastoma, craniopharyngioma, ependymoma, pinealoma, hemangioblastoma, acoustic neuroma, oligodenroglioma, schwannoma, meningioma, melanoma, neuroblastoma, and retinoblastoma). In particular embodiments, the neoplasia is multiple myeloma, beta-cell lymphoma, urothelial/bladder carcinoma, or melanoma. As used herein, “obtaining” as in “obtaining an agent” includes synthesizing, purchasing, or otherwise acquiring the agent.

By “reduces” is meant a negative alteration of at least 5%, 10%, 25%, 50%, 75%, or 100%.

By “reference” is meant a standard or control condition.

A "reference sequence" is a defined sequence used as a basis for sequence comparison. A reference sequence may be a subset of or the entirety of a specified sequence; for example, a segment of a full-length cDNA or gene sequence, or the complete cDNA or gene sequence. For polypeptides, the length of the reference polypeptide sequence will generally be at least about 16 amino acids, preferably at least about 20 amino acids, more preferably at least about 25 amino acids, and even more preferably about 35 amino acids, about 50 amino acids, or about 100 amino acids. For nucleic acids, the length of the reference nucleic acid sequence will generally be at least about 50 nucleotides, preferably at least about 60 nucleotides, more preferably at least about 75 nucleotides, and even more preferably about 100 nucleotides or about 300 nucleotides or any integer thereabout or therebetween.

By "specifically binds" is meant a compound or antibody that recognizes and binds a polypeptide of the invention, but which does not substantially recognize and bind other molecules in a sample, for example, a biological sample, which naturally includes a polypeptide of the invention.

Nucleic acid molecules useful in the methods of the invention include any nucleic acid molecule that encodes a polypeptide of the invention or a fragment thereof. Such nucleic acid molecules need not be 100% identical with an endogenous nucleic acid sequence, but will typically exhibit substantial identity. Polynucleotides having "substantial identity" to an endogenous sequence are typically capable of hybridizing with at least one strand of a double-stranded nucleic acid molecule. Nucleic acid molecules useful in the methods of the invention include any nucleic acid molecule that encodes a polypeptide of the invention or a fragment thereof. Such nucleic acid molecules need not be 100% identical with an endogenous nucleic acid sequence, but will typically exhibit substantial identity. Polynucleotides having "substantial identity" to an endogenous sequence are typically capable of hybridizing with at least one strand of a double-stranded nucleic acid molecule. By "hybridize" is meant pair to form a double-stranded molecule between complementary polynucleotide sequences (e.g., a gene described herein), or portions thereof, under various conditions of stringency. (See, e.g., Wahl, G. M. and S. L. Berger (1987) Methods Enzymol. 152:399; Kimmel, A. R. (1987) Methods Enzymol. 152:507).

For example, stringent salt concentration will ordinarily be less than about 750 mM NaCl and 75 mM trisodium citrate, preferably less than about 500 mM NaCl and 50 mM trisodium

citrate, and more preferably less than about 250 mM NaCl and 25 mM trisodium citrate. Low stringency hybridization can be obtained in the absence of organic solvent, e.g., formamide, while high stringency hybridization can be obtained in the presence of at least about 35% formamide, and more preferably at least about 50% formamide. Stringent temperature conditions will ordinarily include temperatures of at least about 30° C, more preferably of at least about 37° C, and most preferably of at least about 42° C. Varying additional parameters, such as hybridization time, the concentration of detergent, e.g., sodium dodecyl sulfate (SDS), and the inclusion or exclusion of carrier DNA, are well known to those skilled in the art. Various levels of stringency are accomplished by combining these various conditions as needed. In a preferred: embodiment, hybridization will occur at 30° C in 750 mM NaCl, 75 mM trisodium citrate, and 1% SDS. In a more preferred embodiment, hybridization will occur at 37° C in 500 mM NaCl, 50 mM trisodium citrate, 1% SDS, 35% formamide, and 100 .mu.g/ml denatured salmon sperm DNA (ssDNA). In a most preferred embodiment, hybridization will occur at 42° C in 250 mM NaCl, 25 mM trisodium citrate, 1% SDS, 50% formamide, and 200 µg/ml ssDNA. Useful variations on these conditions will be readily apparent to those skilled in the art.

For most applications, washing steps that follow hybridization will also vary in stringency. Wash stringency conditions can be defined by salt concentration and by temperature. As above, wash stringency can be increased by decreasing salt concentration or by increasing temperature. For example, stringent salt concentration for the wash steps will preferably be less than about 30 mM NaCl and 3 mM trisodium citrate, and most preferably less than about 15 mM NaCl and 1.5 mM trisodium citrate. Stringent temperature conditions for the wash steps will ordinarily include a temperature of at least about 25° C, more preferably of at least about 42° C, and even more preferably of at least about 68° C. In a preferred embodiment, wash steps will occur at 25° C in 30 mM NaCl, 3 mM trisodium citrate, and 0.1% SDS. In a more preferred embodiment, wash steps will occur at 42 C in 15 mM NaCl, 1.5 mM trisodium citrate, and 0.1% SDS. In a more preferred embodiment, wash steps will occur at 68° C in 15 mM NaCl, 1.5 mM trisodium citrate, and 0.1% SDS. Additional variations on these conditions will be readily apparent to those skilled in the art. Hybridization techniques are well known to those skilled in the art and are described, for example, in Benton and Davis (Science 196:180, 1977); Grunstein and Hogness (Proc. Natl. Acad. Sci., USA 72:3961, 1975); Ausubel et al. (Current Protocols in Molecular Biology, Wiley Interscience, New York, 2001); Berger and Kimmel (Guide to

Molecular Cloning Techniques, 1987, Academic Press, New York); and Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, New York.

By "substantially identical" is meant a polypeptide or nucleic acid molecule exhibiting at least 50% identity to a reference amino acid sequence (for example, any one of the amino acid sequences described herein) or nucleic acid sequence (for example, any one of the nucleic acid sequences described herein). Preferably, such a sequence is at least 60%, more preferably 80% or 85%, and more preferably 90%, 95% or even 99% identical at the amino acid level or nucleic acid to the sequence used for comparison.

Sequence identity is typically measured using sequence analysis software (for example, Sequencher, Gene Codes Corporation, 775 Technology Drive, Ann Arbor, MI; Vector NTI, Life Technologies, 3175 Staley Rd. Grand Island, NY). Such software matches identical or similar sequences by assigning degrees of homology to various substitutions, deletions, and/or other modifications. Conservative substitutions typically include substitutions within the following groups: glycine, alanine; valine, isoleucine, leucine; aspartic acid, glutamic acid, asparagine, glutamine; serine, threonine; lysine, arginine; and phenylalanine, tyrosine. In an exemplary approach to determining the degree of identity, a BLAST program may be used, with a probability score between e^{-3} and e^{-100} indicating a closely related sequence.

By "subject" is meant a mammal, including, but not limited to, a human or non-human mammal, such as a bovine, equine, canine, ovine, or feline. The subject is preferably a mammal in need of such treatment, e.g., a subject that has been diagnosed with B cell lymphoma or a predisposition thereto. The mammal is any mammal, e.g., a human, a primate, a mouse, a rat, a dog, a cat, a horse, as well as livestock or animals grown for food consumption, e.g., cattle, sheep, pigs, chickens, and goats. In a preferred embodiment, the mammal is a human.

Ranges provided herein are understood to be shorthand for all of the values within the range. For example, a range of 1 to 50 is understood to include any number, combination of numbers, or sub-range from the group consisting 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, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50.

The terms "treating" and "treatment" as used herein refer to the administration of an agent or formulation to a clinically symptomatic individual afflicted with an adverse condition, disorder, or disease, so as to affect a reduction in severity and/or frequency of symptoms,

eliminate the symptoms and/or their underlying cause, and/or facilitate improvement or remediation of damage. It will be appreciated that, although not precluded, treating a disorder or condition does not require that the disorder, condition, or symptoms associated therewith be completely eliminated.

The terms “preventing” and “prevention” refer to the administration of an agent or composition to a clinically asymptomatic individual who is susceptible or predisposed to a particular adverse condition, disorder, or disease, and thus relates to the prevention of the occurrence of symptoms and/or their underlying cause.

Unless specifically stated or obvious from context, as used herein, the term “or” is understood to be inclusive. Unless specifically stated or obvious from context, as used herein, the terms “a”, “an”, and “the” are understood to be singular or plural.

Unless specifically stated or obvious from context, as used herein, the term “about” is understood as within a range of normal tolerance in the art, for example within 2 standard deviations of the mean. About can be understood as within 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, 1%, 0.5%, 0.1%, 0.05%, or 0.01% of the stated value. Unless otherwise clear from context, all numerical values provided herein are modified by the term about.

The recitation of a listing of chemical groups in any definition of a variable herein includes definitions of that variable as any single group or combination of listed groups. The recitation of an embodiment for a variable or aspect herein includes that embodiment as any single embodiment or in combination with any other embodiments or portions thereof.

Any compositions or methods provided herein can be combined with one or more of any of the other compositions and methods provided herein.

The transitional term “comprising,” which is synonymous with “including,” “containing,” or “characterized by,” is inclusive or open-ended and does not exclude additional, unrecited elements or method steps. By contrast, the transitional phrase “consisting of” excludes any element, step, or ingredient not specified in the claim. The transitional phrase “consisting essentially of” limits the scope of a claim to the specified materials or steps “and those that do not materially affect the basic and novel characteristic(s)” of the claimed invention.

Other features and advantages of the invention will be apparent from the following description of the preferred embodiments thereof, and from the claims. 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. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, suitable methods and materials are described below. All published foreign patents and patent applications cited herein are incorporated herein by reference.

Genbank and NCBI submissions indicated by accession number cited herein are incorporated herein by reference. All other published references, documents, manuscripts, and scientific literature cited herein are incorporated herein by reference. In the case of conflict, the present specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a schematic diagram illustrating the activity of the PD-L1 TxM complex comprising anti-PD-L1 scAb/huIL-15N72D and anti-PD-L1 scAb/huIL-15RαSu/Fc fusion proteins, and its immune-mediated effects against disease cells expressing PD-L1 antigen.

FIG. 2 is a schematic diagram illustrating different TxM complexes comprising the IL-15/ IL-15RαSu/Fc scaffold fused to binding domains that recognize immune checkpoint molecules, immune signaling molecule and/or disease antigens.

FIG. 3 is a photograph showing a sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis of the TxM complex following disulfide bond reduction. Right lane: PD-L1 TxM; left lane: marker.

FIG. 4A is a line graph showing the binding activity of an anti-human PD-L1 TxM complex to antibodies specific to human IL-15 and human IgG. FIG. 4B is a line graph showing the binding activity of a second anti-human PD-L1 TxM complex to antibodies specific to human IL-15 and human IgG. FIG. 4C is a line graph showing the binding activity of an anti-mouse PD-L1 TxM complex to antibodies specific to human IL-15 and mouse IgG.

FIG. 5A is a line graph showing the binding activity of a PD-L1 TxM complex to PD-L1-bearing human MB231 tumor cells. FIG. 5B is a line graph showing the blocking activity of a PD-L1 TxM complex of PD-L1 expressed on human MB231 tumor cells. FIG. 5C is a line graph showing the binding activity of a PD-L1 TxM complex to PD-L1-bearing human MB231 tumor cells.

FIG. 6A is a line graph showing the blocking activity of a PD-L1 TxM complex of PD-L1 expressed on mouse 5T33P tumor cells. FIG. 6B is a line graph showing the blocking activity of a PD-L1 TxM complex of PD-L1 expressed on mouse MB49luc tumor cells.

FIG. 7A and FIG. 7B are line graphs comparing the blocking activities of an anti-PD-L1 Ab and a PD-L1 TxM complex of PD-L1 expressed on mouse A20 tumor cells.

FIG. 8 is a line graph illustrating the proliferation of IL-15-dependent 32Dβ cells mediated by a PD-L1 TxM complex.

FIG. 9A is a schematic diagram illustrating "4 headed" and "2 headed" PD-L1 TxM complexes. FIG. 9B is a photograph showing SDS-PAGE analysis of four- and two-headed mouse-specific PD-L1 TxM complexes following disulfide bond reduction. FIG. 9C and FIG. 9D show line graphs representing the chromatographic profiles of two- and four-headed mouse-specific PD-L1 TxM complexes, respectively, following elution on an analytical size exclusion column, demonstrating separation of TxM complexes from protein aggregates.

FIG. 10A is a line graph showing the binding activity of two- and four-headed mouse-specific PD-L1 TxM complexes to IL-2Rβγ-bearing 32Dβ cells. FIG. 10B and FIG. 10C show line graphs demonstrating the blocking activity of the two- and four-headed mouse-specific PD-L1 TxM complexes of PD-L1 expressed on 5T33P myeloma cells.

FIG. 11A is a line graph illustrating the proliferation of IL-15-dependent 32Dβ cells mediated by a two-headed mouse-specific PD-L1 TxM complex compared to ALT-803 (IL-15N72D:IL-15Rα/Fc complex). FIG. 11B is a line graph illustrating the proliferation of IL-15-dependent 32Dβ cells mediated by a four-headed mouse-specific PD-L1 TxM complex compared to ALT-803.

FIG. 12A is a photograph showing SDS-PAGE analysis of the two and four headed human-specific PD-L1 TxM complexes following disulfide bond reduction. FIG. 12B and FIG. 12C show line graphs representing the chromatographic profiles of two- and four-headed human-specific PD-L1 TxM complexes, respectively, following elution on an analytical size exclusion column, demonstrating separation of TxM complexes from protein aggregates.

FIG. 13 is a line graph showing the blocking activity of the two- and four-headed human-specific PD-L1 TxM complexes of PD-L1 expressed on PC-3 human prostate cancer cells.

FIG. 14A is a line graph illustrating the proliferation of IL-15-dependent 32Dβ cells mediated by a two-headed human-specific PD-L1 TxM complex compared to ALT-803. FIG.

14B is a line graph illustrating the proliferation of IL-15-dependent 32Dβ cells mediated by a four-headed human-specific PD-L1 TxM complex compared to ALT-803.

FIG. 15A is a bar chart showing the spleen weights of mice treated with PBS, ALT-803, four-headed mouse-specific PD-L1 TxM (T4M-mPD-L1), and two-headed mouse-specific PD-L1 TxM (T2M-mPD-L1). FIG. 15B and FIG. 15C show bar charts illustrating the percentage of different immune cell subsets in the spleens and lymph nodes, respectively, of mice treated with PBS, ALT-803, four-headed mouse-specific PD-L1 TxM (T4M-mPD-L1), and two-headed mouse-specific PD-L1 TxM (T2M-mPD-L1).

FIG. 16 is a bar chart illustrating the cytotoxicity of immune cells against 5T33 myeloma cells induced by PD-L1 TxM, anti-PD-L1 Ab or ALT-803.

FIG. 17 is a bar chart illustrating the cytotoxicity of human immune cells against PD-L1-positive SW1990 human pancreatic cancer cells induced by anti-human PD-L1 Ab, two-headed human-specific PD-L1 TxM (T4M-mPD-L1), or four-headed human-specific PD-L1 TxM (T2M-mPD-L1) compared to medium alone.

FIG. 18 is a line graph illustrating the survival of mice bearing 5T33 myeloma tumors following treatment with PD-L1 TxM complex, ALT-803, ALT-803+anti-PD-L1 Ab or PBS.

FIG. 19 is a line graph illustrating the survival of mice bearing orthotopic MB49luc bladder tumors following treatment with 2H PD-L1 TxM complex, ALT-803, ALT-803+anti-PD-L1 Ab or PBS.

FIG. 20 shows line graphs representing the chromatographic profiles of different purified TxM proteins following elution on an analytical size exclusion column, demonstrating separation of TxM complexes from protein aggregates.

FIG. 21A is a line graph showing the blocking activity of a CTLA-4 TxM complex of CTLA-4 expressed on mouse lymphocytes. FIG. 21B is a line graph showing the blocking activity of a CTLA-4 TxM complex of CTLA-4 expressed on human lymphocytes.

FIG. 22A is a line graph showing the blocking activity of a PD-L1/CTLA-4 TxM complex of PD-L1 expressed on mouse 5T33P tumor cells. FIG. 22B is a line graph showing the blocking activity of a PD-L1/CTLA-4 TxM complex of CTLA-4 expressed on mouse lymphocytes.

FIG. 23A is a line graph showing the binding activity of a CD47 TxM complex to CD47-bearing mouse B16F10 melanoma tumor cells. FIG. 23B is a line graph showing the binding activity of a CD47 TxM complex to CD47-bearing human Jurkat T cells.

FIG. 24A is a line graph demonstrating the binding activity of an TNT scAb TxM complex to single stranded DNA. FIG24B is a line graph demonstrating the binding activity of an TNT scAb/anti-PD-L1 scAb TxM complex to single stranded DNA.

FIG. 25A is a line graph showing the binding activity of TNT scAb TxM, TNT scAb/anti-PD-L1 scAb TxM and 2-headed anti-PD-L1 scAb TxM complexes to permeabilized human MB231 breast cancer cells. FIG. 25B is a line graph showing the binding activity of TNT scAb TxM, TNT scAb/anti-PD-L1 scAb TxM and 2-headed anti-PD-L1 scAb TxM complexes to permeabilized human A549 lung cancer cells.

FIG. 26 is a line graph showing the binding activity of 2-headed hOAT scAb TxM, anti-human PD-L1 scAb/hOAT scAb TxM, 2-headed anti-human PD-L1 scAb TxM complexes and hOAT and anti-human PD-L1 control Abs to human TF-positive PD-L1-positive SW1990 human pancreatic cancer cells

FIG. 27A is a line graph demonstrating the binding activity of an LFA-1 TxM complex to antibodies specific to human IL-15 and human IgG. FIG27B is a bar graph showing the binding activity of an LFA-1 TxM complex to ICAM-1.

FIG. 28 is a line graph demonstrating the binding activity of a Notch1-specific TxM complex to antibodies specific to human IL-15 and human IgG.

FIG. 29 is a line graph demonstrating the binding activity of an anti-human TIM3 scAb TxM complex to antibodies specific to human IL-15 and human IgG.

FIG. 30A and FIG. 30B are line graphs demonstrating the binding activity of HIV-specific bNAb scFv TxM complexes to antibodies specific to human IL-15 and human IgG. FIG. 30C through FIG. 30F show line graphs demonstrating the binding activity of HIV-specific bNAb TxM complexes to HIV envelope proteins.

FIG. 31 is a bar chart illustrating the cytotoxicity of human immune cells against human TF-positive SW1990 human pancreatic cancer cells induced by 2-headed hOAT scAb TxM or hOAT control Ab compared to medium alone.

DETAILED DESCRIPTION

The invention is based, at least in part, on the surprising discovery that multi-specific IL-15-based protein complexes enhance the activity of immune cells and promote their activity against disease cells, thereby resulting in reduction or prevention of disease. These protein complexes also show increased binding to disease and target antigens. Provided herein are multi-specific protein complexes with one domain comprising IL-15 or a functional variant and a binding domain comprising a disease-specific binding domain, immune checkpoint inhibitor or immune agonist. Such protein complexes have utility in methods for treating a neoplasia, infectious disease, or autoimmune disease in a subject. Specifically, as described in detail below, a soluble anti-PD-L1 scAb/huIL-15N72D:anti-PD-L1 scAb/huIL-15RαSu/huIgG1 Fc complex ("PD-L1 TxM") stimulated immune cells to kill tumor target cells (FIG. 1). Thus, provided herein are compositions featuring PD-L1 TxM and methods of using such compositions to enhance an immune response against a neoplasia (e.g., solid and hematologic tumors).

As described herein, the use of proteins with the capability of targeting diseased cells for host immune recognition and response is an effective strategy for treating cancer, infectious diseases, and autoimmune diseases. As described in U.S. Patent No. 8,507,222 (incorporated herein by reference), a protein scaffold comprising IL-15 and IL-15 receptor α domains has been used to generate multi-specific proteins capable of recognizing antigens on disease cells and receptors on immune cells. *See*, U.S. Patent No. 8,507,222 at Example 15. Described herein is the generation of soluble multi-specific protein complexes comprising IL-15 and IL-15 receptor α linked to one or more binding domains recognizing immune checkpoint or signaling molecules. In some cases, these complexes also comprise binding domains that recognize antigens, such as PD-L1, ssDNA, CD20, HER2, EGFR, CD19, CD38, CD52, GD2, CD33, Notch1, intercellular adhesion molecule 1 (ICAM-1), tissue factor, HIV envelope or other tumor antigens, expressed on disease cells.

In some cases, the binding domain comprises a single chain antibody wherein an immunoglobulin light chain variable domain covalently linked to an immunoglobulin heavy chain variable domain by a polypeptide linker sequence. The single chain antibody domain can be arranged in either the VH-linker-VL or VL-linker-VH format. Alternatively, the binding domain comprises a soluble or extracellular ligand or receptor domain capable of acting as an immune checkpoint inhibitor or immune agonist. The binding domains recognizing an immune

checkpoint or signaling molecule are linked to either the N- or C-termini of the IL-15 or IL-15 receptor α proteins with or without an additional linker sequence so long as binding activity is maintained. Preferably, the binding domain is linked to the N-terminus of the human IL-15N72D superagonist protein (huIL-15N72D). Alternatively, the binding domain is linked to the C-terminus of the human IL-15N72D protein. Preferably, the binding domain is linked to the N-terminus of the human IL-15 receptor α sushi domain (huIL-15RαSu). Alternatively, the binding domain is linked to the C-terminus of the huIL-15RαSuFc protein. In some cases, the multi-specific protein complexes of the invention further comprise an IgG Fc domain for protein dimerization and recognition of CD16 receptors on immune cells. Such a domain mediates stimulation of antibody-dependent cellular cytotoxicity (ADCC), antibody-dependent cellular phagocytosis (ADCP) and complement-dependent cytotoxicity (CDC) against target cells. In some examples, it is useful to employ Fc domains with enhanced or decreased CD16 binding activity. In one aspect, the Fc domain contains amino acid substitutions L234A and L235A (LALA) (number based on Fc consensus sequence) that reduce ADCC activity, but retain the ability to form disulfide-bound dimers.

Interleukin-15

Interleukin-15 (IL-15) is an important cytokine for the development, proliferation, and activation of effector NK cells and $CD8^+$ memory T cells. IL-15 binds to the IL-15 receptor α (IL-15Rα) and is presented in *trans* to the IL-2/IL-15 receptor β - common γ chain (IL-15R$\beta\gamma_c$) complex on effector cells. IL-15 and IL-2 share binding to the IL-15R$\beta\gamma_c$, and signal through STAT3 and STAT5 pathways. However, unlike IL-2, IL-15 does not support maintenance of $CD4^+CD25^+FoxP3^+$ regulatory T (Treg) cells or induce cell death of activated $CD8^+$ T cells, effects that may have limited the therapeutic activity of IL-2 against multiple myeloma. Additionally, IL-15 is the only cytokine known to provide anti-apoptotic signaling to effector $CD8^+$ T cells. IL-15, either administered alone or as a complex with the IL-15Rα, exhibits potent anti-tumor activities against well-established solid tumors in experimental animal models and, thus, has been identified as one of the most promising immunotherapeutic drugs that could potentially cure cancer.

To facilitate clinical development of an IL-15-based cancer therapeutic, an IL-15 mutant (IL-15N72D) with increased biological activity compared to IL-15 was identified (Zhu et al., J Immunol, 183: 3598-3607, 2009). The pharmacokinetics and biological activity of this IL-15

super-agonist (IL-15N72D) was further improved by the creation of IL-15N72D:IL-15Rα/Fc fusion complex (ALT-803), such that the super agonist complex has at least 25-times the activity of the native cytokine *in vivo* (Han et al., Cytokine, 56: 804-810, 2011).

Immune Checkpoint Inhibitor and Immune Agonist Domains

In other embodiments, the binding domain is specific to an immune checkpoint or signaling molecule or its ligand and acts as an inhibitor of immune checkpoint suppressive activity or as an agonist of immune stimulatory activity. Such immune checkpoint and signaling molecules and ligands include PD-1, PD-L1, PD-L2, CTLA-4, CD28, CD80, CD86, B7-H3, B7-H4, B7-H5, ICOS-L, ICOS, BTLA, CD137L, CD137, HVEM, KIR, 4-1BB, OX40L, CD70, CD27, CD47, CIS, OX40, GITR, IDO, TIM3, GAL9, VISTA, CD155, TIGIT, LIGHT, LAIR-1, Siglecs and A2aR (Pardoll DM. 2012. Nature Rev Cancer 12:252-264, Thaventhiran T, et al. 2012. J Clin Cell Immunol S12:004). Additionally, preferred antibody domains of the invention may include ipilimumab and/or tremelimumab (anti-CTLA4), nivolumab, pembrolizumab, pidilizumab, TSR-042, ANB011, AMP-514 and AMP-224 (a ligand-Fc fusion) (anti-PD1), atezolizumab (MPDL3280A), avelumab (MSB0010718C), durvalumab (MEDI4736), MEDI0680, and BMS-9365569 (anti-PDL1), MEDI6469 (anti-OX40 agonist), BMS-986016, IMP701, IMP731, IMP321 (anti-LAG3) and GITR ligand.

Antigen-specific Binding Domains

Antigen-specific binding domains consist of polypeptides that specifically bind to targets on diseased cells. Alternatively, these domains may bind to targets on other cells that support the diseased state, such as targets on stromal cells that support tumor growth or targets on immune cells that support disease-mediated immunosuppression. Antigen-specific binding domains include antibodies, single chain antibodies, Fabs, Fv, T-cell receptor binding domains, ligand binding domains, receptor binding domains, domain antibodies, single domain antibodies, minibodies, nanobodies, peptibodies, or various other antibody mimics (such as affimers, affitins, alphabodies, atrimers, CTLA4-based molecules, adnectins, anticalins, Kunitz domain-based proteins, avimers, knottins, fynomers, darpins, affibodies, affilins, monobodies and armadillo repeat protein-based proteins (Weidle, UH, et al. 2013. Cancer Genomics & Proteomics 10: 155-168)) known in the art.

In certain embodiments, the antigen for the antigen-specific binding domain comprises a cell surface receptor or ligand. In a further embodiment, the antigen comprises a CD antigen, cytokine or chemokine receptor or ligand, growth factor receptor or ligand, tissue factor, cell adhesion molecule, MHC/MHC-like molecules, Fc receptor, Toll-like receptor, NK receptor, TCR, BCR, positive/negative co-stimulatory receptor or ligand, death receptor or ligand, tumor associated antigen, or virus encoded antigen.

Preferably, the antigen-specific binding domain is capable of binding to an antigen on a tumor cell. Tumor-specific binding domain may be derived from antibodies approved for treatment of patients with cancer include rituximab, ofatumumab, and obinutuzumab (anti-CD20 Abs); trastuzumab and pertuzumab (anti-HER2 Abs); cetuximab and panitumumab (anti-EGFR Abs); and alemtuzumab (anti-CD52 Ab). Similarly, binding domains from approved antibody-effector molecule conjugates specific to CD20 (^{90}Y-labeled ibritumomab tiuxetan, ^{131}I-labeled tositumomab), HER2 (ado-trastuzumab emtansine), CD30 (brentuximab vedotin) and CD33 (gemtuzumab ozogamicin) (Sliwkowski MX, Mellman I. 2013 Science 341:1192) could be used.

Additionally, preferred binding domains of the invention may include various other tumor-specific antibody domains known in the art. The antibodies and their respective targets for treatment of cancer include but are not limited to nivolumab (anti-PD-1 Ab), TA99 (anti-gp75), 3F8 (anti-GD2), 8H9 (anti-B7-H3), abagovomab (anti-CA-125 (imitation)), adecatumumab (anti-EpCAM), afutuzumab (anti-CD20), alacizumab pegol (anti-VEGFR2), altumomab pentetate (anti-CEA), amatuximab (anti-mesothelin), AME-133 (anti-CD20), anatumomab mafenatox (anti-TAG-72), apolizumab (anti-HLA-DR), arcitumomab (anti-CEA), bavituximab (anti-phosphatidylserine), bectumomab (anti-CD22), belimumab (anti-BAFF), besilesomab (anti-CEA-related antigen), bevacizumab (anti-VEGF-A), bivatuzumab mertansine (anti-CD44 v6), blinatumomab (anti-CD19), BMS-663513 (anti-CD137), brentuximab vedotin (anti-CD30 (TNFRSF8)), cantuzumab mertansine (anti-mucin CanAg), cantuzumab ravtansine (anti-MUC1), capromab pendetide (anti-prostatic carcinoma cells), carlumab (anti-MCP-1), catumaxomab (anti-EpCAM, CD3), cBR96-doxorubicin immunoconjugate (anti-Lewis-Y antigen), CC49 (anti-TAG-72), cedelizumab (anti-CD4), Ch.14.18 (anti-GD2), ch-TNT (anti-DNA associated antigens), citatuzumab bogatox (anti-EpCAM), cixutumumab (anti-IGF-1 receptor), clivatuzumab tetraxetan (anti-MUC1), conatumumab (anti-TRAIL-R2), CP-870893 (anti-CD40), dacetuzumab (anti-CD40), daclizumab (anti-CD25), dalotuzumab (anti-insulin-like

growth factor I receptor), daratumumab (anti-CD38 (cyclic ADP ribose hydrolase)), demcizumab (anti-DLL4), detumomab (anti-B-lymphoma cell), drozitumab (anti-DR5), duligotumab (anti-HER3), dusigitumab (anti-ILGF2), ecromeximab (anti-GD3 ganglioside), edrecolomab (anti-EpCAM), elotuzumab (anti-SLAMF7), elsilimomab (anti-IL-6), enavatuzumab (anti-TWEAK receptor), enoticumab (anti-DLL4), ensituximab (anti-5AC), epitumomab cituxetan (anti-episialin), epratuzumab (anti-CD22), ertumaxomab (anti-HER2/neu, CD3), etaracizumab (anti-integrin αvβ3), faralimomab (anti-Interferon receptor), farletuzumab (anti-folate receptor 1), FBTA05 (anti-CD20), ficlatuzumab (anti-HGF), figitumumab (anti-IGF-1 receptor), flanvotumab (anti-TYRP1(glycoprotein 75)), fresolimumab (anti-TGF β), futuximab (anti-EGFR), galiximab (anti-CD80), ganitumab (anti-IGF-I), gemtuzumab ozogamicin (anti-CD33), girentuximab (anti-carbonic anhydrase 9 (CA-IX)), glembatumumab vedotin (anti-GPNMB), guselkumab (anti-IL13), ibalizumab (anti-CD4), ibritumomab tiuxetan (anti-CD20), icrucumab (anti-VEGFR-1), igovomab (anti-CA-125), IMAB362 (anti-CLDN18.2), IMC-CS4 (anti-CSF1R), IMC-TR1 (TGFβRII), imgatuzumab (anti-EGFR), inclacumab (anti-selectin P), indatuximab ravtansine (anti-SDC1), inotuzumab ozogamicin (anti-CD22), intetumumab (anti-CD51), ipilimumab (anti-CD152), iratumumab (anti-CD30 (TNFRSF8)), KM3065 (anti-CD20), KW-0761 (anti-CD194), LY2875358 (anti-MET) labetuzumab (anti-CEA), lambrolizumab (anti-PDCD1), lexatumumab (anti-TRAIL-R2), lintuzumab (anti-CD33), lirilumab (anti-KIR2D), lorvotuzumab mertansine (anti-CD56), lucatumumab (anti-CD40), lumiliximab (anti-CD23 (IgE receptor)), mapatumumab (anti-TRAIL-R1), margetuximab (anti-ch4D5), matuzumab (anti-EGFR), mavrilimumab (anti-GMCSF receptor α-chain), milatuzumab (anti-CD74), minretumomab (anti-TAG-72), mitumomab (anti-GD3 ganglioside), mogamulizumab (anti-CCR4), moxetumomab pasudotox (anti-CD22), nacolomab tafenatox (anti-C242 antigen), naptumomab estafenatox (anti-5T4), narnatumab (anti-RON), necitumumab (anti-EGFR), nesvacumab (anti-angiopoietin 2), nimotuzumab (anti-EGFR), nivolumab (anti-IgG4), nofetumomab merpentan, ocrelizumab (anti-CD20), ocaratuzumab (anti-CD20), olaratumab (anti-PDGF-R α), onartuzumab (anti-c-MET), ontuxizumab (anti-TEM1), oportuzumab monatox (anti-EpCAM), oregovomab (anti-CA-125), otlertuzumab (anti-CD37), pankomab (anti-tumor specific glycosylation of MUC1), parsatuzumab (anti-EGFL7), pascolizumab (anti-IL-4), patritumab (anti-HER3), pemtumomab (anti-MUC1), pertuzumab (anti-HER2/neu), pidilizumab (anti-PD-1), pinatuzumab vedotin (anti-CD22), pintumomab (anti-adenocarcinoma antigen),

polatuzumab vedotin (anti-CD79B), pritumumab (anti-vimentin), PRO131921 (anti-CD20), quilizumab (anti-IGHE), racotumomab (anti-N-glycolylneuraminic acid), radretumab (anti-fibronectin extra domain-B), ramucirumab (anti-VEGFR2), rilotumumab (anti-HGF), robatumumab (anti-IGF-1 receptor), roledumab (anti-RHD), rovelizumab (anti-CD11 & CD18), samalizumab (anti-CD200), satumomab pendetide (anti-TAG-72), seribantumab (anti-ERBB3), SGN-CD19A (anti-CD19), SGN-CD33A (anti-CD33), sibrotuzumab (anti-FAP), siltuximab (anti-IL-6), solitomab (anti-EpCAM), sontuzumab (anti-episialin), tabalumab (anti-BAFF), tacatuzumab tetraxetan (anti-alpha-fetoprotein), taplitumomab paptox (anti-CD19), telimomab aritox, tenatumomab (anti-tenascin C), teneliximab (anti-CD40), teprotumumab (anti-CD221), TGN1412 (anti-CD28), ticilimumab (anti-CTLA-4), tigatuzumab (anti-TRAIL-R2), TNX-650 (anti-IL-13), tositumomab (anti-CS20), tovetumab (anti-CD140a), TRBS07 (anti-GD2), tregalizumab (anti-CD4), tremelimumab (anti-CTLA-4), TRU-016 (anti-CD37), tucotuzumab celmoleukin (anti-EpCAM), ublituximab (anti-CD20), urelumab (anti-4-1BB), vantictumab (anti-Frizzled receptor), vapaliximab (anti-AOC3 (VAP-1)), vatelizumab (anti-ITGA2), veltuzumab (anti-CD20), vesencumab (anti-NRP1), visilizumab (anti-CD3), volociximab (anti-integrin $\alpha5\beta1$), vorsetuzumab mafodotin (anti-CD70), votumumab (anti-tumor antigen CTAA16.88), zalutumumab (anti-EGFR), zanolimumab (anti-CD4), zatuximab (anti-HER1), ziralimumab (anti-CD147 (basigin)), RG7636 (anti-ETBR), RG7458 (anti-MUC16), RG7599 (anti-NaPi2b), MPDL3280A (anti-PD-L1), RG7450 (anti-STEAP1), and GDC-0199 (anti-Bcl-2).

Other antibody domains or tumor target binding proteins useful in the invention (e.g. TCR domains) include, but are not limited to, those that bind the following antigens (note, the cancer indications indicated represent non-limiting examples): aminopeptidase N (CD13), annexin Al, B7-H3 (CD276, various cancers), CA125 (ovarian cancers), CA15-3 (carcinomas), CA19-9 (carcinomas), L6 (carcinomas), Lewis Y (carcinomas), Lewis X (carcinomas), alpha fetoprotein (carcinomas), CA242 (colorectal cancers), placental alkaline phosphatase (carcinomas), prostate specific antigen (prostate), prostatic acid phosphatase (prostate), epidermal growth factor (carcinomas), CD2 (Hodgkin's disease, NHL lymphoma, multiple myeloma), CD3 epsilon (T cell lymphoma, lung, breast, gastric, ovarian cancers, autoimmune diseases, malignant ascites), CD19 (B cell malignancies), CD20 (non-Hodgkin's lymphoma, B-cell neoplasmas, autoimmune diseases), CD21 (B-cell lymphoma), CD22 (leukemia, lymphoma,

multiple myeloma, SLE), CD30 (Hodgkin's lymphoma), CD33 (leukemia, autoimmune diseases), CD38 (multiple myeloma), CD40 (lymphoma, multiple myeloma, leukemia (CLL)), CD51 (metastatic melanoma, sarcoma), CD52 (leukemia), CD56 (small cell lung cancers, ovarian cancer, Merkel cell carcinoma, and the liquid tumor, multiple myeloma), CD66e (carcinomas), CD70 (metastatic renal cell carcinoma and non-Hodgkin lymphoma), CD74 (multiple myeloma), CD80 (lymphoma), CD98 (carcinomas), CD123 (leukemia), mucin (carcinomas), CD221 (solid tumors), CD227 (breast, ovarian cancers), CD262 (NSCLC and other cancers), CD309 (ovarian cancers), CD326 (solid tumors), CEACAM3 (colorectal, gastric cancers), CEACAM5 (CEA, CD66e) (breast, colorectal and lung cancers), DLL4 (A-like-4), EGFR (various cancers), CTLA4 (melanoma), CXCR4 (CD 184, heme-oncology, solid tumors), Endoglin (CD 105, solid tumors), EPCAM (epithelial cell adhesion molecule, bladder, head, neck, colon, NHL prostate, and ovarian cancers), ERBB2 (lung, breast, prostate cancers), FCGR1 (autoimmune diseases), FOLR (folate receptor, ovarian cancers), FGFR (carcinomas), GD2 ganglioside (carcinomas), G-28 (a cell surface antigen glycolipid, melanoma), GD3 idiotype (carcinomas), heat shock proteins (carcinomas), HER1 (lung, stomach cancers), HER2 (breast, lung and ovarian cancers), HLA-DR10 (NHL), HLA-DRB (NHL, B cell leukemia), human chorionic gonadotropin (carcinomas), IGF1R (solid tumors, blood cancers), IL-2 receptor (T-cell leukemia and lymphomas), IL-6R (multiple myeloma, RA, Castleman's disease, IL6 dependent tumors), integrins (αvβ3, α5β1, α6β4, α11β3, α5β5, αvβ5, for various cancers), MAGE-1 (carcinomas), MAGE-2 (carcinomas), MAGE-3 (carcinomas), MAGE 4 (carcinomas), anti-transferrin receptor (carcinomas), p97 (melanoma), MS4A1 (membrane-spanning 4-domains subfamily A member 1, Non-Hodgkin's B cell lymphoma, leukemia), MUC1 (breast, ovarian, cervix, bronchus and gastrointestinal cancer), MUC16 (CA125) (ovarian cancers), CEA (colorectal cancer), gp100 (melanoma), MARTI (melanoma), MPG (melanoma), MS4A1 (membrane-spanning 4-domains subfamily A, small cell lung cancers, NHL), nucleolin, Neu oncogene product (carcinomas), P21 (carcinomas), nectin-4 (carcinomas), paratope of anti-(N-glycolylneuraminic acid, breast, melanoma cancers), PLAP-like testicular alkaline phosphatase (ovarian, testicular cancers), PSMA (prostate tumors), PSA (prostate), ROB04, TAG 72 (tumour associated glycoprotein 72, AML, gastric, colorectal, ovarian cancers), T cell transmembrane protein (cancers), Tie (CD202b), tissue factor, TNFRSF10B (tumor necrosis factor receptor superfamily member 10B, carcinomas), TNFRSF13B (tumor necrosis factor receptor

superfamily member 13B, multiple myeloma, NHL, other cancers, RA and SLE), TPBG (trophoblast glycoprotein, renal cell carcinoma), TRAIL-R1 (tumor necrosis apoptosis inducing ligand receptor 1, lymphoma, NHL, colorectal, lung cancers), VCAM-1 (CD106, Melanoma), VEGF, VEGF-A, VEGF-2 (CD309) (various cancers). Some other tumor associated antigen targets have been reviewed (Gerber, et al, mAbs 2009 1:247-253; Novellino et al, Cancer Immunol Immunother. 2005 54:187-207, Franke, et al, Cancer Biother Radiopharm. 2000, 15:459-76, Guo, et al., Adv Cancer Res. 2013; 119: 421–475, Parmiani et al. J Immunol. 2007 178:1975-9). Examples of these antigens include Cluster of Differentiations (CD4, CD5, CD6, CD7, CD8, CD9, CD10, CDl la, CDl lb, CDl lc, CD12w, CD14, CD15, CD16, CDwl7, CD18, CD21, CD23, CD24, CD25, CD26, CD27, CD28, CD29, CD31, CD32, CD34, CD35, CD36, CD37, CD41, CD42, CD43, CD44, CD45, CD46, CD47, CD48, CD49b, CD49c, CD53, CD54, CD55, CD58, CD59, CD61, CD62E, CD62L, CD62P, CD63, CD68, CD69, CD71, CD72, CD79, CD81, CD82, CD83, CD86, CD87, CD88, CD89, CD90, CD91, CD95, CD96, CD100, CD103, CD105, CD106, CD109, CD117, CD120, CD127, CD133, CD134, CD135, CD138, CD141, CD142, CD143, CD144, CD147, CD151, CD152, CD154, CD156, CD158, CD163, CD166, .CD168, CD184, CDwl86, CD195, CD202 (a, b), CD209, CD235a, CD271, CD303, CD304), annexin Al, nucleolin, endoglin (CD105), ROB04, amino-peptidase N, -like-4 (DLL4), VEGFR-2 (CD309), CXCR4 (CD184), Tie2, B7-H3, WT1, MUC1, LMP2, HPV E6 E7, EGFRvIII, HER-2/neu, idiotype, MAGE A3, p53 nonmutant, NY-ESO-1, GD2, CEA, MelanA/MARTl, Ras mutant, gp100, p53 mutant, proteinase3 (PR1), bcr-abl, tyrosinase, survivin, hTERT, sarcoma translocation breakpoints, EphA2, PAP, ML-IAP, AFP, EpCAM, ERG (TMPRSS2 ETS fusion gene), NA17, PAX3, ALK, androgen receptor, cyclin B l, polysialic acid, MYCN, RhoC, TRP-2, GD3, fucosyl GMl , mesothelin, PSCA, MAGE Al, sLe(a), CYPIB I, PLACl, GM3, BORIS, Tn, GloboH, ETV6-AML, NY-BR-1, RGS5, SART3, STn, carbonic anhydrase IX, PAX5, OY-TES1, sperm protein 17, LCK, HMWMAA, AKAP-4, SSX2, XAGE 1, B7H3, legumain, Tie 2, Page4, VEGFR2, MAD- CT-1, FAP, PDGFR-β, MAD-CT-2, Notch1, ICAM1 and Fos-related antigen 1.

Additionally, preferred binding domains of the invention include those specific to antigens and epitope targets associated with infected cells that are known in the art. Such targets include but are not limited those derived from the following infectious agents are of interest: HIV virus (particularly antigens derived from the HIV envelope spike and/or gp120 and gp41

epitopes), Human papilloma virus (HPV), *Mycobacterium tuberculosis*, *Streptococcus agalactiae*, methicillin-resistant *Staphylococcus aureus*, *Legionella pneumophilia*, *Streptococcus pyogenes*, *Escherichia coli*, *Neisseria gonorrhoeae*, *Neisseria meningitidis*, *Pneumococcus*, *Cryptococcus neoformans*, *Histoplasma capsulatum*, - *influenzae* B, *Treponema pallidum*, Lyme disease spirochetes, *Pseudomonas aeruginosa*, *Mycobacterium leprae*, *Brucella abortus*, rabies virus, *influenza virus*, *cytomegalovirus*, herpes simplex virus I, herpes simplex virus II, human serum parvo-like virus, respiratory syncytial virus, varicella-zoster virus, hepatitis B virus, hepatitis C virus, measles virus, adenovirus, human T-cell leukemia viruses, Epstein-Barr virus, murine leukemia virus, mumps virus, vesicular stomatitis virus, sindbis virus, lymphocytic choriomeningitis virus, wart virus, blue tongue virus, Sendai virus, feline leukemia virus, reovirus, polio virus, simian virus 40, mouse mammary tumor virus, dengue virus, rubella virus, West Nile virus, *Plasmodium falciparum*, *Plasmodium vivax*, *Toxoplasma gondii*, *Trypanosoma rangeli*, *Trypanosoma cruzi*, *Trypanosoma rhodesiensei*, *Trypanosoma brucei*, *Schistosoma mansoni*, *Schistosoma japonicum*, *Babesia bovis*, *Elmeria tenella*, *Onchocerca volvulus*, *Leishmania tropica*, *Trichinella spiralis*, *Theileria parva*, *Taenia hydatigena*, *Taenia ovis*, *Taenia saginata*, *Echinococcus granulosus*, *Mesocestoides corti*, *Mycoplasma arthritidis*, *M. hyorhinis*, *M. orale*, *M arginini*, *Acholeplasma laidlawii*, *M. salivarium* and *M. pneumoniae*.

T-Cell Receptors (TCRs)

T-cells are a subgroup of cells which together with other immune cell types (polymorphonuclear cells, eosinophils, basophils, mast cells, B-cells, NK cells), constitute the cellular component of the immune system. Under physiological conditions, T-cells function in immune surveillance and in the elimination of foreign antigen. However, under pathological conditions, there is compelling evidence that T-cells play a major role in the causation and propagation of disease. In these disorders, breakdown of T-cell immunological tolerance, either central or peripheral is a fundamental process in the causation of autoimmune disease.

The TCR complex is composed of at least seven transmembrane proteins. The disulfide-linked (αβ or γδ) heterodimer forms the monotypic antigen recognition unit, while the invariant chains of CD3, consisting of ε, γ, δ, ζ, and η chains, are responsible for coupling the ligand binding to signaling pathways that result in T-cell activation and the elaboration of the cellular immune responses. Despite the gene diversity of the TCR chains, two structural features are

common to all known subunits. First, they are transmembrane proteins with a single transmembrane spanning domain--presumably alpha-helical. Second, all TCR chains have the unusual feature of possessing a charged amino acid within the predicted transmembrane domain. The invariant chains have a single negative charge, conserved between the mouse and human, and the variant chains possess one (TCR-β) or two (TCR-α) positive charges. The transmembrane sequence of TCR-α is highly conserved in a number of species and thus phylogenetically may serve an important functional role. The octapeptide sequence containing the hydrophilic amino acids arginine and lysine is identical between the species.

A T-cell response is modulated by antigen binding to a TCR. One type of TCR is a membrane bound heterodimer consisting of an α and β chain resembling an immunoglobulin variable (V) and constant (C) region. The TCR α chain includes a covalently linked V-α and C-α chain, whereas the β chain includes a V-β chain covalently linked to a C-β chain. The V-α and V-β chains form a pocket or cleft that can bind a superantigen or antigen in the context of a major histocompatibility complex (MHC) (known in humans as an HLA complex). See, Davis *Ann. Rev. of Immunology* 3: 537 (1985); *Fundamental Immunology* 3rd Ed., W. Paul Ed. Rsen Press LTD. New York (1993).

The extracellular domains of the TCR chains (αβ or γδ) can also engineered as fusions to heterologous transmembrane domains for expression on the cell surface. Such TCRs may include fusions to CD3, CD28, CD8, 4-1BB and/or chimeric activation receptor (CAR) transmembrane or activation domains. TCRs can also be the soluble proteins comprising one or more of the antigen binding domains of αβ or γδ chains. Such TCRs may include the TCR variable domains or function fragments thereof with or without the TCR constant domains. Soluble TCRs may be heterodimeric or single-chain molecules.

<u>Fc Domain</u>

Protein complexes of the invention may contain an Fc domain. For example, PD-L1 TxM comprises an anti-PD-L1 scAb/huIL-15N72D:anti-PD-L1 scAb/huIL-15RαSu/huIgG1 Fc fusion complex. Fusion proteins that combine the Fc regions of IgG with the domains of another protein, such as various cytokines and soluble receptors have been reported (see, for example, Capon et al., Nature, 337:525-531, 1989; Chamow et al., Trends Biotechnol., 14:52-60, 1996); U.S. Pat. Nos. 5,116,964 and 5,541,087). The prototype fusion protein is a homodimeric protein

linked through cysteine residues in the hinge region of IgG Fc, resulting in a molecule similar to an IgG molecule without the heavy chain variable and C_H1 domains and light chains. The dimeric nature of fusion proteins comprising the Fc domain may be advantageous in providing higher order interactions (i.e. bivalent or bispecific binding) with other molecules. Due to the structural homology, Fc fusion proteins exhibit an *in vivo* pharmacokinetic profile comparable to that of human IgG with a similar isotype. Immunoglobulins of the IgG class are among the most abundant proteins in human blood, and their circulation half-lives can reach as long as 21 days. To extend the circulating half-life of IL-15 or an IL-15 fusion protein and/or to increase its biological activity, fusion protein complexes containing the IL-15 domain non-covalently bound to IL-15Rα covalently linked to the Fc portion of the human heavy chain IgG protein are described herein.

The term "Fc" refers to the fragment crystallizable region which is the constant region of an antibody that interacts with cell surface receptors called Fc receptors and some proteins of the complement system. Such an "Fc" is in dimeric form. The original immunoglobulin source of the native Fc is preferably of human origin and may be any of the immunoglobulins, although IgG1 and IgG2 are preferred. Native Fc's are made up of monomeric polypeptides that may be linked into dimeric or multimeric forms by covalent (i.e., disulfide bonds) and non-covalent association. The number of intermolecular disulfide bonds between monomeric subunits of native Fc molecules ranges from 1 to 4 depending on class (e.g., IgG, IgA, IgE) or subclass (e.g., IgG1, IgG2, IgG3, IgA1, IgGA2). One example of a native Fc is a disulfide-bonded dimer resulting from papain digestion of an IgG (see Ellison et al. (1982), Nucleic Acids Res. 10: 4071-9). The term "native Fc" as used herein is generic to the monomeric, dimeric, and multimeric forms. Fc domains containing binding sites for Protein A, Protein G, various Fc receptors and complement proteins. In some embodiments, Fc domain of the complex is capable of interacting with Fc receptors to mediate antibody-dependent cell-mediated cytotoxicity (ADCC) and/or antibody dependent cellular phagocytosis (ADCP). In other applications, the complex comprises an Fc domain (e.g., IgG4 Fc) that is incapable of effectively mediating ADCC or ADCP.

In some embodiments, the term "Fc variant" refers to a molecule or sequence that is modified from a native Fc, but still comprises a binding site for the salvage receptor, FcRn. International applications WO 97/34631 and WO 96/32478 describe exemplary Fc variants, as well as interaction with the salvage receptor, and are hereby incorporated by reference. Thus, the

term “Fc variant” comprises a molecule or sequence that is humanized from a non-human native Fc. Furthermore, a native Fc comprises sites that may be removed because they provide structural features or biological activity that are not required for the fusion molecules of the present invention. Thus, in certain embodiments, the term “Fc variant” comprises a molecule or sequence that alters one or more native Fc sites or residues that affect or are involved in (1) disulfide bond formation, (2) incompatibility with a selected host cell (3) N-terminal heterogeneity upon expression in a selected host cell, (4) glycosylation, (5) interaction with complement, (6) binding to an Fc receptor other than a salvage receptor, (7) antibody-dependent cellular cytotoxicity (ADCC) or (8) antibody-dependent cellular phagocytosis (ADCP). Such alterations can increase or decrease any one or more of these Fc properties. Fc variants are described in further detail hereinafter.

The term “Fc domain” encompasses native Fc and Fc variant molecules and sequences as defined above. As with Fc variants and native Fc's, the term “Fc domain” includes molecules in monomeric or multimeric form, whether digested from whole antibody or produced by recombinant gene expression or by other means.

Fusions Protein Complexes

The invention provides for fusion protein complexes (FIG. 1 and FIG. 2). In some cases, the first protein comprises a first biologically active polypeptide covalently linked to interleukin-15 (IL-15) or functional fragment thereof; and the second protein comprises a second biologically active polypeptide covalently linked to soluble interleukin-15 receptor alpha (IL-15Rα) polypeptide or functional fragment thereof, where the IL-15 domain of a first protein binds to the soluble IL-15Rα domain of the second protein to form a soluble fusion protein complex. Fusion protein complexes of the invention also comprise immunoglobulin Fc domain or a functional fragment thereof linked to one or both of the first and second proteins. Preferably, the Fc domains linked to the fusion proteins interact to form a fusion protein complex. Such a complex may be stabilized by disulfide bond formation between the immunoglobulin Fc domains. In one aspect, the soluble fusion protein complexes of the invention include an IL-15 polypeptide, IL-15 variant or a functional fragment thereof and a soluble IL-15Rα polypeptide or a functional fragment thereof, wherein one or both of the IL-15

and IL-15Rα polypeptides further include an immunoglobulin Fc domain or a functional fragment thereof.

In certain examples, one or both of the first and second proteins comprises an antibody or functional fragment thereof. For example, one of the binding domain comprises a soluble anti-PD-L1 single chain antibody or functional fragment thereof. In another example, the other or second binding domain comprises an anti-CTLA4 single chain antibody or a disease antigen-specific antibody or functional fragment thereof. In one embodiment, the invention provides PD-L1 TxM, comprising a soluble anti-PD-L1 scAb/huIL-15N72D:anti-PD-L1 scAb/huIL-15RαSu/huIgG1 Fc fusion protein complex. In this complex, the huIL-15N72D and huIL-15RαSu domains interact and the huIgG1 Fc domains on two anti-PD-L1 scAb/huIL-15RαSu/huIgG1 Fc fusion protein to form a multichain fusion protein complex.

As used herein, the term “biologically active polypeptide” or “effector molecule” is meant an amino acid sequence such as a protein, polypeptide, or peptide; a sugar or polysaccharide; a lipid or a glycolipid, glycoprotein, or lipoprotein that can produce the desired effects as discussed herein. Effector molecules also include chemical agents. Also contemplated are effector molecule nucleic acids encoding a biologically active or effector protein, polypeptide, or peptide. Thus, suitable molecules include regulatory factors, enzymes, antibodies, or drugs as well as DNA, RNA, and oligonucleotides. The biologically active polypeptides or effector molecule can be naturally-occurring or it can be synthesized from known components, e.g., by recombinant or chemical synthesis and can include heterologous components. A biologically active polypeptide or effector molecule is generally between about 0.1 to 100 KD or greater up to about 1000 KD, preferably between about 0.1, 0.2, 0.5, 1, 2, 5, 10, 20, 30 and 50 KD as judged by standard molecule sizing techniques such as centrifugation or SDS-polyacrylamide gel electrophoresis. Desired effects of the invention include, but are not limited to, for example, forming a fusion protein complex of the invention with increased binding activity, killing a target cell, e.g. either to induce cell proliferation or cell death, initiate an immune response, in preventing or treating a disease, or to act as a detection molecule for diagnostic purposes. For such detection, an assay could be used, for example an assay that includes sequential steps of culturing cells to proliferate same, and contacting the cells with a fusion complex of the invention and then evaluating whether the fusion complex inhibits further development of the cells.

Covalently linking the effector molecule to the fusion protein complexes of the invention in accordance with the invention provides a number of significant advantages. Fusion protein complexes of the invention can be produced that contain a single effector molecule, including a peptide of known structure. Additionally, a wide variety of effector molecules can be produced in similar DNA vectors. That is, a library of different effector molecules can be linked to the fusion protein complexes for recognition of infected or diseased cells. Further, for therapeutic applications, rather than administration of a fusion protein complex of the invention to a subject, a DNA expression vector coding for the fusion protein complex can be administered for *in vivo* expression of the fusion protein complex. Such an approach avoids costly purification steps typically associated with preparation of recombinant proteins and avoids the complexities of antigen uptake and processing associated with conventional approaches.

As noted, components of the fusion proteins disclosed herein, e.g., effector molecule such as cytokines, chemokines, growth factors, protein toxins, immunoglobulin domains or other bioactive molecules and any peptide linkers, can be organized in nearly any fashion provided that the fusion protein has the function for which it was intended. In particular, each component of the fusion protein can be spaced from another component by at least one suitable peptide linker sequence if desired. Additionally, the fusion proteins may include tags, e.g., to facilitate modification, identification and/or purification of the fusion protein. More specific fusion proteins are in the Examples described below.

Linkers

The fusion complexes of the invention preferably also include a flexible linker sequence interposed between the IL-15 or IL-15Rα domains and the biologically active polypeptide. The linker sequence should allow effective positioning of the biologically active polypeptide with respect to the IL-15 or IL-15Rα domains to allow functional activity of both domains.

In certain cases, the soluble fusion protein complex has a linker wherein the first biologically active polypeptide is covalently linked to IL-15 (or functional fragment thereof) by polypeptide linker sequence. In other aspects, the soluble fusion protein complex as described herein has a linker wherein the second biologically active polypeptide is covalently linked to IL-15Rα polypeptide (or functional fragment thereof) by polypeptide linker sequence.

The linker sequence is preferably encoded by a nucleotide sequence resulting in a peptide that can effectively position the binding groove of a TCR molecule for recognition of a presenting antigen or the binding domain of an antibody molecule for recognition of an antigen. As used herein, the phrase "effective positioning of the biologically active polypeptide with respect to the IL-15 or IL-15Rα domains", or other similar phrase, is intended to mean the biologically active polypeptide linked to the IL-15 or IL-15Rα domains is positioned so that the IL-15 or IL-15Rα domains are capable of interacting with each other to form a protein complex. For example, the IL-15 or IL-15Rα domains are effectively positioned to allow interactions with immune cells to initiate or inhibit an immune reaction, or to inhibit or stimulate cell development.

The fusion complexes of the invention preferably also include a flexible linker sequence interposed between the IL-15 or IL-15Rα domains and the immunoglobulin Fc domain. The linker sequence should allow effective positioning of the Fc domain, biologically active polypeptide and IL-15 or IL-15Rα domains to allow functional activity of each domain. For example, the Fc domains are effectively positioned to allow proper fusion protein complex formation and/or interactions with Fc receptors on immune cells or proteins of the complement system to stimulate Fc-mediated effects including opsonization, cell lysis, degranulation of mast cells, basophils, and eosinophils, and other Fc receptor-dependent processes; activation of the complement pathway; and enhanced in vivo half-life of the fusion protein complex.

Linker sequences can also be used to link two or more polypeptides of the biologically active polypeptide to generate a single-chain molecule with the desired functional activity.

Preferably, the linker sequence comprises from about 7 to 20 amino acids, more preferably from about 10 to 20 amino acids. The linker sequence is preferably flexible so as not hold the biologically active polypeptide or effector molecule in a single undesired conformation. The linker sequence can be used, e.g., to space the recognition site from the fused molecule. Specifically, the peptide linker sequence can be positioned between the biologically active polypeptide and the effector molecule, e.g., to chemically cross-link same and to provide molecular flexibility. The linker preferably predominantly comprises amino acids with small side chains, such as glycine, alanine, and serine, to provide for flexibility. Preferably, about 80 or 90 percent or greater of the linker sequence comprises glycine, alanine, or serine residues, particularly glycine and serine residues.

Different linker sequences could be used including any of a number of flexible linker designs that have been used successfully to join antibody variable regions together (see, Whitlow, M. et al., (1991) Methods: A Companion to Methods in Enzymology, 2:97-105).

Pharmaceutical Therapeutics

The invention provides pharmaceutical compositions comprising fusion protein complexes for use as a therapeutic. In one aspect, fusion protein complex of the invention is administered systemically, for example, formulated in a pharmaceutically-acceptable buffer such as physiological saline. Preferable routes of administration include, for example, instillation into the bladder, subcutaneous, intravenous, intraperitoneal, intramuscular, intratumoral or intradermal injections that provide continuous, sustained, or effective levels of the composition in the patient. Treatment of human patients or other animals is carried out using a therapeutically effective amount of a therapeutic identified herein in a physiologically-acceptable carrier. Suitable carriers and their formulation are described, for example, in Remington's Pharmaceutical Sciences by E. W. Martin. The amount of the therapeutic agent to be administered varies depending upon the manner of administration, the age and body weight of the patient, and with the clinical symptoms of the neoplasia. Generally, amounts will be in the range of those used for other agents used in the treatment of other diseases associated with neoplasia, autoimmune or infectious diseases, although in certain instances lower amounts will be needed because of the increased specificity of the compound. A compound is administered at a dosage that enhances an immune response of a subject, or that reduces the proliferation, survival, or invasiveness of a neoplastic, infected, or autoimmune cell as determined by a method known to one skilled in the art.

Formulation of Pharmaceutical Compositions

The administration of the fusion protein complex of the invention for the treatment of a neoplasia, infectious or autoimmune disease is by any suitable means that results in a concentration of the therapeutic that, combined with other components, is effective in ameliorating, reducing, or stabilizing said neoplasia, infectious or autoimmune disease. The fusion protein complex of the invention may be contained in any appropriate amount in any suitable carrier substance, and is generally present in an amount of 1-95% by weight of the total weight of the composition. The composition may be provided in a dosage form that is suitable

for parenteral (e.g., subcutaneous, intravenous, intramuscular, intravesicular, intratumoral or intraperitoneal) administration route. For example, the pharmaceutical compositions are formulated according to conventional pharmaceutical practice (see, e.g., Remington: The Science and Practice of Pharmacy (20th ed.), ed. A. R. Gennaro, Lippincott Williams & Wilkins, 2000 and Encyclopedia of Pharmaceutical Technology, eds. J. Swarbrick and J. C. Boylan, 1988-1999, Marcel Dekker, New York).

Human dosage amounts are initially determined by extrapolating from the amount of compound used in mice or non-human primates, as a skilled artisan recognizes it is routine in the art to modify the dosage for humans compared to animal models. For example, the dosage may vary from between about 1 μg compound/kg body weight to about 5000 mg compound/kg body weight; or from about 5 mg/kg body weight to about 4,000 mg/kg body weight or from about 10 mg/kg body weight to about 3,000 mg/kg body weight; or from about 50 mg/kg body weight to about 2000 mg/kg body weight; or from about 100 mg/kg body weight to about 1000 mg/kg body weight; or from about 150 mg/kg body weight to about 500 mg/kg body weight. For example, the dose is about 1, 5, 10, 25, 50, 75, 100, 150, 200, 250, 300, 350, 400, 450, 500, 550, 600, 650, 700, 750, 800, 850, 900, 950, 1,000, 1,050, 1,100, 1,150, 1,200, 1,250, 1,300, 1,350, 1,400, 1,450, 1,500, 1,600, 1,700, 1,800, 1,900, 2,000, 2,500, 3,000, 3,500, 4,000, 4,500, or 5,000 mg/kg body weight. Alternatively, doses are in the range of about 5 mg compound/Kg body weight to about 20 mg compound/kg body weight. In another example, the doses are about 8, 10, 12, 14, 16 or 18 mg/kg body weight. Preferably, the fusion protein complex is administered at 0.5 mg/kg-about 10 mg/kg (e.g., 0.5, 1, 3, 5, 10 mg/kg). Of course, this dosage amount may be adjusted upward or downward, as is routinely done in such treatment protocols, depending on the results of the initial clinical trials and the needs of a particular patient.

Pharmaceutical compositions are formulated with appropriate excipients into a pharmaceutical composition that, upon administration, releases the therapeutic in a controlled manner. Examples include single or multiple unit tablet or capsule compositions, oil solutions, suspensions, emulsions, microcapsules, microspheres, molecular complexes, nanoparticles, patches, and liposomes. Preferably, the fusion protein complex is formulated in an excipient suitable for parenteral administration.

<u>Parenteral Compositions</u>

The pharmaceutical composition comprising a fusion protein complex of the invention are administered parenterally by injection, infusion, or implantation (subcutaneous, intravenous, intramuscular, intratumoral, intravesicular, intraperitoneal) in dosage forms, formulations, or via suitable delivery devices or implants containing conventional, non-toxic pharmaceutically acceptable carriers and adjuvants. The formulation and preparation of such compositions are well known to those skilled in the art of pharmaceutical formulation. Formulations can be found in Remington: The Science and Practice of Pharmacy, supra.

Compositions comprising a fusion protein complex of the invention for parenteral use are provided in unit dosage forms (e.g., in single-dose ampoules). Alternatively, the composition is provided in vials containing several doses and in which a suitable preservative may be added (see below). The composition is in the form of a solution, a suspension, an emulsion, an infusion device, or a delivery device for implantation, or it is presented as a dry powder to be reconstituted with water or another suitable vehicle before use. Apart from the active agent that reduces or ameliorates a neoplasia, infectious or autoimmune disease, the composition includes suitable parenterally acceptable carriers and/or excipients. The active therapeutic agent(s) may be incorporated into microspheres, microcapsules, nanoparticles, liposomes for controlled release. Furthermore, the composition may include suspending, solubilizing, stabilizing, pH-adjusting agents, tonicity adjusting agents, and/or dispersing, agents.

As indicated above, the pharmaceutical compositions comprising a fusion protein complex of the invention may be in a form suitable for sterile injection. To prepare such a composition, the suitable active therapeutic(s) are dissolved or suspended in a parenterally acceptable liquid vehicle. Among acceptable vehicles and solvents that may be employed are water, water adjusted to a suitable pH by addition of an appropriate amount of hydrochloric acid, sodium hydroxide or a suitable buffer, 1,3-butanediol, Ringer's solution, and isotonic sodium chloride solution and dextrose solution. The aqueous formulation may also contain one or more preservatives (e.g., methyl, ethyl, or n-propyl p-hydroxybenzoate). In cases where one of the compounds is only sparingly or slightly soluble in water, a dissolution enhancing or solubilizing agent can be added, or the solvent may include 10-60% w/w of propylene glycol.

The present invention provides methods of treating neoplasia, infectious or autoimmune diseases or symptoms thereof which comprise administering a therapeutically effective amount of a pharmaceutical composition comprising a compound of the formulae herein to a subject

(e.g., a mammal such as a human). Thus, one embodiment is a method of treating a subject suffering from or susceptible to a neoplasia, infectious or autoimmune disease or symptom thereof. The method includes the step of administering to the mammal a therapeutic amount of an amount of a compound herein sufficient to treat the disease or disorder or symptom thereof, under conditions such that the disease or disorder is treated.

The methods herein include administering to the subject (including a subject identified as in need of such treatment) an effective amount of a compound described herein, or a composition described herein to produce such effect. Identifying a subject in need of such treatment can be in the judgment of a subject or a health care professional and can be subjective (e.g. opinion) or objective (e.g. measurable by a test or diagnostic method).

The therapeutic methods of the invention (which include prophylactic treatment) in general comprise administration of a therapeutically effective amount of the compounds herein, such as a compound of the formulae herein to a subject (e.g., animal, human) in need thereof, including a mammal, particularly a human. Such treatment will be suitably administered to subjects, particularly humans, suffering from, having, susceptible to, or at risk for a neoplasia, infectious disease, autoimmune disease, disorder, or symptom thereof. Determination of those subjects "at risk" can be made by any objective or subjective determination by a diagnostic test or opinion of a subject or health care provider (e.g., genetic test, enzyme or protein marker, Marker (as defined herein), family history, and the like). The fusion protein complexes of the invention may be used in the treatment of any other disorders in which an increase in an immune response is desired.

The invention also provides a method of monitoring treatment progress. The method includes the step of determining a level of diagnostic marker (Marker) (e.g., any target delineated herein modulated by a compound herein, a protein or indicator thereof, etc.) or diagnostic measurement (e.g., screen, assay) in a subject suffering from or susceptible to a disorder or symptoms thereof associated with neoplasia in which the subject has been administered a therapeutic amount of a compound herein sufficient to treat the disease or symptoms thereof. The level of Marker determined in the method can be compared to known levels of Marker in either healthy normal controls or in other afflicted patients to establish the subject's disease status. In some cases, a second level of Marker in the subject is determined at a time point later than the determination of the first level, and the two levels are compared to monitor the course of

disease or the efficacy of the therapy. In certain aspects, a pre-treatment level of Marker in the subject is determined prior to beginning treatment according to this invention; this pre-treatment level of Marker can then be compared to the level of Marker in the subject after the treatment commences, to determine the efficacy of the treatment.

Combination Therapies

Optionally, the fusion protein complex of the invention is administered in combination with any other standard therapy; such methods are known to the skilled artisan and described in Remington's Pharmaceutical Sciences by E. W. Martin. If desired, fusion protein complexes of the invention is administered in combination with any conventional anti-neoplastic therapy, including but not limited to, immunotherapy, therapeutic antibodies, targeted therapy, surgery, radiation therapy, or chemotherapy.

Kits or Pharmaceutical Systems

Pharmaceutical compositions comprising the fusion protein complex of the invention may be assembled into kits or pharmaceutical systems for use in ameliorating a neoplasia, infectious or autoimmune disease. Kits or pharmaceutical systems according to this aspect of the invention comprise a carrier means, such as a box, carton, tube, having in close confinement therein one or more container means, such as vials, tubes, ampoules, bottles, and the like. The kits or pharmaceutical systems of the invention may also comprise associated instructions for using the fusion protein complex of the invention.

Recombinant Protein Expression

In general, preparation of the fusion protein complexes of the invention (e.g., components of a TxM complex) can be accomplished by procedures disclosed herein and by recognized recombinant DNA techniques.

In general, recombinant polypeptides are produced by transformation of a suitable host cell with all or part of a polypeptide-encoding nucleic acid molecule or fragment thereof in a suitable expression vehicle. Those skilled in the field of molecular biology will understand that any of a wide variety of expression systems may be used to provide the recombinant protein. The precise host cell used is not critical to the invention. A recombinant polypeptide may be produced in virtually any eukaryotic host (e.g., *Saccharomyces cerevisiae*, insect cells, e.g., Sf21

cells, or mammalian cells, e.g., NIH 3T3, HeLa, or preferably COS cells). Such cells are available from a wide range of sources (e.g., the American Type Culture Collection, Rockland, Md.; also, see, e.g., Ausubel et al., Current Protocol in Molecular Biology, New York: John Wiley and Sons, 1997). The method of transfection and the choice of expression vehicle will depend on the host system selected. Transformation methods are described, e.g., in Ausubel et al. (supra); expression vehicles may be chosen from those provided, e.g., in Cloning Vectors: A Laboratory Manual (P. H. Pouwels et al., 1985, Supp. 1987).

A variety of expression systems exist for the production of recombinant polypeptides. Expression vectors useful for producing such polypeptides include, without limitation, chromosomal, episomal, and virus-derived vectors, e.g., vectors derived from bacterial plasmids, from bacteriophage, from transposons, from yeast episomes, from insertion elements, from yeast chromosomal elements, from viruses such as baculoviruses, papova viruses, such as SV40, vaccinia viruses, adenoviruses, fowl pox viruses, pseudorabies viruses and retroviruses, and vectors derived from combinations thereof.

Once the recombinant polypeptide is expressed, it is isolated, e.g., using affinity chromatography. In one example, an antibody (e.g., produced as described herein) raised against the polypeptide may be attached to a column and used to isolate the recombinant polypeptide. Lysis and fractionation of polypeptide-harboring cells prior to affinity chromatography may be performed by standard methods (see, e.g., Ausubel et al., supra). Once isolated, the recombinant protein can, if desired, be further purified, e.g., by high performance liquid chromatography (see, e.g., Fisher, Laboratory Techniques in Biochemistry and Molecular Biology, eds., Work and Burdon, Elsevier, 1980).

As used herein, biologically active polypeptides or effector molecules of the invention may include factors such as cytokines, chemokines, growth factors, protein toxins, immunoglobulin domains or other bioactive proteins such as enzymes. Also, biologically active polypeptides may include conjugates to other compounds such as non-protein toxins, cytotoxic agents, chemotherapeutic agents, detectable labels, radioactive materials, and such.

Cytokines of the invention are defined by any factor produced by cells that affect other cells and are responsible for any of a number of multiple effects of cellular immunity. Examples of cytokines include but are not limited to the IL-2 family, interferon (IFN), IL-10, IL-1, IL-17,

TGF and TNF cytokine families, and to IL-1 through IL-35, IFN-α, IFN-β, IFNγ, TGF-β, TNF-α, and TNFβ.

In an aspect of the invention, the first protein comprises a first biologically active polypeptide covalently linked to interleukin-15 (IL-15) domain or a functional fragment thereof. IL-15 is a cytokine that affects T-cell activation and proliferation. IL-15 activity in affecting immune cell activation and proliferation is similar in some respects to IL-2, although fundamental differences have been well characterized (Waldmann, T A, 2006, *Nature Rev. Immunol.* 6:595-601).

In another aspect of the invention, the first protein comprises an interleukin-15 (IL-15) domain that is an IL-15 variant (also referred to herein as IL-15 mutant). The IL-15 variant preferably comprises a different amino acid sequence that the native (or wild type) IL-15 protein. The IL-15 variant preferably binds the IL-15Rα polypeptide and functions as an IL-15 agonist or antagonist. Preferably, IL-15 variants with agonist activity have super agonist activity. The IL-15 variant can function as an IL-15 agonist or antagonist independent of its association with IL-15Rα. IL-15 agonists are exemplified by comparable or increased biological activity compared to wild type IL-15. IL-15 antagonists are exemplified by decreased biological activity compared to wild type IL-15 or by the ability to inhibit IL-15-mediated responses. In some examples, the IL-15 variant binds with increased or decreased activity to the IL-15RβγC receptors. In some cases, the sequence of the IL-15 variant has at least one amino acid change, e.g. substitution or deletion, compared to the native IL-2 sequence, such changes resulting in IL-15 agonist or antagonist activity. Preferably, the amino acid substitutions/deletions are in the domains of IL-15 that interact with IL-15Rβ and/or γC. More preferably, the amino acid substitutions/deletions do not affect binding to the IL-15Rα polypeptide or the ability to produce the IL-15 variant. Suitable amino acid substitutions/deletions to generate IL-15 variants can be identified based on putative or known IL-15 structures, comparisons of IL-15 with homologous molecules such as IL-2 with known structure, through rational or random mutagenesis and functional assays, as provided herein, or other empirical methods. Additionally, suitable amino acid substitutions can be conservative or non-conservative changes and insertions of additional amino acids. Preferably, IL-15 variants of the invention contain one or more than one amino acid substitutions/deletions at position 6, 8, 10, 61, 65, 72, 92, 101, 104, 105, 108, 109, 111, or 112 of the mature human IL-15 sequence; particularly, D8N ("D8" refers to the amino acid and residue

position in the native mature human IL-15 sequence and "N" refers to the substituted amino acid residue at that position in the IL-15 variant), I6S, D8A, D61A, N65A, N72R, V104P or Q108A substitutions result in IL-15 variants with antagonist activity and N72D substitutions result in IL-15 variants with agonist activity.

Chemokines, similar to cytokines, are defined as any chemical factor or molecule which when exposed to other cells are responsible for any of a number of multiple effects of cellular immunity. Suitable chemokines may include but are not limited to the CXC, CC, C, and CX_3C chemokine families and to CCL-1 through CCL-28, CXC-1 through CXC-17, XCL-1, XCL-2, CX3CL1, MIP-1b, IL-8, MCP-1, and Rantes.

Growth factors include any molecules which when exposed to a particular cell induce proliferation and/or differentiation of the affected cell. Growth factors include proteins and chemical molecules, some of which include: GM-CSF, G-CSF, human growth factor and stem cell growth factor. Additional growth factors may also be suitable for uses described herein.

Toxins or cytotoxic agents include any substance that has a lethal effect or an inhibitory effect on growth when exposed to cells. More specifically, the effector molecule can be a cell toxin of, e.g., plant or bacterial origin such as, e.g., diphtheria toxin (DT), shiga toxin, abrin, cholera toxin, ricin, saporin, pseudomonas exotoxin (PE), pokeweed antiviral protein, or gelonin. Biologically active fragments of such toxins are well known in the art and include, e.g., DT A chain and ricin A chain. Additionally, the toxin can be an agent active at the cell surface such as, e.g., phospholipase enzymes (e.g., phospholipase C).

Further, the effector molecule can be a chemotherapeutic drug such as, e.g., vindesine, vincristine, vinblastin, methotrexate, adriamycin, bleomycin, or cisplatin.

Additionally, the effector molecule can be a detectably-labeled molecule suitable for diagnostic or imaging studies. Such labels include biotin or streptavidin/avidin, a detectable nanoparticles or crystal, an enzyme or catalytically active fragment thereof, a fluorescent label such as green fluorescent protein, FITC, phycoerythrin, cychome, texas red or quantum dots; a radionuclide e.g., iodine-131, yttrium-90, rhenium-188 or bismuth-212; phosphorescent or chemiluminescent molecules or a label detectable by PET, ultrasound, or MRI such as Gd-- or paramagnetic metal ion-based contrast agents. See e.g., Moskaug, et al. *J. Biol. Chem.* 264, 15709 (1989); Pastan, I. et al. *Cell* 47, 641, 1986; Pastan et al., Recombinant Toxins as Novel Therapeutic Agents, *Ann. Rev. Biochem.* 61, 331, (1992); "Chimeric Toxins" *Olsnes and Phil,*

Pharmac. Ther., 25, 355 (1982); published PCT application no. WO 94/29350; published PCT application no. WO 94/04689; published PCT application no. WO2005046449 and U.S. Pat. No. 5,620,939 for disclosure relating to making and using proteins comprising effectors or tags.

A protein fusion or conjugate complex that includes a covalently linked IL-15 and IL-15Rα domains has several important uses. For example, the protein fusion or conjugate complex comprising an anti-PD-L1 scAb can be employed to deliver the IL-15:IL-15Rα complex to certain cells, e.g., tumor cells that express PD-L1. Accordingly, the protein fusion or conjugate complex provides means of selectively damaging or killing cells comprising the ligand. Examples of cells or tissue capable of being damaged or killed by the protein fusion or conjugate complexes include tumors and virally or bacterially infected cells expressing one or more ligands. Cells or tissue susceptible to being damaged or killed can be readily assayed by the methods disclosed herein.

The IL-15 and IL-15Rα polypeptides of the invention suitably correspond in amino acid sequence to naturally occurring IL-15 and IL-15Rα molecules, e.g. IL-15 and IL-15Rα molecules of a human, mouse or other rodent, or other mammals. Sequences of these polypeptides and encoding nucleic acids are known in the literature, including human interleukin 15 (IL15) mRNA - GenBank: U14407.1 (incorporated herein by reference), Mus musculus interleukin 15 (IL15) mRNA - GenBank: U14332.1 (incorporated herein by reference), human interleukin-15 receptor alpha chain precursor (IL15RA) mRNA - GenBank: U31628.1 (incorporated herein by reference), Mus musculus interleukin 15 receptor, alpha chain - GenBank: BC095982.1 (incorporated herein by reference).

In some settings, it can be useful to make the protein fusion or conjugate complexes of the present invention polyvalent, e.g., to increase the valency of the sc-antibody. In particular, interactions between the IL-15 and IL-15Rα domains of the fusion protein complex provide a means of generating polyvalent complexes. In addition, the polyvalent fusion protein can be made by covalently or non-covalently linking together between one and four proteins (the same or different) by using e.g., standard biotin-streptavidin labeling techniques, or by conjugation to suitable solid supports such as latex beads. Chemically cross-linked proteins (for example cross-linked to dendrimers) are also suitable polyvalent species. For example, the protein can be modified by including sequences encoding tag sequences that can be modified such as the biotinylation BirA tag or amino acid residues with chemically reactive side chains such as Cys or

His. Such amino acid tags or chemically reactive amino acids may be positioned in a variety of positions in the fusion protein, preferably distal to the active site of the biologically active polypeptide or effector molecule. For example, the C-terminus of a soluble fusion protein can be covalently linked to a tag or other fused protein which includes such a reactive amino acid(s). Suitable side chains can be included to chemically link two or more fusion proteins to a suitable dendrimer or other nanoparticle to give a multivalent molecule. Dendrimers are synthetic chemical polymers that can have any one of a number of different functional groups of their surface (D. Tomalia, Aldrichimica Acta, 26:91:101 (1993)). Exemplary dendrimers for use in accordance with the present invention include e.g. E9 starburst polyamine dendrimer and E9 combust polyamine dendrimer, which can link cystine residues. Exemplary nanoparticles include liposomes, core-shell particles, or PLGA-based particles.

In another aspect, one or both of the polypeptides of the fusion protein complex comprises an immunoglobulin domain. Alternatively, the protein binding domain-IL-15 fusion protein can be further linked to an immunoglobulin domain. The preferred immunoglobulin domains comprise regions that allow interaction with other immunoglobulin domains to form multichain proteins as provided above. For example, the immunoglobulin heavy chain regions, such as the IgG1 C_H2-C_H3, are capable of stably interacting to create the Fc region. Preferred immunoglobulin domains including Fc domains also comprise regions with effector functions, including Fc receptor or complement protein binding activity, and/or with glycosylation sites. In some aspects, the immunoglobulin domains of the fusion protein complex contain mutations that reduce or augment Fc receptor or complement binding activity or glycosylation or dimerization, thereby affecting the biological activity of the resulting protein. For example, immunoglobulin domains containing mutations that reduce binding to Fc receptors could be used to generate fusion protein complex of the invention with lower binding activity to Fc receptor-bearing cells, which may be advantageous for reagents designed to recognize or detect specific antigens.

Nucleic Acids and Vectors

The invention further provides nucleic acid sequences and particularly DNA sequences that encode the present fusion proteins (e.g., components of TxM). Preferably, the DNA sequence is carried by a vector suited for extrachromosomal replication such as a phage, virus, plasmid, phagemid, cosmid, YAC, or episome. In particular, a DNA vector that encodes a desired fusion protein can be used to facilitate preparative methods described herein and to

obtain significant quantities of the fusion protein. The DNA sequence can be inserted into an appropriate expression vector, i.e., a vector that contains the necessary elements for the transcription and translation of the inserted protein-coding sequence. A variety of host-vector systems may be utilized to express the protein-coding sequence. These include mammalian cell systems infected with virus (e.g., vaccinia virus, adenovirus, etc.); insect cell systems infected with virus (e.g., baculovirus); microorganisms such as yeast containing yeast vectors, or bacteria transformed with bacteriophage DNA, plasmid DNA or cosmid DNA. Depending on the host-vector system utilized, any one of a number of suitable transcription and translation elements may be used. See, Sambrook et al., supra and Ausubel et al. supra.

Included in the invention are methods for making a soluble fusion protein complex, the method comprising introducing into a host cell a DNA vector as described herein encoding the first and second proteins, culturing the host cell in media under conditions sufficient to express the fusion proteins in the cell or the media and allow association between IL-15 domain of a first protein and the soluble IL-15Rα domain of a second protein to form the soluble fusion protein complex, purifying the soluble fusion protein complex from the host cells or media.

In general, a preferred DNA vector according to the invention comprises a nucleotide sequence linked by phosphodiester bonds comprising, in a 5' to 3' direction a first cloning site for introduction of a first nucleotide sequence encoding a biologically active polypeptide, operatively linked to a sequence encoding an effector molecule.

The fusion protein components encoded by the DNA vector can be provided in a cassette format. By the term “cassette” is meant that each component can be readily substituted for another component by standard recombinant methods. In particular, a DNA vector configured in a cassette format is particularly desirable when the encoded fusion complex is to be used against pathogens that may have or have capacity to develop serotypes.

To make the vector coding for a fusion protein complex, the sequence coding for the biologically active polypeptide is linked to a sequence coding for the effector peptide by use of suitable ligases. DNA coding for the presenting peptide can be obtained by isolating DNA from natural sources such as from a suitable cell line or by known synthetic methods, e.g. the phosphate triester method. See, e.g., Oligonucleotide Synthesis, IRL Press (M. J. Gait, ed., 1984). Synthetic oligonucleotides also may be prepared using commercially available automated oligonucleotide synthesizers. Once isolated, the gene coding for the biologically active

polypeptide can be amplified by the polymerase chain reaction (PCR) or other means known in the art. Suitable PCR primers to amplify the biologically active polypeptide gene may add restriction sites to the PCR product. The PCR product preferably includes splice sites for the effector peptide and leader sequences necessary for proper expression and secretion of the biologically active polypeptide-effector fusion complex. The PCR product also preferably includes a sequence coding for the linker sequence, or a restriction enzyme site for ligation of such a sequence.

The fusion proteins described herein are preferably produced by standard recombinant DNA techniques. For example, once a DNA molecule encoding the biologically active polypeptide is isolated, sequence can be ligated to another DNA molecule encoding the effector polypeptide. The nucleotide sequence coding for a biologically active polypeptide may be directly joined to a DNA sequence coding for the effector peptide or, more typically, a DNA sequence coding for the linker sequence as discussed herein may be interposed between the sequence coding for the biologically active polypeptide and the sequence coding for the effector peptide and joined using suitable ligases. The resultant hybrid DNA molecule can be expressed in a suitable host cell to produce the fusion protein complex. The DNA molecules are ligated to each other in a 5' to 3' orientation such that, after ligation, the translational frame of the encoded polypeptides is not altered (i.e., the DNA molecules are ligated to each other in-frame). The resulting DNA molecules encode an in-frame fusion protein.

Other nucleotide sequences also can be included in the gene construct. For example, a promoter sequence, which controls expression of the sequence coding for the biologically active polypeptide fused to the effector peptide, or a leader sequence, which directs the fusion protein to the cell surface or the culture medium, can be included in the construct or present in the expression vector into which the construct is inserted. An immunoglobulin or CMV promoter is particularly preferred.

In obtaining variant biologically active polypeptide, IL-15, IL-15Rα or Fc domain coding sequences, those of ordinary skill in the art will recognize that the polypeptides may be modified by certain amino acid substitutions, additions, deletions, and post-translational modifications, without loss or reduction of biological activity. In particular, it is well-known that conservative amino acid substitutions, that is, substitution of one amino acid for another amino acid of similar size, charge, polarity, and conformation, are unlikely to significantly alter protein function. The

20 standard amino acids that are the constituents of proteins can be broadly categorized into four groups of conservative amino acids as follows: the nonpolar (hydrophobic) group includes alanine, isoleucine, leucine, methionine, phenylalanine, proline, tryptophan and valine; the polar (uncharged, neutral) group includes asparagine, cysteine, glutamine, glycine, serine, threonine and tyrosine; the positively charged (basic) group contains arginine, histidine and lysine; and the negatively charged (acidic) group contains aspartic acid and glutamic acid. Substitution in a protein of one amino acid for another within the same group is unlikely to have an adverse effect on the biological activity of the protein. In other instance, modifications to amino acid positions can be made to reduce or enhance the biological activity of the protein. Such changes can be introduced randomly or via site-specific mutations based on known or presumed structural or functional properties of targeted residue(s). Following expression of the variant protein, the changes in the biological activity due to the modification can be readily assessed using binding or functional assays.

Homology between nucleotide sequences can be determined by DNA hybridization analysis, wherein the stability of the double-stranded DNA hybrid is dependent on the extent of base pairing that occurs. Conditions of high temperature and/or low salt content reduce the stability of the hybrid, and can be varied to prevent annealing of sequences having less than a selected degree of homology. For instance, for sequences with about 55% G-C content, hybridization, and wash conditions of 40-50 C, 6 x SSC (sodium chloride/sodium citrate buffer) and 0.1% SDS (sodium dodecyl sulfate) indicate about 60-70% homology, hybridization, and wash conditions of 50-65 C, 1 x SSC and 0.1% SDS indicate about 82-97% homology, and hybridization, and wash conditions of 52 C, 0.1 x SSC and 0.1% SDS indicate about 99-100% homology. A wide range of computer programs for comparing nucleotide and amino acid sequences (and measuring the degree of homology) are also available, and a list providing sources of both commercially available and free software is found in Ausubel et al. (1999). Readily available sequence comparison and multiple sequence alignment algorithms are, respectively, the Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1997) and ClustalW programs. BLAST is available on the world wide web at ncbi.nlm.nih.gov and a version of ClustalW is available at 2.ebi.ac.uk.

The components of the fusion protein can be organized in nearly any order provided each is capable of performing its intended function. For example, in one embodiment, the biologically active polypeptide is situated at the C or N terminal end of the effector molecule.

Preferred effector molecules of the invention will have sizes conducive to the function for which those domains are intended. The effector molecules of the invention can be made and fused to the biologically active polypeptide by a variety of methods including well-known chemical cross-linking methods. See, e.g., Means, G. E. and Feeney, R. E. (1974) in *Chemical Modification of Proteins*, Holden-Day. See also, S. S. Wong (1991) in *Chemistry of Protein Conjugation and Cross-Linking*, CRC Press. However, it is generally preferred to use recombinant manipulations to make the in-frame fusion protein.

As noted, a fusion molecule or a conjugate molecule in accord with the invention can be organized in several ways. In an exemplary configuration, the C-terminus of the biologically active polypeptide is operatively linked to the N-terminus of the effector molecule. That linkage can be achieved by recombinant methods if desired. However, in another configuration, the N-terminus of the biologically active polypeptide is linked to the C-terminus of the effector molecule.

Alternatively, or in addition, one or more additional effector molecules can be inserted into the biologically active polypeptide or conjugate complexes as needed.

Vectors and Expression

A number of strategies can be employed to express the components of fusion protein complex of the invention (e.g., TxM). For example, a construct encoding one or more components of fusion protein complex of the invention can be incorporated into a suitable vector using restriction enzymes to make cuts in the vector for insertion of the construct followed by ligation. The vector containing the gene construct is then introduced into a suitable host for expression of the fusion protein. See, generally, Sambrook et al., *supra*. Selection of suitable vectors can be made empirically based on factors relating to the cloning protocol. For example, the vector should be compatible with, and have the proper replicon for the host that is being employed. The vector must be able to accommodate the DNA sequence coding for the fusion protein complex that is to be expressed. Suitable host cells include eukaryotic and prokaryotic cells, preferably those cells that can be easily transformed and exhibit rapid growth in culture medium. Specifically, preferred hosts cells include prokaryotes such as *E. coli, Bacillus*

subtillus, etc. and eukaryotes such as animal cells and yeast strains, e.g., *S. cerevisiae*. Mammalian cells are generally preferred, particularly J558, NSO, SP2-O or CHO. Other suitable hosts include, e.g., insect cells such as Sf9. Conventional culturing conditions are employed. See, Sambrook, supra. Stable transformed or transfected cell lines can then be selected. Cells expressing a fusion protein complex of the invention can be determined by known procedures. For example, expression of a fusion protein complex linked to an immunoglobulin can be determined by an ELISA specific for the linked immunoglobulin and/or by immunoblotting. Other methods for detecting expression of fusion proteins comprising biologically active polypeptides linked to IL-15 or IL-15Rα domains are disclosed in the Examples.

As mentioned generally above, a host cell can be used for preparative purposes to propagate nucleic acid encoding a desired fusion protein. Thus, a host cell can include a prokaryotic or eukaryotic cell in which production of the fusion protein is specifically intended. Thus, host cells specifically include yeast, fly, worm, plant, frog, mammalian cells and organs that are capable of propagating nucleic acid encoding the fusion. Non-limiting examples of mammalian cell lines which can be used include CHO dhfr-cells (Urlaub and Chasm, *Proc. Natl. Acad. Sci. USA*, 77:4216 (1980)), 293 cells (Graham et al., *J Gen. Virol*., 36:59 (1977)) or myeloma cells like SP2 or NSO (Galfre and Milstein, *Meth. Enzymol*., 73(B):3 (1981)).

Host cells capable of propagating nucleic acid encoding a desired fusion protein complexes encompass non-mammalian eukaryotic cells as well, including insect (e.g., *Sp. frugiperda*), yeast (e.g., *S. cerevisiae, S. pombe, P. pastoris., K. lactis, H. polymorpha*; as generally reviewed by Fleer, R., *Current Opinion in Biotechnology*, 3(5):486496 (1992)), fungal and plant cells. Also contemplated are certain prokaryotes such as *E. coli* and *Bacillus*.

Nucleic acid encoding a desired fusion protein can be introduced into a host cell by standard techniques for transfecting cells. The term "transfecting" or "transfection" is intended to encompass all conventional techniques for introducing nucleic acid into host cells, including calcium phosphate co-precipitation, DEAE-dextran-mediated transfection, lipofection, electroporation, microinjection, viral transduction and/or integration. Suitable methods for transfecting host cells can be found in Sambrook et al. supra, and other laboratory textbooks.

Various promoters (transcriptional initiation regulatory region) may be used according to the invention. The selection of the appropriate promoter is dependent upon the proposed

expression host. Promoters from heterologous sources may be used as long as they are functional in the chosen host.

Promoter selection is also dependent upon the desired efficiency and level of peptide or protein production. Inducible promoters such as tac are often employed in order to dramatically increase the level of protein expression in *E. coli*. Overexpression of proteins may be harmful to the host cells. Consequently, host cell growth may be limited. The use of inducible promoter systems allows the host cells to be cultivated to acceptable densities prior to induction of gene expression, thereby facilitating higher product yields.

Various signal sequences may be used according to the invention. A signal sequence which is homologous to the biologically active polypeptide coding sequence may be used. Alternatively, a signal sequence which has been selected or designed for efficient secretion and processing in the expression host may also be used. For example, suitable signal sequence/host cell pairs include the *B. subtilis* sacB signal sequence for secretion in *B. subtilis*, and the *Saccharomyces cerevisiae* α-mating factor or *P. pastoris* acid phosphatase phoI signal sequences for *P. pastoris* secretion. The signal sequence may be joined directly through the sequence encoding the signal peptidase cleavage site to the protein coding sequence, or through a short nucleotide bridge consisting of usually fewer than ten codons, where the bridge ensures correct reading frame of the downstream TCR sequence.

Elements for enhancing transcription and translation have been identified for eukaryotic protein expression systems. For example, positioning the cauliflower mosaic virus (CaMV) promoter 1,000 bp on either side of a heterologous promoter may elevate transcriptional levels by 10- to 400-fold in plant cells. The expression construct should also include the appropriate translational initiation sequences. Modification of the expression construct to include a Kozak consensus sequence for proper translational initiation may increase the level of translation by 10-fold.

A selective marker is often employed, which may be part of the expression construct or separate from it (e.g., carried by the expression vector), so that the marker may integrate at a site different from the gene of interest. Examples include markers that confer resistance to antibiotics (e.g., bla confers resistance to ampicillin for *E. coli* host cells, nptII confers kanamycin resistance to a wide variety of prokaryotic and eukaryotic cells) or that permit the host to grow on minimal medium (e.g., HIS4 enables *P. pastoris* or His^- *S. cerevisiae* to grow in

the absence of histidine). The selectable marker has its own transcriptional and translational initiation and termination regulatory regions to allow for independent expression of the marker. If antibiotic resistance is employed as a marker, the concentration of the antibiotic for selection will vary depending upon the antibiotic, generally ranging from 10 to 600 µg of the antibiotic/mL of medium.

The expression construct is assembled by employing known recombinant DNA techniques (Sambrook et al., 1989; Ausubel et al., 1999). Restriction enzyme digestion and ligation are the basic steps employed to join two fragments of DNA. The ends of the DNA fragment may require modification prior to ligation, and this may be accomplished by filling in overhangs, deleting terminal portions of the fragment(s) with nucleases (e.g., ExoIII), site directed mutagenesis, or by adding new base pairs by PCR. Polylinkers and adaptors may be employed to facilitate joining of selected fragments. The expression construct is typically assembled in stages employing rounds of restriction, ligation, and transformation of *E. coli*. Numerous cloning vectors suitable for construction of the expression construct are known in the art (λZAP and pBLUESCRIPT SK-1, Stratagene, La Jolla, CA, pET, Novagen Inc., Madison, WI, cited in Ausubel et al., 1999) and the particular choice is not critical to the invention. The selection of cloning vector will be influenced by the gene transfer system selected for introduction of the expression construct into the host cell. At the end of each stage, the resulting construct may be analyzed by restriction, DNA sequence, hybridization, and PCR analyses.

The expression construct may be transformed into the host as the cloning vector construct, either linear or circular, or may be removed from the cloning vector and used as is or introduced onto a delivery vector. The delivery vector facilitates the introduction and maintenance of the expression construct in the selected host cell type. The expression construct is introduced into the host cells by any of a number of known gene transfer systems (e.g., natural competence, chemically mediated transformation, protoplast transformation, electroporation, biolistic transformation, transfection, or conjugation) (Ausubel et al., 1999; Sambrook et al., 1989). The gene transfer system selected depends upon the host cells and vector systems used.

For instance, the expression construct can be introduced into *S. cerevisiae* cells by protoplast transformation or electroporation. Electroporation of *S. cerevisiae* is readily accomplished, and yields transformation efficiencies comparable to spheroplast transformation.

The present invention further provides a production process for isolating a fusion protein of interest. In the process, a host cell (e.g., a yeast, fungus, insect, bacterial or animal cell), into which has been introduced a nucleic acid encoding the protein of the interest operatively linked to a regulatory sequence, is grown at production scale in a culture medium to stimulate transcription of the nucleotides sequence encoding the fusion protein of interest. Subsequently, the fusion protein of interest is isolated from harvested host cells or from the culture medium. Standard protein purification techniques can be used to isolate the protein of interest from the medium or from the harvested cells. In particular, the purification techniques can be used to express and purify a desired fusion protein on a large-scale (i.e. in at least milligram quantities) from a variety of implementations including roller bottles, spinner flasks, tissue culture plates, bioreactor, or a fermentor.

An expressed protein fusion complex can be isolated and purified by known methods. Typically, the culture medium is centrifuged or filtered and then the supernatant is purified by affinity or immunoaffinity chromatography, e.g. Protein-A or Protein-G affinity chromatography or an immunoaffinity protocol comprising use of monoclonal antibodies that bind the expressed fusion complex. The fusion proteins of the present invention can be separated and purified by appropriate combination of known techniques. These methods include, for example, methods utilizing solubility such as salt precipitation and solvent precipitation, methods utilizing the difference in molecular weight such as dialysis, ultra-filtration, gel-filtration, and SDS-polyacrylamide gel electrophoresis, methods utilizing a difference in electrical charge such as ion-exchange column chromatography, methods utilizing specific affinity such as affinity chromatography, methods utilizing a difference in hydrophobicity such as reverse-phase high performance liquid chromatography and methods utilizing a difference in isoelectric point, such as isoelectric focusing electrophoresis, metal affinity columns such as Ni-NTA. See generally Sambrook et al. and Ausubel et al. supra for disclosure relating to these methods.

It is preferred that the fusion proteins of the present invention be substantially pure. That is, the fusion proteins have been isolated from cell substituents that naturally accompany it so that the fusion proteins are present preferably in at least 80% or 90% to 95% homogeneity (w/w). Fusion proteins having at least 98 to 99% homogeneity (w/w) are most preferred for many pharmaceutical, clinical and research applications. Once substantially purified the fusion protein should be substantially free of contaminants for therapeutic applications. Once purified partially

or to substantial purity, the soluble fusion proteins can be used therapeutically, or in performing in vitro or in vivo assays as disclosed herein. Substantial purity can be determined by a variety of standard techniques such as chromatography and gel electrophoresis.

The present fusion protein complexes are suitable for *in vitro* or *in vivo* use with a variety of cells that are cancerous or are infected or that may become infected by one or more diseases.

Human interleukin-15 (huIL-15) is trans-presented to immune effector cells by the human IL-15 receptor α chain (huIL-15Rα) expressed on antigen presenting cells. IL-15Rα binds huIL-15 with high affinity (38 pM) primarily through the extracellular sushi domain (huIL-15RαSu). As described herein, the huIL-15 and huIL-15RαSu domains can be used as a scaffold to construct multi-domain fusion complexes.

IgG domains, particularly the Fc fragment, have been used successfully as dimeric scaffolds for a number of therapeutic molecules including approved biologic drugs. For example, etanercept is a dimer of soluble human p75 tumor necrosis factor-α (TNF-α) receptor (sTNFR) linked to the Fc domain of human IgG1. This dimerization allows etanercept to be up to 1,000 times more potent at inhibiting TNF-α activity than the monomeric sTNFR and provides the fusion with a five-fold longer serum half-life than the monomeric form. As a result, etanercept is effective at neutralization of the pro-inflammatory activity of TNF-α *in vivo* and improving patient outcomes for a number of different autoimmune indications.

In addition to its dimerization activity, the Fc fragment also provides cytotoxic effector functions through the complement activation and interaction with Fcγ receptors displayed on natural killer (NK) cells, neutrophils, phagocytes, and dendritic cells. In the context of anti-cancer therapeutic antibodies and other antibody domain-Fc fusion proteins, these activities likely play an important role in efficacy observed in animal tumor models and in cancer patients. However, these cytotoxic effector responses may not be sufficient in a number of therapeutic applications. Thus, there has been considerable interest in improving and expanding on the effector activity of the Fc domain and developing other means of recruiting cytolytic immune responses, including T cell activity, to the disease site via targeted therapeutic molecules. IgG domains have been used as a scaffold to form bispecific antibodies to improve the quality and quantity of products generated by the traditional hybridoma fusion technology. Although these methods bypass the shortcomings of other scaffolds, it has been difficult to produce bispecific antibodies in mammalian cells at levels sufficient to support clinical development and use.

In an effort to develop human-derived immunostimulatory multimeric scaffold, human IL-15 (huIL-15) and IL-15 receptor domains were used. huIL-15 is a member of the small four α-helix bundle family of cytokines that associates with the huIL-15 receptor α-chain (huIL-15Rα) with a high binding affinity (equilibrium dissociation constant (KD) ~10^{-11} M). The resulting complex is then trans-presented to the human IL-2/15 receptor β/common γ chain (huIL-15RβγC) complexes displayed on the surface of T cells and NK cells. This cytokine/receptor interaction results in expansion and activation of effector T cells and NK cells, which play an important role in eradicating virally infected and malignant cells. Normally, huIL-15 and huIL-15Rα are co-produced in dendritic cells to form complexes intracellularly that are subsequently secreted and displayed as heterodimeric molecules on cell surfaces. Thus, the characteristics of huIL-15 and huIL-15Rα interactions suggest that these inter chain binding domains could serve as a human-derived immunostimulatory scaffold to make soluble dimeric molecules capable of target-specific binding.

As described in detail below, an huIL-15:huIL-15RαSu-based scaffold was used to create PD-L1 TxM. The dimeric fusion protein complexes retained immunostimulatory and target-specific biological activity of their huIL-15 domains and binding domains, indicating that the addition of huIL-15 and huIL-15Rα did not significantly alter the spatial arrangement of the fusion domains and provided an adequate degree of conformational flexibility without impacting cytokine activity. Thus, this scaffold could be used to form multivalent fusion complexes, such as the PD-L1 TxM, to increase the overall binding affinity of molecules. The soluble fusion proteins were produced at relatively high levels in recombinant CHO cell culture (mgs per liter in cell culture supernatant without extensive cell line screening or optimization) and could be readily purified from the cell culture supernatants.

The practice of the present invention employs, unless otherwise indicated, conventional techniques of molecular biology (including recombinant techniques), microbiology, cell biology, biochemistry, and immunology, which are well within the purview of the skilled artisan. Such techniques are explained fully in the literature, such as, "Molecular Cloning: A Laboratory Manual", second edition (Sambrook, 1989); "Oligonucleotide Synthesis" (Gait, 1984); "Animal Cell Culture" (Freshney, 1987); "Methods in Enzymology" "Handbook of Experimental Immunology" (Weir, 1996); "Gene Transfer Vectors for Mammalian Cells" (Miller and Calos, 1987); "Current Protocols in Molecular Biology" (Ausubel, 1987); "PCR: The Polymerase

Chain Reaction", (Mullis, 1994); "Current Protocols in Immunology" (Coligan, 1991). These techniques are applicable to the production of the polynucleotides and polypeptides of the invention, and, as such, may be considered in making and practicing the invention. Particularly useful techniques for particular embodiments will be discussed in the sections that follow.

Lymphoma

Lymphoma is a type of blood cancer that occurs when B or T lymphocytes divide faster than normal cells or live longer than intended. For example, B cell lymphomas include both Hodgkin's lymphomas and most non-Hodgkin's lymphomas. B cell lymphomas express CD20.

Lymphoma may develop in the lymph nodes, spleen, bone marrow, blood, or other organs. These malignant cells often originate in the lymph nodes, presenting as an enlargement of the node, i.e., a solid tumor of lymphoid cells. Lymphoma is definitively diagnosed by a lymph node biopsy, i.e., a partial or total excision of a lymph node, which is examined under a microscope. This examination may reveal histopathological features that may indicate lymphoma. Treatment might involve chemotherapy, radiotherapy, and/or bone marrow transplantation.

The following examples are put forth so as to provide those of ordinary skill in the art with a complete disclosure and description of how to make and use the assay, screening, and therapeutic methods of the invention, and are not intended to limit the scope of what the inventors regard as their invention.

EXAMPLES

Example 1: Generation and Characterization of IL-15-Based Fusion Protein Complexes Comprising Anti-PD-L1 Binding Domains (PD-L1 TxM)

Cancer cells are able to turn on various immune inhibitory pathways which are regulated by immune checkpoint molecules and ligands, such as PD-L1. Antibodies that block these checkpoint molecules have been shown to enhance anti-tumor immunity. IL-15 activates and expands NK and $CD8^+$ cells while increasing their cytolytic activity. The Fc region of Ig molecules can interact with Fcγ receptors on NK cells and macrophages and mediate antibody-dependent cellular cytotoxicity (ADCC) or antibody-dependent cellular phagocytosis (ADCP) against target disease cells. As described in detail below, protein complexes comprising an IL-15N72D:IL-15RαSu/Fc scaffold fused to anti-PD-L1 binding domains were generated. These

complexes recognize tumor cells via the anti-PD-L1 binding domain, induce NK and T cells responses via IL-15 activity, and stimulate ADCC and CDC via the Fc binding domain.

Specifically, constructs were made linking a single-chain anti-PD-L1 antibody to the huIL-15N72D and IL-15RαSu/Fc chains. The anti-PD-L1 single chain antibody (anti-PD-L1 scAb) sequence comprises the coding regions of the heavy and light chain V antibody domains antibody linked via a flexible linker sequence. The single chain antibody domain can be arranged in either the VH-linker-VL or VL-linker-VH format. In some cases, the anti-PD-L1 scAb is linked to the C-terminus of the IL-15N72D and/or IL-15RαSu/Fc chains. In other cases, the anti-PD-L1 is linked to the N-terminus of IL-15N72D and/or IL-15RαSu/Fc chains. Anti-PD-L1 scAbs specific to either the mouse or human PD-L1 molecules were used in these constructs.

1) The nucleic acid and protein sequences of constructs comprising an anti-human PD-L1 scAb linked to the N-terminus of the huIL-15N72D and huIL-15RαSu/huIgG1 Fc chains are shown below. The nucleic acid sequence of anti-human PD-L1 scAb/IL-15N72D construct (including signal peptide sequence and stop codon) is as follows (SEQ ID NO: 1):

(Signal peptide)

ATGAAGTGGGTGACCTTCATCAGCCTGCTGTTCCTGTTCTCCAGCGCCTACTCC

(Anti-human PD-L1 scAb)

(VL)

AACATCCAGATGACCCAGTCCCCTAGCTCCGTGTCCGCCTCCGTGGGAGATCGGGTG
ACCATCACCTGTAGGGCCTCCCAGGACATCTCCAGGTGGCTGGCCTGGTACCAGCAG
AAGCCCGGCAAGGCCCCCAAGCTGCTGATCTACGCCGCCTCCTCCCTGCAGTCCGGA
GTGCCTAGCAGGTTCTCCGGCTCCGGATCCGGCACAGACTTCGCCCTGACCATCTCC
TCCCTGCAGCCCGAGGACTTCGCCACCTACTACTGCCAGCAGGCCGACTCCAGGTTC
TCCATCACCTTCGGCCAGGGCACCAGGCTGGAGATCAAGAGG

(Linker)

GGAGGTGGCGGATCCGGAGGTGGAGGTTCTGGTGGAGGTGGGAGT

(VH)

GAGGTGCAGCTGGTGCAGTCCGGAGGAGGACTGGTGCAGCCTGGCGGATCCCTGAG
GCTGTCCTGTGCCGCTTCCGGCTTCACCTTCAGCTCCTACTCCATGAACTGGGTGAG
GCAGGCCCCTGGAAAGGGCCTGGAGTGGGTGTCCTACATCTCCAGCTCCTCCTCCAC
CATCCAGTACGCCGACTCCGTGAAGGGCAGGTTCACCATCTCCAGGGACAACGCCA
AGAACTCCCTGTACCTGCAGATGAACAGCCTGAGGGACGAGGACACCGCCGTGTAC
TACTGCGCCAGGGGCGACTATTACTACGGCATGGACGTGTGGGGCCAGGGAACCAC
CGTGACCGTGTCCTCC

(Human IL-15N72D)

AACTGGGTTAACGTAATAAGTGATTTGAAAAAAATTGAAGATCTTATTCAATCTATG
CATATTGATGCTACTTTATATACGGAAAGTGATGTTCACCCCAGTTGCAAAGTAACA
GCAATGAAGTGCTTTCTCTTGGAGTTACAAGTTATTTCACTTGAGTCCGGAGATGCA
AGTATTCATGATACAGTAGAAAATCTGATCATCCTAGCAAACGACAGTTTGTCTTCT
AATGGGAATGTAACAGAATCTGGATGCAAAGAATGTGAGGAACTGGAGGAAAAA
ATATTAAAGAATTTTTGCAGAGTTTTGTACATATTGTCCAAATGTTCATCAACACTTC
TTAA

The amino acid sequence of the anti-human PD-L1 scAb/IL-15N72D fusion protein (including signal peptide sequence) is as follows (SEQ ID NO: 2):

(Signal peptide)

MKWVTFISLLFLFSSAYS

(Anti-human PD-L1 scAb)

(VL)

NIQMTQSPSSVSASVGDRVTITCRASQDISRWLAWYQQKPGKAPKLLIYAASSLQSGVPS
RFSGSGSGTDFALTISSLQPEDFATYYCQQADSRFSITFGQGTRLEIKR

(Linker)

GGGGSGGGGSGGGGS

(VH)

EVQLVQSGGGLVQPGGSLRLSCAASGFTFSSYSMNWVRQAPGKGLEWVSYISSSSSTIQ
YADSVKGRFTISRDNAKNSLYLQMNSLRDEDTAVYYCARGDYYYGMDVWGQGTTVT
VSS

(Human IL-15N72D)

NWVNVISDLKKIEDLIQSMHIDATLYTESDVHPSCKVTAMKCFLLELQVISLESGDASIH
DTVENLIILANDSLSSNGNVTESGCKECEELEEKNIKEFLQSFVHIVQMFINTS

In some cases, the leader peptide is cleaved from the mature polypeptide.

The nucleic acid sequence of an anti-human PD-L1 scAb/huIL-15RαSu/huIgG1 Fc construct (including leader sequence) is as follows (SEQ ID NO: 3):

(Leader sequence)
ATGAAGTGGGTGACCTTCATCAGCCTGCTGTTCCTGTTCTCCAGCGCCTACTCC
(Anti-human PD-L1 scAb)
(VL)
AACATCCAGATGACCCAGTCCCCTAGCTCCGTGTCCGCCTCCGTGGGAGATCGGGTG
ACCATCACCTGTAGGGCCTCCCAGGACATCTCCAGGTGGCTGGCCTGGTACCAGCAG
AAGCCCGGCAAGGCCCCCAAGCTGCTGATCTACGCCGCCTCCTCCCTGCAGTCCGGA
GTGCCTAGCAGGTTCTCCGGCTCCGGATCCGGCACAGACTTCGCCCTGACCATCTCC
TCCCTGCAGCCCGAGGACTTCGCCACCTACTACTGCCAGCAGGCCGACTCCAGGTTC
TCCATCACCTTCGGCCAGGGCACCAGGCTGGAGATCAAGAGG
(Linker)
GGAGGTGGCGGATCCGGAGGTGGAGGTTCTGGTGGAGGTGGGAGT
(VH)
GAGGTGCAGCTGGTGCAGTCCGGAGGAGGACTGGTGCAGCCTGGCGGATCCCTGAG
GCTGTCCTGTGCCGCTTCCGGCTTCACCTTCAGCTCCTACTCCATGAACTGGGTGAG
GCAGGCCCCTGGAAAGGGCCTGGAGTGGGTGTCCTACATCTCCAGCTCCTCCTCCAC
CATCCAGTACGCCGACTCCGTGAAGGGCAGGTTCACCATCTCCAGGGACAACGCCA
AGAACTCCCTGTACCTGCAGATGAACAGCCTGAGGGACGAGGACACCGCCGTGTAC
TACTGCGCCAGGGGCGACTATTACTACGGCATGGACGTGTGGGGCCAGGGAACCAC
CGTGACCGTGTCCTCC
(Human IL-15R α sushi domain)
ATCACGTGCCCTCCCCCCATGTCCGTGGAACACGCAGACATCTGGGTCAAGAGCTAC
AGCTTGTACTCCAGGGAGCGGTACATTTGTAACTCTGGTTTCAAGCGTAAAGCCGGC

ACGTCCAGCCTGACGGAGTGCGTGTTGAACAAGGCCACGAATGTCGCCCACTGGAC
AACCCCCAGTCTCAAATGCATTAGA

(Human IgG1 CH2-CH3 (Fc) domain)

GAGCCGAAATCTTGTGACAAAACTCACACATGCCCACCGTGCCCAGCACCTGAACT
CCTGGGGGGACCGTCAGTCTTCCTCTTCCCCCCAAAACCCAAGGACACCCTCATGAT
CTCCCGGACCCCTGAGGTCACATGCGTGGTGGTGGACGTGAGCCACGAAGACCCTG
AGGTCAAGTTCAACTGGTACGTGGACGGCGTGGAGGTGCATAATGCCAAGACAAAG
CCGCGGGAGGAGCAGTACAACAGCACGTACCGTGTGGTCAGCGTCCTCACCGTCCT
GCACCAGGACTGGCTGAATGGCAAGGAGTACAAGTGCAAGGTCTCCAACAAAGCCC
TCCCAGCCCCCATCGAGAAAACCATCTCCAAAGCCAAAGGGCAGCCCCGAGAACCA
CAGGTGTACACCCTGCCCCCATCCCGGGATGAGCTGACCAAGAACCAGGTCAGCCT
GACCTGCCTGGTCAAAGGCTTCTATCCCAGCGACATCGCCGTGGAGTGGGAGAGCA
ATGGGCAGCCGGAGAACAACTACAAGACCACGCCTCCCGTGCTGGACTCCGACGGC
TCCTTCTTCCTCTACAGCAAGCTCACCGTGGACAAGAGCAGGTGGCAGCAGGGGAA
CGTCTTCTCATGCTCCGTGATGCATGAGGCTCTGCACAACCACTACACGCAGAAGAG
CCTCTCCCTGTCTCCTGGTAAATAA

The amino acid sequence of the anti-human PD-L1 scAb/huIL-15RαSu/huIgG1 Fc fusion protein (including leader sequence) is as follows (SEQ ID NO: 4):

(Leader peptide)

MKWVTFISLLFLFSSAYS

(Anti-human PD-L1 scAb)

(VL)

NIQMTQSPSSVSASVGDRVTITCRASQDISRWLAWYQQKPGKAPKLLIYAASSLQSGVPS
RFSGSGSGTDFALTISSLQPEDFATYYCQQADSRFSITFGQGTRLEIKR

(Linker)

GGGGSGGGGSGGGGS

(VH)

EVQLVQSGGGLVQPGGSLRLSCAASGFTFSSYSMNWVRQAPGKGLEWVSYISSSSSTIQ
YADSVKGRFTISRDNAKNSLYLQMNSLRDEDTAVYYCARGDYYYGMDVWGQGTTVT
VSS

(Human IL-15R α sushi domain)

ITCPPPMSVEHADIWVKSYSLYSRERYICNSGFKRKAGTSSLTECVLNKATNVAHWTTPS
LKCIR

(Human IgG1 CH2-CH3 (Fc) domain)

EPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKF
NWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPI
EKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNY
KTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK

In some cases, the leader peptide is cleaved from the mature polypeptide.

2) The nucleic acid and protein sequences of constructs comprising a second anti-human PD-L1 (avelumab) scAb linked to the N-terminus of the huIL-15N72D and huIL-15RαSu/huIgG1 Fc chains are shown below. The nucleic acid sequence of anti-human PD-L1 scAb/IL-15N72D construct (including signal peptide sequence and stop codon) is as follows (SEQ ID NO: 5):

(Signal peptide)

ATGAAGTGGGTGACCTTCATCAGCCTGCTGTTCCTGTTCTCCAGCGCCTACTCC

(Anti-human PD-L1 scAb)

(VL)

CAGTCCGCTCTGACCCAGCCTGCTTCCGTGTCCGGCTCCCCTGGACAGTCCATCACC
ATCTCCTGTACCGGCACCTCCTCCGATGTGGGCGGCTACAACTACGTGTCCTGGTAC
CAGCAGCACCCCGGCAAAGCCCCCAAGCTGATGATCTATGACGTGTCCAACCGGCC
CTCCGGCGTGTCCAACAGGTTCTCCGGCTCCAAGTCCGGCAACACCGCCTCCCTGAC
AATCTCCGGCCTGCAGGCCGAGGATGAGGCTGACTACTACTGCTCCTCCTACACCTC
CTCCTCCACCAGGGTGTTCGGCACCGGCACCAAGGTGACCGTGCTG

(Linker)

GGAGGTGGCGGATCCGGAGGTGGAGGTTCTGGTGGAGGTGGGAGT

(VH)

GAGGTGCAGCTGCTGGAGTCCGGAGGCGGACTGGTGCAGCCTGGAGGATCCCTGAG
GCTGTCCTGCGCTGCCTCCGGCTTCACCTTCTCCTCCTACATCATGATGTGGGTGAGG
CAGGCTCCTGGCAAGGGCCTGGAGTGGGTGTCCTCCATCTACCCCTCCGGCGGCATC
ACCTTCTACGCCGATACCGTGAAGGGCAGGTTCACCATCTCCCGGGACAACTCCAAG
AACACCCTGTACCTGCAGATGAACTCCCTGAGGGCTGAGGACACCGCCGTGTACTA
CTGCGCCAGGATCAAGCTGGGCACCGTGACCACAGTGGACTACTGGGGACAGGGCA
CCCTGGTGACCGTGTCCTCC

(Human IL-15N72D)

AACTGGGTTAACGTAATAAGTGATTTGAAAAAAATTGAAGATCTTATTCAATCTATG
CATATTGATGCTACTTTATATACGGAAAGTGATGTTCACCCCAGTTGCAAAGTAACA
GCAATGAAGTGCTTTCTCTTGGAGTTACAAGTTATTTCACTTGAGTCCGGAGATGCA
AGTATTCATGATACAGTAGAAAATCTGATCATCCTAGCAAACGACAGTTTGTCTTCT
AATGGGAATGTAACAGAATCTGGATGCAAAGAATGTGAGGAACTGGAGGAAAAAA
ATATTAAAGAATTTTTGCAGAGTTTTGTACATATTGTCCAAATGTTCATCAACACTTC
TTAA

The amino acid sequence of the anti-human PD-L1 scAb/IL-15N72D fusion protein (including signal peptide sequence) is as follows (SEQ ID NO: 6):

(Signal peptide)

MKWVTFISLLFLFSSAYS

(Anti-human PD-L1 scAb)

(VL)

QSALTQPASVSGSPGQSITISCTGTSSDVGGYNYVSWYQQHPGKAPKLMIYDVSNRPSG
VSNRFSGSKSGNTASLTISGLQAEDEADYYCSSYTSSSTRVFGTGTKVTVL

(Linker)

GGGGSGGGGSGGGGS

(VH)

EVQLLESGGGLVQPGGSLRLSCAASGFTFSSYIMMWVRQAPGKGLEWVSSIYPSGGITF
YADTVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCARIKLGTVTTVDYWGQGTLVT
VSS

(Human IL-15N72D)

NWVNVISDLKKIEDLIQSMHIDATLYTESDVHPSCKVTAMKCFLLELQVISLESGDASIH
DTVENLIILANDSLSSNGNVTESGCKECEELEEKNIKEFLQSFVHIVQMFINTS

In some cases, the leader peptide is cleaved from the mature polypeptide.

The nucleic acid sequence of an anti-human PD-L1 scAb/huIL-15RαSu/huIgG1 Fc construct (including leader sequence) is as follows (SEQ ID NO: 7):

(Leader sequence)

ATGAAGTGGGTGACCTTCATCAGCCTGCTGTTCCTGTTCTCCAGCGCCTACTCC

(Anti-human PD-L1 scAb)

(VL)

CAGTCCGCTCTGACCCAGCCTGCTTCCGTGTCCGGCTCCCCTGGACAGTCCATCACC
ATCTCCTGTACCGGCACCTCCTCCGATGTGGGCGGCTACAACTACGTGTCCTGGTAC
CAGCAGCACCCCGGCAAAGCCCCCAAGCTGATGATCTATGACGTGTCCAACCGGCC
CTCCGGCGTGTCCAACAGGTTCTCCGGCTCCAAGTCCGGCAACACCGCCTCCCTGAC
AATCTCCGGCCTGCAGGCCGAGGATGAGGCTGACTACTACTGCTCCTCCTACACCTC
CTCCTCCACCAGGGTGTTCGGCACCGGCACCAAGGTGACCGTGCTG

(Linker)

GGAGGTGGCGGATCCGGAGGTGGAGGTTCTGGTGGAGGTGGGAGT

(VH)

GAGGTGCAGCTGCTGGAGTCCGGAGGCGGACTGGTGCAGCCTGGAGGATCCCTGAG
GCTGTCCTGCGCTGCCTCCGGCTTCACCTTCTCCTCCTACATCATGATGTGGGTGAGG
CAGGCTCCTGGCAAGGGCCTGGAGTGGGTGTCCTCCATCTACCCCTCCGGCGGCATC
ACCTTCTACGCCGATACCGTGAAGGGCAGGTTCACCATCTCCCGGGACAACTCCAAG
AACACCCTGTACCTGCAGATGAACTCCCTGAGGGCTGAGGACACCGCCGTGTACTA
CTGCGCCAGGATCAAGCTGGGCACCGTGACCACAGTGGACTACTGGGGACAGGGCA
CCCTGGTGACCGTGTCCTCC

(Human IL-15R α sushi domain)

ATCACGTGCCCTCCCCCCATGTCCGTGGAACACGCAGACATCTGGGTCAAGAGCTAC
AGCTTGTACTCCAGGGAGCGGTACATTTGTAACTCTGGTTTCAAGCGTAAAGCCGGC
ACGTCCAGCCTGACGGAGTGCGTGTTGAACAAGGCCACGAATGTCGCCCACTGGAC
AACCCCCAGTCTCAAATGCATTAGA

(Human IgG1 CH2-CH3 (Fc) domain)

GAGCCGAAATCTTGTGACAAAACTCACACATGCCCACCGTGCCCAGCACCTGAACT
CCTGGGGGGACCGTCAGTCTTCCTCTTCCCCCCAAAACCCAAGGACACCCTCATGAT
CTCCCGGACCCCTGAGGTCACATGCGTGGTGGTGGACGTGAGCCACGAAGACCCTG
AGGTCAAGTTCAACTGGTACGTGGACGGCGTGGAGGTGCATAATGCCAAGACAAAG
CCGCGGGAGGAGCAGTACAACAGCACGTACCGTGTGGTCAGCGTCCTCACCGTCCT
GCACCAGGACTGGCTGAATGGCAAGGAGTACAAGTGCAAGGTCTCCAACAAAGCCC
TCCCAGCCCCCATCGAGAAAACCATCTCCAAAGCCAAAGGGCAGCCCCGAGAACCA
CAGGTGTACACCCTGCCCCCATCCCGGGATGAGCTGACCAAGAACCAGGTCAGCCT
GACCTGCCTGGTCAAAGGCTTCTATCCCAGCGACATCGCCGTGGAGTGGGAGAGCA
ATGGGCAGCCGGAGAACAACTACAAGACCACGCCTCCCGTGCTGGACTCCGACGGC
TCCTTCTTCCTCTACAGCAAGCTCACCGTGGACAAGAGCAGGTGGCAGCAGGGGAA
CGTCTTCTCATGCTCCGTGATGCATGAGGCTCTGCACAACCACTACACGCAGAAGAG
CCTCTCCCTGTCTCCTGGTAAATAA

The amino acid sequence of the anti-human PD-L1 scAb/huIL-15RαSu/huIgG1 Fc fusion protein (including leader sequence) is as follows (SEQ ID NO: 8):

(Leader peptide)

MKWVTFISLLFLFSSAYS

(Anti-human PD-L1 scAb)

(VL)

QSALTQPASVSGSPGQSITISCTGTSSDVGGYNYVSWYQQHPGKAPKLMIYDVSNRPSG
VSNRFSGSKSGNTASLTISGLQAEDEADYYCSSYTSSSTRVFGTGTKVTVL

(Linker)

GGGGSGGGGSGGGGS

(VH)

EVQLLESGGGLVQPGGSLRLSCAASGFTFSSYIMMWVRQAPGKGLEWVSSIYPSGGITF
YADTVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCARIKLGTVTTVDYWGQGTLVT
VSS

(Human IL-15R α sushi domain)

ITCPPPMSVEHADIWVKSYSLYSRERYICNSGFKRKAGTSSLTECVLNKATNVAHWTTPS
LKCIR

(Human IgG1 CH2-CH3 (Fc) domain)

EPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKF
NWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPI
EKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNY
KTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK

In some cases, the leader peptide is cleaved from the mature polypeptide.

3) The nucleic acid and protein sequences of constructs comprising am anti-mouse PD-L1 scAb linked to the N-terminus of the huIL-15N72D and huIL-15RαSu/muIgG2A Fc chains are shown below. The nucleic acid sequence of anti-mouse PD-L1 scAb/IL-15N72D construct (including signal peptide sequence and stop codon) is as follows (SEQ ID NO: 9):

(Signal peptide)

ATGACATGGACTCTACTATTCCTTGCCTTCCTTCATCACTTAACAGGGTCATGTGCCC
AGTTTGTGCTTACTCAGCCAAACTCT

(Anti-mouse PD-L1 scAb)

(VL)

GTGTCTACGAATCTCGGAAGCACAGTCAAGCTGTCTTGCAACCGCAGCACTGGTAAC
ATTGGAAACAATTATGTGAACTGGTACCAGCAGCATGAAGGAAGATCTCCCACCAC
TCTGATTTATTGGGATGATAGGAGACCAGATGGAGTTCCTGACAGGTTCTCTGGCTC
CATTGACAGATCTTCCAACTCAGCCCTCCTGACAATCAATAATGTGCAGACTGAGGA
TGAAACTGACTACTTCTGTCAGTCTTACAGTAGTGGTATGTATATTTTCGGCGGTGG
AACCAAGCTCACTGTCCTA

(Linker)

GGAGGTGGCGGATCCGGAGGTGGAGGTTCTGGTGGAGGTGGGAGT

(VH)

GAGGTTCAGCTGCAGCAGTCTGGGGCTGAGCTGGTGAAGCCTGGGGCTTCAGTAAA
GTTGTCCTGCAAAACTTCTGGTTACACCTTCAGCAATTACTATATGAGTTGGTTGAA
GCAGATGCCTGGACAGAATATTGAGTGGATCGGAAACATTTATGGTGGAAATGGTG
GTGCTGGCTATAATCAGAAGTTCAAGGGCAAGGCCACACTGACAGTGGACAAATCT
TCCAGCACAGCGTACATGGATCTCAGCAGCCTGACATCTGAGGCCTCTGCAGTCTAT
TTTTGTGCAAGGGTCGGACTTCCCGGCCTTTTTGATTACTGGGGCCAGGGAGTCATG
GTCACAGTCTCCTCA

(Human IL-15N72D)

AACTGGGTGAATGTAATAAGTGATTTGAAAAAAATTGAAGATCTTATTCAATCTATG
CATATTGATGCTACTTTATATACGGAAAGTGATGTTCACCCCAGTTGCAAAGTAACA
GCAATGAAGTGCTTTCTCTTGGAGTTACAAGTTATTTCACTTGAGTCCGGAGATGCA
AGTATTCATGATACAGTAGAAAATCTGATCATCCTAGCAAACGACAGTTTGTCTTCT
AATGGGAATGTAACAGAATCTGGATGCAAAGAATGTGAGGAACTGGAGGAAAAAA
ATATTAAAGAATTTTTGCAGAGTTTTGTACATATTGTCCAAATGTTCATCAACACTTC
TTAA

The amino acid sequence of the anti-mouse PD-L1 scAb/IL-15N72D fusion protein (including signal peptide sequence) is as follows (SEQ ID NO: 10):

(Signal peptide)

MTWTLLFLAFLHHLTGSCAQFVLTQPNS

(Anti-mouse PD-L1 scAb)

(VL)

VSTNLGSTVKLSCNRSTGNIGNNYVNWYQQHEGRSPTTLIYWDDRRPDGVPDRFSGSID
RSSNSALLTINNVQTEDETDYFCQSYSSGMYIFGGGTKLTVL

(Linker)

GGGGSGGGGSGGGGS

(VH)

EVQLQQSGAELVKPGASVKLSCKTSGYTFSNYYMSWLKQMPGQNIEWIGNIYGGNGGA
GYNQKFKGKATLTVDKSSSTAYMDLSSLTSEASAVYFCARVGLPGLFDYWGQGVMVT
VSS

(Human IL-15N72D)

NWVNVISDLKKIEDLIQSMHIDATLYTESDVHPSCKVTAMKCFLLELQVISLESGDASIH
DTVENLIILANDSLSSNGNVTESGCKECEELEEKNIKEFLQSFVHIVQMFINTS

In some cases, the leader peptide is cleaved from the mature polypeptide.

The nucleic acid sequence of an anti-mouse PD-L1 scAb/huIL-15RαSu/muIgG2A Fc construct (including leader sequence) is as follows (SEQ ID NO: 11):

(Signal peptide)

ATGACATGGACTCTACTATTCCTTGCCTTCCTTCATCACTTAACAGGGTCATGTGCCC
AGTTTGTGCTTACTCAGCCAAACTCT

(Anti-mouse PD-L1 scAb)

(VL)

GTGTCTACGAATCTCGGAAGCACAGTCAAGCTGTCTTGCAACCGCAGCACTGGTAAC
ATTGGAAACAATTATGTGAACTGGTACCAGCAGCATGAAGGAAGATCTCCCACCAC
TCTGATTTATTGGGATGATAGGAGACCAGATGGAGTTCCTGACAGGTTCTCTGGCTC
CATTGACAGATCTTCCAACTCAGCCCTCCTGACAATCAATAATGTGCAGACTGAGGA
TGAAACTGACTACTTCTGTCAGTCTTACAGTAGTGGTATGTATATTTTCGGCGGTGG
AACCAAGCTCACTGTCCTA

(Linker)

GGAGGTGGCGGATCCGGAGGTGGAGGTTCTGGTGGAGGTGGGAGT

(VH)

GAGGTTCAGCTGCAGCAGTCTGGGGCTGAGCTGGTGAAGCCTGGGGCTTCAGTAAA
GTTGTCCTGCAAAACTTCTGGTTACACCTTCAGCAATTACTATATGAGTTGGTTGAA
GCAGATGCCTGGACAGAATATTGAGTGGATCGGAAACATTTATGGTGGAAATGGTG
GTGCTGGCTATAATCAGAAGTTCAAGGGCAAGGCCACACTGACAGTGGACAAATCT
TCCAGCACAGCGTACATGGATCTCAGCAGCCTGACATCTGAGGCCTCTGCAGTCTAT

TTTTGTGCAAGGGTCGGACTTCCCGGCCTTTTTGATTACTGGGGCCAGGGAGTCATG
GTCACAGTCTCCTCA

(Human IL-15R α sushi domain)

ATCACGTGCCCTCCCCCCATGTCCGTGGAACACGCAGACATCTGGGTCAAGAGCTAC
AGCTTGTACTCCAGGGAGCGGTACATTTGTAACTCTGGTTTCAAGCGTAAAGCCGGC
ACGTCCAGCCTGACGGAGTGCGTGTTGAACAAGGCCACGAATGTCGCCCACTGGAC
AACCCCCAGTCTCAAATGCATTAGA

(Mouse IgG2a CH2-CH3 domain)

GAACCAAGAGGGCCCACAATCAAGCCCTGTCCTCCATGCAAATGCCCAGCACCTAA
CCTCTTGGGTGGACCATCCGTCTTCATCTTCCCTCCAAAGATCAAGGATGTACTCATG
ATCTCCCTGAGCCCCATAGTCACATGTGTGGTGGTGGATGTGAGCGAGGATGACCCA
GATGTCCAGATCAGCTGGTTTGTGAACAACGTGGAAGTACACACAGCTCAGACACA
AACCCATAGAGAGGATTACAACAGTACTCTCCGGGTGGTCAGTGCCCTCCCCATCCA
GCACCAGGACTGGATGAGTGGCAAGGAGTTCAAATGCAAGGTCAACAACAAAGACC
TCCCAGCGCCCATCGAGAGAACCATCTCAAAACCCAAAGGGTCAGTAAGAGCTCCA
CAGGTATATGTCTTGCCTCCACCAGAAGAAGAGATGACTAAGAAACAGGTCACTCT
GACCTGCATGGTCACAGACTTCATGCCTGAAGACATTTACGTGGAGTGGACCAACA
ACGGGAAAACAGAGCTAAACTACAAGAACACTGAACCAGTCCTGGACTCTGATGGT
TCTTACTTCATGTACAGCAAGCTGAGAGTGGAAAAGAAGAACTGGGTGGAAAGAAA
TAGCTACTCCTGTTCAGTGGTCCACGAGGGTCTGCACAATCACCACACGACTAAGAG
CTTCTCCCGGACTCCAGGTAAATAA

The amino acid sequence of the anti-mouse PD-L1 scAb/huIL-15RαSu/muIgG2A Fc fusion protein (including leader sequence) is as follows (SEQ ID NO: 12):

(Signal peptide)

MTWTLLFLAFLHHLTGSCAQFVLTQPNS

(Anti-mouse PD-L1 scAb)

(VL)

VSTNLGSTVKLSCNRSTGNIGNNYVNWYQQHEGRSPTTLIYWDDRRPDGVPDRFSGSID
RSSNSALLTINNVQTEDETDYFCQSYSSGMYIFGGGTKLTVL

(Linker)

GGGGSGGGGSGGGGS

(VH)

EVQLQQSGAELVKPGASVKLSCKTSGYTFSNYYMSWLKQMPGQNIEWIGNIYGGNGGA
GYNQKFKGKATLTVDKSSSTAYMDLSSLTSEASAVYFCARVGLPGLFDYWGQGVMVT
VSS

(Human IL-15R α sushi domain)

ITCPPPMSVEHADIWVKSYSLYSRERYICNSGFKRKAGTSSLTECVLNKATNVAHWTTPS
LKCIR

(Mouse IgG2a CH2-CH3 domain)

EPRGPTIKPCPPCKCPAPNLLGGPSVFIFPPKIKDVLMISLSPIVTCVVVDVSEDDPDVQIS
WFVNNVEVHTAQTQTHREDYNSTLRVVSALPIQHQDWMSGKEFKCKVNNKDLPAPIER
TISKPKGSVRAPQVYVLPPPEEEMTKKQVTLTCMVTDFMPEDIYVEWTNNGKTELNYK
NTEPVLDSDGSYFMYSKLRVEKKNWVERNSYSCSVVHEGLHNHHTTKSFSRTPGK

In some cases, the leader peptide is cleaved from the mature polypeptide.

The anti-PD-L1 scAb/IL-15N72D and anti-PD-L1 scAb/IL-15RαSu/Fc sequences were cloned into expression vectors as described previously (U.S. Patent No. 8,507,222, incorporated herein by reference), and the expression vectors transfected into CHO cells. Co-expression of the two constructs in CHO cells allowed formation and secretion of a soluble anti-PD-L1 scAb/IL-15N72D:anti-PD-L1 scAb/IL-15RαSu/Fc complex which was purified from the CHO cell culture supernatant using Protein A affinity chromatography.

SDS-PAGE analysis of the purified anti-PD-L1 scAb/IL-15N72D:anti-PD-L1 scAb/IL-15RαSu/Fc protein complexes is shown in FIG. 3. Bands corresponding to the soluble anti-mouse PD-L1 scAb/huIL-15RαSu/muIgG2A and anti-mouse PD-L1 scAb/IL-15N72D proteins at ~60 kDa and ~40 kDa, respectively, were observed.

<u>Example 2: In Vitro Characterization of the Activities of PD-L1 TxM</u>

ELISA-based methods confirmed the formation of a PD-L1 TxM complex. In FIG. 4A, the anti-human PD-L1 scAb/IL-15N72D:anti-human PD-L1 scAb/huIL-15RαSu/huIgG1 Fc fusion protein complexes in the culture supernatant from transfected CHO cells were detected

using a huIgG1/huIL15-specific ELISA with a capture antibody, anti-human IgG antibody (Jackson ImmunoResearch), and a detection antibody, biotinylated anti-human IL-15 antibody (BAM 247, R&D Systems). This is compared to a control sample using only the supernatant of media containing untransfected CHO cells. The increased signal observed in the culture supernatant from transfected CHO cells verifies formations of the PD-L1 TxM complex. Similar results were obtained from a second anti-human PD-L1 scAb/IL-15N72D:anti-human PD-L1 scAb/huIL-15RαSu/huIgG1 Fc fusion protein complex (avelumab TxM) (FIG. 4B).

For the mouse specific PD-L1 TxM, the fusion protein complexes were detected using a mIgG2a-specific or huIL15-specific ELISA with a capture antibody, affinipure donkey anti-mouse IgG (Jackson ImmunoResearch) or human/primate IL15 antibody (MAB647, R&D system) and a detection antibody horseradish peroxidase-affinipure donkey anti-mouse IgG (Jackson ImmunoResearch) or biotinylated anti-human IL-15 antibody (BAM 247, R&D Systems), respectively (FIG. 4C). Compared to the positive controls, antibody reactivity to the purified mouse specific PD-L1 TxM verified formation of the complex.

The ability of these fusion protein complexes to bind PD-L1 on tumor cells was also examined. The binding of human specific PD-L1 TxM was assessed by flow cytometry using receptor bearing MB231 tumor cells. In these studies, 1×10^5 cells were stained with PD-L1 TxM complexes. As shown in FIG. 5A, flow cytometry analysis demonstrated binding of the PD-L1 TxM complex to MB231 cells when detected using an APC conjugated mouse anti-human Fc Ab (Biolegend). In FIG. 5B, specificity of this binding was tested by using the PD-L1 TxM complex to block the staining of MB231 cells with a commercially available APC conjugated anti-human PD-L1 Ab (Biolegend). Similarly, flow cytometry analysis demonstrated binding of the avelumab-based PD-L1 TxM complex to MB231 tumor cells (FIG. 5C).

To assess binding of the mouse-specific PD-L1 TxM complexes, PD-L1-positive 5T33P myeloma and MB49luc bladder tumor cells (5×10^5 cells/test) initially were stained with PE or Brilliant Violet labeled anti-mouse PD-L1 Ab (2 μg/test in 100 μL) (FIG. 6 A and FIG. 6B). Specificity of this binding was tested by addition of purified mouse-specific PD-L1 TxM complexes to block antibody binding to PD-L1 ligand. A purified anti-mPD-L1 Ab (S1-PD-L1) was used as a positive control. Interestingly, the PD-L1 TxM complexes were found to block PD-L1 staining on the tumor cell better than the equivalent amount of anti-PD-L1 Ab. This was further assessed in blocking studies using A20 B-cell lymphoma cells. Titration analysis

indicated that PD-L1 TxM was at least 5-fold more effective than anti-PD-L1 Ab at blocking interactions with PD-L1 expressed on the tumor cell surface (FIG. 7A and FIG. 7B).

To assess the IL-15 immunostimulatory activity of PD-L1 TxM complexes, proliferation of IL-15-dependent 32Dβ cells, a mouse hematopoietic cell line, was assessed. Increasing levels of PD-L1 TxM were added to 32Dβ cells (10^4 cell/well) and cell proliferation was determined 2 days later using WST-1 proliferation reagent. As shown in FIG. 8, a dose dependent increase in 32Dβ cell proliferation was mediated by PD-L1 TxM verifying the immunostimulatory activity of the complex.

Further studies were conducted to assess the characteristics and activity of different forms of the PD-L1 TxM complex. Complexes comprising anti-PD-L1 scAb/IL-15N72D and anti-PD-L1 scAb/IL-15RαSu/Fc proteins are expected to have four anti-PD-L1 scAb binding domains (i.e., 4 headed (4H)) whereas complexes comprising IL-15N72D and anti-PD-L1 scAb/IL-15RαSu/Fc proteins are expected to have two anti-PD-L1 scAb binding domains (i.e., 2 headed (2H)) (FIG. 9A). These complexes are expected to have different activities based on the higher avidity binding to target cells of the 4H TxM compared to the 2H TxM. Protein fusion to the IL-15N72D has also been shown to reduce the IL-15 biological activity. Thus, the 4H TxM format is expected to have lower IL-15 activity than the 2H TxM. These differences are expected to provide advantages where high (antibody-like) targeting and lower immunostimulatory activity is preferred (i.e., 4H TxM format) or where lower targeting and higher immunostimulatory activity (immunocytokine) is preferred.

To evaluate these formats, 4H mouse-specific PD-L1 TxM (2C2) and 2H mouse-specific PD-L1 TxM (PDN3) were generated by transfecting CHO cells with anti-mouse PD-L1 scAb/IL-15N72D and anti-mouse PD-L1 scAb-/IL-15RαSu/Fc expression vectors or IL-15N72D and anti-mouse PD-L1 scAb-/IL-15RαSu/Fc expression vectors, respectively. The TxM complexes were then purified from the transfected CHO cell supernatant by Protein A chromatography and the purified proteins evaluated by reduced SDS-PAGE (FIG. 9B). Bands corresponding to the soluble anti-mouse PD-L1 scAb/huIL-15RαSu/muIgG2A, anti-mouse PD-L1 scAb/IL-15N72D and IL-15N72D proteins at ~60 kDa, ~40 kDa and ~16 kDa, respectively, were observed. Additionally, the purified 4H PD-L1 TxM (2C2) and 2H PD-L1 TxM (PDN3) complexes migrate as a single protein peak when analyzed by analytical size exclusion chromatography (SEC) (FIG. 9C and FIG. 9D). These results indicate that the two different PD-L1 TxM

complexes can be produced and purified as soluble proteins with the expected structural properties.

Similar to studies described above, the ability of these fusion protein complexes to bind IL-2Rβ/γ on immune cells and PD-L1 on tumor cells was examined. IL-2Rβ/γ-positive 32Dβ cells were incubated with 4H PD-L1 TxM (2C2), 2H PD-L1 TxM (PDN3) or control ALT-803 complexes. Followed by a wash step, anti-human IL-15 Ab-PE (or isotype control Ab) was added to detect bound TxM/ALT-803 complexes by flow cytometry. As shown in FIG. 10A, 4H PD-L1 TxM (2C2), 2H PD-L1 TxM (PDN3) and ALT-803 proteins were capable of 32Dβ cells compared to the controls. To assess binding to PD-L1, PD-L1-positive 5T33P myeloma cells initially were stained with Brilliant Violet 421 (BV421)-labeled anti-mouse PD-L1 Ab (10F.9G2). Specificity of this binding was tested by addition of purified 4H PD-L1 TxM (2C2) and 2H PD-L1 TxM (PDN3) complexes to block BV421 antibody binding to PD-L1 ligand (FIG. 10B and FIG. 10C). A purified anti-mPD-L1 Ab (NJI6) was used as a positive control. Consistent with its higher avidity, 1 μg of 4H PD-L1 TxM was as effective at blocking anti-PD-L1 Ab staining as 6 μg of 2H PD-L1 TxM. These results confirm that the 4H PD-L1 and 2H PD-L1 TxM complexes retain IL-2Rβ/γ and PD-L1 target binding activity.

As described above, the IL-15 immunostimulatory activity of the mouse-specific 4H PD-L1 and 2H PD-L1 TxM complexes was determined based on proliferation of IL-15-dependent 32Dβ cells. As shown in FIG. 11A and FIG. 11B, a dose dependent increase in 32Dβ cell proliferation was mediated by either 4H PD-L1 and 2H PD-L1 TxM complexes, verifying the immunostimulatory activity of these TxM formats. The 2H PD-L1 TxM complex (PDN-3) exhibits a slight decrease in IL-15 bioactivity compared to ALT-803 whereas the 4H PD-L1 TxM complex (2C2) exhibits approximately a 30-fold decrease in IL-15 bioactivity. This is consistent with the lower IL-15 activity of previous binding domain-IL-15N72D fusion proteins.

Similar studies were conducted on 4H human-specific PD-L1 TxM and 2H human-specific PD-L1 TxM complexes. These proteins were generated by transfecting CHO cells with anti-human PD-L1 scAb/IL-15N72D and anti-human PD-L1 scAb-/IL-15RαSu/Fc expression vectors or IL-15N72D and anti-human PD-L1 scAb-/IL-15RαSu/Fc expression vectors, respectively, followed by purification from cell culture supernatants via Protein A chromatography. Reduced SDS-PAGE analysis confirmed the expected protein bands in the purified 4H human-specific PD-L1 TxM and 2H human-specific PD-L1 TxM preparations (FIG.

12A). Similarly, analytical SEC indicated that the purified 4H human-specific PD-L1 TxM and 2H human-specific PD-L1 TxM complexes migrated as single protein peaks (FIG. 12B and FIG. 12C).

The ability of these fusion protein complexes to bind PD-L1 on tumor cells was examined. PD-L1-positive PC-3 human prostate cancer cells were stained with APC labeled anti-mouse PD-L1 Ab in the presence or absence of 10 nM of purified human-specific 4H PD-L1 TxM, 2H PD-L1 TxM or control 2H anti-CD20 scAb (2B8) TxM complexes (FIG. 13). The results show that the human-specific 4H PD-L1 and 2H PD-L1 TxM complexes were capable of blocking anti-PD-L1 Ab binding to human tumor cells, whereas the control TxM complex was not. Consistent with previous results the 4H PD-L1 TxM complex showed better binding activity than the 2H PD-L1 TxM complexes. These results confirm that the human-specific 4H PD-L1 and 2H PD-L1 TxM complexes retain PD-L1 target binding activity on human tumor cells.

As described above, the IL-15 immunostimulatory activity of the human-specific 4H PD-L1 and 2H PD-L1 TxM complexes was determined based on proliferation of IL-15-dependent 32Dβ cells. As shown in FIG. 14A and FIG. 14B, a dose dependent increase in 32Dβ cell proliferation was mediated by either 4H PD-L1 and 2H PD-L1 TxM complexes, verifying the immunostimulatory activity of these TxM formats. The 2H PD-L1 TxM complex exhibits a slight decrease in IL-15 bioactivity compared to ALT-803 whereas the 4H PD-L1 TxM complex exhibits approximately a 5-fold decrease in IL-15 bioactivity compared to ALT-803. This is consistent with the lower IL-15 activity of previous binding domain-IL-15N72D fusion proteins.

Example 3: Immunostimulatory and Anti-Tumor Activity of PD-L1 TxM In Vitro and In Vivo

The ability of PD-L1 TxM to stimulate immune responses in vivo was assessed in mice. C57BL/6 mice were injected subcutaneously with 200 μl of PBS, ALT-803 (0.4 mg/kg, control), 4H mouse-specific PD-L1 TxM (200 μg, 2C2 (T4M-mPD-L1)), or 2H mouse-specific PD-L1 TxM (200 μg, PDN3 (T2M-mPD-L1)). Three days after treatment, spleens and lymph nodes were collected. Splenocytes and lymphocytes were prepared for flow cytometry following staining of immune cell subsets using anti-CD4, CD8, NK, and CD19 Abs. As shown in FIG. 15A, treatment with ALT-803, 2H PD-L1 TxM and 4H PD-L1 TxM induce an increase in spleen weights consistent with the immunostimulatory activities of these proteins. In particular, 2H PD-

L1 TxM treatment induced a greater increase in spleen weights than 4H PD-L1 TxM, consistent with the difference in IL-15 activity observed with these complexes. Treatment with 2H PD-L1 TxM and 4H PD-L1 TxM also resulted in an increase in the percentage of CD8 T cells and NK cells in the spleen and lymph nodes of mice compared to the PBS control group (FIG. 15B and FIG. 15C). These immune responses are consistent with the IL-15 bioactivity of these TxM complexes.

Additionally, the ability of PD-L1 TxM to stimulate immune cell cytotoxicity against tumor cells was assessed in vitro. PD-L1-positive cells were labeled with CellTrace Violet (Invitrogen) according to the manufacturer's instructions, and cultured with immune effector cells (i.e. splenocytes) at effector:5T33P myeloma target ratio of 10:1 in R10 media (RPMI-1640 with 10% fetal calf serum) at 37°C with 5% CO_2. The effector cells were prepared by stimulation of P-mel mice splenocytes for 3 days with anti-CD3 Ab (2C11: 4 μg/ml). The tumor and effector cells were incubated for 4 days with mouse specific PD-L1 TxM and then analyzed by flow cytometry to determine target cells survival. PBS served as a negative control and ALT-803 (IL-15N72D:IL-15Rα/Fc), anti-PD-L1 Ab and ALT-803 + anti-PD-L1 Ab served as positive controls. As shown in FIG. 16, significant killing of 5T33P tumor cells was found in the group containing 2.1 μg of PD-L1 TxM compared to PBS treatment.

Similar *in vitro* anti-tumor activity was assessed using human-specific 2H PD-L1 TxM and 4H PD-L1 TxM complexes. Human NK cells from two different donors were purified from blood buffy coats with NK cell isolation kit from Stemcell Technologies and used as effector cells. PD-L1-positive human pancreatic tumor cells, SW1990, were labeled with Celltrace-violet and used as target cells. The human NK cells and SW1990 tumor cells were mixed at an E:T ratio of 5:1 in media alone or media containing 50nM anti-human PD-L1 Ab (control), human-specific 2H PD-L1 TxM complex or 4H PD-L1 TxM complex. After 40 hrs, the percent of target cell death was assessed by flow cytometry based on propidium iodide staining of violet-labeled target cells. As shown in FIG. 17, human NK cells incubated with either human-specific 2H PD-L1 TxM or 4H PD-L1 TxM complexes were capable of mediating greater cytotoxicity against PD-L1-positive human tumor cells than untreated NK cells or NK cells treated with anti-human PD-L1 Ab (i.e., traditional ADCC). These results represent a significant improvement in immune cell-mediated targeted anti-tumor activity of the PD-L1 TxM complexes compared to anti-PD-L1 Abs.

An orthotopic 5T33P myeloma model was used to assess the efficacy of PD-L1 TxM in tumor bearing animals. Female C57BL/6NHsd mice (4 mice/group) were injected i.v. with 5T33P myeloma cells (1 x 10^7/mouse) on day 0. Low dose PD-L1 TxM (0.11 mg/kg, a molar equivalent dose to 0.05 mg/kg ALT-803) or high dose PD-L1 TxM (52.5 µg/dose, a molar equivalent dose to 25 µg/dose anti-PD-L1 Ab) was then administered subcutaneously on days 7 and 14. ALT-803 (0.15 mg/kg) and ALT-803 (0.05 mg/kg) + anti-PD-L1 Ab (25 µg/dose) served as positive controls and PBS served as a negative control. Survival (or morbidity due to hind leg paralysis) was monitored as a study endpoint. Clearly, high dose PD-L1 TxM (52.5 µg/mouse) treatment was found to prolong survival of tumor-bearing mice compared to PBS treatment (FIG. 18). This effect was equivalent to that observed with the comparable ALT-803 + anti-PD-L1 Ab combination therapy. No apparent toxicity was observed following PD-L1 TxM treatment of tumor bearing animals.

Additionally, the antitumor activity of PD-L1 TxM complexes was assessed in mice bearing orthotopic MB49luc tumor. C57BL/6NHsd mice (6 mice/group) were instilled intravesically into the bladder with MB49luc bladder tumor cells (3 x 10^4/mouse) on day 0. Tumor bearing mice were treated subcutaneously with mouse-specific 2H PD-L1 TxM (2.8 mg/kg) on days 7, 11, 14 and 18. ALT-803 treatment (0.2 mg/kg, subQ) and ALT-803 (0.2 mg/kg, subQ) + anti-PD-L1 Ab (50 µg/dose, subQ) served as positive controls and PBS served as a negative control. Survival (or morbidity) was monitored as a study endpoint. As shown in FIG. 19, 2H PD-L1 TxM treatment was found to prolong survival of MB49luc tumor-bearing mice compared to PBS treatment. The anti-tumor effects of 2H PD-L1 TxM were as good or better than that observed with the ALT-803 and ALT-803+anti-PD-L1 Ab positive controls.

Example 4: Generation of IL-15-Based Fusion Protein Complexes Comprising Anti-CTLA4 Binding Domains (CTLA4 TxM) and Anti-PD-1 Binding Domains (PD-1 TxM)

Similar to the fusion protein complexes described in Examples 1 – 3, fusion protein complexes of the invention have been generated comprising binding domains that recognize CTLA4 and PD-1. Specifically, constructs were made linking a single-chain anti-CTLA4 antibody to the huIL-15N72D and IL-15RαSu/Fc chains. The anti-CTLA4 single chain antibody (anti-CTLA4scAb) sequence comprises the coding regions of the heavy and light chain V antibody domains antibody linked via a flexible linker sequence. The single chain antibody

domain can be arranged in either the VH-linker-VL or VL-linker-VH format. In some cases, the anti-CTLA4scAb is linked to the C-terminus of the IL-15N72D and/or IL-15RαSu/Fc chains. In other cases, the anti-CTLA4 is linked to the N-terminus of IL-15N72D and/or IL-15RαSu/Fc chains. Anti-CTLA4scAbs specific to either the mouse or human CTLA4 molecules were used in these constructs.

The nucleic acid sequence of anti-human CTLA-4 scAb/huIL-15RαSu/huIgG1 Fc construct (including leader sequence) is as follows (SEQ ID NO: 13):

(Signal peptide)

ATGAAGTGGGTGACCTTTATCTCCCTGCTGTTCCTGTTTTCCTCCGCCTACAGC-

(anti-human CTLA-4 scAb)

(VL)

ATCGTGATGACCCAGTCCCCTAGCTCCCTGAGCGCTAGCGTGGGAGACCGGGTGAC
CATCACCTGTCGGGCCTCCCAGAGCATTTCCAGCTACCTGAACTGGTACCAGCAGAA
GCCCGGCAAGGCCCCTAAGCTGCTGATTTACGCTGCCAGCAGCCTGCAGTCCGGAGT
GCCTCCCAGGTTTAGCGGCTCCGGATCCGGCACCGAGTTCACCCTGACCATCTCCTC
CCTGCAGCCCGAGGACTTCGCCACCTACTACTGTCAGCAGGCCAACAGCTTTCCCCC
CACCTTTGGCCAAGGAACCAAGGTGGACATCAAGAGGACCGTGGCC

(Linker)

GGAGGCGGAGGCTCCGGCGGCGGCGGCTCCGGCGGCGGCGGCTCC

(VH)

CTGGTGCAGTCCGGCGCTGAAGTGAAGAAGCCTGGCGCCTCCGTGAAGGTGTCCTG
CGAGGCCTCCGGCTACACCTTCACCAACTACTACATCCACTGGCTGAGGCAGGCTCC
TGGACAGGGCCTGGAGTGGATGGGCATCATCAACCCCTCCGGAGGCTCCACCACCT
ACGCCCAGAAGTTCCAGGGCAGGATCACCATGACAAGGGACACCTCCACCAACACC
CTGTACATGGAACTGTCCTCCCTCCGGTCCGAGGACACCGCCATCTACTACTGCGCC
AGGAGGGATTGCAGGGGCCCTAGCTGCTACTTCGCTTACTGGGGCCAGGGAACCAC
CGTGACCGTGTCCTCCGCCTCCACCAAGGGC

(Human IL-15R α sushi domain)

ATCACGTGCCCTCCCCCCATGTCCGTGGAACACGCAGACATCTGGGTCAAGAGCTAC
AGCTTGTACTCCAGGGAGCGGTACATTTGTAACTCTGGTTTCAAGCGTAAAGCCGGC

ACGTCCAGCCTGACGGAGTGCGTGTTGAACAAGGCCACGAATGTCGCCCACTGGAC
AACCCCCAGTCTCAAATGCATTAGA

(Human IgG1 CH2-CH3 (Fc) domain)

GAGCCGAAATCTTGTGACAAAACTCACACATGCCCACCGTGCCCAGCACCTGAACT
CCTGGGGGGACCGTCAGTCTTCCTCTTCCCCCCAAAACCCAAGGACACCCTCATGAT
CTCCCGGACCCCTGAGGTCACATGCGTGGTGGTGGACGTGAGCCACGAAGACCCTG
AGGTCAAGTTCAACTGGTACGTGGACGGCGTGGAGGTGCATAATGCCAAGACAAAG
CCGCGGGAGGAGCAGTACAACAGCACGTACCGTGTGGTCAGCGTCCTCACCGTCCT
GCACCAGGACTGGCTGAATGGCAAGGAGTACAAGTGCAAGGTCTCCAACAAAGCCC
TCCCAGCCCCCATCGAGAAAACCATCTCCAAAGCCAAAGGGCAGCCCCGAGAACCA
CAGGTGTACACCCTGCCCCCATCCCGGGATGAGCTGACCAAGAACCAGGTCAGCCT
GACCTGCCTGGTCAAAGGCTTCTATCCCAGCGACATCGCCGTGGAGTGGGAGAGCA
ATGGGCAGCCGGAGAACAACTACAAGACCACGCCTCCCGTGCTGGACTCCGACGGC
TCCTTCTTCCTCTACAGCAAGCTCACCGTGGACAAGAGCAGGTGGCAGCAGGGGAA
CGTCTTCTCATGCTCCGTGATGCATGAGGCTCTGCACAACCACTACACGCAGAAGAG
CCTCTCCCTGTCTCCTGGTAAATAA

The amino acid sequence of the human CTLA-4 scAb/huIL-15RαSu/huIgG1 Fc protein (including leader sequence) is as follows (SEQ ID NO: 14):

(Signal peptide)

MKWVTFISLLFLFSSAYS-

(anti-human CTLA-4 scAb)

(VL)

IVMTQSPSSLSASVGDRVTITCRASQSISSYLNWYQQKPGKAPKLLIYAASSLQSGVPPRF
SGSGSGTEFTLTISSLQPEDFATYYCQQANSFPPTFGQGTKVDIKRTVA

(Linker)

GGGGSGGGGSGGGGS

(VH)

LVQSGAEVKKPGASVKVSCEASGYTFTNYYIHWLRQAPGQGLEWMGIINPSGGSTTYA
QKFQGRITMTRDTSTNTLYMELSSLRSEDTAIYYCARRDCRGPSCYFAYWGQGTTVTVS
SASTKG

(Human IL-15R α sushi domain)

ITCPPPMSVEHADIWVKSYSLYSRERYICNSGFKRKAGTSSLTECVLNKATNVAHWTTPS
LKCIR

(Human IgG1 CH2-CH3 (Fc) domain)

EPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKF
NWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPI
EKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNY
KTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK

In some cases, the leader peptide is cleaved from the mature polypeptide.

Similarly, the nucleic acid sequence of anti-mouse CTLA-4 scAb/huIL-15RαSu/mIgG2a construct (including leader sequence) is as follows (SEQ ID NO: 15):

(Signal peptide)

ATGAAGTGGGTAACCTTTATTTCCCTTCTTTTTCTCTTTAGCTCGGCTTATTCC

(anti-mouse CTLA-4 scAb)

(VL)

GACATCATGATGACCCAGTCCCCTTCCTCCCTGTCCGTGAGCGCTGGCGAGAAGGCT
ACCATCAGCTGCAAGTCCTCCCAGTCCCTGTTCAACAGCAACGCCAAGACCAACTAC
CTGAACTGGTACCTGCAGAAGCCCGGCCAGTCCCCCAAGCTGCTGATCTATTACGCT
AGCACCAGGCATACCGGCGTGCCCGACAGGTTTAGGGGATCCGGCAGCGGCACCGA
CTTCACCCTGACCATCTCCAGCGTGCAGGACGAGGACCTCGCTTTCTACTACTGCCA
GCAATGGTACGATTACCCTTACACCTTCGGCGCTGGCACCAAGGTGGAGATTAAGA
GG

(Linker)

GGCGGAGGCGGATCCGGCGGCGGCGGCTCCGGCGGCGGAGGCTCC

(VH)

CAGATTCAGCTGCAGGAGTCCGGCCCTGGACTGGTCAACCCTAGCCAGTCCCTGAGC
CTGTCCTGTTCCGTGACAGGCTATAGCATCACCAGCGGCTACGGCTGGAACTGGATC
AGGCAGTTTCCCGGCCAGAAAGTGGAGTGGATGGGCTTCATCTACTACGAGGGCTC
CACCTACTATAACCCCTCCATCAAGTCCCGGATCAGCATCACCAGGGATACCTCCAA

GAACCAGTTCTTCCTGCAAGTCAACTCCGTGACCACCGAAGACACCGCCACCTACTA
CTGCGCCAGGCAGACAGGCTACTTCGACTACTGGGGCCAGGGCACAATGGTGACCG
TCAGCAGCGCC

(Human IL-15R α sushi domain)

ATCACGTGCCCTCCCCCCATGTCCGTGGAACACGCAGACATCTGGGTCAAGAGCTAC
AGCTTGTACTCCAGGGAGCGGTACATTTGTAACTCTGGTTTCAAGCGTAAAGCCGGC
ACGTCCAGCCTGACGGAGTGCGTGTTGAACAAGGCCACGAATGTCGCCCACTGGAC
AACCCCCAGTCTCAAATGCATTAGA

(Mouse IgG2a CH2-CH3 domain)

GAACCAAGAGGGCCCACAATCAAGCCCTGTCCTCCATGCAAATGCCCAGCACCTAA
CCTCTTGGGTGGACCATCCGTCTTCATCTTCCCTCCAAAGATCAAGGATGTACTCATG
ATCTCCCTGAGCCCCATAGTCACATGTGTGGTGGTGGATGTGAGCGAGGATGACCCA
GATGTCCAGATCAGCTGGTTTGTGAACAACGTGGAAGTACACACAGCTCAGACACA
AACCCATAGAGAGGATTACAACAGTACTCTCCGGGTGGTCAGTGCCCTCCCCATCCA
GCACCAGGACTGGATGAGTGGCAAGGAGTTCAAATGCAAGGTCAACAACAAAGACC
TCCCAGCGCCCATCGAGAGAACCATCTCAAAACCCAAAGGGTCAGTAAGAGCTCCA
CAGGTATATGTCTTGCCTCCACCAGAAGAAGAGATGACTAAGAAACAGGTCACTCT
GACCTGCATGGTCACAGACTTCATGCCTGAAGACATTTACGTGGAGTGGACCAACA
ACGGGAAAACAGAGCTAAACTACAAGAACACTGAACCAGTCCTGGACTCTGATGGT
TCTTACTTCATGTACAGCAAGCTGAGAGTGGAAAAGAAGAACTGGGTGGAAAGAAA
TAGCTACTCCTGTTCAGTGGTCCACGAGGGTCTGCACAATCACCACACGACTAAGAG
CTTCTCCCGGACTCCAGGTAAATAA

The amino acid sequence of the anti-mouse CTLA-4 scAb/huIL-15RαSu/mIgG2a fusion protein (including leader sequence) is as follows (SEQ ID NO: 16):

(Signal peptide)

MKWVTFISLLFLFSSAYS-

(Anti-mouse CTLA-4 scAb)

(VL)

DIMMTQSPSSLSVSAGEKATISCKSSQSLFNSNAKTNYLNWYLQKPGQSPKLLIYYASTR
HTGVPDRFRGSGSGTDFTLTISSVQDEDLAFYYCQQWYDYPYTFGAGTKVEIKR

(Linker)

GGGGSGGGGSGGGGS

(VH)

QIQLQESGPGLVNPSQSLSLSCSVTGYSITSGYGWNWIRQFPGQKVEWMGFIYYEGSTY
YNPSIKSRISITRDTSKNQFFLQVNSVTTEDTATYYCARQTGYFDYWGQGTMVTVSSA-

(Human IL-15R α sushi domain)

ITCPPPMSVEHADIWVKSYSLYSRERYICNSGFKRKAGTSSLTECVLNKATNVAHWTTPS
LKCIR

(Mouse IgG2a CH2-CH3 domain)

EPRGPTIKPCPPCKCPAPNLLGGPSVFIFPPKIKDVLMISLSPIVTCVVVDVSEDDPDVQIS
WFVNNVEVHTAQTQTHREDYNSTLRVVSALPIQHQDWMSGKEFKCKVNNKDLPAPIER
TISKPKGSVRAPQVYVLPPPEEEMTKKQVTLTCMVTDFMPEDIYVEWTNNGKTELNYK
NTEPVLDSDGSYFMYSKLRVEKKNWVERNSYSCSVVHEGLHNHHTTKSFSRTPGK

In some cases, the leader peptide is cleaved from the mature polypeptide.

As indicated above, the anti-human and mouse CTLA4 scAb domains have also been generated as fusions to the IL-15N72D protein.

Similarly, the nucleic acid sequence of anti-human PD1 scAb/IL-15N72D construct (including signal peptide sequence and stop codon) is as follows (SEQ ID NO: 17):

(Signal peptide)

ATGGAATGGAGCTGGGTGTTCCTGTTCTTTCTGTCCGTGACCACCGGTGTCCACTCC

(Anti-human PD1 scAb)

(VL)

CTGCCTGTGCTGACTCAACCACCCTCGGTGTCTGAAGTCCCCGGGCAGAGGGTCACC
ATTTCCTGTTCTGGAGGCATCTCCAACATCGGAAGCAATGCTGTAAACTGGTACCAG
CACTTCCCAGGAAAGGCTCCCAAACTCCTCATCTATTATAATGATCTGCTGCCCTCA
GGGGTCTCTGACCGATTCTCTGCCTCCAAGTCTGGCACCTCAGCCTCCCTGGCCATC

AGTGGGCTCCGGTCCGAGGATGAGGCTGATTATTACTGTGCAGCATGGGATGACAA TCTGAGTGCTTATGTCTTCGCAACTGGGACCAAGGTCACCGTCCTGAGT

(Linker)

GGAGGTGGCGGATCCGGAGGTGGAGGTTCTGGTGGAGGTGGGAGT

(VH)

CAGGTTCAGCTGGTGCAGTCTGGGGCTGAGGTGAAGAAGCCTGGGGCCTCAGTGAA GGTCTCCTGCAAGGCTTCTGGTTACACCTTTACCAGCTATGGTATCAGCTGGGTGCG ACAGGCCCCTGGACAAGGGCTTGAGTGGATGGGATGGATCAGCGCTTACAATGGTA ACACAAACTATGCACAGAAGCTCCAGGGCAGAGTCACCATGACCACAGACACATCC ACGAGCACAGCCTACATGGAGCTGAGGAGCCTGAGATCTGACGACACGGCCGTGTA TTACTGTGCGAGAGGGTTATACGGTGACGAGGACTACTGGGGCCAGGGAACCCTGG TCACCGTGAGCTCA

(Human IL-15N72D)

AACTGGGTTAACGTAATAAGTGATTTGAAAAAAATTGAAGATCTTATTCAAT CTATGCATATTGATGCTACTTTATATACGGAAAGTGATGTTCACCCCAGTTGCAAAG TAACAGCAATGAAGTGCTTTCTCTTGGAGTTACAAGTTATTTCACTTGAGTCCGGAG ATGCAAGTATTCATGATACAGTAGAAAATCTGATCATCCTAGCAAACGACAGTTTGT CTTCTAATGGGAATGTAACAGAATCTGGATGCAAAGAATGTGAGGAACTGGAGGAA AAAAATATTAAAGAATTTTTGCAGAGTTTTGTACATATTGTCCAAATGTTCATCAAC ACTTCTTAA

The amino acid sequence of the anti-PD1 scAb-IL-15N72D fusion protein (including signal peptide sequence) is as follows (SEQ ID NO: 18):

(Signal peptide)

MEWSWVFLFFLSVTTGVHS-

(Anti-human PD1 scAb)

(*VL*)

LPVLTQPPSVSEVPGQRVTISCSGGISNIGSNAVNWYQHFPGKAPKLLIYYNDLLPSGVSD RFSASKSGTSASLAISGLRSEDEADYYCAAWDDNLSAYVFATGTKVTVLS

(Linker)

GGGGSGGGGSGGGGS

(*VH*)

QVQLVQSGAEVKKPGASVKVSCKASGYTFTSYGISWVRQAPGQGLEWMGWISAYNGN
TNYAQKLQGRVTMTTDTSTSTAYMELRSLRSDDTAVYYCARGLYGDEDYWGQGTLVT
VSS

(Human IL-15N72D)

NWVNVISDLKKIEDLIQSMHIDATLYTESDVHPSCKVTAMKCFLLELQVISLESGDASIH
DTVENLIILANDSLSSNGNVTESGCKECEELEEKNIKEFLQSFVHIVQMFINTS

In some cases, the leader peptide is cleaved from the mature polypeptide. As indicated above, the anti-human PD-1 scAb domain has also been generated as fusions to the huIL-15RαSu/Fc construct.

The sequences were cloned into expression vectors as described in Example 1 and previously (U.S. Patent No. 8,507,222, at Examples 1 and 2, incorporated herein by reference), and the expression vectors transfected into CHO cells. In some cases, the CHO cells were transfected with vectors encoding both huIL-15N72D and huIL-15RαSu/Fc fusion proteins with the same or different (i.e. anti-PD-L1 and anti-CTLA4 scAb) binding domains. The fusion protein complexes were purified from the CHO cell culture supernatant using Protein A affinity chromatography as described above.

In addition of anti-human PD-1 scAb/anti-human CTLA-4 scAb TxM complexes described above, an anti-human PD-L1 scAb/anti-human CTLA-4 scAb TxM complex was generated by co-transfecting CHO cells with expression vectors comprising the anti-human PD-L1 scAb/IL-15N72D (SEQ. ID NO: 1) and anti-human CTLA-4 scAb/huIL-15RαSu/huIgG1 Fc ((SEQ ID NO: 15) constructs. These fusion protein complexes were purified from the CHO cell culture supernatant using Protein A affinity chromatography as described above.

<u>Example 5: Generation of IL-15-Based Fusion Protein Complexes Comprising Other Binding Domains</u>

Similar to the fusion protein complexes described in Examples 1-4, fusion protein complexes of the invention have been generated comprising binding domains that recognize CD47, GITR, ssDNA and other disease related targets (i.e., CD20, CD19, etc).

CD47 is a cell-surface molecule that promotes immune evasion by engaging signal-regulatory protein alpha (SIRPα), which serves as an inhibitory receptor on macrophages. This "don't eat me signal" can be disrupted by blocking the interaction of CD47 and SIRPα, thus restoring antibody-dependent cellular phagocytosis (ADCP) by macrophages. The IL-15 domains of the invention activate and expand NK and $CD8^+$ cells while increasing their cytolytic activity. At high enough concentrations, the Fc region of the invention may interact with Fcγ receptors on NK cells and macrophages for ADCC or ADCP, respectively. This example describes the generation and initial characterization of a fusion protein complex that comprises a Vh region of a nanobody (NbVh; PNAS 2016 113 (19) E2646-E2654) to block the CD47/ SIRPα pathway, activation of NK and $CD8^+$ cells through the IL-15 domain, and allow for tumor clearance via Fc-mediated ADCC/ADCP. As described in detail below, a protein complex comprising an anti-mouse CD47 NbVh/huIL-15N72D and an anti-mouse CD47 NbVh/huIL-15RαSu/mIgG2a Fc was generated.

Specifically, constructs were made linking anti-mouse CD47 NbVh to the huIL-15N72D chains. The anti-mouse CD47 NbVh sequence comprises the coding regions of the heavy chain variable domain of an alpaca nanobody. The anti-mouse CD47 NbVh is linked to the N-terminus of huIL-15N72D. The nucleic acid and protein sequences of a construct comprising the anti-mouse CD47 NbVh linked to the N-terminus of the huIL-15N72D are shown below.

The nucleic acid sequence of the anti-mouse CD47 NbVh/huIL-15N72D construct (including signal peptide sequence) is as follows (SEQ ID NO: 19):

(Signal peptide)

ATGAAGTGGGTGACCTTCATCAGCCTGCTGTTCCTGTTCTCCAGCGCCTACTCC

(Anti-mouse CD47 Vh chain of nanobody)

CAGGTGCAGCTGGTGGAGTCCGGAGGAGGCCTGGTGGAGCCTGGAGGATCCCTGAG
GCTGTCCTGTGCCGCCAGCGGCATCATCTTCAAGATCAACGACATGGGCTGGTATCG
GCAGGCCCCTGGCAAAGGAGGGAGTGGGTGGCCGCTTCCACAGGAGGCGATGAG
GCCATCTACAGGGACTCCGTGAAGGACAGGTTCACCATCTCCAGGGACGCCAAGAA
CTCCGTGTTCCTGCAGATGAACTCCCTGAAGCCCGAGGATACCGCCGTGTACTACTG
CACCGCCGTGATCTCCACCGATAGGGACGGCACCGAGTGGAGGAGGTACTGGGGCC
AGGGCACACAGGTGACTGTGTCCTCCGGCGGC

(Human IL-15N72D)

AACTGGGTTAACGTAATAAGTGATTTGAAAAAAATTGAAGATCTTATTCAATCTATG
CATATTGATGCTACTTTATATACGGAAAGTGATGTTCACCCCAGTTGCAAAGTAACA
GCAATGAAGTGCTTTCTCTTGGAGTTACAAGTTATTTCACTTGAGTCCGGAGATGCA
AGTATTCATGATACAGTAGAAAATCTGATCATCCTAGCAAACGACAGTTTGTCTTCT
AATGGGAATGTAACAGAATCTGGATGCAAAGAATGTGAGGAACTGGAGGAAAAAA
ATATTAAAGAATTTTTGCAGAGTTTTGTACATATTGTCCAAATGTTCATCAACACTTC
TTAA

The amino acid sequence of the anti-mouse CD47 NbVh/IL-15N72D fusion protein (including signal peptide sequence) is as follows (SEQ ID NO: 20):

(Signal peptide)

MKWVTFISLLFLFSSAYS

(Anti-mouse CD47 Vh chain of nanobody)

QVQLVESGGGLVEPGGSLRLSCAASGIIFKINDMGWYRQAPGKRREWVAASTGGDEAI
YRDSVKDRFTISRDAKNSVFLQMNSLKPEDTAVYYCTAVISTDRDGTEWRRYWGQGTQ
VTVSSGG

(Human IL-15N72D)

NWVNVISDLKKIEDLIQSMHIDATLYTESDVHPSCKVTAMKCFLLELQVISLESGDASIH
DTVENLIILANDSLSSNGNVTESGCKECEELEEKNIKEFLQSFVHIVQMFINTS

The nucleic acid sequence of the anti-mouse CD47 NbVh/huIL-15RαSu/mIgG2a Fc construct (including leader sequence) is as follows (SEQ ID NO: 21):

(Signal peptide)

ATGAAGTGGGTGACCTTCATCAGCCTGCTGTTCCTGTTCTCCAGCGCCTACTCC

(Anti-mouse CD47 Vh chain of nanobody)

CAGGTGCAGCTGGTGGAGTCCGGAGGAGGCCTGGTGGAGCCTGGAGGATCCCTGAG
GCTGTCCTGTGCCGCCAGCGGCATCATCTTCAAGATCAACGACATGGGCTGGTATCG
GCAGGCCCCTGGCAAAAGGAGGGAGTGGGTGGCCGCTTCCACAGGAGGCGATGAG

GCCATCTACAGGGACTCCGTGAAGGACAGGTTCACCATCTCCAGGGACGCCAAGAA
CTCCGTGTTCCTGCAGATGAACTCCCTGAAGCCCGAGGATACCGCCGTGTACTACTG
CACCGCCGTGATCTCCACCGATAGGGACGGCACCGAGTGGAGGAGGTACTGGGGCC
AGGGCACACAGGTGACTGTGTCCTCCGGCGGC

(Human IL-15R α sushi domain)

ATCACGTGTCCTCCTCCTATGTCCGTGGAACACGCAGACATCTGGGTCAAGAGCTAC
AGCTTGTACTCCAGGGAGCGGTACATTTGTAACTCTGGTTTCAAGCGTAAAGCCGGC
ACGTCCAGCCTGACGGAGTGCGTGTTGAACAAGGCCACGAATGTCGCCCACTGGAC
AACCCCCAGTCTCAAATGCATTAGA

(Mouse IgG2a CH2-CH3 (Fc) domain)

GAACCAAGAGGGCCCACAATCAAGCCCTGTCCTCCATGCAAATGCCCAGCACCTAA
CCTCTTGGGTGGACCATCCGTCTTCATCTTCCCTCCAAAGATCAAGGATGTACTCATG
ATCTCCCTGAGCCCCATAGTCACATGTGTGGTGGTGGATGTGAGCGAGGATGACCCA
GATGTCCAGATCAGCTGGTTTGTGAACAACGTGGAAGTACACACAGCTCAGACACA
AACCCATAGAGAGGATTACAACAGTACTCTCCGGGTGGTCAGTGCCCTCCCCATCCA
GCACCAGGACTGGATGAGTGGCAAGGAGTTCAAATGCAAGGTCAACAACAAAGACC
TCCCAGCGCCCATCGAGAGAACCATCTCAAAACCCAAAGGGTCAGTAAGAGCTCCA
CAGGTATATGTCTTGCCTCCACCAGAAGAAGAGATGACTAAGAAACAGGTCACTCT
GACCTGCATGGTCACAGACTTCATGCCTGAAGACATTTACGTGGAGTGGACCAACA
ACGGGAAAACAGAGCTAAACTACAAGAACACTGAACCAGTCCTGGACTCTGATGGT
TCTTACTTCATGTACAGCAAGCTGAGAGTGGAAAAGAAGAACTGGGTGGAAAGAAA
TAGCTACTCCTGTTCAGTGGTCCACGAGGGTCTGCACAATCACCACACGACTAAGAG
CTTCTCCCGGACTCCAGGTAAA

The amino acid sequence of the anti-mouse CD47 NbVh/huIL-15RαSu/mIgG2a Fc fusion protein (including leader sequence) is as follows (SEQ ID NO: 22):

(Signal peptide)

MKWVTFISLLFLFSSAYS

(Anti-mouse CD47 Vh chain of nanobody)

QVQLVESGGGLVEPGGSLRLSCAASGIIFKINDMGWYRQAPGKRREWVAASTGGDEAI
YRDSVKDRFTISRDAKNSVFLQMNSLKPEDTAVYYCTAVISTDRDGTEWRRYWGQGTQ
VTVSSGG

(Human IL-15R α sushi domain)

ITCPPPMSVEHADIWVKSYSLYSRERYICNSGFKRKAGTSSLTECVLNKATNVAHWTTPS
LKCIR

(Mouse IgG2a CH2-CH3 (Fc) domain)

EPRGPTIKPCPPCKCPAPNLLGGPSVFIFPPKIKDVLMISLSPIVTCVVVDVSEDDPDVQIS
WFVNNVEVHTAQTQTHREDYNSTLRVVSALPIQHQDWMSGKEFKCKVNNKDLPAPIER
TISKPKGSVRAPQVYVLPPPEEEMTKKQVTLTCMVTDFMPEDIYVEWTNNGKTELNYK
NTEPVLDSDGSYFMYSKLRVEKKNWVERNSYSCSVVHEGLHNHHTTKSFSRTPGK

As indicated above, in some cases, the leader peptide is cleaved from the mature polypeptide.

Similar constructs were generated using a single chain antibody domain derived from an antibody specific to human CD47. The nucleic acid sequence of the anti-human CD47 scAb/huIL-15N72D construct (including signal peptide sequence) is as follows (SEQ ID NO: 23):

(Signal peptide)

ATGAAGTGGGTGACCTTCATCAGCCTGCTGTTCCTGTTCTCCAGCGCCTACTCC

(Anti-human CD47 scAb)

(VL)

AACATCCAGATGACCCAGTCCCCTTCCGCCATGAGCGCTTCCGTGGGCGACAGGGTG
ACCATCACCTGCAAGGCCTCCCAGGACATCCACAGGTACCTGTCCTGGTTCCAGCAG
AAGCCCGGCAAGGTGCCCAAGCACCTGATCTACAGGGCTAACAGGCTGGTGTCCGG
CGTGCCTTCCAGGTTTTCCGGCTCCGGCTCCGGCACCGAGTTCACCCTGACCATCTCC
AGCCTGCAGCCCGAGGACTTCGCCACCTACTACTGCCTGCAGTACGACGAGTTCCCC
TACACCTTCGGCGGCGGCACCAAGGTGGAGATCAAG

(Linker)

GGAGGTGGCGGATCCGGAGGTGGAGGTTCTGGTGGAGGTGGGAGT

(VH)

CAGATGCAGCTGGTACAGTCCGGCGCCGAGGTGAAGAAGACCGGCTCCAGCGTGAA
GGTGTCCTGCAAGGCCTCCGGCTTCAACATCAAGGACTACTACCTGCACTGGGTGAG
GCAGGCCCCTGGACAAGCCCTGGAGTGGATGGGCTGGATCGACCCCGACAACGGCG
ACACCGAGTACGCCCAGAAGTTCCAGGACAGGGTGACCATCACCAGGGACAGGTCC
ATGAGCACCGCCTACATGGAGCTGTCCTCCCTGAGGTCCGAGGACACCGCCATGTAC
TACTGCAACGCCGCCTACGGCTCCTCCTCCTACCCCATGGACTACTGGGGCCAGGGC
ACCACCGTGACCGTG

(Human IL-15N72D)

AACTGGGTTAACGTAATAAGTGATTTGAAAAAAATTGAAGATCTTATTCAATCTATG
CATATTGATGCTACTTTATATACGGAAAGTGATGTTCACCCCAGTTGCAAAGTAACA
GCAATGAAGTGCTTTCTCTTGGAGTTACAAGTTATTTCACTTGAGTCCGGAGATGCA
AGTATTCATGATACAGTAGAAAATCTGATCATCCTAGCAAACGACAGTTTGTCTTCT
AATGGGAATGTAACAGAATCTGGATGCAAAGAATGTGAGGAACTGGAGGAAAAAA
ATATTAAAGAATTTTTGCAGAGTTTTGTACATATTGTCCAAATGTTCATCAACACTTC
TTAA

The amino acid sequence of the anti-human CD47 scAb/huIL-15N72D fusion protein (including signal peptide sequence) is as follows (SEQ ID NO: 24):

(Signal peptide)

MKWVTFISLLFLFSSAYS

(Anti-human CD47 scAb)

(VL)

NIQMTQSPSAMSASVGDRVTITCKASQDIHRYLSWFQQKPGKVPKHLIYRANRLVSGVP
SRFSGSGSGTEFTLTISSLQPEDFATYYCLQYDEFPYTFGGGTKVEIK

(Linker)

GGGGSGGGGSGGGGS

(VH)

QMQLVQSGAEVKKTGSSVKVSCKASGFNIKDYYLHWVRQAPGQALEWMGWIDPDNG
DTEYAQKFQDRVTITRDRSMSTAYMELSSLRSEDTAMYYCNAAYGSSSYPMDYWGQG
TTVTV

(human IL-15N72D)

NWVNVISDLKKIEDLIQSMHIDATLYTESDVHPSCKVTAMKCFLLELQVISLESGDASIH
DTVENLIILANDSLSSNGNVTESGCKECEELEEKNIKEFLQSFVHIVQMFINTS

The nucleic acid sequence of the anti-human CD47 scAb /huIL-15RαSu/hIgG1 Fc construct (including leader sequence) is as follows (SEQ ID NO: 25):

(Signal peptide)

ATGAAGTGGGTGACCTTCATCAGCCTGCTGTTCCTGTTCTCCAGCGCCTACTCC

(Anti-human CD47 scAb)

(VL)

AACATCCAGATGACCCAGTCCCCTTCCGCCATGAGCGCTTCCGTGGGCGACAGGGTG
ACCATCACCTGCAAGGCCTCCCAGGACATCCACAGGTACCTGTCCTGGTTCCAGCAG
AAGCCCGGCAAGGTGCCCAAGCACCTGATCTACAGGGCTAACAGGCTGGTGTCCGG
CGTGCCTTCCAGGTTTTCCGGCTCCGGCTCCGGCACCGAGTTCACCCTGACCATCTCC
AGCCTGCAGCCCGAGGACTTCGCCACCTACTACTGCCTGCAGTACGACGAGTTCCCC
TACACCTTCGGCGGCGGCACCAAGGTGGAGATCAAG

(Linker)

GGAGGTGGCGGATCCGGAGGTGGAGGTTCTGGTGGAGGTGGGAGT

(VH)

CAGATGCAGCTGGTACAGTCCGGCGCCGAGGTGAAGAAGACCGGCTCCAGCGTGAA
GGTGTCCTGCAAGGCCTCCGGCTTCAACATCAAGGACTACTACCTGCACTGGGTGAG
GCAGGCCCCTGGACAAGCCCTGGAGTGGATGGGCTGGATCGACCCCGACAACGGCG
ACACCGAGTACGCCCAGAAGTTCCAGGACAGGGTGACCATCACCAGGGACAGGTCC
ATGAGCACCGCCTACATGGAGCTGTCCTCCCTGAGGTCCGAGGACACCGCCATGTAC
TACTGCAACGCCGCCTACGGCTCCTCCTCCTACCCCATGGACTACTGGGGCCAGGGC
ACCACCGTGACCGTG

(Human IL-15R α sushi domain)

ATCACGTGTCCTCCTCCTATGTCCGTGGAACACGCAGACATCTGGGTCAAGAGCTAC
AGCTTGTACTCCAGGGAGCGGTACATTTGTAACTCTGGTTTCAAGCGTAAAGCCGGC

ACGTCCAGCCTGACGGAGTGCGTGTTGAACAAGGCCACGAATGTCGCCCACTGGAC
AACCCCCAGTCTCAAATGCATTAGA

(Human IgG1 CH2-CH3 (Fc) domain)

GAGCCGAAATCTTGTGACAAAACTCACACATGCCCACCGTGCCCAGCACCTGAACT
CCTGGGGGGACCGTCAGTCTTCCTCTTCCCCCCAAAACCCAAGGACACCCTCATGAT
CTCCCGGACCCCTGAGGTCACATGCGTGGTGGTGGACGTGAGCCACGAAGACCCTG
AGGTCAAGTTCAACTGGTACGTGGACGGCGTGGAGGTGCATAATGCCAAGACAAAG
CCGCGGGAGGAGCAGTACAACAGCACGTACCGTGTGGTCAGCGTCCTCACCGTCCT
GCACCAGGACTGGCTGAATGGCAAGGAGTACAAGTGCAAGGTCTCCAACAAAGCCC
TCCCAGCCCCCATCGAGAAAACCATCTCCAAAGCCAAAGGGCAGCCCCGAGAACCA
CAGGTGTACACCCTGCCCCCATCCCGGGATGAGCTGACCAAGAACCAGGTCAGCCT
GACCTGCCTGGTCAAAGGCTTCTATCCCAGCGACATCGCCGTGGAGTGGGAGAGCA
ATGGGCAGCCGGAGAACAACTACAAGACCACGCCTCCCGTGCTGGACTCCGACGGC
TCCTTCTTCCTCTACAGCAAGCTCACCGTGGACAAGAGCAGGTGGCAGCAGGGGAA
CGTCTTCTCATGCTCCGTGATGCATGAGGCTCTGCACAACCACTACACGCAGAAGAG
CCTCTCCCTGTCTCCTGGTAAATAA

The amino acid sequence of the anti-human CD47 scAb /huIL-15RαSu/hIgG1 Fc fusion protein (including leader sequence) is as follows (SEQ ID NO: 26):

(Signal peptide)

MKWVTFISLLFLFSSAYS

(Anti-human CD47 scAb)

(VL)

NIQMTQSPSAMSASVGDRVTITCKASQDIHRYLSWFQQKPGKVPKHLIYRANRLVSGVP
SRFSGSGSGTEFTLTISSLQPEDFATYYCLQYDEFPYTFGGGTKVEIK

(Linker)

GGGGSGGGGSGGGGS

(VH)

QMQLVQSGAEVKKTGSSVKVSCKASGFNIKDYYLHWVRQAPGQALEWMGWIDPDNG
DTEYAQKFQDRVTITRDRSMSTAYMELSSLRSEDTAMYYCNAAYGSSSYPMDYWGQG
TTVTV

(Human IL-15R α sushi domain)

ITCPPPMSVEHADIWVKSYSLYSRERYICNSGFKRKAGTSSLTECVLNKATNVAHWTTPS
LKCIR

(Human IgG1 CH2-CH3 (Fc) domain)

EPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKF
NWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPI
EKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNY
KTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK

Interactions between GITR ligand and GITR are known to provide stimulatory signaling to immune cells, thus GITR ligand (GITRL) is known to be an immune agonist molecule that could potentially act synergistically with the immune stimulatory activity of IL-15. Thus, constructs were made linking human GIRTL to the huIL-15N72D chains.

The nucleic acid sequence of the human GITRL/huIL-15N72D construct (including signal peptide sequence) is as follows (SEQ ID NO: 27):

(Signal peptide)

ATGAAGTGGGTGACCTTCATCAGCCTGCTGTTCCTGTTCTCCAGCGCCTACTCC

(Human GITRL)

ACCGCCAAGGAGCCCTGCATGGCCAAGTTCGGCCCTCTGCCCTCCAAGTGGCAGAT
GGCCTCCTCCGAGCCTCCCTGTGTGAACAAGGTGTCCGACTGGAAGCTGGAGATCCT
GCAGAACGGCCTGTACCTGATCTACGGCCAGGTGGCCCCCAACGCCAACTACAACG
ACGTGGCCCCCTTCGAGGTGCGGCTGTACAAGAACAAGGACATGATCCAGACCCTG
ACCAACAAGTCCAAGATCCAGAACGTGGGCGGCACCTATGAGCTGCACGTGGGCGA
CACCATCGACCTGATCTTCAACTCCGAGCACCAGGTGCTGAAGAACAACACCTACTG
GGGCATC

(Human IL-15N72D)

AACTGGGTTAACGTAATAAGTGATTTGAAAAAAATTGAAGATCTTATTCAATCTATG
CATATTGATGCTACTTTATATACGGAAAGTGATGTTCACCCCAGTTGCAAAGTAACA
GCAATGAAGTGCTTTCTCTTGGAGTTACAAGTTATTTCACTTGAGTCCGGAGATGCA
AGTATTCATGATACAGTAGAAAATCTGATCATCCTAGCAAACGACAGTTTGTCTTCT
AATGGGAATGTAACAGAATCTGGATGCAAAGAATGTGAGGAACTGGAGGAAAAA
ATATTAAAGAATTTTTGCAGAGTTTTGTACATATTGTCCAAATGTTCATCAACACTTC
TTAA

The amino acid sequence of the human GITRL/IL-15N72D fusion protein (including signal peptide sequence) is as follows (SEQ ID NO: 28):

(Signal peptide)
MKWVTFISLLFLFSSAYS
(Human GITRL)
TAKEPCMAKFGPLPSKWQMASSEPPCVNKVSDWKLEILQNGLYLIYGQVAPNANYNDV
APFEVRLYKNKDMIQTLTNKSKIQNVGGTYELHVGDTIDLIFNSEHQVLKNNTYWGI
(Human IL-15N72D)
NWVNVISDLKKIEDLIQSMHIDATLYTESDVHPSCKVTAMKCFLLELQVISLESGDASIH
DTVENLIILANDSLSSNGNVTESGCKECEELEEKNIKEFLQSFVHIVQMFINTS

The nucleic acid sequence of the human GITRL/huIL-15RαSu/hIgG1 Fc construct (including leader sequence) is as follows (SEQ ID NO: 29):

(Signal peptide)
ATGAAGTGGGTGACCTTCATCAGCCTGCTGTTCCTGTTCTCCAGCGCCTACTCC
(Human GITRL)
ACCGCCAAGGAGCCCTGCATGGCCAAGTTCGGCCCTCTGCCCTCCAAGTGGCAGAT
GGCCTCCTCCGAGCCTCCCTGTGTGAACAAGGTGTCCGACTGGAAGCTGGAGATCCT
GCAGAACGGCCTGTACCTGATCTACGGCCAGGTGGCCCCCAACGCCAACTACAACG
ACGTGGCCCCCTTCGAGGTGCGGCTGTACAAGAACAAGGACATGATCCAGACCCTG
ACCAACAAGTCCAAGATCCAGAACGTGGGCGGCACCTATGAGCTGCACGTGGGCGA

CACCATCGACCTGATCTTCAACTCCGAGCACCAGGTGCTGAAGAACAACACCTACTG
GGGCATC

(Human IL-15R α sushi domain)

ATCACGTGTCCTCCTCCTATGTCCGTGGAACACGCAGACATCTGGGTCAAGAGCTAC
AGCTTGTACTCCAGGGAGCGGTACATTTGTAACTCTGGTTTCAAGCGTAAAGCCGGC
ACGTCCAGCCTGACGGAGTGCGTGTTGAACAAGGCCACGAATGTCGCCCACTGGAC
AACCCCCAGTCTCAAATGCATTAGA

(Human IgG1 CH2-CH3 (Fc) domain)

GAGCCGAAATCTTGTGACAAAACTCACACATGCCCACCGTGCCCAGCACCTGAACT
CCTGGGGGGACCGTCAGTCTTCCTCTTCCCCCCAAAACCCAAGGACACCCTCATGAT
CTCCCGGACCCCTGAGGTCACATGCGTGGTGGTGGACGTGAGCCACGAAGACCCTG
AGGTCAAGTTCAACTGGTACGTGGACGGCGTGGAGGTGCATAATGCCAAGACAAAG
CCGCGGGAGGAGCAGTACAACAGCACGTACCGTGTGGTCAGCGTCCTCACCGTCCT
GCACCAGGACTGGCTGAATGGCAAGGAGTACAAGTGCAAGGTCTCCAACAAAGCCC
TCCCAGCCCCCATCGAGAAAACCATCTCCAAAGCCAAAGGGCAGCCCCGAGAACCA
CAGGTGTACACCCTGCCCCCATCCCGGGATGAGCTGACCAAGAACCAGGTCAGCCT
GACCTGCCTGGTCAAAGGCTTCTATCCCAGCGACATCGCCGTGGAGTGGGAGAGCA
ATGGGCAGCCGGAGAACAACTACAAGACCACGCCTCCCGTGCTGGACTCCGACGGC
TCCTTCTTCCTCTACAGCAAGCTCACCGTGGACAAGAGCAGGTGGCAGCAGGGGAA
CGTCTTCTCATGCTCCGTGATGCATGAGGCTCTGCACAACCACTACACGCAGAAGAG
CCTCTCCCTGTCTCCTGGTAAATAA

The amino acid sequence of the human GITRL/huIL-15RαSu/hIgG1 Fc fusion protein (including leader sequence) is as follows (SEQ ID NO: 30):

(Signal peptide)

MKWVTFISLLFLFSSAYS

(Human GITRL)

TAKEPCMAKFGPLPSKWQMASSEPPCVNKVSDWKLEILQNGLYLIYGQVAPNANYNDV
APFEVRLYKNKDMIQTLTNKSKIQNVGGTYELHVGDTIDLIFNSEHQVLKNNTYWGI

(Human IL-15R α sushi domain)

ITCPPPMSVEHADIWVKSYSLYSRERYICNSGFKRKAGTSSLTECVLNKATNVAHWTTPS
LKCIR

(Human IgG1 CH2-CH3 (Fc) domain)

EPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKF
NWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPI
EKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNY
KTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK

Fusion protein complexes of the invention could also be generated comprising binding domains that target antigens expressed by diseased cells. Such antigens could comprise single stranded DNA (ssDNA) released by disease cells including tumor cells. Thus, fusion protein complexes of the invention were generated with single chain Ab domains that recognize ssDNA (TNT scAb from Hu51-4 antibody).

The nucleic acid sequence of the TNT scAb/huIL-15N72D construct (including signal peptide sequence) is as follows (SEQ ID NO: 31):

(Signal peptide)

ATGAAGTGGGTGACCTTCATCAGCCTGCTGTTCCTGTTCTCCAGCGCCTACTCC

(TNT scAb)

(VL)

GAGATCGTGCTGACCCAGTCCCCTGCTACCCTGTCCCTGTCCCCTGGCGAGAGGGCT
ACCCTGTCCTGCAGGGCCAGGCAATCCATCTCCAACTACCTGCACTGGTACCAGCAG
AAACCTGGCCAGGCCCCCAGGCTGCTGATCTACTACGCCTCCCAGTCCATCTCCGGC
ATCCCTGACAGGTTCAGCGGATCCGGCTCCGGCACCGACTTCACCCTGACCATCTCC
AGGCTGGAGCCTGAGGACTTCGCCGTGTACTACTGCCAGCAGTCCAACTCCTGGCCT
CTGACCTTCGGCCAGGGCACCAAGGTGGAGATCAAGCGG

(Linker)

GGAGGTGGCGGATCCGGAGGTGGAGGTTCTGGTGGAGGTGGGAGT

(VH)

GAGGTGCAGCTGGTGCAGTCCGGCGCCGAAGTGAAGAAGCCCGGAGCCTCCGTGAA
GGTGTCCTGCAAGGCCTCCGGCTACACCTTCACCAGGTACTGGATGCACTGGGTGAG

GCAGGCCCCTGGACAGGGACTGGAGTGGATCGGCGCCATCTACCCCGGCAACTCCG
ACACCTCCTACAACCAGAAGTTCAAGGGCAAGGCCACCATCACCGCCGACACCTCC
ACCAACACCGCCTACATGGAGCTGTCCTCCCTGAGGTCCGAGGACACCGCCGTGTAC
TACTGCGCTAGGGGCGAGGAGATCGGCGTGAGGAGGTGGTTCGCCTACTGGGGACA
GGGCACCCTGGTGACCGTGTCCAGC

(Human IL-15N72D)

AACTGGGTTAACGTAATAAGTGATTTGAAAAAAATTGAAGATCTTATTCAATCTATG
CATATTGATGCTACTTTATATACGGAAAGTGATGTTCACCCCAGTTGCAAAGTAACA
GCAATGAAGTGCTTTCTCTTGGAGTTACAAGTTATTTCACTTGAGTCCGGAGATGCA
AGTATTCATGATACAGTAGAAAATCTGATCATCCTAGCAAACGACAGTTTGTCTTCT
AATGGGAATGTAACAGAATCTGGATGCAAAGAATGTGAGGAACTGGAGGAAAAA
ATATTAAAGAATTTTTGCAGAGTTTTGTACATATTGTCCAAATGTTCATCAACACTTC
TTAA

The amino acid sequence of the TNT scAb/IL-15N72D fusion protein (including signal peptide sequence) is as follows (SEQ ID NO: 32):

(Signal peptide)

MKWVTFISLLFLFSSAYS

(TNT scAb)

(VL)

EIVLTQSPATLSLSPGERATLSCRARQSISNYLHWYQQKPGQAPRLLIYYASQSISGIPDRF
SGSGSGTDFTLTISRLEPEDFAVYYCQQSNSWPLTFGQGTKVEIKR

(Linker)

GGGGSGGGGSGGGGS

(VH)

EVQLVQSGAEVKKPGASVKVSCKASGYTFTRYWMHWVRQAPGQGLEWIGAIYPGNSD
TSYNQKFKGKATITADTSTNTAYMELSSLRSEDTAVYYCARGEEIGVRRWFAYWGQGT
LVTVSS

(Human IL-15N72D)

NWVNVISDLKKIEDLIQSMHIDATLYTESDVHPSCKVTAMKCFLLELQVISLESGDASIH
DTVENLIILANDSLSSNGNVTESGCKECEELEEKNIKEFLQSFVHIVQMFINTS

The nucleic acid sequence of the TNT/huIL-15RαSu/hIgG1 Fc construct (including leader sequence) is as follows (SEQ ID NO: 33):

(Signal peptide)
ATGAAGTGGGTGACCTTCATCAGCCTGCTGTTCCTGTTCTCCAGCGCCTACTCC
(TNT scAb)
(VL)
GAGATCGTGCTGACCCAGTCCCCTGCTACCCTGTCCCTGTCCCCTGGCGAGAGGGCT
ACCCTGTCCTGCAGGGCCAGGCAATCCATCTCCAACTACCTGCACTGGTACCAGCAG
AAACCTGGCCAGGCCCCCAGGCTGCTGATCTACTACGCCTCCCAGTCCATCTCCGGC
ATCCCTGACAGGTTCAGCGGATCCGGCTCCGGCACCGACTTCACCCTGACCATCTCC
AGGCTGGAGCCTGAGGACTTCGCCGTGTACTACTGCCAGCAGTCCAACTCCTGGCCT
CTGACCTTCGGCCAGGGCACCAAGGTGGAGATCAAGCGG
(Linker)
GGAGGTGGCGGATCCGGAGGTGGAGGTTCTGGTGGAGGTGGGAGT
(VH)
GAGGTGCAGCTGGTGCAGTCCGGCGCCGAAGTGAAGAAGCCCGGAGCCTCCGTGAA
GGTGTCCTGCAAGGCCTCCGGCTACACCTTCACCAGGTACTGGATGCACTGGGTGAG
GCAGGCCCCTGGACAGGGACTGGAGTGGATCGGCGCCATCTACCCCGGCAACTCCG
ACACCTCCTACAACCAGAAGTTCAAGGGCAAGGCCACCATCACCGCCGACACCTCC
ACCAACACCGCCTACATGGAGCTGTCCTCCCTGAGGTCCGAGGACACCGCCGTGTAC
TACTGCGCTAGGGGCGAGGAGATCGGCGTGAGGAGGTGGTTCGCCTACTGGGGACA
GGGCACCCTGGTGACCGTGTCCAGC
(Human IL-15R α sushi domain)
ATCACGTGTCCTCCTCCTATGTCCGTGGAACACGCAGACATCTGGGTCAAGAGCTAC
AGCTTGTACTCCAGGGAGCGGTACATTTGTAACTCTGGTTTCAAGCGTAAAGCCGGC
ACGTCCAGCCTGACGGAGTGCGTGTTGAACAAGGCCACGAATGTCGCCCACTGGAC
AACCCCCAGTCTCAAATGCATTAGA

(Human IgG1 CH2-CH3 (Fc) domain)

GAGCCGAAATCTTGTGACAAAACTCACACATGCCCACCGTGCCCAGCACCTGAACT
CCTGGGGGGACCGTCAGTCTTCCTCTTCCCCCCAAAACCCAAGGACACCCTCATGAT
CTCCCGGACCCCTGAGGTCACATGCGTGGTGGTGGACGTGAGCCACGAAGACCCTG
AGGTCAAGTTCAACTGGTACGTGGACGGCGTGGAGGTGCATAATGCCAAGACAAAG
CCGCGGGAGGAGCAGTACAACAGCACGTACCGTGTGGTCAGCGTCCTCACCGTCCT
GCACCAGGACTGGCTGAATGGCAAGGAGTACAAGTGCAAGGTCTCCAACAAAGCCC
TCCCAGCCCCCATCGAGAAAACCATCTCCAAAGCCAAAGGGCAGCCCCGAGAACCA
CAGGTGTACACCCTGCCCCCATCCCGGGATGAGCTGACCAAGAACCAGGTCAGCCT
GACCTGCCTGGTCAAAGGCTTCTATCCCAGCGACATCGCCGTGGAGTGGGAGAGCA
ATGGGCAGCCGGAGAACAACTACAAGACCACGCCTCCCGTGCTGGACTCCGACGGC
TCCTTCTTCCTCTACAGCAAGCTCACCGTGGACAAGAGCAGGTGGCAGCAGGGGAA
CGTCTTCTCATGCTCCGTGATGCATGAGGCTCTGCACAACCACTACACGCAGAAGAG
CCTCTCCCTGTCTCCTGGTAAATAA

The amino acid sequence of the TNT scAb/huIL-15RαSu/hIgG1 Fc fusion protein (including leader sequence) is as follows (SEQ ID NO: 34):

(Signal peptide)

MKWVTFISLLFLFSSAYS

(TNT scAb)

(VL)

EIVLTQSPATLSLSPGERATLSCRARQSISNYLHWYQQKPGQAPRLLIYYASQSISGIPDRF
SGSGSGTDFTLTISRLEPEDFAVYYCQQSNSWPLTFGQGTKVEIKR

(Linker)

GGGGSGGGGSGGGGS

(VH)

EVQLVQSGAEVKKPGASVKVSCKASGYTFTRYWMHWVRQAPGQGLEWIGAIYPGNSD
TSYNQKFKGKATITADTSTNTAYMELSSLRSEDTAVYYCARGEEIGVRRWFAYWGQGT
LVTVSS

(Human IL-15R α sushi domain)

ITCPPPMSVEHADIWVKSYSLYSRERYICNSGFKRKAGTSSLTECVLNKATNVAHWTTPS
LKCIR

(Human IgG1 CH2-CH3 (Fc) domain)
EPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKF
NWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPI
EKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNY
KTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK

Fusion protein complexes of the invention could also be generated comprising binding domains that target other antigens expressed by diseased cells. Such antigens could comprise tissue factor or CD33 expressed on disease cells including tumor cells or checkpoint inhibitors expressed on immune cells.

Tissue Factor (TF) is a transmembrane glycoprotein reported to be overexpressed in several tumor cell types. Importantly, increased TF expression has been implicated in cancer cell signaling, tumor cell migration, and decreased apoptosis leading to enhanced prospect of metastasis. Therefore, targeting of TF may be beneficial in immunotherapeutic strategies against tumor cell types that overexpress this protein. A chimeric anti-tissue factor antibody, ALT-836, has been previously generated and clinically tested. Humanized variable chains of this antibody (hOAT) have also been characterized. Thus, fusion protein complexes of the invention were generated with single chain Ab domains that recognize human tissue factor (hOAT scAb).

The nucleic acid sequence of the hOAT scAb/huIL-15RαSu/hIgG1 Fc construct (including leader sequence) is as follows (SEQ ID NO: 35):

(Signal peptide)
ATGAAGTGGGTGACCTTCATCAGCCTGCTGTTCCTGTTCTCCAGCGCCTACTCC

(hOAT scAb)

(VL)
GACATCCAGATGACCCAGTCCCCTGCTTCCCTGTCCGCTTCCGTGGGCGACAGGGTG
ACCATCACCTGCCTGGCCTCCCAGACCATCGACACCTGGCTGGCCTGGTACCTGCAG
AAGCCCGGCAAGTCCCCCCAGCTGCTGATCTACGCCGCTACCAACCTGGCCGACGG
CGTGCCTAGCAGGTTTTCCGGCTCCGGCTCCGGCACCGACTTCTCCTTCACCATCTCC

TCCCTGCAGCCCGAGGACTTCGCCACCTACTACTGCCAGCAGGTGTACTCCTCCCCC
TTCACCTTCGGCCAGGGCACCAAGCTGGAGATCAAG

(Linker)

GGAGGTGGCGGATCCGGAGGTGGAGGTTCTGGTGGAGGTGGGAGT

(VH)

CAGATCCAGCTGGTGCAGTCCGGCGGCGAAGTGAAAAAGCCCGGCGCCAGCGTGAG
GGTGTCCTGTAAGGCCTCCGGCTACTCCTTCACCGACTACAACGTGTACTGGGTGAG
GCAGTCCCCCGGCAAGGGACTGGAGTGGATCGGCTACATCGACCCCTACAACGGCA
TCACCATCTACGACCAGAACTTCAAGGGCAAGGCCACCCTGACCGTGGACAAGTCC
ACCTCCACAGCCTACATGGAGCTGTCCTCCCTGAGGTCCGAGGACACCGCCGTGTAC
TTCTGCGCCAGGGACGTGACCACCGCTCTGGACTTCTGGGGACAGGGCACCACCGT
GACCGTGAGCTCC

(Human IL-15R α sushi domain)

ATCACGTGTCCTCCTCCTATGTCCGTGGAACACGCAGACATCTGGGTCAAGAGCTAC
AGCTTGTACTCCAGGGAGCGGTACATTTGTAACTCTGGTTTCAAGCGTAAAGCCGGC
ACGTCCAGCCTGACGGAGTGCGTGTTGAACAAGGCCACGAATGTCGCCCACTGGAC
AACCCCCAGTCTCAAATGCATTAGA

(Human IgG1 CH2-CH3 (Fc) domain)

GAGCCGAAATCTTGTGACAAAACTCACACATGCCCACCGTGCCCAGCACCTGAACT
CCTGGGGGGACCGTCAGTCTTCCTCTTCCCCCCAAAACCCAAGGACACCCTCATGAT
CTCCCGGACCCCTGAGGTCACATGCGTGGTGGTGGACGTGAGCCACGAAGACCCTG
AGGTCAAGTTCAACTGGTACGTGGACGGCGTGGAGGTGCATAATGCCAAGACAAAG
CCGCGGGAGGAGCAGTACAACAGCACGTACCGTGTGGTCAGCGTCCTCACCGTCCT
GCACCAGGACTGGCTGAATGGCAAGGAGTACAAGTGCAAGGTCTCCAACAAAGCCC
TCCCAGCCCCCATCGAGAAAACCATCTCCAAAGCCAAAGGGCAGCCCCGAGAACCA
CAGGTGTACACCCTGCCCCCATCCCGGGATGAGCTGACCAAGAACCAGGTCAGCCT
GACCTGCCTGGTCAAAGGCTTCTATCCCAGCGACATCGCCGTGGAGTGGGAGAGCA
ATGGGCAGCCGGAGAACAACTACAAGACCACGCCTCCCGTGCTGGACTCCGACGGC
TCCTTCTTCCTCTACAGCAAGCTCACCGTGGACAAGAGCAGGTGGCAGCAGGGGAA
CGTCTTCTCATGCTCCGTGATGCATGAGGCTCTGCACAACCACTACACGCAGAAGAG
CCTCTCCCTGTCTCCTGGTAAA

The amino acid sequence of the hOAT scAb/ huIL-15RαSu/hIgG1 Fc fusion protein (including signal peptide sequence) is as follows (SEQ ID NO: 36):

(Signal peptide)

MKWVTFISLLFLFSSAYS

(hOAT scAb)

(VL)

DIQMTQSPASLSASVGDRVTITCLASQTIDTWLAWYLQKPGKSPQLLIYAATNLADGVPS
RFSGSGSGTDFSFTISSLQPEDFATYYCQQVYSSPFTFGQGTKLEIK

(Linker)

GGGGSGGGGSGGGGS

(VH)

QIQLVQSGGEVKKPGASVRVSCKASGYSFTDYNVYWVRQSPGKGLEWIGYIDPYNGITI
YDQNFKGKATLTVDKSTSTAYMELSSLRSEDTAVYFCARDVTTALDFWGQGTTVTVSS

(Human IL-15R α sushi domain)

ITCPPPMSVEHADIWVKSYSLYSRERYICNSGFKRKAGTSSLTECVLNKATNVAHWTTPS
LKCIR

(Human IgG1 CH2-CH3 (Fc) domain)

EPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKF
NWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPI
EKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNY
KTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK

In some cases, the leader peptide is cleaved from the mature polypeptide.

Similar constructs could be generated to express the hOAT scAb/huIL-15N72D fusion protein.

The sequences were cloned into expression vectors as described in Example 1 and previously (U.S. Patent No. 8,507,222, at Examples 1 and 2, incorporated herein by reference), and the expression vectors transfected into CHO cells. In some cases, the CHO cells were transfected with vectors encoding both huIL-15N72D and huIL-15RαSu/Fc fusion proteins with

the same or different binding domains of the invention. The fusion protein complexes were purified from the CHO cell culture supernatant using Protein A affinity chromatography as described above.

Fusion protein complexes of the invention were also generated with single chain Ab domains that recognize CD33 (CD33 scAb). The nucleic acid sequence of the CD33 scAb/huIL-15N72D construct (including signal peptide sequence) is as follows (SEQ ID NO: 37):

(Signal peptide)

ATGGATTTTCAGGTGCAGATTATCAGCTTCCTGCTAATCAGTGCTTCAGTCATAATGT
CAAGAGGA

(CD33 scAb)

(VL)

CAGGTGCAGCTGGTTCAGAGCGGTGCGGAAGTTAAAAAGCCGGGCTCTTCCGTGAA
AGTTAGCTGCAAAGCGTCTGGTTATACCTTCACCGACTACAACATGCACTGGGTCCG
CCAGGCCCCAGGCCAGGGTCTGGAATGGATCGGTTATATTTACCCGTACAACGGTGG
CACGGGATATAACCAGAAATTCAAATCCAAAGCTACCATCACTGCGGACGAAAGCA
CCAACACCGCATATATGGAATTGTCTTCTCTGCGTAGCGAAGATACCGCGGTTTACT
ATTGCGCTCGTGGTCGTCCAGCGATGGATTACTGGGGTCAGGGCACCCTGGTGACCG
TGAGCTCT

(Linker)

GGCGGAGGCGGATCTGGTGGTGGCGGATCCGGTGGAGGCGGAAGC

(VH)

GATATCCAGATGACCCAGTCCCCGAGCTCCCTGTCTGCCAGCGTGGGCGACCGCGTG
ACTATCACCTGCCGTGCGTCCGAAAGCGTGGATAACTACGGCATTTCCTTTATGAAC
TGGTTCCAGCAGAAACCGGGTAAAGCCCCGAAACTGCTGATTTATGCGGCCTCTAAC
CAGGGCAGCGGTGTGCCGAGCCGCTTTTCCGGCAGCGGTTCGGGGACCGATTTCACT
CTGACCATTTCTAGCCTGCAGCCAGATGACTTCGCGACCTACTACTGCCAACAGTCT
AAAGAAGTTCCGTGGACCTTCGGTCAGGGTACCAAAGTTGAAATTAAA

(Human IL-15N72D)

AACTGGGTTAACGTAATAAGTGATTTGAAAAAAATTGAAGATCTTATTCAATCTATG
CATATTGATGCTACTTTATATACGGAAAGTGATGTTCACCCCAGTTGCAAAGTAACA

GCAATGAAGTGCTTTCTCTTGGAGTTACAAGTTATTTCACTTGAGTCCGGAGATGCA
AGTATTCATGATACAGTAGAAAATCTGATCATCCTAGCAAACGACAGTTTGTCTTCT
AATGGGAATGTAACAGAATCTGGATGCAAAGAATGTGAGGAACTGGAGGAAAAA
ATATTAAAGAATTTTTGCAGAGTTTTGTACATATTGTCCAAATGTTCATCAACACTTC
TTAA

The amino acid sequence of the CD33 scAb/IL-15N72D fusion protein (including signal peptide sequence) is as follows (SEQ ID NO: 38):

(Signal peptide)
MDFQVQIISFLLISASVIMSRG
(CD33 scAb)
(VL)
QVQLVQSGAEVKKPGSSVKVSCKASGYTFTDYNMHWVRQAPGQGLEWIGYIYPYNGG
TGYNQKFKSKATITADESTNTAYMELSSLRSEDTAVYYCARGRPAMDYWGQGTLVTVS
S
(Linker)
GGGGSGGGGSGGGGS
(VH)
DIQMTQSPSSLSASVGDRVTITCRASESVDNYGISFMNWFQQKPGKAPKLLIYAASNQGS
GVPSRFSGSGSGTDFTLTISSLQPDDFATYYCQQSKEVPWTFGQGTKVEIK
(Human IL-15N72D)
NWVNVISDLKKIEDLIQSMHIDATLYTESDVHPSCKVTAMKCFLLELQVISLESGDASIH
DTVENLIILANDSLSSNGNVTESGCKECEELEEKNIKEFLQSFVHIVQMFINTS

In some cases, the leader peptide is cleaved from the mature polypeptide.

The nucleic acid sequence of the CD33 scAb/huIL-15RαSu/hIgG1 Fc construct (including leader sequence) is as follows (SEQ ID NO: 39):

(Signal peptide)

ATGGATTTTCAGGTGCAGATTATCAGCTTCCTGCTAATCAGTGCTTCAGTCATAATGT
CAAGAGGA

(CD33 scAb)

(VL)

CAGGTGCAGCTGGTTCAGAGCGGTGCGGAAGTTAAAAAGCCGGGCTCTTCCGTGAA
AGTTAGCTGCAAAGCGTCTGGTTATACCTTCACCGACTACAACATGCACTGGGTCCG
CCAGGCCCCAGGCCAGGGTCTGGAATGGATCGGTTATATTTACCCGTACAACGGTGG
CACGGGATATAACCAGAAATTCAAATCCAAAGCTACCATCACTGCGGACGAAAGCA
CCAACACCGCATATATGGAATTGTCTTCTCTGCGTAGCGAAGATACCGCGGTTTACT
ATTGCGCTCGTGGTCGTCCAGCGATGGATTACTGGGGTCAGGGCACCCTGGTGACCG
TGAGCTCT

(Linker)

GGCGGAGGCGGATCTGGTGGTGGCGGATCCGGTGGAGGCGGAAGC

(VH)

GATATCCAGATGACCCAGTCCCCGAGCTCCCTGTCTGCCAGCGTGGGCGACCGCGTG
ACTATCACCTGCCGTGCGTCCGAAAGCGTGGATAACTACGGCATTTCCTTTATGAAC
TGGTTCCAGCAGAAACCGGGTAAAGCCCCGAAACTGCTGATTTATGCGGCCTCTAAC
CAGGGCAGCGGTGTGCCGAGCCGCTTTTCCGGCAGCGGTTCGGGGACCGATTTCACT
CTGACCATTTCTAGCCTGCAGCCAGATGACTTCGCGACCTACTACTGCCAACAGTCT
AAAGAAGTTCCGTGGACCTTCGGTCAGGGTACCAAAGTTGAAATTAAA

(Human IL-15R α sushi domain)

ATCACGTGTCCTCCTCCTATGTCCGTGGAACACGCAGACATCTGGGTCAAGAGCTAC
AGCTTGTACTCCAGGGAGCGGTACATTTGTAACTCTGGTTTCAAGCGTAAAGCCGGC
ACGTCCAGCCTGACGGAGTGCGTGTTGAACAAGGCCACGAATGTCGCCCACTGGAC
AACCCCCAGTCTCAAATGCATTAGA

(Human IgG1 C_H2-C_H3 (Fc) domain)

GAGCCGAAATCTTGTGACAAAACTCACACATGCCCACCGTGCCCAGCACCTGAACT
CCTGGGGGGACCGTCAGTCTTCCTCTTCCCCCCAAAACCCAAGGACACCCTCATGAT
CTCCCGGACCCCTGAGGTCACATGCGTGGTGGTGGACGTGAGCCACGAAGACCCTG
AGGTCAAGTTCAACTGGTACGTGGACGGCGTGGAGGTGCATAATGCCAAGACAAAG
CCGCGGGAGGAGCAGTACAACAGCACGTACCGTGTGGTCAGCGTCCTCACCGTCCT

GCACCAGGACTGGCTGAATGGCAAGGAGTACAAGTGCAAGGTCTCCAACAAAGCCC
TCCCAGCCCCCATCGAGAAAACCATCTCCAAAGCCAAAGGGCAGCCCCGAGAACCA
CAGGTGTACACCCTGCCCCCATCCCGGGATGAGCTGACCAAGAACCAGGTCAGCCT
GACCTGCCTGGTCAAAGGCTTCTATCCCAGCGACATCGCCGTGGAGTGGGAGAGCA
ATGGGCAGCCGGAGAACAACTACAAGACCACGCCTCCCGTGCTGGACTCCGACGGC
TCCTTCTTCCTCTACAGCAAGCTCACCGTGGACAAGAGCAGGTGGCAGCAGGGGAA
CGTCTTCTCATGCTCCGTGATGCATGAGGCTCTGCACAACCACTACACGCAGAAGAG
CCTCTCCCTGTCTCCTGGTAAATAA

The amino acid sequence of the CD33 scAb/huIL-15RαSu/hIgG1 Fc fusion protein (including leader sequence) is as follows (SEQ ID NO: 40):

(Signal peptide)
MDFQVQIISFLLISASVIMSRG
(CD33 scAb)
(VL)
QVQLVQSGAEVKKPGSSVKVSCKASGYTFTDYNMHWVRQAPGQGLEWIGYIYPYNGG
TGYNQKFKSKATITADESTNTAYMELSSLRSEDTAVYYCARGRPAMDYWGQGTLVTVS
S
(Linker)
GGGGSGGGGSGGGGS
(VH)
DIQMTQSPSSLSASVGDRVTITCRASESVDNYGISFMNWFQQKPGKAPKLLIYAASNQGS
GVPSRFSGSGSGTDFTLTISSLQPDDFATYYCQQSKEVPWTFGQGTKVEIK
(Human IL-15R α sushi domain)
ITCPPPMSVEHADIWVKSYSLYSRERYICNSGFKRKAGTSSLTECVLNKATNVAHWTTPS
LKCIR
(Human IgG1 CH2-CH3 (Fc) domain)
EPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKF
NWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPI

EKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNY
KTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK

In some cases, the leader peptide is cleaved from the mature polypeptide.

The sequences were cloned into expression vectors as described in Example 1 and previously (U.S. Patent No. 8,507,222, at Examples 1 and 2, incorporated herein by reference), and the expression vectors transfected into CHO cells. In some cases, the CHO cells were transfected with vectors encoding both huIL-15N72D and huIL-15RαSu/Fc fusion proteins with the same or different binding domains of the invention. The fusion protein complexes were purified from the CHO cell culture supernatant using Protein A affinity chromatography as described above.

Intercellular adhesion molecule 1 (ICAM-1) is a cell surface glycoprotein in the immunoglobulin superfamily. It has been demonstrated that the level of ICAM-1 protein expression on the cell surface positively correlated with metastatic potential of various solid tumors. Lymphocyte function-associated antigen 1 (LFA-1) is found on all T-cells and on B-cells, macrophages, neutrophils, and NK cells. It is known to bind to ICAM-1, specifically through the “I domain”, to sustain cellular adhesion (immunological/cytolytic synapse formation) or rolling (to slow the movement of cells in the bloodstream prior to extravasation). The I domain alone can support high affinity binding to ICAM-1 with the addition of two mutations: K287C and K294C. Therefore, a TxM was created comprising the LFA-1 I domain, along with the mutations, in order to target tumors and facilitate activation and localization of effector immune cells via the huIL-15N72D: huIL-15RαSu complex.

The nucleic acid sequence of the human LFA-1 I domain(K287C/K294C)/huIL-15RαSu/huIgG1 Fc construct (including leader sequence) is as follows (SEQ ID NO: 41):

(Signal peptide)

ATGAAGTGGGTGACCTTCATCAGCCTGCTGTTCCTGTTCTCCAGCGCCTACTCC

(human LFA-1 I domain(K287C/K294C))

ATGAAGTGGGTGACCTTCATCAGCCTGCTGTTCCTGTTCTCCAGCGCCTACTC
CGATTTAGTGTTTCTGTTCGACGGCTCCATGTCTTTACAGCCCGATGAGTTCCAGAAG
ATTTTAGACTTCATGAAGGACGTGATGAAGAAACTGTCCAACACCAGCTACCAGTTC

GCTGCCGTGCAGTTCTCCACCTCCTACAAGACCGAGTTCGACTTCTCCGACTACGTG
AAGCGGAAGGACCCCGATGCTTTACTGAAGCACGTCAAGCACATGCTGCTGCTCAC
CAACACCTTTGGCGCCATCAACTACGTGGCCACCGAGGTGTTTCGTGAGGAACTGGG
AGCTCGGCCCGATGCCACCAAGGTGCTGATTATCATCACCGACGGCGAAGCCACCG
ATAGCGGAAACATCGATGCCGCCAAGGACATCATCCGGTACATTATCGGCATCGGC
AAGCACTTCCAGACCAAGGAGAGCCAAGAGACTTTACACAAGTTCGCCTCCAAGCC
CGCTTCCGAGTTCGTGTGCATTTTAGACACCTTCGAGTGTTTAAAGGATTTATTTACC
GAGCTGCAGAAGAAGATCTACGTGATTGAGGGCACCAGCAAGCAAGATCTGACCTC
CTTCAACATGGAGCTGTCCAGCAGCGGCATTTCCGCTGATTTATCTCGTGGCCACGC
C

(Human IL-15R α sushi domain)

ATCACGTGTCCTCCTCCTATGTCCGTGGAACACGCAGACATCTGGGTCAAGAGCTAC
AGCTTGTACTCCAGGGAGCGGTACATTTGTAACTCTGGTTTCAAGCGTAAAGCCGGC
ACGTCCAGCCTGACGGAGTGCGTGTTGAACAAGGCCACGAATGTCGCCCACTGGAC
AACCCCCAGTCTCAAATGCATTAGA

(Human IgG1 CH2-CH3 (Fc) domain)

GAGCCGAAATCTTGTGACAAAACTCACACATGCCCACCGTGCCCAGCACCTGAACT
CCTGGGGGGACCGTCAGTCTTCCTCTTCCCCCCAAAACCCAAGGACACCCTCATGAT
CTCCCGGACCCCTGAGGTCACATGCGTGGTGGTGGACGTGAGCCACGAAGACCCTG
AGGTCAAGTTCAACTGGTACGTGGACGGCGTGGAGGTGCATAATGCCAAGACAAAG
CCGCGGGAGGAGCAGTACAACAGCACGTACCGTGTGGTCAGCGTCCTCACCGTCCT
GCACCAGGACTGGCTGAATGGCAAGGAGTACAAGTGCAAGGTCTCCAACAAAGCCC
TCCCAGCCCCCATCGAGAAAACCATCTCCAAAGCCAAAGGGCAGCCCCGAGAACCA
CAGGTGTACACCCTGCCCCCATCCCGGGATGAGCTGACCAAGAACCAGGTCAGCCT
GACCTGCCTGGTCAAAGGCTTCTATCCCAGCGACATCGCCGTGGAGTGGGAGAGCA
ATGGGCAGCCGGAGAACAACTACAAGACCACGCCTCCCGTGCTGGACTCCGACGGC
TCCTTCTTCCTCTACAGCAAGCTCACCGTGGACAAGAGCAGGTGGCAGCAGGGGAA
CGTCTTCTCATGCTCCGTGATGCATGAGGCTCTGCACAACCACTACACGCAGAAGAG
CCTCTCCCTGTCTCCTGGTAAATAA

The amino acid sequence of the mature human LFA-1 I domain(K287C/K294C)/huIL-15RαSu/huIgG1 Fc fusion protein (including leader sequence) is as follows (SEQ ID NO: 42):

(Signal peptide)
MKWVTFISLLFLFSSAYS
(human LFA-1 I domain(K287C/K294C)
DLVFLFDGSMSLQPDEFQKILDFMKDVMKKLSNTSYQFAAVQFSTSYKTEFDFSDYVKR
KDPDALLKHVKHMLLLTNTFGAINYVATEVFREELGARPDATKVLIIITDGEATDSGNID
AAKDIIRYIIGIGKHFQTKESQETLHKFASKPASEFVCILDTFECLKDLFTELQKKIYVIEGT
SKQDLTSFNMELSSSGISADLSRGHA
(Human IL-15R α sushi domain)
ITCPPPMSVEHADIWVKSYSLYSRERYICNSGFKRKAGTSSLTECVLNKATNVAHWTTPS
LKCIR
(Human IgG1 CH2-CH3 (Fc) domain)
EPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKF
NWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPI
EKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNY
KTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK

In some cases, the leader peptide is cleaved from the mature polypeptide.

Similar constructs could be generated to express the human LFA-1 I domain (K287C/K294C)/huIL-15N72D fusion protein.

The sequences were cloned into expression vectors as described in Example 1 and previously (U.S. Patent No. 8,507,222, at Examples 1 and 2, incorporated herein by reference), and the expression vectors transfected into CHO cells. In some cases, the CHO cells were transfected with vectors encoding both huIL-15N72D and huIL-15RαSu/Fc fusion proteins with the same or different binding domains of the invention. The fusion protein complexes were purified from the CHO cell culture supernatant using Protein A affinity chromatography as described above.

For example. CHO cells were transfected with the huIL-15N72D expression vector. Cells were also transfected with vectors expressing the human LFA-1 I domain(K287C/K294C)

/huIL-15RαSu/huIgG1 Fc construct. Co-expression of the two constructs in CHO cells allowed formation and secretion of a soluble huIL-15N72D: human LFA-1 I domain(K287C/K294C) /huIL-15RαSu/huIgG1 Fc complex (referred to as 2*hLFA1/TxM).

Notch1 is a member of the Type I transmembrane protein family, which shares structural characteristics including an extracellular domain consisting of multiple epidermal growth factor-like (EGF) repeats, and an intracellular domain consisting of multiple different domain types. Its overexpression has been demonstrated in several tumor types making it an attractive target for immunotherapy. Delta-like protein 4 (DLL4) is one several ligands for Notch1 and has been shown to have the highest affinity. Therefore, the extracellular domain of DLL4 (positions 27-529) was used for targeting of Notch1 in the creation of a TxM complex.

The nucleic acid sequence of the hDLL4/huIL-15RαSu/huIgG1 Fc construct (including leader sequence) is as follows (SEQ ID NO: 43):

(Signal peptide)
ATGAAGTGGGTGACCTTCATCAGCCTGCTGTTCCTGTTCTCCAGCGCCTACTCC
(hDLL4)
AGCGGCGTGTTCCAGCTGCAGCTGCAAGAGTTTATCAACGAGAGGGGCGTGCTGGC
TTCCGGTCGTCCTTGTGAGCCCGGTTGTAGGACCTTTTTCCGGGTGTGTTTAAAGCAT
TTTCAAGCTGTGGTGTCCCCCGGACCTTGTACCTTCGGCACCGTGTCCACCCCCGTTC
TGGGCACCAACTCCTTCGCCGTTCGTGACGACAGCTCCGGAGGAGGTCGTAATCCTT
TACAGCTGCCTTTCAACTTTACTTGGCCCGGCACCTTCTCCCTCATCATCGAAGCTTG
GCATGCCCCCGGTGACGATCTGCGGCCCGAAGCTCTGCCCCCCGATGCTTTAATCAG
CAAGATTGCCATTCAAGGTTCTTTAGCCGTGGGCCAGAACTGGCTGCTGGACGAGCA
GACCAGCACACTCACTCGTCTGAGGTACTCCTATCGTGTGATCTGCAGCGACAACTA
CTACGGCGACAATTGCAGCCGGCTGTGCAAGAAGAGGAACGACCACTTCGGCCATT
ACGTCTGCCAGCCCGACGGCAATTTATCTTGTCTGCCCGGTTGGACCGGCGAGTACT
GTCAGCAGCCCATCTGTTTAAGCGGCTGCCACGAGCAGAACGGCTACTGCAGCAAG
CCCGCTGAGTGTCTGTGTAGGCCCGGCTGGCAAGGTAGGCTGTGCAACGAGTGCATC
CCCCACAATGGCTGTCGGCACGGCACTTGTTCCACCCCTTGGCAGTGCACTTGTGAC
GAGGGCTGGGGAGGTTTATTCTGCGACCAAGATCTGAACTACTGCACCCACCACAG
CCCTTGTAAGAACGGAGCTACTTGTTCCAACAGCGGCCAGAGGTCCTACACTTGTAC

TTGTAGGCCCGGTTACACCGGCGTCGACTGCGAACTGGAACTGAGCGAATGCGATA
GCAACCCTTGTCGTAACGGCGGCAGCTGCAAGGACCAAGAAGACGGCTACCACTGT
TTATGCCCTCCCGGATACTACGGTTTACACTGCGAGCACTCCACACTGTCTTGTGCC
GACTCCCCTTGTTTCAACGGCGGAAGCTGTCGTGAGAGGAACCAAGGTGCCAACTA
CGCTTGTGAGTGCCCTCCCAACTTCACCGGCTCCAACTGCGAGAAGAAGGTGGATCG
TTGCACCTCCAACCCTTGCGCCAACGGCGGCCAGTGTTTAAATAGGGGCCCTTCCCG
GATGTGTCGTTGTCGTCCCGGTTTTACCGGCACCTACTGCGAGCTGCACGTCAGCGA
TTGCGCCCGGAATCCTTGCGCTCACGGCGGAACTTGTCACGATTTAGAGAACGGTTT
AATGTGCACTTGTCCCGCTGGATTCAGCGGTCGTAGGTGTGAGGTGAGGACCTCCAT
CGACGCTTGTGCCAGCAGCCCTTGCTTCAATCGTGCCACTTGTTACACCGATTTATCC
ACCGACACCTTCGTGTGCAACTGCCCCTACGGCTTCGTGGGATCTCGTTGCGAGTTC
CCCGTTGGCCTGCCTCCTAGCTTTCCCTGG

(Human IL-15R α sushi domain)

ATCACGTGTCCTCCTCCTATGTCCGTGGAACACGCAGACATCTGGGTCAAGA
GCTACAGCTTGTACTCCAGGGAGCGGTACATTTGTAACTCTGGTTTCAAGCGTAAAG
CCGGCACGTCCAGCCTGACGGAGTGCGTGTTGAACAAGGCCACGAATGTCGCCCAC
TGGACAACCCCCAGTCTCAAATGCATTAGA

(Human IgG1 CH2-CH3 (Fc) domain)

GAGCCGAAATCTTGTGACAAAACTCACACATGCCCACCGTGCCCAGCACCTGAACT
CCTGGGGGGACCGTCAGTCTTCCTCTTCCCCCCAAAACCCAAGGACACCCTCATGAT
CTCCCGGACCCCTGAGGTCACATGCGTGGTGGTGGACGTGAGCCACGAAGACCCTG
AGGTCAAGTTCAACTGGTACGTGGACGGCGTGGAGGTGCATAATGCCAAGACAAAG
CCGCGGGAGGAGCAGTACAACAGCACGTACCGTGTGGTCAGCGTCCTCACCGTCCT
GCACCAGGACTGGCTGAATGGCAAGGAGTACAAGTGCAAGGTCTCCAACAAAGCCC
TCCCAGCCCCCATCGAGAAAACCATCTCCAAAGCCAAAGGGCAGCCCCGAGAACCA
CAGGTGTACACCCTGCCCCCATCCCGGGATGAGCTGACCAAGAACCAGGTCAGCCT
GACCTGCCTGGTCAAAGGCTTCTATCCCAGCGACATCGCCGTGGAGTGGGAGAGCA
ATGGGCAGCCGGAGAACAACTACAAGACCACGCCTCCCGTGCTGGACTCCGACGGC
TCCTTCTTCCTCTACAGCAAGCTCACCGTGGACAAGAGCAGGTGGCAGCAGGGGAA
CGTCTTCTCATGCTCCGTGATGCATGAGGCTCTGCACAACCACTACACGCAGAAGAG
CCTCTCCCTGTCTCCTGGTAAATAA

The amino acid sequence of the mature hDLL4/huIL-15RαSu/huIgG1 Fc fusion protein (including leader sequence) is as follows (SEQ ID NO: 44):

(Signal peptide)
MKWVTFISLLFLFSSAYS
(hDLL4)
SGVFQLQLQEFINERGVLASGRPCEPGCRTFFRVCLKHFQAVVSPGPCTFGTVSTPVLGT
NSFAVRDDSSGGGRNPLQLPFNFTWPGTFSLIIEAWHAPGDDLRPEALPPDALISKIAIQG
SLAVGQNWLLDEQTSTLTRLRYSYRVICSDNYYGDNCSRLCKKRNDHFGHYVCQPDGN
LSCLPGWTGEYCQQPICLSGCHEQNGYCSKPAECLCRPGWQGRLCNECIPHNGCRHGTC
STPWQCTCDEGWGGLFCDQDLNYCTHHSPCKNGATCSNSGQRSYTCTCRPGYTGVDC
ELELSECDSNPCRNGGSCKDQEDGYHCLCPPGYYGLHCEHSTLSCADSPCFNGGSCRER
NQGANYACECPPNFTGSNCEKKVDRCTSNPCANGGQCLNRGPSRMCRCRPGFTGTYCE
LHVSDCARNPCAHGGTCHDLENGLMCTCPAGFSGRRCEVRTSIDACASSPCFNRATCYT
DLSTDTFVCNCPYGFVGSRCEFPVGLPPSFPW
(Human IL-15R α sushi domain)
ITCPPPMSVEHADIWVKSYSLYSRERYICNSGFKRKAGTSSLTECVLNKATNVAHWTTPS
LKCIR
(Human IgG1 CH2-CH3 (Fc) domain)
EPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKF
NWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPI
EKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNY
KTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK

In some cases, the leader peptide is cleaved from the mature polypeptide.

Similar constructs could be generated to express hDLL4 domain/huIL-15N72D fusion protein.

The sequences were cloned into expression vectors as described in Example 1 and previously (U.S. Patent No. 8,507,222, at Examples 1 and 2, incorporated herein by reference), and the expression vectors transfected into CHO cells. In some cases, the CHO cells were

transfected with vectors encoding both huIL-15N72D and huIL-15RαSu/Fc fusion proteins with the same or different binding domains of the invention. The fusion protein complexes were purified from the CHO cell culture supernatant using Protein A affinity chromatography as described above.

For example, co-expression of huIL-15N72D and hDLL4/huIL-15RαSu/huIgG1 Fc expression vectors in CHO cells allowed formation and secretion of a soluble TxM complex referred to as 2*hDLL4/TxM.

T-cell immunoglobulin and mucin-domain containing-3 (Tim-3) is an immune checkpoint receptor found on IFN-γ–producing $CD4^+$ T helper 1 (Th1) and $CD8^+$ T cytotoxic 1 (Tc1) T cells. Therefore, it is an attractive target for cancer immunotherapy. Thus, fusion protein complexes of the invention were generated with single chain Ab domains that recognize human Tim-3 (haTIM3scFv).

The nucleic acid sequence of the haTIM3scFv/huIL-15RαSu/huIgG1 Fc construct (including leader sequence) is as follows (SEQ ID NO: 45):

(Signal peptide)

ATGGAATGGAGCTGGGTCTTTCTCTTCTTCCTGTCAGTAACCACCGGTGTCCACTCC

(haTIM3scFv: VL-linker-VH scFv)

(VL)

TCCTATGTGCTGACTCAGCCTCCCTCCGCGTCCGGGTCTCCTGGACAGTCAGTCACC
ATCTCCTGCACTGGAACCAGCAGTGACGTTGGTAATAATAACTATGTCTCCTGGTAC
CAACAGCACCCAGGCAAAGCCCCCAAACTCATGATTTATGATGTCAGTAATCGGCC
CTCAGGGGTTTCTACTCGCTTCTCTGGCTCCAAGTCTGGCAACACGGCCTCCCTGAC
CATCTCTGGGCTCCAGGCTGAGGACGAGGCTGATTATTACTGCAGCTCATATACAAC
CAGCAGTACTTATGTCTTCGGAACTGGGACCAAGCTGACCGTCCTGGGGCAGCCAA
AGGCG

(linker)

GGAGGTGGCGGATCCGGAGGTGGAGGTTCTGGTGGAGGTGGGAGT

(VH)

CTGGTGCAATCTGGGGCTGAGGTGAAGAAGCCTGGGGCCTCAGTGAAGGTCTCCTG
CAAGGCTTCTGGATACACCTTCACCGGCTACTATATGCACTGGGTGCGACAGGCCCC

TGGACAAGGGCTTGAGTGGATGGGATGGATCAACCCTAACAGTGGTGGCACAAACT
ATGCACAGAAGTTCCAGGGCAGAGTCACCATGACCAGGAACACCTCCATAAGCACA
GCCTACATGGAGTTGAGCAGCCTGAGATCTGACGACACGGCCGTGTATTACTGTGCG
AGAGAGATGTATTACTATGGTTCGGGGTACAACTGGTTCGACCCCTGGGGCCAGGG
AACCCTGGTCACCGTGAGCTCA

(Human IL-15R α sushi domain)

ATCACGTGTCCTCCTCCTATGTCCGTGGAACACGCAGACATCTGGGTCAAGAGCTAC
AGCTTGTACTCCAGGGAGCGGTACATTTGTAACTCTGGTTTCAAGCGTAAAGCCGGC
ACGTCCAGCCTGACGGAGTGCGTGTTGAACAAGGCCACGAATGTCGCCCACTGGAC
AACCCCCAGTCTCAAATGCATTAGA

(Human IgG1 CH2-CH3 (Fc) domain)

GAGCCGAAATCTTGTGACAAAACTCACACATGCCCACCGTGCCCAGCACCTGAACT
CCTGGGGGGACCGTCAGTCTTCCTCTTCCCCCCAAAACCCAAGGACACCCTCATGAT
CTCCCGGACCCCTGAGGTCACATGCGTGGTGGTGGACGTGAGCCACGAAGACCCTG
AGGTCAAGTTCAACTGGTACGTGGACGGCGTGGAGGTGCATAATGCCAAGACAAAG
CCGCGGGAGGAGCAGTACAACAGCACGTACCGTGTGGTCAGCGTCCTCACCGTCCT
GCACCAGGACTGGCTGAATGGCAAGGAGTACAAGTGCAAGGTCTCCAACAAAGCCC
TCCCAGCCCCCATCGAGAAAACCATCTCCAAAGCCAAAGGGCAGCCCCGAGAACCA
CAGGTGTACACCCTGCCCCCATCCCGGGATGAGCTGACCAAGAACCAGGTCAGCCT
GACCTGCCTGGTCAAAGGCTTCTATCCCAGCGACATCGCCGTGGAGTGGGAGAGCA
ATGGGCAGCCGGAGAACAACTACAAGACCACGCCTCCCGTGCTGGACTCCGACGGC
TCCTTCTTCCTCTACAGCAAGCTCACCGTGGACAAGAGCAGGTGGCAGCAGGGGAA
CGTCTTCTCATGCTCCGTGATGCATGAGGCTCTGCACAACCACTACACGCAGAAGAG
CCTCTCCCTGTCTCCTGGTAAATAA

The amino acid sequence of the mature haTIM3scFv/huIL-15RαSu/huIgG1 Fc fusion protein (including leader sequence) is as follows (SEQ ID NO: 46):

(Signal peptide)

MEWSWVFLFFLSVTTGVHS

(haTIM3scFv: VL-linker-VH scFv)

(VL)

SYVLTQPPSASGSPGQSVTISCTGTSSDVGNNNYVSWYQQHPGKAPKLMIYDVSNRPSG
VSTRFSGSKSGNTASLTISGLQAEDEADYYCSSYTTSSTYVFGTGTKLTVLGQPKA

(linker)

GGGGSGGGGSGGGGS

(VH)

LVQSGAEVKKPGASVKVSCKASGYTFTGYYMHWVRQAPGQGLEWMGWINPNSGGTN
YAQKFQGRVTMTRNTSISTAYMELSSLRSDDTAVYYCAREMYYYGSGYNWFDPWGQG
TLVTVSS

(Human IL-15R α sushi domain)

ITCPPPMSVEHADIWVKSYSLYSRERYICNSGFKRKAGTSSLTECVLNKATNVAHWTTPS
LKCIR

(Human IgG1 CH2-CH3 (Fc) domain)

EPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKF
NWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPI
EKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNY
KTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK

In some cases, the leader peptide is cleaved from the mature polypeptide.

Similar constructs could be generated to express the haTIM3scFv/huIL-15N72D fusion protein.

The sequences were cloned into expression vectors as described in Example 1 and previously (U.S. Patent No. 8,507,222, at Examples 1 and 2, incorporated herein by reference), and the expression vectors transfected into CHO cells. In some cases, the CHO cells were transfected with vectors encoding both huIL-15N72D and huIL-15RαSu/Fc fusion proteins with the same or different binding domains of the invention. The fusion protein complexes were purified from the CHO cell culture supernatant using Protein A affinity chromatography as described above.

For example. CHO cells were transfected with the huIL-15N72D expression vector. Cells were also transfected with vectors expressing the haTIM3scFv/huIL-15RαSu/huIgG1 Fc construct. Co-expression of the two constructs in CHO cells allowed formation and secretion of

a soluble huIL-15N72D: haTIM3scFv/huIL-15RαSu/huIgG1 Fc complex (referred to as 2*haTIM3/TxM).

In addition to tumor targeting molecules, TxM complexes can be created that detect and act against virally infected cells. The recent discovery of highly potent, broadly neutralizing, HIV-specific monoclonal antibodies (bNAbs) provides a novel class of potential therapeutic agents. It has long been known that neutralizing antibodies can target the HIV envelope (Env) and effectively suppress viral replication in vitro. To combine this Ab mediated suppression with the "kick and kill" approach of waking up latent virus replication and killing it with activated effector cells (via IL-15 stimulation), TxM complexes have been created comprising single chain antibody domains (scFvs) of bNAbs. The creation and characterization of four different anti-HIV TxMs comprising scFvs from bNAbs N6, 2G12, VRC07 and 10-1074 are described below.

The nucleic acid sequence of N6scFv/huIL-15RαSu/huIgG1 Fc construct (including leader sequence) is as follows (SEQ ID NO: 47):

(Signal peptide)

ATGAAGTGGGTGACCTTCATCAGCCTGCTGTTCCTGTTCTCCAGCGCCTACTCC

(N6 scFv: VL-linker-VH scFv)

(VL)

TACATCCACGTGACCCAGTCCCCCTCCTCTTTAAGCGTGAGCATCGGAGATCGTGTG
ACCATCAACTGCCAGACCTCCCAAGGTGTGGGCTCCGATTTACACTGGTACCAGCAC
AAGCCCGGTCGGGCCCCCAAGCTGCTGATCCACCACACCAGCTCCGTGGAGGATGG
CGTGCCCTCTCGTTTCTCCGGCTCCGGCTTCCATACCTCCTTCAATTTAACCATCAGC
GATTTACAAGCTGACGACATCGCCACCTACTACTGCCAAGTTCTCCAGTTCTTCGGC
CGGGGCTCTCGTCTGCATATCAAG

(linker)

GGAGGCGGCGGATCCGGCGGCGGAGGCAGCGGCGGAGGCGGATCT

(VH)

CGTGCTCATCTGGTGCAGAGCGGAACCGCCATGAAGAAGCCCGGTGCTAGCGTGCG
GGTGTCTTGTCAGACCAGCGGATACACCTTCACCGCCCACATTTTATTCTGGTTTCGT
CAAGCTCCCGGTCGTGGACTGGAATGGGTGGGCTGGATCAAGCCCCAGTATGGCGC

CGTGAACTTTGGCGGCGGCTTTCGTGATCGGGTGACTTTAACTCGTGACGTGTATCG
GGAGATCGCCTACATGGACATTAGGGGTTTAAAGCCCGACGATACCGCCGTGTACT
ACTGCGCTCGTGATCGTTCCTACGGCGATAGCAGCTGGGCTTTAGATGCTTGGGGCC
AAGGTACCACAGTGTGGTCCGCC

(Human IL-15R α sushi domain)

ATCACGTGTCCTCCTCCTATGTCCGTGGAACACGCAGACATCTGGGTCAAGAGCTAC
AGCTTGTACTCCAGGGAGCGGTACATTTGTAACTCTGGTTTCAAGCGTAAAGCCGGC
ACGTCCAGCCTGACGGAGTGCGTGTTGAACAAGGCCACGAATGTCGCCCACTGGAC
AACCCCCAGTCTCAAATGCATTAGA

(Human IgG1 CH2-CH3 (Fc) domain)

GAGCCGAAATCTTGTGACAAAACTCACACATGCCCACCGTGCCCAGCACCTGAACT
CCTGGGGGGACCGTCAGTCTTCCTCTTCCCCCCAAAACCCAAGGACACCCTCATGAT
CTCCCGGACCCCTGAGGTCACATGCGTGGTGGTGGACGTGAGCCACGAAGACCCTG
AGGTCAAGTTCAACTGGTACGTGGACGGCGTGGAGGTGCATAATGCCAAGACAAAG
CCGCGGGAGGAGCAGTACAACAGCACGTACCGTGTGGTCAGCGTCCTCACCGTCCT
GCACCAGGACTGGCTGAATGGCAAGGAGTACAAGTGCAAGGTCTCCAACAAAGCCC
TCCCAGCCCCCATCGAGAAAACCATCTCCAAAGCCAAAGGGCAGCCCCGAGAACCA
CAGGTGTACACCCTGCCCCCATCCCGGGATGAGCTGACCAAGAACCAGGTCAGCCT
GACCTGCCTGGTCAAAGGCTTCTATCCCAGCGACATCGCCGTGGAGTGGGAGAGCA
ATGGGCAGCCGGAGAACAACTACAAGACCACGCCTCCCGTGCTGGACTCCGACGGC
TCCTTCTTCCTCTACAGCAAGCTCACCGTGGACAAGAGCAGGTGGCAGCAGGGGAA
CGTCTTCTCATGCTCCGTGATGCATGAGGCTCTGCACAACCACTACACGCAGAAGAG
CCTCTCCCTGTCTCCTGGTAAATAA

The nucleic acid sequence of 2G12scFv/huIL-15RαSu/huIgG1 Fc construct (including leader sequence) is as follows (SEQ ID NO: 48):

(Signal peptide)

ATGAAGTGGGTGACCTTCATCAGCCTGCTGTTCCTGTTCTCCAGCGCCTACTCC

(2G12 scFv: VL-linker-VH scFv)

(VL)

GTGGTGATGACCCAGTCCCCTTCCACCCTGTCCGCTTCCGTGGGCGACACCATCACC
ATCACCTGCAGGGCCTCCCAGTCCATCGAGACCTGGCTGGCCTGGTACCAGCAGAA
GCCCGGCAAGGCCCCCAAGCTGCTGATCTACAAGGCCTCCACCCTGAAGACCGGCG
TGCCCTCCAGGTTTTCCGGATCCGGCTCCGGCACCGAGTTCACCCTGACCATCAGCG
GCCTGCAGTTCGACGACTTCGCCACCTACCACTGCCAGCACTACGCCGGCTACTCCG
CCACCTTTGGACAGGGCACCAGGGTGGAGATCAAG

(linker)

GGAGGTGGCGGATCCGGAGGTGGAGGTTCTGGTGGAGGTGGGAGT

(VH)

GAGGTGCAGCTGGTGGAATCCGGAGGCGGCCTGGTGAAAGCTGGCGGAAGCCTGAT
CCTGAGCTGCGGCGTGTCCAACTTCAGGATCTCCGCCCACACCATGAACTGGGTGAG
GAGGGTGCCTGGAGGAGGACTGGAGTGGGTGGCCAGCATCTCCACCTCCTCCACCT
ACAGGGACTACGCCGACGCCGTGAAGGGCAGGTTCACCGTGAGCAGGGACGACCTG
GAGGACTTCGTGTACCTGCAGATGCACAAGATGCGGGTGGAGGACACCGCCATCTA
CTACTGCGCCAGGAAGGGCTCCGACAGGCTGTCCGACAACGACCCCTTTGACGCCT
GGGGCCCTGGAACCGTGGTGACAGTGTCCCCC

(Human IL-15R α sushi domain)

ATCACGTGTCCTCCTCCTATGTCCGTGGAACACGCAGACATCTGGGTCAAGAGCTAC
AGCTTGTACTCCAGGGAGCGGTACATTTGTAACTCTGGTTTCAAGCGTAAAGCCGGC
ACGTCCAGCCTGACGGAGTGCGTGTTGAACAAGGCCACGAATGTCGCCCACTGGAC
AACCCCCAGTCTCAAATGCATTAGA

(Human IgG1 CH2-CH3 (Fc) domain)

GAGCCGAAATCTTGTGACAAAACTCACACATGCCCACCGTGCCCAGCACCTGAACT
CCTGGGGGGACCGTCAGTCTTCCTCTTCCCCCCAAAACCCAAGGACACCCTCATGAT
CTCCCGGACCCCTGAGGTCACATGCGTGGTGGTGGACGTGAGCCACGAAGACCCTG
AGGTCAAGTTCAACTGGTACGTGGACGGCGTGGAGGTGCATAATGCCAAGACAAAG
CCGCGGGAGGAGCAGTACAACAGCACGTACCGTGTGGTCAGCGTCCTCACCGTCCT
GCACCAGGACTGGCTGAATGGCAAGGAGTACAAGTGCAAGGTCTCCAACAAAGCCC
TCCCAGCCCCCATCGAGAAAACCATCTCCAAAGCCAAAGGGCAGCCCCGAGAACCA
CAGGTGTACACCCTGCCCCCATCCCGGGATGAGCTGACCAAGAACCAGGTCAGCCT
GACCTGCCTGGTCAAAGGCTTCTATCCCAGCGACATCGCCGTGGAGTGGGAGAGCA

ATGGGCAGCCGGAGAACAACTACAAGACCACGCCTCCCGTGCTGGACTCCGACGGC
TCCTTCTTCCTCTACAGCAAGCTCACCGTGGACAAGAGCAGGTGGCAGCAGGGGAA
CGTCTTCTCATGCTCCGTGATGCATGAGGCTCTGCACAACCACTACACGCAGAAGAG
CCTCTCCCTGTCTCCTGGTAAATAA

The nucleic acid sequence of VRC07(523)scFv/huIL-15RαSu/huIgG1 Fc construct (including leader sequence) is as follows (SEQ ID NO: 49):

(Signal peptide)
ATGAAGTGGGTGACCTTCATCAGCCTGCTGTTCCTGTTCTCCAGCGCCTACTCC
(VRC07(523) scFv: VL-linker-VH scFv)
(VL)
TCCTCCCTGACCCAGAGCCCCGGAACACTCTCCCTCTCCCCCGGTGAGACCGCTATC
ATCTCTTGTAGGACCAGCCAGTACGGCTCTTTAGCTTGGTATCAACAGAGGCCCGGC
CAAGCTCCTAGGCTGGTCATTTACAGCGGCAGCACAAGGGCCGCCGGCATCCCCGA
TAGGTTCTCCGGCTCCCGGTGGGGCCCCGATTACAATTTAACAATCTCCAATTTAGA
GTCCGGAGACTTCGGCGTCTACTACTGCCAGCAGTACGAGTTCTTCGGCCAAGGTAC
CAAAGTGCAAGTTGATATCAAG
(linker)
GGCGGCGGAGGCTCCGGCGGCGGCGGATCCGGCGGAGGAGGATCC
(VH)
CAAGTTAGGCTGTCCCAGAGCGGAGGCCAGATGAAGAAGCCCGGTGACTCCATGCG
GATCAGCTGTCGTGCCAGCGGCTACGAGTTCATCAACTGCCCCATCAACTGGATTCG
TCTGGCCCCCGGTAAGCGGCCCGAATGGATGGGCTGGATGAAACCTCGTCACGGCG
CTGTGTCCTACGCTCGTCAGCTGCAAGGTCGTGTGACCATGACTCGTGACATGTACA
GCGAGACCGCCTTTTTAGAGCTGAGGTCTTTAACCTCCGACGACACCGCTGTGTACT
TCTGCACCCGGGGCAAGTACTGCACCGCTCGGGACTACTACAACTGGGACTTCGAG
CACTGGGGCCAAGGTACACCCGTGACAGTGTCCTCC
(Human IL-15R α sushi domain)
ATCACGTGTCCTCCTCCTATGTCCGTGGAACACGCAGACATCTGGGTCAAGAGCTAC
AGCTTGTACTCCAGGGAGCGGTACATTTGTAACTCTGGTTTCAAGCGTAAAGCCGGC

ACGTCCAGCCTGACGGAGTGCGTGTTGAACAAGGCCACGAATGTCGCCCACTGGAC
AACCCCCAGTCTCAAATGCATTAGA

(Human IgG1 CH2-CH3 (Fc) domain)

GAGCCGAAATCTTGTGACAAAACTCACACATGCCCACCGTGCCCAGCACCTGAACT
CCTGGGGGGACCGTCAGTCTTCCTCTTCCCCCCAAAACCCAAGGACACCCTCATGAT
CTCCCGGACCCCTGAGGTCACATGCGTGGTGGTGGACGTGAGCCACGAAGACCCTG
AGGTCAAGTTCAACTGGTACGTGGACGGCGTGGAGGTGCATAATGCCAAGACAAAG
CCGCGGGAGGAGCAGTACAACAGCACGTACCGTGTGGTCAGCGTCCTCACCGTCCT
GCACCAGGACTGGCTGAATGGCAAGGAGTACAAGTGCAAGGTCTCCAACAAAGCCC
TCCCAGCCCCCATCGAGAAAACCATCTCCAAAGCCAAAGGGCAGCCCCGAGAACCA
CAGGTGTACACCCTGCCCCCATCCCGGGATGAGCTGACCAAGAACCAGGTCAGCCT
GACCTGCCTGGTCAAAGGCTTCTATCCCAGCGACATCGCCGTGGAGTGGGAGAGCA
ATGGGCAGCCGGAGAACAACTACAAGACCACGCCTCCCGTGCTGGACTCCGACGGC
TCCTTCTTCCTCTACAGCAAGCTCACCGTGGACAAGAGCAGGTGGCAGCAGGGGAA
CGTCTTCTCATGCTCCGTGATGCATGAGGCTCTGCACAACCACTACACGCAGAAGAG
CCTCTCCCTGTCTCCTGGTAAATAA

The nucleic acid sequence of 10-1074scFv/huIL-15RαSu/huIgG1 Fc construct (including leader sequence) is as follows (SEQ ID NO: 50):

(Signal peptide)

ATGAAGTGGGTGACCTTCATCAGCCTGCTGTTCCTGTTCTCCAGCGCCTACTCC

(10-1074 scFv: VL-linker-VH scFv)

(VL)

TCCAGCTACGTGAGGCCTCTCTCCGTGGCTCTGGGCGAAACAGCTCGTATCAGCTGC
GGTCGTCAAGCTCTGGGATCTCGTGCTGTGCAGTGGTACCAGCACCGGCCCGGTCAA
GCTCCCATTTTACTGATCTACAACAACCAAGATCGGCCCTCCGGCATCCCCGAAAGG
TTTAGCGGCACCCCCGATATCAACTTCGGCACAAGGGCCACTTTAACCATTAGCGGA
GTGGAGGCCGGCGACGAGGCCGACTACTACTGCCACATGTGGGACTCCCGGTCCGG
CTTTTCTTGGAGCTTTGGCGGCGCTACTCGTCTGACAGTGCTG

(linker)

GGCGGAGGCGGCTCCGGAGGCGGCGGCAGCGGAGGAGGCGGATCC

(VH)

CAAGTTCAGCTGCAAGAATCCGGACCCGGTTTAGTGAAGCCCAGCGAGACTTTAAG
CGTGACTTGTAGCGTGAGCGGCGACAGCATGAACAACTACTACTGGACTTGGATTCG
TCAGAGCCCCGGTAAGGGTTTAGAGTGGATCGGCTACATCTCCGACCGGGAGTCCG
CCACCTACAACCCCTCTTTAAACTCCCGGGTGGTGATCTCTCGTGACACCTCCAAGA
ACCAGCTGTCTTTAAAGCTGAACTCCGTGACCCCCGCTGACACCGCCGTGTACTACT
GCGCTACCGCTAGGCGGGGCCAGAGGATCTACGGCGTGGTGAGCTTCGGCGAGTTC
TTCTACTACTACAGCATGGACGTGTGGGGCAAAGGCACCACCGTGACCGTGTCCTCC

(Human IL-15R α sushi domain)

ATCACGTGTCCTCCTCCTATGTCCGTGGAACACGCAGACATCTGGGTCAAGAGCTAC
AGCTTGTACTCCAGGGAGCGGTACATTTGTAACTCTGGTTTCAAGCGTAAAGCCGGC
ACGTCCAGCCTGACGGAGTGCGTGTTGAACAAGGCCACGAATGTCGCCCACTGGAC
AACCCCCAGTCTCAAATGCATTAGA

(Human IgG1 CH2-CH3 (Fc) domain)

GAGCCGAAATCTTGTGACAAAACTCACACATGCCCACCGTGCCCAGCACCTGAACT
CCTGGGGGGACCGTCAGTCTTCCTCTTCCCCCCAAAACCCAAGGACACCCTCATGAT
CTCCCGGACCCCTGAGGTCACATGCGTGGTGGTGGACGTGAGCCACGAAGACCCTG
AGGTCAAGTTCAACTGGTACGTGGACGGCGTGGAGGTGCATAATGCCAAGACAAAG
CCGCGGGAGGAGCAGTACAACAGCACGTACCGTGTGGTCAGCGTCCTCACCGTCCT
GCACCAGGACTGGCTGAATGGCAAGGAGTACAAGTGCAAGGTCTCCAACAAAGCCC
TCCCAGCCCCCATCGAGAAAACCATCTCCAAAGCCAAAGGGCAGCCCCGAGAACCA
CAGGTGTACACCCTGCCCCCATCCCGGGATGAGCTGACCAAGAACCAGGTCAGCCT
GACCTGCCTGGTCAAAGGCTTCTATCCCAGCGACATCGCCGTGGAGTGGGAGAGCA
ATGGGCAGCCGGAGAACAACTACAAGACCACGCCTCCCGTGCTGGACTCCGACGGC
TCCTTCTTCCTCTACAGCAAGCTCACCGTGGACAAGAGCAGGTGGCAGCAGGGGAA
CGTCTTCTCATGCTCCGTGATGCATGAGGCTCTGCACAACCACTACACGCAGAAGAG
CCTCTCCCTGTCTCCTGGTAAATAA

The amino acid sequence of the mature N6scFv/huIL-15RαSu/huIgG1 Fc fusion protein (including leader sequence) is as follows (SEQ ID NO: 51):

(Signal peptide)

MKWVTFISLLFLFSSAYS

(N6 scFv: VL-linker-VH scFv)

(VL)

YIHVTQSPSSLSVSIGDRVTINCQTSQGVGSDLHWYQHKPGRAPKLLIHHTSSVEDGVPS
RFSGSGFHTSFNLTISDLQADDIATYYCQVLQFFGRGSRLHIK

(linker)

GGGGSGGGGSGGGGS

(VH)

RAHLVQSGTAMKKPGASVRVSCQTSGYTFTAHILFWFRQAPGRGLEWVGWIKPQYGA
VNFGGGFRDRVTLTRDVYREIAYMDIRGLKPDDTAVYYCARDRSYGDSSWALDAWGQ
GTTVWSA

(Human IL-15R α sushi domain)

ITCPPPMSVEHADIWVKSYSLYSRERYICNSGFKRKAGTSSLTECVLNKATNVAHWTTPS
LKCIR

(Human IgG1 CH2-CH3 (Fc) domain)

EPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKF
NWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPI
EKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNY
KTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK

The amino acid sequence of the mature 2G12scFv/huIL-15RαSu/huIgG1 Fc fusion protein (including leader sequence) is as follows (SEQ ID NO: 52):

(Signal peptide)

MKWVTFISLLFLFSSAYS

(2G12 scFv: VL-linker-VH scFv)

(VL)

VVMTQSPSTLSASVGDTITITCRASQSIETWLAWYQQKPGKAPKLLIYKASTLKTGVPSR
FSGSGSGTEFTLTISGLQFDDFATYHCQHYAGYSATFGQGTRVEIK

(linker)

GGGGSGGGGSGGGGS

(VH)

EVQLVESGGGLVKAGGSLILSCGVSNFRISAHTMNWVRRVPGGGLEWVASISTSSTYRD
YADAVKGRFTVSRDDLEDFVYLQMHKMRVEDTAIYYCARKGSDRLSDNDPFDAWGPG
TVVTVSP

(Human IL-15R α sushi domain)

ITCPPPMSVEHADIWVKSYSLYSRERYICNSGFKRKAGTSSLTECVLNKATNVAHWTTPS
LKCIR

(Human IgG1 CH2-CH3 (Fc) domain)

EPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKF
NWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPI
EKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNY
KTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK

The amino acid sequence of the mature VRC07(523)scFv/huIL-15RαSu/huIgG1 Fc fusion protein (including leader sequence) is as follows (SEQ ID NO: 53):

(Signal peptide)

MKWVTFISLLFLFSSAYS

(VRC07(523) scFv: VL-linker-VH scFv)

(VL)

SSLTQSPGTLSLSPGETAIISCRTSQYGSLAWYQQRPGQAPRLVIYSGSTRAAGIPDRFSGS
RWGPDYNLTISNLESGDFGVYYCQQYEFFGQGTKVQVDIK

(linker)

GGGGSGGGGSGGGGS

(VH)

QVRLSQSGGQMKKPGDSMRISCRASGYEFINCPINWIRLAPGKRPEWMGWMKPRHGAV
SYARQLQGRVTMTRDMYSETAFLELRSLTSDDTAVYFCTRGKYCTARDYYNWDFEHW
GQGTPVTVSS

(Human IL-15R α sushi domain)

ITCPPPMSVEHADIWVKSYSLYSRERYICNSGFKRKAGTSSLTECVLNKATNVAHWTTPS
LKCIR

(Human IgG1 CH2-CH3 (Fc) domain)

EPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKF
NWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPI
EKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNY
KTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK

The amino acid sequence of the mature 10-1074scFv/huIL-15RαSu/huIgG1 Fc fusion protein (including leader sequence) is as follows (SEQ ID NO: 54):

(Signal peptide)

MKWVTFISLLFLFSSAYS

(10-1074) scFv: VL-linker-VH scFv)

(VL)

SSYVRPLSVALGETARISCGRQALGSRAVQWYQHRPGQAPILLIYNNQDRPSGIPERFSG
TPDINFGTRATLTISGVEAGDEADYYCHMWDSRSGFSWSFGGATRLTVL

(linker)

GGGGSGGGGSGGGGS

(VH)

QVQLQESGPGLVKPSETLSVTCSVSGDSMNNYYWTWIRQSPGKGLEWIGYISDRESATY
NPSLNSRVVISRDTSKNQLSLKLNSVTPADTAVYYCATARRGQRIYGVVSFGEFFYYYS
MDVWGKGTTVTVSS

(Human IL-15R α sushi domain)

ITCPPPMSVEHADIWVKSYSLYSRERYICNSGFKRKAGTSSLTECVLNKATNVAHWTTPS
LKCIR

(Human IgG1 CH2-CH3 (Fc) domain)

EPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKF
NWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPI
EKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNY
KTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK

In some cases, the leader peptide is cleaved from the mature polypeptide.

Similar constructs could be generated to express bNAb scFv/huIL-15N72D fusion proteins as described above.

The sequences were cloned into expression vectors as described in Example 1 and previously (U.S. Patent No. 8,507,222, at Examples 1 and 2, incorporated herein by reference), and the expression vectors transfected into CHO cells. In some cases, the CHO cells were transfected with vectors encoding both huIL-15N72D and huIL-15RαSu/Fc fusion proteins with the same or different binding domains of the invention. The fusion protein complexes were purified from the CHO cell culture supernatant using Protein A affinity chromatography as described above.

For example, co-expression of the huIL-15N72D and N6scFv/huIL-15RαSu/huIgG1 Fc expression vectors in CHO cells allowed formation and secretion of a soluble TxM complex referred to as 2*hN6/TxM. Co-expression of the huIL-15N72D and 2G12scFv/huIL-15RαSu/huIgG1 Fc expression vectors in CHO cells allowed formation and secretion of a soluble TxM complex referred to as 2*h2G12/TxM . Co-expression of the huIL-15N72D and VRC07(523)scFv/huIL-15RαSu/huIgG1 Fc expression vectors in CHO cells allowed formation and secretion of a soluble TxM complex referred to as 2*hVRC07(523)/TxM. Co-expression of the huIL-15N72D and 10-1074scFv/huIL-15RαSu/huIgG1 Fc expression vectors in CHO cells allowed formation and secretion of a soluble TxM complex referred to as 2*h10-1074/TxM.

As indicated above, the TxM proteins can be purified from CHO cell supernatants by Protein A chromatography and other separation methods (i.e., ion exchange, hydrophobic, and/or size exclusion chromatography, and filtration methods). Furthermore, the purified proteins can be characterized by gel, chromatography, and other analytical methods. For example, FIG. 20 shows size exclusion chromatography analysis of various TxM complexes including those with 2 scAb or binding domains (i.e. 2 headed (2H) IL-15N72D:anti-PD-L1 scAb/IL-15RαSu/Fc complexes) or 4 scAb or binding domains (i.e. 4 headed (4H) anti-PD-L1 scAb/IL-15N72D:anti-PD-L1 scAb/IL-15RαSu/Fc complexes) or combinations of different targeting domains (i.e., tumor targeting domains/anti-PDL1scAb TxM complexes). The SEC chromatographs indicate that the Protein A-purified TxM proteins are primarily comprised of a major protein peak with a migration pattern consistent with the IL-15N72D:IL-15RαSu/Fc complex.

Similar TxM constructs comprising scAb or binding domains could be readily generated with antibody sequences specific to other CD antigens, cytokines or chemokine receptors or ligands, growth factor receptors or ligands, cell adhesion molecules, MHC/MHC-like molecules, Fc receptors, Toll-like receptors, NK receptors, TCRs, BCRs, positive/negative co-stimulatory receptors or ligands, death receptors or ligands, tumor associated antigens, virus-encoded and bacterial-encoded antigens, and bacterial-specific. Of particular interest are TxM with disease specific binding domains (e.g. scAbs) to antigens of CD4, CD19, CD20, CD21, CD22, CD23, CD25, CD30, CD33, CD38, CD40, CD44, CD51, CD52, CD70, CD74, CD80, CD123, CD152, CD147, CD221, EGFR, HER-2/neu, HER-1, HER-3, HER-4, CEA, OX40 ligand, cMet, tissue factor, Nectin-4, PSA, PSMA, EGFL7, FGFR, IL-6 receptor, IGF-1 receptor, GD2, CA-125, EpCam, death receptor 5, MUC1, VEGFR1, VEGFR2, PDGFR, Trail R2, folate receptor, angiopoietin-2, alphavbeta3 integrin receptor, HLA-DR antigens and other disease targets described herein. Antibody domains against viral antigens from HIV, HCV, HBC, CMV, HTLV, HPV, EBV, RSV and other virus are also of interest, particularly those recognizing the HIV envelope spike and/or gp120 and gp41 epitopes. Such antibody domains can be generated from sequences known in the art or isolated de novo from a variety of sources (i.e., vertebrate hosts or cells, combinatorial libraries, random synthetic libraries, computational modeling, etc.) know in the art.

Example 6: Characterization of Activities of Other TxM

The binding activities of CTLA-4 TxM were assessed using CTLA-4-positive immune cells. In studies on mouse specific CTLA-4 TxM, the expression of CTLA-4 in mouse lymphocytes was first induced by anti-CD3 Ab (2C11, 4 μg/ml) for 4 days. CTLA-4 expression was assessed by staining with PE anti-mouse CTLA-4 antibody (clone UC10-4B9) or PE Armenian Hamster IgG isotype control. As shown in FIG. 21A, flow cytometry analysis demonstrated that mouse CTLA-4 was markedly induced. Addition of mouse specific CTLA-4 TxM (100 μl sup) was capable of blocking anti-mouse CTLA-4 antibody binding as did a positive control anti-mCTLA-4 antibody (clone HB304). For studies with human specific CTLA-4 TxM, the expression of CTLA-4 in human PBMC was induced by anti-CD3 Ab (OKT3 :4 μg/ml) for 3 days. Cells were then stained with PE anti-human CTLA-4 antibody (clone BNI3, Biolegend) or PE mouse IgG2a, κ isotype control. Consistent with the results described

above, human specific CTLA-4 TxM (CL-8-100 ul) was capable of blocking CTLA-4 on the surface of human immune cells (FIG. 21B). These results demonstrate the specificity of the CTLA-4 TxM complex.

Similarly, the binding activity of a mouse specific PD-L1/CTLA-4 TxM complex was assessed on PD-L1-positive 5T33 myeloma tumor cells (per methods described in Example 2) and CTLA-4-positive immune cells. As shown in FIG. 22A and FIG. 22B, the PD-L1/CTLA-4 TxM (sup) was capable of blocking binding to both PD-L1 and CTLA-4 expressed on the cell surface. This also indicates that multispecific TxM complexes retain reactivity of each of the linked binding domains.

Direct binding of the CD47 TxM constructs was assessed using CD47-positive cells. As shown in FIG. 23A and FIG. 23B, mouse and human specific CD47 TxM complexes were able to stain mouse B16F10 melanoma and human Jurkat T cells, respectively. These results indicate that these complexes retained CD47 binding activity.

A single stranded DNA ELISA method (ALPCO ssDNA ELISA kit 35-SSSHU-E01) was used to assess binding of TxM complexes comprising the TNT scAb domain. Briefly, purified TxM protein comprising TNT scAb domains were serially diluted and 100 μL was added to ELISA wells coated with human recombinant single stranded DNA. After 30 min incubation, the wells were washed and 100 μL of HRP-anti-human IgG antibody was added. After additional incubation and wash steps, the bound TxM protein was detected with TMB substrate. Absorbance of the wells was read at 450 nM. As shown in FIG. 24A and FIG. 24B, TNT scAb TxM and TNT scAb/anti-human PD-L1 scAb TxM complexes were capable of binding single stranded DNA, with TNT scAb TxM having a lower Kd (188 pM) compare to that of TNT scAb/anti-human PD-L1 scAb TxM (10279 pM), potentially because of the higher avidity of the 4H TNT scAb in TNT scAb TxM compared to 2H TNT scAb in TNT scAb/anti-human PD-L1 scAb TxM.

The ability of TNT scAb TxM complexes to bind tumor cells was also evaluated, exposing the tumor cell DNA by fixing and permeabilizing the cells. In the initial study, MB231 breast cancer cells were first fixed with 1.5% paraformaldehyde and permeabilized with 0.1% saponin and then 10^5 cells (10^6 cells/mL) were incubated for 30 min at room temperature with 0.01-100nM of TNT scAb TxM, TNT scAb/anti-human PD-L1 scAb TxM or 2H-anti-human PD-L1 scAb TxM (negative control). The cells were washed and stained with anti-human IgG

Fc-APC and then were analyzed by flow cytometry. FIG. 25A shows the mean fluorescence intensity (MFI) of MB231 cell staining at different TxM concentrations, confirming specific and concentration dependent binding of TNT scAb TxM and TNT scAb/anti-human PD-L1 scAb TxM to permeabilized breast tumor cells. Minimal binding was seen with the negative control PD-L1 scAb TxM complex, consistent with low level expression of PD-L1 on the MB231 cell line. A similar study was conducted with fixed and permeabilized PD-L1-negative A549 human lung tumor cells. Again, the results (FIG. 25B) confirm specific and concentration dependent binding of TNT scAb TxM and TNT scAb/anti-human PD-L1 scAb TxM to permeabilized lung tumor cells.

Furthermore, the ability of TxM complexes comprising hOAT scAb and/or anti-human PD-L1 scAb domains to bind tumor cells was assessed. The SW1990 human pancreatic cancer cell line expresses high levels of human TF and low levels of human PD-L1. In this study, 10^5 SW1990 cells (10^6 cells/mL) were incubated for 30 min at room temperature with 0.01-100nM of 2 headed (h2) hOATscAb/TxM, anti-human PD-L1scAb/hOATscAb/TxM, h2*anti-human PD-L1scAb/TxM or control hOAT Ab or control anti-human PD-L1 Ab (Avelumab). The cells were washed and stained with anti-human IgG Fc-APC and then were analyzed by flow cytometry. FIG. 26 shows the mean fluorescence intensity (MFI) of SW1990 cell staining at different protein concentrations. The results confirm that the TxM complexes comprising hOAT scAb (h2*hOATscAb/TxM and anti-human PD-L1scAb/hOATscAb/TxM) exhibit similar high-level staining of human TF on SW1990 tumor cells as the control hOAT Ab. TxM complexes comprising anti-human PD-L1 scAb (h2*anti-human PD-L1scAb/TxM) exhibit lower level staining of human PD-L1 on SW1990 tumor cells similar to the control anti-human PD-L1 Ab (Avelumab).

ELISA-based methods were used to confirm the formation of the huIL-15N72D: human LFA-1 I domain(K287C/K294C)/huIL-15RαSu/huIgG1 Fc complex. Binding activity was assessed in culture supernatant from CHO cells co-transfected with the huIL-15N72D and human LFA-1 I domain(K287C/K294C)/huIL-15RαSu/huIgG1 Fc expression vectors. In FIG. 27A, the fusion protein complexes were detected using a huIL15/huIgG1-specific ELISA with a capture antibody, anti-human IL-15 antibody (R&D Systems) and a detection antibody, anti-human IgG antibody (Jackson ImmunoResearch). This binding was compared to a control sample using only

the supernatant of media containing untransfected CHO cells. The results indicate production and proper complex formation for the 2*hLFA1/TxM.

Additionally, 2*hLFA1/TxM binding to ICAM-1 was assessed by ELISA (FIG. 27B). Wells of an immunoplate were coated with 1 µg of human ICAM-1-Fc (Biolegend). After wash steps, CHO culture supernatant containing 2*hLFA1/TxM was added to the cells. Following incubation and additional wash steps, binding of the fusion protein complexes was detected using an HRP-conjugated anti-human IL-15 antibody (R&D Systems). The absorbance in the wells was read at 405 nm after incubation with ABTS. The results in FIG. 27B indicate that this complex recognizes ICAM-1.

Similar ELISA-based methods confirmed the formation of a huIL-15N72D: hDLL4/huIL-15RαSu/huIgG1 Fc complex in the transfected CHO cell culture supernatant. In FIG. 28, the fusion protein complexes in the culture supernatant were detected using a huIL15/huIgG1-specific ELISA with a capture antibody, anti-human IL-15 antibody (R&D Systems) and a detection antibody, anti-human IgG antibody (Jackson ImmunoResearch). The sample is compared to a control sample using only the supernatant of media containing untransfected CHO cells. The results indicate production and proper complex formation of the 2*hDLL4/TxM complexes.

ELISA-based methods also confirmed the formation of the huIL-15N72D: haTIM3scFv/huIL-15RαSu/huIgG1 Fc complex. In FIG. 29, the fusion protein complexes in the transfected CHO culture supernatant were detected using a huIL15/huIgG1-specific ELISA with a capture antibody, anti-human IL-15 antibody (R&D Systems) and a detection antibody, anti-human IgG antibody (Jackson ImmunoResearch). This binding was compared to a control sample using only the supernatant of media containing untransfected CHO cells. The results indicate production and proper complex formation of 2*haTIM3/TxM complexes.

Supernatant from CHO cells co-transfected with bNAb scFv TxM expression vectors was used to determine the expression and binding capabilities of the TxM complexes. ELISA-based methods confirmed the formation of the bNAb scFv TxM complexes. In FIG. 30A and FIG. 30B, the fusion protein complexes in the transfected CHO culture supernatant were detected using a huIL15/huIgG1-specific ELISA with a capture antibody, anti-human IL-15 antibody (R&D Systems) and a detection antibody, anti-human IgG antibody (Jackson ImmunoResearch). The

positive control TxM is one that recognizes hCD20. The results indicate production and proper complex formation for four different bNAb scFv TxM complexes.

Additionally, bNAb scFv TxM binding to HIV protein targets (gp120(SF162.LS) and gp140 (SF162.LS)) was assessed by ELISA. For this study, wells of an immunoplate were coated with 0.1 μg of HIV gp120(SF162.LS) or gp140(SF162.LS) (ProtTech, Inc.). After wash steps, CHO culture supernatants containing bNAb scFv TxMs were added to the cells. The negative control TxM is one that recognizes hCD20. Following incubation and additional wash steps, binding of the fusion protein complexes was detected using an HRP-conjugated anti-human IgG antibody (Jackson ImmunoResearch). The absorbance in the wells was read at 405 nm after incubation with ABTS. The results in FIG. 30C to FIG. 30F indicate that the bNAb scFv TxM complexes recognizes HIV protein targets.

Overall these results demonstrate that TxM complexes with binding domains specific to a variety of immune checkpoint and signaling molecules can be generated and provide enhanced binding activities to target molecules. These complexes exhibit IL-15 immunostimulatory activity and are capable of directing immune mediated cytotoxicity against target antigens on cells. These complexes also are highly efficacious in animal tumor models.

Example 7: Immunostimulatory and Anti-tumor Activities of TxM Complexes

As indicated in Example 2, the IL-15 immunostimulatory activity of TxM complexes has been assessed based on proliferation of IL-2Rβ/γ-bearing immune cells such as the 32Dβ cell line. Briefly, increasing concentrations of purified TxM proteins were added to 32Dβ cells (10^4 cells/well) in 200 μL IMDM:10% FBS media and cells were incubated for 3 days at 37°C. PrestoBlue cell viability reagent (20 μL/well) then was added. After 4 hours, absorbance was measured at 570 nm (with a 600-nm reference wavelength for normalization) to determine cell proliferation based on reduction of PrestoBlue, a resazurin-based solution, by metabolically active cells. The half maximal effective concentration (EC50) of IL-15 bioactivity for the TxM complexes was then determined based on the relationship between absorbance and TxM protein concentration. Table 1 show the IL-15 EC50 values for various TxM complexes comprising binding domains of the invention. The results confirm the immunostimulatory activity of various purified TxM complexes including those with two scAb/binding domains (i.e., 2 headed (2H) anti-PD-L1 scAb/IL-15N72D:anti-PD-L1 scAb/IL-15RαSu/Fc complexes) or four scAb/binding

domains (i.e., 4 headed (4H) anti-PD-L1 scAb/IL-15N72D:anti-PD-L1 scAb/IL-15RαSu/Fc complexes) or combinations of different targeting domains (i.e., tumor targeting domains/anti-PDL1scAb TxM complexes).

Table 1. IL-15 Activity of TxM Complexes

	EC50 (pM TxM)
2H-anti-human PD-L1 scAb TxM	14
4H-anti-human PD-L1 scAb TxM	220
2H-anti-mouse PD-L1 scAb TxM	58
anti-human CTLA-4 scAb/anti-human PD-L1 scAb TxM	73
TNT scAb TxM	1013
TNT scAb/anti-human PD-L1 scAb TxM	8498
2H-hOAT TxM	115
hOAT/anti-human PD-L1 scAb TxM	202

The ability of hOAT scAb TxM to stimulate immune cell cytotoxicity against tumor cells was assessed in vitro. Human NK cells were purified from blood buffy coats with NK cell isolation kit from Stemcell Technologies and used as effector cells. TF-positive human pancreatic tumor cells, SW1990, were labeled with Celltrace-violet and used as target cells. The human NK cells and SW1990 tumor cells were mixed at an E:T ratio of 1:1 in media alone or media containing 10nM hOAT Ab (control) or 2H hOAT scAb TxM complex. After 40 hrs, the percent of target cell death was assessed by flow cytometry based on propidium iodide staining of violet-labeled target cells. As shown in FIG. 31, human NK cells incubated with 2H hOAT scAb TxM complex were capable of mediating greater cytotoxicity against TF-positive human tumor cells than untreated NK cells or NK cells treated with hOAT Ab (i.e., traditional ADCC). These results represent a significant improvement in immune cell-mediated targeted anti-tumor activity of the anti-TF TxM complexes compared to anti-TF Abs.

The ability of TxM complexes to overcome checkpoint-mediated inhibition of T cell activity is assessed in previously described in vitro assays (Steward, R, et al Cancer Immunol Res 2015 3(9):1052-1062). For example, freshly isolated primary human T cells are cultured together with anti-CD3 and anti–CD28-coated beads to demonstrate increased immune cell proliferation (measured by BrDU incorporation) and IFNγ release (measured by ELISA). Addition of PD-L1 antibody on the beads significantly reduces T cell proliferation and IFNγ release due inhibitory signaling of PD-L1/PD-1 interactions. Addition of soluble PD-L1 TxM or PD-1 TxM in the context of anti-CD3, anti–CD28, PD-L1-coated beads and T cells increases T cell proliferation and IFNγ release due to blockade of PD-L1/PD-1 interactions. Similar assays with CTLA-4 TxM in the context of anti-CD3, anti–CD28, anti-CTLA-4-coated beads and T cells also demonstrate the immune checkpoint inhibitory activity of CTLA-4 TxM.

The anti-tumor activity of these complexes is assessed in mouse xenograft models using human tumor cell lines and patient derived tumor cells (Morton, J.J., et al. Cancer Research 2016 doi: 10.1158/0008-5472). Commercially available humanized mouse models (i.e., Hu-CD34 NSG™, Jackson laboratory) have been developed to assess the activity of immunotherapies on human immune cell responses against tumors derived from human tumor cell lines and patient derived tumor cells. For example, Hu-CD34 NSG™ mice bearing PD-L1-positive subcutaneous human MDA-MB-231 breast cancer tumors is treated with PBS or increasing dose levels of PD-L1 TxM or PD-1 TxM (i.e., subcutaneous administration twice weekly for 2 weeks) and tumor growth is assessed. Dose dependent decreases in tumor volume provides evidence of the therapeutic activity of PD-L1 TxM and/or PD-1 TxM against PD-L1-positive human tumors. Solid tumor mouse models are also available using patient derived PD-L1-postive tumor cells (i.e., BR1126(TM00098), LG1306(TM00302)). The activity of PD-L1 TxM and/or PD-1 TxM in BR1126 tumor-bearing Hu-CD34 NSG™ mice is assessed by evaluating tumor growth or mouse survival post-treatment. In addition, treatment dependent changes in T cell responses in the blood and tumor microenvironment is evaluated in these models. An increase in T cells levels or activity (i.e., IFNγ-positive cells) in the blood or tumor post PD-L1 TxM and/or PD-1 TxM treatment provide evidences of immunostimulatory activity of these complexes in tumor-bearing mice. Together, these studies demonstrate the immune cell-mediated activity of PD-L1 TxM and/or PD-1 TxM against human tumors *in vivo*.

OTHER EMBODIMENTS

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.

The patent and scientific literature referred to herein establishes the knowledge that is available to those with skill in the art. All United States patents and published or unpublished United States patent applications cited herein are incorporated by reference. All published foreign patents and patent applications cited herein are hereby incorporated by reference. Genbank and NCBI submissions indicated by accession number cited herein are hereby incorporated by reference. All other published references, documents, manuscripts, and scientific literature cited herein are hereby incorporated by reference.

While this invention has been particularly shown and described with references to preferred embodiments thereof, it will be understood by those skilled in the art that various changes in form and details may be made therein without departing from the scope of the invention encompassed by the appended claims.

What is claimed is:

1. An isolated soluble fusion protein complex comprising at least two soluble proteins, wherein

a first soluble protein comprises an interleukin-15 (IL-15) polypeptide domain and a second soluble protein comprises a soluble IL-15 receptor alpha sushi-binding domain (IL-15RαSu) fused to an immunoglobulin Fc domain,

wherein one of the first or second soluble protein further comprises a binding domain substantially identical to SEQ ID NO:2 or SEQ ID NO:6, and

wherein the IL-15 domain of the first soluble protein binds to the IL-15RαSu domain of the second soluble protein to form a soluble fusion protein complex.

2. The soluble fusion protein complex of claim 1, wherein one of the first or second soluble protein further comprises a second binding domain that specifically binds to a disease antigen, immune checkpoint molecule, or immune signaling molecule.

3. The soluble fusion protein complex of claim 1, wherein the IL-15 polypeptide is an IL-15 variant comprising an N72D mutation (IL-15N72D).

4. The soluble fusion protein complex of claim 1, wherein the binding domain comprises an immunoglobulin light chain variable domain covalently linked to an immunoglobulin heavy chain variable domain by a polypeptide linker sequence.

5. The soluble fusion protein complex of claim 1, wherein the binding domain specifically binds to one or more molecules comprising: programmed death ligand 1 (PD-L1), programmed death 1 (PD-1), cytotoxic T-lymphocyte associated protein 4 (CTLA-4), cluster of differentiation 33 (CD33), cluster of differentiation 47 (CD47), glucocorticoid-induced tumor necrosis factor receptor (TNFR) family related gene (GITR), lymphocyte function-associated antigen 1 (LFA-1), tissue factor (TF), delta-like protein 4 (DLL4), single strand DNA or T-cell immunoglobulin and mucin-domain containing-3 (Tim-3).

6. The soluble fusion protein complex of claim 1, wherein the first soluble protein comprises the amino acid sequence set forth in one of SEQ ID Nos: 2, 6, 10, 18, 20, 24, 28, 32, or 38.

7. The soluble fusion protein complex of claim 1, wherein the second soluble protein comprises the amino acid sequence set forth in one of SEQ ID NOs: 4, 8, 12, 14, 16, 22, 26, 30, 34, 36, 40, 42, 44, 46, 51, 52, 53, or 54.

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8. An isolated soluble fusion protein comprising an IL-15 peptide domain fused to a binding domain substantially identical to SEQ ID NO:2 or SEQ ID NO:6.

9. An isolated soluble fusion protein comprising IL-15RαSu fused to both: (a) an immunoglobulin Fc domain; and (b) a binding domain substantially identical to SEQ ID NO:2 or SEQ ID NO:6.

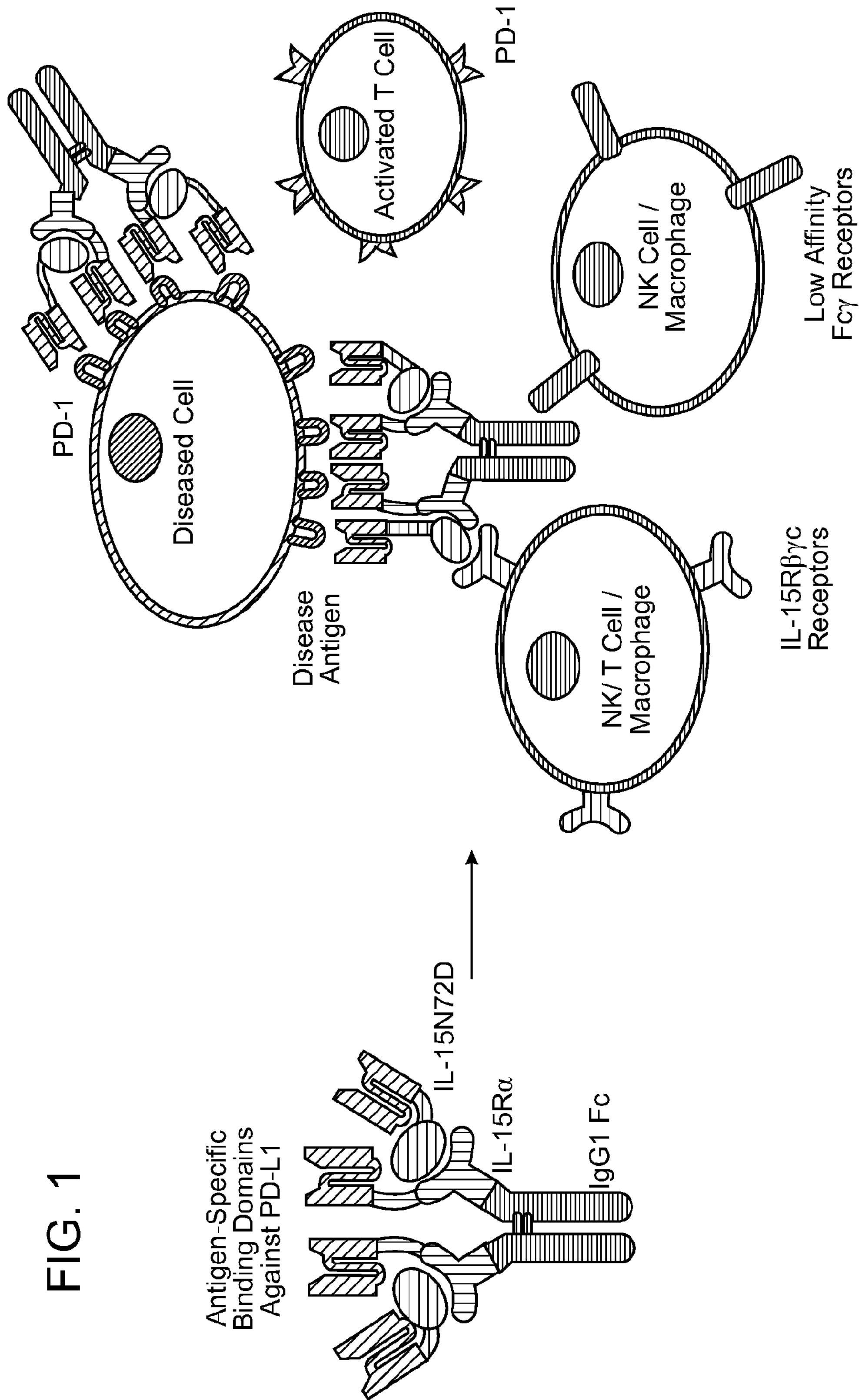


FIG. 1

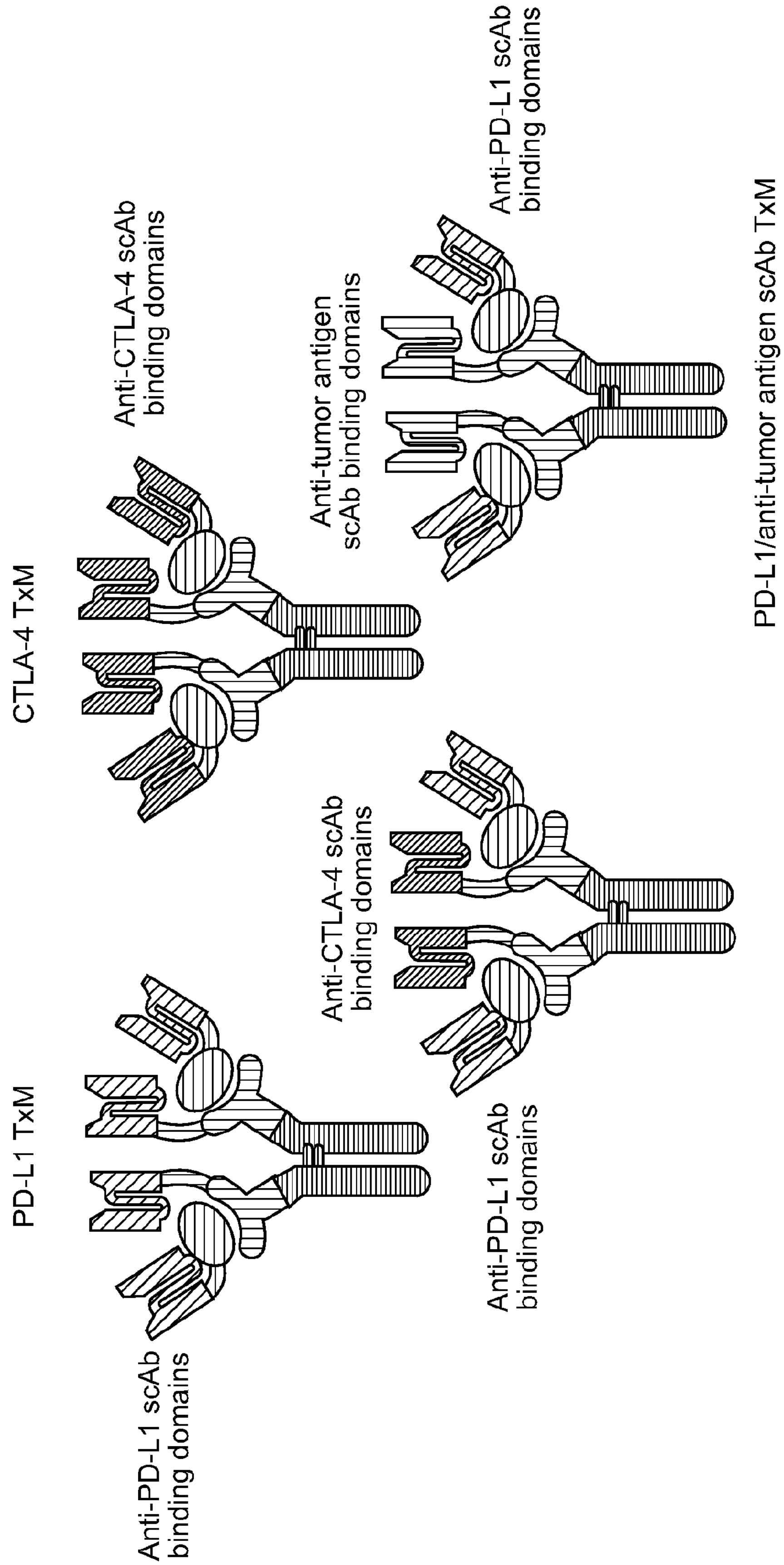
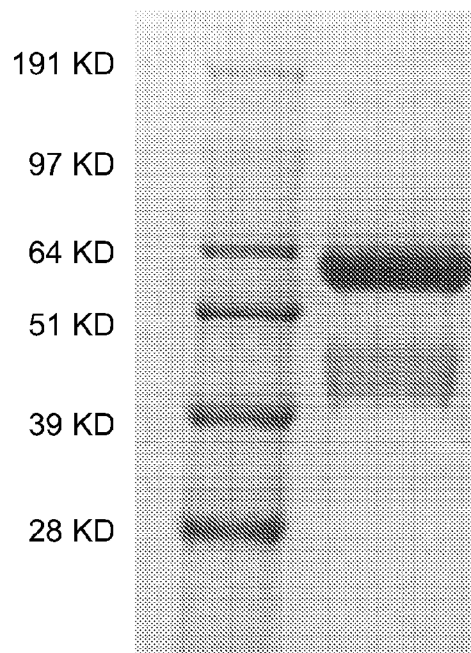


FIG. 2

FIG. 3



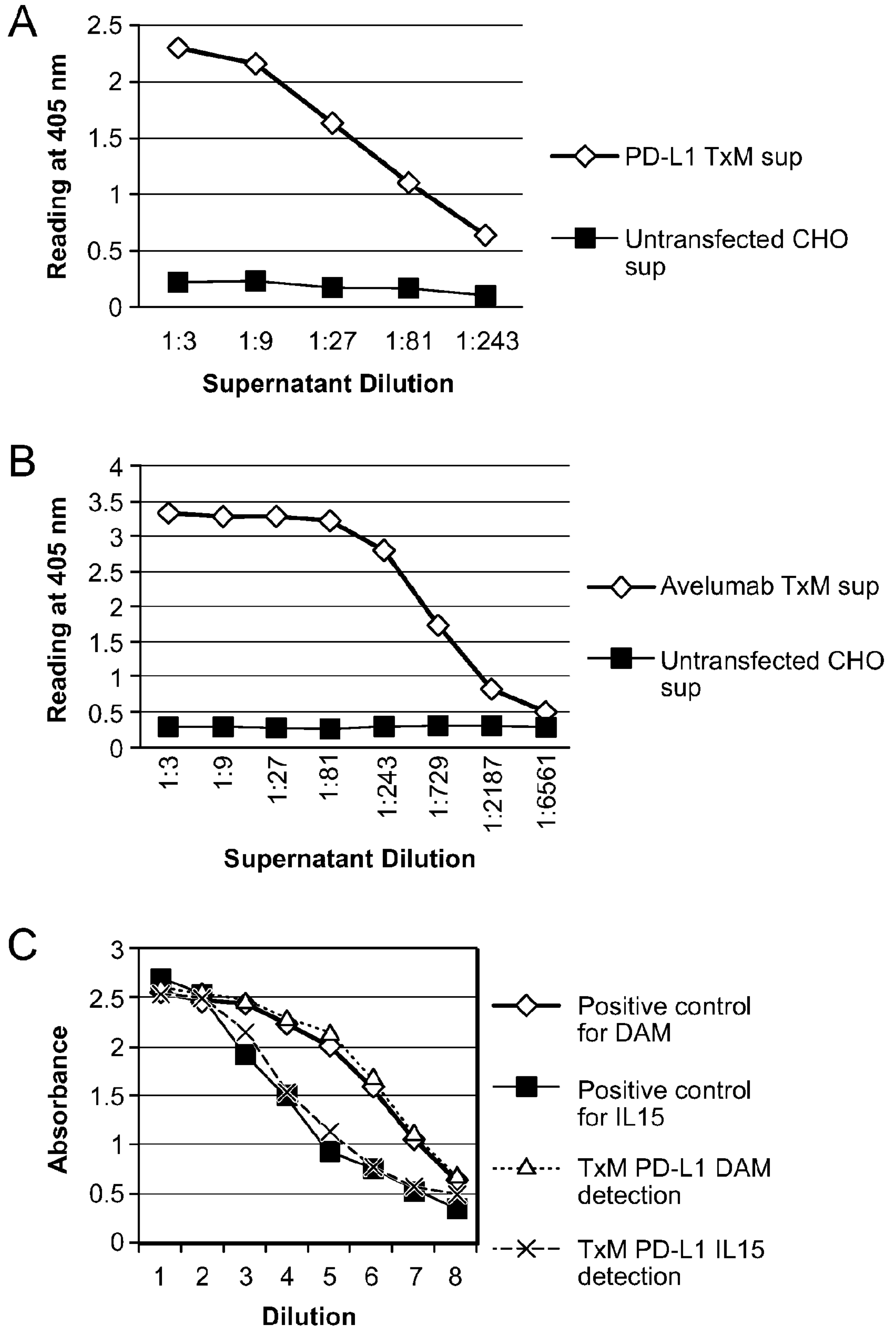


FIG. 4
A
Reading at 405 nm
2.5
2
1.5
1
0.5
0
1:3
1:9
1:27
1:81
1:243
Supernatant Dilution
PD-L1 TxM sup
Untransfected CHO sup
B
Reading at 405 nm
4
3.5
3
2.5
2
1.5
1
0.5
0
1:3
1:9
1:27
1:81
1:243
1:729
1:2187
1:6561
Supernatant Dilution
Avelumab TxM sup
Untransfected CHO sup
C
Absorbance
3
2.5
2
1.5
1
0.5
0
1
2
3
4
5
6
7
8
Dilution
Positive control for DAM
Positive control for IL15
TxM PD-L1 DAM detection
TxM PD-L1 IL15 detection

FIG. 5

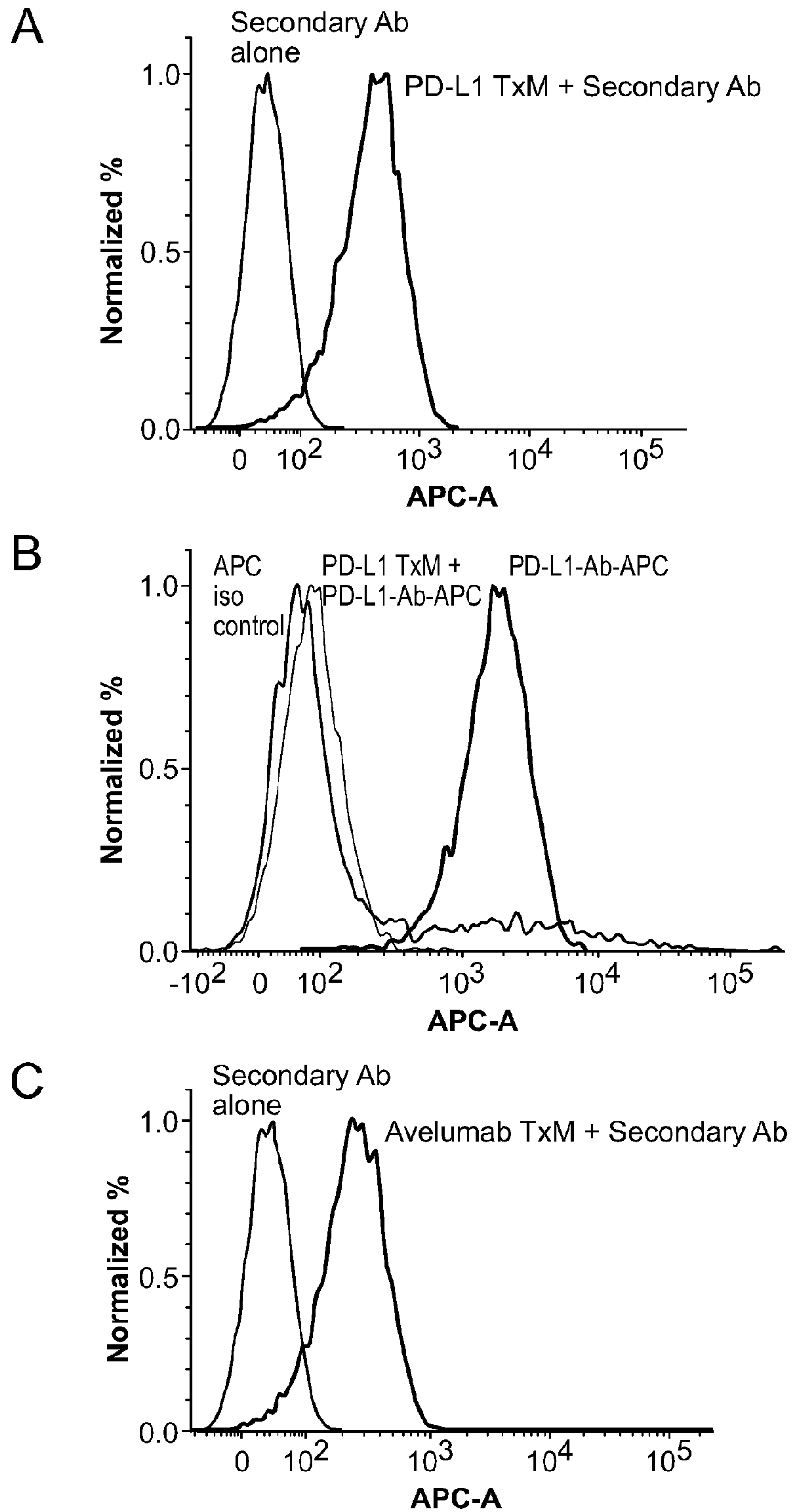
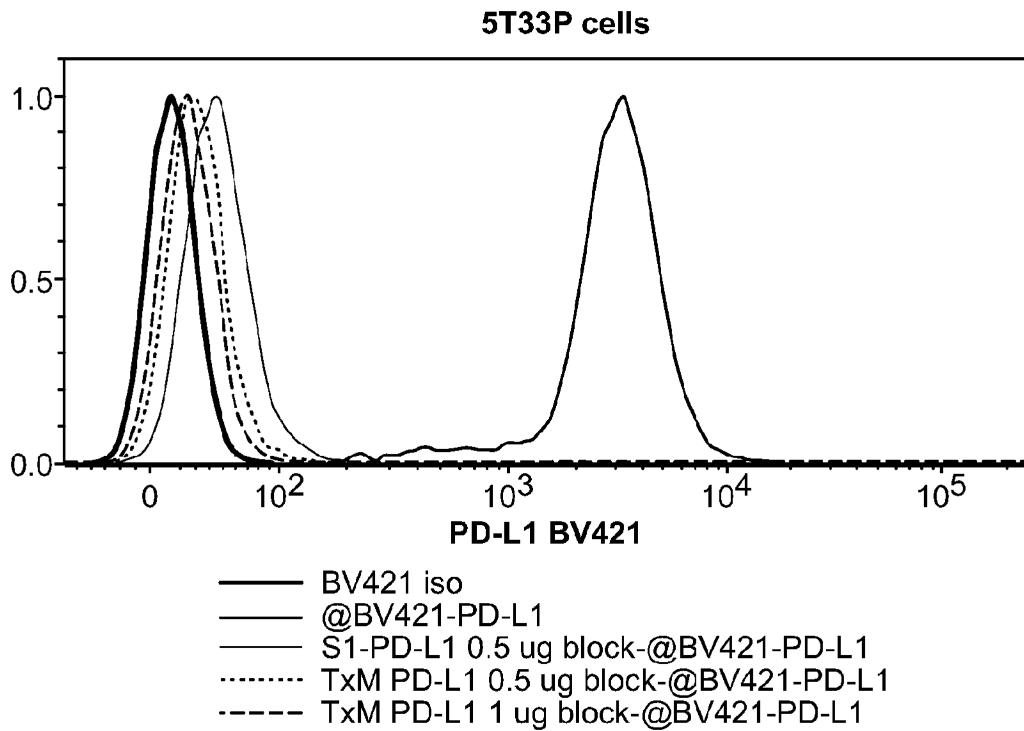
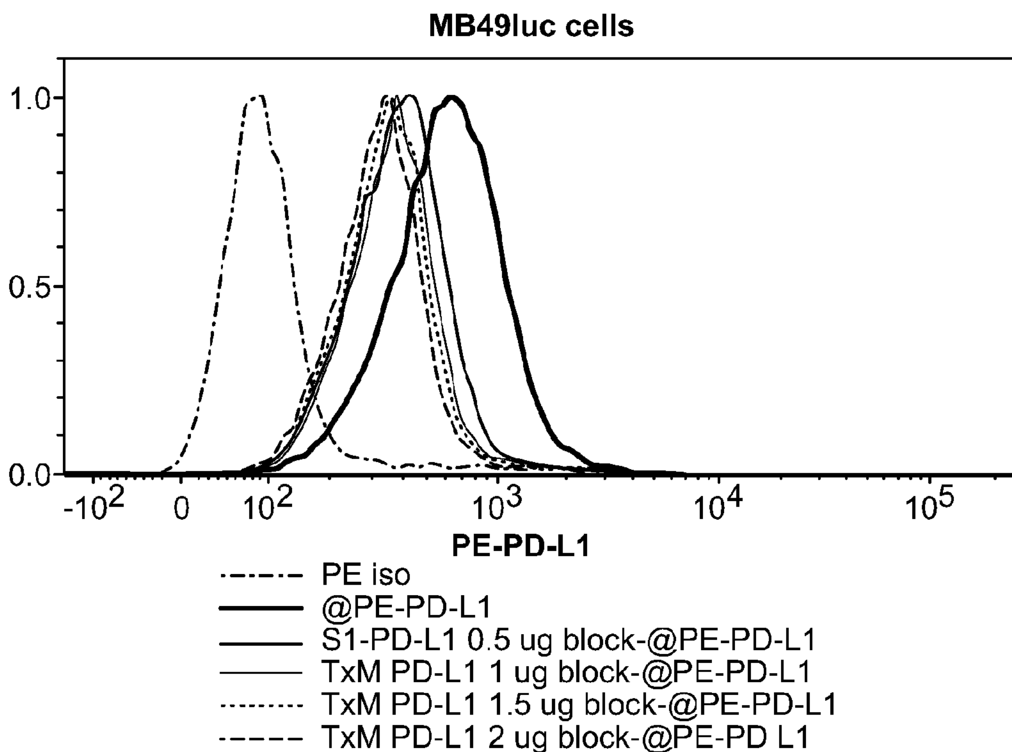


FIG. 6

A



B



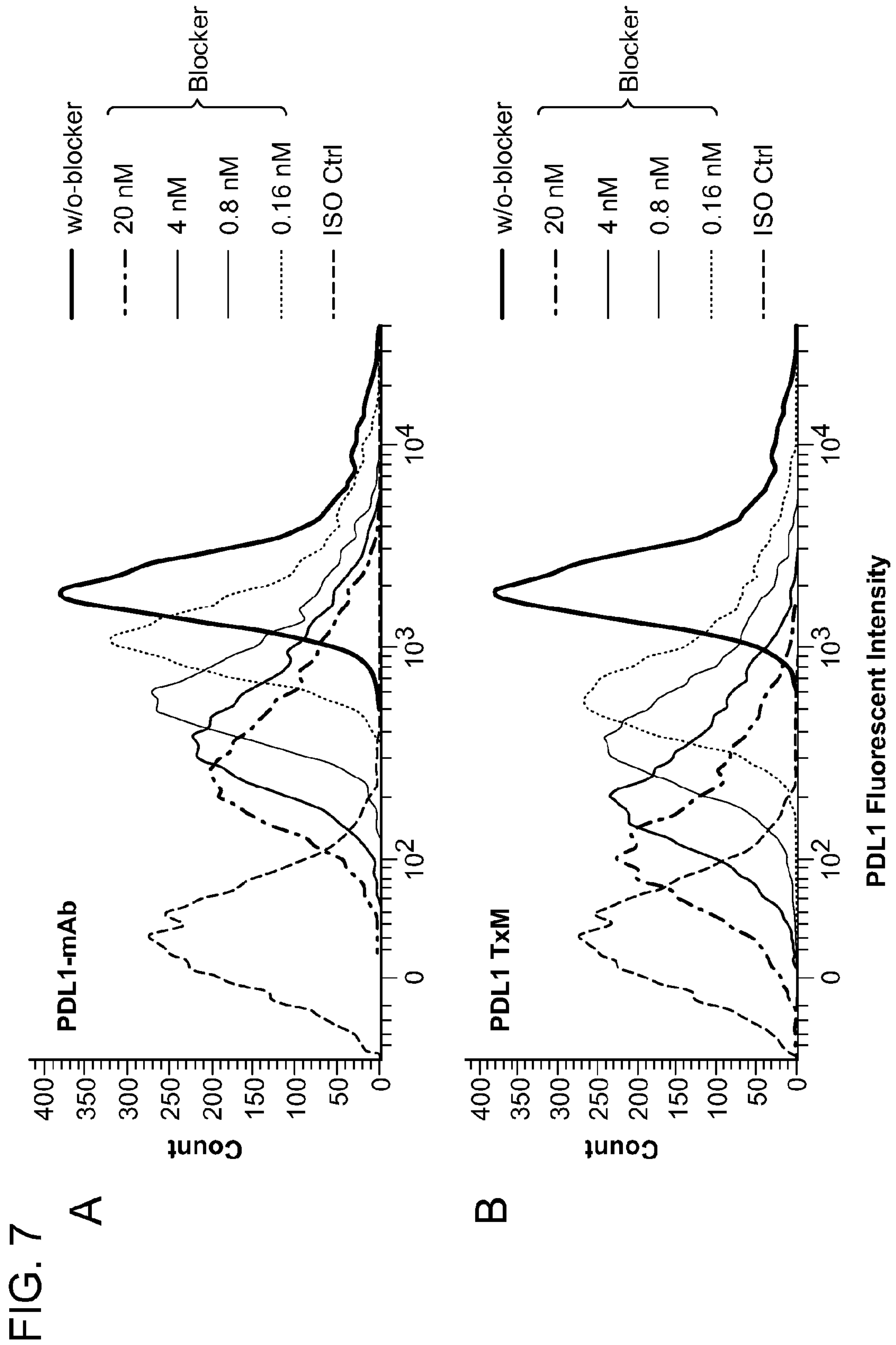


FIG. 7
A
PDL1-mAb
Count
400
350
300
250
200
150
100
50
0
0
10^2
10^3
10^4
w/o-blocker
20 nM
4 nM
0.8 nM
0.16 nM
ISO Ctrl
Blocker
B
PDL1 TxM
Count
PDL1 Fluorescent Intensity

FIG. 8

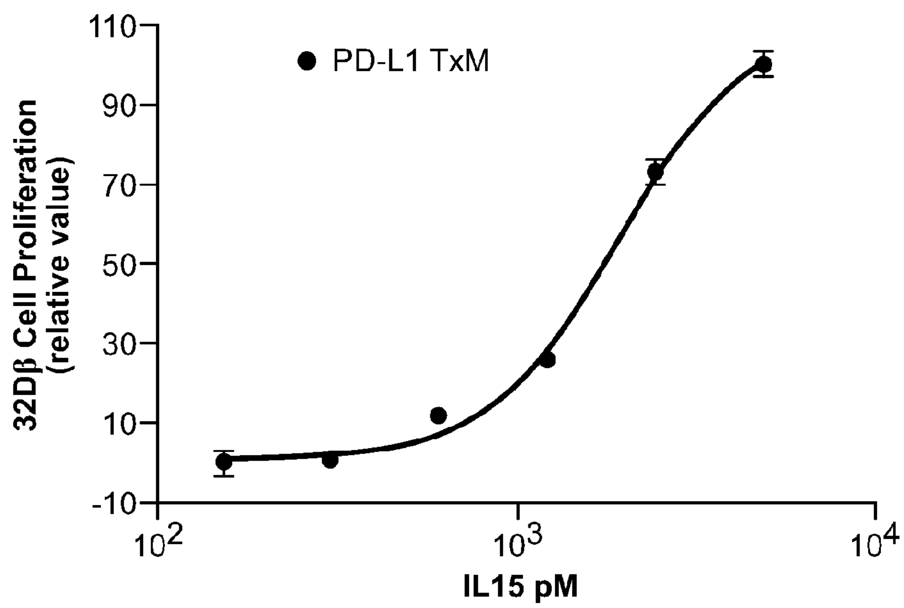
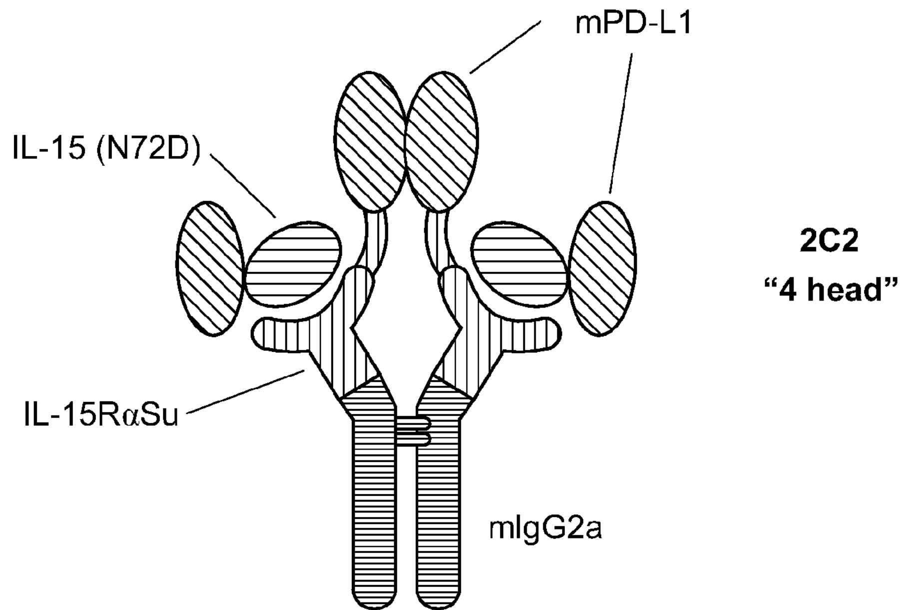


FIG. 9

A



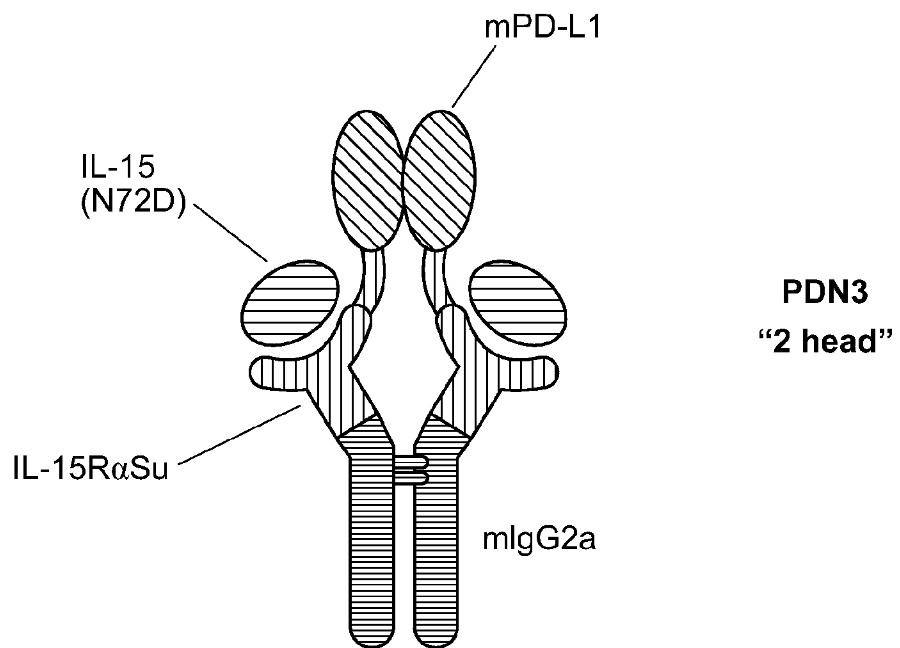


FIG. 9 (cont.)

B

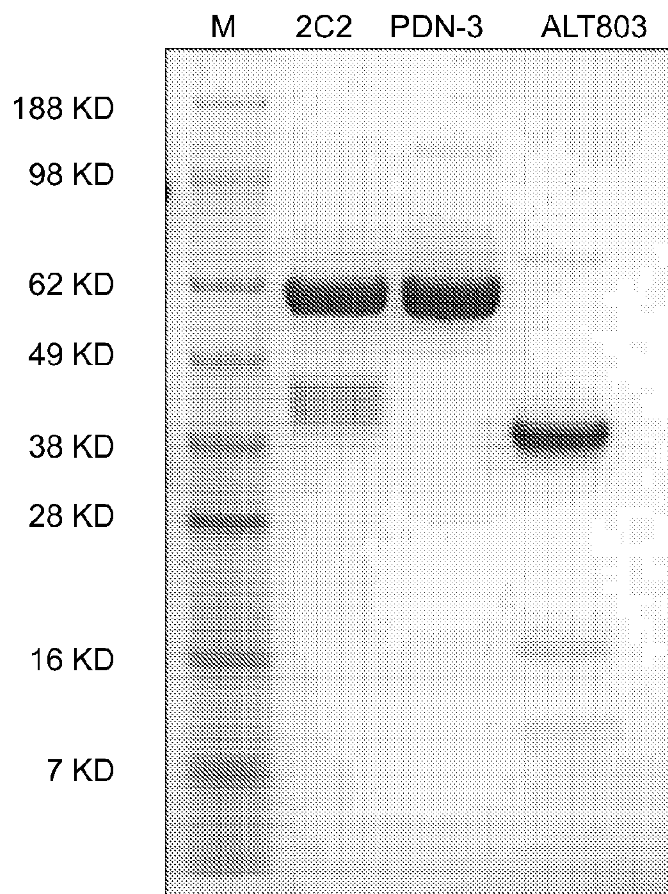
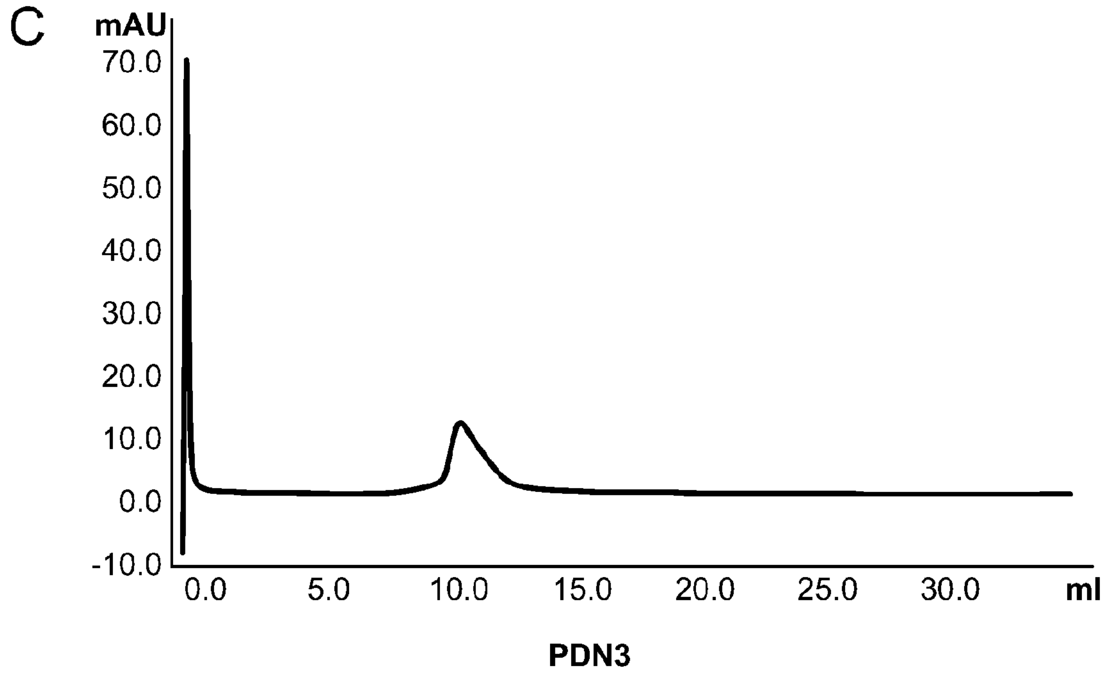


FIG. 9 (cont.)



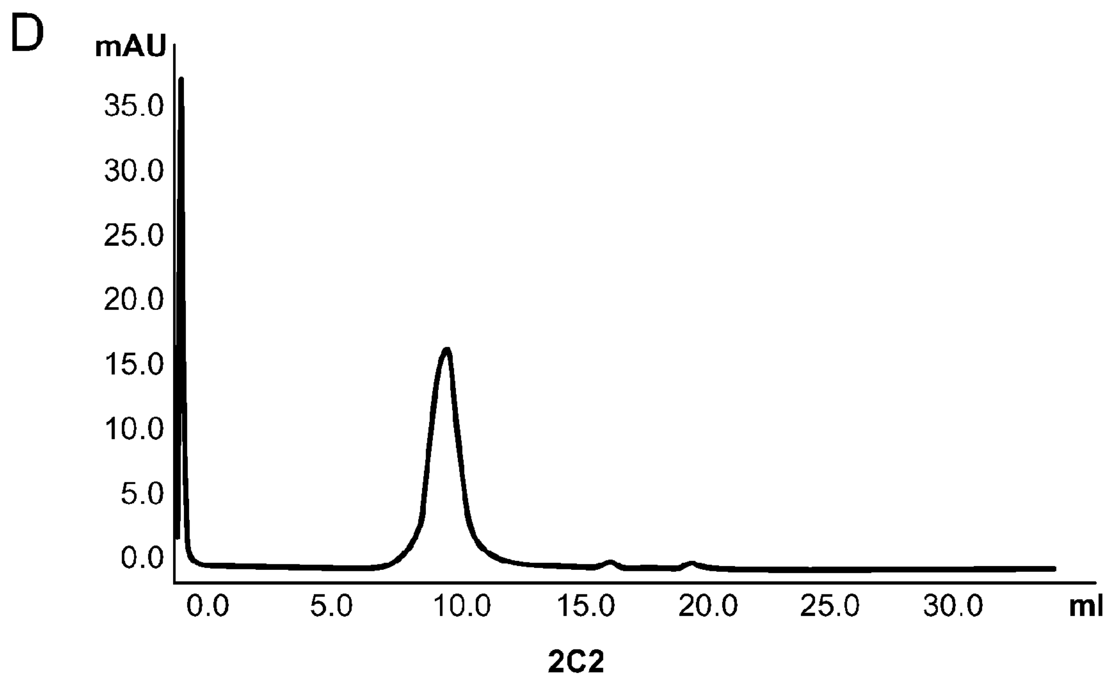


FIG. 10

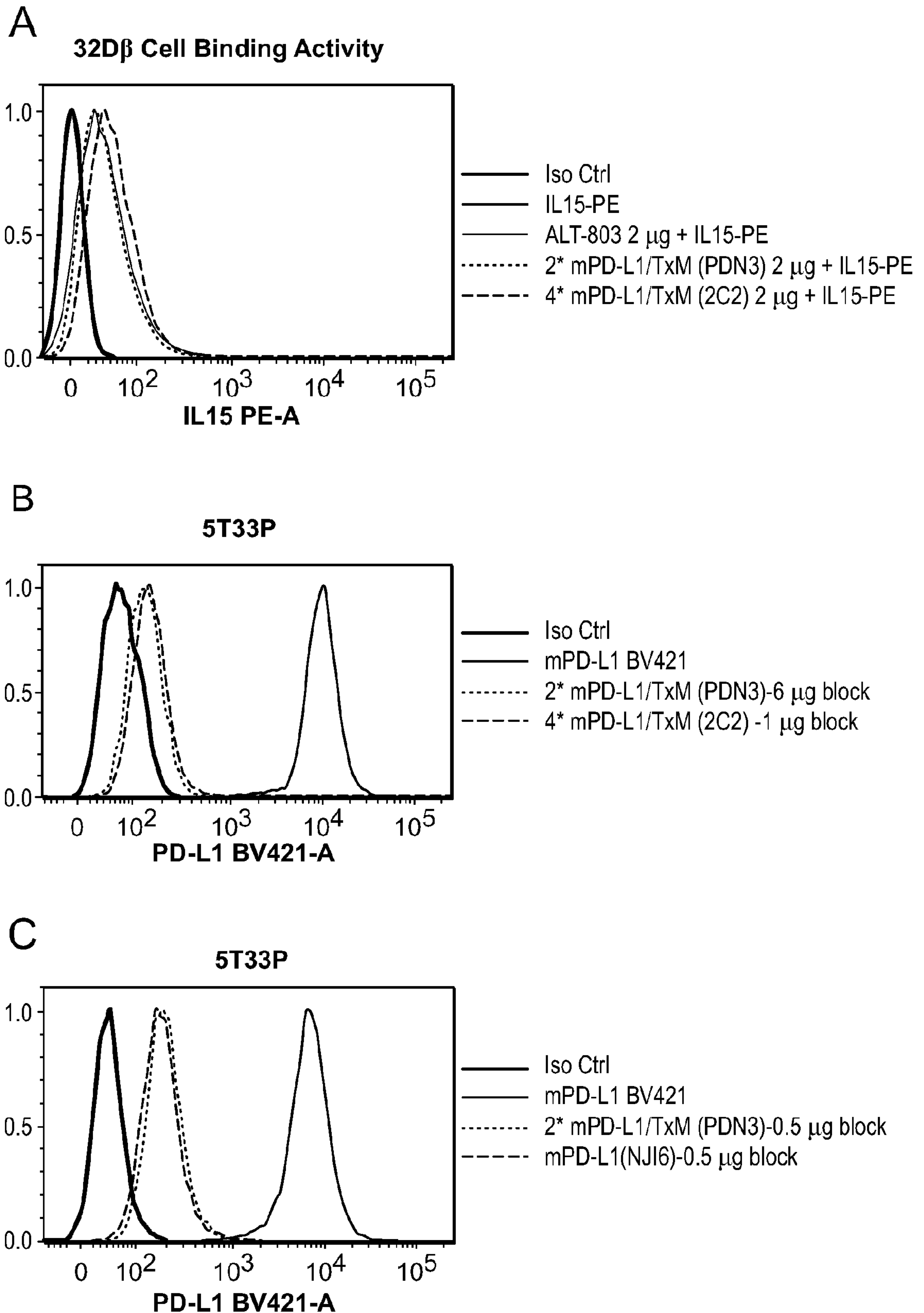
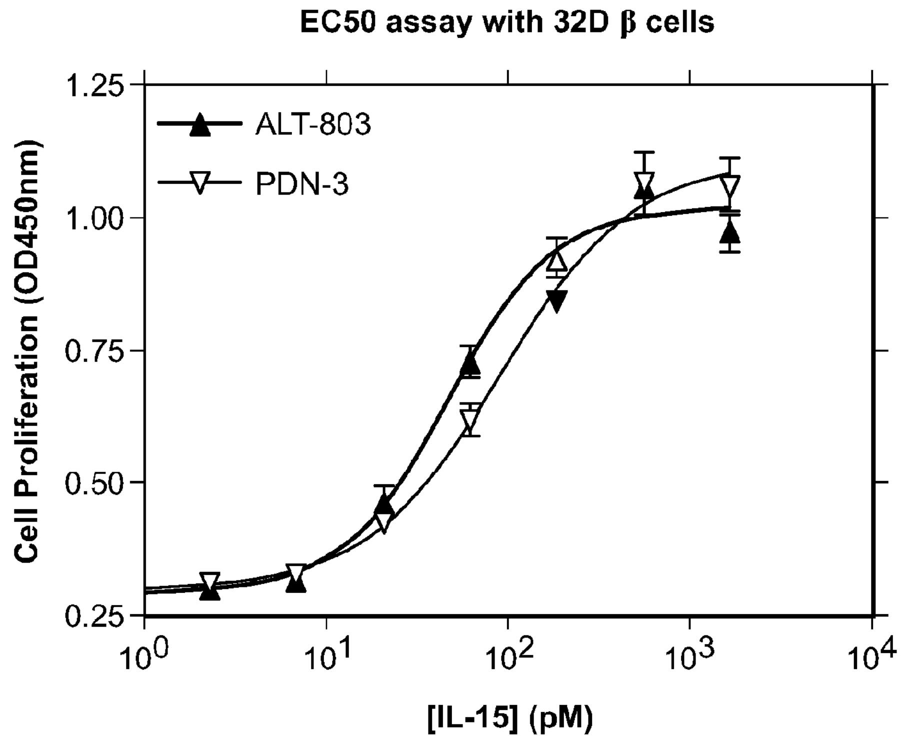
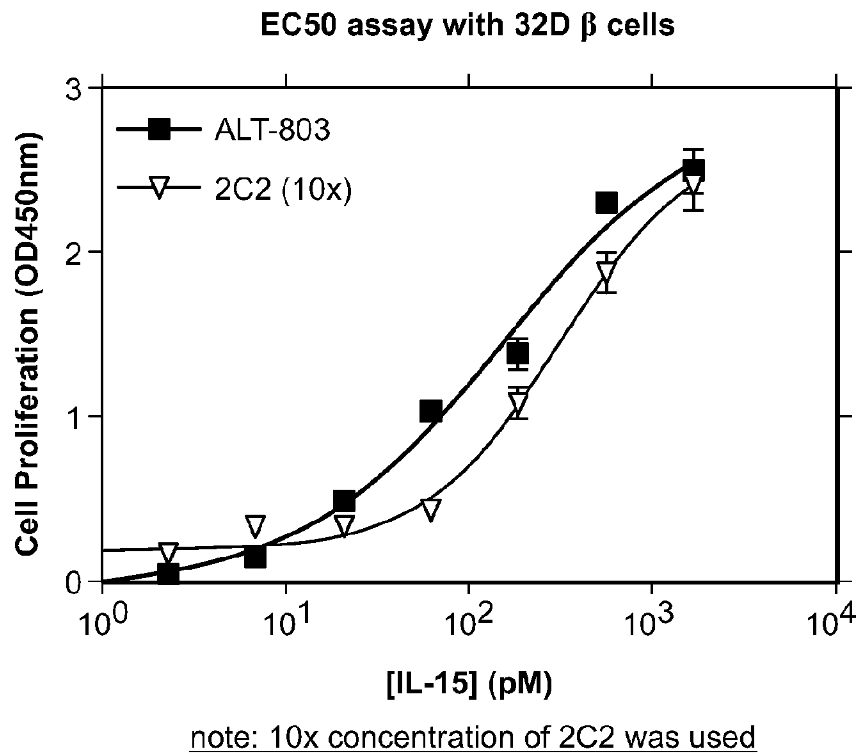


FIG. 11

A



B



note: 10x concentration of 2C2 was used

FIG. 12

A

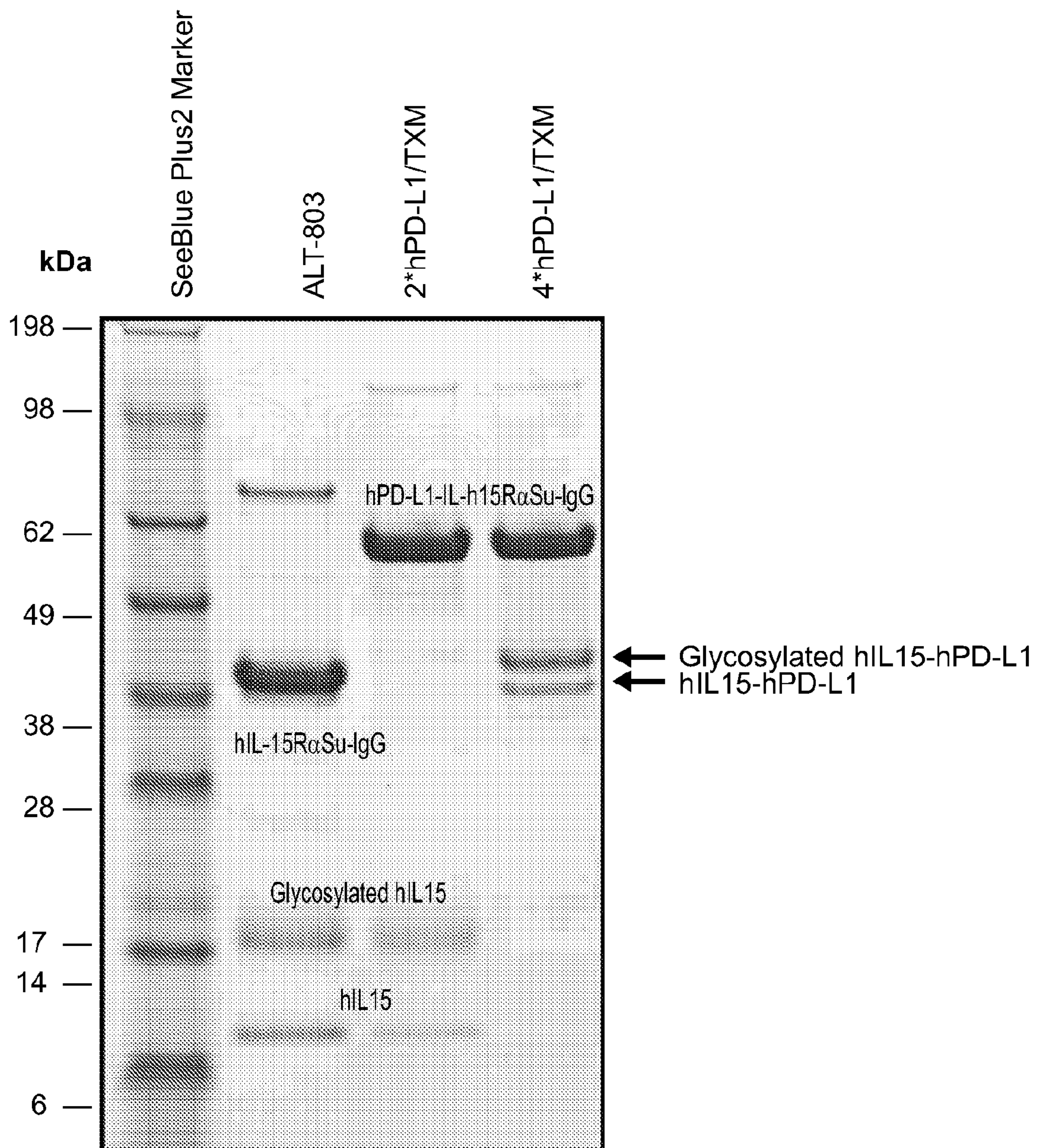
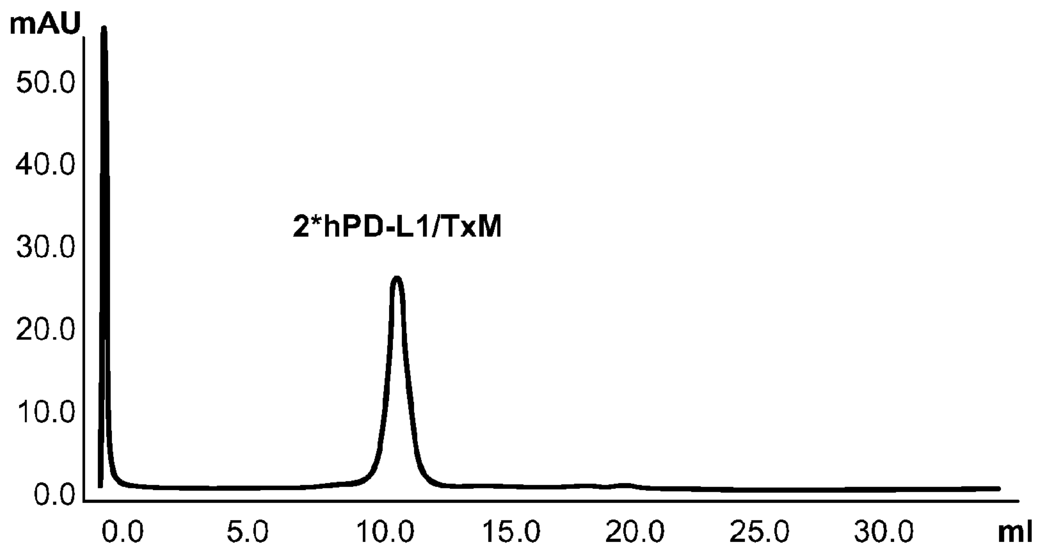
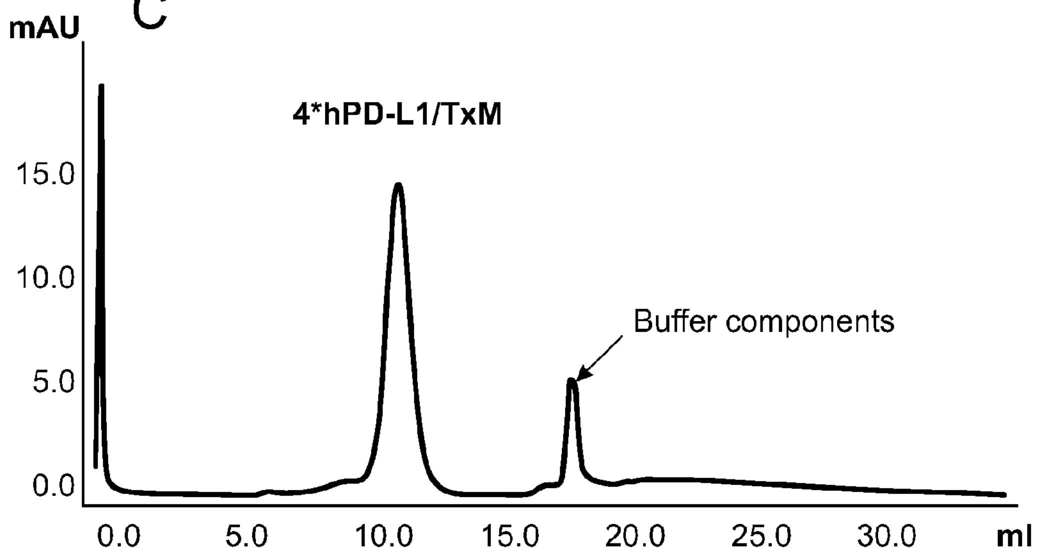


FIG. 12 (cont.)

B



C



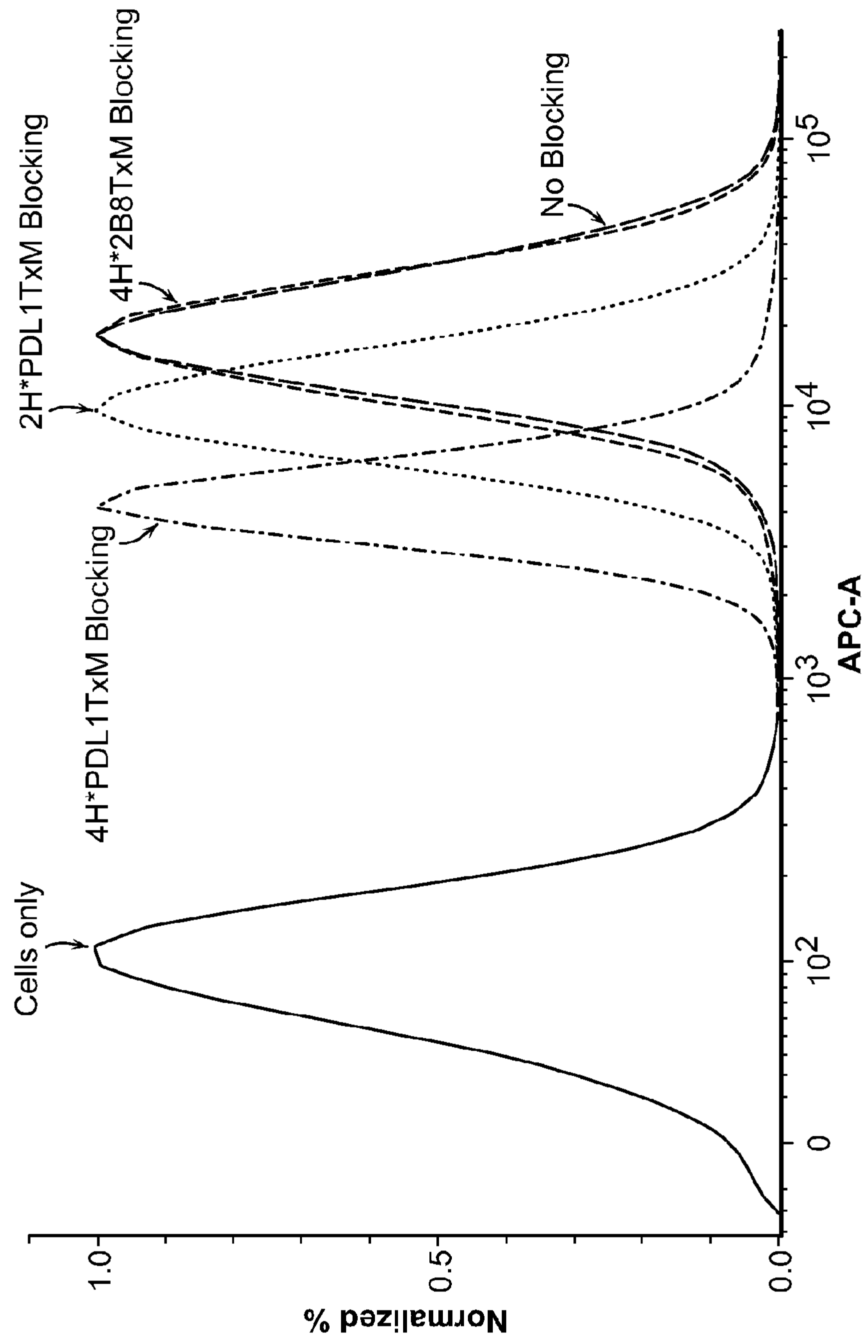
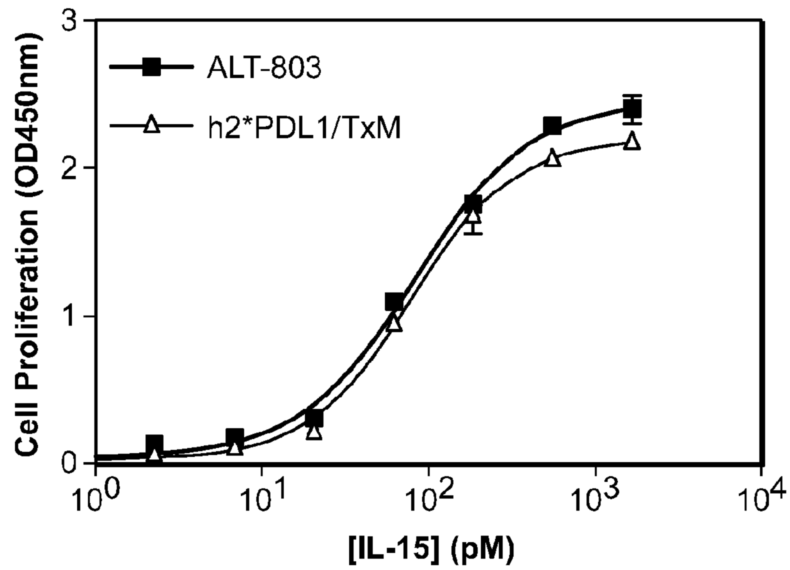


FIG. 13
Cells only
4H*PDL1TxM Blocking
2H*PDL1TxM Blocking
4H*2B8TxM Blocking
No Blocking
Normalized %
1.0
0.5
0.0
0
10^2
10^3
10^4
10^5
APC-A

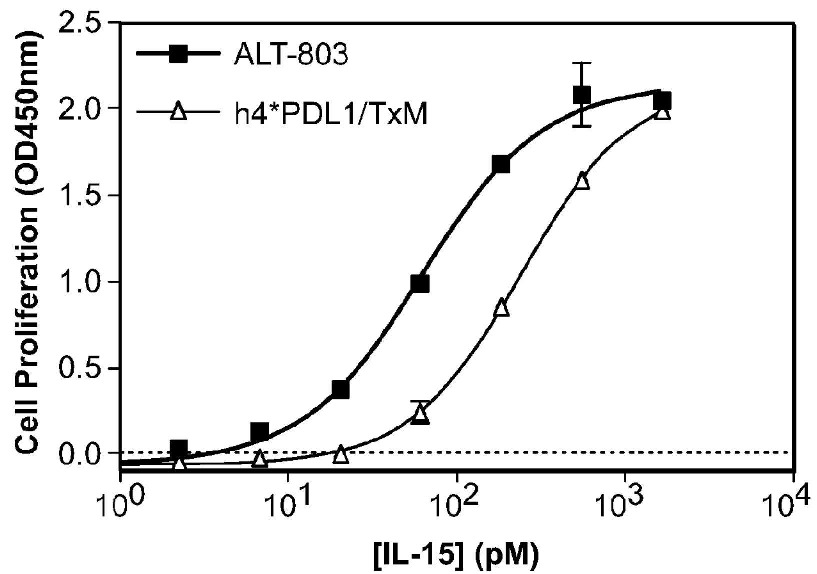
FIG. 14

A



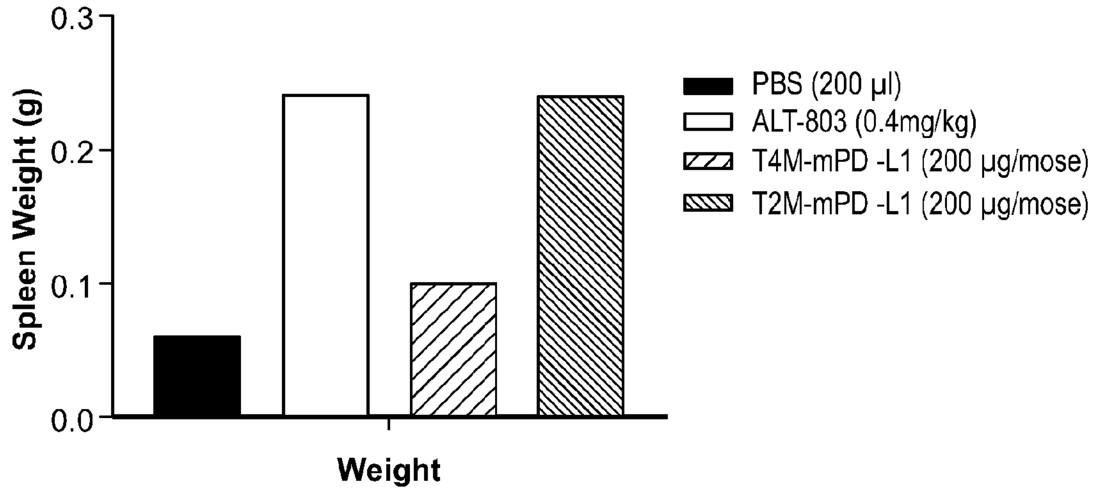
EC50 value of h2*PDL1/TxM is ~1-1.5 fold higher than ALT-803

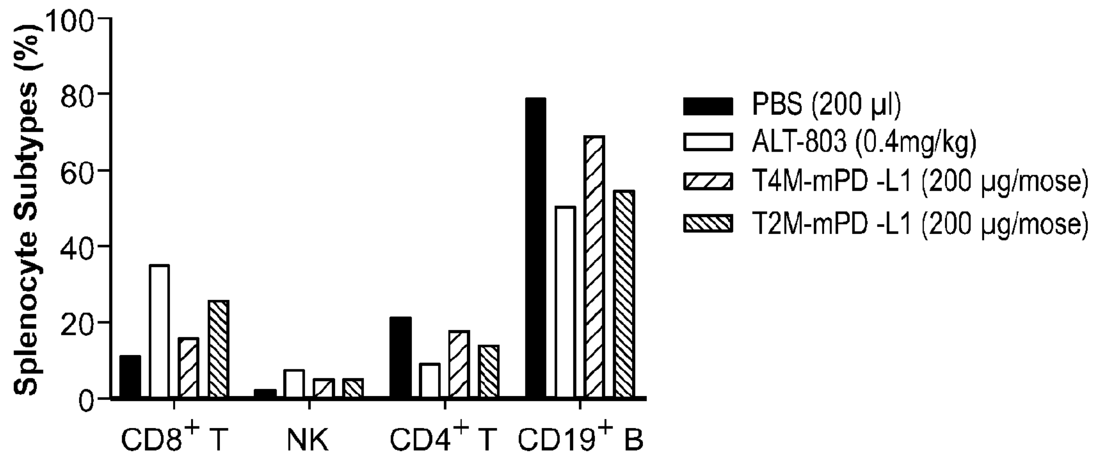
B



EC50 value of h4*PDL1/TxM is ~ 5 fold higher than ALT-803

FIG. 15





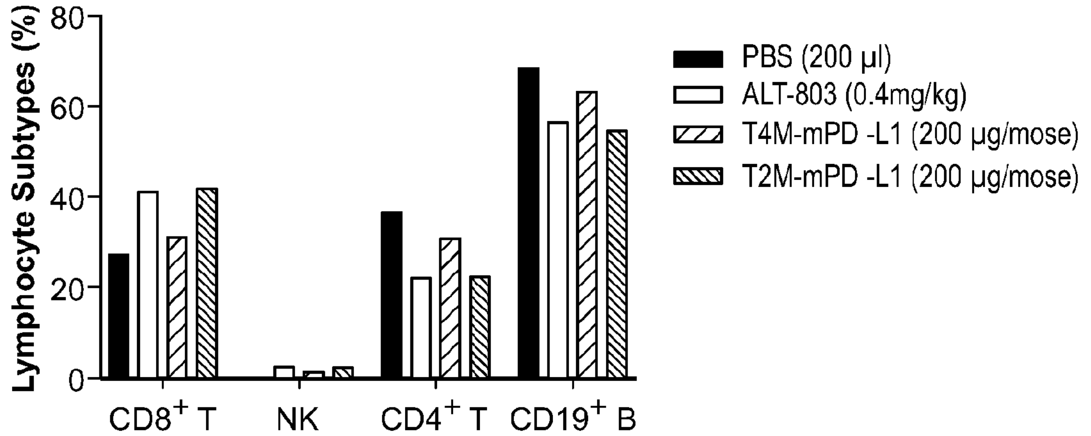


FIG. 16

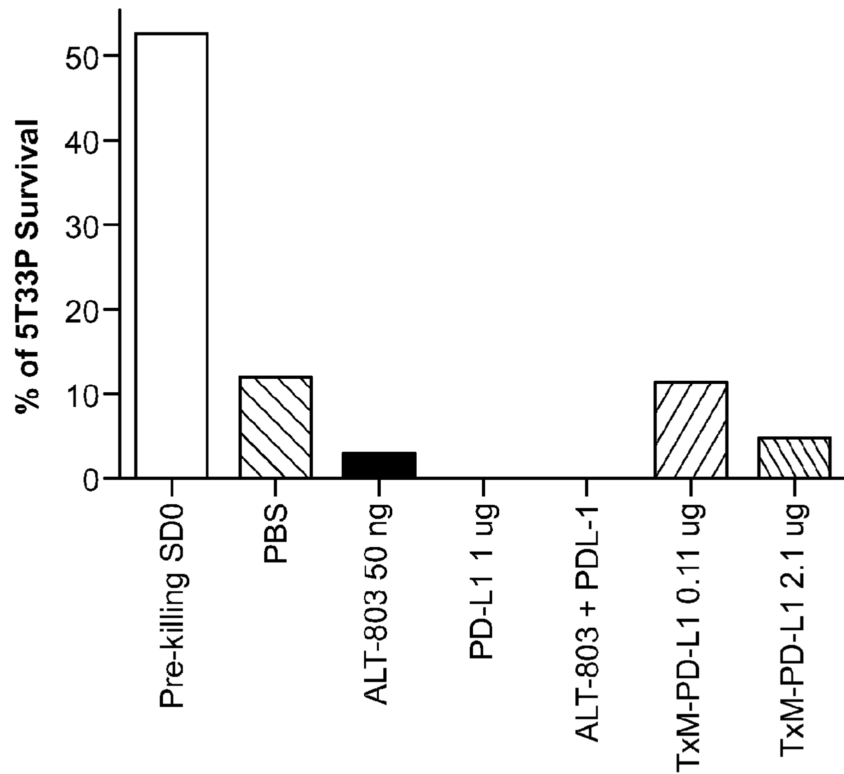


FIG. 17

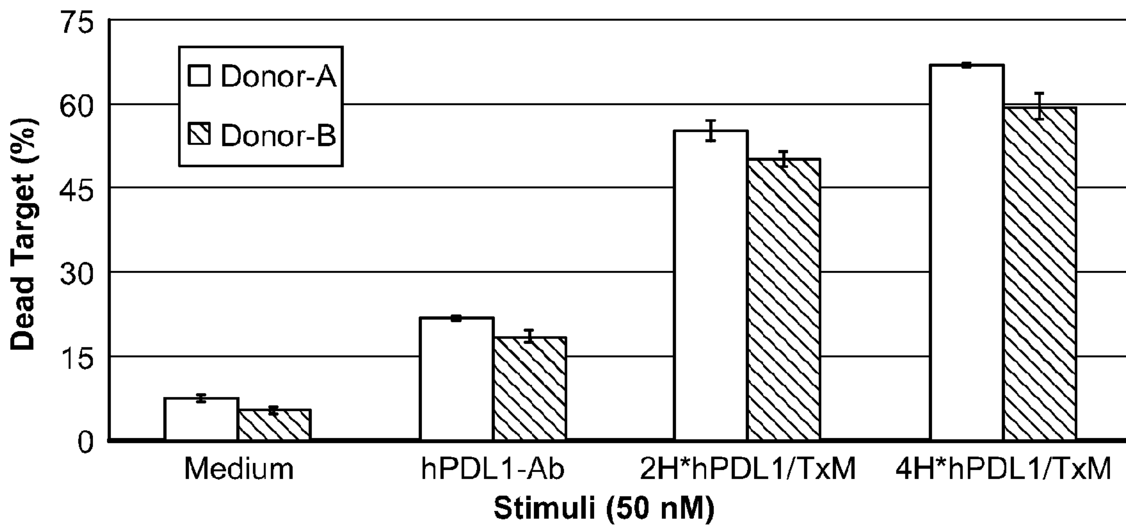


FIG. 18

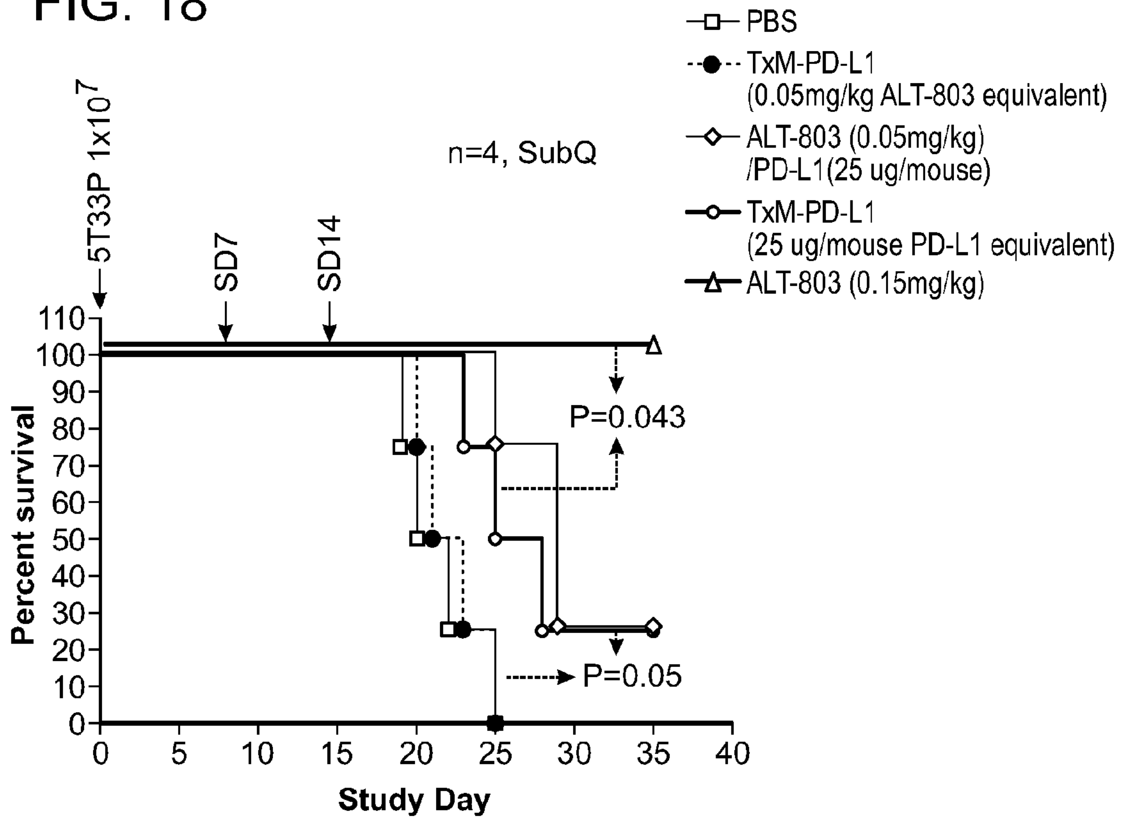


FIG. 19

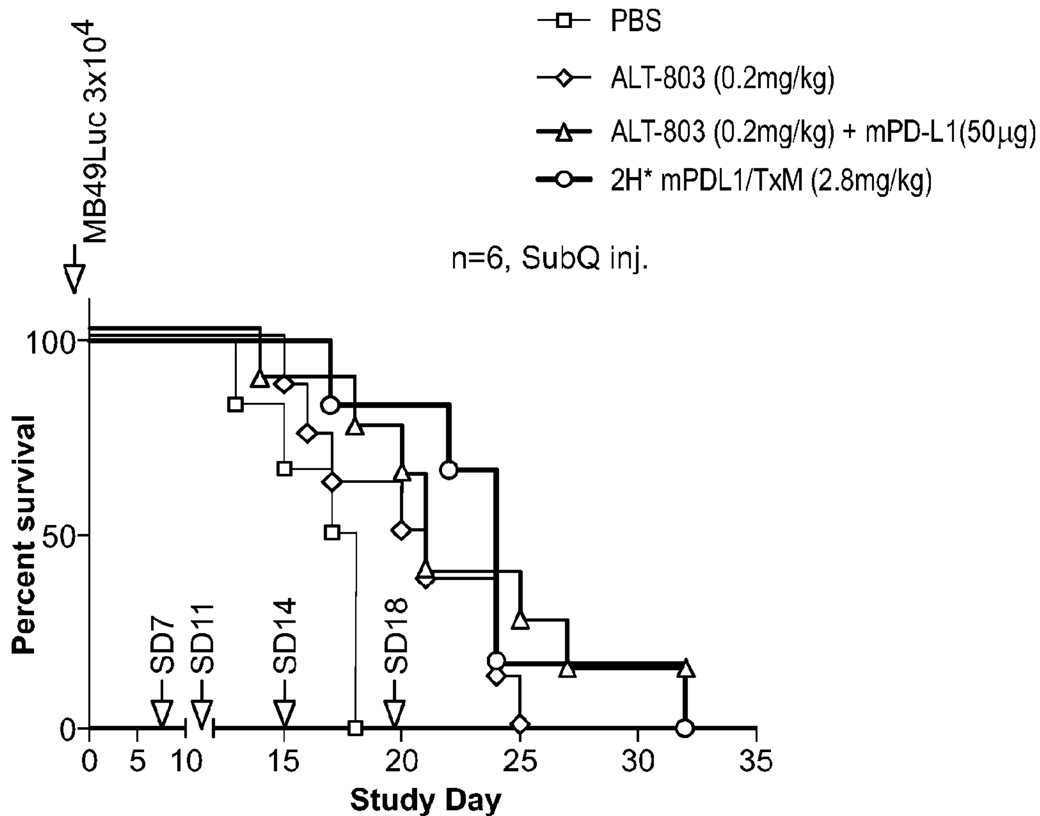


FIG. 20

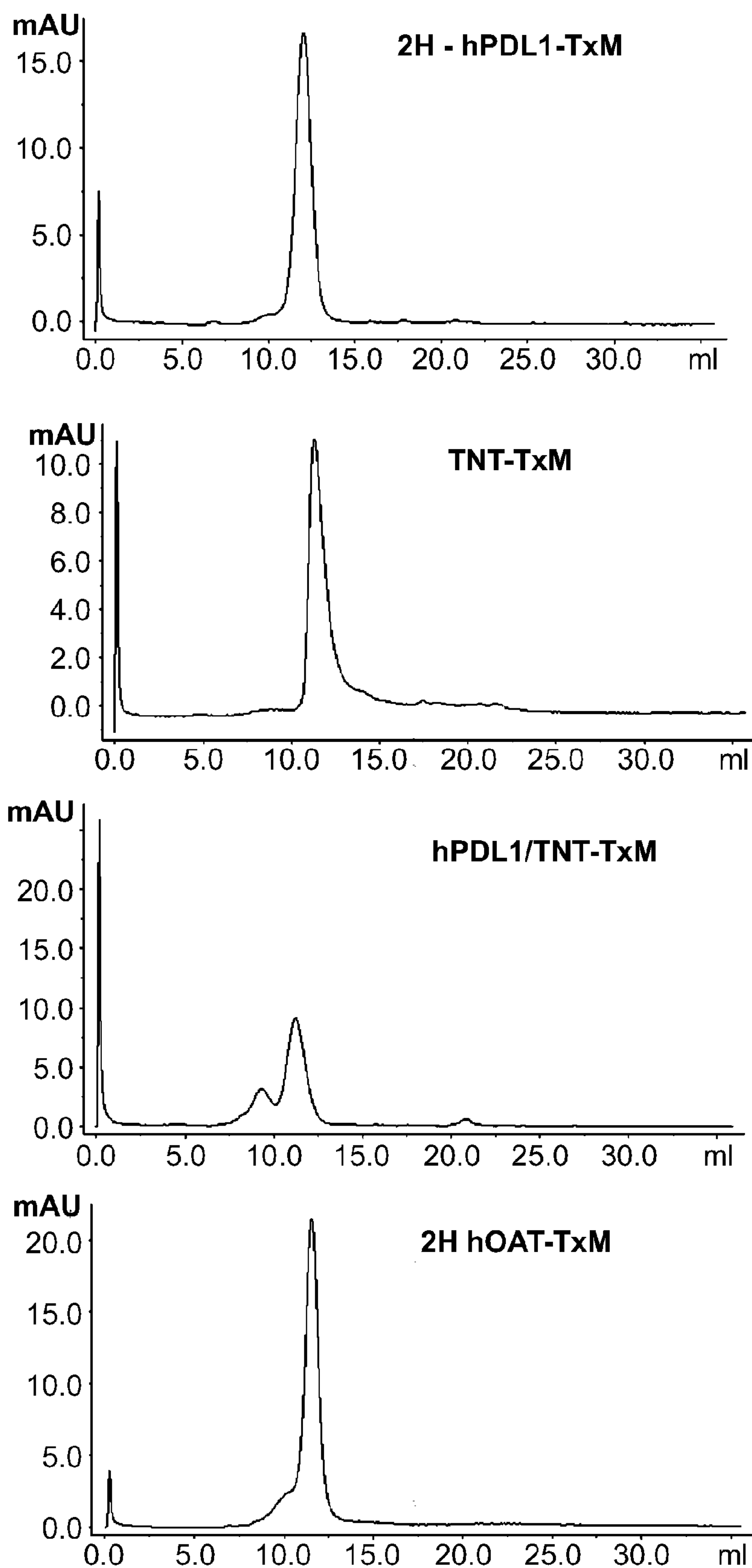
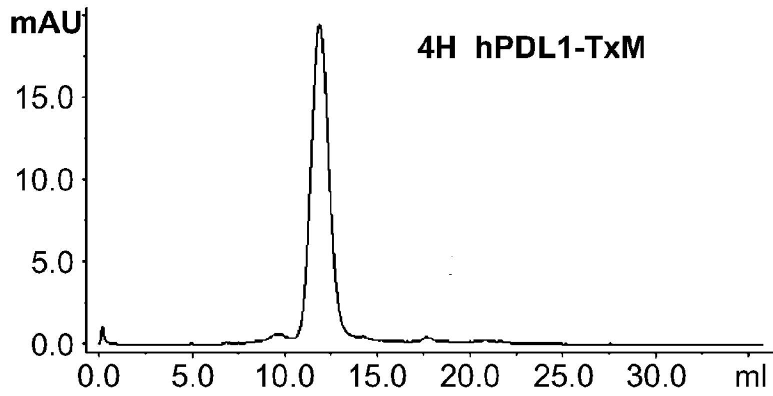
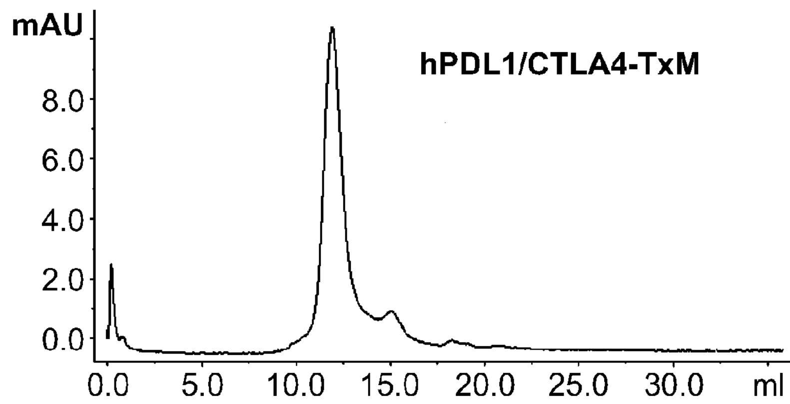
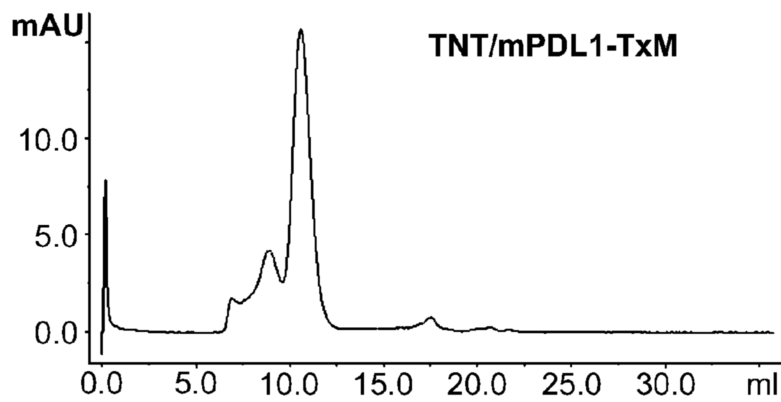


FIG. 20 (cont.) 22/34







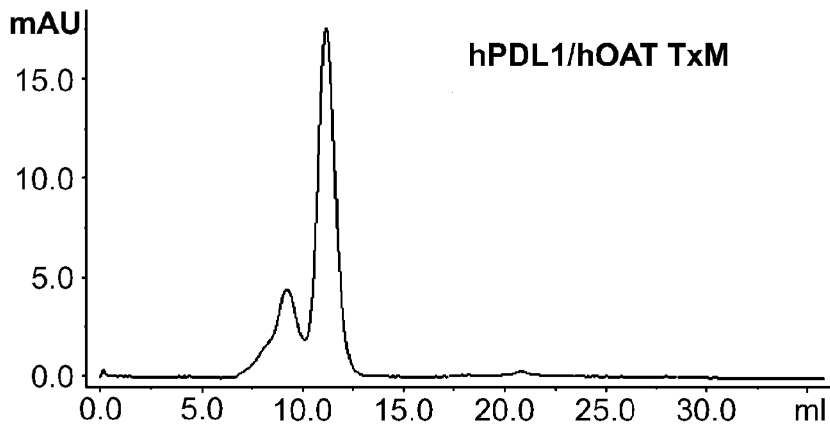
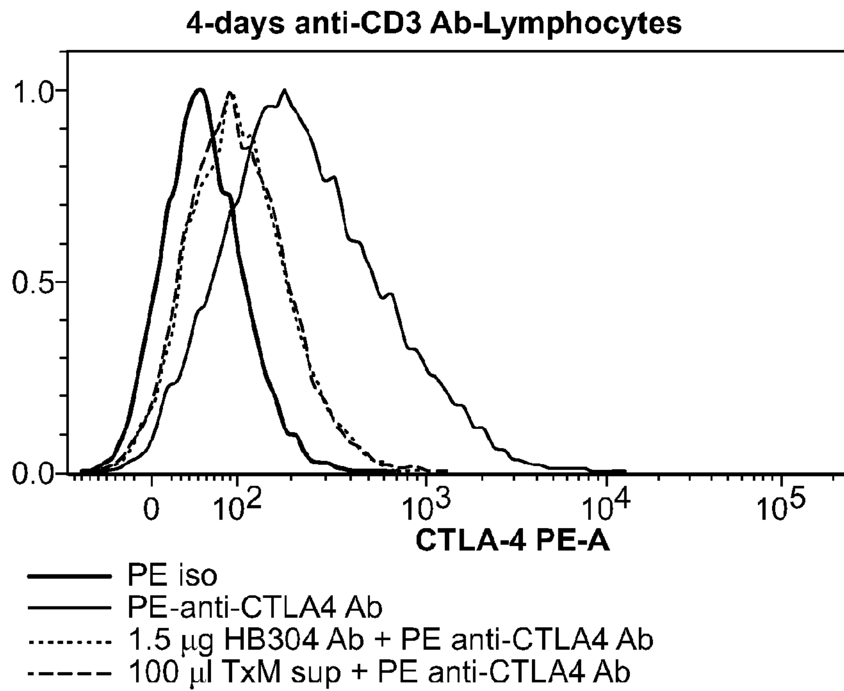


FIG. 21

A



B

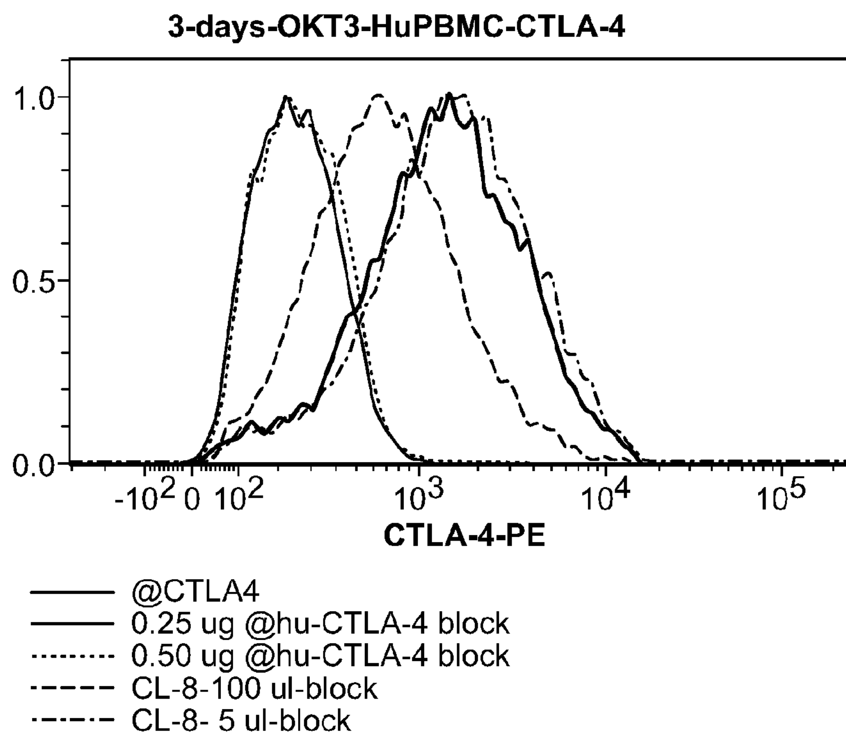
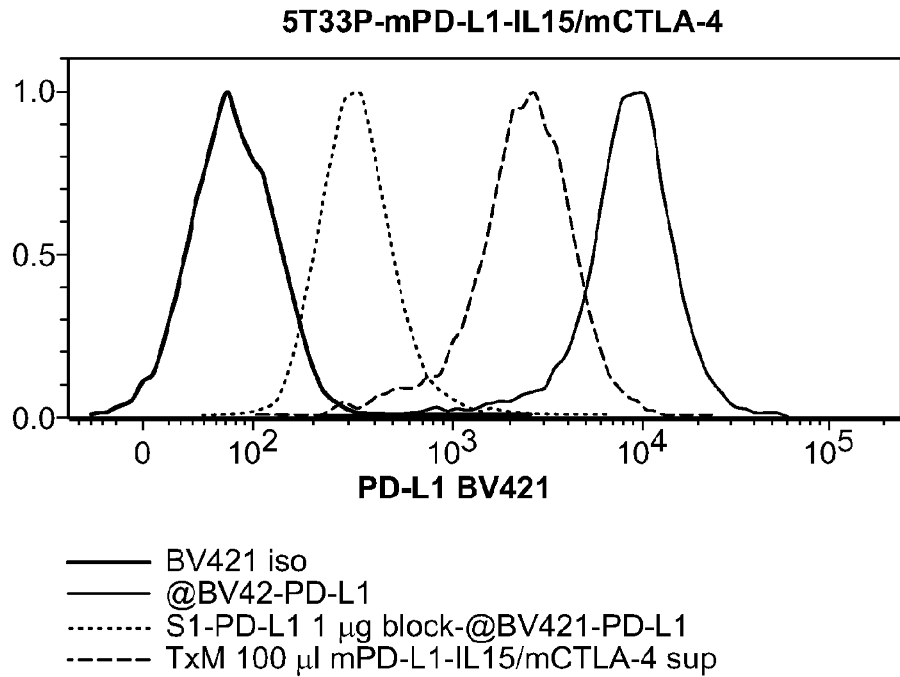


FIG. 22

A



B

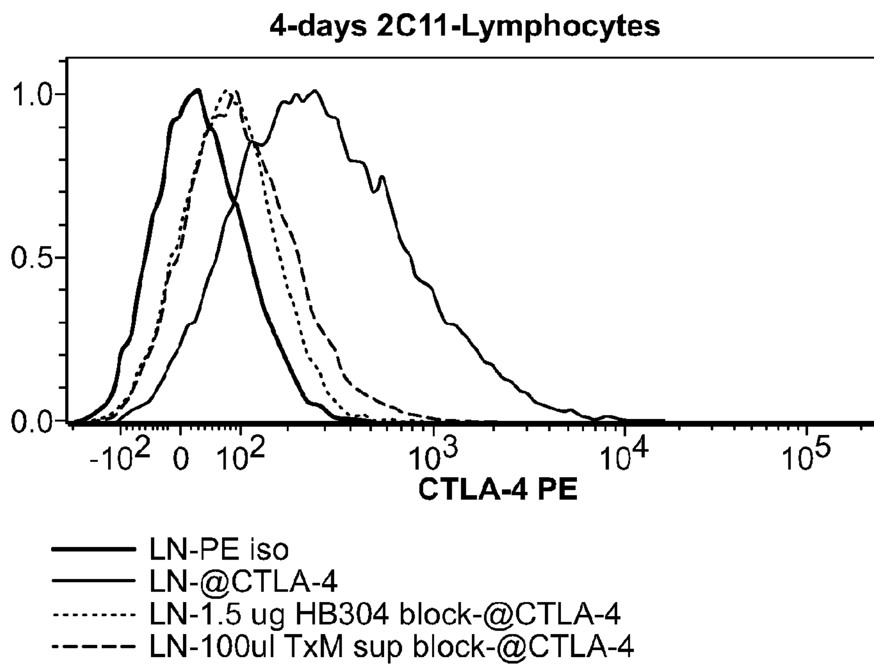
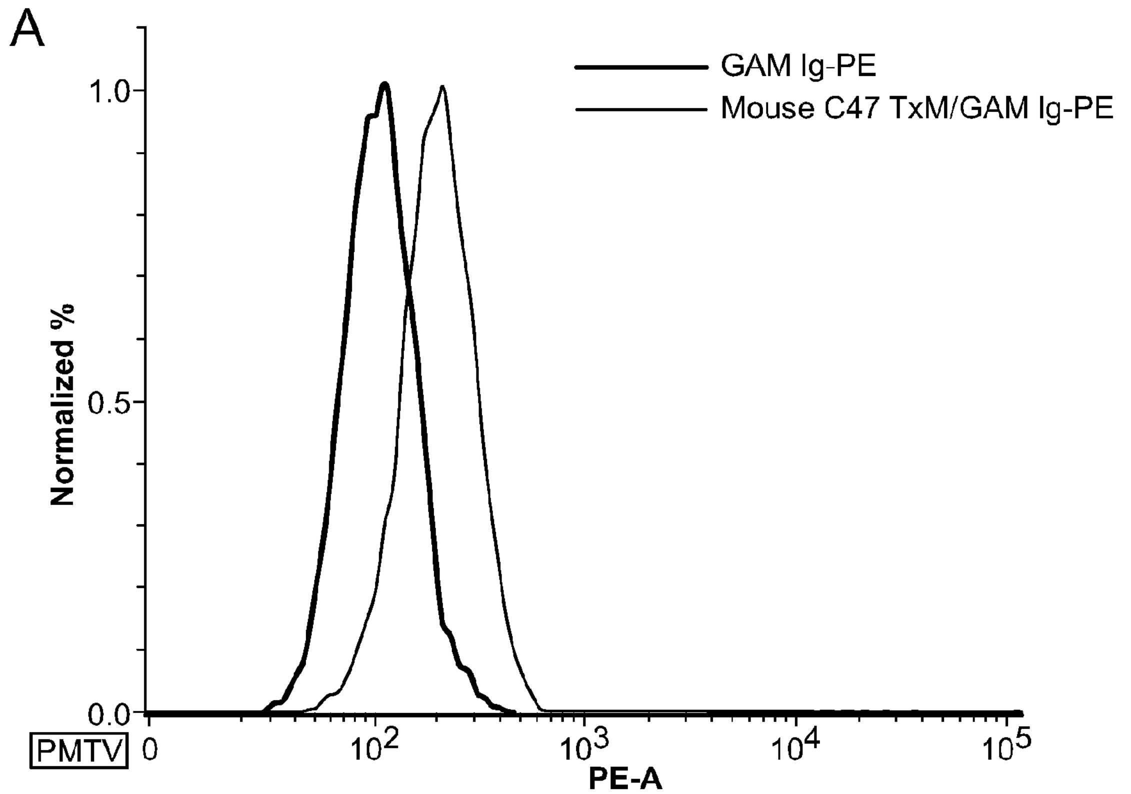


FIG. 23



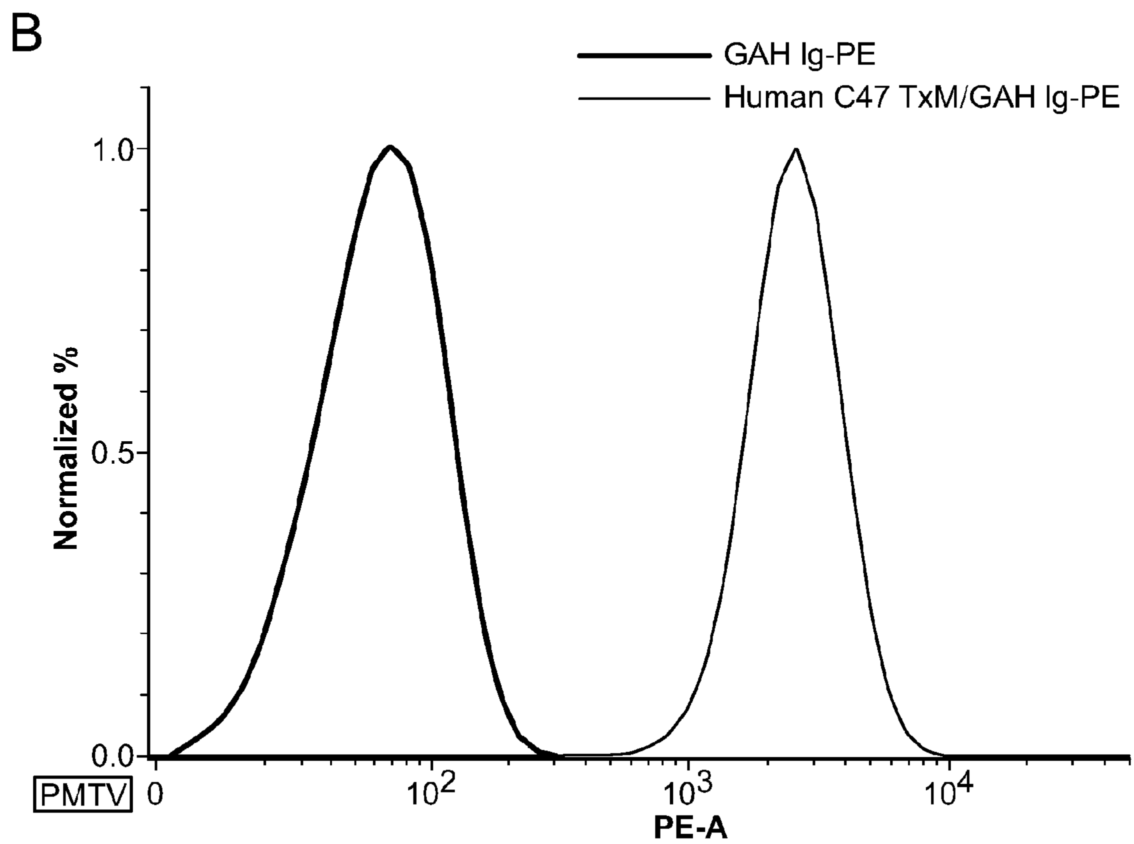


FIG. 24

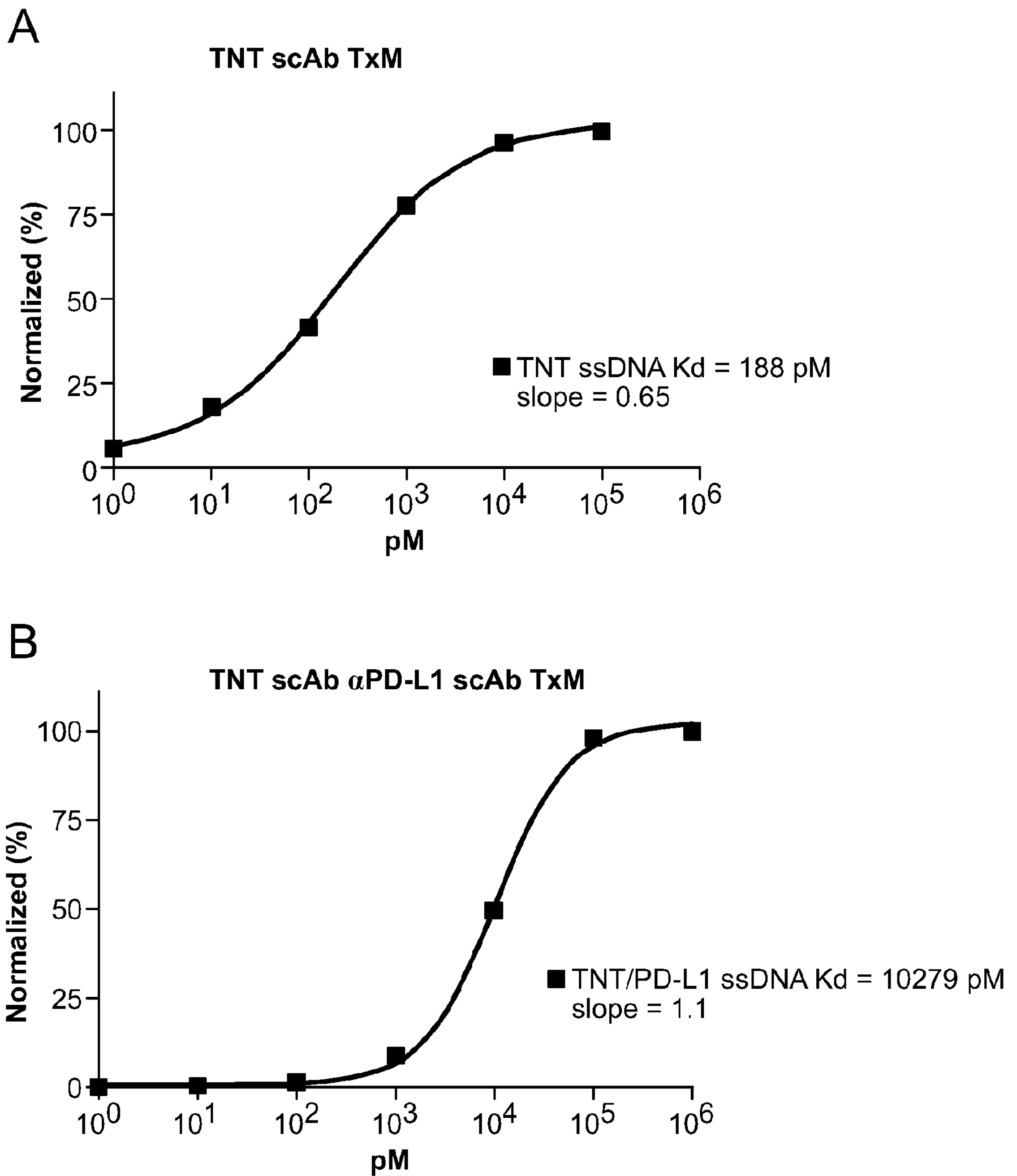
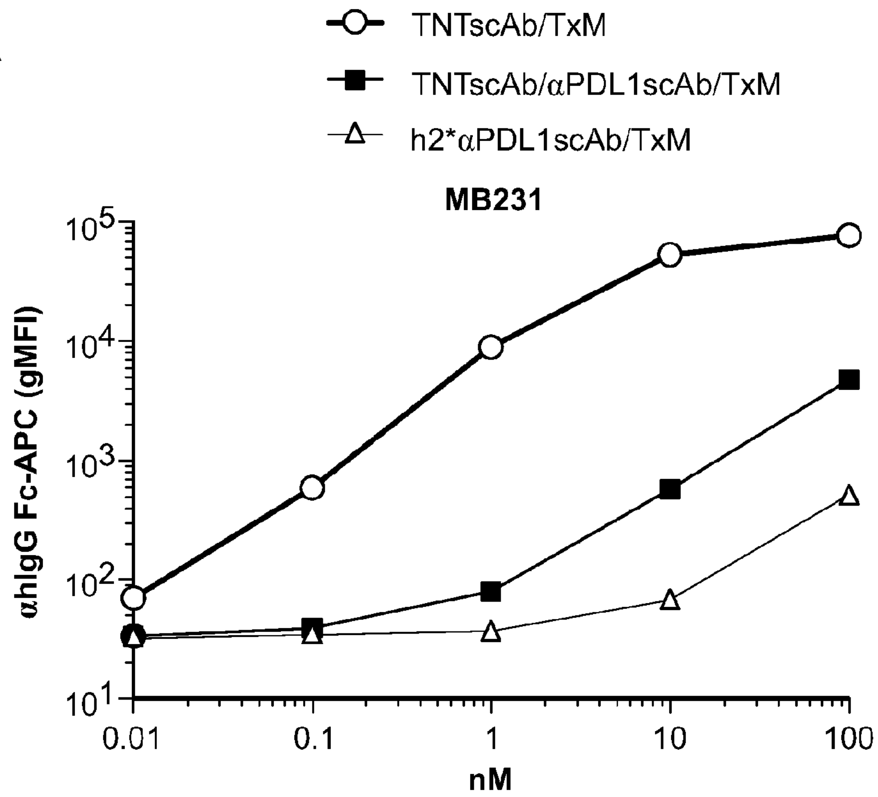
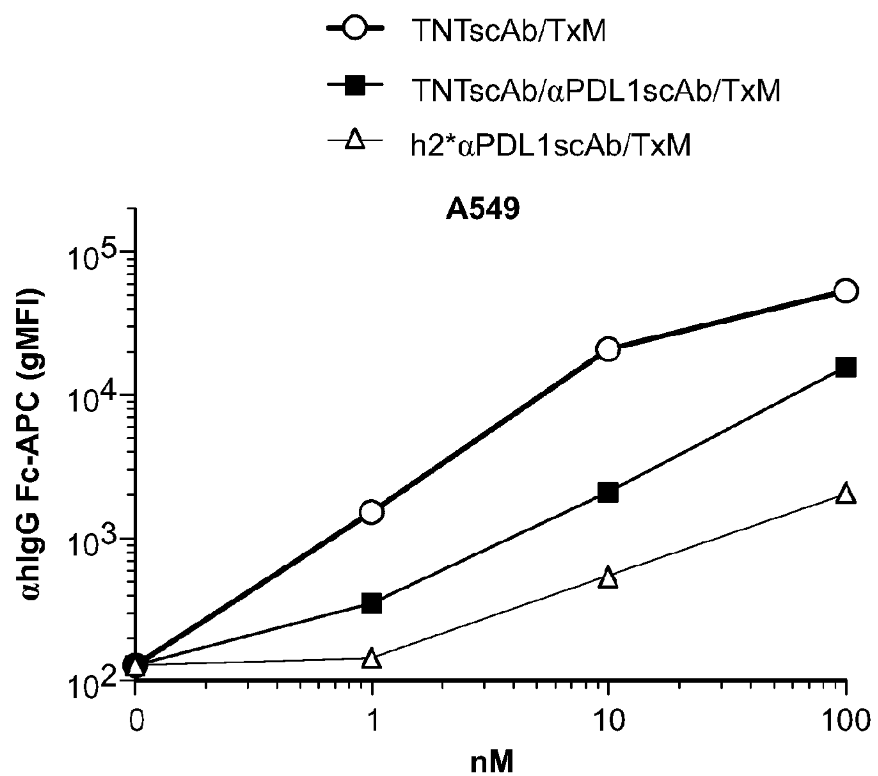


FIG. 25

A



B



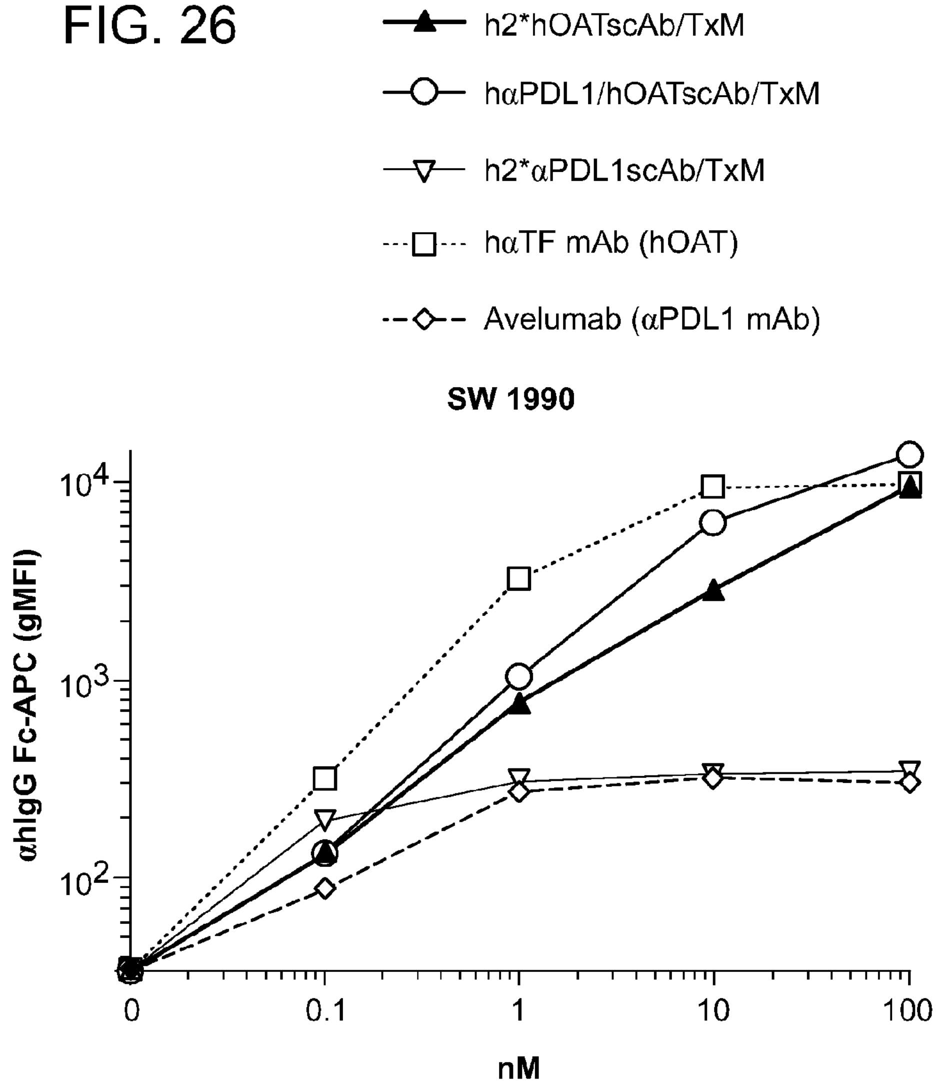
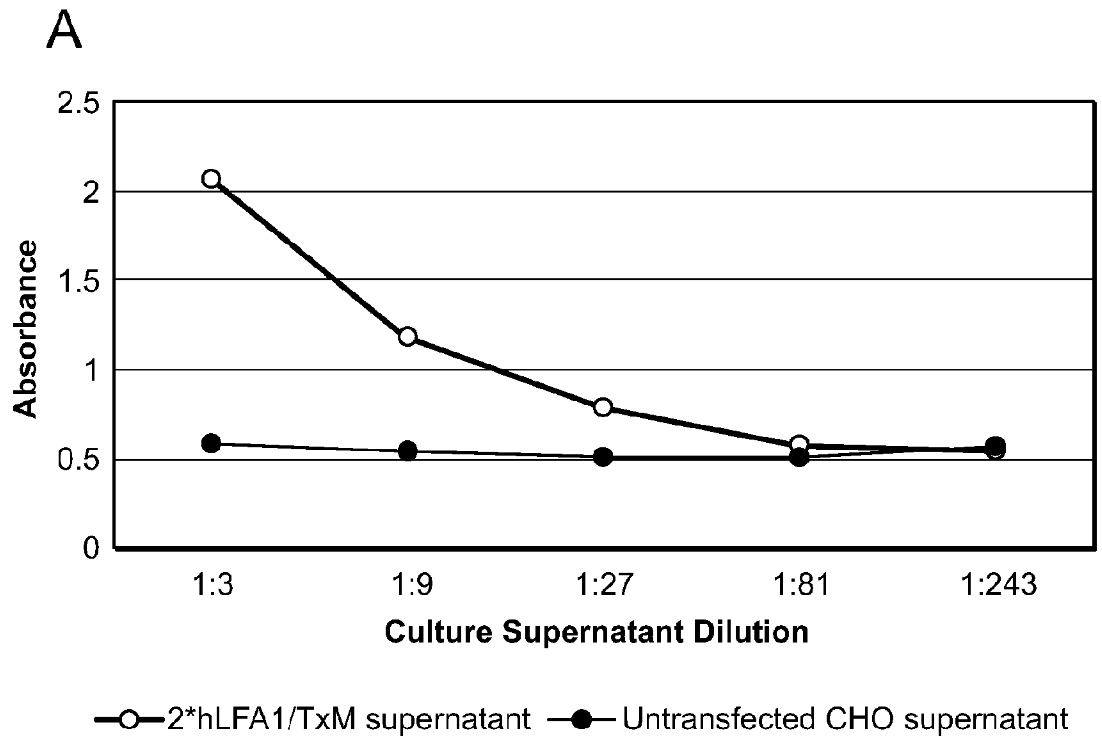


FIG. 26
h2*hOATscAb/TxM
hαPDL1/hOATscAb/TxM
h2*αPDL1scAb/TxM
hαTF mAb (hOAT)
Avelumab (αPDL1 mAb)
SW 1990
αhIgG Fc-APC (gMFI)
10^4
10^3
10^2
0
0.1
1
10
100
nM

FIG. 27



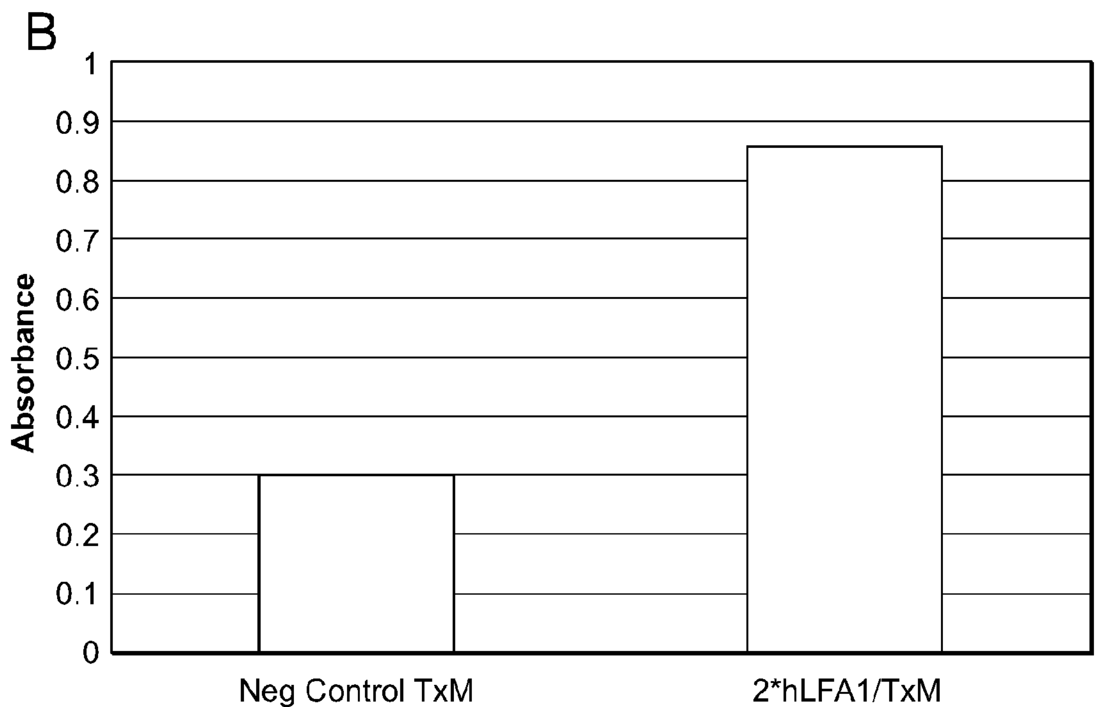


FIG. 28

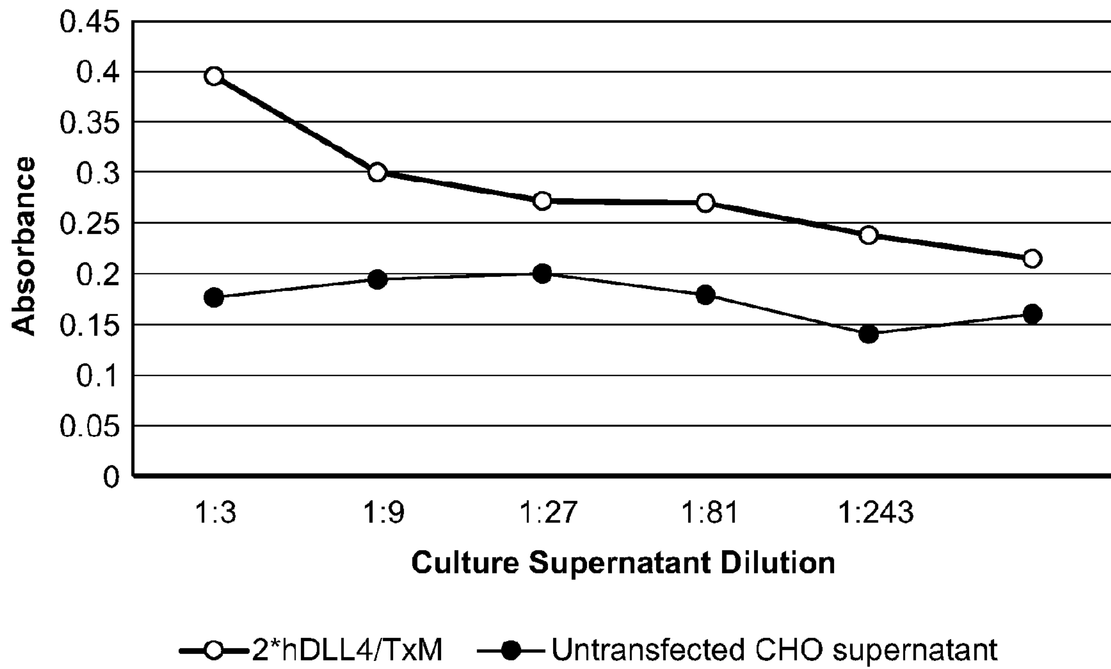


FIG. 29

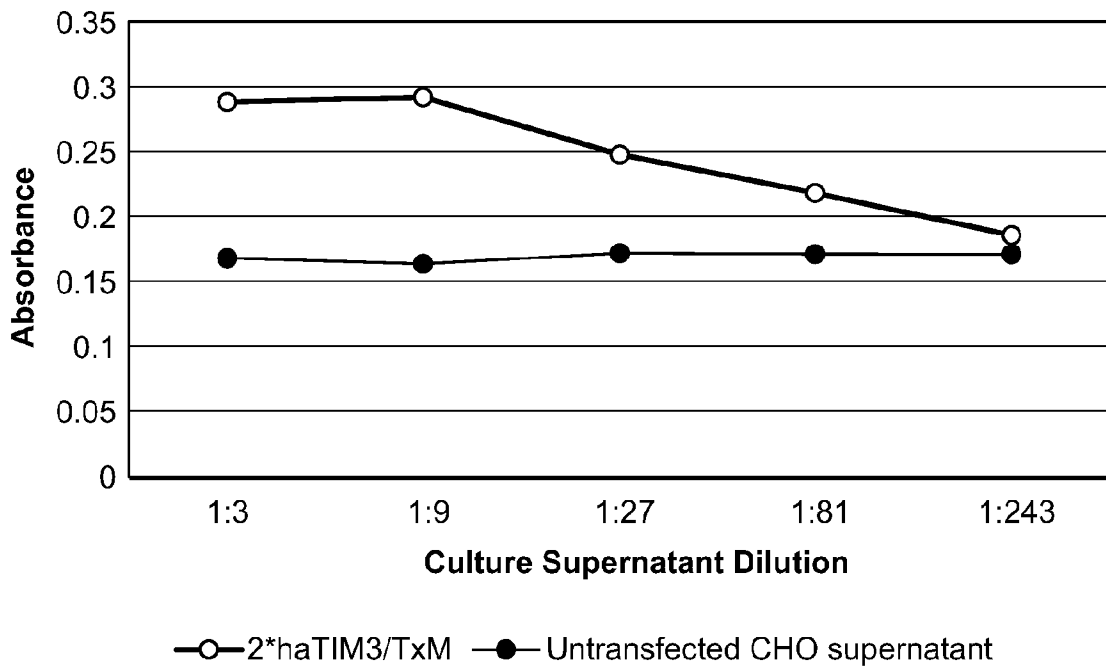
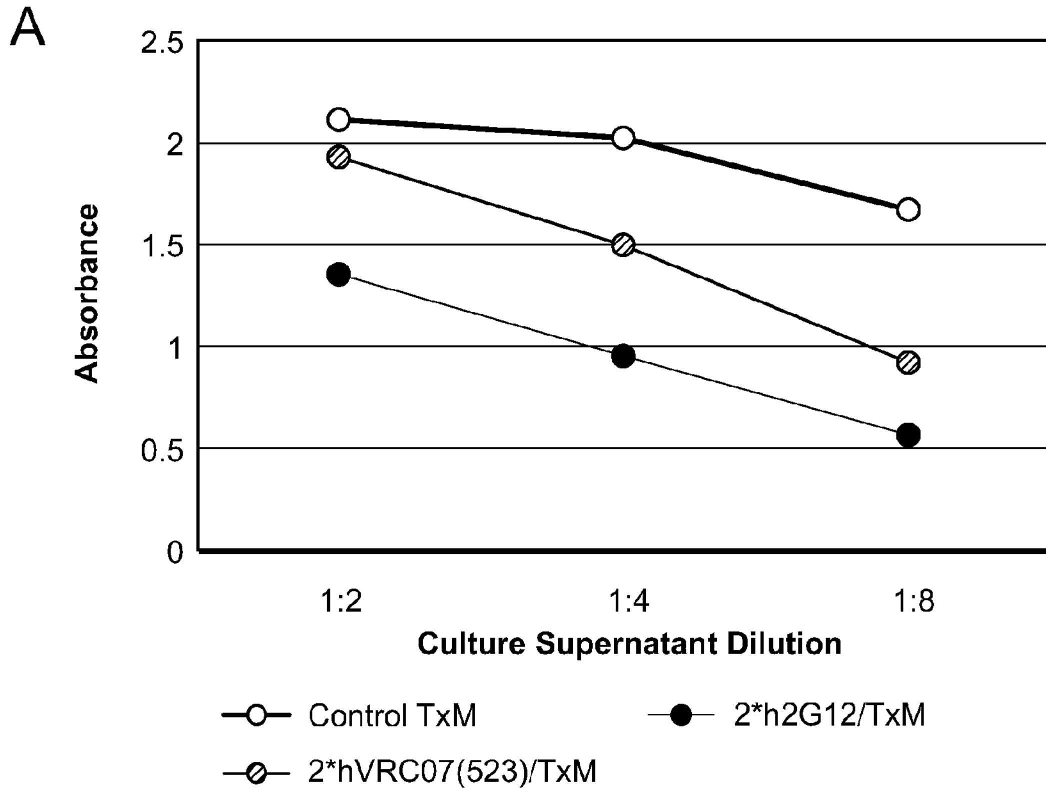


FIG. 30

A



B

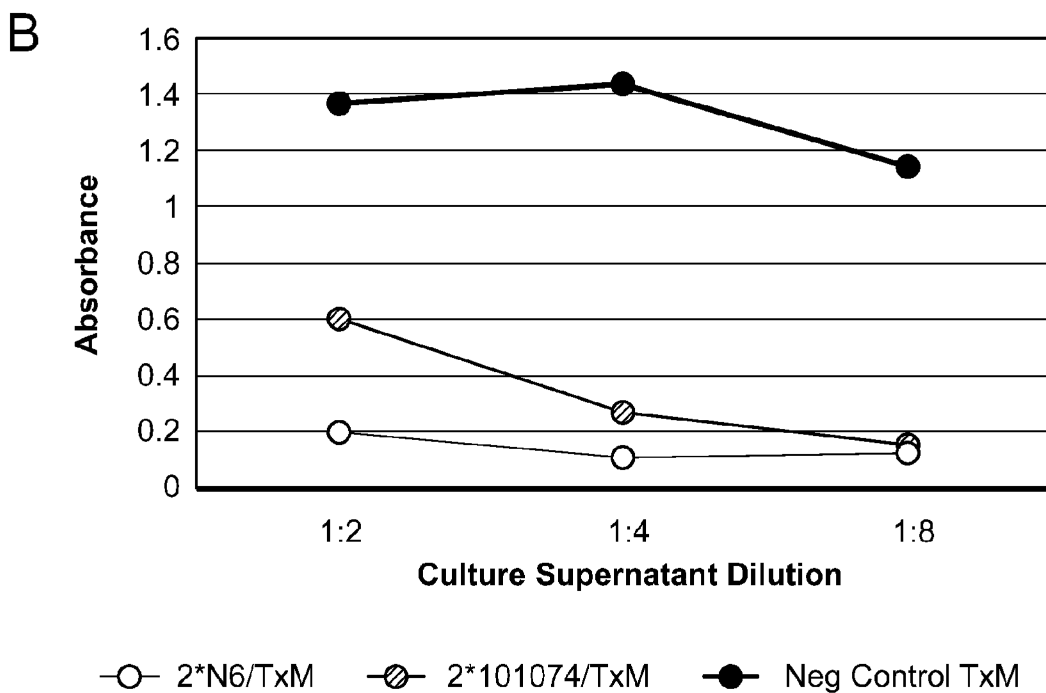
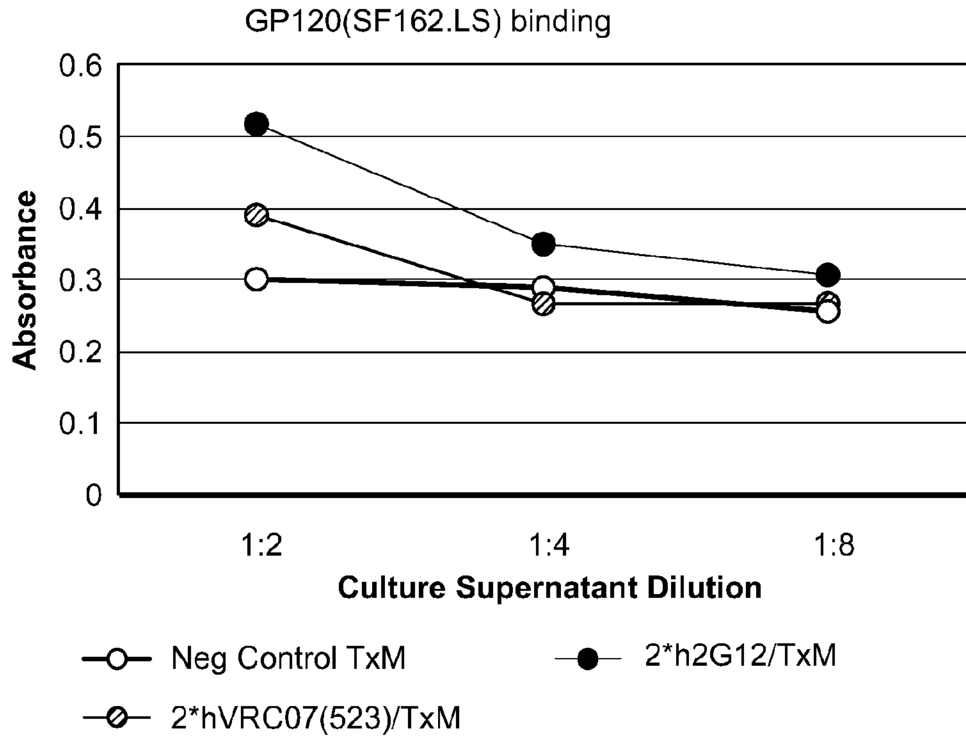


FIG. 30 (cont.)

C



D

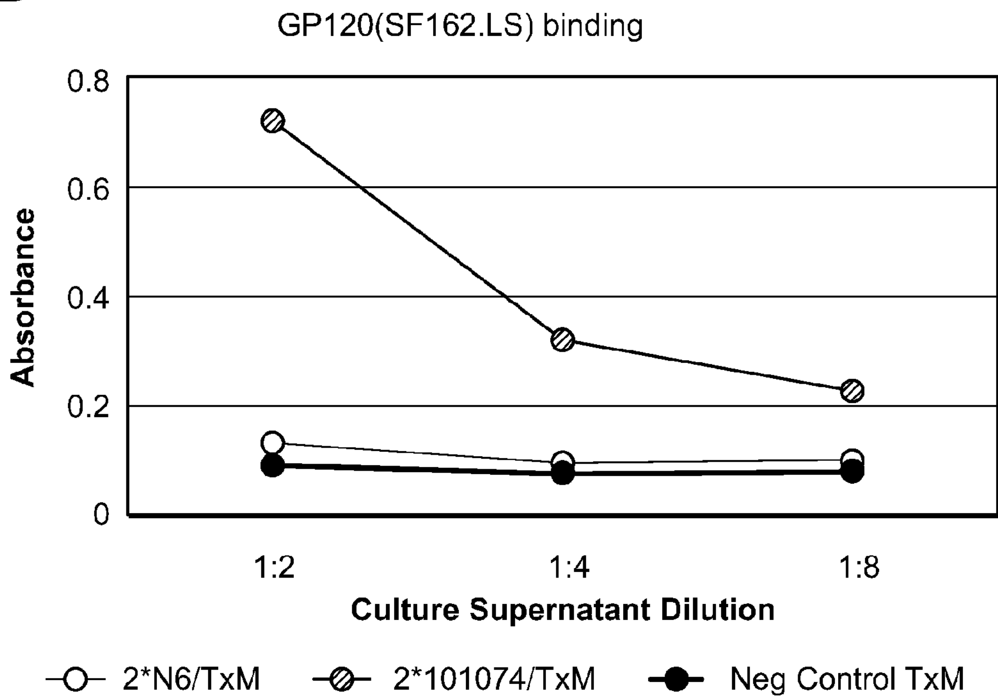
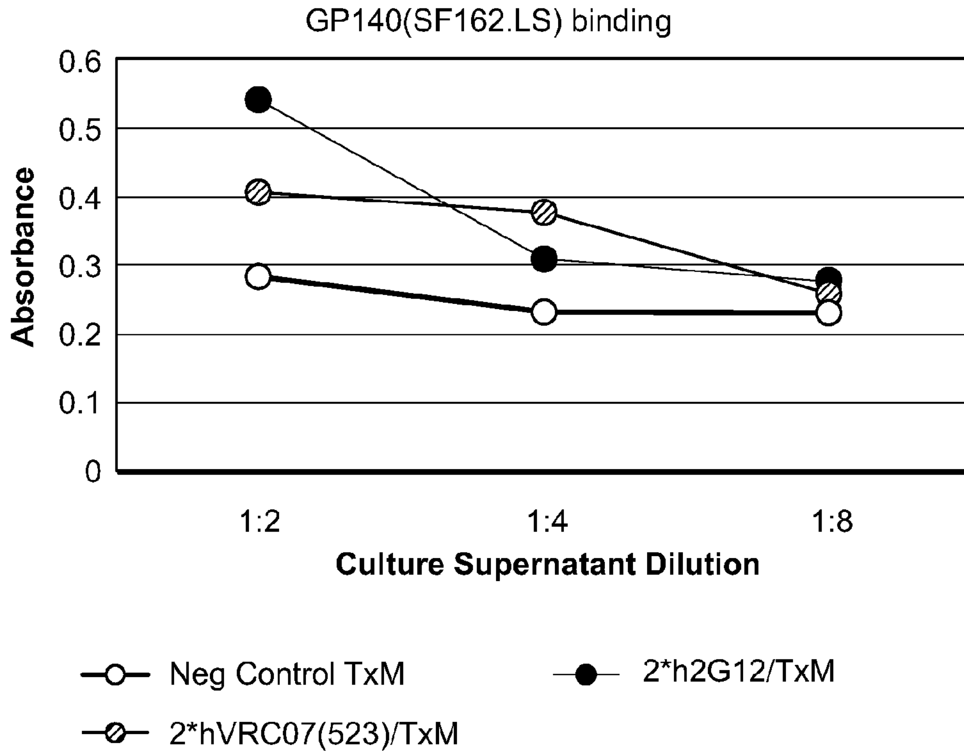


FIG. 30 (cont.)

E



F

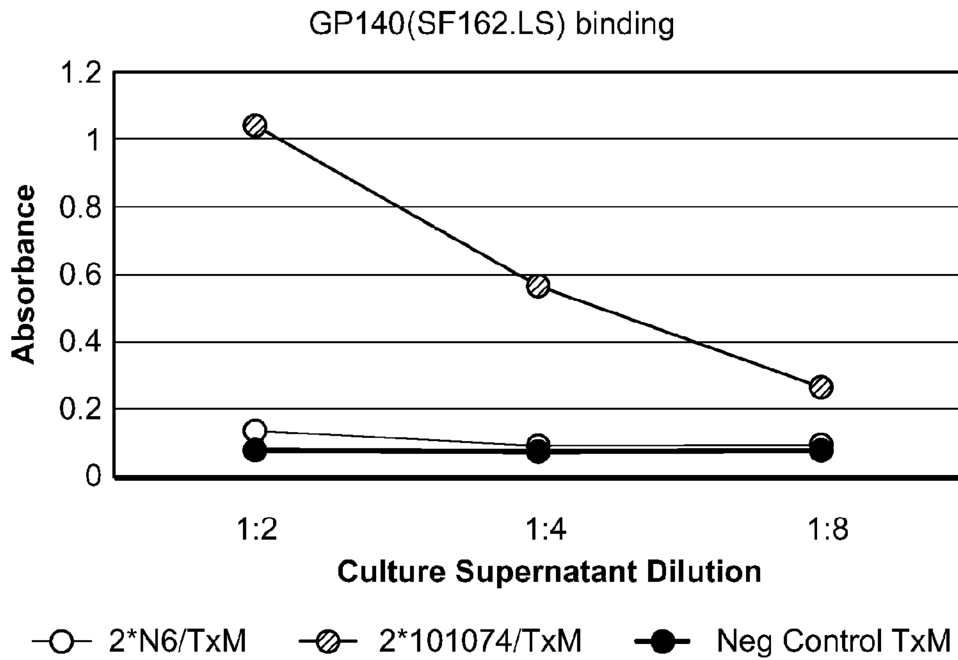


FIG. 31