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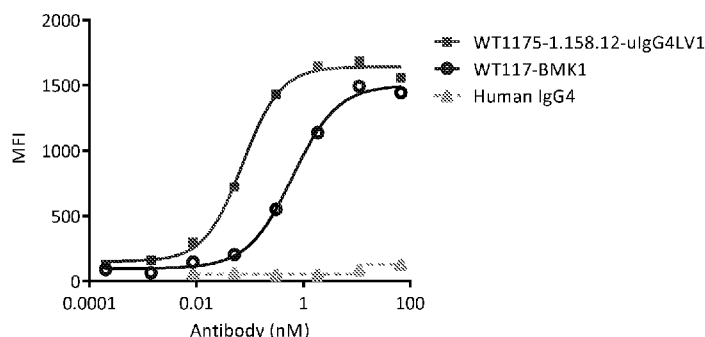


Figure 1

(57) Abstract: Provided in the present disclosure are anti-PVRIG antibodies, the nucleic acid molecules encoding the anti-PVRIG antibodies, expression vectors and host cells used for the expression of anti-PVRIG antibodies. The disclosure further provides the methods for preventing and treating cancers and immune disorders by administering the anti-PVRIG antibodies.

ANTI-PVRIG ANTIBODIES AND USES THEREOF

CROSS REFERENCE

This application claims the benefit of International patent application PCT/CN2023/098601, filed on June 6, 2023, which is incorporated herein by reference in its entirety.

SEQUENCE LISTING

The instant application contains a sequence listing which is hereby incorporated by reference in its entirety.

FIELD

This application generally relates to antibodies. More specifically, the application relates to monoclonal antibodies against PVRIG, a method of preparing the same, and the use of the antibodies.

BACKGROUND

Cancerous cells can exploit natural control mechanisms that limit T-cell activation in the body to prevent unrestrained T-cell activity, to evade or suppress immune response. Restoring the capacity of immune effector cells, especially T cells, to recognize and eliminate cancer is the goal of immunotherapy. In the tumor microenvironment, persisting antigenic stimulation can result in T cell exhaustion, a state of T cell dysfunction, and high expression of co-inhibitory receptors including PD-1, LAG-3, TIM3, and TIGIT. Currently, multiple strategies are being explored to reinvigorate exhausted T cells by either small molecule or therapeutic antibody approaches alone or in combination.

Poliovirus receptor-related Ig domain containing (PVRIG, a.k.a CD112R) is one of the co-inhibitory immune-checkpoint proteins. It plays an important role in reversion of T cell exhaustion and increasing NK cell activation. PVRIG belongs to the nectin and nectin-like family, among which the family members also includes TIGIT, DNAM-1 (CD226) and CD96. PVRIG is expressed on NK and T cells and is further up-regulated in T cells upon activation. The interaction of PVRIG and its ligand PVRL2 (CD112) expressed on APC and multiple tumor cells results in suppression of T cell and NK cell activation. PVRL2 is also a ligand for CD226, which upon ligand interaction activates human T and NK cells.

It was shown in several preclinical mouse tumor models that PVRIG blockade is a promising strategy for treating cancer. COM-701, developed by Compugen, is the first anti-PVRIG antagonistic antibody entering clinical development. The phase 1 clinical results suggested that COM-701 was well tolerated with a manageable safety profile as monotherapy and in combination with nivolumab. The best response of complete response (CR), partial response (PR) or stable

disease (SD) was observed in 11/21 (52%) patients with prior treatment refractory disease, and 13/18 (52%) patients with prior treatment with immune checkpoint inhibitor. GSK4381562, co-developed by GSK and Surface Oncology, is another anti-PVRIG antibody under clinical trials. GSK4381562 binds to a distinct epitope on PVRIG and blocks the interaction of PVRIG with CD112, and it promotes the activation of both NK cells and T cells, with the potential to elicit a strong anti-tumor response and promote immunological memory.

PVRIG could be a promising therapeutic target for tumor immunotherapy as single agent or in combination with other immune modulators.

SUMMARY

These and other objectives are provided for by the present disclosure which, in a broad sense, is directed to compounds, methods, compositions and articles of manufacture that provide antibodies with improved efficacy. The benefits provided by the present disclosure are broadly applicable in the field of antibody therapeutics and diagnostics and may be used in conjunction with other antibodies that react with a variety of targets.

The present disclosure provides antagonistic antibodies against PVRIG. The antibodies as disclosed herein can suppress the signaling pathway triggered by the binding interaction of PVRIG and PRLR2, leading to increased T cell and NK cell activation, among other things. Further provided are methods of treating a subject having cancer or immune disorder or pathogen infection by administering the anti-PVRIG antibodies as disclosed herein. The disclosure also provides methods for validating the function of antibodies in vitro and in vivo.

In some aspects, the present disclosure provides an isolated antibody or an antigen-binding portion thereof, comprising:

- a heavy chain CDR (HCDR) 1 comprising the amino acid sequence of SEQ ID NO: 1;
- a HCDR2 comprising the amino acid sequence of SEQ ID NO: 2;
- a HCDR3 comprising the amino acid sequence of SEQ ID NO: 3;
- a light chain CDR (LCDR) 1 comprising the amino acid sequence of SEQ ID NO: 4;
- a LCDR2 comprising the amino acid sequence of SEQ ID NO: 5; and
- a LCDR3 comprising the amino acid sequence of SEQ ID NO: 6.

In some embodiments, the isolated antibody or the antigen-binding portion thereof comprises a heavy chain variable region (VH) and a light chain variable region (VL),

wherein the VH comprises or consists of:

- (i) the amino acid sequence as set forth in SEQ ID NO: 7;
- (ii) an amino acid sequence at least 85%, 90%, or 95% identical to SEQ ID NO: 7; or
- (iii) an amino acid sequence with addition, deletion and/or substitution of one or more (e.g. 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10) amino acids compared with SEQ ID NO: 7; and/or

the VL comprises or consists of:

- (i) the amino acid sequence as set forth in any of SEQ ID NOs: 8-9;

(ii) an amino acid sequence at least 85%, at least 90%, or at least 95% identical to any of SEQ ID NOs: 8-9; or

(iii) an amino acid sequence with addition, deletion and/or substitution of one or more (e.g. 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10) amino acids compared with any of SEQ ID NOs: 8-9.

5 In some embodiments, the isolated antibody or the antigen-binding portion thereof comprises the HCDR1, HCDR2 and HCDR3 of the VH region as set forth in SEQ ID NO: 7, and the LCDR1, LCDR2 and LCDR3 of the VL region as set forth in any of SEQ ID NOs: 8-9.

In some embodiments, the isolated antibody or the antigen-binding portion thereof comprises: a HCDR1 as set forth in SEQ ID NO: 1; a HCDR2 as set forth in SEQ ID NO: 2; a
10 HCDR3 as set forth in SEQ ID NO: 3; a LCDR1 as set forth in SEQ ID NO: 4; a LCDR2 as set forth in SEQ ID NO: 5; and a LCDR3 as set forth in SEQ ID NO: 6.

In some embodiments, the isolated antibody or the antigen-binding portion thereof comprises a VH region comprising the amino acid sequence of SEQ ID NO: 7 and a VL region comprising the amino acid sequence of SEQ ID NO: 8.

15 In some embodiments, the isolated antibody or the antigen-binding portion thereof further comprises a human IgG constant region, such as a human IgG1, IgG4, IgG2 or IgG3 constant region, which may be native or a variant thereof. Specifically, the antibody may comprise a human IgG1 Fc region or a human IgG4 Fc region.

In some embodiments, the isolated antibody or the antigen-binding portion thereof
20 comprises a heavy chain comprising the amino acid sequence of SEQ ID NO: 12, 15 or 17, and a light chain comprising the amino acid sequence of SEQ ID NO: 13, 16 or 18.

In some embodiments, the anti-PVRIG antibody as disclosed herein is a human antibody or a humanized antibody. In some embodiments, the antibodies herein are anti-PVRIG antagonist antibodies.

25 In some aspects, the present disclosure provides an isolated nucleic acid molecule, comprising a nucleic acid sequence encoding the heavy chain variable region and/or the light chain variable region of the isolated antibody or the antigen-binding portion thereof as disclosed herein. In some embodiments, the nucleic acid molecule comprises the nucleic acid sequence as set forth in SEQ ID NO: 10, and/or the nucleic acid sequence as set forth in SEQ ID NO: 11.

30 In some aspects, the present disclosure provides an expression vector(s) comprising the nucleic acid molecule(s) as disclosed herein.

In some aspects, the present disclosure provides a host cell comprising the expression vector as disclosed herein.

35 In some aspects, the present disclosure provides a pharmaceutical composition comprising the antibody or antigen-binding portion thereof as disclosed herein and a pharmaceutically acceptable carrier.

In some aspects, the present disclosure provides a method for preparing the antibody or antigen-binding portion thereof which comprises expressing the antibody or antigen-binding

portion thereof in a host cell and isolating the antibody or antigen-binding portion thereof from the host cell. In some embodiments, the host cell has been transfected or transformed with an expression vector(s) encoding the heavy chain and light chain of the antibody disclosed herein. The heavy chain encoding nucleic acid sequence and the light chain encoding nucleic acid sequence may be in the same vector or in separate vectors.

In some aspects, the present disclosure provides a method of modulating an immune response in a subject, comprising administering the antibody or antigen-binding portion thereof as disclosed herein to the subject. The immune response may be PVRIG related, T cell related and/or NK cell related.

In some aspects, the present disclosure provides a method for inhibiting growth of tumor cells in a subject, comprising administering an effective amount of the antibody or antigen-binding portion thereof or the pharmaceutical composition as disclosed herein, alone or combined with another anti-cancer agent, to the subject.

In some aspects, the present disclosure provides a method for treating or preventing a cancer or an immune related disorder in a subject, comprising administering an effective amount of the antibody or antigen-binding portion thereof as disclosed herein to the subject. The method may further comprise administering an additional anti-cancer agent, such as a chemotherapeutic agent(s), a monoclonal antibody, an antibody-drug conjugate etc. In some embodiments, the anti-cancer agent is an anti-PD-1 antibody, anti-PD-L1 antibody or an anti-CTLA-4 antibody.

Said cancer can be selected from colon cancer, lung cancer (such as NSCLC), breast cancer, prostate cancer, bladder cancer, ovarian cancer, gastric cancer, colorectal cancer, esophageal cancer, head and neck cancer, cervical cancer, pancreatic cancer, testis cancer, lymphoma, leukemia malignant melanoma and soft-tissue cancer. The immune related disorder may be a T cell dysfunctional disorder, an infection, or an inflammatory disease.

In some aspects, the present disclosure provides use of the antibody or antigen-binding portion thereof as disclosed herein, alone or combined with another anti-cancer agent, in the manufacture of a medicament for treating or preventing diseases such as cancers and immune related disorders.

In some aspects, the present disclosure provides the use of the antibody or antigen-binding portion thereof as disclosed herein in the manufacture of a diagnostic agent for diagnosing diseases related to PVRIG overexpression.

In some aspects, the present disclosure provides the antibody or antigen-binding portion thereof as disclosed herein for use in treating or preventing cancers and immune related disorders.

In some aspects, the present disclosure provides a method for detecting the presence of PVRIG antigen in a sample or measuring the amount of PVRIG antigen, comprising contacting the sample with the anti-PVRIG antibody or an antigen binding portion thereof as disclosed herein.

In some aspects, the present disclosure provides kits or devices that comprise the antibody or antigen-binding portion thereof as disclosed herein in one or more containers.

The foregoing is a summary and thus contains, by necessity, simplifications, generalizations, and omissions of detail; consequently, those skilled in the art will appreciate that the summary is illustrative only and is not intended to be in any way limiting. This summary is not intended to identify key features or essential features of the claimed subject matter, nor is it intended to be used as an aid in determining the scope of the claimed subject matter.

BRIEF DESCRIPTION OF THE FIGURES

Figures 1-2 show the binding of antibodies to human PVRIG engineered cells (Figure 1) and human CD8⁺ T cells (Figure 2), as determined by FACS.

Figure 3 shows the binding of antibodies to cynomolgus monkey PVRIG engineered cells, as determined by FACS.

Figure 4 shows the binding result of antibodies to mouse PVRIG, as determined by ELISA.

Figure 5 shows the affinity of anti-PVRIG antibodies to human PVRIG in an FACS affinity test.

Figures 6 shows the binding of antibodies to PVRIG paralog proteins, as determined by ELISA.

Figure 7 shows the result of antibodies blocking PVRL2 binding to PVRIG, as determined by ELISA.

Figure 8 shows the result of antibodies in NFAT (Nuclear Factor of Activated T-cells) reporter gene assay.

Figure 9 shows the effect of antibodies in stimulation of human CD8⁺ T cells.

Figure 10 shows the stability of the antibodies in human serum.

Figure 11 shows binding of IgG1 and IgG4 version antibodies to human PVRIG engineered cells, as determined by FACS.

Figure 12 shows the activity of IgG1 and IgG4 version antibodies in NFAT reporter gene assay.

Figure 13 shows the activity of IgG1 and IgG4 version antibodies in T cell activation assay.

Figure 14 shows the pharmacokinetics result of anti-PVRIG antibodies in rat.

Figure 15 shows binding of WT1175-1.158.12-m1-uIgG1L to human PVRIG engineered cells, as determined by FACS.

Figure 16 shows the activity of WT1175-1.158.12-m1-uIgG1L in NFAT reporter gene assay.

DETAILED DESCRIPTION

While the present invention may be embodied in many different forms, disclosed herein are specific illustrative embodiments thereof that exemplify the principles of the invention. It should be emphasized that the present invention is not limited to the specific embodiments illustrated. Moreover, any section headings used herein are for organizational purposes only and are not to be construed as limiting the subject matter described.

Unless otherwise defined herein, scientific and technical terms used in connection with the present invention shall have the meanings that are commonly understood by those of ordinary skill in the art. Further, unless otherwise required by context, singular terms shall include pluralities and plural terms shall include the singular. More specifically, as used in this specification and the appended claims, the singular forms “a”, “an” and “the” include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to “a protein” includes a plurality of proteins; reference to “a cell” includes mixtures of cells, and the like. In this application, the use of “or” means “and/or” unless stated otherwise. Furthermore, the use of the term “comprising,” as well as other forms, such as “comprises” and “comprised,” is not limiting. In addition, ranges provided in the specification and appended claims include both end points and all points between the end points.

Generally, nomenclature used in connection with, and techniques of, cell and tissue culture, molecular biology, immunology, microbiology, genetics and protein and nucleic acid chemistry and hybridization described herein are those well-known and commonly used in the art. The methods and techniques of the present disclosure are generally performed according to conventional methods well known in the art and as described in various general and more specific references that are cited and discussed throughout the present specification unless otherwise indicated. See, e.g., Abbas *et al.*, *Cellular and Molecular Immunology*, 6th ed., W.B. Saunders Company (2010); Sambrook J. & Russell D. *Molecular Cloning: A Laboratory Manual*, 3rd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (2000); Ausubel *et al.*, *Short Protocols in Molecular Biology: A Compendium of Methods from Current Protocols in Molecular Biology*, Wiley, John & Sons, Inc. (2002); Harlow and Lane *Using Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1998); and Coligan *et al.*, *Short Protocols in Protein Science*, Wiley, John & Sons, Inc. (2003). The nomenclature used in connection with, and the laboratory procedures and techniques of, analytical chemistry, synthetic organic chemistry, and medicinal and pharmaceutical chemistry described herein are those well-known and commonly used in the art.

Definitions

In order to better understand the disclosure, the definitions and explanations of the relevant terms are provided as follows.

The term “antibody” or “Ab”, as used herein, generally refers to a Y-shaped tetrameric protein comprising two heavy (H) and two light (L) polypeptide chains held together by covalent disulfide bonds and non-covalent interactions. Light chains of an antibody may be classified into κ and λ light chain. Heavy chains may be classified into μ , δ , γ , α and ϵ , which define isotypes of an antibody as IgM, IgD, IgG, IgA and IgE, respectively. Each heavy chain consists of a heavy chain variable region (V_H) and a heavy chain constant region (C_H). A heavy chain constant region consists of 3 domains (C_{H1} , C_{H2} and C_{H3}). Each light chain

consists of a light chain variable region (V_L) and a light chain constant region (C_L). V_H and V_L regions can further be divided into hypervariable regions (called complementary determining regions (CDR)), which are interspaced by relatively conservative regions (called framework region (FR)). Each V_H and V_L consists of 3 CDRs and 4 FRs in the following order: FR1, CDR1, FR2, CDR2, FR3, CDR3, FR4 from N-terminal to C-terminal. The variable region (V_H and V_L) of each heavy/light chain pair forms antigen binding sites, respectively. Antibodies may be of different antibody isotypes, for example, IgG (e.g., IgG1, IgG2, IgG3 or IgG4 subtype), IgA1, IgA2, IgD, IgE or IgM antibody.

The term “antigen-binding portion” or “antigen-binding fragment” of an antibody, which can be interchangeably used in the context of the application, refers to polypeptides comprising fragments of a full-length antibody, which retain the ability of specifically binding to an antigen that the full-length antibody specifically binds to, and/or compete with the full-length antibody for binding to the same antigen. Generally, see Fundamental Immunology, Ch. 7 (Paul, W., ed., the second edition, Raven Press, N.Y. (1989), which is incorporated herein by reference for all purposes. Antigen binding fragments of an antibody may be produced by recombinant DNA techniques or by enzymatic or chemical cleavage of an intact antibody. Under some conditions, antigen binding fragments include Fab, Fab', F(ab')₂, Fd, Fv, dAb and complementary determining region (CDR) fragments, single chain antibody (e.g. scFv), chimeric antibody, diabody and such polypeptides that comprise at least part of antibody sufficient to confer the specific antigen binding ability on the polypeptides. Antigen binding fragments of an antibody may be obtained from a given antibody (e.g., the monoclonal anti-human PVRIG antibody provided herein) by conventional techniques known by a person skilled in the art (e.g., recombinant DNA technique or enzymatic or chemical cleavage methods), and may be screened for specificity in the same manner by which intact antibodies are screened.

The term “monoclonal antibody” or “mAb”, as used herein, refer to a preparation of antibody molecules of single molecular composition. A monoclonal antibody displays a single binding specificity and affinity for a particular epitope.

The term “chimeric antibody”, as used herein, refers to an antibody in which the variable region sequences are derived from one species and the constant region sequences are derived from another species, such as an antibody in which the variable region sequences are derived from a murine antibody and the constant region sequences are derived from a human antibody.

The term “humanized antibody”, as used herein, refers to antibodies in which CDR sequences derived from the germline of another mammalian species, such as a rat or mouse, have been grafted onto human framework sequences. Additional framework region modifications may be made within the human framework sequences. The humanized antibody optionally will also comprise at least a portion of an immunoglobulin constant region (e.g. Fc), typically that of a human immunoglobulin.

The term “human antibody” or “fully human antibody,” as used herein, is intended to include antibodies having variable regions in which both the framework and CDR regions are derived from human germline immunoglobulin sequences. Furthermore, if the antibody contains a constant region, the constant region also is derived from human germline immunoglobulin sequences.

The term “PVRIG” or “Poliovirus Receptor Related Immunoglobulin Domain Containing Protein” includes known or wild type PVRIG, or variants, conjugates or fragments (in particular the ECD fragment) thereof. PVRIG is a transmembrane domain protein with a signal peptide, an extracellular domain, a transmembrane domain and a cytoplasmic domain. PVRIG is expressed on the cell surface of NK and T-cells and shares several similarities to other known immune checkpoints. The identification and methods used to show that PVRIG is a checkpoint receptor can be found in WO2016/134333, incorporated herein by reference. An example of human PVRIG amino acid sequence is shown in SEQ ID NO: 14 (MRTEAQVPALQPPEPGLEGAMGHRTLVL PWVLLTLCV TAGTPEVWVQVRMEATELSS FTIRCGFLGSGSISLVT VSWGGPNGAGGTTLAVLH PERGIRQWAPARQARWETQSSISLI LEGSGASSPCANTTFCKFASFPEGSWEACGSLPPSSDPGLSAPPTPAPILRADLAGILGVS GVLLFGCVYLLHLLRRHKHRPAPRLQPSRTSPQAPRARAWAPSQASQAALHVPYATINT SCR PATLDTAHPHGGPSWWASLP THAAHRPQGPAAWASTPIPARGSFVSVENGLYAQA GERPPHTGPGLTLFPDPRGPRAMEGPLGVR).

The term “binding affinity” is herein used as a measure of the strength of a non-covalent interaction between two molecules, e.g., an antibody or antigen-portion thereof, and an antigen. Binding affinity between two molecules may be quantified by a variety of assays including surface plasmon resonance (SPR), flow cytometry (FACS) and kinetic exclusion assay (KinExA). Flow cytometry is one common technique which analyzes ligand binding to proteins presented on the surface of a cell. In flow cytometry affinity assay, the equilibrium association constant (K_a , i.e. k_{on}/k_{off}) and equilibrium dissociation constant (KD , i.e. K_{off}/K_{on}) may be determined to evaluate protein-binding affinities. The binding kinetics and binding affinity of the antibody can be assessed by standard assays known in the art or as described in the Example section 3.4 below.

The term “ EC_{50} ,” as used herein, which is also termed as “half maximal effective concentration” refers to the concentration of a drug, antibody or toxicant which induces a response halfway between the baseline and maximum after a specified exposure time.

The term “ IC_{50} ”, as used herein, which is also termed as “half maximal inhibitory concentration” refers to the half maximal inhibitory concentration of a drug, antibody or other substances. It is a measure of the potency of a substance in inhibiting a specific biological or biochemical function.

The term “isolated,” as used herein, refers to a state obtained from natural state by artificial means. If a certain “isolated” substance or component is present in nature, it is possible because its natural environment changes, or the substance is isolated from natural environment, or both. For example, a certain un-isolated polynucleotide or polypeptide naturally exists in a certain living

animal body, and the same polynucleotide or polypeptide with a high purity isolated from such a natural state is called isolated polynucleotide or polypeptide. The term “isolated” excludes neither the mixed artificial or synthesized substance nor other impure substances that do not affect the activity of the isolated substance.

5 The term “isolated antibody,” as used herein, is intended to refer to an antibody that is substantially free of other antibodies having different antigenic specificities (e.g., an isolated antibody that specifically binds an PVRIG protein is substantially free of antibodies that specifically bind antigens other than PVRIG proteins). An isolated antibody that specifically binds a human PVRIG protein may, however, have cross- reactivity to other antigens, such as PVRIG
10 proteins from other species. Moreover, an isolated antibody can be substantially free of other cellular material and/or chemicals.

 The term “vector,” as used herein, refers to a nucleic acid vehicle which can have a polynucleotide inserted therein. When the vector allows for the expression of the protein encoded by the polynucleotide inserted therein, the vector is called an expression vector. The vector can
15 have the carried genetic material elements expressed in a host cell by transformation, transduction, or transfection into the host cell. Vectors are well known by a person skilled in the art, including, but not limited to plasmids, phages, cosmids, artificial chromosome such as yeast artificial chromosome (YAC), bacterial artificial chromosome (BAC) or P1-derived artificial chromosome (PAC); phage such as λ phage or M13 phage and animal virus. The animal viruses that can be used
20 as vectors, include, but are not limited to, retrovirus (including lentivirus), adenovirus, adeno-associated virus, herpes virus (such as herpes simplex virus), pox virus, baculovirus, papillomavirus, papova virus (such as SV40). A vector may comprise multiple elements for controlling expression, including, but not limited to, a promoter sequence, a transcription initiation sequence, an enhancer sequence, a selection element and a reporter gene. In addition, a vector may
25 comprise origin of replication.

 The term “host cell,” as used herein, refers to a cellular system which can be engineered to generate proteins, protein fragments, or peptides of interest. Host cells include, without limitation, cultured cells, e.g., mammalian cultured cells derived from rodents (rats, mice, guinea pigs, or hamsters) such as CHO, BHK, NSO, SP2/0, YB2/0; or human tissues or hybridoma cells, yeast
30 cells, and insect cells, and cells comprised within a transgenic animal or cultured tissue. The term encompasses not only the particular subject cell but also the progeny of such a cell. Because certain modifications may occur in succeeding generations due to either mutation or environmental influences, such progeny may not be identical to the parent cell, but are still included within the scope of the term “host cell.”

35 The term “identity,” as used herein, refers to a relationship between the sequences of two or more polypeptide molecules or two or more nucleic acid molecules, as determined by aligning and comparing the sequences. “Percent identity” means the percent of identical residues between the amino acids or nucleotides in the compared molecules and is calculated based on the size of the

smallest of the molecules being compared. For these calculations, gaps in alignments (if any) are preferably addressed by a particular mathematical model or computer program (i.e., an “algorithm”). Methods that can be used to calculate the identity of the aligned nucleic acids or polypeptides include those described in Computational Molecular Biology, (Lesk, A. M., ed.), 1988, New York: Oxford University Press; Biocomputing Informatics and Genome Projects, (Smith, D. W., ed.), 1993, New York: Academic Press; Computer Analysis of Sequence Data, Part I, (Griffin, A. M., and Griffin, H. G., eds.), 1994, New Jersey: Humana Press; von Heinje, G., 1987, Sequence Analysis in Molecular Biology, New York: Academic Press; Sequence Analysis Primer, (Gribskov, M. and Devereux, J., eds.), 1991, New York: M. Stockton Press; and Carillo et al, 1988, SIAMJ. Applied Math. 48:1073.

The term “immunogenicity,” as used herein, refers to ability of stimulating the formation of specific antibodies or sensitized lymphocytes in organisms. It not only refers to the property of an antigen to stimulate a specific immunocyte to activate, proliferate and differentiate so as to finally generate immunologic effector substance such as antibody and sensitized lymphocyte, but also refers to the specific immune response that antibody or sensitized T lymphocyte can be formed in immune system of an organism after stimulating the organism with an antigen. Immunogenicity is the most important property of an antigen. Whether an antigen can successfully induce the generation of an immune response in a host depends on three factors, properties of an antigen, reactivity of a host, and immunization means.

The term “transfection,” as used herein, refers to the process by which nucleic acids are introduced into eukaryotic cells, particularly mammalian cells. Protocols and techniques for transfection include but not limited to lipid transfection and chemical and physical methods such as electroporation. A number of transfection techniques are well known in the art and are disclosed herein. See, e.g., Graham et al., 1973, Virology 52:456; Sambrook et al., 2001, Molecular Cloning: A Laboratory Manual, supra; Davis et al., 1986, Basic Methods in Molecular Biology, Elsevier; Chu et al, 1981, Gene 13:197. In a specific embodiment of the invention, human PVRIG gene was transfected into 293F cells.

The term “SPR” or “surface plasmon resonance,” as used herein, refers to and includes an optical phenomenon that allows for the analysis of real-time biospecific interactions by detection of alterations in protein concentrations within a biosensor matrix, for example using the BIAcore system (Pharmacia Biosensor AB, Uppsala, Sweden and Piscataway, N.J.). For further descriptions, see Example 5 and Jönsson, U., et al. (1993) *Ann. Biol. Clin.* 51:19-26; Jönsson, U., et al. (1991) *Biotechniques* 11:620-627; Johnsson, B., et al. (1995) *J. Mol. Recognit.* 8:125-131; and Johnsson, B., et al. (1991) *Anal. Biochem.* 198:268-277.

The term “fluorescence-activated cell sorting” or “FACS,” as used herein, refers to a specialized type of flow cytometry. It provides a method for sorting a heterogeneous mixture of biological cells into two or more containers, one cell at a time, based upon the specific light

scattering and fluorescent characteristics of each cell (FlowMetric. "Sorting Out Fluorescence Activated Cell Sorting". Retrieved 2017-11-09.). Instruments for carrying out FACS are known to those of skill in the art and are commercially available to the public. Examples of such instruments include FACS Star Plus, FACScan and FACSsort instruments from Becton Dickinson (Foster City, Calif.) Epics C from Coulter Epics Division (Hialeah, Fla.) and MoFlo from Cytomation (Colorado Springs, Colo.).

The term "antibody-dependent cell-mediated cytotoxicity" or "ADCC," as used herein, refers to a form of cytotoxicity in which secreted Ig bound onto Fc receptors (FcRs) present on certain cytotoxic cells (e.g. Natural Killer (NK) cells, neutrophils, and macrophages) enable these cytotoxic effector cells to bind specifically to an antigen-bearing target cell and subsequently kill the target cell with cytotoxins. The antibodies "arm" the cytotoxic cells and are absolutely required for such killing. The primary cells for mediating ADCC, NK cells, express FcγRIII only, whereas monocytes express FcγRI, FcγRII and FcγRIII. FcR expression on hematopoietic cells is summarized in Table 3 on page 464 of Ravetch and Kinet, *Annu. Rev. Immunol* 9:457-92 (1991). To assess ADCC activity of a molecule of interest, an in vitro ADCC assay, such as that described in US Patent No. 5,500,362 or 5,821,337 may be performed. Useful effector cells for such assays include peripheral blood mononuclear cells (PBMC) and Natural Killer (NK) cells. Alternatively, or additionally, ADCC activity of the molecule of interest may be assessed in vivo, e.g., in an animal model such as that disclosed in Clynes et al. *PNAS (USA)* 95:652-656 (1998).

The terms "subject" and "patient" are used interchangeably and include mammals such as humans and non-human primates, as well as rabbits, rats, mice, goats, pigs, and other mammalian species. The term does not necessarily indicate that the subject has been diagnosed with a particular disease, but typically refers to an individual under medical supervision.

The term "prevent," "prevention" or "preventing," as used herein, with reference to a certain disease condition in a mammal, refers to preventing or delaying the onset of the disease, or preventing the manifestation of clinical or subclinical symptoms thereof.

The term "treatment," "treating" or "treated," as used herein in the context of treating a condition, pertains generally to treatment and therapy, whether of a human or an animal, in which some desired therapeutic effect is achieved, for example, the inhibition of the progress of the condition, and includes a reduction in the rate of progress, a halt in the rate of progress, regression of the condition, amelioration of the condition, and cure of the condition. For cancer, "treating" may refer to dampen or slow the tumor or malignant cell growth, proliferation, or metastasis, or some combination thereof.

The term "an effective amount," as used herein, pertains to that amount of an active compound, or a material, composition or dosage form comprising an active compound, which is effective for producing some desired therapeutic effect, commensurate with a reasonable benefit/risk ratio, when administered in accordance with a desired treatment regimen. For instance, the "an effective amount," when used in connection with treatment of a disease or condition, refers

to an antibody or antigen-binding portion thereof in an amount or concentration effective to treat the said disease or condition.

The term “pharmaceutically acceptable,” as used herein, means that the vehicle, diluent, excipient and/or salts thereof, are chemically and/or physically is compatible with other ingredients in the formulation, and the physiologically compatible with the recipient.

As used herein, the term “a pharmaceutically acceptable carrier and/or excipient” refers to a carrier and/or excipient pharmacologically and/or physiologically compatible with a subject and an active agent, which is well known in the art (see, e.g., Remington's Pharmaceutical Sciences. Edited by Gennaro AR, 19th ed. Pennsylvania: Mack Publishing Company, 1995), and includes, but is not limited to pH adjuster, surfactant, adjuvant and ionic strength enhancer. For example, the pH adjuster includes, but is not limited to, phosphate buffer; the surfactant includes, but is not limited to, cationic, anionic, or non-ionic surfactant, e.g., Tween-80; the ionic strength enhancer includes, but is not limited to, sodium chloride.

As used herein, the term “adjuvant” refers to a non-specific immunopotentiator, which can enhance immune response to an antigen or change the type of immune response in an organism when it is delivered together with the antigen to the organism or is delivered to the organism in advance. There are a variety of adjuvants, including, but not limited to, aluminium adjuvants (for example, aluminum hydroxide), Freund's adjuvants (for example, Freund's complete adjuvant and Freund's incomplete adjuvant), coryne bacterium parvum, lipopolysaccharide, cytokines, and the like. Freund's adjuvant is the most commonly used adjuvant in animal experiments now. Aluminum hydroxide adjuvant is more commonly used in clinical trials.

Anti-PVRIG Antibodies

In some aspects, the disclosure provides an antibody or antigen-binding portion thereof against PVRIG (such as human, mouse or cyno PVRIG). Preferably, the antibodies are capable of binding PVRIG with sufficient affinity such that they substantially or completely block the interaction and/or binding of PVRIG with PVLR2. When PVRIG is bound by its ligand PVRL2, an inhibitory signal is elicited which acts to attenuate the immune response of NK and T cells against a target cell. Blocking the binding of PVRL2 to PVRIG shuts-off this inhibitory signal of PVRIG and as a result modulates the immune response of NK and T cells.

In some embodiments, the anti-PVRIG antibody as disclosed herein are fully human antibodies. In some embodiments, the anti-PVRIG antibody as disclosed herein are humanized antibodies. The antigen-binding portion of the antibody may be a Fab, a Fab', a F(ab')₂, a single chain variable fragment (scFv), or a diabody, etc. In some embodiments, human antibodies may comprise further modifications made to CDR residues and framework residues to remove potential post translational modifications, or to refine antibody performance, such as binding affinity.

The antibodies as disclosed herein can bind to at least one of human, mouse and cynomolgus monkey PVRIG with high affinity. The binding of an antibody of the disclosure to PVRIG can be

assessed using one or more techniques well established in the art, for instance, ELISA. The binding specificity of an antibody of the disclosure can also be determined by monitoring binding of the antibody to cells expressing an PVRIG protein, e.g., flow cytometry. For example, an antibody can be tested by a flow cytometry assay in which the antibody is reacted with a cell line that expresses human PVRIG, such as HEK293 cells that have been transfected to express PVRIG on their cell surface. Additionally or alternatively, the binding of the antibody, including the binding kinetics (e.g., K_D value) can be tested in BIAcore binding assays.

In some embodiments, the antibody or antigen-binding portion thereof is capable of specifically binding to human PVRIG as well as cynomolgus PVRIG. For instance, the antibody or antigen-binding portion thereof can bind to human PVRIG expressing cells with an EC_{50} of no more than 0.5 nM, no more than 0.4 nM, no more than 0.3 nM, no more than 0.2 nM, no more than 0.1 nM, no more than 0.09 nM, or no more than 0.08 nM; bind to cyno PVRIG expressing cells with an EC_{50} of no more than 0.5 nM, no more than 0.3 nM, no more than 0.1 nM, no more than 0.08 nM, no more than 0.06 nM, or no more than 0.04 nM; as measured by FACS. In some embodiments, the antibody or antigen-binding portion thereof of the disclosure binds to human PVRIG with a K_D of 1×10^{-9} M or less, 5×10^{-10} M or less, 1×10^{-10} M or less, 5×10^{-11} M or less, 4×10^{-11} M or less, 3×10^{-11} M or less, or 2×10^{-11} M or less, as measured in a FACS affinity test.

The anti-PVRIG antibody as disclosed herein can inhibit interaction between PVRIG and PVRL2 (CD112). Blocking the signaling through PVRL2 can restore a functional response by T-cells (e.g., proliferation, cytokine production, target cell killing) from a dysfunctional state to antigen stimulation. The ability of an anti-PVRIG antibody to inhibit such interactions can be evaluated by measuring whether physical interactions between PVRIG and CD112 decrease in a binding assay. The binding assay is often a competitive binding assay. The assay may be performed in various formats, such as but not limited to an ELISA assay, flow cytometry, a surface plasmon resonance (SPR) assay (e.g., Biacore™), or BioLayer interferometry (e.g., ForteBio Octet™). In some embodiments, the anti-PVRIG antibodies herein can block human PVRL2 binding to PVRIG with an IC_{50} of no more than 0.5 nM, no more than 0.4 nM, or no more than 0.3 nM, as measured by ELISA.

Anti-PVRIG antibodies comprising CDRs

In some embodiments, the present disclosure provides an isolated antibody or the antigen-binding portion thereof comprising:

A) one or more heavy chain CDRs (HCDRs) selected from the group consisting of:

a HCDR1 comprising SEQ ID NO: 1 or an amino acid sequence that differs from SEQ ID NO: 1 by an amino acid addition, deletion and/or substitution of not more than 2 amino acids; a HCDR2 comprising SEQ ID NO: 2 or an amino acid sequence that differs from SEQ ID NO: 2 by an amino acid addition, deletion and/or substitution of not more than 2 amino acids; and a HCDR3

comprising SEQ ID NO: 3 or an amino acid sequence that differs from SEQ ID NO: 3 by an amino acid addition, deletion and/or substitution of not more than 2 amino acids;

B) one or more light chain CDRs (LCDRs) selected from the group consisting of:

a LCDR1 comprising SEQ ID NO: 4 or an amino acid sequence that differs from SEQ ID NO: 4 by an amino acid addition, deletion and/or substitution of not more than 2 amino acids; a LCDR2 comprising SEQ ID NO: 5 or an amino acid sequence that differs from any of SEQ ID NO: 5 by an amino acid addition, deletion and/or substitution of not more than 2 amino acids; and a LCDR3 comprising SEQ ID NO: 6 or an amino acid sequence that differs from SEQ ID NO: 6 by an amino acid addition, deletion and/or substitution of not more than 2 amino acids; or

C) one or more HCDRs of A) and one or more LCDRs of B).

In some embodiments, the CDR identification is according to the IMGT/Kabat definition.

In some embodiments, the present disclosure provides an isolated antibody or antigen-binding portion thereof comprising: a HCDR1 as set forth in SEQ ID NO: 1, a HCDR2 as set forth in SEQ ID NO: 2, a HCDR3 as set forth in SEQ ID NO: 3, a LCDR1 as set forth in SEQ ID NO: 4, a LCDR2 as set forth in SEQ ID NO: 5, and a LCDR3 as set forth in SEQ ID NO: 6.

The extent of the framework region and CDRs can be precisely identified using methodology known in the art, for example, by the Kabat definition, the Chothia definition, the AbM definition, the contact definition, the IMGT definition (all of which are well known in the art) and any combinations thereof. See, *e.g.*, Kabat, E.A., *et al.* (1991) *Sequences of Proteins of Immunological Interest, Fifth Edition*, U.S. Department of Health and Human Services, NIH Publication No. 91-3242, Chothia *et al.*, (1989) *Nature* 342:877; Chothia, C. *et al.* (1987) *J. Mol. Biol.* 196:901-917, Al-lazikani *et al.* (1997) *J. Molec. Biol.* 273:927-948; Edelman *et al.*, *Proc Natl Acad Sci U S A.* 1969 May, 63(1):78-85; and Martin and Allen, in “*Handbook of Therapeutic Antibodies*”, chapter 5, 2007. See also hgmp.mrc.ac.uk and bioinf.org.uk/abs. Correspondence or alignments between numberings according to different definitions can for example be found at www.imgt.org/ (see also Giudicelli V *et al.* IMGT, the international ImMunoGeneTics database. *Nucleic Acids Res.* (1997) 25:206–11; and Lefranc MP *et al.*, IMGT unique numbering for immunoglobulin and T cell receptor variable domains and Ig superfamily V-like domains. *Dev Comp Immunol.* (2003) 27:55–77).

As will be appreciated by those in the art, the exact numbering and placement of the CDRs can be different among different numbering systems. However, it should be understood that the disclosure of a variable heavy sequence and/or a variable light sequence includes the disclosure of the associated (inherent) CDRs, regardless of which numbering approach or definition scheme is adopted. Accordingly, the disclosure of each variable region is a disclosure of the CDRs (*e.g.*, HCDR1, HCDR2 and HCDR3). Two antibodies having the same VH and VL means that their CDRs are identical when determined by the same approach (*e.g.*, the Kabat, AbM, Chothia, Contact, and IMGT definitions as known in the art). The same antibody as disclosed herein may have a different set of CDRs when determined by a different definition scheme.

In some embodiments, the present disclosure provides an isolated antibody or antigen-binding portion thereof comprising one, two, or all three HCDRs of the amino acid sequence as set forth in SEQ ID NO: 7, and one, two, or all three LCDRs of the amino acid sequence as set forth in SEQ ID NO: 8. In some embodiments, provided herein is an anti-PVRIG antibody comprising one, two, or all three HCDRs of the amino acid sequence as set forth in SEQ ID NO: 7, and one, two, or all three LCDRs of the amino acid sequence as set forth in SEQ ID NO: 9.

In some embodiments, the present disclosure provides an isolated antibody or antigen-binding portion thereof comprising HCDR1, HCDR2 and HCDR3 of the VH sequence as set forth in SEQ ID NO: 7, and LCDR1, LCDR2 and LCDR3 of the VL sequence as set forth in SEQ ID NO: 8. In some embodiments, provided herein is an anti-PVRIG antibody comprising HCDR1, HCDR2 and HCDR3 of the VH sequence as set forth in SEQ ID NO: 7, and LCDR1, LCDR2 and LCDR3 of the VL sequence as set forth in SEQ ID NO: 9.

Variable regions and CDRs in an antibody sequence can be identified according to general rules that have been developed in the art or by aligning the sequences against a database of known variable regions. Methods for identifying these regions are described in Kontermann and Dubel, eds., *Antibody Engineering*, Springer, New York, NY, 2001 and Dinarello *et al.*, *Current Protocols in Immunology*, John Wiley and Sons Inc., Hoboken, NJ, 2000. Exemplary databases of antibody sequences are described in, and can be accessed through, the "Abysis" website at www.bioinf.org.uk/abs (maintained by A.C. Martin in the Department of Biochemistry & Molecular Biology University College London, London, England) and the VBASE2 website at www.vbase2.org, as described in Retter *et al.*, *Nucl. Acids Res.*, 33 (Database issue): D671 -D674 (2005). Sequences may be analyzed using the Abysis database, which integrates sequence data from Kabat, IMGT and the Protein Data Bank (PDB) with structural data from the PDB. See Dr. Andrew C. R. Martin's book chapter *Protein Sequence and Structure Analysis of Antibody Variable Domains*. In: *Antibody Engineering Lab Manual* (Ed.: Duebel, S. and Kontermann, R., Springer-Verlag, Heidelberg, ISBN-13: 978-3540413547, also available on the website bioinforg.uk/abs). The Abysis database website further includes general rules that have been developed for identifying CDRs which can be used in accordance with the teachings herein.

In some embodiments, the anti-PVRIG antibodies as disclosed herein comprise the following VH region and a VL region, wherein the VH region comprises FRW1-HCDR1-FRW2-HCDR2-FRW3-HCDR3-FRW4, and wherein HCDR1 has an amino acid sequence as set forth in SEQ ID NO: 1, HCDR2 has an amino acid sequence as set forth in SEQ ID NO: 2, and HCDR3 has an amino acid sequence as set forth in SEQ ID NO: 3, and/or wherein the VL region comprises FRW1-LCDR1-FRW2-LCDR2-FRW3-LCDR3-FRW4, and wherein LCDR1 has an amino acid sequence as set forth in SEQ ID NO: 4, LCDR2 has an amino acid sequence as set forth in SEQ ID NO: 5, and LCDR3 has an amino acid sequence as set forth in SEQ ID NO: 6.

In some embodiments, the framework regions are derived from human germline, e.g. a human immunoglobulin. In some embodiments, certain residues in the framework regions are mutated to

improve antibody performance, such as stability, binding affinity, isomerization, immunogenicity, etc. In some embodiments, S7 in FRW1 of the VL region and/or T43 of FRW2 of the VL region (according to Kabat numbering) are mutated. In some specific embodiments, the VL region of the antibody comprises S7P/T43A substitutions. In some embodiments, the FRW1 and FRW4 at the N and C terminal of the VH and/or VL region may be truncated such that it comprises only a partial FRW1 and/or FRW4. In some embodiments, the CDRs and FR regions have undergone a PTM-removal optimization.

In some embodiments, provided herein is an anti-PVRIG antibody comprising at least one of the FRW1, FRW2, FRW3 and FRW4 of the amino acid sequence as set forth in SEQ ID NO: 7, and at least one of the FRW1, FRW2, FRW3 and FRW4 of the amino acid sequence as set forth in SEQ ID NO: 8. In some embodiments, provided herein is an anti-PVRIG antibody comprising at least one of the FRW1, FRW2, FRW3 and FRW4 of the amino acid sequence as set forth in SEQ ID NO: 7, and at least one of the FRW1, FRW2, FRW3 and FRW4 of the amino acid sequence as set forth in SEQ ID NO: 9.

Anti-PVRIG antibodies comprising a heavy chain variable region and a light chain variable region

In some embodiments, the isolated antibody or the antigen-binding portion thereof comprises:

(A) a heavy chain variable region (VH):

- (i) comprising the amino acid sequence of SEQ ID NO: 7;
- (ii) comprising an amino acid sequence having the same set of CDRs as SEQ ID NO: 7 and with at least 85%, 90%, or 95% identity in the framework regions; or
- (iii) comprising an amino acid sequence with addition, deletion and/or substitution of one or more (e.g. 10, 9, 8, 7, 6, 5, 4, 3, 2) amino acids in the framework regions compared with the amino acid sequence of SEQ ID NO: 7; and/or

(B) a light chain variable region (VL):

- (i) comprising the amino acid sequence of SEQ ID NO: 8 or 9;
- (ii) comprising an amino acid sequence having the same set of CDRs as SEQ ID NO: 8 or 9 and with at least 85%, 90%, or 95% identity in the framework regions; or
- (iii) comprising an amino acid sequence with addition, deletion and/or substitution of one or more (e.g. 10, 9, 8, 7, 6, 5, 4, 3, 2) amino acids in the framework regions compared with the amino acid sequence of SEQ ID NO: 8 or 9.

The percent identity between two amino acid sequences can be determined using the algorithm of E. Meyers and W. Miller (Comput. Appl. Biosci., 4:11-17 (1988)) which has been incorporated into the ALIGN program (version 2.0), using a PAM120 weight residue table, a gap length penalty of 12 and a gap penalty of 4. In addition, the percentage of identity between two amino acid sequences can be determined by the algorithm of Needleman and Wunsch (J. Mol.

Biol. 48:444-453 (1970)) which has been incorporated into the GAP program in the GCG software package (available at <http://www.gcg.com>), using either a Blossum 62 matrix or a PAM250 matrix, and a gap weight of 16, 14, 12, 10, 8, 6, or 4 and a length weight of 1, 2, 3, 4, 5, or 6.

Additionally or alternatively, the protein sequences of the present disclosure can further be used as a “query sequence” to perform a search against public databases to, for example, identify related sequences. Such searches can be performed using the XBLAST program (version 2.0) of Altschul, et al. (1990) J. Mol. Biol. 215:403-10. BLAST protein searches can be performed with the XBLAST program, score = 50, wordlength = 3 to obtain amino acid sequences homologous to the antibody molecules of the disclosure. To obtain gapped alignments for comparison purposes, Gapped BLAST can be utilized as described in Altschul et al. (1997) Nucleic Acids Res. 25(17):3389-3402. When utilizing BLAST and Gapped BLAST programs, the default parameters of the respective programs (e.g., XBLAST and NBLAST) can be used. See www.ncbi.nlm.nih.gov.

In some further embodiments, the isolated antibody or the antigen-binding portion thereof may contain conservative substitution or modification of amino acids in the variable regions of the heavy chain and/or light chain. It is understood in the art that certain conservative sequence modification can be made which do not remove antigen binding. See, e.g., Brummell et al. (1993) Biochem 32:1180-8; de Wildt et al. (1997) Prot. Eng. 10:835-41; Komissarov et al. (1997) J. Biol. Chem. 272:26864- 26870; Hall et al. (1992) J. Immunol. 149:1605-12; Kelley and O’Connell (1993) Biochem. 32:6862-35; Adib-Conquy et al. (1998) Int. Immunol. 10:341-6 and Beers et al. (2000) Clin. Can. Res. 6:2835-43.

The term “conservative substitution,” as used herein, refers to amino acid substitutions which would not disadvantageously affect or change the essential properties of a protein/polypeptide comprising the amino acid sequence. For example, a conservative substitution may be introduced by standard techniques known in the art such as site-directed mutagenesis and PCR-mediated mutagenesis. Conservative amino acid substitutions include substitutions wherein an amino acid residue is substituted with another amino acid residue having a similar side chain, for example, a residue physically or functionally similar (such as, having similar size, shape, charge, chemical property including the capability of forming covalent bond or hydrogen bond, etc.) to the corresponding amino acid residue. The families of amino acid residues having similar side chains have been defined in the art. These families include amino acids having alkaline side chains (for example, lysine, arginine and histidine), amino acids having acidic side chains (for example, aspartic acid and glutamic acid), amino acids having uncharged polar side chains (for example, glycine, asparagine, glutamine, serine, threonine, tyrosine, cysteine, tryptophan), amino acids having nonpolar side chains (for example, alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine), amino acids having β -branched side chains (such as threonine, valine, isoleucine) and amino acids having aromatic side chains (for example, tyrosine, phenylalanine, tryptophan, histidine). Therefore, a corresponding amino acid residue is preferably substituted with another amino

acid residue from the same side-chain family. Methods for identifying amino acid conservative substitutions are well known in the art (see, for example, Brummell et al., Biochem. 32: 1180-1187 (1993); Kobayashi et al., Protein Eng. 12(10): 879-884 (1999); and Burks et al., Proc. Natl. Acad. Sci. USA 94: 412-417 (1997), which are incorporated herein by reference). In some further embodiments, the antibody or the antigen-binding portion thereof as disclosed herein has a conservative substitution at S7 and/or T43 positions (according to Kabat numbering) in the VL region.

In a specific embodiment, the isolated antibody or the antigen-binding portion thereof comprises: a heavy chain variable region comprising or consisting of the amino acid sequence of SEQ ID NO: 7 and a light chain variable region comprising or consisting of the amino acid sequence of SEQ ID NO: 8 or 9.

In other embodiments, the amino acid sequences of the heavy chain variable region and/or the light chain variable region can be at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% identical to the respective sequences set forth above.

Fc region

Anti-PVRIG antibodies and antigen-binding portions provided herein further comprise an immunoglobulin constant region, such as a human IgG1, IgG2, IgG3 or IgG4 immunoglobulin constant region (native or variant thereof), comprising a Fc region and optionally a hinge region. The anti-PVRIG antibodies herein may be of IgG1 or IgG4 isotype. In some embodiments, the Fc region is a native Fc region or an Fc variant. An Fc variant can possess at least about 80% homology with a native sequence Fc region, or at least about 90% homology therewith, for example, at least about 95% homology therewith. In some embodiments, the Fc region is a human IgG4 Fc region, such as a wild-type Fc region or a Fc variant comprising a S228P substitution. In certain embodiments, the antibodies disclosed herein comprise wild-type human IgG1 Fc region.

In some embodiments, the Fc region comprises one or more amino acid changes (e.g., insertions, deletions or substitutions) that results in a modified binding interaction between Fc and FcRn or FcγR.

In certain embodiments, the Fc region is a IgG4 Fc region comprising a S228P mutation (according to EU numbering as in Kabat et al.) that prevents Fab arm exchange and stabilizes IgG4 molecule. In certain embodiments, the Fc region is a IgG1 Fc region and comprises a LALA mutation, i.e. mutations of L234A and L235A. LALA mutation is perhaps the most commonly used mutation for disrupting antibody effector function, e.g. eliminate Fc binding to specific FcγRs, reduce ADCC activity mediated by PBMCs and monocytes. The “EU numbering system” or “EU index” is generally used when referring to a residue in an immunoglobulin heavy chain constant region (e.g., the EU index reported in Kabat et al., supra). The “EU numbering as in Kabat” or “EU index as in Kabat” refers to the residue numbering of the human IgG1 EU antibody. Unless

stated otherwise herein, references to residue numbers in the constant domain of antibodies means residue numbering by the EU numbering system.

In certain embodiments, the hinge region may be derived from the same human IgG immunoglobulin as the Fc region.

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Anti-PVRIG antibodies with certain properties

The antibodies of the present disclosure are characterized by particular functional features or properties of the antibodies. Based on the action mechanism against the target, the *in vitro* functional properties and pharmacological activity of the antibody were fully evaluated at both the molecular and cellular levels. In some embodiments, the isolated antibody or the antigen-binding portion thereof has one or more of the following properties:

- (a) specifically binding to at least one of human PVRIG protein and cyno PVRIG protein;
- (b) having no cross binding to PVRIG paralog proteins;
- (c) block binding between PVRIG and its ligand PVRL2 (CD112);
- 15 (d) activation of immune cells such as T cells and NK cells;
- (e) having good serum stability and thermo stability; and
- (f) showing significantly better efficacy in treating cancer than benchmark antibodies.

As shown in the Examples, CTLs (cytotoxic T Lymphocyte, e.g. CD8⁺ T cells) and NK cells can be used in functional assays for assessing antibody functions. CTLs express a T cell receptor (TCR) recognizing a specific antigen (Ag) presented on MHC molecules. Upon TCR Ag engagement, CTLs undergo activation as manifested by cell proliferation, upregulation of activation markers (e.g. CD35, CD137), cytokine secretion and cytotoxic activity. Upon contact with PVRL2 expressed on cancer cells or antigen presenting cells, PVRIG mediates a negative signal to CTLs thereby causing down-regulation of CTL activation. Thus, contacting anti-PVRIG antibodies that show binding and/or inhibition of receptor-ligand binding with CTLs will interrupt the PVRIG-PVRL2 interaction and thereby release the negative signal mediated by PVRIG and enhances antigen specific CTL activation as manifested by cell proliferation, up-regulation of activation markers (e.g. CD25, CD137 etc.), and cytokine secretion (e.g. interferon gamma, IL2, TNF alfa etc.). In some embodiments, human CD8⁺ T cells treated with anti-PVRIG antibodies resulted in greater IFN- γ secretion than that observed with isotype control and the reference antibody.

The NK cells express various activating and inhibitory receptors. Upon contact with PVRL2 expressed on cancer target cells, PVRIG mediates a negative signal to NK cells thereby causing down-regulation of NK cell activation. Contacting anti-PVRIG antibodies with NK cells will interrupt the PVRIG-PVRL2 interaction and thereby release the negative signal mediated by PVRIG and enhances NK cell activation as manifested by cell proliferation, cytokine secretion (e.g. interferon gamma, IL2, TNF alfa etc.) and/or cytotoxic activity.

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Methods for Producing Antibodies

Monoclonal antibodies can be prepared using a wide variety of techniques known in the art including hybridoma techniques, recombinant techniques, phage display technologies, transgenic animals (e.g., a XenoMouse[®]) or some combination thereof. For example, monoclonal antibodies can be produced using hybridoma and art-recognized biochemical and genetic engineering techniques such as described in more detail in An, Zhigiang (ed.) *Therapeutic Monoclonal Antibodies: From Bench to Clinic*, John Wiley and Sons, 1st ed. 2009; Shire et. al. (eds.) *Current Trends in Monoclonal Antibody Development and Manufacturing*, Springer Science + Business Media LLC, 1st ed. 2010; Harlow *et al.*, *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, 2nd ed. 1988; Hammerling, *et al.*, in: *Monoclonal Antibodies and T-Cell Hybridomas* 563-681 (Elsevier, N.Y., 1981) each of which is incorporated herein in its entirety by reference. It should be understood that a selected binding sequence can be further altered, for example, to improve affinity for the target, to humanize the target binding sequence, to improve its production in cell culture, to reduce its immunogenicity in vivo, to create a multispecific antibody, etc., and that an antibody comprising the altered target binding sequence is also an antibody of this invention.

To obtain human antibodies with minimal immunogenicity, transgenic mice technology, which allows for the generation of fully human therapeutic mAbs, has been developed. OmniRat (Open Monoclonal Technology Company) is transgenic rats carrying a chimeric human/rat IgH locus (comprising 22 human V_{HS}, all human D and J_H segments in natural configuration linked to the rat C_H locus) together with fully human IgL loci (12 V_{KS} linked to J_K-C_K and 16 V_{LS} linked to J_L-C_L). The endogenous Ig loci were silenced using designer zinc finger nucleases. OmniRat rat could produce antibodies with human idiotypes as efficiently as wild-type animals produce rat antibodies.

Nucleic Acid Molecules Encoding Antibodies of the Disclosure

In some aspects, the disclosure is directed to an isolated nucleic acid molecule, comprising a nucleic acid sequence encoding the heavy chain variable region and/or the light chain variable region of the isolated antibody as disclosed herein.

Nucleic acids of the disclosure can be obtained using standard molecular biology techniques. For antibodies expressed by hybridomas (e.g., hybridomas prepared from transgenic mice carrying human immunoglobulin genes), cDNAs encoding the light and heavy chains of the antibody made by the hybridoma can be obtained by standard PCR amplification or cDNA cloning techniques. For antibodies obtained from an immunoglobulin gene library (e.g., using phage display techniques), a nucleic acid encoding such antibodies can be recovered from the gene library.

The isolated nucleic acid encoding the V_H region can be converted to a full-length heavy chain gene by operatively linking the V_H-encoding nucleic acid to another DNA molecule encoding heavy chain constant domains (CH1, CH2 and CH3). The sequences of human heavy

chain constant region genes are known in the art (see e.g., Kabat et al. (1991), supra) and DNA fragments encompassing these regions can be obtained by standard PCR amplification. The heavy chain constant region can be an IgG1, IgG2, IgG3, IgG4, IgA, IgE, IgM or IgD constant region, but more preferably is an IgG1 or IgG4 constant region.

5 The isolated nucleic acid encoding the VL region can be converted to a full-length light chain gene (as well as a Fab light chain gene) by operatively linking the VL-encoding DNA to another DNA molecule encoding the light chain constant region, CL. The sequences of human light chain constant region genes are known in the art (see e.g., Kabat et al., supra) and DNA fragments encompassing these regions can be obtained by standard PCR amplification. The light
10 chain constant region can be a kappa or lambda constant region.

Once DNA fragments encoding VH and VL segments are obtained, these DNA fragments can be further manipulated by standard recombinant DNA techniques, for example to convert the variable region genes to full-length antibody chain genes, to Fab fragment genes or to a scFv gene. In these manipulations, a VL- or VH-encoding DNA fragment is operatively linked to another
15 DNA fragment encoding another protein, such as an antibody constant region or a flexible linker. The term “operatively linked”, as used in this context, is intended to mean that the two DNA fragments are joined such that the amino acid sequences encoded by the two DNA fragments remain in-frame.

In some embodiments, the disclosure is directed to an isolated nucleic acid molecule,
20 comprising a nucleic acid sequence encoding the heavy chain variable region of the isolated antibody as disclosed herein. In some specific embodiments, the isolated nucleic acid molecule encodes the heavy chain variable region of the isolated antibody and comprises a nucleic acid sequence selected from the group consisting of:

(A) a nucleic acid sequence that encodes a heavy chain variable region as set forth in SEQ
25 ID NO: 7;

(B) a nucleic acid sequence as set forth in SEQ ID NO: 10; or

(C) a nucleic acid sequence that hybridized under high stringency conditions to the complementary strand of the nucleic acid sequence of (A) or (B).

In some embodiments, the disclosure is directed to an isolated nucleic acid molecule,
30 comprising a nucleic acid sequence encoding the light chain variable region of the isolated antibody as disclosed herein.

In some specific embodiments, the isolated nucleic acid molecule encodes the light chain variable region of the isolated antibody comprises a nucleic acid sequence selected from the group consisting of:

35 (A) a nucleic acid sequence that encodes a light chain variable region as set forth in SEQ ID NO: 8 or 9;

(B) a nucleic acid sequence as set forth in SEQ ID NO: 11; or

(C) a nucleic acid sequence that hybridized under high stringency conditions to the complementary strand of the nucleic acid sequence of (A) or (B).

For example, the nucleic acid molecule comprises SEQ ID NO: 10 and 11. In some other embodiments, the nucleic acid molecule shares at least 80% (e.g. at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99%) sequence identity to SEQ ID NO: 10 or 11. In some specific embodiments, the percentage of identity is derived from the degeneracy of the genetic code, and the encoded protein sequences remain unchanged.

Exemplary high stringency conditions include hybridization at 45°C in 5X SSPE and 45% formamide, and a final wash at 65°C in 0.1 X SSC. It is understood in the art that conditions of equivalent stringency can be achieved through variation of temperature and buffer, or salt concentration as described Ausubel, et al. (Eds.), *Protocols in Molecular Biology*, John Wiley & Sons (1994), pp. 6.0.3 to 6.4.10. Modifications in hybridization conditions can be empirically determined or precisely calculated based on the length and the percentage of guanosine/cytosine (GC) base pairing of the probe. The hybridization conditions can be calculated as described in Sambrook, et al. (Eds.), *Molecular Cloning: A laboratory Manual*. Cold Spring Harbor Laboratory Press: Cold Spring Harbor, New York (1989), pp. 9.47 to 9.51.

Host Cells

Host cells as disclosed in the present disclosure may be any cell which is suitable for expressing the antibodies of the present disclosure, for example, yeast, bacterial, fungal, plant and animal cells, preferably mammalian cells. Mammalian host cells for expressing the antibodies of the present disclosure include Chinese Hamster Ovary (CHO cells) (including dhfr CHO cells, described in Urlaub and Chasin, (1980) *Proc. Natl. Acad. Sci. USA* 77:4216-4220, used with a DHFR selectable marker, e.g., as described in R. J. Kaufman and P. A. Sharp (1982) *J. Mol. Biol.* 159:601-621), 293F cells, NSO myeloma cells, COS cells and SP2 cells. In particular, for use with NSO myeloma cells, another expression system is the GS gene expression system disclosed in WO 87/04462, WO 89/01036 and EP 338,841. Also included are monkey kidney CV1 line transformed by SV40 (COS-7, ATCC CRL 1651); human embryonic kidney line (293 or 293 cells subcloned for growth in suspension culture, Graham et al., *J. Gen. Virol.* 36:59 (1977)); baby hamster kidney cells (BHK, ATCC CCL 10); Chinese hamster ovary cells/-DHFR (CHO, Urlaub et al., 1980, *Proc. Natl. Acad. Sci. USA* 77:4216); mouse sertoli cells (TM4, Mather, 1980, *Biol. Reprod.* 23:243-251); monkey kidney cells (CV1 ATCC CCL 70); African green monkey kidney cells (VERO-76, ATCC CRL-1587); human cervical carcinoma cells (HELA, ATCC CCL 2); canine kidney cells (MDCK, ATCC CCL 34); buffalo rat liver cells (BRL 3A, ATCC CRL 1442); human lung cells (W138, ATCC CCL 75); human liver cells (Hep G2, HB 8065); mouse mammary tumor (MMT 060562, ATCC CCL51); TRI cells (Mather et al., 1982, *Annals N.Y. Acad. Sci.* 383:44-68); MRC 5 cells; FS4 cells; mouse myeloma cells, such as NSO (e.g. RCB0213, 1992, *Bio/Technology* 10:169) and SP2/0 cells (e.g. SP2/0-Ag14 cells, ATCC CRL 1581); rat myeloma cells, such as

YB2/0 cells (e.g. YB2/3HL.P2.G11.16Ag.20 cells, ATCC CRL 1662); PER.C6 cells; and a human hepatoma line (Hep G2). CHO cells are one of the cell lines that can be used herein, with CHO-K1, DUK-B11, CHO-DP12, CHO-DG44 (Somatic Cell and Molecular Genetics 12:555 (1986)), and Lec13 being exemplary host cell lines. In the case of CHO-K1, DUK-B11, DG44 or CHO-DP12 host cells, these may be altered such that they are deficient in their ability to fucosylate proteins expressed therein. In some embodiments, the host cells herein are selected from CHO, CHO-S, HEK, HEK293, HEK-293F, Expi293F, PER.C6 or NSO cells or lymphocytic cells.

Suitable prokaryotes for this purpose include eubacteria, such as Gram-negative or Gram-positive organisms, for example, Enterobacteriaceae such as Escherichia, e.g., E. coli, Enterobacter, Erwinia, Klebsiella, Proteus, Salmonella, e.g., Salmonella typhimurium, Serratia, e.g., Serratia marcescans, and Shigella, as well as Bacilli such as B. subtilis and B. licheniformis, Pseudomonas such as P. aeruginosa, and Streptomyces.

In addition to prokaryotes, eukaryotic microbes such as filamentous fungi or yeast are also suitable cloning or expression hosts for antibody-encoding vectors. Saccharomyces cerevisiae, or common baker's yeast, is the most commonly used among lower eukaryotic host microorganisms. However, a number of other genera, species, and strains are commonly available and useful herein, such as Schizosaccharomyces pombe; Kluyveromyces hosts such as, e.g., K. lactis, K. fragilis (ATCC 12,424), K. bulgaricus (ATCC 16,045), K. wickerhamii (ATCC 24,178), K. waltii (ATCC 56,500), K. drosophilae (ATCC 36,906), K. thermotolerans, and K. marxianus; Yarrowia (EP 402,226); Pichia pastoris (EP 183,070); Candida; Trichoderma reesei (EP 244,234); Neurospora crassa; Schwannomyces such as Schwannomyces occidentalis; and filamentous fungi such as, e.g., Neurospora, Penicillium, Tolypocladium, and Aspergillus hosts such as A. nidulans and A. niger.

When recombinant expression vectors encoding an antibody are introduced into mammalian host cells, the antibody is produced by culturing the host cells for a period of time sufficient to allow for expression of the antibody in the host cells or, secretion of the antibody into the culture medium in which the host cells are grown. Antibodies can be recovered from the culture medium using standard protein purification methods.

Pharmaceutical Compositions

In some aspects, the disclosure is directed to a pharmaceutical composition comprising at least one antibody or antigen-binding portion thereof as disclosed herein and a pharmaceutically acceptable carrier. In some aspects, the present disclosure provides a pharmaceutical composition comprising a nucleic acid encoding the antibody or antigen-binding portion thereof as disclosed herein and a pharmaceutically acceptable carrier. In some aspects, the present disclosure provides a pharmaceutical composition comprising a cell expressing the antibody or antigen-binding portion thereof as disclosed herein and a pharmaceutically acceptable carrier.

Components of the compositions

The pharmaceutical composition may optionally contain one or more additional pharmaceutically active ingredients, such as another antibody or a drug. The pharmaceutical compositions of the disclosure also can be administered in a combination therapy with, for example, another immune-stimulatory agent, anti-cancer agent, an antiviral agent, or a vaccine. A pharmaceutically acceptable carrier can include, for example, a pharmaceutically acceptable liquid, gel or solid carriers, an aqueous medium, a non-aqueous medium, an anti-microbial agent, isotonic agents, buffers, antioxidants, anesthetics, suspending/dispersing agent, a chelating agent, a diluent, adjuvant, excipient or a nontoxic auxiliary substance, other components known in the art, various combinations thereof etc.

Suitable components may include, for example, antioxidants, fillers, binders, disintegrating agents, buffers, preservatives, lubricants, flavorings, thickening agents, coloring agents, emulsifiers or stabilizers such as sugars and cyclodextrin. Suitable anti-oxidants may include, for example, methionine, ascorbic acid, EDTA, sodium thiosulfate, platinum, catalase, citric acid, cysteine, mercapto glycerol, thioglycolic acid, Mercapto sorbitol, butyl methyl anisole, butylated hydroxy toluene and/or propyl gallate. For example, a composition containing an antibody or an antigen-binding fragment of the present disclosure may include one or more anti-oxidants such as methionine, reducing antibody or antigen binding fragment thereof may be oxidized. The oxidation reduction may prevent or reduce a decrease in binding affinity, thereby enhancing antibody stability and extended shelf life. Thus, in some embodiments, the present disclosure provides a composition comprising one or more antibodies or antigen binding fragment thereof and one or more anti-oxidants such as methionine. The present disclosure further provides a variety of methods, wherein an antibody or antigen binding fragment thereof is mixed with one or more anti-oxidants, such as methionine, so that the antibody or antigen binding fragment thereof can be prevented from oxidation, to extend their shelf life and/or increase activity.

To further illustrate, pharmaceutical acceptable carriers may include, for example, aqueous vehicles such as sodium chloride injection, Ringer's injection, isotonic dextrose injection, sterile water injection, or dextrose and lactated Ringer's injection, nonaqueous vehicles such as fixed oils of vegetable origin, cottonseed oil, corn oil, sesame oil, or peanut oil, antimicrobial agents at bacteriostatic or fungistatic concentrations, isotonic agents such as sodium chloride or dextrose, buffers such as phosphate or citrate buffers, antioxidants such as sodium bisulfate, local anesthetics such as procaine hydrochloride, suspending and dispersing agents such as sodium carboxymethylcellulose, hydroxypropyl methylcellulose, or polyvinylpyrrolidone, emulsifying agents such as Polysorbate 80 (TWEEN-80), sequestering or chelating agents such as EDTA (ethylenediaminetetraacetic acid) or EGTA (ethylene glycol tetraacetic acid), ethyl alcohol, polyethylene glycol, propylene glycol, sodium hydroxide, hydrochloric acid, citric acid, or lactic acid. Antimicrobial agents utilized as carriers may be added to pharmaceutical compositions in multiple-dose containers that include phenols or cresols, mercurials, benzyl alcohol, chlorobutanol, methyl and propyl p-hydroxybenzoic acid esters, thimerosal, benzalkonium chloride and

benzethonium chloride. Suitable excipients may include, for example, water, saline, dextrose, glycerol, or ethanol. Suitable non-toxic auxiliary substances may include, for example, wetting or emulsifying agents, pH buffering agents, stabilizers, solubility enhancers, or agents such as sodium acetate, sorbitan monolaurate, triethanolamine oleate, or cyclodextrin.

5

Administration, Formulation and Dosage

The pharmaceutical composition of the disclosure may be administered *in vivo*, to a subject in need thereof, by various routes, including, but not limited to, oral, intravenous, intra-arterial, subcutaneous, parenteral, intranasal, intramuscular, intracranial, intracardiac, intraventricular, 10 intratracheal, buccal, rectal, intraperitoneal, intradermal, topical, transdermal, and intrathecal, or otherwise by implantation or inhalation. The subject compositions may be formulated into preparations in solid, semi-solid, liquid, or gaseous forms; including, but not limited to, tablets, capsules, powders, granules, ointments, solutions, suppositories, enemas, injections, inhalants, and aerosols. The appropriate formulation and route of administration may be selected according to 15 the intended application and therapeutic regimen.

Suitable formulations for enteral administration include hard or soft gelatin capsules, pills, tablets, including coated tablets, elixirs, suspensions, syrups or inhalations and controlled release forms thereof.

Formulations suitable for parenteral administration (e.g., by injection), include aqueous or 20 non-aqueous, isotonic, pyrogen-free, sterile liquids (e.g., solutions, suspensions), in which the active ingredient is dissolved, suspended, or otherwise provided (e.g., in a liposome or other microparticulate). Such liquids may additionally contain other pharmaceutically acceptable ingredients, such as anti-oxidants, buffers, preservatives, stabilizers, bacteriostats, suspending agents, thickening agents, and solutes which render the formulation isotonic with the blood (or 25 other relevant bodily fluid) of the intended recipient. Examples of excipients include, for example, water, alcohols, polyols, glycerol, vegetable oils, and the like. Examples of suitable isotonic carriers for use in such formulations include Sodium Chloride Injection, Ringer's Solution, or Lactated Ringer's Injection. Similarly, the particular dosage regimen, including dose, timing and repetition, will depend on the particular individual and that individual's medical history, as well as 30 empirical considerations such as pharmacokinetics (e.g., half-life, clearance rate, etc.).

Frequency of administration may be determined and adjusted over the course of therapy, and is based on reducing the number of proliferative or tumorigenic cells, maintaining the reduction of such neoplastic cells, reducing the proliferation of neoplastic cells, or delaying the development of metastasis. In some embodiments, the dosage administered may be adjusted or attenuated to 35 manage potential side effects and/or toxicity. Alternatively, sustained continuous release formulations of a subject therapeutic composition may be appropriate.

It will be appreciated by one of skill in the art that appropriate dosages can vary from patient to patient. Determining the optimal dosage will generally involve the balancing of the level of

therapeutic benefit against any risk or deleterious side effects. The selected dosage level will depend on a variety of factors including, but not limited to, the activity of the particular compound, the route of administration, the time of administration, the rate of excretion of the compound, the duration of the treatment, other drugs, compounds, and/or materials used in combination, the severity of the condition, and the species, sex, age, weight, condition, general health, and prior medical history of the patient. The amount of compound and route of administration will ultimately be at the discretion of the physician, veterinarian, or clinician, although generally the dosage will be selected to achieve local concentrations at the site of action that achieve the desired effect without causing substantial harmful or deleterious side-effects.

In general, the antibody or the antigen binding portion thereof of the disclosure may be administered in various ranges. These include about 5 µg/kg body weight to about 40 mg/kg body weight per dose; about 50 µg/kg body weight to about 5 mg/kg body weight per dose; about 100 µg/kg body weight to about 10 mg/kg body weight per dose. Other ranges include about 100 µg/kg body weight to about 20 mg/kg body weight per dose and about 0.5 mg/kg body weight to about 20 mg/kg body weight per dose. In certain embodiments, the dosage is at least about 100 µg/kg body weight, at least about 250 µg/kg body weight, at least about 750 µg/kg body weight, at least about 3 mg/kg body weight, at least about 5 mg/kg body weight, at least about 10 mg/kg body weight.

In any event, the antibody or the antigen binding portion thereof of the disclosure is preferably administered as needed to subjects in need thereof. Determination of the frequency of administration may be made by persons skilled in the art, such as an attending physician based on considerations of the condition being treated, age of the subject being treated, severity of the condition being treated, general state of health of the subject being treated and the like.

In certain preferred embodiments, the course of treatment involving the antibody or the antigen-binding portion thereof of the present disclosure will comprise multiple doses of the selected drug product over a period of weeks or months. More specifically, the antibody or the antigen-binding portion thereof of the present disclosure may be administered once every day, every two days, every four days, every week, every ten days, every two weeks, every three weeks, every month, every six weeks, every two months, every ten weeks or every three months. In this regard, it will be appreciated that the dosages may be altered or the interval may be adjusted based on patient response and clinical practices.

Dosages and regimens may also be determined empirically for the disclosed therapeutic compositions in individuals who have been given one or more administration(s). For example, individuals may be given incremental dosages of a therapeutic composition produced as described herein. In selected embodiments, the dosage may be gradually increased or reduced or attenuated based respectively on empirically determined or observed side effects or toxicity. To assess efficacy of the selected composition, a marker of the specific disease, disorder or condition can be followed as described previously. For cancer, these include direct measurements of tumor size via

palpation or visual observation, indirect measurement of tumor size by x-ray or other imaging techniques; an improvement as assessed by direct tumor biopsy and microscopic examination of the tumor sample; the measurement of an indirect tumor marker (e.g., PSA for prostate cancer) or a tumorigenic antigen identified according to the methods described herein, a decrease in pain or paralysis; improved speech, vision, breathing or other disability associated with the tumor; increased appetite; or an increase in quality of life as measured by accepted tests or prolongation of survival.

Compatible formulations for parenteral administration (e.g., intravenous injection) may comprise the antibody or antigen-binding portion thereof as disclosed herein in concentrations of from about 10 µg/ml to about 100 mg/ml. It will be apparent to one of skill in the art that the dosage of the antibody or antigen-binding portion thereof as disclosed herein may vary depending on the individual, the type of neoplastic condition, the stage of neoplastic condition, whether the neoplastic condition has begun to metastasize to other location in the individual, the past and concurrent treatments being used, and the dosage of therapeutic agents used in combination with the antibody as disclosed herein.

Applications of the Disclosure

The antibodies, antibody compositions and methods of the present disclosure have numerous in vitro and in vivo utilities involving, for example, detection of PVRIG or enhancement of immune response. For example, these molecules can be administered to cells in culture, in vitro or ex vivo, or to human subjects, e.g., in vivo, to enhance immunity in a variety of situations. The immune response can be modulated, for instance, augmented, stimulated or up-regulated.

For instance, the subjects include human patients in need of enhancement of an immune response. The methods are particularly suitable for treating human patients having a disorder that can be treated by augmenting an immune response (e.g., the T-cell mediated immune response). In a particular embodiment, the methods are particularly suitable for treatment of cancer in vivo, including the immunosuppression induced by cancers.

The antibodies of the present disclosure may be combined with an additional therapeutic agent, such as an anti-cancer agent, including an anti-cancer antibody and a chemotherapeutic agent. The additional therapeutic agent may also be an antagonist or an inhibitor of a T cell coinhibitor, an agonist of a T cell coactivator or an immune stimulatory cytokine. When the anti-PVRIG antibodies are administered together with another agent such as an anti-PD-1 agent, the two can be administered in either order or simultaneously.

The present disclosure further provides methods for detecting the presence of PVRIG antigen in a sample, or measuring the amount of PVRIG antigen, comprising contacting the sample, and a control sample, with the anti-PVRIG antibody or an antigen binding portion thereof, under conditions that allow for formation of a complex between the antibody or portion thereof and PVRIG. The formation of a complex is then detected, wherein a difference in complex formation

between the sample compared to the control sample is indicative of the presence of PVRIG antigen in the sample. Moreover, the anti-PVRIG antibodies of the disclosure can be used to purify PVRIG via immunoaffinity purification.

5 ***Treatment of disorders including cancers***

In some aspects, the present disclosure provides a method of treating a disorder or a disease in a mammal, which comprises administering to the subject (for example, a human) in need of treatment a therapeutically effective amount of the anti-PVRIG antibody or antigen-binding portion thereof as disclosed herein. The disorder or disease comprises but not limited to, proliferative disorders (such as cancers), immune disorders, inflammatory disease or infectious diseases. For example, the disorder may be a cancer.

In some embodiments, the cancer is a cancer that is enriched for expression of PRVL2. In some embodiments, the cancer is a cancer that is enriched for T cells or natural killer (NK) cells that express PVRIG.

15 Examples of cancer include, but are not limited to, carcinoma, lymphoma, blastoma, sarcoma, and leukemia or lymphoid malignancies. More particular examples of such cancers include, but are not limited to, lung cancer, such as non-small cell lung cancer (NSCLC), which includes squamous NSCLC or non-squamous NSCLC, including locally advanced unresectable NSCLC (e.g., Stage IIIB NSCLC), or recurrent or metastatic NSCLC (e.g., Stage IV NSCLC), adenocarcinoma of the lung, or squamous cell cancer (e.g., epithelial squamous cell cancer); esophageal cancer; cancer of the peritoneum; hepatocellular cancer; gastric or stomach cancer, including gastrointestinal cancer and gastrointestinal stromal cancer; pancreatic cancer; glioblastoma; cervical cancer; ovarian cancer; liver cancer; bladder cancer (e.g., urothelial bladder cancer (UBC), muscle invasive bladder cancer (MIBC), and BCG-refractory non-muscle invasive bladder cancer (NMIBC)); cancer of the urinary tract; hepatoma; breast cancer; colon cancer; rectal cancer; colorectal cancer; endometrial or uterine carcinoma; salivary gland carcinoma; kidney or renal cancer (e.g., renal cell carcinoma (RCC)); prostate cancer; vulval cancer; thyroid cancer; hepatic carcinoma; anal carcinoma; penile carcinoma; melanoma, including superficial spreading melanoma, lentigo maligna melanoma, acral lentiginous melanomas, and nodular melanomas; multiple myeloma and B-cell lymphoma (including low grade/follicular non-Hodgkin's lymphoma (NHL)); small lymphocytic (SL) NHL; intermediate grade/follicular NHL; intermediate grade diffuse NHL; high grade immunoblastic NHL; high grade lymphoblastic NHL; high grade small non-cleaved cell NHL; bulky disease NHL; mantle cell lymphoma; AIDS-related lymphoma; and Waldenstrom's Macroglobulinemia); chronic lymphocytic leukemia (CLL); acute lymphoblastic leukemia (ALL); acute myelogenous leukemia (AML); hairy cell leukemia; chronic myeloblastic leukemia (CML); post-transplant lymphoproliferative disorder (PTLD); and myelodysplastic syndromes (MDS), as well as abnormal vascular proliferation associated with

phakomatoses, edema (such as that associated with brain tumors), Meigs' syndrome, brain cancer, head and neck cancer, and associated metastases.

As a co-inhibitory receptor on a wide variety of immune cells, PVRIG is implicated in a variety of cancers, whether malignant or benign and whether primary or secondary, which may be treated or prevented with a method provided by the disclosure. Preferably, the anti-PVRIG antibody as disclosed herein is administered in combination with another anti-cancer agent. The cancers may be solid cancers or hematologic malignancies. Examples of such cancers include lung cancers such as bronchogenic carcinoma (e.g., non-small cell lung cancer, squamous cell carcinoma, small cell carcinoma, large cell carcinoma, and adenocarcinoma), alveolar cell carcinoma, bronchial adenoma, chondromatous hamartoma (noncancerous), and sarcoma (cancerous); heart cancer such as myxoma, fibromas, and rhabdomyomas; bone cancers such as osteochondromas, condromas, chondroblastomas, chondromyxoid fibromas, osteoid osteomas, giant cell tumors, chondrosarcoma, multiple myeloma, osteosarcoma, fibrosarcomas, malignant fibrous histiocytoomas, Ewing's tumor (Ewing's sarcoma), and reticulum cell sarcoma; brain cancer such as gliomas (e.g., glioblastoma multiforme), anaplastic astrocytomas, astrocytomas, oligodendrogliomas, medulloblastomas, chordoma, Schwannomas, ependymomas, meningiomas, pituitary adenoma, pinealoma, osteomas, hemangioblastomas, craniopharyngiomas, chordomas, germinomas, teratomas, dermoid cysts, and angiomas; cancers in digestive system such as colon cancer, leiomyoma, epidermoid carcinoma, adenocarcinoma, leiomyosarcoma, stomach adenocarcinomas, intestinal lipomas, intestinal neurofibromas, intestinal fibromas, polyps in large intestine, and colorectal cancers; liver cancers such as hepatocellular adenomas, hemangioma, hepatocellular carcinoma, fibrolamellar carcinoma, cholangiocarcinoma, hepatoblastoma, and angiosarcoma; kidney cancers such as kidney adenocarcinoma, renal cell carcinoma, hypernephroma, and transitional cell carcinoma of the renal pelvis; bladder cancers; skin cancers such as basal cell carcinoma, squamous cell carcinoma, melanoma, Kaposi's sarcoma, and Paget's disease; head and neck cancers; eye-related cancers such as retinoblastoma and intraocular melanocarcinoma; male reproductive system cancers such as benign prostatic hyperplasia, prostate cancer, and testicular cancers (e.g., seminoma, teratoma, embryonal carcinoma, and choriocarcinoma); breast cancer; female reproductive system cancers such as uterine cancer (endometrial carcinoma), cervical cancer (cervical carcinoma), cancer of the ovaries (ovarian carcinoma), vulvar carcinoma, vaginal carcinoma, fallopian tube cancer, and hydatidiform mole; thyroid cancer (including papillary, follicular, anaplastic, or medullary cancer); pheochromocytomas (adrenal gland); noncancerous growths of the parathyroid glands; pancreatic cancers. In a specific embodiment, the cancer is colon cancer.

In some other embodiments, the disorder or a disease to be treated or prevented is an immune related disease. The immune related disease may be associated with a T cell dysfunctional disorder. In some embodiments, the T cell dysfunctional disorder is characterized by decreased responsiveness to antigenic stimulation. In some embodiments, the T cell dysfunctional disorder

is characterized by T cell anergy, or decreased ability to secrete cytokines, proliferate or execute cytolytic activity. In some embodiments, the T cell dysfunctional disorder is characterized by T cell exhaustion. In some embodiments, the T cells are CD4⁺ and CD8⁺ T cells. In some embodiments, the immune related disease is selected from the group consisting of unresolved acute infection, chronic infection and reduced tumor immunity.

Stimulation of an immune response

In some aspects, the disclosure also provides a method of enhancing (for example, stimulating) an immune response in a subject comprising administering an antibody or an antigen binding portion thereof of the disclosure to the subject such that an immune response in the subject is enhanced. For example, the subject is a mammal. In a specific embodiment, the subject is a human.

The term “enhancing an immune response” or its grammatical variations, means stimulating, evoking, increasing, improving, or augmenting any response of a mammal’s immune system. The immune response may be a cellular response (i.e. cell-mediated, such as cytotoxic T lymphocyte mediated) or a humoral response (i.e. antibody mediated response), and may be a primary or secondary immune response. Examples of enhancement of immune response include increased CD4⁺ helper T cell activity and generation of cytolytic T cells. The enhancement of immune response can be assessed using a number of in vitro or in vivo measurements known to those skilled in the art, including, but not limited to, cytotoxic T lymphocyte assays, release of cytokines (for example IL-2 production or IFN- γ production), regression of tumors, survival of tumor bearing animals, antibody production, immune cell proliferation, expression of cell surface markers, and cytotoxicity. Typically, methods of the disclosure enhance the immune response by a mammal when compared to the immune response by an untreated mammal or a mammal not treated using the methods as disclosed herein. In one embodiment, the antibody or an antigen binding portion thereof is used to enhance the immune response of a human to a microbial pathogen (such as a virus). In another embodiment, the antibody or an antigen binding portion thereof is used to enhance the immune response of a human to a vaccine. In one embodiment, the method enhances a cellular immune response, particularly a cytotoxic T cell response. In another embodiment, the cellular immune response is a T helper cell response. In still another embodiment, the immune response is a cytokine production, particularly IFN- γ production or IL-2 production. The antibody or an antigen binding portion thereof may be used to enhance the immune response of a human to a microbial pathogen (such as a virus) or to a vaccine.

The antibody or the antigen-binding portion thereof may be used alone as a monotherapy, or may be used in combination with other antibodies (such as anti-PD-1 or anti-PD-L1 antibodies), chemical therapies, radiotherapies, targeting therapy or cell immunotherapy etc.

Combined use with chemotherapies

The antibody or the antigen-binding portion thereof may be used in combination with an anti-cancer agent, a cytotoxic agent or chemotherapeutic agent.

The term “anti-cancer agent” or “anti-proliferative agent” means any agent that can be used to treat a cell proliferative disorder such as cancer, and includes, but is not limited to, cytotoxic agents, cytostatic agents, anti-angiogenic agents, debulking agents, chemotherapeutic agents, radiotherapy and radiotherapeutic agents, targeted anti-cancer agents, BRMs, therapeutic antibodies, cancer vaccines, cytokines, hormone therapies, radiation therapy and anti-metastatic agents and immunotherapeutic agents. It will be appreciated that, such anti-cancer agents may comprise conjugates and may be associated with the disclosed antibodies prior to administration. More specifically, in certain embodiments selected anti-cancer agents will be linked to the unpaired cysteines of the engineered antibodies to provide engineered conjugates. Accordingly, such engineered conjugates are expressly contemplated as being within the scope of the present disclosure. In some other embodiments, the anti-cancer agents will be given in combination with antibody-drug conjugates comprising a different therapeutic agent.

As used herein the term “cytotoxic agent” means a substance that is toxic to the cells and decreases or inhibits the function of cells and/or causes destruction of cells. In certain embodiments, the substance is a naturally occurring molecule derived from a living organism. Examples of cytotoxic agents include, but are not limited to, small molecule toxins or enzymatically active toxins of bacteria (e.g., Diphtheria toxin, Pseudomonas endotoxin and exotoxin, Staphylococcal enterotoxin A), fungal (e.g., α -sarcin, restrictocin), plants (e.g., abrin, ricin, modeccin, viscumin, pokeweed anti-viral protein, saporin, gelonin, momoridin, trichosanthin, barley toxin, Aleurites fordii proteins, dianthin proteins, Phytolacca mericana proteins (PAPI, PAPII, and PAP-S), Momordica charantia inhibitor, curcin, crotin, saponaria officinalis inhibitor, gelonin, mitegellin, restrictocin, phenomycin, neomycin, and the tricothecenes) or animals, (e.g., cytotoxic RNases, such as extracellular pancreatic RNases; DNase I, including fragments and/or variants thereof).

For the purposes of the present disclosure a “chemotherapeutic agent” comprises a chemical compound that non-specifically decreases or inhibits the growth, proliferation, and/or survival of cancer cells (e.g., cytotoxic or cytostatic agents). Such chemical agents are often directed to intracellular processes necessary for cell growth or division, and are thus particularly effective against cancerous cells, which generally grow and divide rapidly. For example, vincristine depolymerizes microtubules, and thus inhibits cells from entering mitosis. In general, chemotherapeutic agents can include any chemical agent that inhibits, or is designed to inhibit, a cancerous cell or a cell likely to become cancerous or generate tumorigenic progeny (e.g., TIC). Such agents are often administered, and are often most effective, in combination, e.g., in regimens such as CHOP or FOLFIRI.

Examples of anti-cancer agents that may be used in combination with the antibody of the present disclosure (either as a component of a site specific conjugate or in an unconjugated state)

include, but are not limited to, alkylating agents, alkyl sulfonates, aziridines, ethylenimines and methylamelamines, acetogenins, a camptothecin, bryostatin, callystatin, CC-1065, cryptophycins, dolastatin, duocarmycin, eleutherobin, pancratistatin, a sarcodictyin, spongistatin, nitrogen mustards, antibiotics, enediyne antibiotics, dynemicin, bisphosphonates, esperamicin, 5 chromoprotein enediyne antiobiotic chromophores, aclacinomysins, actinomycin, authramycin, azaserine, bleomycins, cactinomycin, carabecin, carminomycin, carzinophilin, chromomycinis, dactinomycin, daunorubicin, detorubicin, 6-diazo-5-oxo-L-norleucine, ADRIAMYCIN[®] doxorubicin, epirubicin, esorubicin, idarubicin, marcellomycin, mitomycins, mycophenolic acid, nogalamycin, olivomycins, peplomycin, potfiromycin, puromycin, quelamycin, rodorubicin, 10 streptonigrin, streptozocin, tubercidin, ubenimex, zinostatin, zorubicin; anti-metabolites, erlotinib, vemurafenib, crizotinib, sorafenib, ibrutinib, enzalutamide, folic acid analogues, purine analogs, androgens, anti-adrenals, folic acid replenisher such as frolinic acid, aceglatone, aldophosphamide glycoside, aminolevulinic acid, eniluracil, amsacrine, bestrabucil, bisantrene, edatraxate, defofamine, demecolcine, diaziquone, elfornithine, elliptinium acetate, an epothilone, etoglucid, 15 gallium nitrate, hydroxyurea, lentinan, lonidainine, maytansinoids, mitoguazone, mitoxantrone, mopidanmol, nitraerine, pentostatin, phenamet, pirarubicin, losoxantrone, podophyllinic acid, 2-ethylhydrazide, procarbazine, PSK[®] polysaccharide complex (JHS Natural Products, Eugene, OR), razoxane; rhizoxin; sizofiran; spirogermanium; tenuazonic acid; triaziquone; 2,2',2"-trichlorotriethylamine; trichothecenes (especially T-2 toxin, verracurin A, roridin A and anguidine); urethan; vindesine; dacarbazine; mannomustine; mitobronitol; mitolactol; pipobroman; gacytosine; arabinoside ("Ara-C"); cyclophosphamide; thiotepa; taxoids, chloranbucil; GEMZAR[®] gemcitabine; 6-thioguanine; mercaptopurine; methotrexate; platinum analogs, vinblastine; platinum; etoposide (VP-16); ifosfamide; mitoxantrone; vincristine; NAVELBINE[®] vinorelbine; novantrone; teniposide; edatrexate; daunomycin; aminopterin; xeloda; ibandronate; 25 irinotecan (Camptosar, CPT-11), topoisomerase inhibitor RFS 2000; difluoromethylornithine; retinoids; capecitabine; combretastatin; leucovorin; oxaliplatin; inhibitors of PKC-alpha, Raf, H-Ras, EGFR and VEGF-A that reduce cell proliferation; and pharmaceutically acceptable salts, acids or derivatives of any of the above. Also included in this definition are anti-hormonal agents that act to regulate or inhibit hormone action on tumors such as anti-estrogens and selective 30 estrogen receptor modulators, aromatase inhibitors that inhibit the enzyme aromatase, which regulates estrogen production in the adrenal glands, and anti-androgens; as well as troxacitabine (a 1,3- dioxolane nucleoside cytosine analog); antisense oligonucleotides, ribozymes such as a VEGF expression inhibitor; vaccines, PROLEUKIN[®] rIL-2; LURTOTECAN[®] topoisomerase 1 inhibitor; ABARELIX[®] rmRH; Vinorelbine and Esperamicins; and pharmaceutically acceptable 35 salts, acids or derivatives of any of the above.

Combined use with radiotherapies

The present disclosure also provides for the combination of the antibody or the antigen-binding portion thereof with radiotherapy (i.e., any mechanism for inducing DNA damage locally within tumor cells such as gamma-irradiation, X-rays, UV-irradiation, microwaves, electronic emissions and the like). Combination therapy using the directed delivery of radioisotopes to tumor cells is also contemplated, and the disclosed antibodies may be used in connection with a targeted anti-cancer agent or other targeting means. Typically, radiation therapy is administered in pulses over a period of time from about 1 to about 2 weeks. The radiation therapy may be administered to subjects having head and neck cancer for about 6 to 7 weeks. Optionally, the radiation therapy may be administered as a single dose or as multiple, sequential doses.

Pharmaceutical packs and kits

Pharmaceutical packs and kits comprising one or more containers, comprising one or more doses of the antibody or the antigen-binding portion thereof are also provided. In certain embodiments, a unit dosage is provided wherein the unit dosage contains a predetermined amount of a composition comprising, for example, the antibody or the antigen-binding portion thereof, with or without one or more additional agents. For other embodiments, such a unit dosage is supplied in single-use prefilled syringe for injection. In still other embodiments, the composition contained in the unit dosage may comprise saline, sucrose, or the like; a buffer, such as phosphate, or the like; and/or be formulated within a stable and effective pH range. Alternatively, in certain embodiments, the composition may be provided as a lyophilized powder that may be reconstituted upon addition of an appropriate liquid, for example, sterile water or saline solution. In certain preferred embodiments, the composition comprises one or more substances that inhibit protein aggregation, including, but not limited to, sucrose and arginine. Any label on, or associated with, the container(s) indicates that the enclosed antibody is used for treating the neoplastic disease condition of choice.

The present disclosure also provides kits comprising single-dose or multi-dose administration units of antibodies and, optionally, one or more anti-cancer agents. The kit comprises a container and a label or package insert on or associated with the container. Suitable containers include, for example, bottles, vials, syringes, etc. The containers may be formed from a variety of materials such as glass or plastic and contain a pharmaceutically effective amount of the disclosed antibodies. In some embodiments, the container(s) comprise a sterile access port (for example the container may be an intravenous solution bag or a vial having a stopper pierceable by a hypodermic injection needle). Such kits will generally contain in a suitable container a pharmaceutically acceptable formulation of the antibodies and, optionally, one or more anti-cancer agents in the same or different containers. The kits may also contain other pharmaceutically acceptable formulations, either for diagnosis or combined therapy. For example, in addition to the antibody or the antigen-binding portion thereof of the disclosure such kits may contain any one or more of a range of anti-cancer agents such as chemotherapeutic or radiotherapeutic drugs; anti-

angiogenic agents; anti-metastatic agents; targeted anti-cancer agents; cytotoxic agents; and/or other anti-cancer agents.

More specifically the kits may have a single container that contains the antibody or the antigen-binding portion thereof, with or without additional components, or they may have distinct containers for each desired agent. Where combined therapeutics are provided for conjugation, a single solution may be pre-mixed, either in a molar equivalent combination, or with one component in excess of the other. Alternatively, the antibody and any optional anti-cancer agent of the kit may be maintained separately within distinct containers prior to administration to a patient. The kits may also comprise a second/third container means for containing a sterile, pharmaceutically acceptable buffer or other diluents such as bacteriostatic water for injection (BWFI), phosphate-buffered saline (PBS), Ringer's solution and dextrose solution.

When the components of the kit are provided in one or more liquid solutions, the liquid solution is preferably an aqueous solution, with a sterile aqueous or saline solution being particularly preferred. However, the components of the kit may be provided as dried powder(s). When reagents or components are provided as a dry powder, the powder can be reconstituted by the addition of a suitable solvent.

As indicated briefly above, the kits may also contain a means by which to administer the antibody or the antigen-binding portion thereof and any optional components to a patient, e.g., one or more needles, I.V. bags or syringes, or even an eye dropper, pipette, or other such like apparatus, from which the formulation may be injected or introduced into the animal or applied to a diseased area of the body. The kits of the present disclosure will also typically include a means for containing the vials, or such like, and other component in close confinement for commercial sale, such as, e.g., injection or blow-molded plastic containers into which the desired vials and other apparatus are placed and retained.

Sequence Listing Summary

Appended to the instant application is a sequence listing comprising a number of nucleic acid and amino acid sequences. The following Table A, B and C provide a summary of the included sequences.

Table A: CDR sequences of antibodies

	HCDR1	HCDR2	HCDR3	LCDR1	LCDR2	LCDR3
WT1175- 1.158.12-m1- uIgG1L; WT1175- 1.158.12- uIgG4LV1; WT1175- 1.158.12-uIgG1L	GGTFSSY TIS (SEQ ID NO: 1)	GIPIFGSADY AQKFQG (SEQ ID NO: 2)	EGLTGYSSF DY (SEQ ID NO: 3)	TGTSSDIGSYK FVS (SEQ ID NO: 4)	EGSKRPS (SEQ ID NO: 5)	SSYLGS TVV (SEQ ID NO: 6)

Table B: Amino acid sequences and nucleic acid sequences of the variable regions

Clone/Antibody		Sequence
WT1175- 1.158.12- m1-uIgG1L	VH	QVQLVQSGAEVKKPGSSSLKVSCKASGGTFSSYTISWVRQAPGQGLEWMGGIPI <u>FGSADYAQKFQGRVTITADESTSTAYMELSSLRSEDTAVYYCAREGLTGYSSFD</u> <u>YWGQGT</u>LTVVSS (SEQ ID NO: 7) CAGGTGCAGCTGGTGCAGTCTGGGGCTGAGGTGAAGAAGCCTGGGTCTCG TTGAAGGTCTCCTGCAAGGCTTCTGGAGGCACCTTCAGCAGCTATACTATCA GTTGGGTGCGACAGGCCCCCTGGACAAGGGCTTGAGTGGATGGGAGGGATCA TCCCTATCTTTGGTTCAGCAGACTACGCACAGAAGTTCCAGGGCAGAGTCAC GATTACCGCGGACGAATCCACGAGCACAGCCTACATGGAGCTGAGCAGCCT GCGATCTGAGGACACGGCCGTTTATTACTGTGCGAGAGAGGGTTTGACTGGT TATTCCTCTTTGACTACTGGGGCCAGGGAACCCTGGTCACCGTCTCCTCA (SEQ ID NO: 10)
	VL	QSALTQPASVSGSPGQSITISCTGTSSDIGSYKFVSWYQQHPGKAPKLMIEGSK <u>RPSGVSNRFSGSKSGNTASLTISGLQTEDEADYYC</u><u>SSYLGSGTVV</u>FGGGTKLTVL (SEQ ID NO: 8) CAGTCTGCCCTGACTCAGCCCGCCTCCGTGTCTGGGTCTCCTGGACAGTCGA TCACCATCTCCTGCACTGGAACCAGCAGTGATATTGGGAGTTATAAGTTTGT CTCCTGGTACCAGCAGCATCCAGGCAAAGCCCCCAAATCATGATTTATGAG GGCAGTAAGCGGCCCTCAGGGGTTTCTAATCGCTTCTCTGGTTCCAAGTCTG GCAACACGGCCTCCCTGACAATCTCTGGGCTCCAGACTGAAGACGAGGCTG ATTATTACTGCTCCTCATATTTAGGTAGTGGTACTGTGGTGTTCGGCGGAGG GACCAAATGACCGTCCTA (SEQ ID NO: 11)
WT1175- 1.158.12- uIgG4LV1; WT1175- 1.158.12- uIgG1L	VH	QVQLVQSGAEVKKPGSSSLKVSCKASGGTFSSYTISWVRQAPGQGLEWMGGIPI <u>FGSADYAQKFQGRVTITADESTSTAYMELSSLRSEDTAVYYCAREGLTGYSSFD</u> <u>YWGQGT</u>LTVVSS (SEQ ID NO: 7)
	VL	QSALTQSASVSGSPGQSITISCTGTSSDIGSYKFVSWYQQHPGKTPKLMIEGSKR <u>PSGVSNRFSGSKSGNTASLTISGLQTEDEADYYC</u><u>SSYLGSGTVV</u>FGGGTKLTVL (SEQ ID NO: 9)

Table C: Sequences of the heavy and light chains

Antibody		Amino acid sequence
WT1175- 1.158.12- m1-uIgG1L	Heavy chain	QVQLVQSGAEVKKPGSSSLKVSCKASGGTFSSYTISWVRQAPGQGLEWMGGIPI FGSADYAQKFQGRVTITADESTSTAYMELSSLRSEDTAVYYCAREGLTGYSSFD YWGQGT LTVVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWN SGALTSGVHTFPAVLQSSGLYSLSSVTVPSSSLGTQTYICNVNHKPSNTKVKDKR VEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVDVSHE DPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYK CKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPS DIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVM HEALHNHYTQKSLSLSPG (SEQ ID NO: 12)

	Light chain	QSALTQPASVSGSPGQSITISCTGTSSDIGSYKFVSWYQQHPGKAPKLMIEGSK RPSGVSNRFSGSKSGNTASLTISGLQTEDEADYYCSSYLGS GTVVFGGGTKLTVL GQPKAAPSVTLFPPSSEELQANKATLVCLISDFYPGAVTVAWKADSSPVKAGVE TTPSKQSNNKYAASSYLSLTPEQWKSHRSYSCQVTHEGSTVEKTVAPTECS (SEQ ID NO: 13)
WT1175- 1.158.12- uIgG4LV1	Heavy chain	<u>QVQLVQSGAEVKKPGSSSLKV</u> <u>SCKASGGTFSSYTISWVRQAPGQGLEWMGGIIPI</u> <u>FGSADYAQKFQGRVTITADEST</u> <u>STAYMELSSLRSEDTAVYYCAREGLTGYSSFD</u> <u>YWGQGT</u> <u>LVTVSSASTKGPSVFPLAPCSRSTSESTAALGCLVKDYFPEPVTVSWN</u> SGALTSGVHTFPAVLQSSGLYSLSSVTVPSSSLGKT Y T C NVDHKPSNTKVDK RVESKYGP PCPP CAPEFLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSQEDPE VQFNWYVDGVEVHNAKTKPREEQFNSTYRVVSVLT V LHQDWLNGKEYKCKV SNKGLPSSIEKTISKAKGQPREPQVYTLPPSQEEMTKNQVSLTCLVKGFYPSDIAV EWESNGQPENNYKTTTPVLDSDGSFFLYSRLTVDKSRWQEGNVFSCSVMHEAL HNHYTQKSLSLGL (SEQ ID NO: 15)
	Light chain	<u>QSALTQSASVSGSPGQSITISCTGTSSDIGSYKFVSWYQQHPGKTPKLMIEGSKR</u> <u>PSGVSNRFSGSKSGNTASLTISGLQTEDEADYYCSSYLGS GTVVFGGGTKLTVLG</u> QPKAAPSVTLFPPSSEELQANKATLVCLISDFYPGAVTVAWKADSSPVKAGVET TTPSKQSNNKYAASSYLSLTPEQWKSHRSYSCQVTHEGSTVEKTVAPTECS (SEQ ID NO: 16)
WT1175- 1.158.12- uIgG1L	Heavy chain	<u>QVQLVQSGAEVKKPGSSSLKV</u> <u>SCKASGGTFSSYTISWVRQAPGQGLEWMGGIIPI</u> <u>FGSADYAQKFQGRVTITADEST</u> <u>STAYMELSSLRSEDTAVYYCAREGLTGYSSFD</u> <u>YWGQGT</u> <u>LVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWN</u> SGALTSGVHTFPAVLQSSGLYSLSSVTVPSSSLGTQ T YICNVNHKPSNTKVDKR VEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHE DPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLT V LHQDWLNGKEYK CKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPS DIAVEWESNGQPENNYKTTTPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVM HEALHNHYTQKSLSLSPG (SEQ ID NO: 17)
	Light chain	<u>QSALTQSASVSGSPGQSITISCTGTSSDIGSYKFVSWYQQHPGKTPKLMIEGSKR</u> <u>PSGVSNRFSGSKSGNTASLTISGLQTEDEADYYCSSYLGS GTVVFGGGTKLTVLG</u> QPKAAPSVTLFPPSSEELQANKATLVCLISDFYPGAVTVAWKADSSPVKAGVET TTPSKQSNNKYAASSYLSLTPEQWKSHRSYSCQVTHEGSTVEKTVAPTECS (SEQ ID NO: 18)

EXAMPLES

The present disclosure, thus generally described, will be understood more readily by reference to the following Examples, which are provided by way of illustration and are not intended to be limiting of the present disclosure. The Examples are not intended to represent that the experiments below are all or the only experiments performed.

EXAMPLE 1

Preparation of Antigens, Benchmark Antibodies and Cell Lines

1.1 Generation of antigens

WT117-hPro1.ECD.His is the extracellular domain of human PVRIG (NP_076975.2) with a C-terminal polyhistidine tag; WT117-hPro1.ECD.hFc is the extracellular domain of human PVRIG (NP_076975.2) with the Fc region of human IgG1 at the C-terminus; WT117-mPro1.ECD.His is the extracellular domain of mouse PVRIG (XP_011239268.1) with a C-terminal polyhistidine tag; WT117-mPro1.ECD.hFc is the extracellular domain of mouse PVRIG (XP_011239268.1) with the Fc region of human IgG1 at the C-terminus; WT117-hPro1L1.ECD.mFc is the extracellular domain of human PVRL2 (NP_001036189.1) with the Fc region of mouse IgG2a at the C-terminus.

1.2 Preparation of benchmark antibodies (BMKs)

Anti-human PVRIG reference antibodies WT117-BMK1 and WT117-BMK3 were prepared according to the disclosed sequences in respective patent, the information of which is summarized in Table 1.

Table 1. Reference antibody information

Antibody Code	Company	Patent NO.	Naming
WT117-BMK1	Compugen	US20180244774	CHA.7.518.1
WT117-BMK3	SurfaceOncology	US20200040081	Antibody 35

1.3 Cell Pool/Line Generation

Human PVRIG-expressing cell line WT117-293F.hPro1.G11 was generated using 293F cells transfected with full-length human PVRIG (NP_076975.2). Cynomolgus monkey PVRIG-expressing cell pool WT117-Flpin293.cPro1.pool was generated using Flpin293 cells transfected with full-length cynomolgus monkey PVRIG (XP_005549281.1).

EXAMPLE 2

Generation of Human Antibodies against PVRIG

2.1 Immunization

Two OmniRats (Open Monoclonal Technology Company), 8~13 weeks of age, both female, were purchased from Charles River and housed in an IACUC approved animal facility. The two animals were immunized with the protein or plasmid of WT117-hPro1.ECD.hFc and WT117-mPro1.ECD.hFc alternately. The immunization was repeated roughly every week for a total of 111 days.

2.2 Serum titer detection

Anti-human/mouse PVRIG antibody titers in serum samples were determined by ELISA. Microplates were coated with WT117-hPro1.ECD.His at 0.5 µg/mL in 100 µL of coating buffer (Na₂CO₃/NaHCO₃, pH9.2) per well and incubated at 4 °C overnight. On the day of assay, diluted rat serum samples (first 1:100, then 3-fold dilution in 1×PBS/2%BSA) and negative control were

added into the plates post 1-hour blocking with 1×PBS/2%BSA, and then the plates were incubated at ambient temperature for 1 hour. After washing with 1×PBST (PBS containing 0.05% Tween-20) for 3 times, HRP-labeled goat anti-rat IgG Fc (Bethyl, cat#A110-236P) was added and incubated at ambient temperature for 1 hour. After removing of the unbound substance, TMB (3,3',5,5'-Tetramethylbenzidine) substrate was added and the reaction was stopped by 2M HCl. Absorbance at 450nm was detected by a microplate spectrophotometer.

The serum titers of the immunized OMT rats are shown in Table 2. The lymph nodes of the two animals were collected and used for fusion.

Table 2. Serum titer of anti-PVRIG antibodies

Animal ID #	Antigen	Titer information				
		Pre-bleed	1 st bleed	2 nd bleed	3 rd bleed	4 th bleed
1	WT117-	<100	300	2700	218700	72900
2	hPro1.ECD.His	<100	8100	8100	8100	218700

2.3 Hybridoma generation, antibody screening and subcloning

Lymph nodes and spleens were collected from OMT rats under sterile condition, and dissociated into single cell suspension, and then mixed with myeloma cell SP2/0 at a ratio of 1:1.2. Electro cell fusion was performed using BTX 2001 Electro cell manipulator according to an optimized electro-fusion procedure. After fusion, the cells were transferred into 96-well plates (1×10⁴ cells/well) with DMEM medium supplemented with 20% FBS and 1% HAT selective reagents. The plates were cultured at 37 °C, 5% CO₂, and were monitored periodically. When the clones reached about 80% confluence in a well, 100 μL of supernatant were transferred from the tissue culture plates to 96-well assay plates for antibody screening.

The positive lines in logarithmic growth was diluted to ~200 cells per 1.5 mL semi-solid HAT media. The cell suspensions were mixed gently on vortex oscillator for 5 to 10 seconds and then seeded in 6-well plates. The plates were kept at 37 °C, 5% CO₂ for 7-8 days. When the cell clusters grew up, each visible single colony was picked and seeded into 96-well plates with DMEM medium supplemented with 10% fetal bovine serum. After 2-3 days, the supernatant of each clone was collected and screened again to obtain positive hybridoma single clones.

2.4 Hybridoma sequencing

RNAs were isolated from monoclonal hybridoma cells and the cDNAs were amplified using SuperScript™ III First-Strand Synthesis SuperMix Kit according to the manufacturer's instructions. The resulting cDNA was used as templates for subsequent PCR amplification using primers specific for the interested genes. PCR product was inserted into the pMD18-T vectors, and the ligation products as well as PCR products were sent for sequencing.

2.5 Fully human antibody generation

The DNA sequences of the VH and VL domains were amplified by PCR and then subcloned into pcDNA expression vectors with constant region of human IgG1 or human IgG4. The plasmids containing VH and VL genes were co-transfected into Expi293 cells, and the cells were cultured for ~5 days until the supernatants were harvested. The antibodies were purified with Protein A column from the supernatants.

Through primary and secondary binding screening, as well as PVRIG/PVRL2 blockade and TCR/NFAT luciferase activation assay, 45 positive cell lines were selected for subcloning. After confirmation of the monoclonal antibodies, 20 hits were selected for sequencing and 4 of them followed by human IgG conversion. After further *in-vitro* characterization (see below), one clone was identified (named as WT1175-1.158.12-uIgG4LV1) and its sequences are shown in Tables A-B above.

EXAMPLE 3

In vitro Characterization

3.1 Human PVRIG binding assay

WT117-293F.hPro1.G11 (1×10^5 cells/well) or activated human CD8⁺ T cells (1×10^5 cells/well) were incubated with various concentrations of anti-PVRIG antibodies at 4 °C for 1 hour. After washing with 1×PBS/1%BSA, the secondary antibody, PE-labeled goat anti-human IgG (JacksonImmunoResearch cat#109-115-098) was added and incubated with cells at 4 °C in dark for 1 hour. Anti-human PVRIG antibody WT117-BMK1 was used as positive control. Human IgG4 isotype antibody was used as isotype control. The cells were then washed and re-suspended in 1×PBS/1%BSA. MFI of the cells was measured by a flow cytometer (BD) and analyzed by FlowJo.

The binding results of anti-PVRIG antibodies on WT117-293F.hPro1.G11 or human CD8⁺ T cells are shown in Figure 1 and Figure 2, which demonstrate that WT1175-1.158.12-uIgG4LV1 can strongly bind to human PVRIG-expressing cells, and the binding potency is much higher than that of the reference antibody. A summary of antibody binding is shown in Table 3 below.

3.2 Cynomolgus monkey PVRIG binding assay

WT117-Flpin293F.cPro1.pool (1×10^5 cells/well) cells were incubated with various concentrations of anti-PVRIG antibodies at 4 °C for 1 hour. After washing with 1×PBS/1%BSA, the secondary antibody, PE-labeled goat anti-human IgG (JacksonImmunoResearch cat#109-115-098) was added and incubated with cells at 4 °C in dark for 1 hour. Anti-human PVRIG antibody WT117-BMK1 was used as positive control. Human IgG4 isotype antibody was used as isotype control. The cells were then washed and re-suspended in 1×PBS/1%BSA. MFI of the cells was measured by a flow cytometer (BD) and analyzed by FlowJo.

The binding result of anti-PVRIG antibodies on WT117-Flpin293F.cPro1.pool cells is shown in Figure 3, which demonstrates that WT1175-1.158.12-uIgG4LV1 can strongly bind to

cynomolgus monkey PVRIG-expressing cells, and the binding potency is much higher than that of the reference antibody. A summary of antibody binding is shown in Table 3 below.

3.3 Mouse PVRIG binding assay

Plate was pre-coated with 0.5 µg/mL of WT117-hPro1.ECD.His or WT117-mPro1.ECD.His in 100 µL coating buffer per well at 4 °C overnight. After blocking with 200 µL of 1×PBS/2%BSA, 100 µL of anti-PVRIG antibodies were added to the plate at a concentration of 6.67 nM and incubated at ambient temperature for 1 hour. After incubation, the plate was washed using 1×PBST for 3 times. HRP-labeled goat anti-human IgG antibody (Bethyl cat#A80-304P) diluted in 1×PBS/2%BSA was added and incubated for 1 hour at ambient temperature. After washing with 1×PBST, the color was developed by dispensing 100 µL of TMB substrate, and then reaction was stopped by adding 100 µL of 2M HCl. Absorbance was read at 450nm and 540nm using M5e microplate reader (Molecule Devices).

The binding result of anti-PVRIG antibodies to mouse PVRIG extracellular domain is shown in Figure 4, which demonstrates that WT1175-1.158.12-uIgG4LV1 does not bind to mouse PVRIG. A summary of antibody binding is shown in Table 3 below.

Table 3. Summary of antibody binding to PVRIG

Assay	WT1175-1.158.12-uIgG4LV1	WT117-BMK1
Binding to human PVRIG engineered cells, EC50 (nM)	0.073	0.63
Binding to human CD8 ⁺ T cells, EC50 (nM)	0.037	0.28
Binding to cynomolgus monkey PVRIG, EC50 (nM)	0.031	0.36
Binding to mouse PVRIG	Negative	Negative

3.4 Human PVRIG affinity assay

The binding affinity of WT1175 antibodies to cell surface human PVRIG was measured by FACS. WT117-293F.hPro1.G11 cells were transferred into a 96-well round bottom plate at a density of 5×10⁴ cells/well. Anti-PVRIG antibodies were serially diluted in 1×PBS/1%BSA and incubated with the cells at 4 °C for 1 hour. The secondary antibody FITC-labeled goat anti-human IgG Fc (JacksonImmunoResearch cat#109-095-098) was added and incubated at 4 °C in dark for 0.5 hour. The cells were then washed once and re-suspended in 1×PBS/1%BSA and analyzed by flow cytometry (BD). Fluorescence intensity was converted to bound molecules per cell based on the quantitative beads (Bangs Laboratories cat#555pB).

The binding affinity result of anti-PVRIG antibodies on WT117-293F.hPro1.G11 cells is shown in Figure 5 and Table 4. Bmax represents the maximum specific binding and KD is the

ligand concentration needed to achieve a half-maximum binding at equilibrium. WT1175-1.158.12-uIgG4LV1 binds to cell surface human PVRIG with high affinity.

Table 4. Affinity constant of anti-PVRIG antibodies to human PVRIG

Parameter	WT1175-1.158.12-uIgG4LV1	WT117-BMK1
r^2	0.9958	0.9651
Bmax (M)	7.46E-12	7.52E-12
KD (M)	1.33E-11	1.31E-10

3.5 PVRIG paralog protein binding assay

Plate was pre-coated with 1 µg/mL of WT117-hPro1.ECD.His, recombinant human TIGIT, CD226, CD96 or PD-1 extracellular domain in 100 µL coating buffer per well at 4 °C overnight. After blocking with 200 µL of 1×PBS/2%BSA, 100 µL of testing antibodies were added to the plate at a concentration of 10 µg/mL and incubated at ambient temperature for 1 hour. After incubation, the plate was washed using 1×PBST for 3 times. HRP-labeled goat anti-human IgG antibody (Bethyl cat#A80-304P) diluted in 1×PBS/2%BSA was added and incubated for 1 hour at ambient temperature. After washing with 1×PBST for 6 times, the color was developed by dispensing 100 µL of TMB substrate, and then reaction was stopped by adding 100 µL of 2M HCl. Absorbance was read at 450nm and 540nm using M5e microplate reader (Molecule Devices).

The binding results of anti-PVRIG antibodies to PVRIG paralog protein are shown in Figure 6, which demonstrate that WT1175-1.158.12-uIgG4LV1 specifically binds to PVRIG without cross-reactivity to human TIGIT, CD226, CD96 or PD-1.

3.6 Human PVRIG/PVRL2 blocking assay

Plates were pre-coated with 2 µg/mL of WT1175-hPro1.ECD.His in 100 µL coating buffer per well overnight at 4 °C. Blocking was done by adding 200 µL of 1×PBS/2%BSA. The serially diluted anti-PVRIG antibodies were mixed with constant concentration of WT1175-hPro1L1.ECD.mFc (final concentration 10 µg/mL) at a volume ratio of 1:1. After 1-hour blocking, the antibody/ligand mixture were added to the plates and incubated at ambient temperature for 2 hours. After washing with 1×PBST for 3 times, HRP-labeled goat anti-mouse IgG (Bethyl cat#A90-231P) was added to the plates and incubated for 1 hour at ambient temperature. After washing with 1×PBST for 6 times, TMB substrate was added and the interaction was stopped by 2M HCl. Absorbance was read at 450nm and 540nm using M5e microplate reader (Molecule Devices).

The human PVRIG/PVRL2 binding blockade result is shown in Figure 7. The result demonstrates that WT1175-1.158.12-uIgG4LV1 can effectively block human PVRL2 binding to PVRIG. A summary of antibody blocking activity is shown in Table 5 below. The maximum

inhibition rate was calculated as $\text{max inhibition\%} = (\text{OD}_{\text{max}} - \text{OD}_{\text{bottom}}) / \text{OD}_{\text{max}} \times 100\%$, where OD_{max} was defined as the value of OD450 minus OD540 in the absence of antibody.

3.7 Jurkat PVRIG/NFAT-luciferase reporter gene assay

Jurkat cells over-expressing human PVRIG and NFAT-luciferase reporter were stimulated by engagement of T cell receptor by co-culturing with CHOK1 cells expressing human PVRL2 and TCR activator. CHOK1/PVRL2/TCR-activator cells were seeded at a density of 4×10^4 cells/well in a 96-well plate overnight at 37 °C, 5% CO₂. The second day, following removal of the supernatants and non-adherent cells, serially diluted anti-PVRIG antibodies and Jurkat/PVRIG/NFAT-luciferase cells (2×10^4 cells/well) were added to the plate, and were co-cultured at 37 °C, 5% CO₂ for 5-6 hours. After incubation, reconstituted luciferase substrate (Promega cat#E6130) was added to each well and mixed well. The luciferase intensity was read using Envision microplate reader (PerkinElmer).

The result of anti-PVRIG antibodies reversing suppression of NFAT signaling induced by PVRIG/PVRL2 interaction is shown in Figure 8, which demonstrates that WT1175-1.158.12-uIgG4LV1 can enhance TCR/NFAT activation. A summary of antibody RGA activity is shown in Table 5.

Table 5. Summary of antibody characterizations

Assay	WT1175-1.158.12-uIgG4LV1	WT117-BMK1
Blocking PVRL2 binding to PVRIG, IC50 (nM), max inhibition%	0.30, 97%	0.72, 94%
NFAT reporter gene assay, EC50 (nM)	0.064	1.0

3.8 Human primary T cell activation assay

Human primary CD8⁺ T cell was stimulated by engagement of T cell receptor by co-culturing with CHOK1 cells expressing human PVRL2 and TCR activator. Human CD8⁺ T cells were isolated from human peripheral blood mononuclear cells (PBMCs) by magnetic selection using human CD8 MicroBeads (Miltenyi Biotec cat#130-045-201) according to the manufacturer's protocol. The isolated human CD8⁺ T cells (1×10^5 per well) were co-cultured with irradiated CHOK1/PVRL2/TCR-activator cells (2×10^4 per well) in the presence of serially diluted anti-PVRIG antibodies at 37 °C, 5% CO₂ for 5 days. After the incubation, the supernatants were collected for IFN-γ measurement by ELISA (capture antibody Thermo cat#M700A, detection antibody Thermo cat#M701B). The absorbance was detected using M5e microplate reader (Molecule Devices).

Human CD8⁺ T cells treated with anti-PVRIG antibodies resulted in greater IFN- γ secretion than that observed with isotype control and the reference antibody. The result demonstrates that WT1175-1.158.12-uIgG4LV1 enhances CD8⁺ T cell activation. The data is shown in Figure 9.

5 3.9 Antibody serum stability assay

Human serum was freshly isolated from healthy donor. Anti-PVRIG antibody was diluted in the serum. The samples were aliquoted to 5 tubes and incubated at 37 °C. Samples were then collected on day 0, day 1, day 4, day 7 and day 14, respectively, quick-frozen and stored at a freezer set to -70 °C until ready for analysis. The binding activity of the samples was evaluated by
10 FACS according to the method described in section 3.1.

The binding of serum incubated WT1175-1.158.12-uIgG4LV1 to WT117-293F.hPro1.G11 is shown in Figure 10. The antibody incubated with serum up to two weeks has maintained the similar binding activity and very similar EC50 as the fresh antibody. The result demonstrates that WT1175-1.158.12-uIgG4LV1 is stable in human serum at 37 °C for at least two weeks.

15

3.10 Antibody thermal stability assay

The conformational stability is a very important property for a successful antibody. Conformational stability can be assessed by measuring thermal stability using differential scanning fluorimetry (DSF), which is sensitive to changes in protein folding. DSF measures the temperature
20 of the protein unfolding transition (T_m) based on the change in fluorescence intensity of the environmentally sensitive dye SYPRO Orange.

DSF was carried out in a Quant Studio 7 Flex Real-Time PCR instrument (Applied Biosystems) in the respective formulation buffer. The SYPRO orange dye (Invitrogen cat#S6651) was added to the antibody and transfer the mixture to a 96-well plate. Put the plate in a Quant
25 Studio® 7 Flex Real-Time PCR system, and set up the temperature range from 26 °C to 95 °C with a heating rate of 0.9 °C/min. The first two temperatures of protein unfolding transitions were recorded as T_{m1} and T_{m2}. The two values were calculated according to the melt curve using QuantStudio® Real Time PCR software (v1.3).

DSF thermogram for the WT1175-1.158.12-uIgG4LV1 antibody displays 2 transitions: first
30 with a lower (T_{m1}) and second with a higher (T_{m2}) melting temperature, 62.5 °C and 64.9 °C, respectively. The result is shown in Table 6.

Table 6. T_m values of antibody

Antibody	T _{m1} (°C)	T _{m2} (°C)
WT1175-1.158.12-uIgG4LV1	62.5	64.9

3.11 Comparison of human IgG1 and IgG4 format

In order to compare the effect of different Fc on antibody activity, we also generated the corresponding IgG1 formatted antibody of WT1175-1.158.12-uIgG4LV1, which was named as WT1175-1.158.12-uIgG1L, and the referenced antibody WT117-BMK3 with IgG1 Fc was also generated and used as positive control.

The binding activity of WT1175-1.158.12-uIgG1L on WT117-293F.hPro1.G11 was evaluated according to the method described in section 3.1. The effect of reversing PVRIG-mediated inhibitory signal of WT1175-1.158.12-uIgG1L was evaluated according to the method described in section 3.7. The activity of WT1175-1.158.12-uIgG1L enhancing CD8⁺ T cell activation was evaluated according to the method described in section 3.8.

The results of binding (Figure 11), TCR/NFAT luciferase activation assay (Figure 12) and enhancement of CD8⁺ T cell activation (Figure 13) are shown. These results demonstrate that WT1175-1.158.12-uIgG4LV1 and WT1175-1.158.12-uIgG1L have comparable binding and functional activity.

3.12 Generation and characterization of variant PVRIG antibody

The positions S7 and T43 (according to Kabat numbering) in VL were identified as potentially unstable residues in antibody WT1175-1.158.12-uIgG1L. Mutations S7P and T43A were made to improve the stability, and the mutated variant was renamed as WT1175-1.158.12-m1-uIgG1L. FACS binding (method in 3.1) and NFAT reporter gene assay (method in 3.7) were used to compare the activity of these two antibodies. The thermal stability of WT1175-1.158.12-m1-uIgG1L was evaluated according to the method described in section 3.10 to demonstrate the improvement.

The results of binding are shown in Figure 15, and the reporter gene assay result is shown in Figure 16. These results demonstrate that WT1175-1.158.12-uIgG1L and WT1175-1.158.12-m1-uIgG1L have comparable binding and functional activity.

The result of DSF is shown in Table 7. Compared with WT1175-1.158.12-uIgG4LV1, WT1175-1.158.12-m1-uIgG1L exhibited improved thermal stability, with Tm1 raised from 62.5 °C to 68.3 °C.

Table 7. Tm values of antibody

Antibody	Tm1 (°C)	Tm2 (°C)
WT1175-1.158.12-m1-uIgG1L	68.3	-

EXAMPLE 4

In vivo Characterization

4.1 Rodent pharmacokinetics study

This study was to determine the pharmacokinetics of WT1175-1.158.12-uIgG1L and WT1175-1.158.12-uIgG4LV1 in naïve rat following a single intravenous bolus administration. Eight animals were randomly grouped into two groups, treated with WT1175-1.158.12-uIgG1L (10 mg/kg) and WT1175-1.158.12-uIgG4LV1 (10 mg/kg), respectively.

PK blood samples were collected at pre-dose, 0.5h, 4h, day 2, day 3, day 5, day 7, day 10, day 12, day 14 and day 21. The serum concentrations of anti-PVRIG antibodies were determined by ELISA. Briefly, goat anti-human IgG Fc was used as the capturing reagent, and the biotinylated goat anti-human IgG Fc was used as the detecting reagent. Streptavidin-HRP and TMB substrate were used for color development, and the reaction was stopped by 2M HCl. Absorbance was read at 450nm and 540nm using a microplate spectrophotometer. The serum concentration of WT1175-1.158.12-uIgG1L and WT1175-1.158.12-uIgG4LV1 was subjected to a non-compartmental pharmacokinetic analysis using Phoenix WinNonlin software. The linear/log trapezoidal rule was applied to obtain the PK parameters.

Since these two antibodies didn't bind to rat PVRIG, there was no target mediated drug disposition effect. A linear pharmacokinetics (Figure 14) was observed at the single dose of 10 mg/kg. The results of the PK parameters were summarized in Table 8. WT1175-1.158.12-uIgG1L showed an average serum clearance of 4.89 mL/day/kg, half-life 429 hours, volume of distribution 123 mL/kg, AUC_{0-t} 25547 h* μ g/mL, while WT1175-1.158.12-uIgG4LV1 showed an average serum clearance of 5.03 mL/day/kg, half-life 368 hours, volume of distribution 108 mL/kg, AUC_{0-t} 29589 h* μ g/mL.

Table 8. Summary of PK parameters

Parameter	WT1175-1.158.12-uIgG1L	WT1175-1.158.12-uIgG4LV1
Dose	10 mg/kg, iv	10 mg/kg, iv
$T_{1/2}$ (h)	429	368
C_{max} (μ g/ml)	212	215
AUC_{0-t} (h* μ g/ml)	25547	29589
Cl_{obs} (ml/day/kg)	4.89	5.03
$MRTINF_{obs}$ (h)	615	516
$V_{ss_{obs}}$ (mL/kg)	123	108

Those skilled in the art will further appreciate that the present disclosure may be embodied in other specific forms without departing from the spirit or central attributes thereof. In that the foregoing description of the present disclosure discloses only exemplary embodiments thereof, it is to be understood that other variations are contemplated as being within the scope of the present disclosure. Accordingly, the present invention is not limited to the particular embodiments that

have been described in detail herein. Rather, reference should be made to the appended claims as indicative of the scope and content of the invention.

CLAIMS

1. An isolated antibody or an antigen-binding portion thereof, comprising:
 - a heavy chain CDR (HCDR) 1 comprising the amino acid sequence of SEQ ID NO: 1;
 - a HCDR2 comprising the amino acid sequence of SEQ ID NO: 2;
 - a HCDR3 comprising the amino acid sequence of SEQ ID NO: 3;
 - a light chain CDR (LCDR) 1 comprising the amino acid sequence of SEQ ID NO: 4;
 - a LCDR2 comprising the amino acid sequence of SEQ ID NO: 5; and
 - a LCDR3 comprising the amino acid sequence of SEQ ID NO: 6.
2. The isolated antibody or the antigen-binding portion thereof of claim 1, comprising:
 - (A) a heavy chain variable region (VH):
 - (i) comprising the amino acid sequence as set forth in SEQ ID NO: 7; or
 - (ii) comprising an amino acid sequence at least 85%, 90%, or 95% identical to SEQ ID NO: 7; and/or
 - (B) a light chain variable region (VL):
 - (i) comprising the amino acid sequence as set forth in any of SEQ ID NOs: 8 and 9; or
 - (ii) comprising an amino acid sequence at least 85%, at least 90%, or at least 95% identical to any of SEQ ID NOs: 8 and 9.
3. The isolated antibody or the antigen-binding portion thereof of claim 1 or 2, comprising a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 7 and a light chain variable region comprising the amino acid sequence as set forth in SEQ ID NO: 8.
4. The isolated antibody or the antigen-binding portion thereof of any of the preceding claims, wherein the isolated antibody further comprises a human IgG constant region.
5. The isolated antibody or the antigen-binding portion thereof of claim 4, wherein the human IgG constant region is a human IgG1, IgG4, IgG2 or IgG3 constant region or a variant thereof.
6. The isolated antibody or the antigen-binding portion thereof of any of the preceding claims, wherein the antibody comprises a human IgG1 Fc region or a human IgG4 Fc region, optionally comprising a S228P mutation.
7. The isolated antibody or the antigen-binding portion thereof of any of the preceding claims, wherein the antibody is a chimeric antibody, a humanized antibody or a human antibody.

8. An isolated nucleic acid molecule, comprising a nucleic acid sequence encoding the heavy chain variable region and/or the light chain variable region of the isolated antibody or the antigen-binding portion thereof of any of claims 1-7.
9. A vector comprising the isolated nucleic acid molecule of claim 8.
10. A host cell comprising the vector of claim 9.
11. A pharmaceutical composition comprising the isolated antibody or the antigen-binding portion thereof as defined in any of claims 1-7 and a pharmaceutically acceptable carrier.
12. A method for producing the antibody or the antigen-binding portion thereof as defined in any of claims 1-7 comprising the steps of:
 - culturing a host cell comprising an expression vector(s) encoding the antibody or the antigen-binding portion thereof under suitable conditions; and
 - harvesting the antibody or antigen-binding portion thereof from the cell culture.
13. A method for inhibiting growth of tumor cells in a subject, comprising administering an effective amount of the antibody or the antigen-binding portion thereof as defined in any of claims 1-7 or the pharmaceutical composition of claim 11 to the subject.
14. A method for treating or preventing a cancer or an immune related disorder in a subject, comprising administering an effective amount of the antibody or the antigen-binding portion thereof as defined in any of claims 1-7 or the pharmaceutical composition of claim 11 to the subject.
15. The method of claim 14, wherein the cancer is selected from colon cancer, lung cancer, breast cancer, ovarian cancer, melanoma, bladder cancer, renal cell carcinoma, liver cancer, prostate cancer, stomach cancer, pancreatic cancer, lymphoma, leukemia, uterine cancer, cervical cancer, testicular cancer, esophageal cancer, gastrointestinal cancer, gastric cancer, colorectal cancer, kidney cancer, clear cell renal carcinoma, head and neck cancer, germ cell cancer, bone cancer, thyroid cancer, skin cancer, neoplasm of the central nervous system, mesothelioma, myeloma, and sarcoma.
16. The method of claim 14, wherein the immune related disorder is a T cell dysfunctional disorder or an infection.

17. The method of any of claims 14-16, wherein the method further comprises administering an additional therapeutic agent.
18. Use of the antibody or the antigen-binding portion thereof as defined in any of claims 1-7 in the manufacture of a medicament for treating or preventing cancer or an immune related disorder.
19. The antibody or the antigen-binding portion thereof as defined in any of claims 1-7 for use in treating or preventing cancer or an immune related disorder.
20. A kit, comprising a container comprising the antibody or the antigen-binding portion thereof as defined in any of claims 1-7.

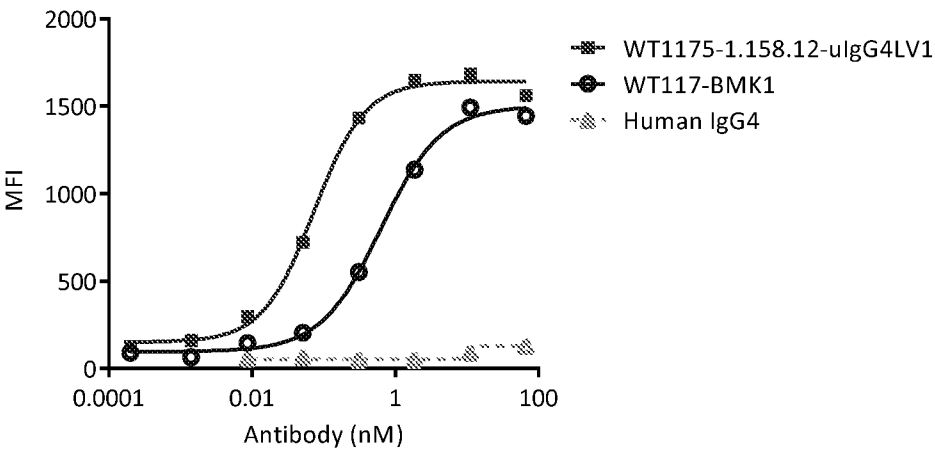


Figure 1

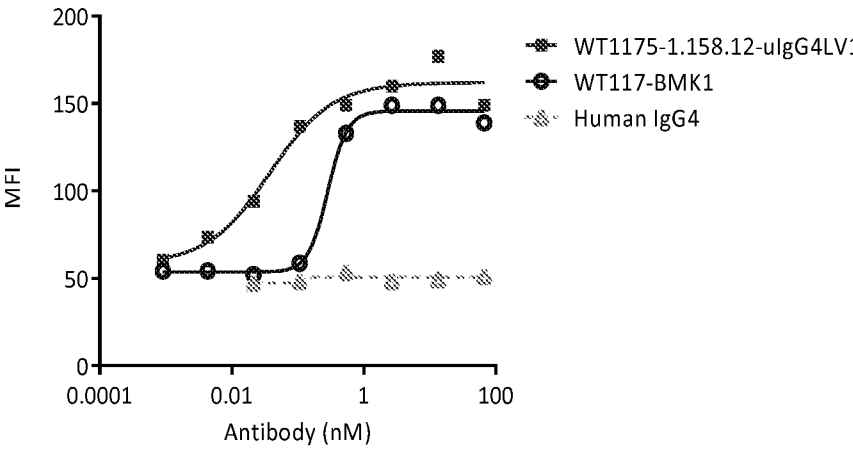


Figure 2

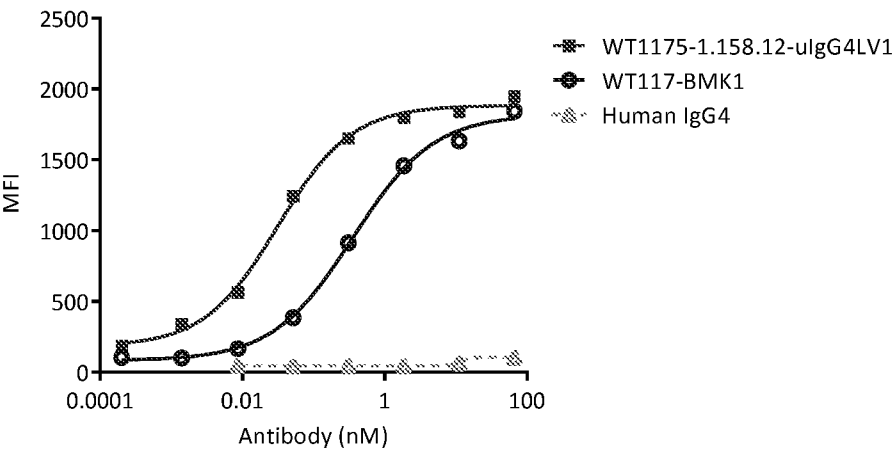


Figure 3

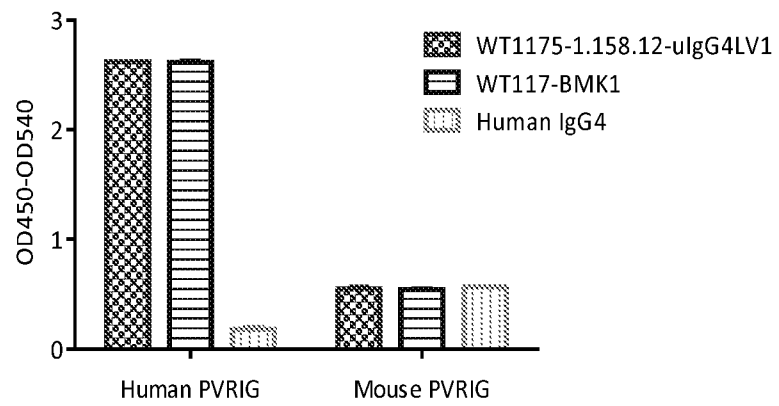


Figure 4

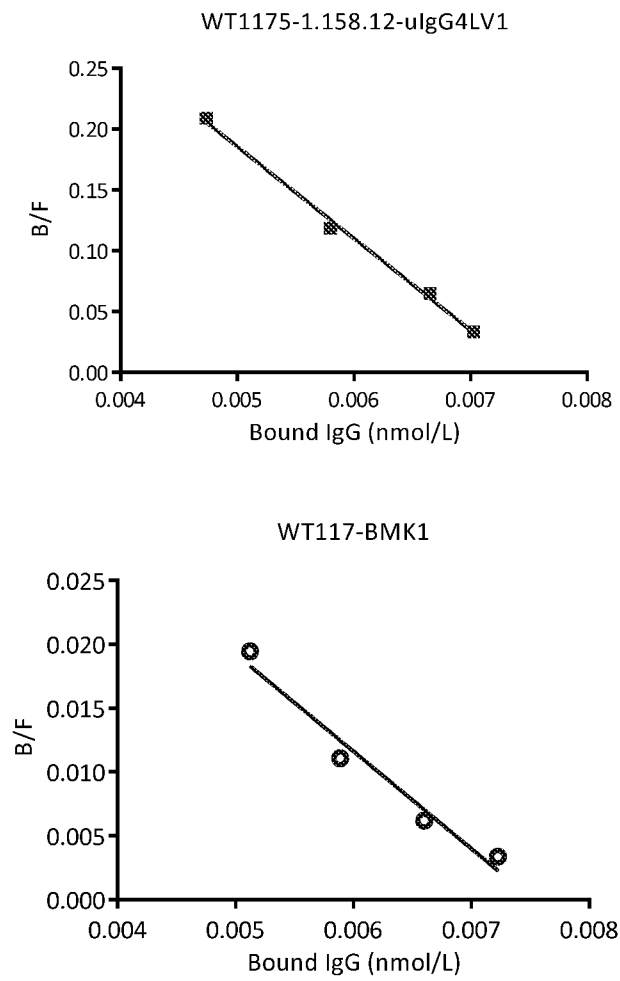


Figure 5

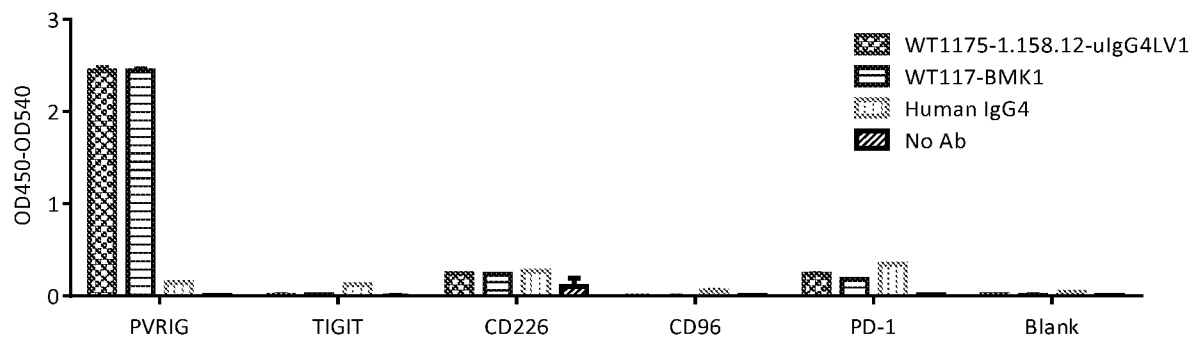


Figure 6

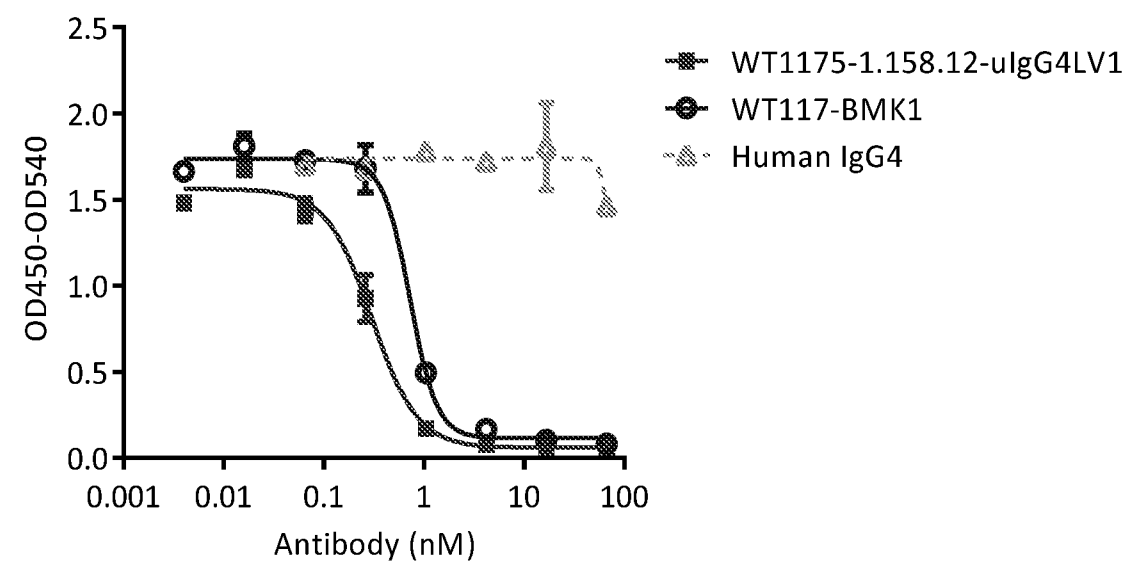


Figure 7

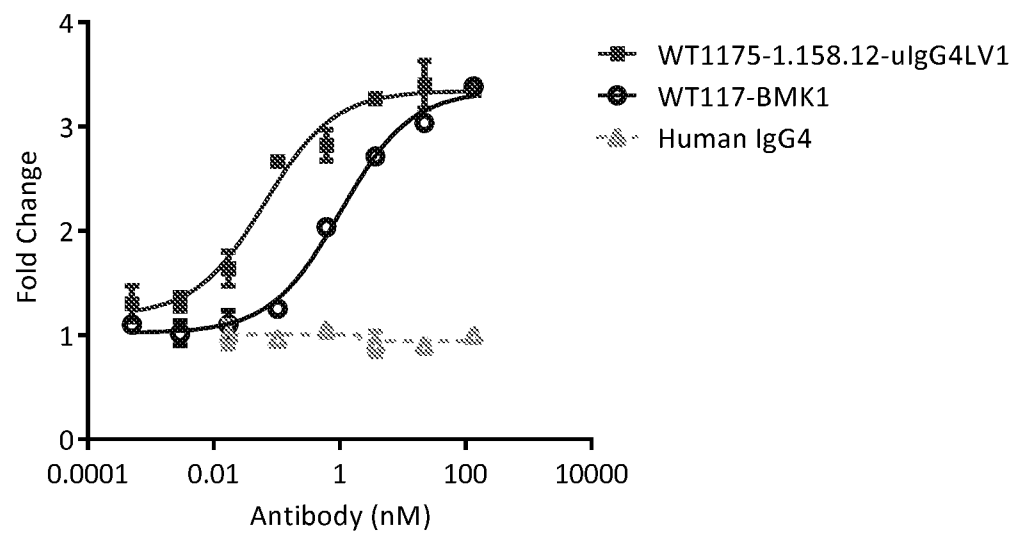


Figure 8

T cell activation assay

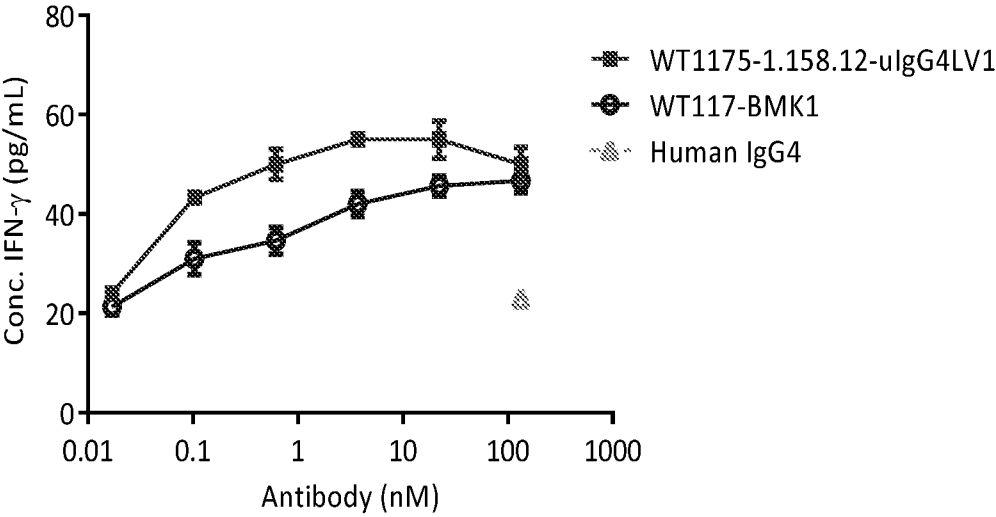


Figure 9

Antibody stability in human serum

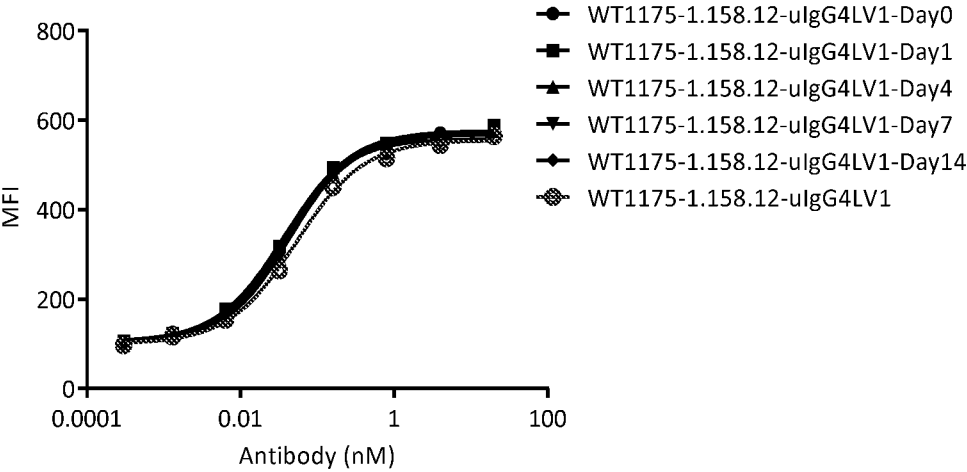


Figure 10

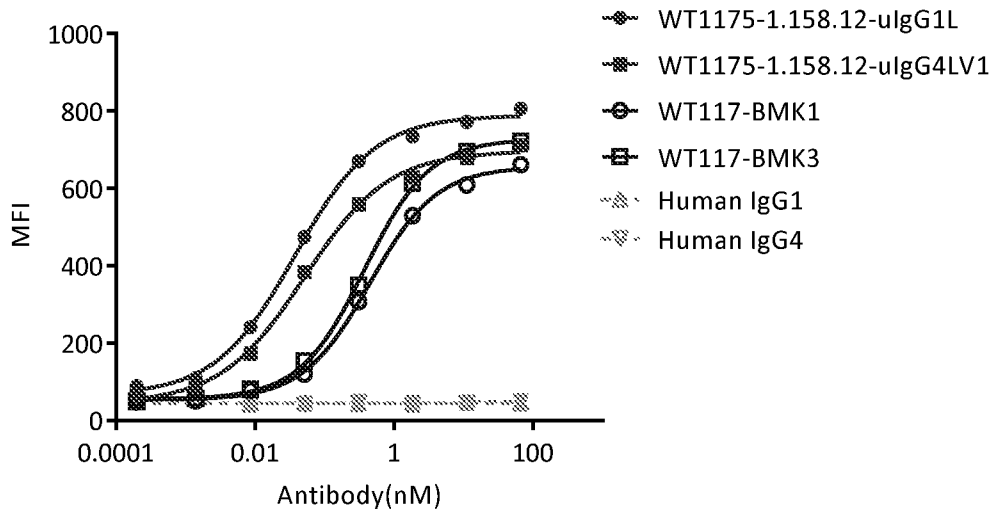


Figure 11

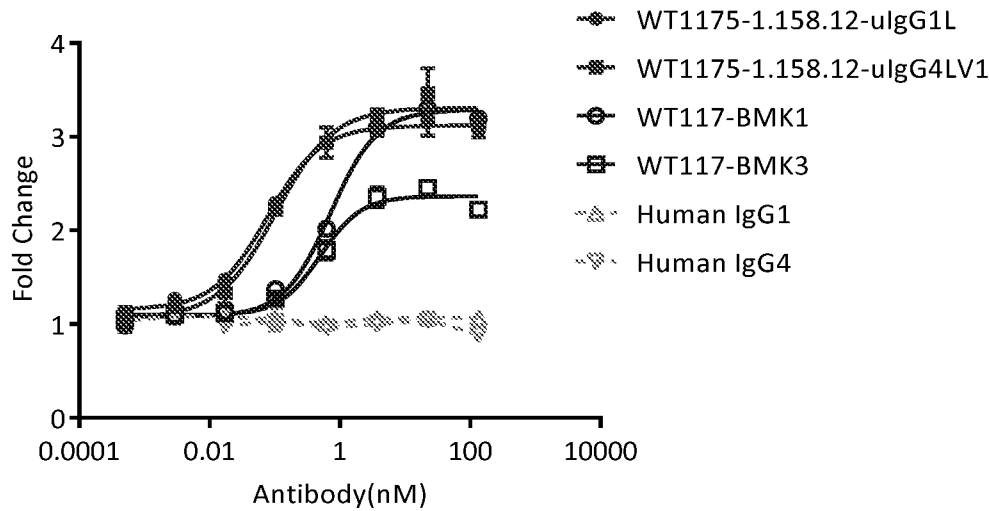


Figure 12

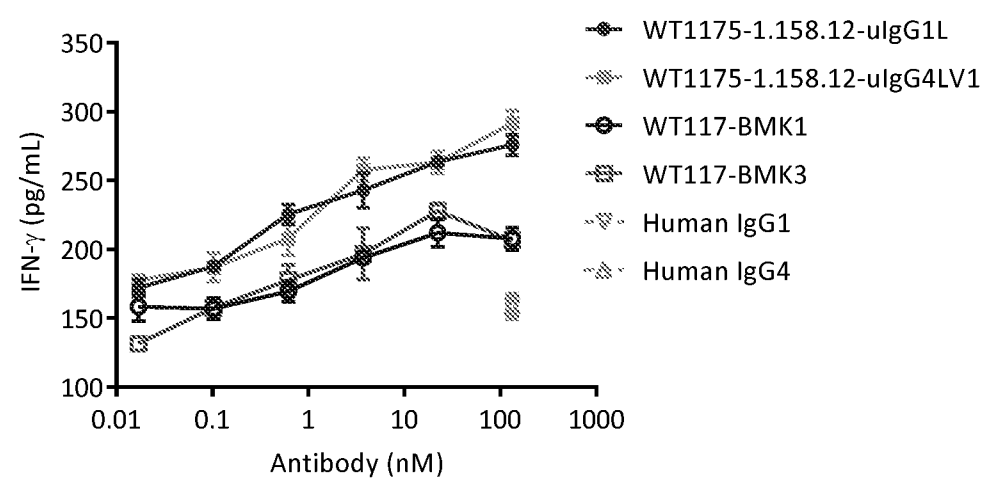


Figure 13

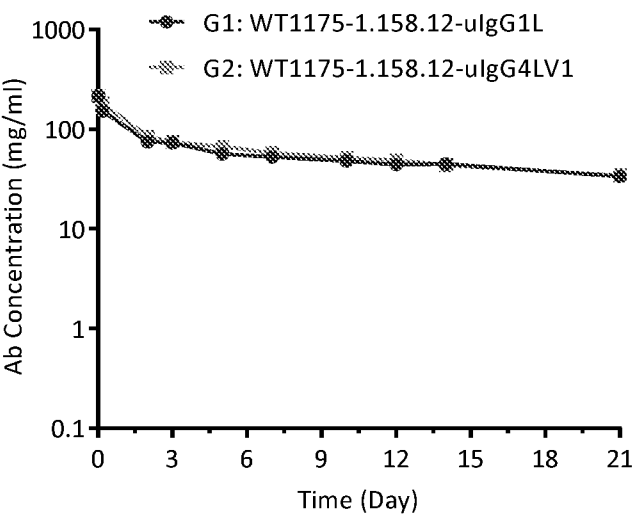


Figure 14

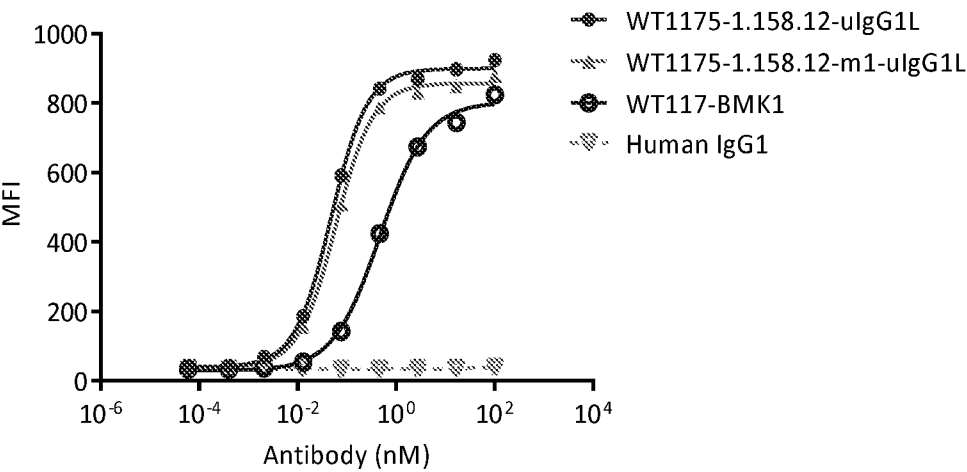


Figure 15

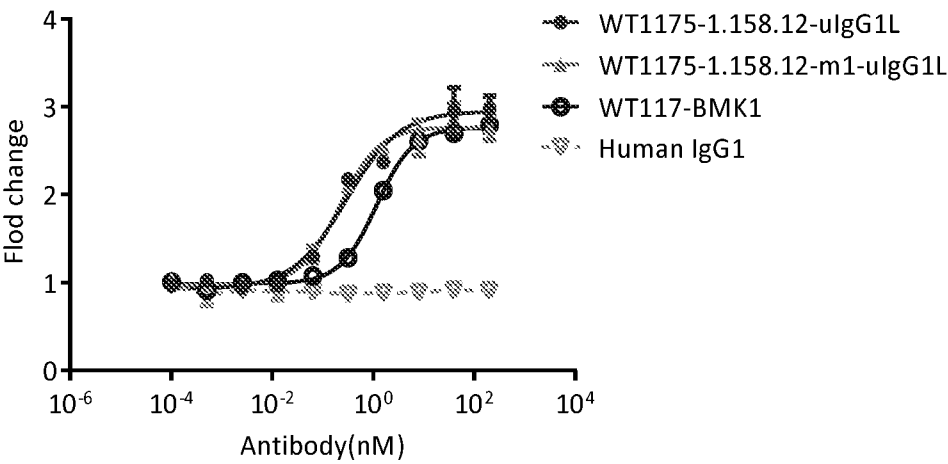


Figure 16

INTERNATIONAL SEARCH REPORT

International application No.

PCT/CN2024/097578

A. CLASSIFICATION OF SUBJECT MATTER

C07K16/28(2006.01)i; A61P35/00(2006.01)i

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC:C07K,A61P.

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

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

VEN,CNABS, PubMed, DWPI, CNTXT, WOTXT, USTXT, EPTXT, JPTXT, CNKI, Web of Science, Patentics,NCBI Genbank,EBI,STNext,Bio-Sequence Database of Chinese Patent:applicant,inventors, 293F,antibody,EGLTGYSSFDY, PVRIG, SSYLGS GTVV,TGTSSDIGSYKFVS, WT117, antibodies,WT117,heavy chain variable region, VH, light chain variable region, VL, SEQ ID NOs:1-8.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 2021021837 A2 (COMPUGEN LTD.) 04 February 2021 (2021-02-04) claims 1-33	1-20
A	WO 2021180205 A1 (JIANGSU HENGRUI MEDICINE CO., LTD. et al) 16 September 2021 (2021-09-16) claims 1-21	1-20
A	WO 2023006040 A1 (JIANGSU SIMCERE PHARMACEUTICAL CO., LTD.) 02 February 2023 (2023-02-02) claims 1-49	1-20
A	WO 2016134333 A1 (COMPUGEN LTD.) 25 August 2016 (2016-08-25) claims 1-37	1-20
A	CN 114644711 A (NANJING NOVOACINE BIOTECHNOLOGY CO., LTD.) 21 June 2022 (2022-06-21) claims 1-24	1-20

☒ Further documents are listed in the continuation of Box C.☒ See patent family annex.

* Special categories of cited documents:

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

“D” document cited by the applicant in the international application

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

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

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

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

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

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

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

“&” document member of the same patent family

Date of the actual completion of the international search

08 September 2024

Date of mailing of the international search report

11 September 2024

Name and mailing address of the ISA/CN

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/CN2024/097578

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	LI, J., et al. "PVRIG is a novel natural killer cell immune checkpoint receptor in acute myeloid leukemia" <i>Haematologica</i> , Vol. 106, No. 12, 05 November 2021 (2021-11-05), pages 3115-3124 pages 3115-3124	1-20

INTERNATIONAL SEARCH REPORT

International application No.

PCT/CN2024/097578

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

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:
 - a. ☒ forming part of the international application as filed.
 - b. ☐ furnished subsequent to the international filing date for the purposes of international search (Rule 13ter.1(a)),
☐ accompanied by a statement to the effect that the sequence listing does not go beyond the disclosure in the international application as filed.
2. ☐ With regard to any nucleotide and/or amino acid sequence disclosed in the international application, this report has been established to the extent that a meaningful search could be carried out without a WIPO Standard ST.26 compliant sequence listing.
3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No.

PCT/CN2024/097578

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

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

1. ☒ Claims Nos.: **13-17, 19**
because they relate to subject matter not required to be searched by this Authority, namely:

Claims 13-17, 19 relate to the treatment of diseases, and therefore do not warrant an international search according to the criteria set out in PCT Rule 39.1(iv). The search has been carried out and based on the use of the antibody in the manufacture of a medicament.
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/CN2024/097578

Patent document cited in search report			Publication date (day/month/year)	Patent family member(s)			Publication date (day/month/year)
WO	2021021837	A2	04 February 2021	JP	2022542505	A	04 October 2022
				MX	2022001146	A	17 March 2022
				EP	4003417	A2	01 June 2022
				IL	290141	A	01 March 2022
				WO	2021021837	A3	11 March 2021
				US	2022280643	A1	08 September 2022
				CA	3149093	A1	04 February 2021
				AU	2020323926	A1	10 March 2022
				BR	112022001575	A2	19 April 2022
				KR	20220041881	A	01 April 2022
WO	2021180205	A1	16 September 2021	TW	202144417	A	01 December 2021
				US	2024043530	A1	08 February 2024
				EP	4119162	A1	18 January 2023
				EP	4119162	A4	23 August 2023
				JP	2023516936	A	21 April 2023
				KR	20220154140	A	21 November 2022
				CA	3170025	A1	16 September 2021
WO	2023006040	A1	02 February 2023	CA	3227972	A1	02 February 2023
				AU	2022320667	A1	14 March 2024
				EP	4378954	A1	05 June 2024
WO	2016134333	A1	25 August 2016	US	2024101669	A1	28 March 2024
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				HRP	20221284	T1	23 December 2022
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				DK	3653221	T3	24 October 2022
				PT	3258951	T	18 August 2020
				LT	3295951	T	25 September 2020
				PL	3653221	T3	19 December 2022
				DK	3258951	T3	06 April 2020
				US	2018244774	A1	30 August 2018
				US	10351625	B2	16 July 2019
				AU	2024202336	A1	16 May 2024
				PT	3653221	T	08 November 2022
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